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Reactions of 2,4,6-Trinitrobenzenesulfonate Ion with Amines and Hydroxide Ion†

Gary E. Means,* W. I. Congdon, and M. L. Bender

ABSTRACT: The reactivity of 2,4,6-trinitrobenzenesulfonate ion (TNBS) with hydroxide ion and a series of primary amines has been examined as a function of pH, nucleophile concentration, ionic strength, and temperature. Its reaction with hydroxide ion is first order in both hydroxide ion and TNBS with a second-order rate constant of $6.33 \pm 0.16 \times 10^{-2}$ M^{-1} sec^{-1} at 25° (μ = 1.0), and appears to follow the usual path for nucleophilic aromatic substitution involving the rate-limiting formation of a tetrasubstituted ring carbon intermediate. The reaction of TNBS with primary amines having pK_a values higher than ~8.7 proceeds by a similar mechanism. Reactivity increases as a function of amine basicity according to the relationship log k (M^{-1} sec^{-1}) = 0.23· pK_a − 1.18. Similar amines with lower pK_a values follow a complex rate law apparently due to partitioning of the tetrasubstituted intermediate between breakdown to products and reversion to reactants. The reaction of TNBS with amines is favored as compared to its reaction with hydroxide ion by low ionic strength, low temperature, and pH values at least 0.5 unit below the amine pK_a value. Complexation of *N*-alkylpicramides by sulfite ion involves its addition to ring carbon number three. The second-order rate constant for complexation of sulfite with *N*-trinitrophenyl-β-alanine is 5.4×10^3 M^{-1} sec^{-1} at pH 8.0 (μ = 0.50) and 25.4°. Reactions of TNBS with proteins are discussed based on these results.

2,4,6-Trinitrobenzenesulfonate (TNBS)[1] was shown by Okuyama and Satake (1960) and Satake *et al.* (1960) to react with amino groups of amino acids, peptides, and proteins. Little or no reaction was observed with several other nucleophilic groups. TNBS has since become an important protein- and peptide-modifying reagent in large part due to this reaction selectivity. Hydrolysis of TNBS, for example, is relatively slow and does not usually interfere greatly with spectrophotometric determination of its reaction with amines (Habeeb, 1966; Freedman and Radda, 1968; Fields, 1971; Means and Feeney, 1971). In this study we have quantitatively evaluated parameters which influence the reactivity of TNBS with amines and with hydroxide ion and also attempted to characterize and assess the importance of complexes formed by *N*-alkylpicramides and sulfite ion, the two products of the TNBS reaction with amines.

Experimental Section

Materials. TNBS (picrylsulfonic acid, sodium salt dihydrate) was purchased from Aldrich Chemical Co. and used without additional purification. Neopentylamine was a product of Alfred Bader Chemicals. Other amines were reagent grade or better, obtained as free amines or as hydrochloride salts. TNP-amines were prepared by the reaction of TNBS with the corresponding amine or amine hydrochloride in water containing a slight excess of sodium carbonate. After the reaction was complete (several hours at room temperature for the less reactive amines) the deep red-orange solutions were acidified with dilute HCl and the resulting precipitate was filtered and washed with cold water. Recrystallization was accomplished in ethanol or ethanol–water. TNP derivatives of *tert*-butylamine, *N*,*N*-dimethylamine, and piperidine were recrystallized from cyclohexane in order to separate them from unknown high melting contaminants. Products were examined by nuclear magnetic resonance (nmr) and their melting points compared to previously published values. Nmr peak positions, intensities, and splittings were consistent with the expected structures.

Methods. Reactions of TNBS with hydroxide ion were monitored spectrophotometrically with a Cary 14 spectrophotometer from the increase in absorbance at 355 nm due to the formation of picrate ion. Pseudo-first-order conditions with hydroxide ion in large excess were maintained in all cases. The formation of product appeared completely first order. Reactions of TNBS with amines were followed similarly at the absorption maximum of the respective TNP–amine sulfite complexes at approximately 410 nm for most amines. Under pseudo-first-order conditions in the presence of excess amine the formation of product appeared first order except during the initial 0.05–0.1 absorbance change wherein the equilibrium between the picramide and sulfite complex was established. Addition of 10^{-4} M sulfite ion rendered the appearance of product first order throughout but was not used routinely as this early part of the reaction was observable only with very slow reactions. Rate constants of the amine reactions determined in phosphate buffer (μ = 0.5, pH 12.6) were identical with those determined in 10^{-3}–10^{-2}

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[1] Abbreviations used are: TNBS, 2,4,6-trinitrobenzenesulfonate ion; TNP, 2,4,6-trinitrophenyl; μ = ionic strength.

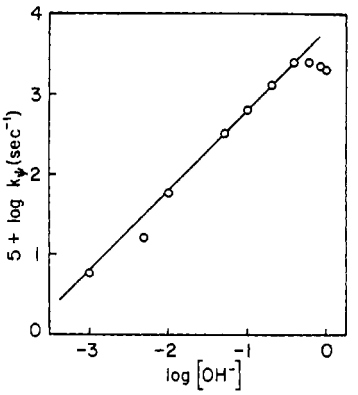


FIGURE 1: First-order rate constants for hydrolysis of TNBS *vs.* log hydroxide ion concentration at 25.4° and an ionic strength of 1.0.

