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DETERMINATION OF DINITROPHENYLAMINO ACIDS IN STRUCTURAL PROTEINS BY CHROMATOGRAPHY ON NYLON POWDER COLUMNS

I. THE WATER-SOLUBLE DINITROPHENYLAMINO ACIDS

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SUMMARY

A procedure is described for the quantitative determination of the water-soluble dinitrophenyl (DNP)-amino acids in structural proteins by column chromatography using nylon powder as adsorbent. The water-soluble DNP-amino acids of α-keratins (O-DNP-serine, S-DNP-cysteine, N_ε-DNP-lysine, O-DNP-tyrosine), silk fibroin (O-DNP-serine, N_ε-DNP-lysine, O-DNP-tyrosine) and collagen (O-DNP-serine, O-DNP-hydroxyproline, N_ε-DNP-hydroxylysine, N_ε-DNP-lysine, O-DNP-tyrosine) were estimated by using a citrate buffer, pH 3.0, at 30° and a flow rate of 30 ml per h.

INTRODUCTION

The reaction of 1-fluoro-2,4-dinitrobenzene (FDNB) with different proteins for the estimation of N-terminal and side-chain amino acids[1] was first applied to wool chemistry by MIDDLEBROOK[2]. Information has also accumulated on methods developed for quantitatively estimating the resultant water-soluble DNP-amino acids following the FDNB reaction and hydrolysis of the dinitrophenylated protein.

Methods used by earlier workers for the separation and estimation of DNP-amino acids included column chromatographic techniques using a wide range of adsorbents[3–5]. Columns of polyamide powder have been used for the separation and quantitative estimation of tanning agents[6] and it was shown that the formation of hydrogen bonds between the phenolic groups and the amide bonds of the polyamide powder facilitated this separation[7,8]. It was also shown that the nitro groups of simple aromatic compounds have an affinity for the amide bonds of collagen, thereby allowing their chromatographic separation on columns of polyamide powder[9].

The first application of nylon powder columns to the separation of DNP-amino acids was made by STEUERLE[5], and later reports in this field were made by ZAHN AND HILLE[10], STEUERLE AND HILLE[11], HILLE[12] and SIEPMANN AND ZAHN[13].

We are now reporting the application of nylon powder columns for the estimation of water-soluble DNP-amino acids for different types of wool, mohair, human

hair, silk fibroin and collagen. The rate of destruction of the DNP-amino acids during hydrolysis was investigated and the standard deviations for each water-soluble DNP-amino acid estimated quantitatively were also determined. We also describe a procedure for preparing a standard nylon powder.

EXPERIMENTAL

Preparation of nylon powder columns

200 g unstretched, TiO_2-treated nylon fibres were covered with 2 l of acetic acid and 100 ml formic acid in a 3 l round-bottomed flask. The flask, fitted with a reflux condensor, was placed in an oil bath and heated up to 180–190° refluxing started within 10–20 min. After 45 min the turbid solution was poured into a beaker and allowed to stand overnight at room temperature. After this, the upper hard layer was removed, and the wet nylon suspension was collected on a Buchner-funnel and allowed to dry for three days. The dry powder was then suspended in 1 l of distilled water and homogenised with a "Starmix" (24000 r.p.m.) for 1 min. The slurry was then filtered and the resulting wet cake washed with distilled water till neutral. After resuspension in distilled water and screening through a 250 mesh/in. sieve (file diameter 0.04 mm) the suspension was collected. This was then diluted to 2.5 l and stored at 4°.

To avoid formation of air bubbles and to facilitate settling of the column, the suspension was then boiled and allowed to cool to 30°. The column (50 × 1 cm) was maintained at 30° and, with pressure of a 3-m high water column to ensure quick and compact settling, filled with the powder to a height of 42 cm. It was then compressed to a total length of 40 cm using compressed air and eluted twice with citrate buffer pH 3.0. The buffer was prepared from 298.5 ml 1 *N* hydrochloric acid, 42.33 g citric acid and 16.12 g sodium hydroxide. This was brought to a volume of 5 l with distilled water.

