Quantitative Micro-analysis of Amino-acid Mixtures on Paper Partition Chromatograms

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The successful separation of amino-acids on paper partition chromatograms (Consden, Gordon & Martin, 1944) naturally led to attempts to employ similar methods quantitatively. The methods described below are presented in a somewhat unfinished state, as external circumstances compelled an interruption of the work. In particular, no attempt has been made so far to devise an analytical method suitable for a normal protein. Only the simpler problem has been attempted of analyzing quantitatively such relatively simple mixtures of amino-acids as can be separated using one-dimensional paper chromatograms. No doubt the method could be applied to more complicated mixtures, but preliminary separations, by paper chromatography or other methods, would be required.

PRELIMINARY EXPERIMENTS

Several methods adapted to micro estimations were investigated. Attempts were made to utilize the colour reaction with ninhydrin upon paper. It was found possible to wash off the colour from the paper with a variety of solvents, but the estimation of colour in a photoelectric microcolorimeter did not give consistent results. These experiments, however, showed that the coloured product consisted of several components whose proportions depended upon the exact conditions under which the colour was developed.

Attempts were next made to utilize the Folin reaction, both the original Folin (1922) technique and the modification of Frame, Russell & Wilhelmi (1943), again using a photoelectric microcolorimeter. Both methods were reasonably satisfactory for such quantities of pure amino-acids as are available on a paper chromatogram, but when amino-acids were washed from the chromatogram, interfering substances, apparently derived both from the paper and the solvents, rendered the results grossly inaccurate.

Nitrogen estimation by the Kjeldahl method was also tried. This method is very attractive theoretically, as it was hoped that after a qualitative analysis the chromatogram could be cut up and a quantitative estimate obtained from the amount of nitrogen in each coloured spot. The

technique used was that of Hawes & Stavinsky (1942). The scale of working was reduced by a factor of 10, and satisfactory results were obtained, if the starting material was $(\text{NH}_4)_2\text{SO}_4$ and the incineration omitted. However, the atmosphere of the laboratory proved to be so heavily-contaminated with the nitrogenous solvents used in the chromatography that satisfactory incinerations could not be performed. It was not even possible to determine whether the nitrogen blank of the paper was low enough to render the method feasible.

Titration in glacial acetic acid (Harris, 1935) was next employed. The amino-acid was washed from the paper into small titration tubes and, after evaporating in a vacuum desiccator and dissolving in 20 μl . glacial acetic acid, it was titrated with 0.01 N-HClO₄ in acetic acid, using crystal violet as indicator (Nadeau & Branchen, 1935). Excellent results were obtained with pure amino-acids, quantities of 15–30 μg . being titratable with an error of 1%. The paper, however, contained such large amounts of acids and bases, which could not be removed by preliminary washing, that the method could not be used on the material washed from the paper.

To avoid interference with contaminating substances, the relatively specific complex formation of the amino-acids with copper was then tried. The technique used was essentially that of Pope & Stevens (1939), which is a modification of the method of Kober (1912). The amino-acid was added to a suspension of copper phosphate in a phosphate-borate mixture, causing the copper to go into solution as the amino-acid-copper complex. After filtration, the copper in solution was estimated iodometrically. Stoichiometric results were obtained for almost all the amino-acids, with a ratio of two amino-acid molecules to one atom of copper. It was not found possible to reduce the scale of the iodometric titration to the micro quantities available, though good values on 100–200 μg . of pure amino-acid were obtainable. However, even in this case, reducing material derived from the paper interfered with the iodometric titration and prevented satisfactory estimation of the material derived from the paper chromatograms.

Finally, the polarograph (see Kolthoff & Lingane, 1941) was used to estimate the copper complex, in place of the iodometric titration. This proved to be sufficiently specific to give useful though not highly accurate results. As has already been said, the method has not been fully worked out and it is hoped that further experience will increase the accuracy and the availability of the method for complex mixtures.

