Observations on the Application of Fluorodinitrobenzene to the Quantitative Analysis of Proteins

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The serial micro-estimation of amino-acids has been effected in two main ways: by partition chromatography of the acetamino-acids (Martin & Synge, 1941), and by separation of amino-acids by partition chromatography on starch (Moore & Stein, 1948). A few amino-acids have also been estimated under special circumstances as their dinitrophenyl (DNP) derivatives, but the available methods of separating these compounds are not suitable for a routine procedure for micro-analysis (Sanger, 1945; Porter & Sanger, 1948; Blackburn, 1949). The quantitative nature of the reaction between fluorodinitrobenzene (FDNB) and amino-acids is very attractive, however, and the discovery of a new method of separating the DNP derivatives has led to a re-investigation of their use in analysis.

The resolution of mixtures of DNP-amino-acids is usually achieved by the serial use of partition chromatographic columns as suggested by Sanger (1945) and by Blackburn (1949). Sanger (1945) has also reported trials with adsorption chromatography, but with the systems he used had only indifferent success. It has now been found that adsorption on kieselguhr permits the discrimination of all the commonly occurring ether-soluble DNP-amino-acids on a single column. This technique has been used as the basis of the analytical procedure which is the subject of this communication.

The absorption method has been applied to several proteins with promising results, but those chosen to illustrate its use here are three for which the composition is known with a considerable degree of probability, namely salmine, insulin and lysozyme. The values obtained have not, however, been fully checked by means of *ad hoc* control mixtures.

EXPERIMENTAL

It will be convenient to describe first of all the coupling of FDNB with a protein hydrolysate, for which certain special pieces of apparatus have been designed.

It is desirable, for reasons to be explained in a following paragraph, to oxidize the protein with 100 vol. hydrogen peroxide in order to convert the cystine to cysteic acid. But if even traces of peroxide are allowed to come into contact with FDNB, a large amount of brown substance is formed which will mask the subsequent chromatographic separation. The oxidation has therefore been done in formic acid according to Sanger (1947), before the protein is hydrolysed. By careful evaporation *in vacuo* most of the hydrogen peroxide can be removed, any that remains being decomposed during the hydrolysis with HCl. The minute amounts of chlorine that are formed are apparently without noticeable effect on the amino-acids.

Hydrolysis. An amount of oxidized protein equivalent to about 0·5 mg. of protein N was boiled with 6 N-HCl on an oil bath at about 130° for 18 hr. The resulting solution was then filtered if necessary and evaporated to dryness *in vacuo*. The amino-acids were dissolved in 2 or 3 ml. of water, and the total N of this solution estimated by micro-Kjeldahl. Based on this value a sample containing approximately 250 μg. N was taken for conversion to the DNP derivatives.

Preparation of the DNP derivatives. The coupling reaction was performed in a reaction tube which was made from a standard *B*14 socket and had a total volume of about 10 ml. This is shown attached to the lower end of the extractor in Fig. 1. Into this tube was pipetted the mixture of amino-acids dissolved in about 1 ml. of water. Residual HCl was neutralized by the careful addition of a slight excess of sodium bicarbonate and followed by about 2 ml. of ethanol. The amino-acids were now in solution in 66% ethanol, not exceeding 5 ml. in volume, and to this were added two drops (0·05 ml.) of FDNB. The reagent used in these experiments was supplied by L. Light and Co. Ltd. and no further purification has been found necessary.

After the tube had been closed with a carefully greased stopper, it was shaken vigorously for a time dependent on the amount of glutamic or aspartic acid present. In several trials with proteins like gliadin, which are rich in glutamic acid, the recoveries of this acid were low, but if the time of reaction with FDNB was increased from 2 to 5 hr. a quantitative recovery resulted. This effect was not found when solutions of pure glutamic acid were used, probably because the quantities taken were too small, but it appears that in a protein hydrolysate the dicarboxylic amino-acids coupled more slowly with FDNB than was expected. Accordingly, if either of the dicarboxylic acids was found to contribute 10% or more of the total protein N, the reaction was allowed to proceed for at least 4 hr. When the amino-N contribution was less than 10%, a coupling time of 2 hr. was sufficient.

It has been found essential during the course of this work to protect the DNP-amino-acids from light at all stages of their preparation and separation. This particularly applies to solutions in alkali and in $CHCl_3$, as has been observed by Blackburn (1949). While being shaken with FDNB, therefore, the reaction tube was enclosed in a short length of the corrugated rubber tubing used on gas-masks.

