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Studies on the Determination of the Sequence of Amino Acids in Peptides and Proteins

3. THE SYNTHESIS OF 3-(4'-DIMETHYLAMINO-3:5'-DINITROPHENYL)HYDANTOIN DERIVATIVES OF VARIOUS AMINO ACIDS, AND THEIR USE FOR THE DETERMINATION OF N-TERMINAL AMINO ACIDS

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The reaction of phenyl isocyanate with amino acids has been known for many years. Some phenyl-carbamyl derivatives obtained in such condensations were described by Paal (1894), and Raper (1907) condensed various fractions of peptones obtained from serum albumin with the same reagent. Bergmann, Miekeley & Kann (1927) condensed dipeptides with phenyl isocyanate, and hydrolysed the products to give hydantoins corresponding to the amino acids which were originally the *N*-terminal residues. Abderhalden & Brockmann (1930) showed that the hydantoins are split from the peptide chain under mild hydrolytic conditions (treatment for 30 min. at 60–65° with methanol saturated with HCl), and that under these conditions the residual peptide was hydrolysed to a small extent only. Edman (1950) found that reaction with phenyl isothiocyanate gives phenylthiocarbamyl peptides, from which under even milder hydrolytic conditions (nitromethane saturated with hydrochloric acid) than those used by Abderhalden & Brockmann (1930) the *N*-terminal amino-acid residue splits off as a hydantoin. Hopkins & Wormald (1934) have shown that phenyl isocyanate reacts with the *N*-terminal amino acids of insulin, and Jensen & Evans (1935) were able to isolate the phenylhydantoin of phenylalanine from the hydrolysate, thus demonstrating for the first time the occurrence of phenylalanine as an *N*-terminal residue in insulin.

In order to facilitate the isolation in a pure state of the individual hydantoins from mixtures representing several *N*-terminal residues, and to allow quantitative determinations to be carried out, we have synthesized a coloured isocyanate, the 4-

dimethylamino-3:5-dinitrophenyl isocyanate. The introduction of the chromophoric groups into the benzene nucleus has also the effect of improving the adsorption characteristics of the hydantoins, and a method of chromatographic separation of their mixtures was found.

The names of the substances described in this paper, when in accordance with the agreed chemical nomenclature, are rather long. We have used abbreviated names as follows: 4-dimethylamino-3:5-dinitrophenyl isocyanate is called DDPI, and reaction products of this with the amino groups of amino acids are called DDP-ureidoamino acids, e.g., DDP-ureidoglycine. The corresponding hydantoin is called glycine-DDP-hydantoin.

EXPERIMENTAL AND RESULTS

Preparation of 4-dimethylamino-3:5-dinitrophenyl isocyanate (DDPI)

4-Dimethylamino-3:5-dinitrobenzoic acid was prepared by a slightly modified method of Ullmann (1909). To a solution of 4-chloro-3:5-dinitrobenzoic acid (10.8 g.) in water (150 ml.) containing K₂CO₃ (6.3 g.) was added a solution of dimethylamine (33%, w/w; 7.5 ml.). After heating the solution on a steam bath for 15 min., and cooling to room temperature, HCl (sp.gr. 1.18; 15 ml.) was added slowly while stirring, when a thick, yellow precipitate appeared, which was filtered off, washed with water and dried. Yield 10.8 g., m.p. 226–236°; recrystallized from ethanol (60 ml.), yield 8.2 g., m.p. 244–246° (decomp.) (75%; Ullmann, 1909, gives m.p. 246°).

This substance (17.3 g.) was refluxed in dry benzene (150 ml.), while SOCl₂ (14 ml.) dissolved in benzene (75 ml.) was slowly added. The mixture was then refluxed for 2 hr., the residue evaporated *in vacuo*, dissolved in dry benzene

(50 ml.), again evaporated and the residue dried overnight in a vacuum desiccator containing pellets of KOH. Yield 18.4 g. (99%), m.p. 117–118°. Recrystallization from light petroleum b.p. 80–100° (800 ml.) yielded 17.0 g. of crystals of 4-dimethylamino-3:5-dinitrobenzoyl chloride, m.p. 120–121°. (Found: C, 39.2; H, 2.9; N, 15.6; Cl, 13.2. Calc. for $C_9H_9N_3O_2Cl$ requires C, 39.5; H, 2.9; N, 15.4; Cl, 13.0%.)

