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

2. THE SEPARATION OF THE METHYL ESTERS OF *N*-2:4-DINITROPHENYL DERIVATIVES OF AMINO ACIDS BY ADSORPTION CHROMATOGRAPHY AND THE FREE AMINO GROUPS OF INSULIN

By C. M. FLETCHER, A. G. LOWTHER AND W. S. REITH
Organic Chemistry Department, The University, Leeds

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N-2:4-Dinitrophenylamino acids were first prepared by Abderhalden & Blumberg (1910). Sanger (1945) showed that mixtures of these compounds could be conveniently separated by partition chromatography on silica columns. He then applied this method to determine the free amino groups of insulin.

Although the use of methods requiring extremely small amounts of material is advantageous for the study of many biochemical problems, it is necessary to use somewhat larger quantities in order to ascertain by physico-chemical means the identity and the homogeneity of chromatographically separated fractions. Our results on the chromatographic analysis of *N*-sulphazoyl derivatives of amino acids (Flowers & Reith, 1953) clearly demonstrated the necessity of such an examination.

As the columns used in partition chromatography are easily overloaded, we investigated adsorption chromatography on columns of activated alumina for the separation of *N*-2:4-dinitrophenyl (DNP) derivatives of amino acids. For this purpose we prepared the methyl esters of these derivatives, and examined their properties. Part of this work was communicated to the Biochemical Society (Lowther & Reith (Reich), 1949).

While this work was in progress, Mills (1952) and Green & Kay (1952) developed methods for adsorption chromatography of DNP-amino acids on kieselguhr and silica.

EXPERIMENTAL AND RESULTS

The preparation of methyl esters of N-2:4-dinitrophenyl derivatives of amino acids

N-2:4-Dinitrophenyl derivatives (DNP derivatives) were prepared by reaction of amino acids with 1-fluoro-2:4-dinitrobenzene (FDNB) by the method of Sanger (1945). They were then esterified either with methanol-HCl or with diazomethane in dioxan solution. Typical esterifications proceeded as follows. DNP-glycine (2.2 g.) was suspended in dry methanol (30 ml.), the suspension was saturated with

dry HCl and refluxed for 10 min. After evaporation *in vacuo* the residue was dissolved in methanol and the solution repeatedly evaporated to dryness *in vacuo*. The dry residue was twice recrystallized from hot ethanol:light petroleum, b.p. 60–80° (1:1, v/v). Yield 1.5 g., m.p. 124–125°.

DNP-glycine (2 g.) was dissolved in dioxan (100 ml.). A freshly prepared solution of diazomethane (from 12 g. of *N*-nitrosomethyl urea) in ether (175 ml.) was added in five portions with shaking at each addition. The red solution was allowed to stand for 1 hr. at room temperature, and was then evaporated *in vacuo*. Yield, 2.1 g. of red crystals (100%). 0.218 g. was dissolved in benzene and purified on an alumina:benzene column. A thin, bright-red zone was retained at the top, and the broad, yellow band which moved down the column was collected, evaporated *in vacuo*, dissolved in dry benzene (2 ml.) and filtered to remove alumina. Evaporation *in vacuo* yielded 0.213 g. of yellow crystals, m.p. 124–125°.

The data on the DNP-amino acid methyl esters prepared in this way are given in Table 1.

α-Di-DNP-L-lysine methyl ester. *α*-Di-DNP-L-lysine (0.501 g.) was esterified by the methanol-HCl method. The preparation yielded a semi-solid material (0.498 g.) which would not crystallize. After chromatographic purification on an alumina:benzene column and elution with acetone, the methyl ester was obtained as a solidified oil melting between 80° and 100°. The substance had a very pronounced tendency to occlude solvent, which was difficult to eliminate. (Found: C, 48.0; H, 4.4; N, 15.5. $C_{19}H_{20}O_{10}N_6$, $CH_3 \cdot CO \cdot CH_2$ requires C, 48.0; H, 4.7; N, 15.3%.)

