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1,2,4-TRINITROBENZENE AS A THIOL REAGENT

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The 2,4-dinitrophenylation of thiol or amino group with 1,2,4-trinitrobenzene proceeded quantitatively at pH 8.5 and 30 °C. The rate of S-dinitrophenylation was $\approx 10^4$ times faster than that of N-dinitrophenylation. So this reaction can be used for both the determination of thiol even in the presence of large excess amine and the specific modification of thiol in proteins.

The 2,4-dinitrophenyl(DNP)ation with 1-fluoro-2,4-dinitrobenzene (FDNB) originally introduced for the N-terminal analysis of peptide,[1] has been used for the chemical modification of proteins.[2-4] FDNB as well as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole,[5] however, reacts with various nucleophiles such as amino, thiol or phenolic hydroxyl group in proteins according to their microenvironmental states. Parker et al.[6] reported that the reactivity of 1,2,4-trinitrobenzene (TNB) is comparable to that of FDNB in the nucleophilic substitution of aniline. This letter describes that TNB is a reagent having high specificity to thiol rather than amine.

TNB was prepared from 2,4-dinitroanisole, according to the Borsche's method[7] with some modifications. After hydroxylaminolysis of the ether linkage, crude DNP-hydroxylamine was oxidized to TNB by refluxing in fuming HNO_3 (d=1.52) for much longer period (20 h) and the oily product was crystallized from 50% HNO_3[6] to give pale yellow crystals with mp 60-61 °C in 87% yield. Found: C, 33.85; H,1.51; N, 19.60%. Calcd for $C_6H_3N_3O_6$: C, 33.83; H, 1.41; N, 19.71%. IR ν_{max}(KBr): 1550 and 1350 cm^{-1} ($-NO_2$). ^{1}H-NMR δ ($CDCl_3$): 8.14 (d, J=8.8 Hz), 8.63 (dd, J=8.8, 2.2 Hz) and 8.84 (d, J=2.2Hz). MS (m/e): 213 (M^+). UV (10% acetonitrile–90% M/10 NaH_2PO_4 -M/20 $Na_2B_4O_7$ buffer pH 8.5, 1 M=1 mol dm^{-3}): λ_{max}=234 nm (ε=1.32x10^4 $M^{-1}cm^{-1}$).

An equimolar mixture of TNB in methanol and L-alanine (Ala) or N-acetyl-L-cysteine (NAC) in 5% $NaHCO_3$ solution gave the NH- or S-DNP derivative in yield more than 75% with stirring for 2 h at room temperature. The IR-spectra of these products (400—4000 cm^{-1}) agreed completely with those of authentic DNP-Ala and DNP-NAC prepared with FDNB, respectively.

The reaction could be monitored directly by the spectrophotometry of a reaction mixture at λ_{max}, 360 nm for DNP-NHR or 339 nm for DNP-SR in lower concentration of substrates (10^{-3}—10^{-5} M) as indicated in the reaction of FDNB.[8] The DNPation with TNB proceeded under the liberation of the equimolar nitrite ion. Table 1 shows the time course of DNPation of NAC with TNB.

The S- or N-DNPation by TNB proceeded with the rate of reaction (v) as v=k[S][R], where [S] and [R] were the concentrations of substrate and reagent, respectively. The apparent second-order rate constant k was dependent on pH of the reaction mixture, and making higher the pH by 1 brought about 8 times increment of k for both S- and N-DNPations, in the pH range from 6 to 9. These results were also similar to the DNPation by FDNB. In Table 2 are summarized the apparent second-order rate constants for the reaction of TNB with SH (NAC), NH_2 (Ala), NH (L-proline), imidazole and phenolic OH (p-hydroxybenzoate) at pH 8.5 and 30 °C, together with the corresponding values obtained on the DNPation with FDNB. These results indicate that the reactivity of TNB with substrates except NAC was similar to or slightly less than that of FDNB. Note that TNB, for the reaction with

Table 1. The amounts of S-DNP-NAC and NO_2^- produced during the reaction of TNB with NAC in M/10 NaH_2PO_4-M/20 $Na_2B_4O_7$ buffer containing 10% acetonitrile at pH 8.5 and 30 °C.

Time min	S-DNP-NAC [a] (10^{-5} M)	NO_2^- [b] (10^{-5} M)
0	0	0
2/3	2.2	2.2
1	2.7	2.8
2	3.8	3.8
4	4.8	4.6
8	5.1	5.1
16	5.4	5.2
32	5.4	5.3

The initial concentrations of TNB and NAC were 5.4×10^{-5} and 9.8×10^{-5} M, respectively.
a) Determined spectrophotometrically: the molar absorption coefficients of S-DNP-NAC and TNB were 1.0×10^{4} and 8.0×10^{2} $M^{-1}cm^{-1}$ at 339 nm in the conditions used, respectively.
b) Determined by the method of Nishimura et al.[9]

Table 2. The reactivity of TNB and FDNB with SH, NH_2, NH, phenolic OH and imidazole group shown as the reaction rate constant k at pH 8.5 and 30 °C.[a]

Substrate	$k/M^{-1}min^{-1}$ TNB	FDNB
NAC	8.5×10^{3}	5.0×10^{2}
Ala	2.8×10^{-1}	3.2×10^{-1}
L-Proline	1.1	2.4
p-Hydroxybenzoate	1.7	1.9
Imidazole	2.5×10^{-2}	1.0×10^{-1}

a) The mixture of 1 ml of reagent (5×10^{-4} M in acetonitrile) and 9 ml of substrate solution (1×10^{-4}—1×10^{-2} M in M/10 NaH_2PO_4-M/20 $Na_2B_4O_7$ pH 8.5) was incubated at 30 °C and the DNPation was monitored spectrophotometrically at 339 nm for S-DNP, 360 nm for NH-DNP or 387 nm for N-DNP. In the case of imidazole or phenolic OH, the reaction rate constant was determined by measuring the amount of remaining reagent, accoding to the method of Rossi et al.[10]

NAC, was 17 times more reactive than FDNB. So TNB will be a reagent for the S- rather than the N-DNPation.