The acid chloride (5.47 g.) was dissolved in acetone (80 ml.), the solution cooled to 0° and, whilst stirring, NaN₃ (1.6 g.) dissolved in water (8 ml.) was added in a continuous stream during 10 min. The stirring was continued for a further 15 min., when water (150 ml.) was added quickly yet dropwise, and the stirring was continued for another 15 min. at 0°. The precipitate was filtered off, washed with water and dried. The yield of 4-dimethylamino-3:5-dinitrobenzoyl azide was 5.43 g. (97%), m.p. 132–133° (decomp.) (Found: C, 38.6; H, 2.9; N, 27.2. $C_9H_9N_5O_2$ requires C, 38.6; H, 2.9; N, 30.0%). Analysis was unsatisfactory owing to violent decomposition when heated.

The acid azide (10 g.) was dissolved in dry toluene (250 ml.) at room temperature in a flask fitted with a mercury-seal stirrer, a reflux condenser closed with a CaCl₂ tube which was connected with a cold trap (cooled in solid CO₂) connected with a nitrometer charged with a KOH solution (30:100, w/v). A stream of dry CO₂ was passed through the apparatus for 30 min. with the nitrometer disconnected. The nitrometer was then connected, and the passage of CO₂ continued until only microbubbles reached the top of the KOH solution (rate of gas collection was then 1 ml. in 5 min.). The passage of CO₂ was then stopped. The flask containing the azide solution was immersed into a glycerol bath, and the temperature was cautiously raised until there was a brisk evolution of gas round the stirrer (80°). The temperature of the reaction mixture was then slowly raised to 95°, and maintained at that temperature until the evolution of gas ceased (about 2 hr. 30 min.; nitrometer reading 30 ml.). The CO₂ was then passed again until the rate of gas collection was again 1 ml. in 5 min. (a further 733 ml. of N₂ was measured). The total gas volume at s.t.p. was 808 ml. (Theory, 800 ml.). The solution was left overnight at room temperature, evaporated *in vacuo* (bath temp. <45°), and the residue dried over H₂SO₄. Yield, 9.5 g., m.p. 96–100°. After two recrystallizations from light petroleum (b.p. 60–80°) orange-red plates of 4-dimethylamino-3:5-dinitrophenyl isocyanate were obtained; yield 8.5 g. (95%), m.p. 101–102°. (Found: C, 42.6; H, 3.2; N, 22.1. $C_9H_9N_4O_2$ requires C, 42.8; H, 3.2; N, 22.2%). The substance was readily soluble in acetone and dioxan, soluble in CHCl₃ and benzene, and sparingly soluble in ether and high-boiling light petroleum. The substance is not wetted by cold water but reacts on boiling.

Reaction of DDPI with aniline

A solution of aniline in benzene (1:10, w/w; 1 ml.) was added to a solution of DDP isocyanate (250 mg.) in dry benzene (2.5 ml.). A solid mass of crystals formed immediately. The reaction was markedly exothermic. Benzene (50 ml.) was added, the solution warmed and the product allowed to crystallize. The crystals (m.p. 190–191°) were recrystallized from benzene when N-(4-dimethylamino-3:5-dinitrophenyl)-N'-phenyl urea, m.p. 190–191°, was obtained. (Found: C, 52.4; H, 4.4; N, 20.2. $C_{18}H_{18}O_2N_6$ requires C, 52.2; H, 4.4; N, 20.3%). The same substituted

diphenyl urea was obtained by refluxing for 5 hr. 4-dimethylamino-3:5-dinitrobenzoyl azide with aniline dissolved in dry benzene; m.p. 189–190°, mixed m.p. 189–191°.

Reaction of DDPI with water

Owing to its insolubility DDP isocyanate reacts with water only very slowly. However, under the conditions of the condensation with the free α -amino groups of amino acids and peptides, where the isocyanate is first dissolved in a solvent miscible with water, a much faster reaction was expected.

