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Method for Determination of the Amino Acid Sequence in Peptides

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The problem of determining the amino acid sequence in peptides has attracted the interest of several investigators in the past because of its bearing on protein structure. The subject has been reviewed by Fox [1]. Since then new approaches, employing partial hydrolysis, have been made by Consden, Gordon, Martin and Synge [2, 3] and by Sanger [4].*

The applicabilities of the available methods are, however, for practical or theoretical reasons restricted and there is a need for alternative methods.

In 1927 Bergmannn, Kann and Miekeley [5] described the following reaction:

$$C_6H_5 \cdot NH \cdot CO \cdot NH \cdot CHR' \cdot CO \cdot NH \cdot CHR'' \cdot COOH \rightarrow$$

(I)

$$C_6H_5 \cdot N\text{——}CO + H_2N \cdot CHR'' \cdot COOH$$

$$\begin{array}{l} | \quad | \\ OC \quad NH \\ \diagdown \; \diagup \\ C \\ \cdot \\ HR' \end{array}$$

(II)

(R', R'' = amino acid side chains)

Refluxing with hydrochloric acid split off the phenyl hydantoin (II) from the phenylcarbamyl peptide (I). The fact that the resistance to acid cleavage was less than that expected for a peptide bond, indicating a labilizing effect of the phenylcarbamyl group, apparently escaped the authors' notice. Later

* Recently Levy [14] has described a new procedure for the removal of terminal groups from peptides as 2-thio-5-thiazolidones.

Abderhalden and Brockmann [6] took advantage of this effect in developing a method for the stepwise degradation of polypeptides. Concomittantly, however, to the desired cleavage other peptide bonds in the chain were also attacked to some extent. This circumstance obviously detracts from the usefulness of the procedure.

The present attempt [7] to employ the above reaction for the determination of peptide structure was guided by several considerations. Firstly, it was postulated that the ease of the reaction parallels the ease of ring closure to hydantoin and from that point of view the phenyl*thio*carbamyl derivatives should be preferable.

Secondly, the cleavage should take place exclusively at the peptide bond adjacent to the carbamyl group, other bonds remaining untouched. Since the hydantoin formation does not require the presence of water, whereas of course peptide linkages are split by hydrolysis or analogous reaction, it was decided to carry out the reaction in an anhydrous, inert solvent. It was found that nitromethane was ideal in this respect. Nitromethane saturated with hydrogen chloride brought about an almost instantaneous and quantitative cleavage of phenylthiocarbamyl (PTC) dipeptides into phenyl thiohydantoin and amino acid at room temperature. It could also be shown for a PTC-tripeptide that the cleavage took place exclusively at the peptide bond adjacent to the PTC-substituent.

Furthermore, since the naturally occurring peptides are usually obtained in small amounts a micromethod was desirable. This requirement almost precluded the identification of the hydantoin by melting point, elementary analyses *etc.* The difficulty was solved by hydrolysis of the hydantoin to the corresponding amino acid and identification of the latter by the paper strip method [8].

The first part of the experimental section describes the formation and mode of cleavage of some PTC-peptides and the second part deals with the application of these reactions in a procedure for stepwise degradation of polypeptides on a micro scale.

EXPERIMENTAL

The peptides used in this work were all synthetic. Leucyltriglycine and alanylleucylglycine were provided as generous gifts by Professor K. Linderstrøm-Lang and Professor G. Ågren, and the dipeptides were the commercial preparations of Hoffmann-La Roche, Basel.

I. Preparation and cleavage of phenylthiocarbamyl (PTC) peptides

Nitrogen determinations were made by the micro-Kjeldahl procedure. Melting points were determined on a heating block (Fisher-Johns) and are uncorrected. Prior to analysis the preparations were dried for 48 hours *in vacuo* (1 mm Hg) over P_2O_5 and pellets of KOH.