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EXPERIMENTAL

Polarographic technique

In the initial stages a simple galvanometer, fitted for visual observation, was used with the dropping mercury cathode. Later, a Cambridge Instrument Company's photographic instrument was used. This is convenient when complete current voltage curves are required, but for routine use, as described below, the simple galvanometer is as satisfactory, since observations are made at one potential only.

The dropping mercury cathode used throughout this investigation had the following characteristics. Height of mercury 62 cm., drop time 2.4 sec., rate of flow 2.70 mg./sec. in $N-KCl$ on open circuit. No attempt has been made to work at a constant temperature, but any given set of measurements has been completed within so short a time that temperature variations have been small. There is, however, an obvious advantage to be gained by working at a constant temperature. The polarograph cells were of two sizes, one 9 mm. internal diameter, 8 cm. long, and the other 3 mm. internal diameter and 3 cm. long; each was fitted with a platinum anode sealed into the bottom. The larger cells required 1–2 ml. and the small ones, which have to be completely filled with solution before the electrode is introduced (to avoid air bubbles), required 0.2 ml.

Throughout, sulphite has been used to remove O_2 , and has proved more convenient than H_2 or other gas, particularly with the smaller volume of solution. Since, with our solutions, the step at about 0.0 V. before the Cu step begins has been too steep to permit accurate measurement, the zero of the instrument, with the dropping cathode disconnected, has been employed as the reference point.

Supporting electrolyte

A solution of 50 g. $CuCl_2 \cdot 2H_2O$ was added with stirring to 200 g. $Na_2HPO_4 \cdot 12H_2O$ in about 1.5 l. of distilled water, and $NaOH$ was added to pH 9.0. The precipitate was filtered on a large Buchner funnel, washed on the filter with 2% borax solution, and then made up to 1 l. with 2% borax solution. This stock suspension can be kept indefinitely. 5 ml. of this suspension, with 1.70 g. $Na_2HPO_4 \cdot 2H_2O$ and 2 ml. of the starch solution of Pope & Stevens (1939) were made up to 200 ml. with 2% borax. This suspension, which should be made up weekly owing to growth of moulds, forms the supporting electrolyte for the polarographic measurements. The amino-acid was added to a known volume of this suspension and left for 30 min. or more (it can be left for 24 hr. without further change); 1 or 2 ml. were now added to the polarograph cell and a small knife point (c. 2 mg.) of Na_2SO_3 was dissolved by stirring with a glass rod (1 mm. diam. with a bent end) which was rolled between fingers and thumb. The dropping electrode was now put into position and allowed to drop for 2 min. with no current passing. The current at -0.5 V. was then observed at 1 min. intervals until steady for 1 min., and then recorded on the photographic drum. The instrumental zero, the dropping electrode having been disconnected, was then immediately recorded on the same segment of the drum. With the large cells the observation was discarded if the current had not become steady within 15 min., and with the small tubes within 5 min. Usually

the current had become steady 3 min. after the electrode had been put in.

The shape and height of the current voltage curve depends upon the composition of the supporting electrolyte. In the absence of starch, two pronounced maxima are shown at about -0.3 and -1.3 V. With about 0.01% of starch, both maxima are suppressed, but the height of the step is not much affected (Fig. 1). With higher concentrations of starch the height of the step is greatly reduced and the shape of the curve changed. With the solution used there is a long straight portion from 0.3 to -1.2 V. Measurements have been made at -0.5 V., which has been arbitrarily chosen within this range.

Fig. 1. Effect of starch on the current-voltage curve of the glutamic acid copper complex. The short cross line near the origin of each curve is the 'instrumental zero'.

The amount of Na_2HPO_4 in the supporting electrolyte affects the height of the step. In its absence, copper phosphate is sufficiently soluble to give a large blank of ionic copper. In the presence of high concentrations of Na_2HPO_4 , the amount of the ionic copper is so small that an appreciable proportion of the amino-acid does not react, and low results are obtained both polarographically and by iodometric titration. This is true also of a CuO suspension in 2% borax.