Purification of the DNP derivatives. When the reaction was completed, the mixture contained excess FDNB, dinitroaniline, dinitrophenol and DNP-amino-acids. The removal of

the first two of these has been done in the extractor (shown in Fig. 1) which has a *B*14 socket at the lower end, on to which the reaction tube was fitted. The extracting solvent could be boiled in a flask attached to the side arm and was returned down the central funnel by a reflux condenser at the top.

After the reaction tube had been opened, the neck was carefully washed down with a few ml. of distilled water. The tube was then plugged on to the bottom of the extractor and the stopper very carefully washed into the top. Sufficient 0·2N-NaOH was added to make the volume in the extractor about 35 ml. and a brisk stream of ether passed through the mixture until no more yellow material was removed. This should not take more than 10 min.

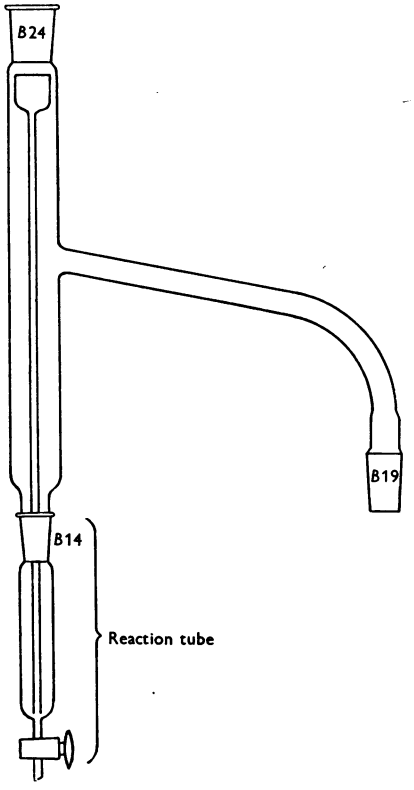

Fig. 1. The special extractor, showing the reaction tube in position.

The apparatus was then tilted, to swill some ether down the side arm and wash away any residue resulting from evaporation of the solvent. The boiling flask was next replaced by one of the type shown in Fig. 2, the ground-glass joint being sealed, as in the first case, with a drop of water. One ml. of 6N-HCl was afterwards pipetted down the central funnel of the extractor and the DNP-amino-acids removed in a brisk stream of ether. When the extraction was nearly complete a small chip of marble was dropped down the funnel, where it fell into the narrow tube above the tap and by its effervescence ensured that this dead space was stirred up. During the whole of the operations in this apparatus the contents were protected from the light by a shield which is not shown in the figure.

When the extraction had been completed, the side arm was again swilled down with ether and the boiling flask removed. The solvents were evaporated over a steam bath to leave a film of yellow DNP compounds round the inner surface of the flask. This mixture contained too much dinitrophenol to be separated on a column, so the amount was reduced by vacuum sublimation in the following way. The cold-finger condenser, which fits the flask containing the DNP-amino-acids as shown in Fig. 2, was loaded with crushed dry ice and the assembly evacuated on an oil pump. When the flask was warmed in a steam bath the dinitrophenol sublimed on to the cold finger. The residue was then redissolved in ether, which was evaporated to leave a new film round the flask, a second sublimation usually being sufficient to reduce the amount of dinitrophenol to manageable proportions.

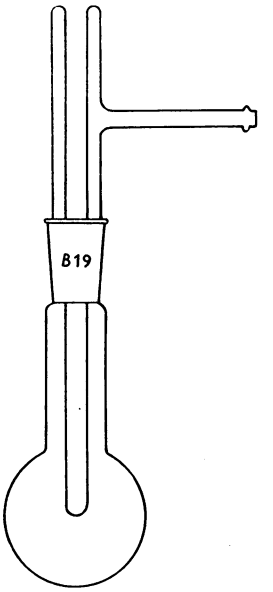

Fig. 2. The cold-finger condenser on to which the dinitrophenol is sublimed.

In the processing of the DNP-amino-acids not extracted by ether, the aqueous phase was drawn into a long-necked flask through the tap at the bottom of the reaction tube. After concentration to small bulk the solution was transferred to a small volumetric flask and this process repeated with the washings from the extractor. Like the ether-soluble compounds, this solution was stored in a refrigerator in the dark.