Structural proteins under investigation

A wide range of structural proteins were used in this study to determine the reactivity of the side-chain amino acids with FDNB. Four wools of different mean diameter were chosen as being representative of commercial wools; they were merino wool (20.9 μm), lincoln wool (38.4 μm), New Zealand wool (29.4 μm) and German land merino (26.7 μm). Three experimentally grown wools were also used; merino wool SW 293 (17.9 μm), lincoln wool SW 308 (35.9 μm) and corriedale wool SW 295 (29.9 μm). These wools were assumed to be uniform in physical and chemical properties[14]. South African mohair (33.5 μm) and human hair (68.8 μm) samples were also included in this study. All samples were subjected to isoionic washing followed by extraction with acetone for 6 h. The sample of silk fibroin studied was prepared according to DRUCKER *et al.*[15] and bovine tendon collagen according to ZAHN AND WEGERLE[16].

The serine, lysine, tyrosine, hydroxyproline and hydroxylysine contents of these samples were determined by ion-exchange chromatography[17]. The cysteine contents were estimated colorimetrically[18]. These values are summarised in Table I.

Control DNP-amino acids for equilibrating the columns

For the quantitative determination of DNP-amino acids in this study it was necessary to synthesise all the water-soluble DNP-amino acids present in the struc-

TABLE I

AMINO ACID COMPOSITION OF THE STRUCTURAL PROTEINS USED

Values are given in μmoles/g.

Sample	*Amino acid*					
	Ser	*Lys*	*Tyr*	*Cys*	*Hyl*	*Hyp*
Wool, merino	988	233	287	27.5	—	—
Wool, lincoln	856	220	192	25.7	—	—
Wool, New Zealand	850	236	279	21.5	—	—
Wool, German land merino	908	218	320	19.7	—	—
Wool, merino SW 293	816	247	324	22.0	—	—
Wool, lincoln SW 308	802	277	252	19.0	—	—
Wool, corriedale SW 295	787	270	283	21.4	—	—
Mohair, South Africa	961	249	252	24.0	—	—
Human hair	847	184	114	24.0	—	—
Silk fibroin	1255	35	632	—	—	—
Collagen	311	215	45	—	86	850

TABLE II

DNP-AMINO ACIDS SYNTHESISED

DNP-amino acid	*Reference*
O-DNP-D,L-serine	19
N_ε-DNP-L-lysine	20
O-DNP-L-tyrosine	21
S-DNP-L-cysteine	22
O-DNP-L-hydroxyproline	23
N_ε-DNP-D,L-hydroxylysine	24
N_{1m}-DNP-L-histidine	25
N_ε-DNP-(2-amino-2-carboxyethyl)-L-lysine	26
N_δ-DNP-L-ornithine	27
N_α-DNP-L-arginine	20
DNP-cysteic acid	28

tural proteins that were to be investigated. Besides this, other water-soluble DNP-amino acids were synthesised, but were not used for quantitative estimation. The derivatives synthesised are listed together with their preparations (reference numbers) in Table II.

From these synthesised DNP-derivatives solutions containing 0.5–1 μmole of DNP-amino acid in 2 ml were made. The DNP-amino acids were first dissolved in 1 ml 1 *N* hydrochloric acid; O-DNP-tyrosine was first dissolved in 1 ml formic acid, and the volume made up with distilled water to 100 ml.

Dinitrophenylation of the samples

200 mg of each sample, dried at 105° for 2 h, was reacted, as reported by MIDDLEBROOK[29], with a solution of 200 mg sodium bicarbonate in 5 ml water and 350 mg FDNB dissolved in 10 ml acetone at 40° with constant stirring. In the case of collagen, however, the reaction was carried out at 20°. After 24 h the solution was renewed and the dinitrophenylation was repeated for another 24 h. The samples were then washed

with water and extracted with acetone. 50 mg of the above dinitrophenylated sample, dried for 2 h at 105°, was then hydrolysed with 10 ml 6 *N* hydrochloric acid in a sealed tube for 16 h at 95°. The hydrolysate was diluted, concentrated in a rotary evaporator and the residue dissolved in 1 ml acetic acid and the volume then made up to 10 ml with citrate buffer, pH 3.0.

It was not necessary to extract the ether-soluble DNP-amino acids or the residual dinitrophenol since these compounds, due to their slow migration down the nylon powder column used, do not hinder the analysis of the faster migrating water-soluble DNP-amino acids.

