The Identification of Lower Peptides in Complex Mixtures

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The mixture of amino-acids and peptides, which is obtained when proteins are only partially hydrolyzed by acids, is so complex that no single method can be expected to achieve the separation and identification of more than a few of the components present. The successive use of methods effecting separations along different lines is required: a scheme of separation is described here which, it is believed, can lead to the identification of a large proportion of the dipeptide species present in partial acid hydrolysates of proteins (cf. Martin, 1946). The scheme includes the use of two previously published methods, ionophoresis in silica jelly (Consden, Gordon & Martin, 1946*a*) and partition chromatography on paper (Consden, Gordon & Martin, 1944), a method of freeing solutions of amino-acids and peptides from salts, a micro-technique for the hydrolysis, and a method for the deamination and subsequent hydrolysis of material obtained from a paper chromatogram.

The partial hydrolysate is first subjected to ionophoresis, at a pH near 6. In this way, a division into about a dozen distinct fractions differing in their mobility in an electric field, is obtained. Method B (Consden *et al.* 1946*a*), in which the silica jelly is set beneath a glass lid, has been adopted because it gives the most complete separation. However, since the jelly is made from sodium silicate and an acid, and replacement by another electrolyte is impracticable, the finally separated fractions are contaminated, not only with silica, but with some sodium salt. Consden *et al.* (1946*a*) discuss various methods of removing salts from amino-acid solutions but none were suitable for the removal of sodium acetate, which has been found so far to be the most suitable electrolyte for a pH near 6. The removal of sodium acetate has been effected in two stages. First, acetate was replaced by sulphate by steam distillation *in vacuo* of a solution acidified with sulphuric acid. Secondly, sodium sulphate and sulphuric acid were removed by a 'desalting apparatus'. This 'desalting apparatus', a preliminary account of which has been communicated (Consden, Gordon & Martin, 1945), employs electrolysis between a carbon anode, enclosed in a cellophan membrane containing dilute sulphuric acid, and a circulating mercury cathode, washed with water, for the removal of strong electrolytes. The problem of removing a given salt is thus changed to the easier one of converting one salt into another, and the alkali metals are no longer troublesome. The field of use of the desalting apparatus thus probably greatly exceeds the use here described. The material obtained from the desalting apparatus is, after evaporation, ready for further fractionation by partition chromatography (Consden *et al.* 1944) or by other methods.

These paper chromatograms, which may be one- or two-dimensional, are carried out in duplicates or in larger sets; one is treated with ninhydrin and is used as a guide to the cutting of the other. The material washed from the cuts may be hydrolyzed or first deaminated by treatment with nitrous fumes and then hydrolyzed. The hydrolysis products are again subjected to chromatography for the identification of the constituent amino-acids. If the cut contains only a single dipeptide, its identity (apart from stereoisomerism) is shown in this way, since of the two amino-acids liberated by hydrolysis, that with the free amino group is destroyed after the deamination treatment. When, however, the cut contains several dipeptides or a heterogeneous mixture of peptides the interpretation of the result is often impossible. Even when only two amino-acids are present, a tripeptide with two residues of the same amino-acid cannot easily be excluded, since the depth of colour given by the ninhydrin is not an accurate measure of the amount of amino-acid present. A reasonably accurate method, applicable to the quantities involved, would be of the greatest assistance. The method of Martin & Mittelmann (to be published) may fulfil these requirements.

Because the R_F values of peptides are more sensitive than those of the amino-acids to changes of pH and to other unknown factors, it is desirable, where possible, to run mixed chromatograms with authentic samples to confirm the results of the analysis described above. For purposes of comparison the R_F values of some synthetic peptides are given in Table 1. In the absence of such confirmation, there is a real danger that with substances containing peptides as part of the molecule only, the other components will be overlooked, since the colour reaction with ninhydrin is often the only guide. Partridge (1946) has, however, been able to demonstrate the presence of reducing sugars by the use of a silver reagent and perhaps later other

Table 1. *R_F values of some peptides* in various solvents on Whatman no. 4 paper*

