QUANTITATIVE ANALYSIS OF AMINO ACIDS USING PAPER CHROMATOGRAPHY

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Summary

A simple method for the quantitative analysis of amino acids present in a protein hydrolysate, or in other mixtures of amino acids, is described. The amino acids are separated on one-dimensional paper chromatograms, four solvent systems being used to resolve 17 amino acids. The chromatograms are treated with a suitable reagent to detect the spots corresponding to each amino acid. Each chromatogram, after being made semi-transparent with dimethylphthalate, is scanned automatically, with a densitometer, and the intensity of the light transmitted by the coloured spots is recorded on light-sensitive paper. A straight line relationship was found to hold, for all amino acids, between concentration and log per cent. transmission. The most useful range of amino acid concentration was 2–5 mM although the method is usable in the range 1–10 mM. The method includes a number of new techniques and the average coefficient of variation for a single reading for an amino acid is 5·7 per cent.

I. Introduction

Work in progress in this Laboratory on the composition of insect haemolymph required a method for the quantitative determination of amino acids in small volumes of biological fluids. Sufficient material is generally not available to enable the use of methods based upon microbiological techniques (Tristram 1953) or column chromatography (Moore and Stein 1949, 1951, 1954). For these reasons the development of a method for quantitative amino acid analysis by paper partition chromatography was undertaken.

Many papers have been published on the use of the techniques of paper chromatography for the quantitative analysis of amino acids in microgram quantities, and much of this work has been reviewed by Balston and Talbot (1952), Block, Durrum, and Zweig (1955), Kofrányi (1955), and Lederer and Lederer (1955). Additional references are given in the various sections below. The amino acids are separated from each other, identified, and the amount of each estimated. This estimation has been accomplished by:

(i) Measurement of the optical densities of the ninhydrin colours on the paper chromatograms (Brown, Kelly, and Watson 1953);

(ii) Elution of the amino acids from the paper followed by measurement of: (*a*) the optical densities of the ninhydrin colours (Chen and Hadorn 1954; Connell Dixon, and Hanes 1955; Gerok 1955); (*b*) nitrogen by the micro-Kjeldahl technique; (*c*) the optical densities of the soluble copper complexes;

(iii) Visual comparison of the ninhydrin colours on the papers with the colours given by known amounts of amino acids;

(iv) Measurement of the area of the ninhydrin spots;

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(v) A radiochemical method based on the incorporation of isotopes (Blackburn and Robson 1953).

Of these methods, development of and measurement of the intensities of the ninhydrin colours appears to be the most promising. It has been applied to both one- and two-dimensional, ascending and descending, chromatograms. In methods based on elution of the amino acids, or on measurements of the areas of the spots, difficulty is experienced in defining the boundary of an amino acid spot, more particularly if the spots lie close together.

The method we have developed is based on direct photometry on one-dimensional chromatograms of the coloured spots, formed by reaction of the amino acids with a suitable reagent. Scanning of the filter paper strips with a densitometer and recording of the intensity of the transmitted light on light-sensitive paper is done automatically. The majority of the published methods for the quantitative amino acid analysis by paper chromatography are unsatisfactory in so far as (1) the expected errors are too great or the method involves a large replication of analyses to obtain a reasonable degree of accuracy; (2) they are too complex for use as a routine analytical tool; or (3) insufficient details are given to enable the results to be repeated with the accuracy quoted.

II. Experimental Methods and Results

(*a*) *Reagents and Paper*

(i) *Amino Acids.*—All amino acids were checked for purity by paper partition chromatography. Particular attention was paid to samples of leucine which, it was found, may contain methionine.

(ii) *Phenol.*—B.D.H. "Analar" phenol was used without further purification. However, any samples which had acquired a pink colouration were rejected.

(iii) n-*Butanol.*—B.D.H. laboratory reagent *n*-butanol was used.

(iv) *Ninhydrin.*—A single large batch of B.D.H. laboratory reagent triketohydrindene hydrate was used throughout the entire work. It was checked for satisfactory colour yields for all amino acids.

(v) *Other Chemicals.*—Analytical reagent grades of acetone, isatin, and buffer reagents were used.