DDPI (500 mg.) was dissolved in acetone (5 ml.) and added dropwise to a Na₂CO₃ solution (2.5:100, w/v; 10 ml.) stirred at 0°. The stirring was continued for 1 hr., the dark-red precipitate filtered off, washed with water and dried *in vacuo*. Yield, 320 mg.; m.p. 215–217° (decomp.). Recrystallized from benzene, deep-red crystals of bis-(4-dimethylamino-3:5-dinitrophenyl)urea (di-DDP-urea) were obtained; m.p. 247° (decomp.). (Found N, 23.5. $C_{17}H_{18}O_2N_8$ requires N, 23.4%.)

The filtrate from the reaction mixture was evaporated *in vacuo*, the residue dissolved in hot water (20 ml.) and left at 0° overnight. The bright-red crystals were filtered off and washed with ice-water. Yield 100 mg.; m.p. 154–156°; recrystallization from CCl₄ yielded 4-dimethylamino-3:5-dinitrophenylamine, m.p. 156–157°. (Found: C, 42.5; H, 4.7; N, 24.7. $C_9H_{10}N_4O_2$ requires C, 42.5; H, 4.4; N, 24.8%). A small sample was dissolved in ether, and to this solution was added an ether solution of a roughly equivalent quantity of DDPI. When the solution was concentrated to a small volume dark-red crystals of di-DDP-urea were obtained.

Reaction of DDPI with esters of amino acids and peptides

A solution of DDPI (1.25 g.; 5.0 mmoles) in CHCl₃ (5 ml.) was shaken at 0° with a solution of glycine ethyl ester (0.51 g.; 5.0 mmoles) in CHCl₃ (2 ml.). After standing overnight at 0° the product was precipitated with light petroleum b.p. 60–80° (30 ml.), the red, oily precipitate solidified on rubbing, was allowed to stand at 0° for 1 hr., and was then filtered off and recrystallized first from ethanol and then from ethanol:water (50:50, v/v); yield 1.5 g. (85%) of 4-dimethylamino-3:5-dinitrophenylcarbamylglycine ethyl ester (DDP-ureidoglycine ethyl ester), m.p. 177–178°. (Found: C, 44.0; H, 4.8; N, 19.7. $C_{15}H_{17}N_5O_5$ requires C, 44.0; H, 4.8; N, 19.7%.)

In a similar way glycylglycine ethyl ester gave DDP-ureidoglycylglycine ethyl ester (yield 78%), m.p. 184–185°. (Found: C, 43.8; H, 4.7; N, 20.1. $C_{15}H_{19}O_5N_5$ requires C, 43.7; H, 4.9; N, 20.4%.)

DL-Alanylglycylglycine (530 mg.; 2.3 mmoles) was suspended in ethanol (15 ml.) previously saturated with dry HCl. The suspension was warmed on a steam bath for 5 min. until the solid was completely in solution, and then cooled quickly to room temperature. The solution was evaporated *in vacuo*, the residue dissolved in ethanol and precipitated with light petroleum (b.p. 60–80°) several times. The precipitate was then dissolved in water (1 ml.), CHCl₃ (5 ml.) was added, and the mixture cooled to –15°; K₂CO₃ was added in small quantities with constant agitation until the aqueous layer became a thick slurry. The CHCl₃ extract was decanted, the slurry thoroughly extracted with small portions of CHCl₃, and the combined CHCl₃ extracts were

shaken for 1 hr. with CaO, filtered and the filtrate cooled to -15° . DDPI (650 mg.; 2.6 mmoles) dissolved in CHCl_3 (10 ml.) and cooled to -15° was then mixed with the above extract; after standing for 2 hr. at 0° , the solution was evaporated *in vacuo*, ether (10 ml.) was added, and the oil when rubbed solidified almost immediately; it was filtered off, washed with ether and light petroleum (b.p. $40-60^{\circ}$), and three times crystallized from ethanol:water (50:50); yield, 560 mg. (45%) of orange crystals of *DDP-ureido-DL-alanylglycylglycine ethyl ester*, m.p. 195° (decomp.). (Found: C, 44.8; H, 5.05; N, 20.4. $\text{C}_{18}\text{H}_{25}\text{O}_8\text{N}_7$ requires C, 44.8; H, 5.2; N, 20.3%.)