Solvent addition	Phenol 0·1% cupron	Phenol coal-gas 0·3% NH_3†	*s*-Collidine	*n*-Butanol 0·1% cupron	Benzyl alcohol 0·1% cupron	*m*-Cresol 0·1% cupron	*m*-Cresol 0·1% cupron 3% NH_3†
Glycylglycine	0·53	0·57	0·28	0·01	0·00	0·10	0·18
Glycyl-*dl*-alanine	0·64	0·63	0·32	0·03	0·01	0·20	0·29
Glycyl-*dl*-valine	0·78	0·74	0·47	0·09	0·02	0·37	0·51
Glycyl-*dl*-leucine	0·87	0·79	0·53	0·24	0·05	0·59	0·66
Glycyl-*l*-proline	0·77	0·69	0·34	0·05	0·03	0·68	0·69
Glycyl-*l*-hydroxyproline	0·57	0·59	0·28	0·02	0·01	0·49	0·25
Glycyl-*l*-phenylalanine	0·78	0·70	0·79	0·18	0·08	0·65	0·72
Glycyl-*l*-tryptophan	—	0·66	0·85	0·29	—	0·44	0·63
Glycylglycylglycine	0·58	0·59	0·32	0·01	0·02	0·08	0·19
Glycylglycyl-*l*-leucylglycine	0·86	0·75	0·71	0·11	0·04	0·67	0·67
dl-Alanylglycine	0·68	0·65	0·32	0·03	0·01	0·21	0·29
l-Alanylglycylglycine	0·67	0·63	0·46	0·01	0·00	0·19	0·29
dl-Valylglycine	0·83	0·73	0·53	0·11	0·04	0·48	0·48
dl-Leucylglycine	0·86	0·78	0·61	0·23	0·08	0·63	0·62
dl-Leucyl-*dl*-leucine	0·95	0·84	0·89	0·65	0·30	0·87	0·85
l-Leucyl-*l*-tryptophan	0·92	0·83	0·95	0·60	0·34	0·87	0·83
l-Leucylglycylglycine	0·88	0·75	0·55	0·11	0·04	0·61	0·60
l-Prolylglycine	0·86	0·68	0·49	0·03	0·03	0·67	0·55
l-Tyrosylglycine	0·59	0·59	0·95	0·09	0·03	0·27	0·33

Peptides in which glycine carries a free amino group, on heating with ninhydrin give, first, a yellow colour, then, grey and finally purple. All the other peptides in the table give a purple colour, except prolylglycine, which is yellow at first, then orange and finally, grey. Diketopiperazines do not give colours with ninhydrin.

* For the R_F values of peptides related to Gramicidin S, see Consden, Gordon, Martin & Synge (1947).

† The % figure refers to the strength of the NH_3 solution present in the tray at the bottom of the chamber.

reagents will allow a large variety of substances to be detected. In many cases, however, evidence of the existence of a non-amino-acid part of the molecule is afforded by an apparently inexplicable ionophoretic and chromatographic behaviour.

EXPERIMENTAL

Removal of silica. The silica jelly, cut from the ionophoresis trough, is reduced to a smooth paste by stirring in a beaker. Water (2 vol.) is added and the suspension is allowed to stand, with occasional stirring, for 2 hr. It is filtered on a Buchner funnel and washed with a further 2 vol. of water.

Removal of acetate ions and acetic acid. After removal of the silica, the filtrate and washings are acidified with 2N-H_2SO_4 until red to thymol blue (pH 2). Acetic acid is then removed by steam distillation under reduced pressure. The apparatus consists of a train of three round-bottomed flasks. The first flask of capacity 1 l. and fitted with a capillary, is immersed in a water bath at 50–60°. The steam is conducted to the bottom of the next flask (500 ml.) which contains the sample (50–100 ml.) and has a Claisen top and a suitable trap to diminish entrainment. The third flask (1 l.) acts as a condenser and is connected to a water pump.

The temperature of the Claisen flask is not allowed to rise above 40° and distillation is carried on until the volume is reduced to 30 ml. When about 500 ml. has been distilled, the condensate is washed out and a further 100 ml. of condensate is titrated against N-NaOH. If less than two drops are required, the removal of acetic acid is considered adequate. The sample is now ready to be transferred to the desalting apparatus, the sample and washings of the Claisen flask together forming a convenient volume.

