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Kinetic Study of the Reaction between Trinitrobenzenesulfonic Acid and Amino Acids with a Trinitrobenzenesulfonate Ion-Selective Electrode

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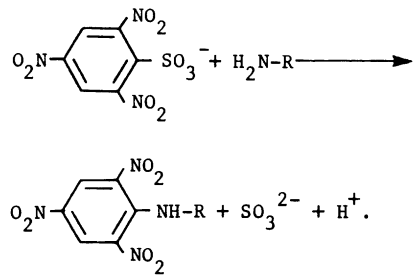
Potentiometric studies of the reaction between trinitrobenzenesulfonic acid (TNBS) and several amino acids with the TNBS electrode indicate that the reaction is first-order with respect to TNBS and amino acid concentration. The reaction is zero-order with respect to hydroxide concentration at pH > 10.5, indicating that the nonprotonated amino group is the reactive species. Rate constants were calculated for each amino acid and a simple mechanism of the reaction is proposed.

Trinitrobenzenesulfonic acid (TNBS)[1] was introduced by Okuyama and Satake (1) as a specific reagent for primary amino groups. This reagent is water-soluble, relatively stable, and reacts with amino groups under comparatively mild conditions according to the scheme



[1]

Freedman and Radda (2) observed that TNBS reacts also with the SH group, this reaction being much faster than the reaction of TNBS with the amino group of several amino acids. Goldfarb (3) observed the reversible association of sulfite with trinitrophenylated amino groups (TNP–NH–R) to form complexes which have absorption maxima near 420 nm. It is also known that TNBS reacts with hydroxyl ions at high pH values (pH > 9) to give a colored product which also absorbs near 420 nm (4). Many manual or automated methods have been described in the literature for the determination of amino acids, amines, peptides, and proteins by use of TNBS as reagent (4–11). In many of them, sulfite is added to the reaction mixture to enhance sensitivity. The kinetics of reaction [1] were also studied spectrophotometrically by some investigators (2–4,12). Recently, we found that this reagent reacts also with sulfide ions and various sugars (13).

The construction and some analytical applications of a liquid membrane TNBS ion-selective electrode have been described elsewhere (14). In this paper the kinetics of the reaction between TNBS and several amino acids are studied with the TNBS electrode. The study included the determination of the order of the reaction with respect to the chemical species involved and the calculation of rate constants. From the results, a possible mechanism of the reaction is also proposed.

EXPERIMENTAL

Reagents. All solutions were prepared in deionized distilled water from reagent-grade materials.

[1] Abbreviations used: TNBS, trinitrobenzenesulfonic acid; TNP–NH–R, trinitrophenylated amino groups.

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Standard 0.1000 M TNBS stock solution was prepared as previously described (14). All solutions for the potentiometric studies were prepared from this solution by dilution. TNBS solutions should be stored in amber-colored bottles.

Amino acid standard solutions, stock 0.1000 or 0.01000 M, were prepared by dissolving pure substances in water. More dilute standard solutions were prepared by dilution.

Phosphate buffers, 0.50 M, were used.

The electrodes, the reaction cell, and the recording system were the same as previously described (14). All measurements were carried out at 25.0 ± 0.1°C under constant magnetic stirring.

THEORY OF MEASUREMENTS

Reaction Kinetics with Amino Acids in Excess

The TNBS electrode exhibits near Nernstian potential behavior for the TNBS anion, at concentrations of 10^{-2} to 5×10^{-5} M, in the pH range 3 to 12, and at constant ionic strength, according to

$$E = E' - \frac{RT}{F} \ln[\text{TNBS}], \qquad [2]$$

where E is the measured total potential of the system. E' is a constant potential and depends on the choice of the reference electrodes, internal solution, ionic strength effects, and junction potentials; R and F are the ideal gas and Faraday constants, respectively; T is the absolute temperature, and [TNBS] is the concentration of TNBS.

When dilute TNBS solutions react with an excess of amino acid, A (concentration of A should be at least five times the TNBS concentration), at a given pH value, and the reaction is first-order with respect to TNBS, then the concentration of TNBS in the reaction mixture, at any time t, is given by

$$[\text{TNBS}] = [\text{TNBS}]_o e^{-k_{obs}t}, \qquad [3]$$

where k_{obs} is the observed pseudo-first-order rate constant. Taking the logarithms of Eq. [3] and combining with Eq. [2] gives

$$E = E'' + \frac{RT}{F} k_{obs}t, \qquad [4]$$

where

$$E'' = E' - \frac{RT}{F} \ln[\text{TNBS}]_o.$$

Thus, Eq. [4] indicates that if reaction [1] is first-order with respect to the TNBS concentration, a plot of E vs t should be a straight line with a slope of $k_{obs}\,RT/F$, independent of initial TNBS concentration, $[\text{TNBS}]_o$. k_{obs} can be calculated conveniently from the E vs t plot and

$$k_{obs} = \frac{\text{slope of the } E \text{ vs } t \text{ plot}}{(RT/F)}$$

$$= \frac{\text{slope of the } E \text{ vs } t \text{ plot}}{\text{slope of the electrode, } S} \times 2.303, \qquad [5]$$

Where $S = 2.303\ RT/F$ is the prelogarithmic term of the Nernst equation, also termed slope of the electrode. The value of S can be calculated from the calibration curve of the TNBS electrode and is equal to 60 mV at 25°C, as has been described in detail elsewhere (14). It can be seen from Eq. [5] that the kinetics of reaction [1] can be studied by monitoring the change of electrode potential E with time (E vs t plot) under pseudo-first-order conditions.