ε-DNP-L-lysine methyl ester hydrochloride. *ε*-DNP-L-lysine HCl, H_2O (76 mg.) was suspended in methanol (5 ml.) previously saturated with dry HCl and cooled to room temperature. Dry HCl was then bubbled through the suspension, care being taken that the temperature did not rise noticeably above room temperature. After about 30 min., a fine, yellow precipitate appeared; HCl was allowed to pass for a further 20 min., and after 2 hr. at 0°, the yellow crystals were filtered off and dried *in vacuo* over conc. H_2SO_4 in the presence of solid KOH. The yield of *ε*-DNP-L-lysine methyl ester hydrochloride was 68 mg.; m.p. 144–147°. (Found: C, 42.6; H, 5.4; N, 15.5; Cl, 9.7. $C_{13}H_{15}N_4O_4Cl$ requires C, 43.0; H, 5.3; N, 15.4; Cl, 9.8%.) Solutions of this substance were heat-labile, and precautions taken in the estimation of the yield will be described under Chromatographic Separations.

Table 1. DNP-Amino acid methyl esters prepared by esterification of DNP-amino acids with methanol-HCl

N-2,4-Dinitrophenylamino acid				Analysis		
	M.p. (corr.) (°)	Yield (%)	M.p. (corr.) (°)	[α] _D ¹⁸	Found	Required
					(%)	(%)
Glycine	205	78	124 (ethanol)	—	C, 42.1; H, 3.6	C ₉ H ₈ O ₆ N ₂ C, 42.3; H, 3.5
DL-Alanine	176	82	121 (ethanol)	—	C, 44.9; H, 4.3	C ₁₀ H ₁₁ O ₆ N ₂ C, 44.5; H, 4.1
L-Alanine	177-8	65	80-81 (ethanol-light petroleum)	-26° (c, 1; l = 1 dm; toluene)	C, 44.6; H, 4.1; N, 15.4	C ₁₀ H ₁₁ O ₆ N ₂ C, 44.5; H, 4.1; N, 15.6
DL-Aspartic acid	197-8 (decomp.)	77	110-111 (ethanol-light petroleum)	—	C, 43.7; H, 3.9	C ₁₁ H ₁₂ O ₆ N ₂ C, 44.1; H, 4.0
DL-Methionine	118-20	40	81-2 (ethanol-light petroleum)	—	C, 44.2; H, 4.5; N, 12.4	C ₁₁ H ₁₃ O ₆ N ₂ S C, 43.6; H, 4.6; N, 12.8
S-Methyl-L-cysteine	Oil	80	57-8 (ethanol)	-99° (c, 1; l = 1 dm; toluene)	C, 42.1; H, 4.4	C ₁₁ H ₁₃ O ₆ N ₂ S C, 41.9; H, 4.2
L-Phenylalanine	185-7	56	109-111 (ethanol)	—	C, 55.6; H, 4.1; N, 12.0	C ₁₆ H ₁₅ O ₆ N ₂ C, 55.6; H, 4.3; N, 12.2

Spectrophotometric estimations

A Unicam S.P. 500 Spectrophotometer was used. The DNP-amino acid methyl esters were estimated in ethanol solution (1 cm. cuvettes). The ethanol was always freshly purified by fractional distillation. For concentrations between 1.5 and 15 mg. of methyl ester/l. Beer's Law was found to be obeyed. Absorption spectra were taken between 200 and 420 mμ., and the molecular extinction coefficients (ε) were calculated at the main absorption maxima. The following values were obtained. DNP-glycine methyl ester: ε = 10300 at 260 mμ.; 17200 at 342 mμ. DNP-L-phenylalanine methyl ester: ε = 9700 at 260 mμ.; 17200 at 342 mμ. ε-DNP-L-lysine methyl ester HCl: ε = 8300 at 260 mμ.; 16650 at 348 mμ.