Nearly all the DNP-amino acids were destroyed to some extent during hydrolysis and it was therefore necessary to estimate the extent of this destruction. Control hydrolysates were prepared containing 50 mg DNP-wool or DNP-collagen, with a known content of DNP-amino acids and known quantities of reference DNP-derivatives. After separation on nylon powder columns, the losses incurred during hydrolysis and column chromatography were determined. The factors for correction are as shown below:

Amino acid	O-DNP-Ser	S-DNP-Cys	N_ε-DNP-Lys	O-DNP-Tyr	O-DNP-Hyp	N_ε-DNP-Hyl
Correction factor	1.2	1.0	1.025	1.0	1.1	1.04

Calibration of the system

2 ml of each solution of the standard mixture of the water-soluble DNP-amino acids were concentrated to 0.5 ml by vacuum distillation under nitrogen. The concentrated solution, taken up twice with 0.5 ml buffer, pH 3.0, was pipetted onto the pre-equilibrated column. 1 ml buffer was used for rinsing the tube and also pipetted onto the column. This 2.5 ml solution was pressed down into the nylon powder column. The column was then filled up with buffer and the chromatographic analysis was started at 30° with a flow rate of 30 ml per h. The chromatogram of the standard calibration mixture was evaluated by planimetry of the peaks obtained by calculation of the plane units for 1 μmole of each DNP-amino acid. This calibration procedure was repeated for every new nylon powder preparation.

Chromatography of the water-soluble DNP-amino acids

2 ml of the hydrolysate was applied by means of the pressure of the water column to the pre-equilibrated column. When the sample had entered the column it was washed with two 0.5 ml aliquots of buffer, pH 3.0. The chromatographic analysis was performed at 30° with a flow rate of 30 ml per h, provided by "Milton Roy" pumps.

When O-DNP-tyrosine had emerged from the column a second sample was applied and chromatographed. However, after two samples had been chromatographed, it was found necessary to regenerate the nylon powder. This regeneration treatment of 200 g nylon powder involved removing the powder and suspending it in 100 ml 5 % ammonia solution for 15 min. This alkaline suspension was then filtered and the residue washed to neutrality. The wet cake was then suspended in 100 ml 0.01 *N* hydrochloric acid, stirred again for 15 min, filtered and washed till neutral. After

sieving the powder was preserved in 2.5 l of distilled water and used for another two tests before being discarded.

Apparatus

The apparatus used was an "Eppendorf" photometer (Netheler and Hinz, Hamburg). It was equipped with an automatic switching device for successive measurements. Three columns were connected to the photometer and the effluent from each passed through two connected flow-cells differing in diameter 1.0 and 0.25 cm, respectively. The measured extinctions at 313 nm were linearized automatically within the photometer unit. A recorder was provided with a six point colour wheel and the signals were printed by closely spaced dots. The recorded curves were evaluated by simple planimetry.

Calculation of the yield of DNP-amino acids

The weight of a non-dinitrophenylated sample E was obtained by the formula:

$$F = \frac{E_1}{E_2} \text{ and } E = E_3 \cdot F$$

where

E_1 = weight of the dry sample before dinitrophenylation;
E_2 = weight of the dry dinitrophenylated sample;
E_3 = weight of the dry sample for hydrolysis;
F = a factor, which should be in the range 0.85 to 0.95.

The value for the yield in μmoles/g non-dinitrophenylated sample was calculated from the formula:

$$G = \frac{A \cdot C \cdot 1000}{B \cdot E}$$

where

E = weight of the analysed sample in mg;
A = μmoles control compound carried on the column;
B = area of the control compound peak measured by the 1.0 cm diameter cell;
C = area of the sample solution peak measured by the 1.0 cm diameter cell;
G = yield in μmole/g non-dinitrophenylated sample.

