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The Free Amino Groups of the Proteus Flagella Protein

Quantitative Determination of Dinitrophenyl Amino Acids Using Paper Chromatography

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The method of Sanger [1] for the identification and estimation of the terminal amino acids of proteins using 1,2,4-fluorodinitrobenzene as a reagent for the free amino groups has now been applied to a great number of proteins. A review of the method is found in [2]. In most cases chromatography on silica gel has been used for the separation of the different dinitrophenyl (DNP-) derivatives. However, this method requires a comparatively large amount of the protein to be investigated. If only small amounts of material are available a possible separation on paper would be more suitable. Actually, some reports on paper chromatography of DNP-amino acids have been published [3–7]. One of them [7] also deals with the quantitative estimation of the derivatives but no figures are given to show the accuracy of the adopted procedure. It will be shown below that at least some of the DNP-amino acids can be accurately determined using paper chromatography when a simple but effective elution of the spots is used. Thus, only about 10 mg of protein are required for the determination of the free amino groups. The method has been applied to the flagella of the bacterium *Proteus vulgaris*, which earlier has been shown to consist almost entirely of protein material [8].

EXPERIMENTAL

The DNP-proteins and DNP-amino acids were prepared essentially according to [2]. In the case of the flagella, a 1—2 % solution of the flagella was added to two volumes of a 10 % (w/v) solution of fluorodinitrobenzene in alcohol. The mixture was shaken for two hours at room temperature, the precipitate sucked off on glass filter, washed and dried [2].

The flagella protein content in the DNP-protein was determined according to [2].

The DNP-proteins were hydrolyzed with 5.7 *N* HCl in an oven at 110° for 12 hours. With small amounts (5—10 mg) of protein in 0.5 ml acid, the hydrolysis was performed in a small test tube, the top end of which had been drawn out so that the opening was reduced to a capillary in order to minimize evaporation.

When the hydrolysis was completed the ether soluble DNP-derivatives were extracted, the acid solution evaporated *in vacuo* and the solid residue dissolved in water. For the qualitative identification of the DNP-amino acids paper chromatography according to [7] and [6] was applied.

For the quantitative determination, chromatography according to [6] was used, since the spots showed the least spreading by this method. Tertiary amyl-alcohol was used as the moving phase. After the chromatogram was run out, the colored spots were cut out and eluted according to the simple method of Aminoff and Morgan [9]. — Usually 10—15 μg of the DNP-derivatives were used for the determinations. They were applied on the paper in the forms of elongated spots, about 0.5 × 5 cm.

The eluates obtained were acidified with dilute H_2SO_4 and the content of DNP amino acids was determined spectrophotometrically at 360 mμ.

RESULTS AND DISCUSSION

In order to estimate the accuracy of the method, chromatograms were first run with pure DNP-amino acids. The derivatives found in the flagella protein, ε-mono-DNP-lysine and DNP-alanine, were used for these quantitative determinations. The results are given in Table 1.

Table 1.

	μg applied	μg found in eluate	Recovery %
DNP-lysin	51.5	52.5	102
»	51.5	51.4	100
DNP-alanine	17.7	16.8	95
»	60.8	56.0	92

The table shows that lysine is quantitatively eluted, while alanine is eluted to about 94 %.

Of the acid soluble DNP-derivatives, DNP-lysine from haemoglobin (cow), albumin from pea [12] and from the *Proteus* flagella, was quantitatively determined. The flagella were treated in the fluorodinitrobenzene without and after disintegration at pH 3. By this disintegration the flagella structure is entirely destroyed and split products having a molecular weight of approximately 40 000 are produced [8].

Table 2 shows the results obtained. (Only DNP-lysine was found in the chromatograms, besides dinitrophenol and dinitroaniline.)

Table 2.

Protein	Sample	Amount used for determination μg	Eq. DNP-lysine found in sample × 10^7	Eq. lysine per 10^5 g original protein
Haemoglobin (cow)	I	628; 628	33.8; 34.7	78.7; 80.7 M = 80
Pea albumin	I	2355; 1570	80.0; 54.0	74.4; 75.5 M = 75
Proteus flagella	I	806; 806	28.5; 28.3	55.5; 56.0 M = 56
» »	II	560; 560	18.0; 18.8	52.0; 54.3 M = 53
» dis-integrated	III	1322	51.2	57.9 M = 58
» »	IV	506; 506	18.9; 18.5	55.1; 53.9 M = 54

The DNP-flagella contained 65—72 % original flagella protein. In calculating the figures in the last column, the decomposition of DNP-lysine during the acid hydrolysis is taken into consideration [2].

The lysine value for haemoglobin is slightly higher than that found by Porter and Sanger, *i. e.*, 73.4 eq. per 10^5 g protein [10]. The lysine contents of the albumin from pea and of the flagella have earlier been determined to 72.5 and 56 eq. per 10^5 g protein by the electrodialysis method [11, 12]. The accordance between the two methods must be considered good.

Further it is of interest to note that the same result is obtained with the DNP-derivatives of whole or disintegrated flagella, respectively. However, by electron microscopical observations, it was found that no flagellar structure could be detected in any DNP-preparation. The flagella structure therefore seems to be destroyed by this reagent as well as by lowering the pH.

Chromatographic determination of the ether soluble DNP-amino acids obtained from the hydrolyzed DNP-flagella have also been carried out. Qualitative chromatography with the solvent mixtures described by Biserte [7] and by Blackburn and Lowther[6] showed that besides dinitrophenol only DNP-alanine was present after hydrolysis of the DNP-flagella. Quantitative determinations were carried out as described above for the DNP-lysine. Table 3 gives the results.

As mentioned earlier, smaller units of a molecular weight of approximately 40 000 are obtained by disintegrating the flagella at pH 3. Since only about

Table 3.

Sample of flagella	Amount used for the chromatography, μg	Eq. DNP-alanine found in sample $\times 10^7$	Eq. alanine per 10^5 g original protein
I	38.5	2.4	1.1
II	32.6	2.2	1.2
III (disintegrated flagella)	3.39	0.22	1.1
IV » »	6.92	0.34	0.8

1 eq. of free amino group is found per 10^5 g flagella protein, ring formations in the peptide chains building up this protein must be assumed.

Determinations of the free amino groups of other fibrous proteins belonging to the keratin-myosin group have been performed by Bailey [13]. No free amino groups are found in these proteins, and ring formations in the peptide chains must therefore occur also here. As is shown above, a relatively low content of free amino groups in the flagella is found. Since the occurrence of small amounts of impurities of a protein nature in the flagella preparations is not quite improbable [8] and since the values obtained above for the DNP-alanine for different samples of flagella seem to vary more than the experimental errors permit [13], the true content of free amino groups in the flagella other than the α-amino groups of the lysine may be still lower than what the figures given above indicate.

SUMMARY

Quantitative determinations of the free amino groups in some proteins have been performed according to the fluorodinitrobenzene method. Using paper chromatography, the determination could be performed on less than 10 mg substance. The *Proteus* flagella protein is found to contain 55 equivalents of free lysine ε-amino-groups per 10^5 g protein, which is in accordance with the earlier determinations by the electrodialysis method. Furthermore, about 1 eq. of free amino groups deriving from alanine is found per 10^5 g protein.

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