For the spectrophotometric determination of thiol, 1 ml of 2.1×10^{-3} M TNB in acetonitrile was mixed with 9 ml of a sample solution containing M/10 NaH_2PO_4-M/20 $Na_2B_4O_7$ pH 8.5, and then increment of the absorbance at 339 nm was measured for 10 min. On a thiol (NAC) solution containing large excess amine (Ala), the rapid increase of the absorbance by the S-DNPation was followed by the gradual increase of the absorbance due to the N-DNPation (Fig. 1(A)). The value of thiol concentration can be obtained by extrapolating the absorbance of 5 to 10 min to 0 time even under these conditions (Fig. 1(A)). As illustrated in Fig. 1(B), a linear relationship between the increment of the absorbance (ΔA) and the initial concentration of NAC was observed up to 1.5×10^{-4} M, indicating that TNB can be used as an effective reagent for the determination of thiol even in the presence of excess amine.

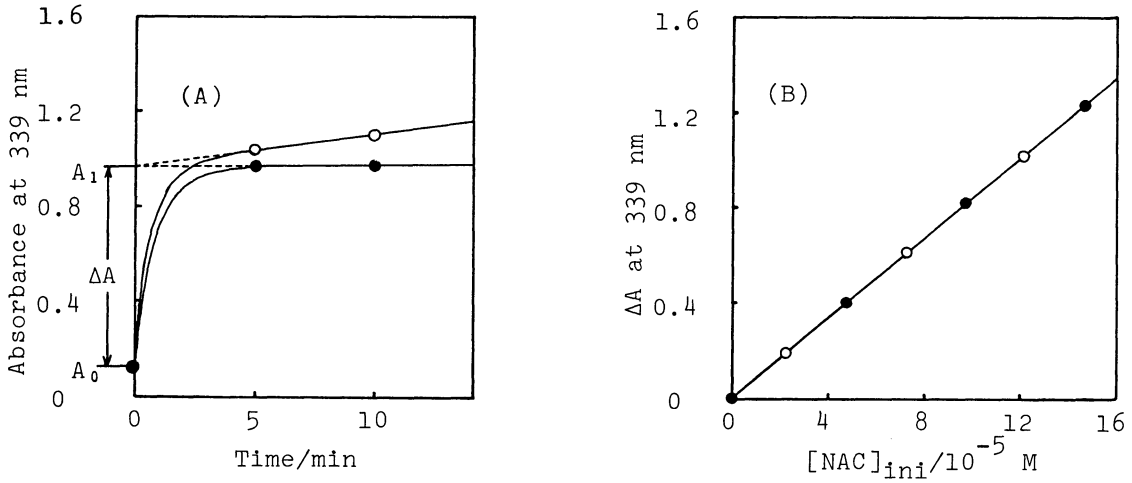

Fig. 1(A)(B). The spectrophotometry of NAC with TNB, in the presence (○) or the absence (●) 1.6×10^{-2} M Ala. (A) $[NAC]_{ini}$ in the sample solution was 9.8×10^{-5} M. $\Delta A=A_1-A_0$, A_1: the absorbance of the extrapolating point, A_0: the absorbance of TNB only. (B) $\frac{\Delta A}{[NAC]_{ini}}\times\frac{10}{9}=9.3\times10^3$ is close to $\Delta\varepsilon$, 9.2×10^3 $M^{-1}cm^{-1}$, the difference between the molar absorption coefficients of S-DNP-NAC (1.0×10^4) and TNB (8.0×10^2) at 339 nm in 10% acetonitrile—90% M/10 NaH_2PO_4-M/20 $Na_2B_4O_7$ buffer pH 8.5.

This procedure could be used for the spectrophotometric assay of thiol residues in proteins, too. All of proteins were reagent grade of Sigma. The values obtained with the TNB method, on proteins denatured with 1% sodium laurylsulfate, were more close to those calculated from their primary structures than those obtained with the Ellman's method,[11] as shown in Table 3.

Table 3. Spectrophotometric determination of thiol residues in proteins.

Protein	Thiol residues (moles per mol)		
	By TNB method	By Ellman's method	From the primary structures
Ovalbumin (hen egg white)	3.9	3.8	4[12]
Serum albumin (bovine)	0.90	0.76	1[13]
Aldolase (rabbit muscle)	30	27	32[14]
Lysozyme (hen egg white)	< 0.05	< 0.1	0[13]
Trypsin (bovine pancreas)	< 0.05	< 0.1	0[13]

In the case of native proteins, on the contrary, most of thiol residues were masked from the S-DNPation with TNB due to the folded structure of proteins under the conditions used (pH 8.5 and 30 °C). For example, on the S-DNPation of native hen ovalbumin and rabbit muscle myosin, were modified only 1 out of 4[15] and 2 out of 44 thiol residues,[16] respectively. Therefore, TNB will be a valuable site specific thiol reagent for protein modification, too.

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