The DNP-amino acids were estimated in NaHCO₃ solution (1%, w/v). The estimations were always performed immediately after the preparation of the solution. All the DNP-amino acids examined were perfectly stable in the NaHCO₃ solution for at least 3 hr. at room temperature. ε was calculated at the main absorption maxima (1 cm. cuvettes):

	265 mμ.	361 mμ.
DNP-glycine	8 800	17 100
DNP-alanine	8 800	17 100
DNP-L-phenylalanine	8 600	17 200
ε-DNP-L-lysine HCl, H ₂ O	8 500	17 700
	263 mμ.	355 mμ.
Di-DNP-L-lysine	15 850	30 850
	265 mμ.	353 mμ.
DNP-glycylglycine	9 020	16 000

For concentrations between 1 and 15 mg./l. Beer's Law was obeyed at 353, 355 or 361 mμ. At 263 or 265 mμ. slight discrepancies occurred.

Chromatographic separation of mixtures of DNP-amino acid methyl esters

Alumina was prepared by the method already described (Flowers & Reith, 1953). With this alumina it was possible to separate mixtures of methyl esters of DNP-amino acids into their individual components. For instance the chromatographic resolution of a mixture of the methyl esters of DL-alanine and S-methyl-L-cysteine was carried out in the following manner.

An alumina column (22 × 320 mm.) was prepared with a slurry of alumina in benzene:light petroleum, b.p. 60-80° (1:3, v/v). To a benzene solution (6 ml.) of the S-methyl-L-cysteine derivative (30.1 mg.) and the DL-alanine derivative (30.9 mg.), light petroleum (12 ml.) was added, and the solution was slowly passed through the column. The mixture of the coloured derivatives adsorbed on the top of the column was developed with benzene:light petroleum, b.p. 60-80° (1:3, v/v, 850 ml.), when two yellow zones formed which were clearly separated by a colourless zone (25 mm.). The yellow zones were separately eluted with a mixture of acetone and methanol (1:1, v/v), the two resulting solutions were filtered and concentrated *in vacuo*. The solution from the top zone gave 31.2 mg. of crystalline material, m.p. 55-6° (m.p. of the S-methyl-L-cysteine derivative 57°), while the bottom zone gave 31.1 mg. of crystalline material, m.p. 122° (m.p. of the DL-alanine derivative 121°).

The high yield, 104 and 103%, was found to be due to small amounts of alumina which in a finely dispersed form passed with the solution through the filter. It was found that this could be eliminated by dissolving the dry residue in a small amount of dry benzene, and filtering the benzene solution through a no. 4 sintered-glass filter. An experiment carried out in this way yielded a 100% recovery of the *S*-methyl-L-cysteine derivative ($[\alpha]_D^{25} = -100 \pm 2$; toluene) and a 98% recovery of the DL-alanine derivative (m.p. 121–122°).

Yields in the esterification of ϵ -DNP-L-lysine. The solution of the methyl ester of ϵ -DNP-L-lysine was heat-labile, and even evaporations *in vacuo* at room temperature decomposed a small amount of the compound. If the evaporations were performed at low temperature under standard conditions, the amount of the decomposition product was constant and very small. Its nature has not yet been investigated.

ϵ -DNP-L-lysine HCl, H₂O (76 mg.), esterified as described above, gave 68.0 mg. of ϵ -DNP-L-lysine methyl ester hydrochloride as yellow crystals and a yellow filtrate. The filtrate was evaporated *in vacuo* in the dark at room temperature, the residue dissolved in a small amount of methanol:CHCl₃ (5:95, v/v) and the solution purified chromatographically on an alumina column (22 × 320 mm.) prepared with benzene. The chromatogram was developed with methanol:benzene (2:98, v/v). An extremely narrow yellow zone remained strongly held at the top of the column (unreacted ϵ -DNP-L-lysine.HCl), a faint narrow zone was washed through the column, while the main broad, bright-yellow zone was eluted with methanol. The methanol solution was evaporated *in vacuo*, the residue dissolved in ethanol, and the amount of substance estimated spectrophotometrically. The amount found was 7.83 mg., thus the total yield from the esterification was 75.9 mg.