RESULTS

The water-soluble DNP-amino acids of α-keratins

Fig. 1 shows the chromatogram of a hydrolysate of DNP-wool containing five peaks. The first peak was identified as N_{1m}-DNP-histidine[25], but the quantitative estimation of histidine as N_{1m}-DNP-histidine showed excessively high values. It was shown[30], that in this peak besides N_{1m}-DNP-histidine free amino acids and other artefacts were present, which makes the quantitative estimation at 313 nm difficult. Therefore, it was not possible to estimate N_{1m}-DNP-histidine using this method. The second peak was shown to be O-DNP-serine by elution and comparison with synthetic O-DNP-serine; O → N rearrangement into N-DNP-serine was followed by identification with chromatographic methods[19]. O-DNP-threonine has the same position in the chromatogram as O-DNP-serine. However, this DNP-amino acid has never been

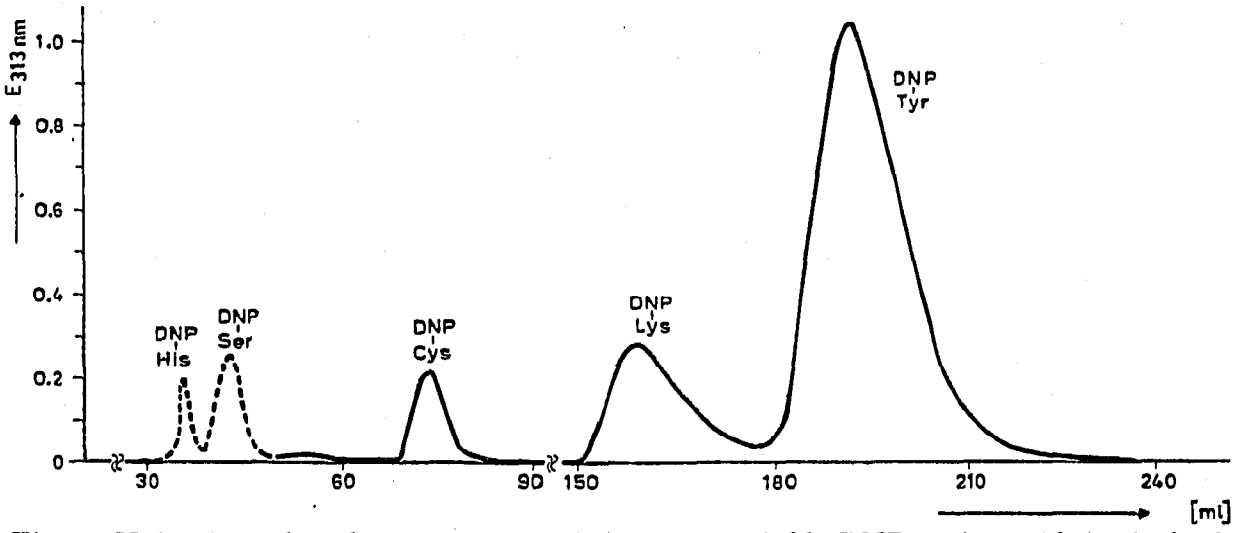

Fig. 1. Nylon powder chromatogram of the water-soluble DNP-amino acids in the hydrolysate of DNP-wool. (– – – – d = 0.25 cm; ——— d = 1.0 cm).

found in hydrolysates of DNP-wool. The substance in the second peak, when treated at pH 8.0 always gave N-DNP-serine. The next three peaks were shown to be S-DNP-cysteine[12], N_ε-DNP-lysine[5] and O-DNP-tyrosine[12]. The resulting values given in Table III are the average of four estimations together with their standard deviations. They have been corrected for losses during hydrolysis.

TABLE III

ESTIMATION OF THE WATER-SOLUBLE DNP-AMINO ACIDS OF DIFFERENT α-KERATINS

Values are given in μmoles/g untreated sample.

Sample	*O-DNP-Ser*	*S-DNP-Cys*	*N_ε-DNP-Lys*	*O-DNP-Tyr*
Wool, merino	164 ± 9.84	25.0 ± 1.25	195 ± 3.00	252 ± 8.82
Wool, lincoln	134 ± 8.71	24.8 ± 1.36	187 ± 2.87	172 ± 6.02
Wool, New Zealand	152 ± 8.54	18.2 ± 0.84	211 ± 2.15	269 ± 10.70
Wool, German land merino	173 ± 10.78	16.5 ± 1.09	169 ± 3.00	260 ± 6.35
Wool, merino SW 293	132 ± 9.24	18.4 ± 1.27	193 ± 4.65	252 ± 7.94
Wool, lincoln SW 308	115 ± 9.20	13.9 ± 1.66	233 ± 3.05	195 ± 10.20
Wool, corriedale SW 295	150 ± 6.55	18.6 ± 0.45	220 ± 4.40	267 ± 7.10
Mohair, South Africa	144 ± 9.56	18.0 ± 1.00	201 ± 4.08	224 ± 8.96
Human hair	76 ± 5.60	16.8 ± 0.37	110 ± 1.46	95 ± 7.06