Chromatographic technique. The kieselguhr used was the 'acid washed' preparation of British Drug Houses Ltd., which was 'activated' before use by shaking up in a large stoppered bottle, inside the stopper of which was fixed a small cloth bag containing a lump of $(NH_4)_2CO_3$. The pH of an aqueous extract of this prepared adsorbent was between 6 and 7.

The series of solvents used with the kieselguhr was of mixtures of methyl ethyl ketone (MEK) and $CHCl_3$, saturated with water. The presence of the water resulted in sharper bands than could be obtained without it, but as the system would also work under completely anhydrous

conditions, the mechanism is presumably one of adsorption rather than partition. The solvents were distilled before use following the observation that old samples of MEK contained a substance reducible by titanous chloride.

For brevity the solvents will be referred to subsequently as 45% MEK, 75% MEK, etc.; this being understood to mean 45% MEK-55% $CHCl_3$ by volume, etc., each mixture being saturated with water at room temperature.

The preparation of columns of kieselguhr was rather more difficult than, for example, in the case of silica gel. Owing to the fact that kieselguhr will not sink very readily in MEK-$CHCl_3$ mixtures, it was necessary to press the solid down with a ramrod and a certain degree of skill had to be acquired before consistently good columns could be made. In a column 1·5 cm. in diameter, it was found that 6·5 g. of dry kieselguhr compressed in 45% MEK-$CHCl_3$ to a height of 12 cm. gave good separations provided great care was taken to ensure that the top of the solid was firm and flat.

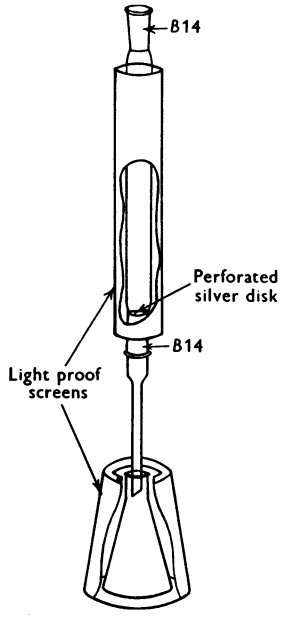

Fig. 3. The chromatographic column, showing the arrangement of light shields.

A column of the kind shown in Fig. 3 has been used in the majority of these experiments. It was made by joining two *B*14 sockets end to end giving a tube about 1·5 cm. in diameter and approximately 24 cm. long. The upper end of the cone leading to the delivery tube was a convenient ledge on which to rest a perforated silver disk covered with a circle of glass cloth. Into the socket at the upper end of the column was fitted a reservoir for solvent, to which a slight pressure of air could be applied.

The solid ether-soluble DNP derivatives were next dissolved by pipetting into the long-necked flask containing them an appropriate volume of 45% MEK. A portion of this solution was then transferred to the column and allowed to sink in. After the sides of the tube had been washed down two or three times with about 1 ml. of solvent, and each allowed to sink in, the reservoir was filled with 45% MEK. A light-proof shield of gas-mask tubing was put round the column, and sufficient pressure applied to cause the delivery of not more than one drop of solvent/sec.

The separate bands of DNP-amino-acids were collected individually, as they reached the bottom of the column, in 50 ml. conical flasks which were stored in a refrigerator. The solvent was evaporated when necessary by directing a stream of air into the flask while it was warmed in a water bath. The solid DNP compounds could then be stored in the cold and dark for several days without apparent deterioration.

The amount of material which was applied to the column depended on the number of components present and their relative abundance. Under most circumstances, however, a column of the size quoted above would carry from 30 to 40 μg. of protein N satisfactorily.

Separation of the water-soluble components. The aqueous phase contained only DNP-cysteic acid and DNP-arginine, which were mixed with various inorganic salts. The latter interfered considerably with the usual chromatograms and were removed in the following way. A portion of the solution was transferred to a porcelain dish and evaporated to dryness on a steam bath. The residue was then washed repeatedly with small quantities of a 10% solution of 6N-HCl in methyl ethyl ketone. This did not dissolve the salts but extracted the DNP derivatives, from which the solvent was afterwards removed by evaporation.