Desalting apparatus

The apparatus (Fig. 1) consists of a Perspex tray, through which mercury is circulated by a water lift pump. A baffle is placed at the top of the exit tube

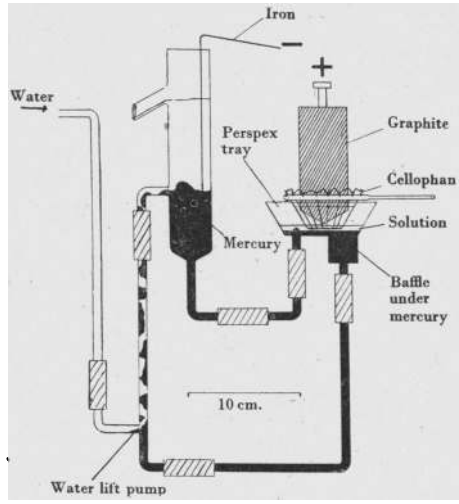

Fig. 1. Diagram of desalting apparatus.

of the tray to prevent the formation of whirlpools in the mercury. An iron cathode dips into the mercury in the pump. The solution to be desalted floats on the mercury. Into this dips a thick cellophan membrane containing 0·1N-H_2SO_4 in which is

immersed a graphite anode. The membrane permits the passage of anions of strong electrolytes to the anode, hydrogen ions passing in the reverse direction. By reason of this high H^+ concentration within the membrane, anions of weak acids lose their charge. Further, since the electro-endosmotic flow is towards the cathode, the diffusion of uncharged molecules into the anode compartment is practically negligible. The amino-acids, even the dicarboxylic amino-acids, acquire a net positive charge within the membrane and are thus less likely than uncharged molecules to reach the anode compartment.

The mercury cathode discharges hydrogen ions and other inorganic cations, hydrogen being liberated and the metals dissolve in the mercury. The alkali metals and earths are washed out of the mercury by the pump water. At high pH sodium, but at low pH hydrogen is preferentially discharged. Thus, if an attempt be made to remove sodium acetate, sodium ions are at first rapidly discharged, but as the amount of free acetic acid increases, the falling pH results in an increased hydrogen evolution, and decreased sodium removal, until at a pH between 3 and 4 practically no more sodium is eliminated. With sodium borate or sodium phenoxide, the sodium (but not the weak acid) can be almost entirely removed. Consider the removal of a sodium sulphate-sulphuric acid mixture. The rate of removal of sulphate ions is governed by the transport number of the sulphate ion in the membrane, and if the pH is low, hydrogen ions will be discharged faster than they come from the anode, causing the pH to rise. Conversely, if the pH is high initially, sodium ions will be removed preferentially and hydrogen ions will arrive from the anode faster than they are discharged, causing a fall of pH. Thus, the pH will attain a value at which the rate of removal of sulphate and sodium ions is identical. As the current continues to pass, the concentration of sodium sulphate drops until, finally, a very dilute solution of sulphuric acid, remains. The concentration of this acid depends on the current density and thickness of the membrane, since, in the absence of diffusion and electro-endosmosis, the concentration would drop to zero. Thus to obtain a very dilute solution, it is necessary to have a high potential between the electrodes, a thick membrane and a small area of contact between the membrane and the liquid.

As the concentration of the solution falls, the resistance of the apparatus rises and the current, falling to a steady small value, can be used as an indication of the end-point. Another indication of the progress of the removal of alkali ions may be obtained by testing the water issuing from the pump with indicators. As explained above, if a weak acid is present, all the salt cannot be removed, and the current becomes steady at a higher value. Ammonia can also be removed in this apparatus, but owing to the decomposition of the ammonium amalgam, the mercury becomes coated with soft lumps which have to be broken up and the current may have to be rather low in order to avoid choking up of the mercury-circulation system. When halides are removed, it is desirable to employ two concentric anode membranes, each containing initially sulphuric acid. In this way the amount of halogen released at the anode is greatly reduced and the amount of free halogen diffusing back to the solution being desalted is negligible.

At the beginning of the desalting, the voltage may be as low as 20 V. but is raised at the end to 300 V., unless weak acids are present. The current at first may rise to 2 amp. and fall at the finish to 50 ma. The circulating mercury effectively cools the solution to be desalted, and a maximum of about 100 W. can be dissipated without excessive rise of temperature. The current efficiency (ratio of equivalents removed to faradays passed) for the removal of salts depends on the nature of the salt to be removed. Thus, with NaOH, where removal of sodium is the limiting factor, the efficiency is about 80–85 %. With Na_2SO_4, where removal of $SO_4^=$ is the limiting factor, the efficiency is 10–15 %; in other words, the efficiency is approximately equal to the transport number of $SO_4^=$ in the membrane, the other ion being the hydrogen ion.