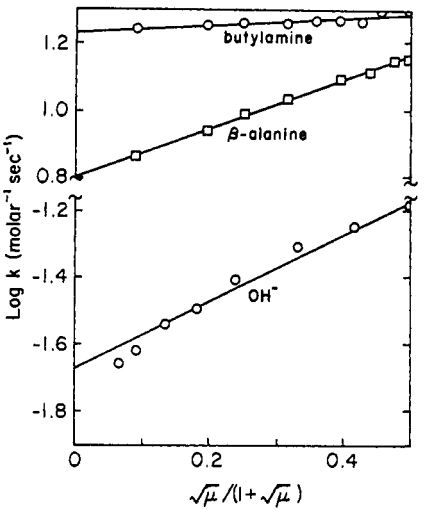


FIGURE 2: Extended Debye–Hückel plot for *n*-butylamine, β-alanine, and hydroxide ion reacting with TNBS. Log of second-order rate constants at 25.4° are shown as a function of ionic strength adjusted with KCl.

M KOH–0.50 M KCl. Amine concentrations were varied from 10^{-4} to 10^{-1} M to achieve convenient reaction rates. Individual amines were varied over at least a tenfold concentration range. Absorption maxima of TNP-piperidine and -proline were at 340 nm indicating they were largely uncomplexed by sulfite ion under the experimental conditions and were followed at that wavelength. All reactions were carried out at 25.4° in a thermostatted cell compartment, except as noted.

Nmr spectra of reaction mixtures, TNBS plus amine and picramide plus sulfite, were obtained with a Brucker HF-90 nmr spectrometer at 25° in 0.1 M Na_2CO_3–D_2O. Nmr spectra of isolated reaction products were obtained with a Varian T-60 nmr spectrometer at 37° in deuteriocholoroform or dimethyl-d_6 sulfoxide with tetramethylsilane as an internal standard.

Reactions of sulfite ion with TNP-β-alanine were followed with a Cary 14 recording spectrophotometer with a 0.1 absorbancy slide-wire under pseudo-first-order conditions with TNP-β-alanine in eightfold excess over sulfite ion and the pseudo-first-order rate constant evaluated for the case of two opposing reactions A + B $\rightleftarrows$ X using the integrated rate equation

$$\frac{x_e}{a_0} \ln \frac{x_e}{x_e - x} = kt$$

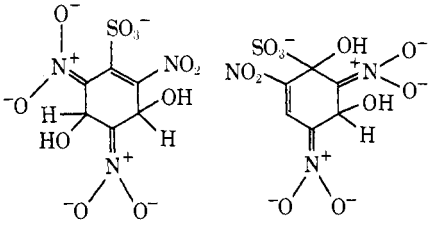
according to Laidler (1965). The integrated second-order rate equation

$$\frac{x_e}{(a_0^2 - x_e^2)} \ln \frac{x_e(a_0^2 - xx_e)}{a_0^2(x_e - x)} = kt$$

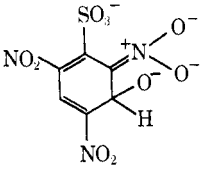
(Laidler, 1965) was used for the same system with the reactants at equal concentration.

Results

Reaction with Hydroxide ion. Plotting logarithms of the observed first-order rate constants for the hydrolysis of TNBS *vs.* log [OH$^-$] at constant ionic strength gives a straight line of unit slope (Figure 1). Second-order rate constants calculated from such plots decreased from 6.33 ± 0.16 × 10^{-2} M^{-1} sec^{-1} at μ = 1.0 to 1.78 × 10^{-2} M^{-1} sec^{-1} extrapolated to μ = 0. Above approximately 0.2 M [OH$^-$], the observed first-order reaction rates plateau and then decrease. In 4 M KOH, μ = 4.0, the apparent first-order rate constant decreases to about one-tenth its maximum value. The presence of an intense absorption centered at ~510 nm and a transient nmr signal at 5.60 ppm observed in these strongly alkaline solutions (*i.e.*, in D_2O: 4 M NaOD) appears to be due to an additional ionization either *via* addition of a second hydroxide ion to give the diadduct Ia or Ib similar to those described by Crampton and Gold (1966), Gitis *et al.* (1968) and Bowden and Cook (1971), or loss of a proton to give the trianionic species II.



Ia Ib



II

TABLE I: Activation Parameters for the Reaction of TNBS with Hydroxide Ion and β-Alanine.

	$\Delta H^{\ddagger}$ (kcal/mole)	$\Delta F^{\ddagger}$ (kcal/mole)[a]	$\Delta S^{\ddagger}$ (eu)
Hydroxide ion[b]	14.2	19.9	−19
β-Alanine[c]	8.5	16.0	−25

[a] 25.0°. [b] μ = 0. [c] pH 12.6 Na_2HPO_4–Na_3PO_4, μ = 0.49.

TABLE II: Second-Order Rate Constants for Reactions of Nucleophiles with Trinitrobenzenesulfonate.[a]

Nucleophile	k (M^{-1} sec^{-1})	pK_a	Nucleophile	k (M^{-1} sec^{-1})	pK_a
Ethylenediamine (H^+)[b]	1.70	7.5	2-Methylalanine	2.8×10^{-2}	10.2
Glycinamide	1.41	7.9	γ-Aminobutyrate	12.7	10.4
Glycylglycine	2.34	8.0	*tert*-Butylamine	7.46×10^{-2}	10.55
2-Bromoethylamine	4.80	8.5	*n*-Butylamine	19.2	10.7
Taurine	6.78	8.7	ε-Aminocaproate	15.5	10.75
2-Methoxyethylamine	8.87	9.2	Tris(hydroxymethyl)-aminomethane[b]	1.52×10^{-3}	8.1
Benzylamine	19.3	9.3	Dimethylamine	1.8×10^{-1}	10.64
Allylamine	12.1	9.5	Diethylamine	7.77×10^{-3}	11.0
Ethanolamine	9.7	9.5	Piperidine	3.45×10^{-2}	11.2
Glycine	11.85	9.6	Sarcosine	8.93×10^{-2}	10.0
Valine	3.05	9.6	Proline	3.81×10^{-2}	10.6
Alanine	6.17	9.7	Imidazole[b]	2.78×10^{-3}	7.0
β-Phenethylamine	23.9	9.8	Hydroxide ion[c]	1.78×10^{-2}	15.7
Neopentylamine	31.4	10.2	Phenol	Not detectable	10
β-Alanine	11.75	10.2			

[a] At 25.4° in μ = 0.49 Na_2HPO_4–Na_3PO_4 (pH 12.6) except as noted. [b] At pK_1 corrected for the degree of ionization. [c] Extrapolated to $\mu = 0$.