A column of kieselguhr prepared in 75% MEK was used for separating these two amino-acid derivatives which were applied by dissolving the solid in the smallest amount of pure MEK saturated with water. Subsequent development with 75% MEK caused the DNP-arginine to run very fast, while the DNP-cysteic acid remained at the top of the column. After the arginine derivative had been collected, the DNP-cysteic acid could be made to move slowly in 100% MEK.

These two derivatives were then estimated in the same way as the others.

Determination of the DNP derivatives. The procedure was that to be found in most text-books on quantitative analysis, and involved the back titration of an excess of titanous chloride with standard ferric alum solution. The concentration of ferric alum in these experiments was arranged so that 1 ml. was equivalent to about 7·5 μg. nitro-N. Commercial titanous chloride was diluted to make 500 ml. batches and standardized frequently during use, since the solution underwent fairly rapid decomposition.

5 ml. of 0·5N-HCl and 2 ml. of 10% potassium thiocyanate were run into each 50 ml. flask and raised to boiling point under a stream of carbon dioxide. After addition of an excess of titanous chloride the solution was boiled for 3–5 min. according to the amount of DNP derivative present. The flask was cooled under running water before titration of the remaining titanous chloride, carbon dioxide being passed the whole time.

RESULTS

Comparative experiments with solutions of known mixtures of DNP-amino-acids indicated that under the optimum conditions all the common derivatives could be separated on one column of kieselguhr. There does seem, however, to be a number of factors governing the resolution obtained which have not been kept under complete control. Thus it has not

always been possible to separate DNP-leucine from DNP-isoleucine or bis-DNP-lysine from bis-DNP-tyrosine. Some evidence was found that the water content of the developing solvent may be involved. Although difficulty was sometimes experienced with other bands also, this was usually interpretational, being especially noticeable when some bands were faint. If one band was not seen, it was easy to identify some of the remainder wrongly.

The order in which the ether-extracted DNP-amino-acids were eluted from the column is shown in Table 1.

Table 1. *Order of elution of the DNP-amino-acids from kieselguhr, the fastest moving bands being those at the bottom of the table*

(Percentage MEK indicates volume proportion of methyl ethyl ketone in methyl ethyl ketone-chloroform mixtures.)

DNP-amino-acid	Eluting solvent
Bis-DNP-cysteine	100% MEK
Aspartic acid	
Glutamic acid	
Serine	75% MEK
Bis-DNP-histidine	
Methionine sulphone	30% MEK followed by 45% MEK
Threonine	
Glycine	
Alanine	
Proline	
Bis-DNP-tyrosine	
Bis-DNP-lysine	
Cystine	
Valine	
Leucine	
Isoleucine	
Phenylalanine	
Dinitrophenol	

It was necessary at some stage to develop the chromatogram with 30% MEK for a short while, in order to resolve DNP-threonine and DNP-glycine which ran at the same speed in 45% MEK. Fortunately 30 and 45% MEK could be mixed without causing water to be precipitated and could be interchanged on the column without ill effect; thus, the separation of DNP-threonine and DNP-glycine could be made at any convenient time.

It will be seen that DNP-cystine ran close to valine in a part of the chromatogram which was already overcrowded. For this reason the cystine of protein hydrolysates was oxidized to cysteic acid which was not extracted into ether.

Under the usual conditions of extraction, bis-DNP-histidine is also looked for in the aqueous phase (Sanger, 1945). By the technique described above, however, it was obtained in the ether extract and when run on kieselguhr was found to develop three bands. Of these the upper was the strongest and the middle one very weak. No explanation of this behaviour has so far been suggested. The DNP derivative of methionine was also found to give three bands which have been tentatively identified as corresponding to the amino-acid, the sulphone and the sulphoxide. Oxidation of the mixture with hydrogen peroxide resulted in one band increasing in strength at the expense of the other two, which disappeared. It was convenient to identify methionine as the DNP derivative of its sulphone obtained in this way as confusion with the histidine bands was thereby lessened. However, as threonine, methionine and histidine often occur in proteins in comparatively small amounts, the bands corresponding to these amino-acids were sometimes difficult to distinguish.

As a preliminary test of the method, known amounts of the amino-acids glutamic acid, glycine and valine were separately estimated. For the glutamic acid a solution was made containing about 1·0 mg. of amino-acid N/ml. and 10 μl. portions spotted on to a piece of filter paper with a calibrated syringe. The paper was then folded concertina-wise and put into the reaction tube where it was allowed to react with FDNB as described above. A separate experiment showed that no interfering substances were derived from the paper. The estimates averaged from 98 to 99% of the 9·3 μg. of glutamic acid N taken.