Recovery of glycine and glutamic acid from the desalting apparatus. To test whether amino-acids are lost either by passage into the anode solution, or by being carried away in droplet form by the mercury, known amounts of glycine and glutamic acid were desalted and then estimated by the copper titration method of Pope & Stevens (1939).

(1) 103 mg. glutamic acid dissolved in 50 ml. of 0·08 N-NaOH were desalted (15 min. minimum current 0·4 amp.; starting voltage 50 V., final voltage 150 V.). The solution was pipetted off; the tray was washed out three times and the solution and washings were made up to volume. Titration showed 101 mg. glutamic acid (i.e. 98 % recovery). (2) 101 mg. glycine dissolved in 25 ml. 0·149 N-NaOH were desalted, washed out, made up to volume and titrated. Recovery 101·3 mg. (100 %). (3) 101 mg. glycine dissolved in 25 ml. 0·08 M-Na_2SO_4 were desalted, washed out, made up to volume and titrated. Recovery 98·2 mg. (97 %). Gas and liquid were visible in the tubes circulating the mercury. This liquid must be picked up as small drops of water in the pump since it did not become coloured when a strong solution of fuchsine was floated on the mercury.

One-dimensional chromatographic analysis

The solution, after desalting, is transferred to a 1 l. Claisen flask together with three 10 ml. washings, by means of a pipette. After evaporation *in vacuo*, below 40°, the sample is transferred to a 25 ml. conical flask, again with a pipette and evaporated to dryness. A known small volume of water is now added and a few μl. of the solution run on a paper chromatogram. After development and colouring with ninhydrin, the complexity of the mixture

can be gauged and a decision taken as to whether a one-dimensional or a two-dimensional chromatogram is needed. An estimate of the size of the measured sample required for further work can also be made.

If it has been decided that chromatography in one dimension will give sufficient separation, a strip of paper is prepared wide enough to have a number of spots (2–8) close together (6 mm. apart) and one spot separated from the others by 2 cm. or more. The number of spots taken should be enough to provide at least 10 μg. of each peptide for each solvent in which the amino-acids are to be run after hydrolysis. Several spots, rather than a single spot made with a larger amount of material, are used in order to avoid local overloading and each spot should contain the same amount of material, so that conditions should be as similar as possible.

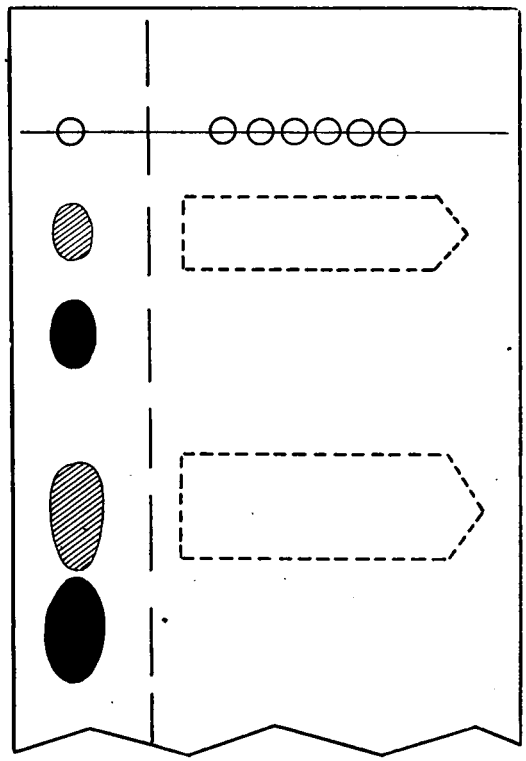

Fig. 2. Diagram showing method of cutting of a one-dimensional chromatogram. The test solution is applied along the top line (open circles). After development of the chromatogram, the left side of the sheet is cut off (broken line) and treated with ninhydrin to show amino-acid spots (black) and peptide spots (shaded). Using this as a guide, the remainder of the sheet is cut as shown by the dotted lines.