The effect of increasing ionic strength on the hydrolysis of TNBS is illustrated in Figure 2. Hydrolysis is enhanced at high ionic strength. The observed dependence indicated by the experimental points in this Debye–Hückel treatment fit a line of unit slope anticipated for rate-determining monoanion attack on a monoanion. Activation parameters for the reaction of TNBS with hydroxide ion are given in Table I.

Reaction with Amines. Second-order rate constants for the reaction of TNBS with a number of amines are given in Table II. As indicated, reactivity increases generally with basicity except that secondary amines and *tert*-alkylamines (primary) are comparatively quite unreactive. Deviations from a simple Brønsted relationship by other amines appear to result from differences in their reaction mechanisms.

Simple primary alkylamines with only one substituent on the β carbon other than hydrogen and with pK_a values greater than approximately 8.7 react with TNBS in proportion to their basicity. Reactivities differ according to the Brønsted relationship

$$\log k\ (M^{-1}\ sec^{-1}) = 0.23pK_a - 1.18 \qquad (1)$$

as indicated by the line shown in Figure 3 for simple neutral primary amines with pK_a values greater than 8.7. The reaction of these amines is first order in both amine and TNBS and is completely explained by the simple second-order rate law.

$$\frac{d[products]}{dt} = k[TNBS][amine] \qquad (2)$$

Only the unprotonated amine reacts as illustrated by the normal pH–log rate constant profile of β-alanine (Figure 4).

Many amines were found to deviate from the above Brønsted relationship. Coulombic interactions, for example, lower the reactivity of negatively charged amines, an effect which is most marked at low ionic strength. This sensitivity to ionic strength is indicated in the Debye–Hückel treatment for the reaction of TNBS with β-alanine (Figure 2). The sensitivity in this case as indicated by the observed slope is only slightly less (*i.e.*, slope = 0.73) than for reaction of TNBS with hydroxide ion although in β-alanine the negative charge is somewhat removed from the attacking amino group. The reactivity of neutral amines was not affected by changes in ionic strength (*i.e.*, see *n*-butylamine, Figure 2).

Amines with pK_a values less than approximately 8.7 also deviate from the given Brønsted relationship being less reactive than expected as compared to similar amines with higher pK_a values. As observed by Goldfarb (1966a,b) and Fields (1971) such amines appear to follow a complex rate law. The pH–log rate constant profile of glycinamide, for example, has an unusually low slope at pH values far below its pK_a (*i.e.* ~0.6), an inflection point suggesting a pK_a well above that of glycinamide and a slightly negative slope at higher pH values (Figure 4). Reactions with glycinamide appeared first order in respect to the appearance of product at all pH values examined; however, plots of first-order rate constants *vs.* glycinamide concentration at pH 8.0 showed slight curvature such that the reaction appears slightly less than first order in glycinamide above ~10^{-3} M. Activation parameters for the reaction of TNBS with β-alanine are given in Table I.

Reaction of Sulfite Ion with TNP-Amino Acids. Crampton (1967) has investigated complexes formed from several substituted trinitrobenzenes and sulfite ion. Picramide, *N*-methylpicramide and *N*,*N*-dimethylpicramide form covalent complexes by addition of sulfite ion to carbon 3 of the aromatic ring. At somewhat higher sulfite concentrations, a second sulfite ion may add to the remaining unsubstituted aromatic ring carbon to give a diadduct (eq 3).

Dilute aqueous solutions of TNBS plus β-alanine and of TNP-β-alanine plus approximately equimolar sulfite ion had essentially identical nmr spectra. With less than the stoichiometric amount of sulfite ion, nmr spectra show the aromatic ring protons of the uncomplexed TNP-β-alanine, a singlet at 8.93 ppm, to be partially eliminated and two new bands formed upfield at 6.22 and 8.44 ppm, coinciding very nearly to the reported chemical shifts for the ring protons of the

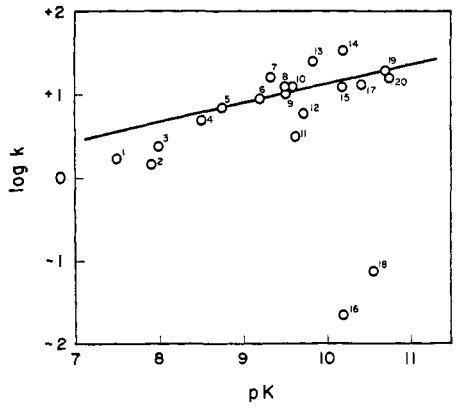


FIGURE 3: Brønsted plot of second-order rate constants for reaction of TNBS with amines *vs.* amine pK_a values at 25.4° and 0.49 ionic strength. (1) Ethylenediamine monocation, (2) glycinamide, (3) glycylglycine, (4) 2-bromoethylamine, (5) taurine, (6) 2-methoxyethylamine, (7) benzylamine, (8) allylamine, (9) ethanolamine, (10) glycine, (11) valine, (12) alanine, (13) β-phenethylamine, (14) neopentylamine, (15) β-alanine, (16) 2-methylalanine, (17) γ-aminobutyrate, (18) *tert*-butylamine, (19) *n*-butylamine, and (20) ε-aminocaproate.