Saponification of the ethyl esters. DDP-ureidoglycine ethyl ester (100 mg.) suspended in 0.1 N-NaOH (5 ml.) was shaken at room temperature for 30 min. when the crystals dissolved. The solution was filtered, acidified with 2 N-HCl, and the mixture kept for 2 hr. at 0° . The precipitate was filtered off and dissolved in ethanol (0.5 ml.); water was added (2 ml.), the mixture dissolved by warming and allowed to cool slowly; the deep-red crystals of *4-dimethylamino-3:5-dinitrophenylcarbamylglycine* (DDP-ureidoglycine) were filtered off; yield 65 mg.; m.p. 178° (decomp.). (Found: C, 40.4; H, 4.0; N, 21.1. $\text{C}_{11}\text{H}_{13}\text{N}_5\text{O}_6$ requires C, 40.4; H, 4.0; N, 21.4%.) The mother liquors were extracted with ethyl acetate, the solution evaporated *in vacuo*, and the residue recrystallized from water; yield 15 mg.; m.p. 175° (decomp.). The total yield was 92%.

In a similar way DDP-ureidoglycylglycine, m.p. $130-136^{\circ}$ (decomp.), and DDP-ureidoalanylglycylglycine, m.p. $140-145^{\circ}$ (decomp.), were obtained. The substances retain small amounts of solvents which were difficult to eliminate, and for this reason were not obtained in an analytically pure state.

Reaction of DDPI with amino acids and peptides

Preparation of DDP-ureidoglycine. Glycine (300 mg.; 4.0 mmoles) was dissolved in dioxan:water (50:50, v/v; 20 ml.) and the solution was brought to pH 9.6 (glass electrode) by addition of 0.1 N-NaOH. The temperature was brought to 0° , and DDPI (1.1 g.; 4.4 mmoles) dissolved in dioxan (10 ml.) was added dropwise with constant stirring. By addition of 0.1 N-NaOH the 'pH' was maintained at 9.6 during the 30 min. required for the addition of the DDPI solution. The stirring was prolonged for another 30 min., during which time the pH reading did not alter appreciably. The solution was brought to 'pH 6', and evaporated *in vacuo*. The residue was taken up in water, the pH adjusted to 9.5 and any DDP-amine present was removed by extraction with ether. An equal volume of dioxan was then added and the solution treated in the same way as before with a solution of DDPI (0.5 g.) in dioxan (5 ml.). After the reaction was completed the solution was acidified with HCl, left overnight at 0° , and the precipitate was filtered off; yield, 1.3 g. (100%) of red crystals, m.p. $160-170^{\circ}$ (decomp.). Recrystallization from boiling water gave 1.2 g. of 4-dimethylamino-3:5-dinitrophenylcarbamylglycine (DDP-ureidoglycine), m.p. 176° . This product (138.4 mg.) titrated with 0.0998 N-NaOH required 4.20 ml. (99%).

Preparation of DDP-ureidoglycylglycine. Glycylglycine hydrochloride monohydrate (95 mg., 0.51 mmole) was dissolved in water, the pH was adjusted to 10 and dioxan (10 ml.) was added. The solution was treated with a solution of DDPI (251 mg., 1 mmole) in dioxan (5 ml.) in the manner described for the preparation of DDP-ureidoglycine. On completion of the addition the solution was stirred for

15 min., and then more DDPI (80 mg., 0.32 mmole) dissolved in dioxan was added in the same way. The reaction mixture was then adjusted to pH 4.5, the precipitate filtered and washed free of water-soluble red components. Yield 130 mg.; m.p. 225° (decomp.). The filtrate was evaporated *in vacuo*, the residue extracted with a NaHCO_3 solution (5:95, w/v), filtered, and the insoluble residue was washed on the filter with the NaHCO_3 solution until no more colour was extracted. The yield of the precipitate was 44 mg. m.p. 230° . The filtrate and washings were acidified with HCl, and the mixture left overnight at 0° . The precipitate was filtered off and washed with ice-water; yield 190 mg. (98%) m.p. $125-130^{\circ}$ (decomp.). Recrystallization from ethanol:water mixtures failed to give a crystalline product. The m.p. remained at $130-136^{\circ}$ (decomp.), the substance retaining small amounts of solvent which could not be eliminated. The mixed m.p. with the DDP-ureidoglycylglycine prepared from the ethyl ester was unchanged.