After the strip has been developed and dried, the chromatogram formed from the single spot, is cut off and treated with ninhydrin. This is then used as a guide for cutting up the rest of the strip. The 'cuts' are then trimmed at one end to a blunt point (Fig. 2). A trough similar to those used for the chromatograms is now provided with a strip of clean filter-paper running the full length of the trough and wide enough just to overlap the side bar. The trough is filled with water. To wash one of the 'cuts', the end opposite the point is held against the wet paper until about half an inch of the end of the 'cut' is wet. One-quarter inch of the wet end is then allowed to overlap the edge of the wet strip. After a few seconds a film of water between the papers anchors the 'cut' firmly in position. The water creeps slowly down the paper carrying the amino-acids, peptides, etc. with it. A thick walled capillary tube of 1 mm. bore and about 12 cm. long, with its ends drawn out is now adjusted so that one end touches the point of the cut, while the other end is slightly lower (Fig. 3). In the course of about 1 hr. the tube becomes filled, and if the cut had an area of not more than 10 sq.cm., washing is usually complete, as shown by testing the dried

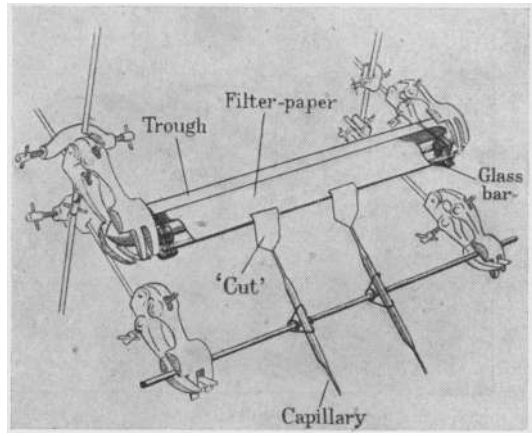

Fig. 3. Diagram of apparatus for washing material off 'cuts' from chromatograms (see Figs. 2 and 5*b*) into capillary tubes.

paper with ninhydrin. It is very important that the organic solvent should be thoroughly removed by drying from the chromatogram before the washing. If not, the water may run down irregularly and washing will be incomplete with this volume. An alternative technique of determining where to cut, by treatment of the strip with very dilute ninhydrin, described below for two-dimensional chromatograms, may be preferable when the spots are very close together. Variation in position of the spots caused by irregularities in the paper can then be followed.

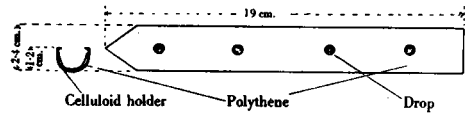

Fig. 4. Plan of polythene strip and cross-section of celluloid holder containing strip.

By attaching and squeezing a piece of cycle valve rubber the solution is forced out of the capillary on to a strip of polythene supported in a celluloid holder of semi-circular cross-section (Fig. 4). Polythene has been employed because its relatively unwettable surface prevents undue spreading of the drops and its flexibility makes for convenient transfer of the drops on to paper by 'printing'. After drying *in*

vacuo, the solid is dissolved in 20 μl. 6N-HCl by rubbing with a fine glass rod. The drop is then sucked, by means of the rubber tube, into another clean capillary whose ends are then sealed in the flame. Hydrolysis is effected by keeping the tube overnight at 105°. The hydrolysate is transferred back on to the polythene, dried *in vacuo* over KOH and taken up in 5 μl. of water. This drop, or a suitably spaced row of drops, if several analyses are being run in parallel, is now ready for transfer to the strip of filter-paper on which the final analysis is to be carried out. This is done by removing the polythene from its holder, inverting it and pressing it down on to the strip of paper ('printing') which lies on a clean sheet of glass.

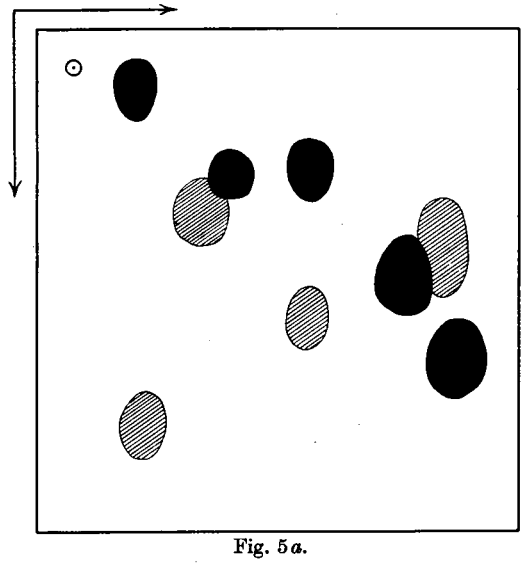

Fig. 5*a*.