This seems to indicate a quantitative yield by the esterification procedure. This cannot be quite true since during chromatographic purifications of crude esterification products there was always a small amount of unreacted ϵ -DNP-L-lysine tightly held at the top of the column. The yield, however, is probably very nearly quantitative, the value of 101% being obtained because the crystalline product used as standard for spectrophotometry was not quite pure. The elementary analysis also points to that.

Yields in the esterification with diazomethane of a mixture of DNP-glycine and DNP-L-phenylalanine. DNP-L-phenylalanine (4.93 mg.) and DNP-glycine (2.96 mg.) were dissolved in dioxan (4 ml.) and esterified with 10 times the theoretical amount of diazomethane. The reaction mixture was evaporated *in vacuo*, the residue dissolved in benzene,

adsorbed on an alumina column (22 × 320 mm.), and the chromatogram developed with benzene:light petroleum, b.p. 60–80° (1:3, v/v). A narrow, very intense red zone was retained at the top of the column, while the rest of the material separated into two bright-yellow zones. These were eluted separately, and after evaporation *in vacuo* each residue was dissolved in ethanol and the amount of material was estimated spectrophotometrically at the 342 m μ . absorption maximum.

Yield of the faster zone $R = 0.185$ (DNP-L-phenylalanine methyl ester) was 5.12 mg. (99.6%), while the yield of the slower zone $R = 0.11$ (DNP-glycine methyl ester) was 3.13 mg. (98.4%).

The '*R* value' is an expression of the relative rate of movement of the adsorption zone (band) to the movement of the solvent.

Stability of the DNP-amino-acid derivatives to acid hydrolysis

Two methods were used for this investigation, both using the same hydrolytic treatment, but each one a different method of recovery of the unchanged substance.

First method. This method was similar to that described by Sanger (1945) and Porter & Sanger (1948). Approximately 0.02% solutions of DNP-amino acids in 6*N*-HCl were boiled under reflux for varying periods of time. After evaporation *in vacuo*, the residue was dissolved in water-saturated ether, the solution passed through a 2 g. silica:ether column to remove HCl, and the material was then purified on a 6 g. silica:CHCl₃ column.

The fast faint-yellow zone (R 1.4) was neglected, and the only other zone obtained in each case was evaporated *in vacuo*, the residue dissolved in an appropriate volume of NaHCO₃ solution (1%, w/v), and estimated spectrophotometrically.

Second method. The DNP-amino acids were subjected to the same conditions of acid hydrolysis as in the first method. After evaporation *in vacuo* the residue was dissolved in dioxan, esterified with diazomethane, the esterification mixture evaporated *in vacuo*, the residue dissolved in CHCl₃ and fractionated chromatographically on alumina columns. A small amount of impurity was completely washed out of the column, the remainder was very strongly adsorbed at the top, whereas the required compound occupied the centre portion of the column. It was eluted with acetone, filtered, and the acetone evaporated to dryness *in vacuo* in a weighed flask. The results obtained by both methods are given in Table 2.

Table 2. *Recovery of DNP-amino acids after exposure to hydrolytic conditions as used for the hydrolysis of proteins*

(The unaltered DNP-amino acids were recovered either as free DNP-amino acids by partition chromatography (1st method), or as DNP-amino acid methyl esters by adsorption chromatography (2nd method).)