Preparation and cleavage of PTC-alanylglycine. DL-alanylglycine (0.29 g) was dissolved in 10 ml pyridine-water (1 : 1). The solution was warmed to 40° and *N* NaOH was added to about pH 9 (glass electrode) which pH was maintained throughout the reaction by the addition in small portions of *N* NaOH. 0.48 ml phenyl isothiocyanate was added with vigorous stirring. The reaction was complete within half an hour. The total amount of *N* NaOH added was 2.4 ml. Pyridine and excess phenyl isothiocyanate were removed by repeated extractions with equal volumes of benzene. On the addition of *N* HCl to pH 3 *phenylthiocarbamyl-alanylglycine* separated as an oil which solidified on standing over night in the icebox. Yield 0.44 g. The material was then recrystallized from ethanol-water. M. p. 155°.

Found: N, 15.00. $C_{12}H_{15}O_3N_3S$ requires N, 14.96.

PTC-alanylglycine (0.28 g) was dissolved in 20 ml anhydrous nitromethane saturated with hydrogen chloride (for preparation see p. 290). Glycine hydrochloride started to crystallize immediately and was filtered off after 15 minutes. Yield 89 %. The material was then recrystallized from ethanol-water.

Found: N, 18.93. Calc. 18.66.

Paper chromatography using pyridine-amyl alcohol [9] showed identity with glycine. The nitromethane solution was evaporated to dryness at reduced pressure. The residue was taken up in a small volume of hot glacial acetic acid and a minor, insoluble residue filtered off. On addition of water to the glacial acetic acid solution and cooling the preparation crystallized. The material was recrystallized from absolute ethanol. M. p. 184—85°. Mixed m. p. with an authentic sample of 5-methyl-3-phenyl-2-thiohydantoin [10] 184—85°.

Preparation and cleavage of PTC-leucylglycine. DL-leucylglycine (0.38 g) was treated as described for DL-alanylglycine. The yield of *phenylthiocarbamyl-leucylglycine* was 0.52 g. The material was then recrystallized from ethanol-water. M. p. 147—48°.

Found: N, 13.07. $C_{15}H_{21}O_3N_3S$ requires N, 13.01.

PTC-leucylglycine (0.34 g) was dissolved in 20 ml nitromethane-HCl. Glycine hydrochloride immediately started to separate and was filtered off after 15 minutes. Yield 93 %. It was then recrystallized from ethanol-water.

Found: N, 18.87. Calc. 18.66.

Paper chromatography using pyridine-amyl alcohol demonstrated identity with glycine. The nitromethane solution was evaporated *in vacuo* to dryness and the residue extracted with a small volume of hot glacial acetic acid. On addition of water and cooling in the ice box crystals appeared, which were recrystallized from ethanol-water. M. p. 177—78°. The mixed m. p. with an authentic sample of 5-isobutyl-3-phenyl-2-thiohydantoin [10] was 178°.

Preparation and cleavage of PTC-leucyltyrosine. L-leucyl-L-tyrosine (0.60 g) was treated as described for DL-alanylglycine. The yield of *phenylthiocarbamyl-leucyltyrosine* was 0.72 g. It was then recrystallized from ethanol-water. M. p. 225—30° with decomposition.

Found: N, 9.60. $C_{22}H_{27}O_4N_3S$ requires N, 9.78.

PTC-leucyltyrosine (0.43 g) was dissolved in 20 ml nitromethane-HCl. Crystallization of tyrosine hydrochloride started immediately. The crystals were filtered off after 15 minutes. Yield 98 %. Recrystallization was carried out by dissolving the crystals in the smallest possible volume of water and neutralizing with sodium hydroxide.

Found: N, 7.68. Calc. 7.73.

Paper chromatography using pyridine-amyl alcohol showed identity with tyrosine. The nitromethane solution was evaporated *in vacuo* to dryness and the residue extracted with a small volume of hot glacial acetic acid. Crystallization occurred on addition of water and cooling in the ice box. The material was then recrystallized from ethanol-water. M. p. 176—77°. The mixed m. p. with an authentic sample of 5-isobutyl-3-phenyl-2-thiohydantoin [10] was 176—77°.