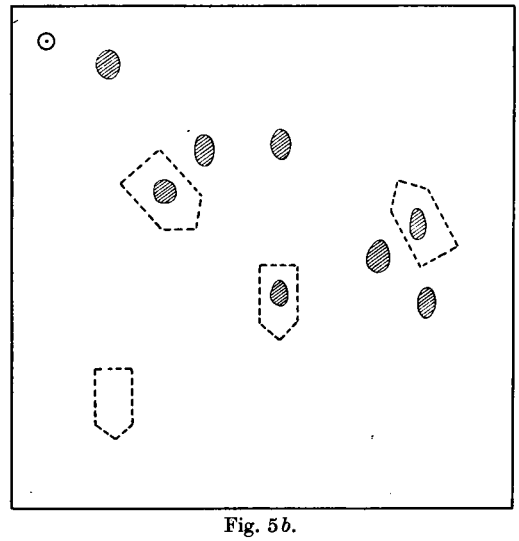

Fig. 5*b*.

Fig. 5. Diagram showing method of cutting of a two-dimensional chromatogram. (*a*) and (*b*) represent duplicate sheets, the solution to be analyzed being applied at the open circles. The arrows indicate the direction of development of the chromatograms in the two solvents. In the guide chromatogram (*a*), amino-acids are shown as black spots and peptides as shaded spots. After treatment with dilute ninhydrin, the duplicate sheet (*b*) shows faint spots (some may be barely visible) and is cut as shown by the dotted lines.

Where necessary, this final analysis can be done in two or more different solvents. Where this is intended 10 μl. or more of water are added to the dry hydrolysate and the solution is distributed between two or more filter-paper strips by means of a micropipette.

Two-dimensional chromatography

Duplicate two-dimensional chromatograms are developed at the same time, in the same chamber, from equal amounts of material. After thorough drying, one sheet is coloured with ninhydrin (0·1% in *n*-butanol). Only the stronger spots thus revealed would have contained enough material for successful analysis. The amount of material obtainable from one sheet is usually not enough for deamination as well as hydrolysis. The second sheet is sprayed with aqueous *n*-butanol containing not more than 0·01% of ninhydrin, dried and heated for 3 min. at 100°. The positions of the stronger amino-acid and peptide spots should be just visible. The areas required for analysis are cut out (Fig. 5*a* and *b*) and washed off as above. The small proportion of each peptide that has reacted with the ninhydrin does not interfere with the analysis. Previously, the positions of the stronger amino-acid spots were found by cutting pieces from one chromatogram using the coloured one as a guide and testing these with ninhydrin. Once the positions of the amino-acid spots had been found, the positions of the peptide spots could be inferred with fair accuracy. This technique has, however, been abandoned, firstly, because it consumed too much time and secondly, because the greater accuracy of the newer method permits smaller cuts to be made, a matter of particular importance where spots are rather near together.

Deamination and hydrolysis

The deamination of peptides is carried out on the polythene strip mentioned above, in 6N-HCl for 10 min. at 30–35°. After drying, each sample is dissolved in about 30 μl. of 6N-HCl (more, of course, if samples for simple hydrolysis are to be taken). The polythene strip, in its celluloid holder, with a number of samples to be deaminated, is pushed into a tube, warmed by a water jacket, through which are led the fumes produced by dropping concentrated HCl solution on solid $NaNO_2$. After 10 min., during which the oxides of nitrogen and NOCl dissolve in the drop (which turns brown at first and evolves gas whether amino-nitrogen is present or not), the strip is removed and dried *in vacuo* over KOH. After drying, more HCl is added and dried again to complete the removal of oxides of nitrogen. Finally, it is taken up in HCl and hydrolyzed and analyzed as described above.

These conditions give complete destruction of the amino-acid possessing the free amino group of peptides, but to ensure complete destruction of certain of the more

resistant free amino-acids, deamination must be continued for 30 min. Unfortunately, with peptides, some destruction of the amino-acids possessing a free amino group, occurs, perhaps as a result of premature hydrolysis and 30 min. treatment should only be employed where free amino-acids are known to be present in the fraction. It is always advisable to take at least twice as much material as for a simple hydrolysis.