(vi) *Paper.*—Whatman No. 1 filter paper sheets were used for all experiments. In any one experiment or series of experiments it is considered advisable to use sheets from a single batch of paper. Except for buffering when required, the paper was not treated in any way. For quantitative work it is most important that the fingers do not come into contact with the paper surface being used in an experiment.

(*b*) *The Densitometer*

The densitometer was of simple construction. Light from a tungsten lamp (operated from a voltage stabilizer) passed through a filter and was "piped" through a "Perspex" rod and through a slit 8×2 mm to a Weston phototronic cell, model 594. The cell was connected directly to the terminals of the galvanometer in a photographically-recording Cambridge polarograph. The "Perspex" rod was covered

loosely with black paper to prevent the entry of stray light. The paper strip to be scanned was drawn by a synchronous electric motor over the photocell, immediately beneath the "Perspex" rod. For ninhydrin colours a green filter was used which transmitted light in the region 560–590 mμ. For the histidine colour a green glass filter transmitting light in the region 500–550 mμ and for the isatin colours a red filter which transmitted light in the region 590–650 mμ were used. The light transmitted by the paper caused the photocell to produce a current which moved the coil of the galvanometer. The movements of the coil were recorded on photographic paper. There was a linear relationship between the intensity of light falling on the photocell and the current produced. The paper strip travelled at the rate of 2·54 cm per min and the photographic paper at the rate of 1·7 cm per min. This enabled the light transmitted by an entire strip of paper to be recorded on one piece of photographic paper. The light intensity was checked before and after each scan by measuring the amount of light transmitted by a suitable glass filter.

(c) *Chromatography*

All the amino acids appearing in a protein hydrolysate were separated in a series of one-dimensional chromatograms using the capillary-ascent method of Williams and Kirby (1948). For ease in handling, ease in replication, and for greater reproducibility the use of large sheets of filter paper was abandoned and the method was standardized on strips of filter paper 6·5 × 47·0 cm. Glass cylinders, 8 cm dia. × 55 cm high, were used as chromatogram chambers and these were kept in a constant temperature room at 25°C. Into each cylinder was placed 100 ml of the required solvent. To obtain good reproducibility every step in the procedure which follows must be rigidly standardized.

Three spots of the amino acid solution, instead of the usual one, were placed on the paper; for 5 μl spots, the centres were 1 cm apart on a straight line drawn 3 cm from one end of the paper strips, the central spot being in the middle of the paper. This technique produced, after development with solvent and colour development, "rectangular" spots *c.* 3 × 1 cm. When these coloured spots were scanned in the direction of their longer dimension it was shown that the intensity of colour was constant, or almost so, over a distance of *c.* 2·5 cm. Each paper strip was immersed to the same depth (*c.* 1 cm) in the solvent contained in the bottom of each cylinder. After the paper strips had been developed with solvent for the required time they were removed from the cylinders and dried. For phenol and butanol solutions this was accomplished by washing them in three separate lots of solvent ether and air drying. A batch of papers was washed a few at a time so that each paper strip was only immersed in ether for a short time. Clean ether was used for each experiment. Excessive washing in ether or the use of ether which had been used for washing papers for a previous experiment brought about variations in the amount of colour subsequently produced by the amino acids. When aqueous acetone was used as the solvent the papers were air dried.

The solutions used to detect the positions of the amino acid spots, i.e. for colour development, were allowed to flow on to the paper strips from a pipette. The pipette was not permitted to touch the paper. The paper strips were hung vertically, the

bottom edge raised above the top edge, and the solution allowed to flow into the trough thus formed at the top of the paper. The bottom edge was slowly lowered so that the pool of liquid moved down the paper, more liquid being added from the pipette to maintain its volume. Surplus solution was removed from the bottom edge of the paper with the aid of a piece of filter paper. This procedure gave more reproducible results than did spraying the papers with the reagent solution and it was quite economical in regard to time and to the volume of solution needed (approx. 3·5 ml per $6{\cdot}5 \times 47.0$ cm strip).