Preparation and cleavage of PTC-glycyltryptophan. Glycyl-L-tryptophan (0.52 g) was treated as described for DL-alanylglycine. The yield of *phenylthiocarbamyl-glycyltryptophan* was 0.71 g. Attempts to crystallize from organic solvent yielded only oils. The preparation was then dissolved in the minimum amount of 0.5 *N* NaOH from which it crystallized on the gradual addition of *N* HCl. M. p. 120—23°.

Found: N, 13.75. $C_{20}H_{20}O_3N_4S$ requires N, 14.14.

PTC-glycyltryptophan (0.40 g) was dissolved in 20 ml nitromethane-HCl. Tryptophan hydrochloride began to crystallize within a few seconds. After 15 minutes the crystals were filtered off. Yield 98 %. It was then recrystallized from ethanol-water.

Found: N, 13.83. Calc. 13.71.

Paper chromatography using pyridine-amyl alcohol showed identity with tryptophan.

The nitromethane solution was evaporated to dryness *in vacuo*. The dark brown residue was taken up in a small volume of hot ethanol and an insoluble residue filtered off. Addition of water to the alcoholic solution brought about precipitation. The material was recrystallized from ethanol. A brownish impurity was, however, very difficult to separate from the preparation. The crystals were then sublimed and resublimed at 150° in a vacuum of 0.05 mm Hg which resulted in a slightly yellowish product. M. p. 240—46° with decomposition. An authentic sample of 3-phenyl-2-thiohydantoin melted at 245—48° with decomposition [10].

Found: N, 14.40. Calc. for $C_9H_8ON_2S$: N, 14.58.

Preparation and cleavage of PTC-alanylleucylglycine. DL-alanyl-DL-leucylglycine (0.13 g) was treated as described for DL-alanylglycine except that only half the amounts of reagents were used. The yield of *phenylthiocarbamylalanylleucylglycine* was 0.16 g. The preparation resisted all attempts at crystallization. It was purified through repeated precipitations with water from its solution in glacial acetic acid.

Found: N, 13.96. $C_{18}H_{26}O_4N_4S$ requires N, 14.22.

PTC-alanylleucylglycine (40 mg) was suspended under anhydrous conditions in 2 ml nitromethane-HCl at 40° by means of a magnetic stirring device (p. 290). After 15 minutes nitromethane and hydrogen chloride were removed by careful evaporation *in vacuo* at room temperature. To the dry residue were added with stirring 1 ml of water and then dilute NaOH to pH 9. The solution was again evaporated to near dryness *in vacuo* at room temperature. The residue was extracted with glacial acetic acid and of this solution aliquots were taken for determination of total nitrogen and amino nitrogen according to Van Slyke with the modifications introduced by Kendrick and Hanke [11].

Found: N, 5.50 mg.; amino-N, 1.35 mg; amino-N/total N, 0.246. The calculated quotient for the cleavage of one peptide bond is 0.25 making the actual cleavage 98 % of the calculated.

PTC-alanylleucylglycine (40 mg) was treated with 2 ml nitromethane-HCl as described in the preceding section. After the removal of nitromethane and hydrogen chloride the residue was thoroughly extracted with 1 ml of water. Paper chromatography using pyridine-amyl alcohol was performed on the aqueous solution. 0.02 ml of the solution was applied to the paper alongside authentic samples of leucylglycine, leucine and glycine. From the test sample was obtained only one spot with the same R_F-value as that of leucylglycine ($R_F = 0.43$) and no trace of leucine ($R_F = 0.58$) or glycine ($R_F = 0.19$). Had the treatment of the PTC-peptide with nitromethane-HCl caused a cleavage to 1 % of the peptide bond between leucine and glycine, there should have been present 2.5 γ of leucine and 1.5 γ of glycine in the applied test sample.

Actual experiments showed that it was easily possible to demonstrate the presence of these amounts on a paper chromatogram. Consequently an eventual cleavage of the bond between leucine and glycine must have been less than 1 %.