Where several investigations, e.g. analyses in different solvents both of simple hydrolysates and hydrolysates after deamination, are to be run on a given fraction, it is preferable to use samples from the pooled fractions of several chromatograms rather than to perform each analysis on a fraction from a separate chromatogram. This avoids the danger that errors of cutting will give fictitious results in certain cases.

Effect of nitrous fumes and hydrolysis on some amino-acids and peptides. Though a fair proportion of pure tryptophan appears to survive hydrolysis, tryptophan which has been washed from a paper strip, no doubt because of the presence of degraded cellulose, is totally destroyed during hydrolysis. Even when the amino group is protected, as in a peptide, tyrosine and tryptophan are destroyed under the conditions of deamination described above. Synthetic tyrosylglycine gave the expected glycine spot, but glycyltyrosine showed a number of yellow and grey spots only. Leucyltryptophan and glycyltryptophan gave after deamination and hydrolysis only a very weak, slow-moving, brownish yellow spot.

When proline and proline peptides containing a free imino group, such as prolylvaline (Synge, to be published), are treated with nitrous fumes and then hydrolyzed, the yellow proline spot appears on the chromatogram of the products. Thus, prolylvaline gave proline and valine. This is presumably due to the formation of the *N*-nitroso derivative of proline during treatment with nitrous fumes, subsequent hydrolysis regenerating the imino group. Arginine and ornithine and peptides with the α-ornithine amino group either free or protected, e.g. ornithylleucine and α-valylornithine (Synge, to be published), gave after deamination and hydrolysis, faint proline spots and a number of faint unidentified spots on the chromatogram, in addition to the amino-acid spots expected from the peptides. The small amounts of proline were presumably formed by ring closure of the ornithine during treatment with nitrous fumes and subsequent hydrolysis. Free lysine gave two medium, unidentified purple spots.

Miscellaneous technical points

The fractions washed off the pieces of paper, as described above, usually contain after hydrolysis only two or three different amino-acids. Thus, identification can almost always be secured by using paper strips in two different solvents without recourse to the more elaborate two-dimensional technique. In the absence of aspartic and glutamic acids, *s*-collidine is a very valuable solvent. If it is run in an alkaline atmosphere, produced by a solution of 0·1% diethylamine dissolved in the water (2 l.) at the bottom of the box, the R_F of phenylalanine is increased so that on strips it almost coincides with tyrosine. On a collidine-diethylamine/phenol-gas-NH_3 two-dimensional chromatogram, phenylalanine does not overlap with any of the other amino-acids. 3% NH_3 is equally effective, but owing to its volatility this concentration is inconvenient to maintain and offensive in a box which has to be occasionally opened. Owing to its low volatility, a single addition of diethylamine will remain effective for several weeks. In the presence of diethylamine, chromatograms developed in *s*-collidine show 'fronts', particularly with the faster moving components. These fronts can be eliminated by a trace of HCN in the atmosphere. It has been found that the effect of one addition of HCN lasts for several weeks.

Small amounts of free acids when run in collidine-diethylamine pick up diethylamine and on treatment with ninhydrin appear as faint purple spots. H_2SO_4, $R_F = 0{\cdot}45$; HCl, $R_F = 0{\cdot}69$; acetic acid, $R_F = 0{\cdot}60$. NaOH has been found to give a yellow spot $R_F = 0{\cdot}24$ with or without diethylamine.

It has been found necessary to use benzyl alcohol only in glass boxes. When a rusty iron container was employed, serious elongation of the spots, which prevented full resolution, often occurred. The reason for this remains unexplained.

Where the amount of amino-acids available is too small for running on more than one strip, the separation of aspartic and glutamic acids in phenol can be achieved, after the separation of the fast running amino-acids in benzyl alcohol, by cutting the strip below the alanine position and developing backwards with the phenol. To do this, the spot to be analyzed is applied one third of the way down the strip. This technique is useful only in the absence of glycine, serine, threonine, alanine and cystine.