The water-soluble DNP-amino acids of silk fibroin

The chromatogram of a hydrolysate of DNP-silk fibroin showed a pattern similar to that in Fig. 1. However, the S-DNP-cysteine peak was not present and the amount of O-DNP-tyrosine had increased while the amount of N_ε-DNP-lysine had decreased. The corresponding values for the DNP-amino acids of silk fibroin are summarised in Table IV. The values are the average of four estimations together with their standard deviations. They have been corrected for losses during hydrolysis.

The water-soluble DNP-amino acids of collagen

Fig. 2 shows the chromatogram of a hydrolysate of DNP-collagen[31]. This chromatogram differs from that of DNP-wool in that two additional peaks were present.

TABLE IV

ESTIMATION OF THE WATER-SOLUBLE DNP-AMINO ACIDS IN SILK FIBROIN
Values are given in μmole/g untreated silk fibroin.

Sample	*O-DNP-Ser*	*N_ε-DNP-Lys*	*O-DNP-Tyr*
Silk fibroin	104 ± 6.24	35 ± 1.08	490 ± 22.7

The first and the second peak were again shown to be N_{1m}-DNP-histidine and O-DNP-serine. The substance of the third peak was shown (by chromatography) to be identical with synthetic O-DNP-hydroxyproline. Further evidence for the existence of O-DNP-hydroxyproline was obtained on treatment with alkali. No rearrangement to N-DNP-hydroxyproline took place because of the ring structure of this amino acid. The

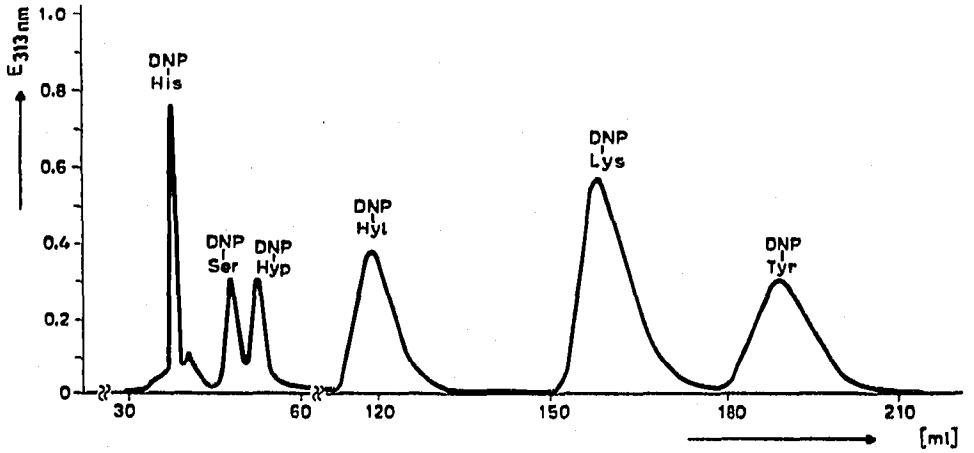

Fig. 2. Nylon powder chromatogram of the water-soluble DNP-amino acids in the hydrolysate of DNP-collagen.

fourth peak was shown to be N_ε-DNP-hydroxylysine, the fifth N_ε-DNP-lysine and the sixth O-DNP-tyrosine. The values found in a hydrolysate of DNP-collagen, together with their standard deviations for four determinations are summarised in Table V. They have been corrected for losses during hydrolysis.

TABLE V

ESTIMATION OF THE WATER-SOLUBLE DNP-AMINO ACIDS OF COLLAGEN
Values are given in μmole/g untreated collagen.