This substance (28.7 mg.), dissolved in dioxan:water (50:50, v/v; 8 ml.), was titrated (glass electrode) with 0.0530 N-NaOH; it required 1.39 ml. (98.5%).

Preparation of DDP-ureido-DL-alanylglycylglycine. DL-Alanylglycylglycine (104 mg.) was treated with a solution of DDPI in the same way as described for glycylglycine. After the reaction was completed, the reaction mixture was concentrated to remove most of the dioxan, acidified and extracted twice with ethyl acetate. The ethyl acetate solution was extracted several times with aqueous NaHCO_3 (5:95, w/v) until no more coloured substance could be extracted. The NaHCO_3 solution was acidified, extracted with ethyl acetate and the ethyl acetate solution evaporated *in vacuo*. The residue was dissolved in dry acetone, filtered, and the acetone solution evaporated *in vacuo*. The residue was rubbed in ether and yielded 228 mg. of DDP-ureido-DL-alanylglycylglycine (98%), m.p. $140-145^{\circ}$ (decomp.). The mixed m.p. with the DDP-ureido-DL-alanylglycylglycine prepared from the ethyl ester was unchanged.

This substance (16.3 mg.) dissolved in dioxan:water (50:50, v/v) and titrated with 0.0265 N-NaOH required 1.34 ml. (99%).

Preparation of DDP-ureido-L-tryptophan. L-Tryptophan (103 mg.) was dissolved in a mixture of 0.1 N-NaOH (5 ml.) and dioxan (5 ml.). DDPI (255 mg.) was added in the manner described above. The residue of di-DDP-urea, when the acid had been extracted with the NaHCO_3 solution, was 122 mg. The bicarbonate extract after washing with ether yielded on acidification 225 mg. (98%) of a very finely divided precipitate which was separated by centrifugation. The substance was dissolved in methanol:water (50:50) and a slow evaporation of the solution at 20° yielded well formed microscopic crystals of 4-dimethylamino-3:5-dinitrophenylcarbamyl-L-tryptophan (DDP-ureido-L-tryptophan), m.p. 155° (decomp.). The m.p. was not very sharp, the crystals were apparently impure and softened at 135° . Analysis was not entirely satisfactory. (Found: C, 52.1; H, 5.0; N, 18.1. $\text{C}_{20}\text{H}_{20}\text{O}_7\text{N}_6$ requires C, 52.7; H, 4.4; N, 18.4%.)

Preparation of the N^3 -(4-dimethylamino-3:5-dinitrophenyl)-substituted hydantoins of various amino acids (amino acid-DDP-hydantoins)

The DDP-hydantoins of six amino acids were prepared. As an example, the preparation of 3-(4'-dimethylamino-3:5'-dinitrophenyl)-5-isopropylhydantoin (valine-DDP-hydantoin) will be described.

Table 1. *DDP-hydantoins prepared from various amino acids by the method given in the text*

Derived from amino acid	M.p. (°)*	Analysis					
		Found (%)			Requires (%)		
		C	H	N	C	H	N
Glycine	213-214	42.9	3.8	22.6	42.7	3.6	22.6
DL-Alanine	174-175	44.5	4.1	21.8	44.6	4.05	21.7
DL-Phenylalanine	173-174	54.2	4.5	17.8	54.1	4.3	17.55
DL-Valine	163-164	47.8	4.1	19.8	47.9	4.34	19.9
L-Tyrosine	200	52.6	4.0	17.0	52.1	4.1	16.9
DL-Serine	177	42.1	3.8	20.9	42.5	3.9	20.6

* All melting points are corrected.

DL-Valine was treated with DDPI as described above for the preparation of DDP-ureido-amino acids. After the reaction the solution was concentrated *in vacuo*, extracted completely into ethyl acetate, and the amino acid derivative extracted from the ethyl acetate with aqueous NaHCO_3 (5:95, w/v). The bicarbonate extract was washed twice with ether, acidified, and evaporated *in vacuo*. The residue dissolved in acetone was filtered, the filtrate evaporated *in vacuo*, and the residue rubbed with light petroleum, b.p. 40-60°. The yellow powder was dissolved in a very small amount of ethanol, N-HCl (10 vol.) was added, and the solution refluxed for 3 hr. Orange crystals formed gradually. After standing at 0° overnight, the substance was filtered off and recrystallized from ethanol:water (50:50, v/v). The data on the *hydantoins* prepared in a similar way are given in Table 1.