The use of Whatman no. 4 filter-paper, while in no way essential, markedly speeds up the development of the chromatograms (Consden *et al.* 1946*a*, *b*). Water-saturated *n*-butanol has been used for the ninhydrin solution because it gives rather stronger colours on the chromatograms. This may be because only in the presence of water can sufficient diffusion occur for all the amino-acids inside the cellulose fibres to react with the ninhydrin. The *s*-collidine should be pure and should remain colourless. Occasionally, a sample has been received from the manufacturers which is yellow and which deepens in colour on standing. Careful fractionation of such material fails to prevent subsequent colour development. To obtain satisfactory material, collidine (1 l.) is shaken with bromine (1–2 ml.), added in small quantities and allowed to stand overnight. It is then shaken with a few ml. of 40% (w/v) NaOH to remove HBr and distilled *in vacuo* over solid $Na_2S_2O_3$. The material so obtained remains colourless indefinitely.

SUMMARY

1. A method is presented for the identification of lower peptides present in protein partial hydrolysates, employing ionophoresis followed by partition chromatography on paper.

2. Peptide spots, obtained after chromatography on paper, are identified, after washing off the paper, by (*a*) hydrolysis, (*b*) deamination and hydrolysis. The amino-acids so formed are identified by chromatography on paper.

3. An apparatus, for the removal of salts from solutions of amino-acids and peptides, is described.

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Gramicidin S: the Sequence of the Amino-acid Residues

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We report here a study of the products of partial hydrolysis of gramicidin S by mineral acid acting at low temperature. Identification of these products has permitted an unequivocal formulation of the sequence of the amino-acid residues in this compound. A preliminary report of this work has been published (Consden, Gordon, Martin & Synge, 1946).

Gramicidin S seemed a particularly favourable object for study, since it had been shown to consist of the residues of the five amino-acids, *l*-valine (*V*), *l*-ornithine (*O*), *l*-proline (*P*), *l*-leucine (*L*) and *d*-phenylalanine (Φ) condensed together in equimolecular proportions; the presence of one free amino group/stoichiometric minimum unit, and the absence of free carboxyl groups, as well as the high optical rotation, were taken to indicate a closed peptide-chain (*cyclo*peptide) structure (Synge, 1945*b*). Subsequently Sanger (1946) established that the free amino group is the δ-amino group of the ornithine residue and the peptide chain therefore consists of amino-acid residues in α-linkage to one another. The crystallographic studies of Crowfoot & Schmidt (to be published) indicate that the molecule comprises either five or ten amino-acid residues; if the latter, the molecule also must possess a twofold axis of symmetry. This could occur only by twofold repetition of a particular sequence of the five different residues. It would be difficult, from studies of the later products of hydrolysis by acid, to distinguish between a given *cyclo*pentapeptide and the corresponding *cyclo*decapeptide structure, and we have not attempted to do so. The hydrolysis products which we have identified could all arise by the breakdown, without rearrangement, of the sequence of residues of the structure:

-α-(*l*-valyl)-*l*-ornithyl-*l*-leucyl-*d*-phenylalanyl-*l*-prolyl-
(-*VOL*Φ*P*-)

occurring either once or more often in a closed peptide chain. The fact that a high proportion of the possible hydrolysis products have been identified (some of them by more than one technique) gives a firm basis for assigning this structure to gramicidin S. That no products have been noted that are inconsistent with this structure, fits with the simple picture of the structure of gramicidin S which the crystallographic data suggest. A decision between the *cyclo*pentapeptide and *cyclo*decapeptide structures could perhaps be obtained by a study of the physical properties of gramicidin S in the dissolved state.

Knowledge of the structure of gramicidin S is likely to prove helpful for the more difficult problem of the nature of tyrocidine, which embodies the same peculiar amino-acid residues (Gordon, Martin & Synge, 1943*c*), and in which the δ-amino group of the ornithine is likewise free (Christensen, 1945). The present demonstration of the occurrence in nature of a *cyclo*peptide raises the question whether such structures may be embodied in proteins as sub-units (Chibnall, 1942, 1946). Such sub-units would have no characteristic terminal groups available for recognition, and would thus escape detection by methods employing this approach (Fox, 1945; Sanger, 1945).

The unequivocal results of the present study give increased confidence in the use of partial hydrolysis products (at least those formed by acid acting at low temperature) for the study of protein structure. The sort of control that is needed for such work was discussed by some of us six years ago (Gordon, Martin & Synge, 1941; cf. Synge, 1943); work with glutathione (reviewed by Fox, 1945) and gramicidin (Synge, 1944) and the results presented here provide further evidence for the assumption that no rearrangement of linkages occurs during acid hydrolysis. The rather