Three reagent solutions were used for the colour development:

(i) For proline and hydroxyproline, 1 per cent. isatin in ethanol was used, the treated papers being heated at 100°C for 10 min. Acetone is a less satisfactory solvent because of its greater volatility. Although the addition of acetic acid to the reagent considerably increased the intensity of the proline colour it gave less satisfactory results with hydroxyproline. It also gave colours with some other amino acids although these did not interfere. Heating the treated papers for a longer time at a lower temperature was not as satisfactory. The recommended method is not entirely specific for proline and hydroxyproline since glutamic acid also gives a dark colour (cf. Saifer and Oreskes 1954).

(ii) Histidine was detected with a diazotized sulphanilamide solution. Sulphanilamide (1 g) was dissolved in ethanol (*c.* 90 ml), concentrated hydrochloric acid (2 ml) was added, and the total volume made up to 100 ml with ethanol. Amyl nitrite (1·3 ml) was dissolved in sufficient ethanol to make 100 ml of solution. Both solutions were cooled to 0°C, equal volumes mixed, and allowed to stand at 0°C for 5 min. The diazotized solution was applied to the paper strips which were air dried and treated with 2N aqueous sodium carbonate. This formed a cerise colour with histidine. The papers were air dried. The above solution was found to be superior to those based on *p*-anisidine instead of sulphanilamide, or sodium nitrite instead of amyl nitrite. Aqueous sodium carbonate gave better colour development than did aqueous sodium or potassium hydroxide, aqueous ammonia, ethanolic sodium ethylate, ethanolic triethylamine, or buffers of pH 12.

(iii) Ninhydrin was used for the detection and estimation of all the other amino acids. The paper strips, after having been treated with an appropriate ninhydrin solution (*vide infra*), were allowed to air dry for a short period of time. Each paper strip was held at both ends in a frame so designed that the papers did not touch the frame except at each end, nor did they touch each other. The frame (with papers) was placed in an incubator at 20°C and 35 per cent. R.H. for 30 hr, by which time the colour development was complete (cf. Wellington 1952, 1953). Aqueous glycerol was used for humidity control.

After colour development was completed, the papers were prepared for automatic scanning in a densitometer. The strips were cut 5·5 cm wide (as required by the scanning apparatus), the coloured spots being centred in this width. Each strip was hung vertically and treated with redistilled dimethylphthalate using a pipette as described above. The paper strip was allowed to hang for a few minutes, blotted with filter paper to remove excess dimethylphthalate, allowed to hang for a further 2 or 3 min, and scanned immediately. Each paper strip was scanned twice,

once on each side of the centre line. The two areas scanned were adjacent and were 8 mm wide. This was accomplished by moving the carrier of the densitometer, in which the paper travelled, over the photocell to pre-determined positions. The automatic scanning of each paper strip in the densitometer gave continuous graphs