Table 2. *Recovery of some amino-acids submitted to the analytical procedure*

Amino-acid	N taken (mg.)	N recovered (mg.)	Recovery (%)	Range (%)
Glutamic acid	0·0093	0·0092	99	±1
Glycine	0·518	0·515	99·4	±0·5
Valine	0·515	0·513	99·7	±0·5

For the glycine and valine 0·5 ml. of a solution containing about 10 mg. N/ml. was treated with FDNB and a portion of the DNP derivative run on the column. Table 2 summarizes the results. The nitrogen in each solution was determined by the micro-Kjeldahl method.

These results were thought sufficiently encouraging to warrant a more stringent trial under the conditions of protein analysis. The proteins examined were a sample of salmine which was the gift of Dr G. R. Tristram, crystalline insulin obtained from Boots Pure Drug Co. Ltd. and crystalline lysozyme marketed by Armour and Co. In each case hydrolysates were prepared as described in the experimental section, and the figures quoted below represent an average of two or three separate analyses on as many different hydrolysates. In all cases the values are the amino-acid N as a percentage of the protein N.

Table 3. *Amino-acids of salmine*

(Amino-acid N expressed as % of protein N.)

Amino-acid	Mills	Tristram (1947)	Block & Bolling (1945)
Arginine	90·6	89·0	92·4
Isoleucine, Valine	1·85	1·76	2·09
Proline	2·16	2·29	3·11
Alanine	0·59	0·56	1·83
Glycine	1·97	1·78	—
Serine	2·70	3·94	3·02
Total	99·87	99·33	102·45

The estimates obtained for salmine are compared in Table 3 with those given by Tristram (1947) and Block & Bolling (1945). The DNP derivatives of valine and isoleucine in this early experiment were not resolved sufficiently well to permit their individual determination, and they were accordingly estimated together. With the exception of serine, the results agree fairly closely with those of Tristram. For serine a much lower recovery was obtained, being in better agreement with that found by Block & Bolling.

Table 4. *Amino-acids of insulin*

(Amino-acid N expressed as % of protein N.)

Amino-acid	Mills	Chibnall (1946)	Brand (1946)	Fromageot (1950)
Phenylalanine	4·4	4·4	4·2	4·6
Isoleucine	2·1	1·7	1·9	1·1
Leucine	8·1	9·3	8·9	10·2
Valine	6·8	5·8	6·6	5·8
Lysine	3·4	3·0	3·1	3·4
Tyrosine	5·6	6·0	5·9	6·1
Proline	1·7	2·0	2·2	1·6
Alanine	4·4	4·4	—	4·7
Glycine	5·5	5·2	5·4	5·5
Threonine	2·0	1·6	2·3	1·8
Histidine	9·0	8·5	9·0	8·8
Serine	4·7	4·5	4·8	4·5
Glutamic acid	11·1	11·4	12·0	12·0
Aspartic acid	4·5	3·8	4·5	3·8
Arginine	6·1	6·2	7·0	6·0
Cystine	8·0	9·4	8·0	9·5
Total	87·4	87·2	85·8	89·4

Very accurate analyses of crystalline insulin have been published by Chibnall (1946), by Brand (1946) and by Fromageot (1950). This protein was consequently of special use as a test substance. The values quoted in Table 4 are in substantial agreement with those previously published, although in some cases there is greater similarity to the results of Brand than to those of Chibnall or Fromageot. This is noticeable, for example, in the amounts of histidine, aspartic acid and cystine. It will be noticed also that the total leucine N is rather lower than the values quoted in the three other analyses, while the value for valine is high, being comparable with that found by Brand. It is thought that the leucine and valine bands were adequately resolved in these experiments, and consequently that the possibility of their intermixture is unlikely. In one experiment a faint, unidentified band was observed close behind the valine band. This may be due to an interfering substance which was not usually separated from the valine derivative and which spuriously raised the recovery of valine. A similar observation was made by Tristram (1946) during his analyses of insulin via the acetamino-acids.

Table 5. *Amino-acids of lysozyme*

(Amino-acid N expressed as % of protein N.)