It is clear that DDPI has not reacted with the hydroxyl groups of tyrosine and serine.

It was found that, at least in the case of the DDP-ureido-amino acids which we have prepared, ring closure leading to the *hydantoins* can most conveniently be effected by refluxing with 2N-HCl for 1-2 hr. Under these conditions the *hydantoins* were perfectly stable and were recovered in 98-100% yields. Refluxing with 6N-HCl leads to decomposition and poor yields. It is probable that the decomposition occurs before ring closure. In the case of DDP-ureido-amino acid esters and in DDP-ureido peptides where ring closure proceeds more easily than in the case of DDP-ureido-amino acids, the refluxing with 6N-HCl gives good yields of *hydantoins*.

DDP-ureidoglycine (8.5 mg.) was suspended in 2N-HCl (2 ml.) and refluxed for 2 hr. The red crystals went completely into solution. After 1 hr. yellow crystals precipitated. The mixture was evaporated *in vacuo*, the residue washed with ethanol and evaporated 4 times to remove HCl and moisture. The residue was dissolved in acetone (1 ml.), benzene (9 ml.) was added, and the solution was adsorbed on an alumina column (see below). After development with benzene:ethanol (99.75:0.25, v/v), the main zone was eluted and estimated colorimetrically. The yield was 99% of theoretical value. The solution was evaporated *in vacuo*, and the m.p. of the residue, and the mixed m.p. with an analytically pure specimen of glycine-DDP-hydantoin were practically the same (208-210°).

DDP-ureidoglycylglycine (9.8 mg.) was refluxed with 2N-HCl (2 ml.) for 2 hr. The chromatographic purification was carried out as in the experiment above. On the alumina column crystals of glycine hydrochloride could be seen on the top of the column. The main zone eluted and estimated

colorimetrically gave a yield of 98.5% of the theoretical yield of glycine-DDP-hydantoin. The m.p. and the mixed m.p. was 207-210°.

It was found that *hydantoin* formation proceeds more easily from DDP-ureido-amino acid esters than from DDP-ureido-amino acids. When, for instance, DDP-ureidoglycine was refluxed with 6N-HCl for 1 hr. the yield of *hydantoin* was very poor, while DDP-ureidoglycine ethyl ester treated in the same way gave a 92% yield of the *hydantoin*.

A similar observation was made for DDP-ureido-amino acids contained in peptides. DDP-ureidoglycine kept for 4 days at 40° with nitromethane saturated with HCl gave a 48% yield of the *hydantoin*, while DDP-ureidoglycylglycine under the same conditions gave a 78% yield. It was found that DDP-ureidoglycylglycine kept for 5-6 days at 40° under the same conditions gave a practically quantitative yield of glycine-DDP-hydantoin.

Stepwise degradation of DL-alanylglycylglycine

DDP-ureido-DL-alanylglycylglycine (36.4 mg.) was mixed with nitromethane previously saturated with dry HCl (10 ml.) and was left standing for 5 days at 40°. After evaporation *in vacuo* (<30°) the residue was washed several times with ethanol and evaporated to dryness after each washing to remove HCl. The residue was then extracted with several small portions of dry acetone and the extract filtered. The filter was washed to dissolve all traces of colour. The filter containing the glycylglycine hydrochloride was kept for further investigation.

The acetone solution was evaporated *in vacuo*, the residue dissolved in as small a volume of acetone as possible, and the solution was diluted with benzene (10 vol.). This solution was then adsorbed on an alumina column, and the chromatogram was developed with benzene:ethanol (99.75:0.25, v/v). There was a thin zone at the top of the column, and a slight fast zone besides the main zone containing the *hydantoin*. The main zone was eluted, made up to 100 ml. with benzene:ethanol (99.75:0.25, v/v) and estimated colorimetrically. The yield was 99.4% of the theoretical amount of alanine-DDP-hydantoin. The solution was evaporated *in vacuo*, and the residue was hydrolysed by refluxing with a saturated solution of $\text{Ba}(\text{OH})_2$ (2 ml.; saturated at room temperature) for 3 hr. The presence of alanine in the hydrolysate was confirmed by paper chromatography (see below).