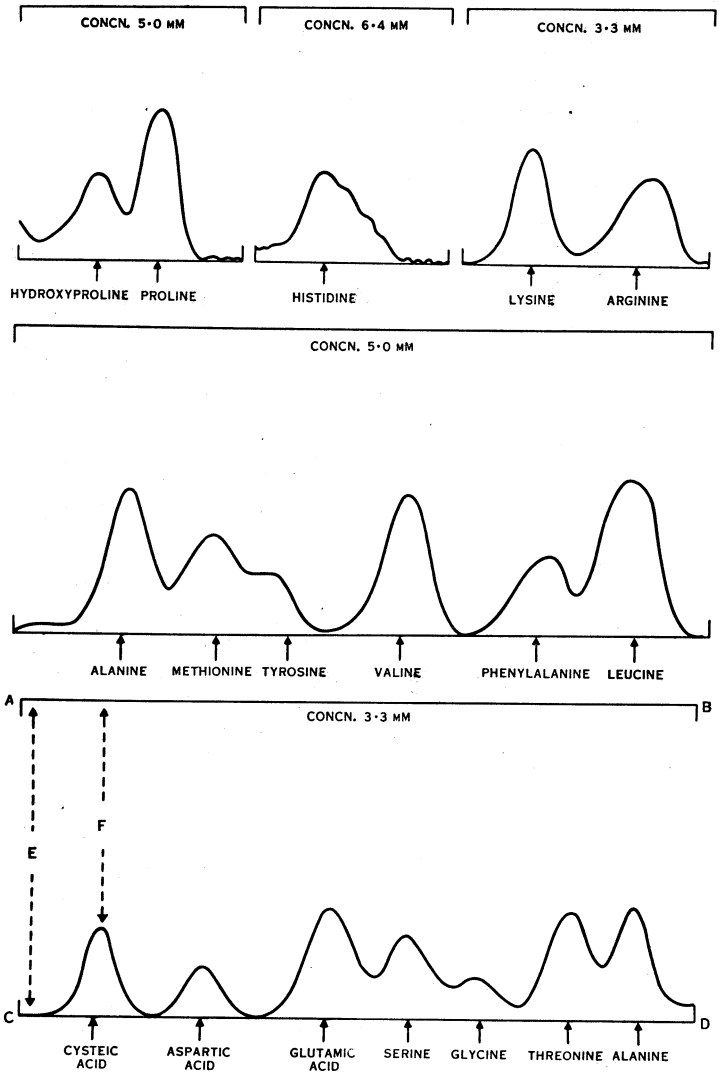

Fig. 1.—Optical transmission curves for mixtures of amino acids.

(see Fig. 1), each peak indicating the position of one amino acid and the height of each peak being a measure of the optical density of each spot. On each sheet of photographic paper is placed a line (*AB*) representing zero transmission (no light entering the photocell) the blank reading (*CD*) being obtained by recording the light transmitted at the beginning and end of each paper strip. Earlier work had shown that,

using the techniques and solutions described in this paper, blank strips of filter paper carried through the entire process gave, on scanning, a straight line parallel to the line representing zero transmission. Making the paper semi-transparent with dimethylphthalate has the effect of producing a straight line for such a blank instead of a wavy line. The percentage of light transmitted by the coloured spots is calculated by dividing the distance E by the distance F (see Fig. 1) and multiplying by 100. The value for E is the mean of the results from the two scans of each paper strip. In practice, both scans are recorded on the same sheet of photographic paper. A straight line relationship was found to hold for all amino acids between concentration and log per cent. transmission.

(*d*) *Solvents Used for Complete Separation*

Four solvents were used to give complete separation of the amino acids found in protein hydrolysates:

(i) Phenol (74 per cent. w/w) and a buffer of pH 10·0 (0·053M boric acid and potassium chloride, 0·047M sodium hydroxide) (cf. McFarren 1951). This solvent gave an excellent separation of cysteic acid, aspartic acid, glutamic acid, serine, glycine, threonine, and alanine. Large sheets of filter paper were hung up and saturated with buffer (pH 10·0) by allowing the buffer to flow on to the paper from a pipette. The sheets were air dried, cut into strips, and the amino acid solution applied. The strips were suspended in the cylinders containing the solvent and allowed to remain overnight so that they came into equilibrium with the atmosphere within the cylinders. The paper strips were lowered into the solvent and developed for 24 hr. For colour development, the strips were treated with 2 per cent. ninhydrin in ethanol containing 2 per cent. acetic acid. The papers were hung by the end where the amino acid solution had been applied before being treated with the ninhydrin solution. This minimized interference from a pale yellow stain which sometimes appeared near the solvent front. The stain was soluble in acidified ethanol and so tended to be washed from the paper.

(ii) *n*-Butanol (77 per cent. v/v), acetic acid (6 per cent. v/v), and water (17 per cent v/v). This solvent provided a good separation of alanine, methionine, tyrosine, valine, phenylalanine, and leucine. The amino acid mixture was first run overnight on unbuffered paper without prior equilibration. The papers were washed in ether, air dried, and run again overnight in the same solvent after the addition of 1·2 ml of a mixture of 10 per cent. aqueous ammonium molybdate (40 ml) and hydrogen peroxide (13·3 ml; 100 vol.) to each 100 ml of butanol-acetic acid-water. For colour development, the papers were treated with 1 per cent. ninhydrin in ethanol containing 0·25 per cent. triethylamine.