Sample	*O-DNP-Ser*	*O-DNP-Hyp*	*N_ε-DNP-Hyl*	*N_ε-DNP-Lys*	*O-DNP-Tyr*
Collagen	36 ± 5.25	57 ± 8.31	74 ± 1.21	178 ± 12.1	43 ± 3.20

Other water-soluble DNP-amino acids

Fig. 3 shows a chromatogram of other synthesised water-soluble DNP-amino acids. The positions of N_{1m}-DNP-histidine and O-DNP-serine were again identified. The third peak was identified as N_ε-DNP-(2-amino-2-carboxyethyl)-L-lysine. The next peak was S-DNP-cysteine followed by $N\delta$-DNP-ornithine, which migrates as far

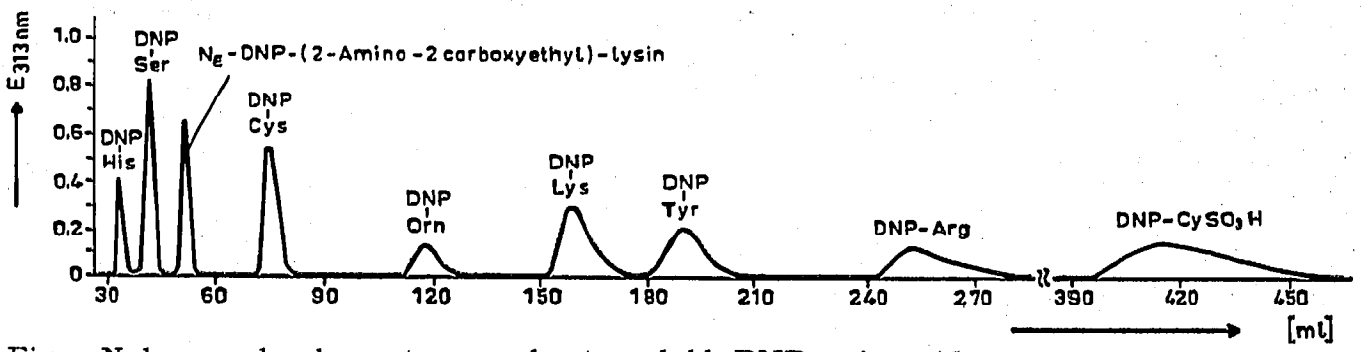

Fig. 3. Nylon powder chromatogram of water-soluble DNP-amino acids.

as N_{ε}-DNP-hydroxylysine. The next peak was N_{ε}-DNP-lysine followed by O-DNP-tyrosine. N_{α}-DNP-arginine and DNP-cysteic acid were also identified as the two last peaks, respectively.

DISCUSSION

Comparing the amino acid composition in Table I with the yield of the DNP-amino acids in Tables III, IV and V it can be seen that none of the side-chain amino acids reacted completely with FDNB. The main reason for the low reactivity of the seryl and hydroxyprolyl residues was due to the high pK-value of the hydroxyl group. The tyrosyl residues also do not react completely with FDNB[32]. It was shown[33] that hydrogen bonds between the tyrosyl residues and aspartyl or glutamyl residues, or main-chain carbonyl groups[34], prevent quantitative dinitrophenylation of the tyrosyl residues. All investigations in this laboratory for a complete dinitrophenylation of the lysyl residues failed.

The simplest method of estimating DNP-amino acids as well as unmodified amino acids would be by ion-exchange chromatography, based on the method of MOORE *et al.*[17]. However, the separation of DNP-amino acids is complicated by the strong interactions between the DNP-residues and the aromatic constituents of the resin. WOFSY AND SINGER[35] and NISHIKAWA *et al.*[36] separated the basic amino acids as well as N_{1m}-DNP-histidine, N_{ε}-DNP-lysine and O-DNP-tyrosine on short columns of an amino acid analyser. However, in a hydrolysate of DNP-wool, for example, the presence of O-DNP-serine, S-DNP-cysteine and N_{ε}-(2-amino-2-carboxyethyl)-L-lysine[33] must be taken into account and the chromatogram becomes more complicated and the estimation of all the DNP-amino acids by ion-exchange chromatography more laborious.

It must be stated here that there is no nylon powder available commercially, which can be used for obtaining the separations described in this paper. Commercial polyamide powders were tested without success. However, our procedure for the preparation of nylon powder gave batches of constant quality and therefore, the chromatographic patterns are reproducible.

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