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IDENTIFICATION OF N-TERMINAL AMINO ACIDS IN THE FORM OF FLUORESCENT THIOHYDANTOINS

ZDENĚK DEYL

Physiological Institute, Czechoslovak Academy of Science, Prague-Krč (Czechoslovakia)

SUMMARY

Out of three different types of aromatic isothiocyanates tested, 1-naphthyliso-thiocyanate is the most suitable for stepwise degradation of proteins. The hydan-toins formed exhibit good chromatographic properties on paper (solvents: 5% acetic acid; 10% pyridine; phosphate buffer, pH 6, μ 0.1) and on electrophoresis (pyridine-acetate buffer). The pH dependence of the emitted fluorescence, which differs for individual amino acids, is of great diagnostic value. The sensitivity is due to the luminescent properties of these derivatives being increased up to the order 10^{-3} to 10^{-4} μmoles, which is comparable to the sensitivity of the 1-dimethylamino-5-naphthalene sulphochloride (DANSYL) derivatives.

INTRODUCTION

Sequential analysis of proteins is very intimately related to the determination of N-terminal amino acids. Since dinitrophenylation and EDMAN's degradation (for a review see ref. 1), several attempts to make the whole procedure more sensitive and to eliminate possible mistakes in the identification of the N-terminal amino acid[2,3] have been published. A big step forward was made by GRAY AND HARTLEY[4] who used the fluorescent derivatives of 1-dimethylaminonaphthalene-4-sulphochlo-ride (DANSYL) and thus increased the sensitivity of the N-terminal amino acid determination up to the range of 10^{-3}–10^{-4} μmoles. Later several procedures, in-volving mainly thin-layer chromatography (TLC) on silica gel plates and high voltage electrophoresis were applied for the identification of DANSYL-amino acids[5-8]. On the other hand, hydantoins split off during EDMAN's procedure received much less attention with regard to their separation; it is only fairly recently that PATAKI AND STRASKY[9] reported the sensitive quantitation of phenylhydantoin derivatives. PATAKI[10] has also published a paper on the so-called double-checking technique using both derivatives, *e.g.* DANSYL-amino acid is identified in an aliquot and the particular hydantoin split off during EDMAN's degradation is also utilised. Phenylthiohydantoins, however, exhibit several disadvantages with respect to their identification: firstly their chromatographic separation is very sensitive towards the total amount spotted on the paper; secondly the sensitivity of their detection is about one order less than that of DANSYL-amino acids. Thus if one expects the stepwise determination of about ten amino acids, the amount of N-terminal amino acid split off in the form of phenyl-

thiohydantoin is just about enough for one run. Our efforts were therefore directed towards the production of hydantoins that were more readily detectable and which possibly exhibited some other diagnostic properties. The importance of such a procedure is based on the fact that there are numerous situations in sequential analysis where it is not possible to control the individual steps of the EDMAN procedure by concurrent amino acid analysis on account of the small amount of the starting material or the large molecular weight of the protein (mol. wt. of $3 \cdot 10^5$ or higher).