(iii) Lysine and arginine were separated on filter paper strips buffered at pH 7·0 (Sorensen phosphate buffer: 0·040M Na_2HPO_4, 0·027M KH_2PO_4) using 60 per cent. (v/v) acetone plus 40 per cent. (v/v) phosphate buffer pH 7·0. This solvent mixture had to be filtered before use as some salts were precipitated. The paper strips were developed overnight in the solvent, removed from the cylinders, and air dried. They were then developed in 80 per cent. aqueous phenol for 22 hr. For colour development, the papers were treated with 2 per cent. ninhydrin in ethanol.

(iv) Sixty per cent. (v/v) aqueous acetone gave a good separation of histidine, proline, and hydroxyproline. The paper strips were developed overnight in the solvent and air dried. Histidine and hydroxyproline were well separated and each paper strip was cut between these two amino acids. The histidine colour was developed on the lower part of the paper and the proline and hydroxyproline colours on the upper part of the papers.

(*e*) *Standardization of the Method*

Mixtures of all the abovementioned amino acids at concentrations of 0·005, 0·00417, 0·0033, 0·0025, and 0·002M were prepared in pH 10 buffer (boric acid-potassium chloride-sodium hydroxide) containing 10 per cent. *iso*propanol. The procedures for separation and estimation, described above, were carried out using eight to 10 replicates. An estimate of the reproducibility among replicates of optical density measurements for the amino acids is given in Table 1.

TABLE 1

REPRODUCIBILITY AMONG REPLICATES OF OPTICAL DENSITY MEASUREMENTS

Amino Acid	Coefficient of Variation for a Single Reading	Amino Acid	Coefficient of Variation for a Single Reading
Cysteic acid	3·2	Valine	4·5
Aspartic acid	5·7	Phenylalanine	7·1
Glutamic acid	5·2	Leucine	4·7
Serine	4·6	Lysine	8·9
Glycine	7·8	Arginine	6·3
Threonine	3·4	Histidine	9·9
Alanine	5·6	Proline	5·6
Methionine	5·5	Hydroxyproline	8·5
Tyrosine	1·4		

(*f*) *Amino Acid Analysis of a Simulated Insulin Hydrolysate*

The method of analysis was applied to a mixture of amino acids designed to reproduce approximately the composition of amino acids as found in a hydrolysate of the B chain of insulin (concentration corresponding to *c.* 1·25 g B chain per 100 ml; cf. Tristram 1953). To bring the concentration of the individual amino acids within the experimental range (viz. *c.* 2–5 mM) aliquots of the solution were diluted 1·5 and 3 times. For those amino acids separated by buffered phenol, the solution diluted 1·5 times was used except for glycine and glutamic acid. For these two amino acids and for those amino acids separated by *n*-butanol-acetic acid the solution diluted 3 times was used. The remaining four amino acids were estimated in the undiluted solution. Five replicates were used and the results are given in Table 2.

(*g*) *Recommended Procedure for Protein Hydrolysates*

After acid hydrolysis of a protein the excess acid must be removed by repeated evaporation *in vacuo*. Cystine, if present, must be converted to cysteic acid by the addition of hydrogen peroxide (100 vol., one-fifth of the volume of hydrolysate taken) to an aliquot of the hydrolysate, allowing the solution to stand for several hours, and evaporating to dryness *in vacuo*. All the hydrogen peroxide must be destroyed before phenol buffered at pH 10 is used as a developing solvent. If insufficient hydrolysate is available to enable an aliquot to be taken for the oxidation, the other three solvents are used first, the remaining hydrolysate treated with hydrogen peroxide and used with phenol as the developing solvent.

TABLE 2

COMPOSITION OF AN AMINO ACID MIXTURE

Amino Acid	Estimate from Regression Line (mM)	95% Confidence Limits	Approx. Standard Error	Actual Value
Cysteic acid	2·13*	2·33–1·90	0·09	2·25*
Aspartic acid	2·36*	2·65–2·02	0·14	2·21*
Threonine	2·56*	2·79–2·39	0·09	2·19*
Serine	2·60*	3·07–2·16	0·20	2·14*
Glycine	2·99†	3·26–2·74	0·11	3·32†
Glutamic acid	3·34†	3·69–3·03	0·14	3·32†
Alanine	2·40†	2·69–2·08	0·14	2·23†
Valine	3·36†	3·58–3·14	0·10	3·30†
Phenylalanine	3·54†	4·07–3·02	0·24	3·31†
Leucine	4·45†	4·76–4·16	0·14	4·32†
Tyrosine	2·22†	2·45–1·89	0·12	2·31†
Arginine	3·39	3·81–2·88	0·21	3·34
Lysine	3·61	4·08–3·15	0·21	3·34
Proline	3·33	3·58–3·08	0·11	3·35
Histidine	6·41	7·55–5·62	0·30	6·62

* Result to be multiplied by 1·5. † Result to be multiplied by 3·0.