The original Pope & Stevens (1939) mixture gave satisfactory results in the polarograph if the solution had been filtered from copper phosphate. If the suspension was used without filtering, at about -0.5 V. the current began to increase, and at higher voltages became very large and independent of the amount of copper in solution. The value of the current in the supporting electrolyte in the absence of amino-acids is very variable. The presence of as little as 2.5×10^{-5} M-amino-acid greatly reduces the variability. For this reason, no blanks are included in the comparisons given. Perhaps because of the variability it has not been possible to demonstrate a consistent curvature of the current/concentration curve at the lower concentrations of amino-acids. When an amino-acid on a paper chromatogram was to be estimated, the paper containing the amino-acid, which had an area of about 40 cm.², was folded, cut into pieces of 10 mm.² and introduced into a centrifuge tube by means of a wide-necked funnel; 3 ml. of the supporting electrolyte were then added, which just covered the paper. It was then allowed to stand overnight to ensure that diffusion of the amino-acid from the paper to the copper

phosphate was complete. The paper and the copper phosphate were centrifuged down and the supernatant liquid was transferred to the polarograph cell and the measurement made as before.

Chromatographic technique

The method described by Consden *et al.* (1944) and Consden, Gordon & Martin (1947) has been followed without essential change. Whatman no. 4 paper has, however, been used and HCN has been employed to eliminate the 'pink fronts', where necessary. Wide strips were used, so that the amino-acids would not approach the edge of the paper where irregularity of running is likely to occur.

A sheet of filter paper was marked out with pencil into, say, ten strips, each 4 cm. wide. The solution to be analyzed was applied with a microburette to strips 3, 5, 7 and 9, about 7 cm. from one end. The amount, the same for each strip, should be enough to contain about 20 $\mu\text{g.}$ of each amino-acid to be estimated. To strips 2, 4, 6 and 8 was applied the control mixture, with the amounts on each strip in arithmetical progression, the mean amount being similar to that believed to have been put on strips 3, 5, 7 and 9. Strips 1 and 10 had amounts of control mixture equal to that on strip 8. After development of the chromatogram, and very thorough drying, strips 1 and 10 were cut off, treated with ninhydrin and used as guides for cutting up the other strips. The guide strips have more amino-acid on them than most of the other strips, and hence the separation is probably better on the other strips.

Since there was no direct indication of the proper place to cut, merely an inference from another chromatogram, the line of cutting carried an appreciable error. In order to ensure that the spots are well separated, the time of running of the chromatogram was increased as compared with that employed in qualitative work. If the guide strips show any considerable difference between each other or if the spots are not well separated, that experiment should be abandoned.

In all the measurements a direct comparison has been made between the material under analysis and a control mixture which simulates it as closely as possible. By such a comparison, rather than by attempting an absolute measure, various errors, e.g. those due to variation of temperature or to adsorption by the paper, were minimized.

After the polarographic measurements had been completed in the way described above, the current at 0.5 V. was plotted against the amount of amino-acid for the control series. The best straight line was then drawn (by inspection) through these points (Figs. 8 and 9). This procedure is justifiable over the range of concentration employed, since no consistent curvature has been discovered. The mean of the current readings of the mixture under analysis was then plotted on this line and hence the amount of amino-acid present was inferred.

RESULTS

The results of application of these methods are presented graphically below. Figs. 2 and 3 show the relation between current and concentration of amino-acid, the solution being added directly to the copper phosphate suspension and used without

filtration after addition of solid sulphite. No explanation has been found of the variation of both zero current and slope from one experiment to the

Fig. 2. Relation between current and concentration of amino-acid at -0.5 V. using large and small cells. The zero of the glutamic acid in small cells has been reduced by 5 mm., and glutamic acid in large cells by 10 mm., to avoid overlapping. Sensitivity of galvanometer $1/50$.

Fig. 3. Relation between current and concentration of amino-acid. The zero of the leucine line has been raised 5 mm. to avoid overlapping. Sensitivity of galvanometer $1/7$.

next, while the results within a given experiment (represented by a single line on the graph) are much more consistent. The copper complex of leucine is too insoluble to be used satisfactorily alone at 10^{-3} M.