MATERIAL AND METHODS

Three different isothiocyanates were used for the modification of EDMAN's procedure:
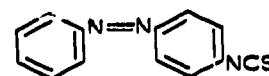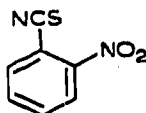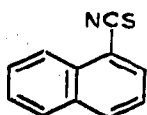
The procedure used was identical with that published for the microdetermination of the N-terminal sequence by SJÖQUIST[11] which is as follows:
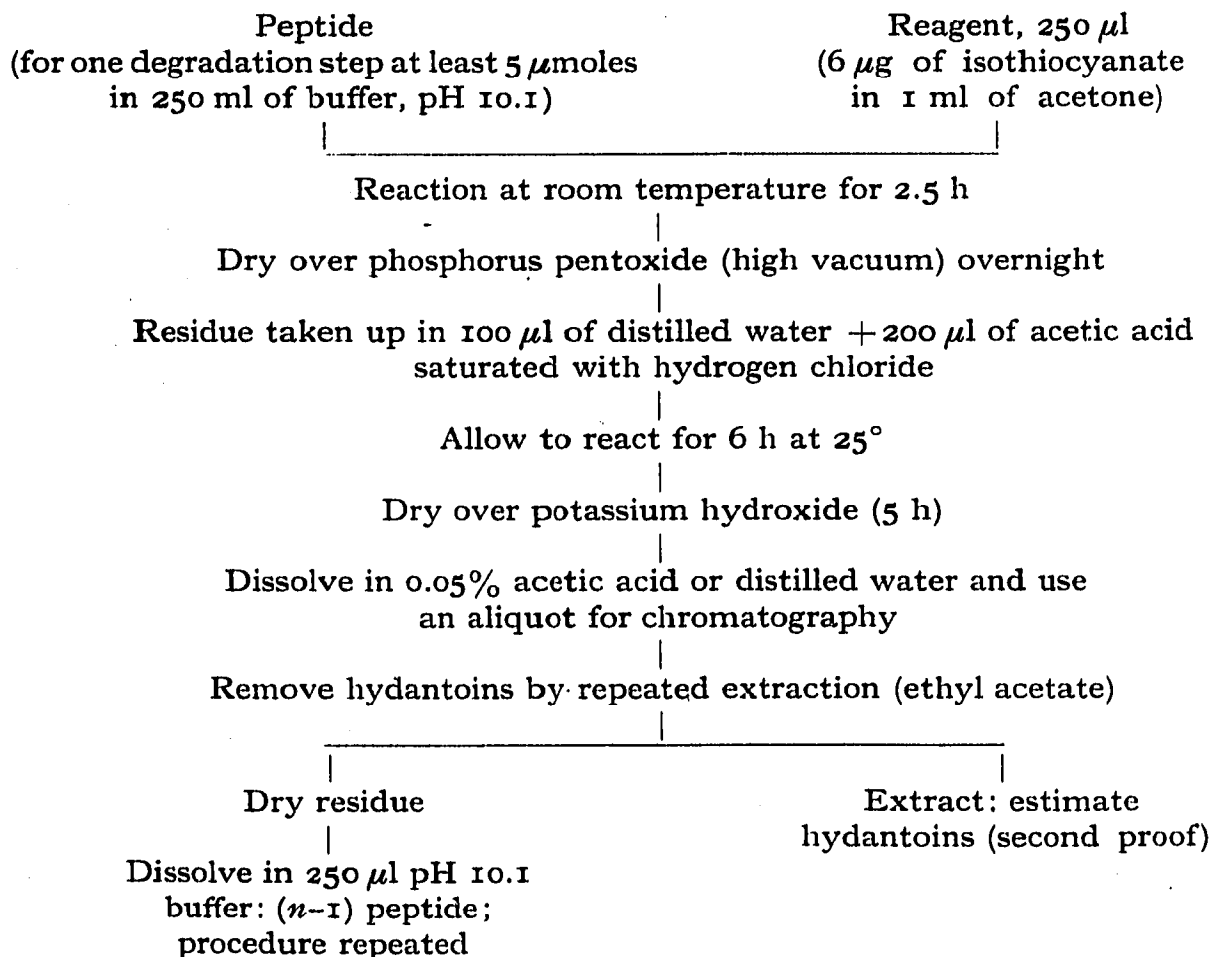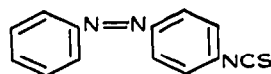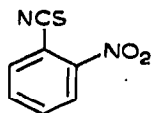
                    Peptide   Reagent, 250 μl
(for one degradation step at least 5 μmoles   (6 μg of isothiocyanate
        in 250 ml of buffer, pH 10.1)   in 1 ml of acetone)
                        |_____|

                Reaction at room temperature for 2.5 h
                                    |

        Dry over phosphorus pentoxide (high vacuum) overnight
                                    |

    Residue taken up in 100 μl of distilled water $+$ 200 μl of acetic acid
                    saturated with hydrogen chloride
                                    |

                    Allow to react for 6 h at 25°
                                    |

                Dry over potassium hydroxide (5 h)
                                    |

        Dissolve in 0.05% acetic acid or distilled water and use
                    an aliquot for chromatography
                                    |