III. DISCUSSION

The success of quantitative analysis of amino acids by paper partition chromatography depends largely on the production of compact and discrete spots. The nature of the developed spots was found to depend on the original volume of solution applied to the paper, the number of such applications, the extent of equilibration, and on the technique used in applying the reagent. It is obvious that the smaller the original spot the more compact are the spots likely to be after development and best results were obtained when the original volume was small. The solubility of the amino acids in water absorbed by the paper leads to the formation of diffuse spots. This was demonstrated when spotted papers were hung for some hours in a moist atmosphere. If such papers were treated with ninhydrin, with or without development, the original spot was seen to have increased considerably in size, e.g. from 1·0 to 1·7 cm in

diameter. Excessive drenching of the papers with a solution of ninhydrin in water-saturated *n*-butanol can also lead to tailing of the spots if the paper is suspended vertically during the operation. The procedure adopted, after testing several variations, was to apply ninhydrin (or other reagent) in absolute ethanol with the aid of a pipette, in order to ensure adequate and even distribution. Occasionally some spots, in particular aspartic and glutamic acids, appeared as ring-like structures or "halos". This appeared to be due to the super-imposition of aliquots of solutions to the original spot and single applications prevented a further occurrence of this source of error.

When buffered phenol at pH 12 was first used in this work, as recommended by McFarren (1951), the results were not satisfactory, as serine consistently had a low R_F value and it overlapped with glutamic acid. An explanation for this phenomenon is that the highly alkaline paper absorbed carbon dioxide causing a decrease in pH for, as McFarren has shown, separations at pH 11 are not satisfactory. Since from McFarren's tables a good separation was to be expected in the region pH 9–10, the paper and solvent were treated with a boric acid-sodium hydroxide buffer of pH 10·0. This procedure eliminated the trouble experienced at pH 12. The separation of glutamic acid and serine was further improved by bringing the paper strips into equilibrium with the atmosphere of the chromatographic chamber. If the paper is equilibrated in the same chamber as is used for the chromatographic run it removes from the solution considerable amounts of water. However, as variations in phenol content from 74 to 79 per cent. caused little, if any, alteration in the R_F values of the amino acids, the loss of water was not important in a single run provided the phenol content did not exceed 79 per cent. and the water could be replaced after each run if necessary.

In the analysis of those acids separated in *n*-butanol-acetic acid-water, use is made of the technique of developing the papers more than once in the same solvent. This procedure leads to better separations of the substances being chromatographed if the R_F values of those substances are less than 0·5. If the R_F values are greater than 0·5, the substances separated in the first development are brought closer together in each subsequent development (Csobán 1950; Jeanes, Wise, and Dimler 1951). The addition of hydrogen peroxide and ammonium molybdate for the second development in *n*-butanol-acetic acid-water converts the methionine to methionine sulphone. Methionine travels on the paper with valine, but methionine sulphone has a low R_F value (*c.* 0·08) and it finally appears between alanine and tyrosine. Residual traces of acetic acid on the paper strips cause the ninhydrin colours to be reddish. The addition of triethylamine to the ninhydrin solution produces blue spots with amino acids as also does the addition of other organic bases such as pyridine. Triethylamine was found to be the best base to use and the optimum concentration was 0·25 per cent. which gave a 20–25 per cent. increase in colour intensity when compared with the same ninhydrin solution without an organic base.