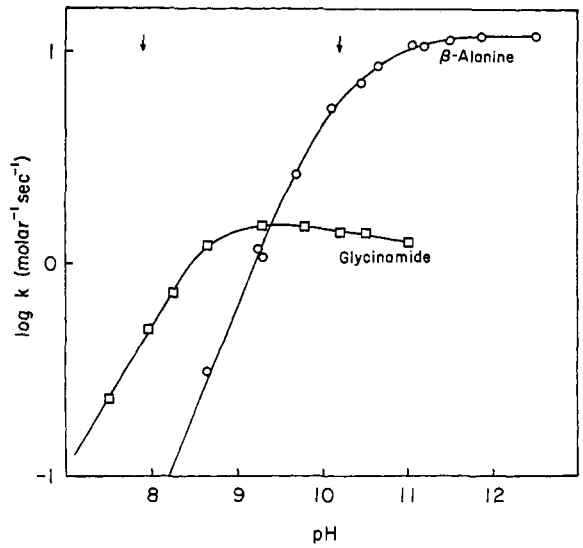
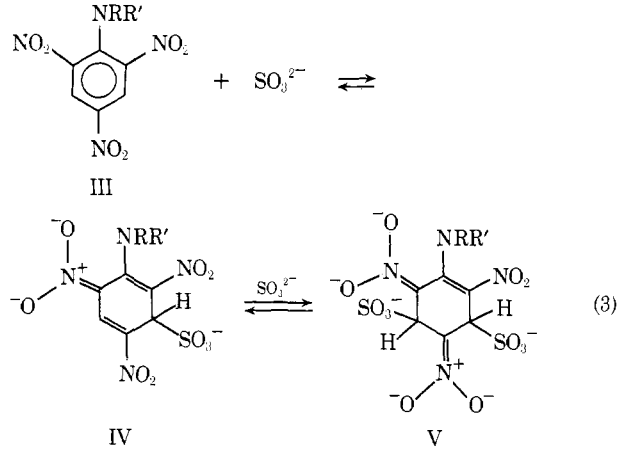


FIGURE 4: Plot of log second-order rate constants (observed first-order rate constants divided by the concentration of amine) *vs.* pH for the reactions of β-alanine and glycinamide with TNBS at 25.4° and 0.49 ionic strength. Arrows indicate literature pK_a values.



(3)

sulfite monoadduct of *N*-methylpicramide IV (R = CH_3, R′ = H) (Crampton, 1967). The uv–visible absorption maximum initially at 340 nm is displaced to longer wavelength (*i.e.*, ~ 420 nm), also indicating formation of a sulfite monoadduct IV. At higher sulfite concentrations resonances at 5.98 and 6.11 ppm appear at the expense of the former and the uv–visible spectrum undergoes a second shift (*i.e.*, λ_{max} = 403 nm) indicating formation of the sulfite diadduct V. Methylene proton resonances at 3.1 and 2.5 ppm were unchanged by addition of sulfite ion. At equimolar TNP-β-alanine and sulfite ion all of the above resonances were detectable, indicating significant formation of diadduct before formation of the monoadduct was complete. The equilibrium constant for formation of TNP-β-alanine sulfite monoadduct was estimated to be 2.6×10^4 M^{-1} on the basis of absorbancy changes with varying sulfite ion concentrations. This compares with 5.4×10^4 M^{-1} for *N*-methylpicramide (Crampton, 1967) and 2.1×10^4 M^{-1} and ~1×10^4 M^{-1} for α-acetyllysine and glycylglycine, respectively (Goldfarb, 1966b).

Complexation of TNP-β-alanine by sulfite ion is very fast. A second-order rate constant for the reaction at pH 8.0, 25.4°, in 0.10 M sodium phosphate was determined under pseudo-first-order conditions and under second-order conditions with reactants at equal concentration (Figure 5). Values of 5.5×10^3 and 5.25×10^3 M^{-1} sec^{-1}, respectively, were obtained.

Discussion

Reactions of TNBS with amino groups of proteins can be followed spectrophotometrically, due in part to its comparatively slow rate of reaction with hydroxide ion. Below pH ~13 the hydrolysis of TNBS is first order in both TNBS and hydroxide ion, obeying the simple rate law: rate = k[TNBS] × [OH^-]. The reaction rate has a strong dependence on ionic strength as expected for a reaction between two ions of like charge (see Figure 2). The relatively low reactivity of TNBS with hydroxide ion as compared to its reactivity with primary amines thus appears partially attributable to coulombic repulsion. The reactions of TNBS with other oxyanions are similarly very slow. In contrast, the electrically neutral activated aryl halides 2,4-dinitrofluoro- and 2,4-dinitrochlorobenzene hydrolyze at rates similar to their rates of reaction with primary amines (Biggi and Pietra, 1971; Dixon and Bruice, 1971). Reactions of picryl chloride with substituted phenolate ions, however, are much slower (*i.e.*, ~10^4) than its reactions with substituted anilines of similar basicity, indicating the importance of factors other than charge (Ryan and Humffray, 1967).