DNP-amino acid	Hydrolysis method	Duration of hydrolysis (hr.)	Initial weight of material (mg.)	DNP-amino acid recovered (mg.)	Method of recovery	Recovery of DNP-amino acid (%)
Glycine	Reflux with 6 <i>N</i> -HCl	8	5.13	1.40	1st	27.3
Glycine	Reflux with 6 <i>N</i> -HCl	8	5.33	1.39	1st	25.1
Glycylglycine	Reflux with 6 <i>N</i> -HCl	8	4.32	0.960	1st	27.5
Glycine	Reflux with 6 <i>N</i> -HCl	8	92.2	43.0	2nd	42.1
Glycine	100° (<i>in vacuo</i>) 6 <i>N</i> -HCl	8	5.14	2.59	1st	50.4
L-Phenylalanine	Reflux with 6 <i>N</i> -HCl	8	3.98	2.87	1st	72.1
L-Phenylalanine	Reflux with 6 <i>N</i> -HCl	8	161.6	128.0	2nd	77.0
ϵ -DNP-L-Lysine.HCl, H ₂ O	Reflux with 6 <i>N</i> -HCl	8	6.9	5.37	1st	77.8

The free amino groups of insulin

Crystalline insulin (Boots Pure Drug Co. Ltd.; 22.05 units/mg.; 1.15% $\text{NH}_2\text{-N}$ (Van Slyke 30 min. reaction period)) was recrystallized following essentially the methods of Romans, Scott & Fisher (1940) and Lens (1948). The product finally obtained had $\text{NH}_2\text{-N} = 1.00\%$ (Van Slyke). (Found: C, 49.1; H, 6.8; N, 15.3; S, 3.1%.) This insulin was treated with FDNB following the procedure of Sanger (1945), when DNP-insulin (Found: C, 47.3; H, 6.1; N, 15.4; S, 2.5%) was obtained as a yellow powder.

DNP-insulin (162.1 mg.) was refluxed with 6N-HCl (25 ml.) for 8 hr. in a glycerol bath (bath temperature 123°). The hydrolysate cooled to room temperature, was extracted six times with ether (15 ml.); after the fourth extraction the ether layers were colourless. The combined ether layers were three times extracted with water (6 ml.); the water layers were colourless after the first extraction. The combined ether layers (the ether-soluble fraction), and the combined water layers (the water-soluble fraction) were separately evaporated *in vacuo*.

The ether-soluble fraction. The yellow residue was dissolved in dioxan (5 ml.) and esterified using approximately 10 times the required amount of diazomethane. The solution was evaporated *in vacuo*, the residue dissolved in benzene (2 ml.), and adsorbed on an alumina column (11 × 250 mm.) prepared with benzene:light petroleum, b.p. 60–80° (1:3, v/v). The rate of flow was 31 ml./hr. After development with 60 ml. of the benzene:light petroleum mixture, an extremely faint buff-coloured zone (R 1.2) was washed out of the column and was discarded. The appearance of the chromatogram was (the bracketed figures indicate the width of zones in mm.): top, extremely thin zone, bright-red, (2) orange, (13) white, (14) yellow (R 0.035; zone A), (30) white, (48) yellow (R 0.115; zone B), (16) white, (70) yellow (R 0.19; zone C).

Zone C. The R value compares well with the value of 0.185 obtained for synthetic DNP-L-phenylalanine methyl ester under similar conditions of chromatographic analysis. A mixed chromatogram with the synthetic material containing approximately equal amounts of both substances was chromatographically homogeneous.

A sample corresponding to 16.21 mg. of DNP-insulin was concentrated to dryness *in vacuo* and dissolved in 100 ml. of ethanol. The spectrum was identical with that of the synthetic DNP-L-phenylalanine methyl ester. An optical density of 0.276 was observed at the 342 m μ . maximum where $\epsilon = 17\,200$; calculation gives 0.554 mg. of DNP-phenylalanine methyl ester for 16.21 mg. of DNP-insulin. Assuming the validity of the experimentally established value of 72.1% for the recovery of DNP-phenylalanine under the hydrolytic conditions used, and a molecular weight of 14800 for the DNP-insulin submolecule (calculated from sulphur contents), calculation leads to 2.03 N -terminal phenylalanine residues per insulin submolecule of mol.wt. 12000.