The method for estimation of the colour intensities involved the automatic scanning of the paper strips. When the paper strips were cut to fit the carrier of the scanning apparatus, it was not possible always to correct for non-vertical travel of the amino acids on the strips which sometimes occurred. Although this source of error was to some extent overcome by rigid standardization of the technique it was largely

eliminated by the use of three spots of amino acid solution instead of the usual one. Slight variations in the direction of travel of this wide spot were not sufficient to cause any trouble. Statistical analysis of results showed that the three-spot method gave much greater precision than was obtained by use of single spots.

The method for the quantitative estimation of amino acids was standardized on replicate runs of five known concentrations which were approximately 5·00, 4·17, 3·33, 2·50, and 2·00 mM. For each of the amino acids the plot of the mean readings of the optical density readings of the spots against concentration appeared to be linear. This linearity was examined statistically and it was shown clearly that over the range of concentrations used there was no evidence of curvature for any amino acid.

An estimate of the reproducibility among replicates of the optical density measurements, each replicate being on a separate strip of filter paper, are given in Table 1. The average coefficients of variation for a single reading for each amino acid are recorded. In our hands the estimation of glycine has always been somewhat unsatisfactory. Statistical analysis of the results showed that the highest precision was obtained by measuring the maximum colour density of the spots and not by estimating the total colour of the spots (i.e. by measurement of the distance E and not by measurement of the area under the curve (Fig. 1)).

These calibration curves were used to estimate the concentrations of the amino acids in a mixture of amino acids designed to reproduce the composition of the hydrolysate obtained from the B chain of insulin. The results are given in Table 2 which also includes the 95 per cent. confidence limits (Eisenhart 1939) for the true concentration of each amino acid and the corresponding approximate standard errors. This procedure for testing the method was chosen because the composition of the "hydrolysate" was known accurately. As the composition of most biological materials varies with the source and as the method of hydrolysis introduces some unknown errors, the use of a biological preparation would not adequately test the method. The errors associated with the estimation of each amino acid are within the expected limits except for serine and threonine. These greater errors are caused by the high concentrations of glutamic acid and alanine (which travel adjacent to serine and threonine respectively) when compared with the concentrations of serine and threonine. This additional source of error will always occur when a small amount of one amino acid is not completely separated from a much larger amount of another amino acid travelling next to it on the paper strips. The error can be eliminated by adding a known amount of that amino acid present in low concentration to raise its concentration to approximately that of the interfering adjacent amino acid. The only error to be anticipated then is that indicated in Table 1. These results indicate very clearly that the coefficient of variation does not always give an indication of the accuracy of an analysis but only of its reproducibility.

Even though the same set of calibration curves can be used for analyses performed on different occasions, where a high degree of accuracy is desired calibration curves should be prepared at the same time as the unknown is analysed. If it is desired to correct for errors introduced by the method of hydrolysis, then the calibration curves would have to be prepared from hydrolysed amino acid mixtures;

however, the amino acid mixture would have to simulate the composition of the protein being analysed. Other amino acids than those mentioned can readily be estimated by slight modification of the described procedures. The isomeric leucines can be separated by use of a suitable solvent system, e.g. pyridine-amyl alcohol (Heynes and Walter 1951) and the coloured spots estimated on the papers. Tryptophan can be separated by a suitable solvent (e.g. *n*-butanol-acetic acid-water) and estimated by reaction with *p*-dimethylaminobenzaldehyde. The yellow colour formed with ninhydrin is not satisfactory. The papers are treated with a solution of *p*-dimethylaminobenzaldehyde (1 g) in a mixture of acetone (90 ml) and concentrated hydrochloric acid (10 ml) and allowed to air dry. Tryptophan gives a purple colour within a few minutes. Since the tryptophan colour changes on standing the colour intensity must be read after a fixed period of time. Asparagine and glutamine can be estimated in biological fluids by first estimating the concentrations of aspartic acid and glutamic acid, subjecting the amino acid mixture to hydrolysis (2N HCl at 100°C for 3 hr), and again analysing for aspartic acid and glutamic acid. The concentrations of asparagine and glutamine can be calculated from the difference in concentration of aspartic acid and glutamic acid before and after hydrolysis. By modifications such as these the concentration of any particular amino acid can be estimated. Although the most useful range of amino acid concentration is 2–5 mM the method is usable in the range 1–10 mM.