Reactions of strong nucleophiles with 1-substituted 2,4-dinitrobenzenes have been shown to proceed *via* a two-step mechanism with a tetrahedral intermediate (*i.e.*, for a Meisenheimer complex, see Buncel *et al.*, 1968, and Miller, 1968). Formation of this intermediate in the case of substituted mono- and dinitrobenzenes is slow as compared to its breakdown, giving rise in most cases to a simple second-order rate law. Hydrolysis of TNBS appears to follow such a simple two-step mechanism as illustrated in eq 4a. The kinetic results do not indicate, however, whether the attack of hydroxide ion (k_1) or breakdown of the intermediate complex to products (k_2) is rate limiting. In either case a simple second-order rate law could be obtained with the observed rate constant corresponding either to k_1 or $k_1k_2/(k_{-1} + k_2)$, re-

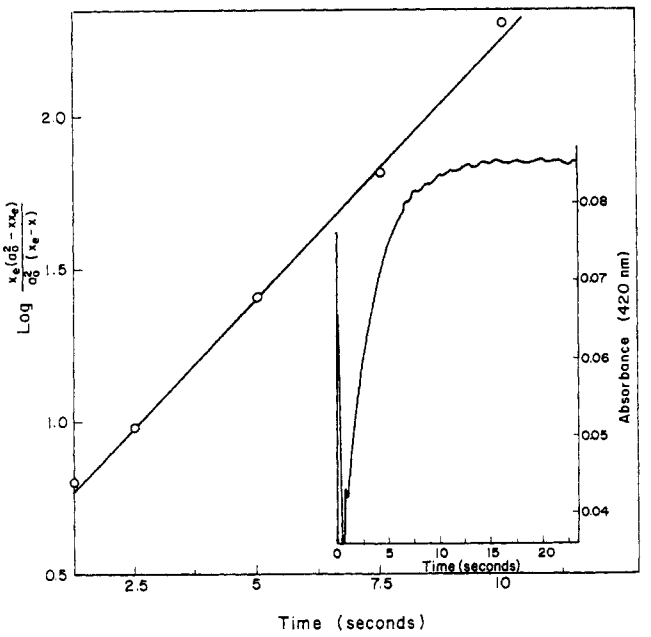
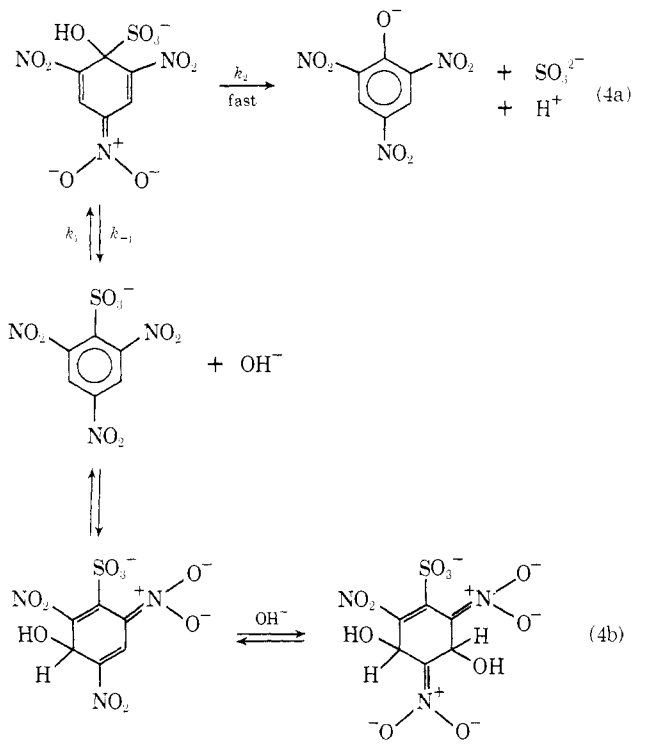


FIGURE 5: Reaction of TNP-β-alanine with sulfite ion showing change in absorbance at 420 nm as a function of time (inset) and plotted according to integrated rate equation for opposing reaction as described in Methods. At 25.4°, pH 8.0, and 0.50 ionic strength.



spectively (see eq 4a). Because hydroxide ion is comparatively much the poorer leaving group, breakdown of the intermediate should be almost exclusively by elimination of bisulfite ion (*i.e.*, $k_{-1} \ll k_2$) in which case the experimental second-order rate constant reduces to k_1k_2/k_2 (*i.e.*, k_1). The Debye–Hückel correlation is compatable with this form of the rate law without indicating anything about the relative magnitudes of k_1 and k_2. As previously noted for nucleophilic aromatic substitution of mono- and dinitro-X-benzenes, the rate-determining step largely depends on the leaving group while the reactivity of the aromatic ring is of little consequence since all steps are affected similarly and are mutually compensatory (Bunnett and Bernasconi, 1965; Kirby and Younas, 1970). In those cases, breakdown of intermediates is fast as compared to their formation (*i.e.*, $k_1 \ll k_{-1}$ and k_2), a situation which may not hold for analogous trinitrobenzene derivatives. Indeed, breakdown of such an intermediate has been reported to be rate limiting in the reaction of picryl chloride with hydroxide ion (Gaboriaud and Schaal, 1967), although more recent results indicate this conclusion is likely in error (Crampton *et al.*, 1971). Our inability to detect such an intermediate in the hydrolysis of TNBS as well as the failure of Crampton *et al.* (1971) to detect the analogous intermediate during hydrolysis of picryl chloride support k_1 being rate determining in the hydrolysis of both compounds.

Rates of TNBS hydrolysis decrease above ~0.2 M hydroxide ion similar to the decreases at high alkalinities reported for hydrolysis of picryl chloride (Gaboriaud and Schaal, 1967) and several 1-substituted-2,4-dinitrobenzenes (Bowden and Cook, 1971). Slower reaction rates under these conditions appears to result from a decrease in the concentration of TNBS brought about by its kinetically favored conversion into a nonproductive 3-hydroxy Meisenheimer complex (Servis, 1967; Bernasconi, 1971a; Crampton *et al.*, 1971) followed by further ionization to unreactive trianionic species (Ia, Ib, or II), for example, as indicated in eq 4b.