The residue of the extraction with acetone which remained in the reaction flask and on the filter was taken up with water (7 ml.) followed by dioxan (7 ml.). The solution

was brought to 'pH 9.6' and treated with a DDPI solution in the usual way. The DDP derivative which was obtained (presumed to be DDP-ureidoglycylglycine) was treated with the nitromethane-HCl solution for 6 days at 40°, and the experiment was continued in the same way as described above. The acetone extract yielded 108% of the theoretical amount of glycine-DDP-hydantoin (from the amount expected if the substance had been pure DDP-ureidoglycylglycine). On hydrolysis of the glycine-DDP-hydantoin with Ba(OH)₂ only the presence of glycine was detected by paper chromatography.

The residue of the extraction with acetone which remained in the reaction flask and on the filter (presumed to be glycine hydrochloride) was dissolved in water and estimated by formal titration with a glass electrode. A yield of 79% of the theoretical yield of glycine was found. A paper chromatogram showed the presence of glycine.

Colorimetric estimation of the amino acid-DDP-hydantoin

A series of concentrations of standard solutions of various hydantoin were made up in benzene:ethanol (99.75:0.25, v/v) and their absorption was measured with an 'Evans Electroselenium' photoelectric absorptiometer using an Evans dark-blue filter no. 622. Fig. 1 is a graphical representation of the results of these measurements. It can be seen that Beer's Law is obeyed.

Chromatographic separation of some amino acid-DDP-hydantoin

Alumina was prepared by the method already described (Flowers & Reith, 1953). As an example of a chromatographic separation, the resolution of a mixture of the DDP-hydantoin of glycine (2.14 μ moles), DL-alanine (3.02 μ moles), DL-phenylalanine (0.90 μ mole), DL-serine (1.93 μ moles), DL-valine (2.62 μ moles) and L-tyrosine (1.39 μ moles) dissolved in benzene:ethanol (95:5, v/v; 1.92 ml.) is described.

The solution was first diluted with light petroleum, b.p. 60–80° (10 ml.), and was then adsorbed on an alumina column (9 $\times$ 530 mm.) prepared with a slurry with light petroleum, b.p. 60–80°. After the adsorption on to the

column was completed, the ethanol-containing solvent was washed with light petroleum (10 ml.), and the column was developed first with light petroleum:ethanol (99.75:0.25, v/v; 150 ml.) when a zone containing valine-DDP-hydantoin (2.59 μ moles) was recovered in the effluent. Continued development with another 20 ml. of the same solvent brought the zone containing the phenylalanine-DDP-hydantoin (0.90 μ mole) into the effluent. On further development with 95 ml. of the same solvent the zone which contained the alanine-DDP-hydantoin (3.04 μ moles) was recovered in the effluent. The development was continued first with light petroleum:ethanol (99:1, v/v; 50 ml.) when a zone containing glycine-DDP-hydantoin (2.09 μ moles) was recovered from the effluent. Further development first with the same solvent (100 ml.), and then with light petroleum:ethanol (95:5, v/v; 43 ml.) brought a zone containing tyrosine-DDP-hydantoin (1.38 μ moles) into the effluent. The final zone which contained serine-DDP-hydantoin (1.97 μ moles) was eluted from the alumina with methanol.

After the colorimetric estimations, the solutions containing the various zones were separately evaporated *in vacuo*, and hydrolysed with a Ba(OH)₂ solution (saturated at room temperature). In each of the hydrolysates the amino acid was identified by paper chromatography.

Hydrolysis of the DDP-hydantoin to amino acids

The DDP-hydantoin were refluxed with the saturated Ba(OH)₂ solution for 3 hr. Excess Ba(OH)₂ was removed by treatment of the solution with CO₂ and filtering, and the DDP-amine and other coloured products were removed by adsorption on a short column of activated alumina (Hopkins and Williams Ltd.). The aqueous solution was evaporated, and the amino acid contained in the residue was identified by paper chromatography with butanol:acetic acid:water (5:1:4, v/v).