Zone B. The R value of 0.115 found for this zone corresponds very well with the value of 0.11 obtained for synthetic DNP-glycine methyl ester under similar chromatographic conditions. A chromatogram after mixing with the synthetic derivative gave a single band.

A sample corresponding to 16.21 mg. of DNP-insulin concentrated *in vacuo* and dissolved in 25 ml. of ethanol showed an optical density of 0.516 at the 342 m μ . absorption maximum. The spectrum was identical with that of the

synthetic derivative. This is equivalent to 0.191 mg. of substance in 16.21 mg. of DNP-insulin. Assuming the correctness of the value of 27.5% for the recovery of DNP-glycine under the hydrolytic conditions used, calculation gives 2.48 N -terminal glycine residues per insulin submolecule of mol.wt. 12000.

Zone A. The R value of 0.035 found for this zone does not correspond to that of any DNP-amino acid methyl ester as yet investigated. Mixed chromatograms with synthetic derivatives of glycine, phenylalanine, ϵ - and di-DNP-L-lysine methyl esters show that the substance contained in this band is a chromatographically distinct compound.

A sample corresponding to 15.20 mg. of DNP-insulin was concentrated *in vacuo*, dissolved in 25 ml. of ethanol and the ultraviolet-spectrum of the solution was examined. The spectrum differed slightly from those of the derivatives of glycine, phenylalanine and lysine. Attempts were made to weigh the residue from this zone, but no significance could be attached to the result owing to the extreme difficulty of completely removing the solvent.

A rough calculation can be made of the number of moles of material present in this zone if one assumes that the substance has at its 338 m μ . maximum a molecular extinction coefficient (17200) similar to the derivatives of glycine and phenylalanine at their 342 m μ . absorption maxima. If the further assumption is made that the compound has a molecular weight of about 300, then the amount of material derived from 15.2 mg. of DNP-insulin would be 0.135 mg. This compares with 0.179 mg. of DNP-glycine methyl ester, and with 0.520 mg. of DNP-phenylalanine methyl ester present in the same amount of DNP-insulin. This necessarily very rough calculation was made in order to give some idea of the relative amount of material present in the unidentified zone.

The water-soluble fraction. The residue from the water-soluble fraction of the DNP-insulin hydrolysate was rehydrolysed as before for another 8 hr., concentrated *in vacuo*, and the residue esterified in the same way as described for the esterification of ϵ -DNP-L-lysine.HCl, H_2O . After evaporation of the esterification mixture *in vacuo* at room temperature, one-fifth of the residue dissolved in a 5% solution of methanol in CHCl_3 (2 ml.) was adsorbed on an alumina column (10 × 267 mm.), prepared with benzene and developed with 2% methanol in benzene. The appearance of the chromatogram was identical with that described for the methyl ester of ϵ -DNP-L-lysine.

The R value (0.14) of the main zone was in fair agreement with the R value of 0.10 obtained for the main zone in the esterification of synthetic ϵ -DNP-L-lysine HCl, H_2O under similar conditions. Mixed chromatograms showed the chromatographic homogeneity with synthetic ϵ -DNP-L-lysine methyl ester.

A sample corresponding to 9.19 mg. of DNP-insulin dissolved in 50 ml. of ethanol had a spectrum identical with that of ϵ -DNP-L-lysine methyl ester hydrochloride, and an optical density of 0.249 at the 348 m μ . absorption maximum corresponding to 0.271 mg. of substance. A correction factor for the loss through decomposition during concentration *in vacuo* followed by rechromatography corrects the value to 0.286 mg. of ϵ -DNP-lysine methyl ester hydrochloride for 9.19 mg. of DNP-insulin. Assuming the correctness of the value obtained for the amount of decomposition of the substance under the hydrolytic conditions, calculation gives 2.10 free ϵ -amino-groups of lysine per insulin submolecule of mol.wt. 12000.