Reactions of TNBS with primary amines are generally two to three orders of magnitude more rapid than its reaction with hydroxide ion. Rates for individual amines differ considerably, however, as a result of steric and electronic effects. The primary amines studied cover a range of slightly more than 3 pK_a units and apparently encompass a change in reaction mechanism. Amines with pK_a values greater than approximately 8.7 and with only one α substituent (other than hydrogen) follow a common rate law and probably a common reaction mechanism. Rate-limiting nucleophilic attack is indicated for these strongly basic amines by the normal Brønsted dependence (Figure 3) similar to those obtained for the reaction of amines with 2,4-dinitrofluorobenzene (Bunnett and Garst, 1965) and 2,4-dinitrophenyl methyl phosphate (Kirby and Younas, 1970). The slope of 0.23 is considerably lower than reported in these other cases (*i.e.*, 0.63 (Biggi and Pietra, 1971) and 0.46 (calculated from the data of Kirby and Younas, 1970)), indicating relatively little bonding and little positive charge on the amine at the transition state, if nucleophilic attack is rate limiting. This would be consistent with the greater intrinsic reactivity of the trinitrobenzene ring, greater steric hindrance at the reaction center and the generally low slopes observed for nucleophilic attack on anions. In sharp contrast to these results, reactions of substituted anilines with picryl chloride have a very high dependence on basicity with a Brønsted slope of 1.1 (calculated from the data of Ryan and Humffray, 1967). The reaction of aniline with TNBS appears complex, however, having an unusual pH dependence and a considerably faster rate than expected for its basicity as compared to alkylamines (Fields, 1971).

The observed Brønsted relationship (eq 1) can be used to calculate the reactivity of similar amines whose pK_a values are known, or to estimate pK_a values of such amines on the basis of their reactivity with TNBS. Because intrinsic reactivities differ very little with differences in pK_a (*i.e.*, p$K_a \geq$ 8.7), observed reactivities in aqueous solutions are much more sensitive to differences in ionization. High reactivity at moderate to low pH values mainly reflects low ionization and hence low basicity rather than exceptional nucleophilicity.

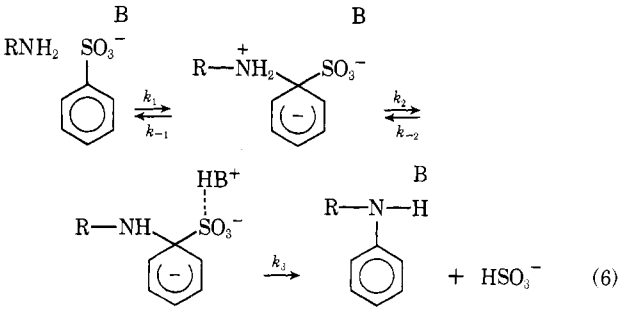
For reactions with low Brønsted slopes it is possible to *estimate* the pK_a value of a reacting group from its rate of reaction using the relationship

$$pK_a = \log\left(\frac{k_{lim}}{k_{obsd}} - 1\right) + pH \qquad (5)$$

for an ionizable group reacting in the unprotonated form, where k_{obsd} is the observed second-order rate constant and k_{lim} the second-order rate constant of the completely unprotonated group.[2]

For fast reacting amino groups in proteins (*e.g.*, see Haynes *et al.*, 1967; Goldfarb, 1970; Freedman and Radda, 1968; Fields, 1971) it is reasonable to assume the absence of large steric accessability factors that lower reactivity, in which case, in the absence of specific binding and other rate accelerating factors, reactivity is largely an expression of the pK_a. Equation 5 may be useful for estimating pK_a values, even though these other factors cannot be evaluated. Rate accelerations due to specific binding of TNBS can sometimes be evaluated from the concentration dependence of the observed rate constant (Marshall and Cohen, 1972), or by studying the binding of the similar but nonreactive compound, trinitrobenzoic acid (Goldfarb, 1970). This approach clearly is not warranted for slow-reacting amino groups. Using 17.0 M^{-1} sec^{-1} as k_{lim} for a lysine side chain[3] and 0.50 M^{-1} sec^{-1} for k_{obsd} reported at pH 7.6 for a single fast-reacting amino group of pancreatic ribonuclease (Freedman and Radda, 1968) gives an estimated pK_a of 9.1. Although it is not possible to identify the amino group involved, this value agrees well with 8.8 for the amino group most reactive with dinitrofluorobenzene determined by a much more laborious procedure (Murdock *et al.*, 1966). This approach ignores specific effects of protein structure on reactivity with TNBS, a potential source of large error, but provides approximate information not otherwise readily available. A more satisfactory, but also more laborious, general approach for determining protein amino group pK_a values has recently been described (Kaplan *et al.*, 1971).

