THIOLYSIS OF SOME DINITROPHENYL DERIVATIVES OF AMINO ACIDS

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FDNB* has been widely used in structural and functional studies of peptides and proteins (Sanger, 1945; Hirs et al., 1961; Sokolovsky et al., 1964; Di Prisco, 1967). Being a reactive aryl halide, this reagent may react with several of the functional groups of proteins such as α- and ε- amino groups, imidazoles, sulfhydryls and aliphatic or phenolic hydroxyls. This paper describes a method for quantitative removal of dinitrophenyl groups from histidine, tyrosine and cysteine side chains. The reaction is referred to as "thiolysis" since cleavage is brought about by thiols (e. g. 2-mercaptoethanol). In view of the mild conditions under which the reaction proceeds (aqueous medium, pH = 8.0 and 22^{o}), it may find a variety of applications in peptide and protein chemistry.

MATERIALS AND METHODS

S-DNP-2-mercaptoethanol was synthesized as follows: A solution of FDNB (1.86 g in 20 ml of ethanol) was mixed with a solution of 2-mercaptoethanol (0.78 g in 10 ml ethanol). The reaction mixture was kept for two hours at room temperature in the dark and then concentrated in vacuo (40^{o}) until a yellow oil was formed. Upon trituration with water (20 ml) yellow crystals were formed which were recrystallized three times with 25% ethanol in water. The crystalline material was dried in vacuo m. p. 97^{o}. Yield 2.44 g (94%).

Analysis: calculated for $C_8H_8N_2O_5S$

calc: C = 39.35%; H = 3.30%; N = 11. 47%; S = 13.10%

found: C = 39.38%; H = 3.21%; N = 11.65%; S = 12.95%

The spectral properties of S-DNP-2-mercaptoethanol are given in figure 1. This compound is readily soluble in ether and can therefore be easily extracted with ether from aqueous

*Abbreviations: FDNB, 1-fluoro-2, 4-dinitrobenzene; DNP, 2, 4-dinitrophenyl; ME, 2-mercaptoethanol.

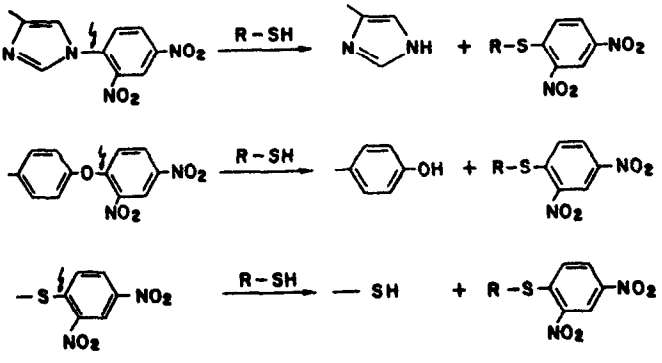
solutions.

$N_{(Im)}$-DNP-histidine and $N_{(\alpha)}$-DNP-histidine were prepared according to the literature (Siepmann and Zahn, 1964; Zahn and Pfannmüller, 1956). All other DNP derivatives were purchased from Mann. Amino acids were obtained from Light and Co., 2-mercaptoethanol, 3-mercaptopropionic acid and cysteamine · HCl from Eastman. DNP-derivatives of amino acids and of 2-mercaptoethanol were identified on paper chromatograms by their strong absorption under ultraviolet light. Their amount in reaction mixtures was determined after chromatography, by extraction with 1% $NaHCO_3$ (1 hour; 37°) and measuring the optical density at 350 mμ (Bailey, 1967). Compounds with a free α-amino group were determined on paper chromatograms by quantitative ninhydrin assay (Shaltiel and Patchornik, 1963). Derivatives of histidine or tyrosine with an unprotected side chain were revealed with the Pauli reagent (Alexander and Block, 1960). $N_{(\alpha)}$-DNP-cysteine was determined as its S-carboxymethyl derivative (Bailey, 1967).

Spectrophotometric measurements were performed with a Cary model 14 spectrophotometer.

RESULTS

When di-DNP-derivatives of histidine, tyrosine or cysteine were incubated with an excess of 2-mercaptoethanol, two yellow compounds were obtained in each case which were identified as S-DNP-2-mercaptoethanol and the appropriate DNP-amino acid with a free imidazole, phenolic or sulfhydryl* group. Schematically, the cleavage that occurs (thiolysis) can be described as follows:



*Together with N-DNP-cysteine some N, N'-di-DNP-cystine was also formed due to partial oxidation.

These reactions were demonstrated using the following model compounds: $N_{(a)}$, $N_{(Im)}$-di-DNP-histidine, $N_{(Im)}$-DNP-histidine, N,O-di-DNP-tyrosine, O-DNP-tyrosine and N,S-di-DNP-cysteine. In each case the formation of S-DNP-2-mercaptoethanol was established by preparative paper chromatography (solvent-isoamyl alcohol : pyridine : water, 35 : 35 : 30), spectrophotometric characterization (figure 1) and determination of the mixed melting point with a synthetic sample after 1-3 recrystallizations. The amino acid derivatives with a non-dinitrophenylated side chain were determined after chromatography using three different solvent systems (n-butanol : acetic acid : water, 4 : 1 : 4; isoamyl alcohol : pyridine : water, 35 : 35 : 30; sec. butanol saturated with 1% NH_4OH).