RESULTS AND DISCUSSION

Recorded curves for the reaction (E vs t plots) of TNBS with glycine at various amino acid concentrations are shown in Fig. 1. The curves are linear, indicating that the assumption that the reaction is first-order with respect to TNBS is correct (Eqs. [3] and [4]). For each amino acid concentration, k_{obs} was calculated from Eq. [5]. The variation of k_{obs} with amino acid concentration for glycine is given in Table 1.

If we assume that $k_{obs} = k[\text{A}]^n$ and take the logarithms, we have $\log k_{obs} = \log k + n \log[\text{A}]$.

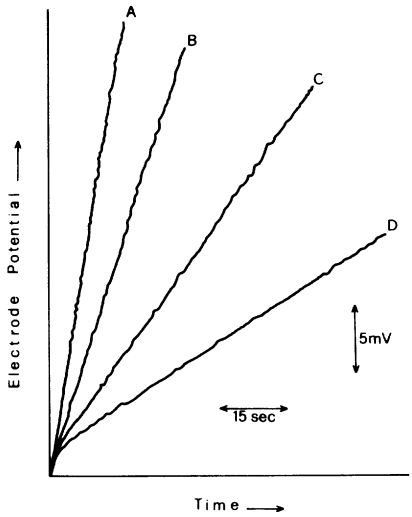

FIG. 1. E vs t plots for the reaction between TNBS and glycine at various glycine concentrations (M). (A) 9.97 $\times 10^{-3}$; (B) 4.98 $\times 10^{-3}$; (C) 1.99 $\times 10^{-3}$; (D) 1.00 $\times 10^{-3}$. The reaction was carried out at 25.0°C and at pH 11.4. $[TNBS]_o = 3.33 \times 10^{-4}$ M.

Thus, if we plot log k_{obs} vs log [A], the constants k and n can be determined from the intercept and the slope of the linear curve, respectively. Such a plot gave the regression equation $y = (0.97 \pm 0.02)X + (0.907 \pm 0.060)$ and correlation coefficient, $r = 0.9995$. It can be seen that the reaction is first-order with respect to amino acid concentration. A more accurate value for k can be obtained from a plot of k versus [A]. When $n = 1$, such a plot is linear, passes through the origin, and has a slope equal to k (in M^{-1} s^{-1}). The plot of k_{obs} vs [A] gave the regression equation $y = (9.57 \pm 0.16)X + (6.6 \times 10^{-5} \pm 8.8 \times 10^{-4})$, $r = 0.9997$.

The variation of k_{obs} with hydroxide concentration (at constant amino acid concentration) is given in Table 2. The values of k_{obs} were calculated from Eq. [5] and from the E vs t plots obtained at constant amino acid concentration and varying the hydroxide concentration.

The value of k_{obs} is independent of pH in the pH region studied (see below for further discussion). For glycine, it has been shown that the reaction is first-order with respect to TNBS and glycine concentration at pH > 10.5, and the rate constant k was calculated to be 9.57 M^{-1} s^{-1} with the results of Table 1 or 9.28 M^{-1} s^{-1} with the results of Table 2 ($k = k_{obs}/[A]$).

The values of n for various amino acids under various experimental conditions are shown in Table 3. The reaction was always found to be first-order with respect to TNBS concentration (that is, the E vs t plots recorded were linear) and zero-order with respect to hydroxide concentration at pH > 10.5.

The overall rate equation for all the amino acids studied is

$$\text{Rate} = k[TNBS][A]. \qquad [6]$$

TNBS reacts with the amino groups of the amino acid in all cases except cysteine, in which the group reacting fast with TNBS is SH, so the rate equation and rate constant value for cysteine refer to this reaction (2). For lysine, our results refer to the reaction of TNBS with the α-amino group since it is known that the ϵ-amino group reacts with TNBS at a much slower rate (4).

The values of k for the various amino acids studied under various experimental conditions are shown in Table 4.

Previous investigators (2–4) studied the kinetics of the reaction with TNBS in excess by spectrophotometric techniques. In our study,

TABLE 1

VARIATION OF k_{obs} WITH GLYCINE CONCENTRATION IN THE TNBS–GLYCINE REACTION AT 25.0°C

Glycine ($\times 10^{-3}$) M	1.00	1.99	4.98	9.97
k_{obs} ($\times 10^{-2}$) s^{-1} [a]	1.04	1.90	4.65	9.60

[a] $[TNBS]_o = 3.33 \times 10^{-4}$ M; pH 11.4 (phosphate).