        Remove hydantoins by repeated extraction (ethyl acetate)
                                    |
        _____|_____

              Dry residue   Extract: estimate
                  | hydantoins (second proof)
        Dissolve in 250 μl pH 10.1
            buffer: (n-1) peptide;
            procedure repeated

TABLE I

DIAGNOSTIC PROPERTIES OF SOME THIOHYDANTOINS

Substance	Colour	Substance	Colour
Trp	Blue fluorescence[a]		
Orn	Two spots: yellow fluorescence one UV absorbing spot	Trp	Blue fluorescence[b]
Lys	One spot: yellow fluorescence two UV absorbing zones	Phe	Blue fluorescence
		Ser	Green fluorescence
		Thr	Green fluorescence
Phe	Green fluorescence	Orn	Needs 10–15 min of UV irradiation before visible
Arg	One spot: yellow fluorescence one UV absorbing zone		
		Others	Yellow fluorescence
Others	UV absorbing zones		

[a] Sensitivity $1–2 \cdot 10^{-1}$ μmoles; fluorescent spots 10^{-2} μmoles.
[b] Sensitivity $10^{-3}–10^{-4}$ μmoles.

RESULTS AND DISCUSSION

All three isothiocyanates result in over 90% splitting off of the N-terminal amino acid and the recovery of hydantoins after extraction is usually within the limits 85–90%. Obviously all the isothiocyanates used are eminently suitable re-

TABLE II

FLUORESCENCE OF THE I-NAPHTHYLISOTHIOCYANATE DERIVATIVES IN DIFFERENT MEDIA
(Sensitivity $10^{-3}–10^{-4}$ μmoles)

Amino acid	Colour of the luminescence			
	5% CH_3COOH	Phosphate buffer $pH = 6.0$	10% Pyridine	Distilled water
Glu	Blue	Green	Yellow	Yellow
Arg	Blue	Yellow + green	Yellow + yellow	Orange + orange
Gly	Blue	Orange	Blue	Yellow
Asp-NH$_2$	Yellow	Orange	Yellow	Yellow
Phe	UV absorbent	UV absorbent	Yellow	Yellow
Asp	Blue	Green	Yellow	Yellow
Ala	Blue	Orange	Orange	Blue
Val	Yellow	Yellow	Blue	Blue
His	Yellow + blue	Blue + yellow	Yellow + yellow	UV absorbent + green
Lys	Blue + blue	Orange + orange	Orange + orange	Red + red
Met	Yellow		Blue	
Norleu	Yellow		Blue	Blue
Orn	Orange + red	Orange + orange	Red + red	UV absorbent + red
Ser	Yellow	Blue		Blue
Trp	Blue	Blue	Blue	Blue
Cys	Blue	Blue	Blue	Blue
Leu	Yellow	Yellow	Blue	Blue

placements for the phenylisothiocyanate used in the classical version of the EDMAN degradation.

Their sensitivity to detection is of the order 10^{-2}–10^{-4} μmoles; some of the derivatives formed are UV absorbing and some show a yellow-to-blue fluorescence in UV light. The important fact about the luminescence of 1-naphthylisothiocyanate derivatives is the change of the colour of the luminescent spot with pH which is of high diagnostic value. Our findings on all three types of derivatives are summarized in Tables I and II. Both chromatography and electrophoresis were used for separation; in the electrophoretic separation pyridine–acetate buffer (0.7 M pyridine, 1.24 M acetic acid) was used. Samples were applied to Whatman No. 1 paper and electrophoresis was carried out for 6 h at 0.6 V/cm. For chromatography (Whatman No. 1 paper) the following solvent systems were used:

(1) phosphate buffer pH 6.0; μ = 0.1
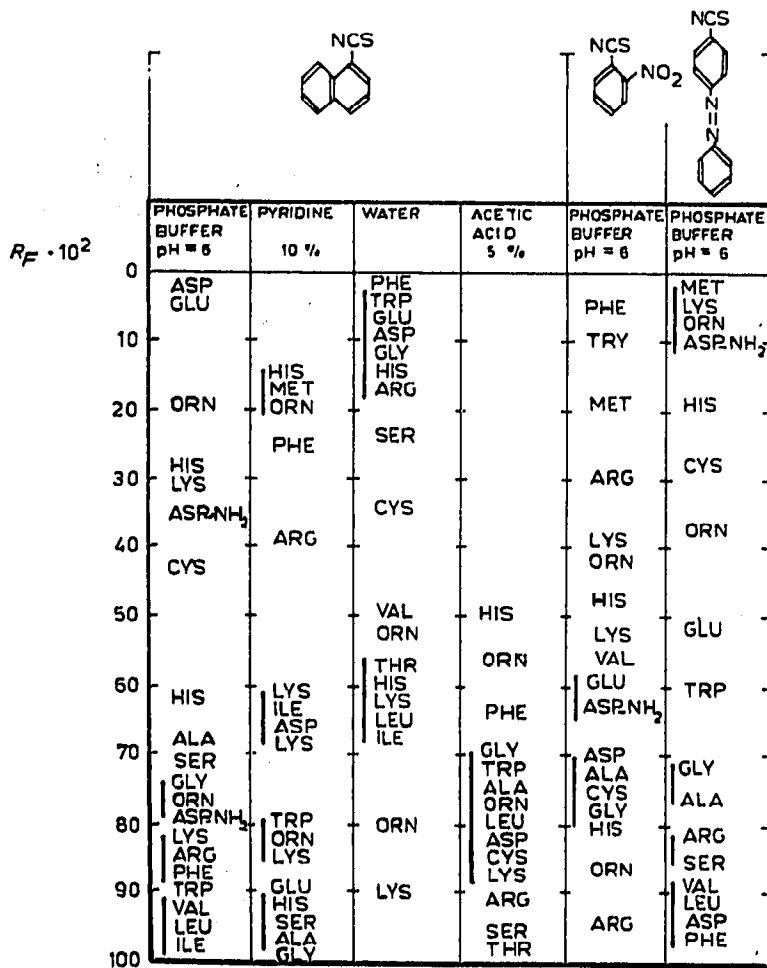(2) 10% pyridine
(3) 5% acetic acid
(4) distilled water

Fig. 1. Chromatographic behaviour of thiohydantoins derived from the compounds indicated in aqueous media. Solid lines indicate amino acid derivatives not separated.

(5) chloroform–ethyl alcohol–acetic acid (8:4:1)
(6) chloroform–n-butyl alcohol–acetic acid (8:4:1)
(7) chloroform–n-amyl alcohol–acetic acid (8:4:1)
(8) chloroform–ethyl alcohol–acetic acid (10:2:1)
(9) benzene–n-amyl alcohol–acetic acid (8:5:1)

Solvents (5)–(9) were used for the separation of 1-naphthylisothiocyanate derivatives only. The overall scheme of chromatographic mobilities is summarized in Figs. 1 and 2. Data related to the electrophoretic behaviour are presented in Fig. 3.