Amino-acid	Mills	Fromageot (1950)
Phenylalanine	1·3	1·1
Isoleucine	2·8	3·6
Leucine	3·7	4·0
Valine	4·2	4·0
Lysine	6·3	6·2
Tyrosine	1·4	1·6
Proline	1·1	0·9
Alanine	5·1	5·2
Glycine	4·8	5·6
Methionine	1·2	1·2
Threonine	2·9	3·3
Histidine	1·9	1·5
Serine	4·5	5·1
Glutamic acid	2·2	1·7
Aspartic acid	6·8	6·7
Arginine	25·8	25·6
Cystine	5·8	5·0
Total	81·8	82·3

As for insulin, the analyses of crystalline lysozyme show a fair measure of agreement with the results published by Fromageot (1950), the most substantial differences being in the values for isoleucine, glycine, serine and cystine. In a more recent paper, however, Fromageot, Acher & Jutisz (1950) have given revised results for some amino-acids, which do not conform so well with those in Table 5. It would appear that for these estimations Armour lysozyme which had been twice recrystallized was used. This may explain the source of the different results, since the protein used in the present study was not recrystallized, and especially as Stein has recently resolved lysozyme into two components (Tallan & Stein, 1951).

The most notable modification in the revised figures is to the values for arginine and aspartic acid, the former being diminished to 22·8% and the latter increased to 9·3% of the protein N. The value for lysine has also been slightly increased to 7·6%, while the values for isoleucine, leucine and valine have been readjusted to 3·0, 4·8 and 3·0% respectively. The results of the DNP method of analysis do not agree with these modifications except in the case of isoleucine. It is worth noting, however, that the total N contributed by isoleucine, leucine and valine is almost the same in each case.

DISCUSSION

The application of a proposed serial method of determining amino-acids will necessarily depend ultimately on the ability of the chromatographic machinery to resolve the amino-acids to an adequate degree. It would appear from experience so far gained in the use of kieselguhr columns that it is possible to separate all the common DNP-amino-acids on this adsorbent. Occasional unexplained failures have been met, however, usually in the resolution of closely running pairs such as DNP-isoleucine and DNP-leucine. In this connexion it seems that the amount of adsorbent/ml. of column may play a critical part. Thus, improved separations of some fast moving derivatives have been obtained by compressing the lower part of the column more tightly than the upper part.

The greatest difficulty in the use of adsorption columns is usually the tendency of the adsorbate to 'tail' during the run. This has been observed under unfavourable conditions on kieselguhr, but does not usually happen with the selected solvents mentioned above. The presence of water in the organic phase has a marked effect in suppressing 'tailing' and some DNP-amino-acids tend to 'beard' if a solvent containing much water is used to elute them. Thus it seems that clear-cut bands depend to some extent on the composition of the solvent, which can in turn be varied, within limits, to obtain the most satisfactory results.

The original separation procedure of Sanger (1945), Porter & Sanger (1948) and the more recent ones of Blackburn (1949), Perrone (1951) and Bell (1949), while giving quantitative separations of the amino-acids, suffer from the disadvantage that several columns are used. In these systems, therefore, numerous quantitative transfers may be necessary. For this reason the kieselguhr column seems to be useful at least for rapid routine analyses of moderate accuracy.

The precision with which estimations can be made is good for single amino-acids, or for simple mixtures, replicate results agreeing to within about $\pm 0{\cdot}5\%$. For protein hydrolysates, for which separation of the components may be more difficult, the results may be duplicated to about $\pm 2{\cdot}5\%$ in unsatisfactory cases, although it is frequently much better than this.

With a column of the size used here, quantities of amino-acid N of the order of $1{\cdot}0\ \mu$g. could be determined with reasonable accuracy, but bands corresponding to $0{\cdot}5\ \mu$g. or less were so faint as to be difficult to see. Generally, between 2 and $3\ \mu$g. of amino N proved the most suitable concentration for each band.

Kieselguhr also has a considerable sensitivity towards differences in structure, which makes it very suitable for the separation of DNP-peptides. Considerable use has been made of columns for this purpose, up to twenty DNP-peptides being separated on a single unit. The method is also applicable to DNP-proteins, with the modifications necessary to get the particular protein derivative into solution.

SUMMARY

1. A method has been described whereby the dinitrophenyl derivatives of the commonly occurring amino-acids may be distinguished on a single chromatographic column.

2. The application of this technique to the quantitative estimation of amino-acids has been discussed.

3. Analyses of the proteins salmine, insulin and lysozyme are reported as examples of the use of the method.

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