DISCUSSION

The examination of the *N*-terminal amino acids carrying the free amino groups in insulin led Sanger (1945) to the conclusion that in each insulin submolecule of molecular weight 12000 there are two *N*-terminal residues of glycine, two of phenylalanine, and two lysine residues carrying free ϵ -amino groups. Sanger concluded that the insulin submolecule was made up of four open peptide chains, two of these having terminal glycyl residues, and the other two having terminal phenylalanyl residues. These conclusions are based on the identification and estimation by partition-chromatographic methods of the DNP-amino acids in the hydrolysate of DNP-insulin. Owing to the instability of DNP-amino acids under the hydrolytic conditions used for the preparation of the hydrolysate, correction factors for the decomposition of the DNP-amino acids during hydrolysis had to be used in those calculations.

In the present investigation an adsorption-chromatographic method for the separation and estimation of methyl esters of DNP-amino acids is described. An application of this method to the study of the free amino groups of insulin led to the following results. In the insulin submolecule of molecular weight 12000 there are two *N*-terminal phenylalanyl residues, but the amount of DNP-glycine found may represent 2 or 3 (2.48) glycyl residues with equal probability. In addition, an unidentified substance was found; a rough calculation of the amount present indicates an order of magnitude similar to that of the amount of DNP-glycine.

It is interesting to note that in experiments with the same DNP-insulin, carried out in a way similar to that used by Sanger (1945), the unidentified substance was not observed. The explanation might be that in separations by partition chromatography the DNP derivative in question formed so wide a zone that the diffuse colour could not be observed.

As the calculation of the number of *N*-terminal residues depends on the amount of decomposition of DNP-amino acids during the hydrolysis of the DNP-protein, we have carefully re-examined the magnitude of those correction factors. Even by standardizing most carefully the hydrolytic conditions the recovery of the unchanged DNP-amino acids varies to a considerable extent. For instance, in the case of DNP-glycine the recoveries vary between 25 and 42% depending on the method used for the estimation of the yield of the unchanged product after the hydrolytic treatment. But there is in this connexion an additional complication. The rate of decomposition of any DNP-amino acid may be determined by

the presence of various products of protein hydrolysis. Porter & Sanger (1948) reported that there is no change in the stability of DNP-amino acids in the presence of added globin. Desnuelle, Rivery & Fabre (1951), however, report a considerable effect of the presence of various proteins on the recovery of DNP-aspartic acid. For all these reasons it seems to be quite uncertain how far the results of calculations using the above-mentioned correction factors really correspond to the amounts of the *N*-terminal amino acid residues actually contained in insulin.

In this preliminary investigation only small amounts of insulin were used, and for this reason an attempt was not made to identify the chromatographically isolated derivatives by their melting points and by other physico-chemical methods. The provisional identification of the isolated substances was made by determinations of the *R* values under standard conditions, and by mixing each isolated substance with pure authentic specimens and ascertaining the chromatographic homogeneity of the mixtures.

SUMMARY

1. Methyl esters of *N*-2:4-dinitrophenyl (DNP) derivatives of a number of amino acids have been prepared.

2. The separation of mixtures of these derivatives by adsorption chromatography on alumina columns was investigated.

3. The stability to acid hydrolysis of some DNP-amino acids was investigated by two different methods.

4. The new method of separation of mixtures of DNP-amino acids (by adsorption chromatography of their methyl esters) was applied to the re-investigation of the free amino groups of insulin.

5. Assuming a molecular weight of 12000 it is shown that two free amino groups are on phenylalanine residues, two or three on glycine residues, and two represent the ϵ -amino groups of lysine. In addition, a new, unidentified substance bearing free amino groups was found.

6. It is suggested that owing to the difficulty of an exact assessment of the magnitude of correction factors for the decomposition of DNP-amino acids during protein hydrolysis, it must be considered as quite uncertain how far the calculated values for the amount of *N*-terminal amino acid residues really correspond to the amount actually contained in insulin.

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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

BY G. G. EVANS AND W. S. REITH

Organic Chemistry Department, The University, Leeds

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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