In Fig. 4 a comparison is made between measured amounts of glycine put on a chromatogram, alone and mixed with glutamic acid. The chromatogram was run for 41 hr. in phenol-coal gas-0.3% NH_3 . The extra manipulation and lower concentration has resulted in a decreased consistency of the measurements. Large cells were used here and throughout the remainder of the work.

Fig. 4. Glycine and glutamic acid separated on chromatogram. Galvanometer sensitivity 1/5.

Fig. 5. Arginine, leucine and alanine separated on chromatogram. Galvanometer sensitivity 1/3.

In Fig. 5 a comparison is made between measured amounts of arginine put on a chromatogram alone and mixed with leucine and alanine. Alanine results were lost. Chromatograms were run 41 hr. in *s*-collidine-diethylamine-HCN.

In Fig. 6 a comparison is made between measured amounts of proline put on a chromatogram alone and as a mixture of equimolar amounts of proline, valine, leucine, glutamic acid, aspartic acid, phenylalanine, tyrosine and ornithine. The chromatogram

was run for 67 hr. in *s*-collidine-diethylamine-HCN.

In Fig. 7 a comparison is made between glutamic acid alone and aspartic acid alone and both, in the same mixture as used in the experiment described

Fig. 6. Proline alone and separated from a mixture. Galvanometer sensitivity 1/3.

Fig. 7. Glutamic and aspartic acids separated from a mixture of amino-acids. Galvanometer sensitivity 1/2. $\times$, controls of the single amino-acids; $\bullet$, amino-acid applied as mixture.

in Fig. 6. The chromatogram was developed for 67 hr. with phenol-0.3% NH_3 . The aspartic and glutamic acids were not, on this occasion, perfectly separated from each other as judged from the guide strips, though well separated from the six other amino-acids. For this reason the sum of the deflections for aspartic and glutamic acids has also been plotted for the mixture and the controls.

Analysis of gramicidin S

Figs. 8 and 9 show an analysis of gramicidin *S*. Syngé (1945) concluded that this substance consists of equimolecular amounts of ornithine, proline, valine, leucine and phenylalanine, perhaps with 1 or 2 mols. of water.

10.8 mg. of air-dry gramicidin *S* were weighed into a small tube with 1 ml. of 6*N*-HCl. The tube was sealed and put in an oven at 105–110° for 48 hr. After opening, it was dried in a vacuum desiccator over KOH. To remove further

fuge tube). The mean values are plotted on the lines given by the control mixture.

Fig. 9 shows results obtained with benzyl alcohol-HCN. The chromatogram was run for 41 hr. Comparison was made between five levels of control mixture and four spots of 10 μ l. of gramicidin solution. The deflexions given for the gramicidin *S* were: leucine, 39.0, 39.0, 39.5, 38.5, mean 39.0 mm.; phenylalanine, 36.0, 35.5, 35.0, 35.6, mean 35.5 mm. The mean values of the gramicidin *S* deflexions were plotted on the lines given by the control mixture.

Fig. 8. Analysis of gramicidin *S*. $\times$, mean of five determinations on 10 μ l. of hydrolysate; other points single values of control mixture. Sensitivity of galvanometer 2/3.

HCl, water was added twice, and evaporated in the desiccator. Finally, 1.013 g. of water were added. 10 μ l. of the resulting solution were used on each chromatogram spot. Since altogether nine spots were used, 0.96 mg. of gramicidin *S* was actually consumed in the analysis.

The control mixture contained 12.8 mg. DL-proline, 9.0 mg. DL-valine, 12.7 mg. L-ornithine hydrochloride, 13.3 mg. DL-phenylalanine, 9.8 mg. DL-leucine made up to 10 ml. The ornithine was a rather dark coloured specimen prepared from arginine but free from other amino-acids. The other amino-acids were supplied by Roche Products Ltd. and believed to be pure.