The steeper Brønsted slopes previously reported for TNBS reacting with a series of amines came about by comparing sterically and mechanistically dissimilar amines (Freedman and Radda, 1968; Fields, 1971). Only amines with pK_a values greater than ~8.7 follow a simple rate law and are amenable to a Brønsted treatment. Those with lower pK_a values follow a complex rate law as revealed by their unusual pH–log rate constant profiles (Figure 4), by being detectably less than first order in amine at low pH values and by their low reactivity as compared to sterically similar amines with higher pK_a values (see Figure 3 and Table II). For these amines the mechanistic complexity appears to arise from partitioning of the intermediate Meisenheimer complex between two possible modes for its breakdown. The rate of its reversion to starting materials being comparable to the rate for its breakdown to products (*i.e.*, in eq 6, k_{-1} is comparable to k_3) and the observed rate constants are of the form



(6)

$$k_{obsd} = \frac{k_1 k_3}{\alpha k_{-1} + k_3}$$

where α is a complex variable containing terms for dissociation of both the attacking amine and the intermediate Meisenheimer complex and terms for general acid promotion[4] of the breakdown to products (see eq 6). The pK_a of such an intermediate is probably not greatly different than that of the attacking amine (Bernasconi, 1971a,b). Similar rate laws are observed for other nucleophilic aromatic substitutions involving the elimination of poor leaving groups (*e.g.*, Bunnett and Garst, 1965; Bunnett and Bernasconi, 1965; Kirby and Jencks, 1965; Bunnett and Hermann, 1970; Kirby and Younas, 1970). General base catalysis as observed in those cases, however, does not explain the unusual pH dependences observed in the present case. Present data are consistent with the mechanism illustrated in eq 6 where B is a base, either hydroxide ion, a buffer or a second molecule of amine, and HB^+ is the corresponding conjugate acid. The observed rate of reaction depends in such a mechanism on both the rate of formation of the intermediate (*i.e.*, k_1) and on the position of the equilibrium k_2/k_{-2} which controls its breakdown by the two alternate pathways k_3 and k_{-1}.

The Brønsted relationship indicated in eq 1 applies only to amines with pK_a values of 8.7 or greater and of the general type RCH_2NH_2 where R is a relatively unbranched, nonaromatic, and nonionic group. More than one substituent on the α carbon (other than hydrogen) retards reactivity, an effect most easily viewed as a steric effect. A similar large effect by α substituents has been observed on the reactivity of amines with 2,4-dinitrochlorobenzene (Brady and Cropper, 1950) and with picryl chloride (Minetti and Bruylants, 1970a,b) while substituents on subsequent carbons have relatively little effect. For reaction with TNBS the importance of α substituents is easily seen by comparing reactivities of glycine, alanine, valine, and 2-methylalanine (10, 12, 11, and 16 in Figure 3) which have similar pK_a values but where reactivity drops more than 500-fold. A comparable difference in reactivity is seen between *n*-butyl- and *tert*-butylamine (19 and 18, respectively, in Figure 3). No decrease in reactivity appears to be associated with β substituents. Neopentylamine, the only amine investigated with multiple β substituents, had unusually high reactivity, a fact which cannot be

[2] This equation is derived for the case of a group reacting as a function of pH only in the least protonated of two possible forms. It does not hold in the event of acid or base catalysis. It pertains in the present sense to cases where nucleophilicity does not vary appreciably with basicity. In most proteins wherein reaction of TNBS is mainly with ϵ-amino side chains of lysine residues it is a good approximation since factors which perturb basicity generally do not have as large an effect on nucleophilicity. In a protein eq 5 should be valid for all pK_a values except those which reflect a difference in nucleophilicity (*i.e.*, terminal α-amino groups as compared to ϵ-amino groups).

[3] This is equivalent to a nucleophilic reactivity of an amine with a pK_a value of 10.5.

[4] Usually observed as base catalysis but thought to involve the conjugate acid of the base following prior rapid proton loss by the Meisenheimer intermediate (*i.e.*, k_2).

explained at this time. The somewhat high reactivity of benzylamine and β-phenethylamine may be due to association between their respective aromatic rings and the electron deficient aromatic system of TNBS.

Secondary amines react very slowly with TNBS as compared to primary amines in contrast to the usual order of nucleophilic reactivity of amines. This low reactivity is shared by all the secondary amines studied (including the sterically less hindered, piperidine and dimethylamine (Table II). This is in contrast with the reactivity of these amines with 2,4-dinitro-1-halobenzenes (Brady and Cropper, 1950; Bunnett and Hermann, 1970), 2,4-dinitrophenyl ethers (Bunnett and Bernasconi, 1965), 2,4-dinitrophenyl methyl phosphate (Kirby and Younas, 1970), 1,3,5-trinitrobenzene[5] (Bernasconi, 1970), and picryl chloride (Minetti and Bruylants, 1970a,b). This low reactivity thus appears to result from the combined effects of two *o*-nitro groups and the bulky sulfonate leaving group. The stability of Meisenheimer complexes of picryl ethers with amines is known to be strongly dependent on steric properties of both the amine and the departing alkoxide (Clapp *et al.*, 1968). The slower rates may not be solely the result of steric crowding, however, as specific effects of the *o*-nitro groups *via* intramolecular hydrogen bonding in the intermediate also may affect the relative rates of decomposition to products and reversion to reactants (Bernasconi, 1971a,b, 1972; Minetti and Bruylants, 1970b). The previously observed failure of TNBS to react with secondary amines (Okuyama and Satake, 1960; Satake *et al.*, 1960) in more quantitative terms amounts to better than three orders of magnitude lower reactivity than primary amines of simlar basicity (see Table II).