II. Procedure for the stepwise degradation of polypeptides

Preparation of the PTC-peptide

The method was worked out for amounts of peptides corresponding to 1/10 millimole. A mixture of equal volumes of pyridine and water was found to be a good solvent for both peptides and phenyl isothiocyanate. The rate of the reaction is within limits higher the higher the pH. A pH of 8.6 was found to give a conveniently rapid reaction and this pH was maintained throughout the reaction by the addition of known amounts of alkali.

Since the alkali consumption is proportional to the amount of PTC-peptide formed, the course of the reaction could be conveniently followed and this was done for a number of amino acids and peptides. The rate varied somewhat but was consistently higher for di- and tripeptides than for amino acids (Fig. 1), the probable explanation being that the *a*-amino group reacts in its uncharged form and that at pH 8.6 this group is less dissociated in the peptides than in the amino acids [12].

Method.

The reaction is carried out in a special tube (Fig. 2) which is immersed in a water bath at 40°. One tenth of a millimole of the peptide is dissolved in 2 ml pyridine-water (1 : 1). The solvent should contain 3 mg bromothymol blue per 100 ml. By the addition of alkali the color is adjusted to that corresponding to pH 8.6. For comparison another tube is placed alongside containing solvent and indicator the pH of which is adjusted to 8.6 by means of a glass electrode. To the solution of the peptide is added 70 μl of phenyl isothiocyanate. The reaction mixture is vigorously stirred with a mechanical stirrer in order to keep the solution saturated in respect to phenyl isothiocyanate. The pH is maintained at 8.6 by addition of *N* sodium hydroxide from a microburette.

After completion of the reaction pyridine and excess phenyl isothiocyanate are removed by repeated extractions with equal volumes of benzene in the same tube as that used for the reaction.

The aqueous solution of the sodium salt of the PTC-peptide (1 ml) is then evaporated to complete dryness by a lyophilizing procedure. The solution is

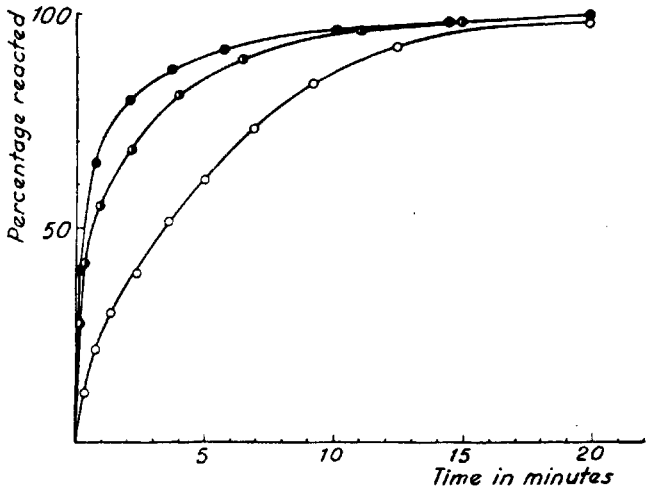

Fig. 1. Course of the reaction between phenyl isothiocyanate and a) leucine (—○—○—), b) leucylglycine (—◑—◑—), and c) leucylglycylglycine (—●—●—) in pyridine-water. pH 8.6. Temperature 40°.

first frozen to about — 15° and then a container with phosphorous pentoxide (Fig. 2) is attached to the tube and the system connected to a good vacuum oil pump. The drying operation generally requires 5—6 hours. On the repeated passage of the peptide through the degradation cycle sodium chloride will gradually accumulate making it difficult to keep the preparation frozen during the lyophilizing procedure. The dry PTC-peptide is then freed from the salt by extraction with glacial acetic acid which leaves the salt as an insoluble residue. The acetic acid is subsequently removed by evaporation *in vacuo*.

Cleavage of the PTC-peptide

The reaction is carried out in anhydrous nitromethane-HCl. The main rate limiting factor appears to be the solubility of the PTC-peptide in nitromethane. For a number of dipeptide derivatives the solubility has been found to be high and the cleavage in then practically instantaneous at room temperature, whereas the tri- and tetrapeptide derivatives were less soluble and as a consequence more time was required for the reaction to reach completeness. In order to increase the speed and to insure complete reaction, the PTC-peptide is finely powdered and suspended in the medium.