Fig. 8 shows the results obtained with *s*-collidine-diethylamine-HCN as solvent. The chromatogram was run for 67 hr. Comparison was made between the synthetic mixture, run at four different levels, and five lots of gramicidin *S* at the same level of 10 μ l. of solution. The deflexions given for the gramicidin *S* were: ornithine, 53.5, 54.0, 54.5, 54.5, 54.5, mean 54.2 mm.; proline, 50.0, 51.5, 51.1, 52.0, 50.0, mean 50.9 mm.; valine, 59.5, 59.0, 59.8, 59.0, mean 59.3 mm. (one lost through cracking of centri-

Fig. 9. Analysis of gramicidin *S*. $\times$, mean of four determinations on 10 μ l. of hydrolysate; other points single values of control mixture. Sensitivity of galvanometer 2/3.

From Syngé's formula, the mols. of each amino-acid contained in 10 μ l. of the hydrolysate are 1.69×10^{-7} assuming 1 mol. H_2O , and 1.64×10^{-7} assuming 2 mols. H_2O . The amounts of amino-acids determined from Figs. 8 and 9, in terms of mols. $\times 10^{-7}$, are: Found: proline, 1.62; valine, 1.65; ornithine, 1.55; leucine, 1.53; phenylalanine, 1.67. Expected for all amino-acids: 1.64 mols.

DISCUSSION

An advantage of the use of the lower concentrations of amino-acids which are described here, compared with those used by Kober (1912) or Pope & Stevens (1939), is that amino-acids forming relatively insoluble copper complexes, e.g. leucine and phenylalanine, can be used without the addition of a solubilizer such as glycine or glutamic acid, the use of which is, because of the large amount which has to be added, attended with a large increase of error. Colorimetric methods of measuring copper are available which could almost certainly be made to work at the micro level required, but the labour involved in these is great compared to the use of the

polarograph directly on the suspension. Apart from its use for amino-acids, the method should be applicable to peptides, provided authentic specimens of these exist for comparison. For the amino-acids, the value of the method lies principally in the small amount of material required for analysis, though at present the whole range of amino-acids have not been dealt with simultaneously. For certain amino-acids the microbiological methods, while using comparable amounts of material, are more accurate. For peptides it is improbable that it will be worth while developing the microbiological methods.

The results obtained in the analysis of gramicidin *S*, though intended primarily as a test of the method of analysis, may be considered to confirm the formula proposed by Syngé (1945). The material consumed in the analysis here (0.96 mg.) is about 10% of that which Syngé employed, using partition chromatography of acetamido-acids on silica columns.

SUMMARY

1. Various methods have been investigated in an attempt to render quantitative amino-acid analysis by partition chromatography on paper.

2. A polarographic modification of Kober's method for the estimation of amino-acids as their copper complexes is described. A few micrograms only are required.

3. Estimations of several amino-acids in a number of simple mixtures are presented. An analysis of gramicidin *S* using 1 mg. only, confirms the formula proposed by Syngé.

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The Amino-acid Composition and Titration Curve of Collagen

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The amino-acid composition of gelatin has been extensively studied, but until recently little attention has been paid to that of its precursor collagen, the natural protein of skin. Some of the analyses of collagen which have been reported were carried out by methods which are now considered unsatisfactory, and in no case have most of the major component amino-acids been determined in one sample of the protein. Further, most of the analyses have been made on collagens which had been given an alkaline or enzyme treatment, or both, to remove the keratins, elastic fibres and reticular tissue which occur in close association with it, and there is a risk

that such treatments will cause some modification of the collagen.

For the present study, a sample of collagen has been prepared with the minimum of chemical treatment, since it was considered that the presence of small amounts of elastic fibres and reticular tissue would lead to less error than the treatments necessary to bring about their removal. Also, it seems doubtful whether it is possible to remove these proteins completely by any of the methods which have been suggested. On this preparation Chibnall and his collaborators (Chibnall, 1946; Macpherson, 1946; Rees, 1946; Tristram, 1946) have determined