Under conditions usually employed to modify proteins with TNBS the initially formed picramide derivatives and sulfite ion, also a product of the reaction, rapidly combine as illustrated in eq 3. To assess the effects of TNBS modification on a protein it is important to know that under some conditions the product is a doubly charged anion rather than a neutral picramide. The equilibrium for complexation with one mole of sulfite ion is extremely favorable under typical conditions for reaction of TNBS with proteins, making the sulfite adduct (IV) the main product. The equilibrium for this reaction can be rapidly displaced, however, by acidification, by dilution (or dialysis, gel filtration) or by any means which decreases the sulfite (dianion) concentration (Goldfarb, 1966a; Fields, 1971). For quantitative determinations of protein amino groups, elimination of sulfite complexes by acidification gives highly reproducible results (Habeeb, 1966; Goldfarb, 1970). To directly follow the reaction of TNBS with amines at higher pH values, addition of exogenous sulfite can be used to stabilize the position of the equilibrium in favor of the complex (Fields, 1971). Absorption spectra of picramides (λ_{max} 340 nm, ϵ 1.4×10^4 cm^{-1} M^{-1}) and the monosulfite complexes (λ_{max} 420 nm, ϵ 2.4×10^4 cm^{-1} M^{-1}) are easily distinguished from each other. Reactions of proteins with TNBS can be followed by monitoring the appearance of either product so long as the position of the equilibrium does not significantly change during the time of measurement.

Reactivity with TNBS in addition to being a sensitive measure of basicity can also be used as a measure of the local environment of amino groups in proteins. The importance of electrostatic interactions with adjacent charged groups to either increase (positive charge) or decrease (negative charge) reactivity is illustrated by the slightly low reactivity of all the negatively charged amines examined (see Figure 3 and Table II). Proximity to surface hydrophobic regions which can bind TNBS can increase reactivity by increasing its effective concentration at the potential reaction center. Present results indicate interaction with nearby aromatic groups can have a similar effect. The combined effect of these factors is susceptible in some cases to evaluation by studying the dependence of the reaction rate on TNBS concentration (*e.g.*, see Marshall and Cohen, 1972). In addition to the factors discussed, accessibility of TNBS to a potentially reactive group is an unknown factor analogous to steric effects on the reactivity of low molecular weight amines. Reactions of TNBS with such amines have been found particularly sensitive to steric hindrance and its reactions with proteins probably are similarly sensitive.

Reactions of TNBS with protein amino groups can be followed most accurately when its competing reaction with hydroxide ion is low. This blank reaction is much lower for TNBS than for similar reagents (*i.e.*, dinitrofluorobenzene) and is its principle advantage over them in spectrophotometric determinations of amino group contents of proteins. Still, under most conditions, the background reaction with hydroxide ion is rather high and a limiting factor on accuracy in determining small numbers of amino groups. Reaction conditions can be chosen, however, to minimize this interfering reaction. Activation parameters (Table I) reveal a decided advantage of lower temperatures to favor the amine reaction over that with hydroxide ion. Thus, if compensated for by longer reaction times, the best results should be obtained at the lowest possible temperature. The reactivity of TNBS with hydroxide ion is enhanced by increasing ionic strength without a compensatory effect on its reactivity with amines. Thus, low ionic strengths are desired. Rates of hydrolysis of TNBS increase with pH in parallel with the rates of its reaction with amines at low pH values. Rates of reaction with amines increase more slowly, however, as the pH approaches their pK_a value. Thus, pH values somewhat lower than the pK_a (*i.e.*, ~0.5 unit) should be used. Also, it should be noted that we have observed significantly lower reaction rates for amines when carbonate buffers were employed. Formation of carbamates from CO_2 (aqueous) and amines appears to account for these decreases. Further study is necessary, however, to establish this. Carbonate buffers should not be used where optimum reactivity of TNBS with amines is desired.

The unusual reactivity pattern of TNBS with nucleophiles distinguishes it from other electrophilic reagents commonly in use to modify proteins. Steric considerations are of primary importance in determining reactivity and appear to account for the low reactivity of sterically hindered nucleophiles and, at least in part, to account for an inversion of the usual order of amine nucleophilic reactivity in the case of TNBS (*i.e.*, $1 \gg 2°$). Coulombic effects appear responsible for a general lowering of its reactivity with anionic nucleophiles (as compared, for example, with fluoro-2,4-dinitrobenzene), but account for only a small part of the total difference in reactivity with oxyanions as compared to primary amine nucleophiles. The remainder, presumably attributable to an increased sensitivity to solvation and polarizability, is more like that for attack on saturated (sp^3) carbon and is compatible with greater steric hindrance at the reaction center and with the increased stability of the tetrahedral intermediate.

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[5] Corresponds to only k_1/k_{-1} of eq 6.

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Evidence for the Compact Conformation of Monomeric Glucagon. Hydrogen–Tritium Exchange Studies†

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ABSTRACT: Glucagon, a polypeptide hormone of 29 amino acids, was subjected to tritium–hydrogen exchange according to the two-column technique of Englander. The primary structure of this molecule does not contain any covalent cross-links such as peptide cyclization or disulfide bonds; thus any slowly exchanging hydrogens would indicate a folding of the peptide chain. The hydrogen exchange of glucagon is described by more than one first-order rate constant. The exchange of the slowest class of hydrogens exhibits specific acid and base catalysis and is composed of approximately 8 hydrogens. The number of hydrogens in this kinetic class was found to be nearly constant (±2 hydrogens) within the entire pH interval investigated, showing that no conformational change had occurred between pH 1.7 and 4.0. It is suggested that most of these slowly exchanging hydrogens result from i to $(i + 3)$ hydrogen bonds of tetrapeptide β bends.

The rates of isotope exchange of hydrogen atoms in proteins have been shown to be a reflection of the conformation of these macromolecules (Hvidt and Nielsen, 1966). Studies with model compounds have been geared toward the elucidation of the factors involved in the kinetics of the exchange process (Berger *et al.*, 1959; Klotz and Frank, 1962, 1965; Nielsen, 1960; Leichtling and Klotz, 1966; Scarpa *et al.*, 1967; Kakuda *et al.*, 1971; Englander and Poulsen, 1969). With the

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