As expected none of the solvent systems produced a complete separation of all the amino acid derivatives; however stepwise chromatography in different systems or two-dimensional chromatography and electrophoresis permits the identification of a particular amino acid derivative. Of the reagents used 1-naphthylisothiocyanate is obviously the best because, in addition to the diagnostic properties mentioned above, the sensitivity in the chromatographic and electrophoretic separation equals that of the DANSYL derivatives. Thus if one takes the amount necessary for the N-terminal amino acid determination and splits off the N-terminal amino acid in the form of the 1-naphthylisothiocyanate derivative, there should be enough for at least
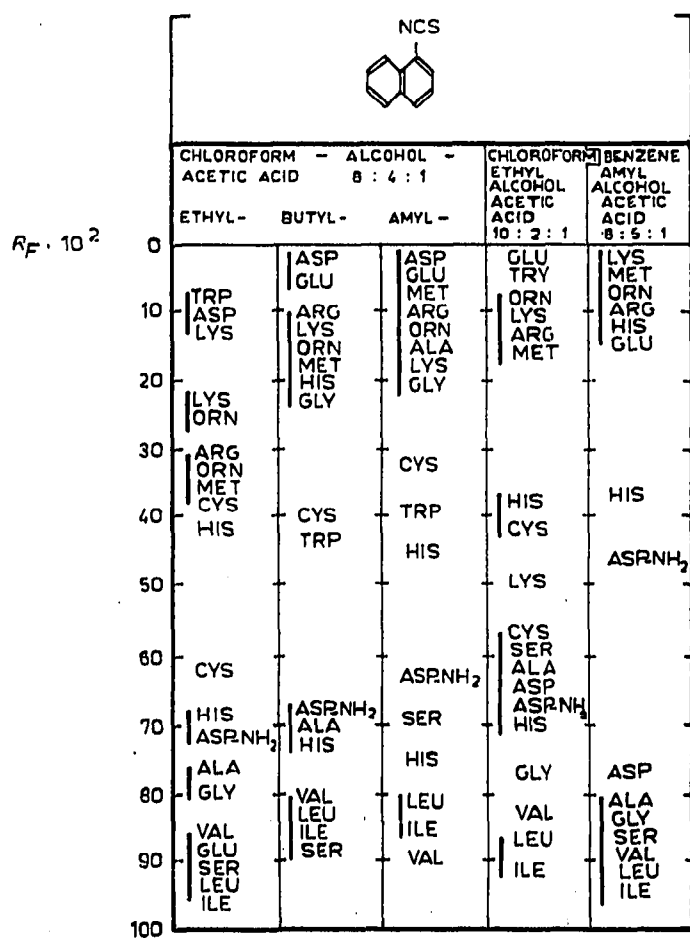

Fig. 2. Chromatographic behaviour of thiohydantoins derived from 1-naphthylisothiocyanate in solvents with lower polarity. Solid lines indicate amino acid derivatives not separated.

six to eight chromatographic tests. Thus there is always plenty of material for identification purposes and, even in cases where it is not possible to control the sequential procedure by concurrent amino acid analysis, the possibility of introducing mistakes into the sequence analyzed is decreased to an absolute minimum.

Some amino acids are recognizable at once: sulphur-containing amino acids, and those with aromatic rings in their molecule, if treated with 1-naphthylisothiocyanate, show a very intensive blue luminescence, while the others are yellow in most
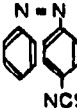
AMINO ACID	NCS	NCS NO	N=N NCS
GLU	0.32	2.54	2.54
HIS	6.7 8.7	4.26 6.66 10.3	8.2
ALA	3.38	2.73	3.5
CYS	6.7	5.5	2.87
ARG	2.2 6.49	3.94 6.26	7.6
VAL	3.0	2.78	3.2
MET			3.2
TRP	2.78	7.1	3.0
LYS	4.9 8.2	2.87 6.3	8.3
PHE		6.9	3.2
ASP		1.2	1.7
ORN	1.99 8.4	2.68 6.52	8.5 9.6
SER			4.4
ASP-NH$_2$	3.1	2.59	
GLY	3.0	2.85	3.38
LEU			3.2

Fig. 3. Electrophoretic mobilities of thiohydantoins derived from aromatic isothiocyanates indicated above (cm/sec·10^5) in 1.24 M acetic acid + 0.7 M pyridine. Paper: Whatman No. 1, 0.6 V/cm.

media (see Table II). Some amino acids result in multiple spots which are again an important indication for the final identification of the amino acid; for instance ornithine and lysine both result in two orange fluorescent spots in phosphate buffer pH 6. However if they are transferred into 5% acetic acid the spots turn blue in the case of lysine while the ornithine spot with the higher chromatographic mobility becomes red and that with lower mobility does not change colour. In most practical cases, three chromatograms (phosphate buffer pH 6; 5% acetic acid and 10% pyridine) together with one electrophoretic run are sufficient for identification. The total amount obtained after degradation allows at least two additional runs and is usually

saved for unpredicted situations. The result of the identification is then compared with the identification obtained *via* the DANSYL derivative.

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