DISCUSSION

It was found that the reaction of DDPI with amino acids and peptides proceeds under mild conditions at a pH near to neutrality. The yields were excellent in all cases so far examined. The DDP-ureido-amino acids are converted into DDP-hydantoin by heating with acid. This conversion proceeds more readily in the case of DDP-ureidopeptides so that a method has been developed which permits ring closure with only slight hydrolysis of the residual peptide. During this process the *N*-terminal amino acid of the peptide chain appears as a free DDP-hydantoin in a quantitative yield, and these hydantoin are deeply coloured compounds suitable for chromatography on alumina; the residual peptide can be recovered in good, though not in quantitative yield because of the slight hydrolysis of peptide bonds.

Thus the experimental conditions under which the cyclization is completed are not as yet quite satisfactory for the use of DDP derivatives for sequence determinations as a continuous stepwise degradation procedure. However, the method was found to be excellent for the determination of the *N*-terminal amino-acid residue in a peptide. The

Fig. 1. Absorptiometric measurements of standard solutions of the DDP-hydantoin of glycine ($\square$), alanine ($\circ$), valine (+) and serine ($\triangle$) in benzene-ethanol (99.75:0.25, v/v) solution.

DDP-substituted *N*-terminal amino acid splits off with such ease that mild hydrolytic conditions may be used under which the hydantoins are perfectly stable. Furthermore, as under these conditions the remaining peptide bonds are split only to a very small extent, a possible catalytic effect of the degradation products of certain amino acids on the stability of the marked *N*-terminal amino acids is eliminated.

Mixtures of the deeply coloured DDP-hydantoins can easily be resolved by adsorption chromatography; the isolated DDP-hydantoins can be identified not only by their chromatographic behaviour and their melting point but also by alkaline hydrolysis to regenerate the free amino acid. Paper chromatography can then provide an additional check of identity.

DDPI is thus an excellent reagent for the determination of the *N*-terminal amino acid residue in a peptide.

SUMMARY

1. A red-coloured *isocyanate*, the 4-dimethyl-amino-3:5-dinitrophenyl *isocyanate* (briefly called DDPI) was synthesized.

2. It was found that this *isocyanate* reacts quantitatively with the free amino groups of amino acids or peptides dissolved in water-dioxan at the apparent pH 9.6 at 0°, giving deep-orange coloured carbamyl derivatives (briefly called DDP-ureido-amino acids or DDP-ureido peptides, for example, DDP-ureido glycine).

3. DDP-ureido-amino acids when refluxed for 2 hr. in 2*N*-hydrochloric acid, or when treated for several days at 40° with nitromethane saturated with hydrochloric acid, give by ring closure and

elimination of water the corresponding hydantoins (briefly called amino acid-DDP-hydantoins, for example, glycine-DDP-hydantoin) in quantitative yields.

4. When DDP-ureido peptides are treated in the same way, the *N*-terminal amino acid carrying the DDP radical splits up, forming by ring closure the corresponding hydantoin which can be recovered in quantitative yields.

5. The amino acid-DDP-hydantoins are deep-orange coloured substances which crystallize well and have sharp melting points. They can be adsorbed on alumina columns, and their mixtures can be conveniently resolved by adsorption chromatography.

6. The scope for the use of these derivatives for the determination of *N*-terminal amino acids in peptides is discussed.

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Studies on the Determination of the Sequence of Amino Acids in Peptides and Proteins

4. THE SYNTHESIS OF 3-(4'-DIMETHYLAMINO-3':5'-DINITROPHENYL)-2-THIOHYDANTOIN DERIVATIVES OF VARIOUS AMINO ACIDS, AND THEIR USE FOR AMINO ACID-SEQUENCE DETERMINATIONS

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The use of *isocyanates* and *isothiocyanates* for the determination of the sequence of amino acids in peptides was discussed in a previous paper (Evans & Reith, 1954). Aschan (1883) synthesized thiohydantoins by heating phenyl *isothiocyanate* with glycine, alanine and leucine at 140°. The method of

forming the thiohydantoic acids in alkaline solution and subsequently obtaining the thiohydantoins by acid catalysis was first used by Marckwald, Neumark & Stelzner (1891). The difficulty experienced in quantitative separation and estimation of mixtures of thiohydantoins, when only small quantities of substance are available, has led us to the synthesis of coloured thiohydantoins. Their preparation and use for the determination of the sequence of amino acids in peptides is described in

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