The reaction products separate spontaneously owing to the fact that the phenyl thiohydantoins are sufficiently soluble in the medium to stay in solu-

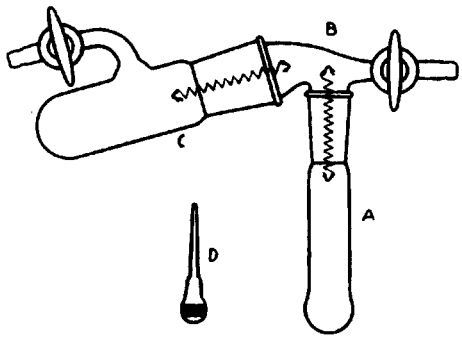

Fig. 2. Assembled apparatus. A, reaction tube; B, midpiece; C, container for drying agent; D, stirrer with sealed in magnet. The apparatus is made of Pyrex glass. (1/4.)

tion, whereas the residual amino acid or peptide (as hydrochloride) is more or less insoluble and therefore precipitates. The phenyl thiohydantoin, however, is always contaminated with a small fraction of the residual peptide. This circumstance has not caused any difficulty in the identification of the hydantoin and the objection is rather the loss of material which eventnally may become considerable after many repetitions of the procedure.

Method.

The anhydrous solution of hydrogen chloride in nitromethane is prepared in the following way. Nitromethane is dried first over calcium sulfate and then over phosphorous pentoxide. It is then distilled at atmospheric pressure and the fraction boiling at 101—02° used. This fraction is saturated with dry hydrogen chloride. The hydrogen chloride is prepared by letting conc. sulfuric acid drip into conc. hydrochloric acid. The gas is passed in a finely divided state through conc. sulfuric acid and then through the nitromethane. The nitromethane rapidly takes up 3—4 % w/w of hydrogen chloride. The solution is stored with protection against atmospheric moisture.

In the tube with the dry PTC-peptide is placed a small magnetic stirrer sealed in glass (Fig. 2) and then 2 ml of nitromethane-HCl is added with protections against atmospheric moisture. The tube is closed, immersed in a water bath at 40° and the contents are thoroughly stirred so that all lumps are broken up and a fine suspension is formed. The time required for the reaction to reach completion depends on the nature of the particular PTC-derivative (p. 289).

At the end of the reaction the insoluble material is filtered off and the filtrate transferred to a thickwalled test tube. This operation is conveniently carried out with the aid of an Emich filter stick to which is sealed a capillary siphon.

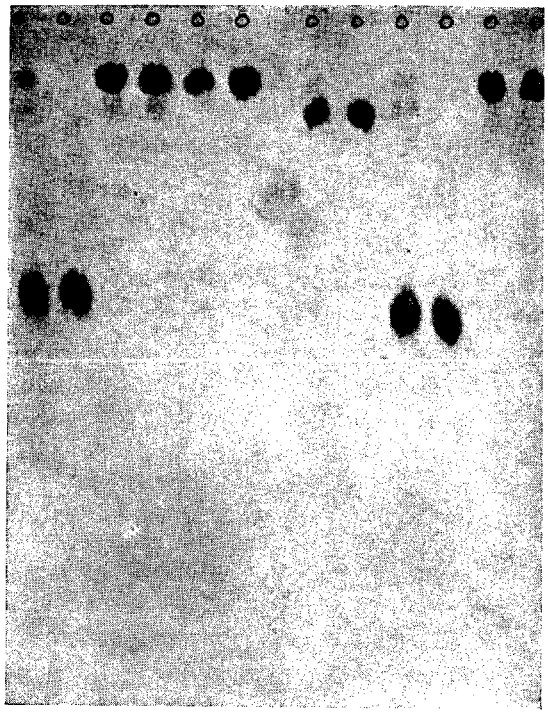

Fig. 3. Paper strip identification of amino acids obtained by stepwise degradation of leucylglycylglycylglycine (left) and alanylleucylglycine (right). Solvent: Pyridine-amyl alcohol. The samples are applied in the order from left to right: 1st amino acid, leucine, 2nd amino acid, 3rd amino acid, 4th amino acid, glycine and 1st amino acid, alanine, 2nd amino acid, leucine, 3rd amino acid, glycine.