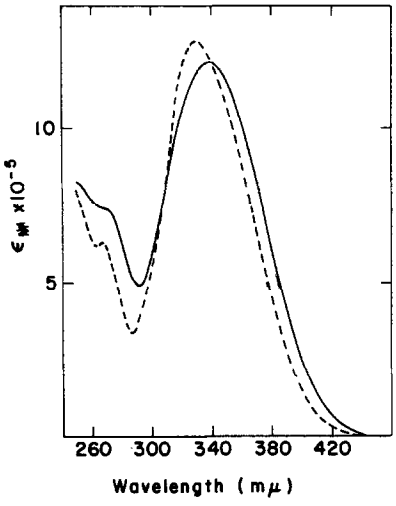


Fig. 1: Spectra of S-DNP-2-mercaptoethanol: (———) in water, pH = 7.0; (- - - -) in ethanol.

Thiolysis proceeds under very mild conditions. At pH = 8.0 and 22° (one hour incubation) the reaction is completed (90-100% yield) with less than 100 moles of 2-mercaptoethanol per mole of the DNP compound (figure 2). In the case of di-DNP-histidine, an excess of 5-6 moles of the thiol was sufficient to attain completion of the cleavage. In addition to 2-mercaptoethanol, 3-mercaptopropionic acid and cysteamine were also shown to cause cleavage of the DNP group from the model DNP compounds mentioned above. In the case of cysteamine, however, the S-DNP product formed may undergo a rearrangement to the N-DNP derivative (Burchfield, 1958).

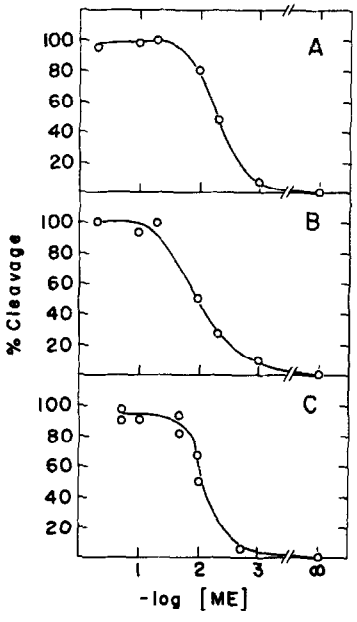


Fig. 2: Extent of thiolysis as a function of the concentration of 2-mercaptoethanol. (A) $N_{(\alpha)}$, $N_{(Im)}$ di-DNP histidine (5×10^{-3}M). % Cleavage followed by determination of $N_{(\alpha)}$-DNP-histidine. (B) O-DNP-Tyrosine (1.15×10^{-3}M). % Cleavage followed determination of free tyrosine. (C) N,S-di-DNP cysteine (2×10^{-3}M). % Cleavage followed determination of S-DNP-2-mercaptoethanol.

Thiolysis of O-DNP-tyrosine or $N_{(Im)}$-DNP-histidine residues can be followed spectro photometrically. As seen in figure 3, thiolysis of such derivatives results in an increase of their U. V. absorption at 340 mμ due to the formation of S-DNP-2-mercaptoethanol. O-DNP-tyrosine and $N_{(Im)}$-DNP-histidine have themselves a low absorption at this wavelength (Alexander and Block, 1960; Siepmann and Zahn, 1964).

It should be emphasized that under the conditions described above no thiolysis of DNP-amino groups occurs. Both DNP-alanine and ε-DNP-lysine were unaffected by treatment with 2-mercaptoethanol.

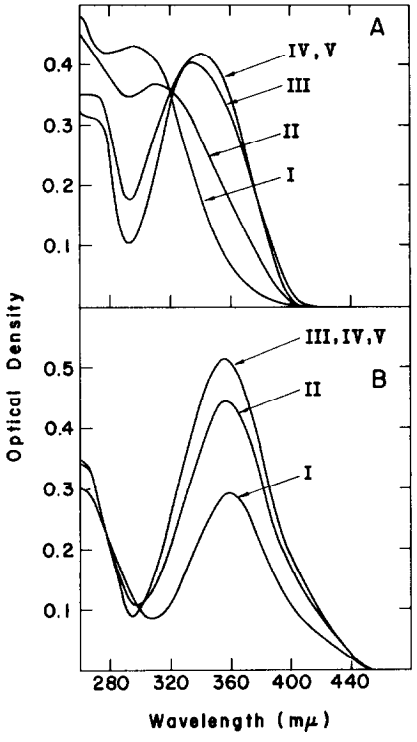


Fig. 3: Changes in the U. V. spectrum of DNP derivatives upon thiolysis. (A) O-DNP-Tyrosine: alone (I) and in the presence of ME, 1 Mole/Mole (II), 5 Moles/Mole (III), 10 Moles/Mole (IV), 50 Moles/Mole (V). (B) $N_{(\alpha)}$, $N_{(Im)}$-di-DNP-histidine: alone (I) and in the presence of ME, 1 Mole/Mole (II), 2 Moles/Mole (III), 10 Moles/Mole (IV), 20 Moles/Mole (V).

The reactions were allowed to proceed for 4 hours (pH = 8.0 and 22°) and then the spectra were recorded.

DISCUSSION

The method described in this paper for mild removal of DNP groups from imidazoles, phenolic hydroxyls, and sulfhydryls converts dinitrophenylation into a reversible technique for the blocking of these functional groups. As such, dinitrophenylation may find a variety of uses in the chemical modification of proteins and in the synthesis of peptides and polyamino acids.

In this connection it should be noted that dinitrophenylation can be selectively directed to SH groups by performing the reaction at pH = 5-6 (Zahn and Traumann, 1954).

The findings presented here should be considered when dinitrophenylated proteins are reduced with thiols, e.g., for the cleavage of disulfide bridges. This treatment would also remove the labeling DNP groups on histidine and tyrosine residues. Preliminary experiments (Shaltiel and Givol, 1967) with dinitrophenylated normal γ-globulin or anti-DNP antibodies indicate indeed that O-DNP-tyrosine residues are unmasked when separation of chains by reduction of S-S bonds with thiols is attempted.

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