IV. Acknowledgment

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V. References

Balston, J. N., and Talbot, B. E. (1952).—"A Guide to Filter Paper and Cellulose Powder Chromatography." (Reeve Angel and Co. Ltd., London, and Balston Ltd., Maidenhead.)

Blackburn, S., and Robson, A. (1953).—A radiochemical method for the microestimation of α-amino-acids separated on paper partition chromatograms. *Biochem. J.* **54**: 295–9.

Block, R. J., Durrum, E. L., and Zweig, G. (1955).—"A Manual of Paper Chromatography and Paper Electrophoresis." (Academic Press Inc.: New York.)

Brown, G. L., Kelly, F. C., and Watson, M. (1953).—"Nature and Structure of Collagen." (Ed. J. T. Randall.) pp. 117–21, 122–3. (Academic Press Inc.: New York.)

Chen, P. S., and Hadorn, E. (1954).—Vergleichende Untersuchungen über die freien Aminosäuren in der larvalen Haemolymphe von *Drosophila ephestia* and *corethra*. *Rev. Suisse Zool.* **61**: 437–51.

Connell, G. E., Dixon, G. H., and Hanes, C. S. (1955).—Quantitative chromatographic methods for the study of enzymic transpeptidation reactions. *Canad. J. Biochem. & Physiol.* **33**: 416–27.

Csobán, G. (1950).—Paper chromatography. *Mag. Kem. Fóly.* **56**: 449–51. (*Chem. Abstr.* **46**: 1384*f* (1952).)

Eisenhart, C. (1939).—The interpretation of certain regression methods and their use in biological and industrial research. *Ann. Math. Stat.* **10**: 162–86.

Gerok, W. (1955).—Die quantitative papierchromatographische Bestimmung der Aminosäuren. *Hoppe-Seyl Z.* **299**: 112–28.

Heynes, K., and Walter, W. (1951).—Über Proteine und deren Abbauprodukte. V. Papierchromatographische Trennung der isomeren Leucine. *Hoppe-Seyl. Z.* **287**: 15–18.

JEANES, A., WISE, C. S., and DIMLER, R. J. (1951).—Improved techniques in paper chromatography of carbohydrates. *Anal. Chem.* **23**: 415–20.

KOFRANYI, E. (1955).—Über quantitative Papierchromatographie von Aminosäuren in Proteinhydrolysaten. *Hoppe-Seyl. Z.* **299**: 129–38.

LEDERER, E., and LEDERER, M. (1955).—"Chromatography. A Review of Principles and Applications." (Elsevier Publ. Co.: Amsterdam.)

MCFARREN, E. F. (1951).—Buffered filter paper chromatography of the amino acids. *Anal. Chem.* **23**: 168–74.

MOORE, S., and STEIN, W. H. (1949).—Chromatography of amino acids on starch columns. Solvent mixtures for the fractionation of protein hydrolysates. *J. Biol. Chem.* **178**: 53–77.

MOORE, S., and STEIN, W. H. (1951).—Chromatography of amino acids on sulfonated polystyrene resins. *J. Biol. Chem.* **192**: 663–81.

MOORE, S., and STEIN, W. H. (1954).—Procedures for the chromatographic determination of amino acids on four per cent. cross-linked sulfonated resins. *J. Biol. Chem.* **211**: 893–906.

SAIFER, A., and ORESKES, I. (1954).—Circular paper chromatography. II. Isatin as a colour reagent for amino acids. *Science* **119**: 124–5.

TRISTRAM, G. R. (1953).—"The Proteins." Vol. 1A. Ch. 3. (Ed. H. Neurath and K. Bailey.) (Academic Press Inc.: New York.)

WELLINGTON, E. F. (1952).—An ultramicro method for quantitative determination of amino acids. *Canad. J. Chem.* **30**: 581–91.

WELLINGTON, E. F. (1953).—The effect of relative humidity on the reaction of ninhydrin with amino acids on paper chromatograms. *Canad. J. Chem.* **31**: 484–7.

WILLIAMS, R. J., and KIRBY, H. (1948).—Paper chromatography using capillary ascent. *Science* **107**: 481–3.