The insoluble residue left in the reaction tube is washed repeatedly with small volumes of dry ether in order to remove excess hydrogen chloride. The sample is then ready for renewed treatment with phenyl isothiocyanate.

The filtrate is evaporated to dryness on the steam bath in a stream of nitrogen.

Identification of the removed amino acid

The phenyl thiohydantoin is subjected to alkaline hydrolysis and the resulting amino acid identified by means of the paper strip method [8,9]. Phenyl thiohydandoin derivatives of a large variety of amino acids [10] were hydrolysed in this way, and it was found that with few exceptions the original amino acid

could be recovered *. The exceptions were arginine and asparagine. The arginine derivative gave rise to two ninhydrin positive compounds of which one was ornithine [13]. The other was not identified **. The asparagine derivative yielded aspartic acid. The hydrolysate of the tryptophan derivative showed in the chromatogram two ninhydrin positive spots of which the more intense spot corresponded to tryptophan itself.

Method.

To the residue after evaporation of the nitromethane is added 2 ml 0.25 *N* barium hydroxide and the tube is sealed. Hydrolysis is carried out at 140° for 48 hours. The tube is then opened and the barium is precipitated as carbonate by passing a stream of carbon dioxide through the solution.

It is not necessary to remove the precipitate of barium carbonate prior to the application to the filter paper but it is advisable to heat the sample shortly in order to dissolve the amino acids with low solubility in water.

Degradation of alanylleucylglycin

The directions given above were followed. The amount of tripeptide used was 26 mg. For the cleavage of the PTC-peptides 15 minutes were sufficient. The glycine remaining after the second cleavage was identified directly. The paper strip identifications of the amino acids are shown in Fig. 3.

Degradation of leucylglycylglycylglycine

The procedure described was followed. The amount of tetrapeptide used was 30 mg. The last amino acid was also transformed to its PTC-derivative, treated with nitromethane-HCl and hydrolysed in the ordinary way. The paper strip identification of the amino acids can be seen in Fig. 3.

DISCUSSION

The applicability of the method can only be briefly discussed in view of the limited number of instances in which it has been employed.

It is obvious from the very nature of the procedure that the presence in the peptide of a free α-amino or α-imino group is a prerequisite for its applicability.

* Phenyl thiohydantoins have been prepared from all the amino acids commonly occuring in proteins with the exception of serine, threonine, cystine and glutamine [10]. The behavior of the hydantoin derivatives from the latter amino acids is therefore not known.

** Immediately after the treatment with ninhydrin the colour of this spot was yellow but later on it turned purple.

Furthermore only molecules exclusively built up of peptide bonds between α-amino (α-imino) and α-carboxyl groups can be expected to lend themselves to a complete degradation.

It is possible that some of the amino acids will behave differently from those investigated. Of particular interest in this respect are the amino acids serine, threonine and cystine because in these cases there is a tendency for other reactions to occur concomittantly with the hydantoin formation [10]. This question is being investigated.

In an accompanying paper [10] it has been shown that optical activity is more or less completely lost on the formation of phenyl thiohydantoins from optically active amino acids. Whether or not racemisation will occur under the different conditions of the degradation procedure is of obvious interest and will be investigated.

SUMMARY

It has been shown that phenylthiocarbamyl peptides are easily cleavable into 3-phenyl-2-thiohydantoins and peptides with one less amino acid residue.

This reaction has been employed in the development of a micromethod for determination of the amino acid sequence of peptides. The details of the method are given and its applications to a tripeptide and a tetrapeptide are described.

The applicability of the method is briefly discussed.

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