TABLE 2

VARIATION OF k_{obs} WITH HYDROXIDE CONCENTRATION IN THE TNBS–GLYCINE REACTION AT 25.0°C

Hydroxide ($\times 10^{-3}$) M	0.40	1.00	2.51	7.94
k_{obs} ($\times 10^{-2}$) s^{-1} [a]	5.33	5.54	5.57	5.75

[a] $[TNBS]_o = 3.98 \times 10^{-4}$ M; [glycine] = 5.98×10^{-3} M.

TABLE 3

ORDER OF THE REACTION (n) WITH RESPECT TO AMINO ACID CONCENTRATION UNDER VARIOUS EXPERIMENTAL CONDITIONS, AT 25.0°C AND pH 11.4 (PHOSPHATE)

Amino acid	n^a	n^b	$\bar{n}$
Cysteine[c]	0.99	1.00	1.00
Tryptophan	0.86	0.88	0.87
Lysine[d]	0.98	0.94	0.96
Histidine	0.96	1.00	0.98
Isoleucine	1.05	0.90	0.98
Phenylalanine	0.90	0.98	0.94
Glycine	0.97	0.96	0.97
Valine	0.92	0.92	0.92
Leucine	0.93	0.90	0.92
Arginine	0.94	0.96	0.95
Glutamic acid	0.93	0.98	0.96
Alanine	0.82	0.88	0.85
Serine	1.07	0.93	1.00
Asparagine	0.92	0.95	0.94

[a] [A] ranged from 24.8×10^{-3} to 2.48×10^{-3} M, $[TNBS]_o = 6.66 \times 10^{-4}$ M.

[b] [A] ranged from 9.97×10^{-3} to 0.97×10^{-3} M; $[TNBS]_o = 3.33 \times 10^{-4}$ M.

[c] Refers to the reaction of TNBS with the SH group of cysteine.

[d] Refers to the reaction of TNBS with the α-amino group of lysine.

the reaction was found to be first-order with respect to TNBS and amino acid concentration, in agreement with results of previous studies (2–4). Reported values for k, for the amino acids glycine, alanine, phenylalanine, and *N*-acetylcysteine are 11.3, 6.00, 6.50, and 166 M^{-1} s^{-1}, respectively (2). Fields (4) reported also k values for some amino acids but he used Na_2SO_3 in the reaction mixture and the reported values are at pH 9.5. If his values are corrected for the pH effect (see below for the discussion of proposed mechanism), then the values of 193, 4.37, 9.47, and 7.80 M^{-1} s^{-1} are obtained for the amino acids cysteine, alanine, glycine, and lysine (α-amino-group), respectively, in fair agreement with our results.

Proposed Mechanism

Freedman and Radda (2) showed that the unprotonated amino group of the amino acid is the reactive species. Goldfarb (3) concluded that the reaction between TNBS and a free amino group could not be used exclusively to explain the mechanism. Fields (4) observed an increase in the reaction rate by rising pH in the case of aniline even at pH values four units or more above its pK_a.

In our results for the reaction of TNBS with the amino acids listed in Tables 3 and 4, we found that at pH values where the amino group is partly protonated, the reaction rate depends on pH. For pH > 10.5, where the amino acid is turned quantitatively to its non-protonated form, the reaction rate is independent of pH, up to pH 12 (maximum pH studied). So, at least for the amino acids studied, the following simple mechanism is proposed:

$$RNH_3^+ \overset{K_a}{\leftrightarrows} RNH_2 + H^+ \quad \text{(rapid)} \quad [7]$$

$$RNH_2 + TNBS \rightarrow \text{Products} \quad \text{(slow)}, \quad [8]$$

TABLE 4

SECOND-ORDER REACTION RATE CONSTANTS FOR SEVERAL AMINO ACIDS[a]

Amino acid	Reaction rate constant k (M^{-1} s^{-1})
Cysteine[b]	108
Tryptophane	29.7
Lysine[c]	12.8
Histidine	10.6
Isoleucine	10.5
Phenylalanine	9.6
Glycine	9.6
Valine	9.5
Leucine	9.4
Arginine	9.2
Glutamic acid	5.0
Alanine	4.3
Serine	4.1
Asparagine	2.2

[a] [A] ranged from 24.8×10^{-3} to 2.48×10^{-3} M, $[TNBS]_o = 6.66 \times 10^{-4}$ M. Temperature = 25.0 °C; pH 11.4.

[b] Reaction of TNBS with the SH group of cysteine.

[c] Reaction of TNBS with the α-amino group of lysine.

where Eq. [8] is the rate-determining step of the reaction and fits with the rate equation found experimentally (Eq. [6]). In order to further support the proposed mechanism, study of the kinetics of the reaction was carried out at various pH values in the pH range 8–11, with the amino acid in great excess (at least 150 time the concentration of TNBS) so that even at pH = pK_a − 1.5, the concentration of unprotonated amino group was in excess over TNBS. The rate constant k was calculated as described above (Eq. [5]) by using as amino acid concentration only the unprotonated fraction [A], which is equal to

$$[A] = \frac{C_A K_a}{K_a + [H^+]}, \qquad [9]$$

where C_A is the total amino acid concentration.

The values of k obtained at various pH values are shown in Table 5 for the amino acids glycine and alanine. These values are similar to those obtained at pH 11.4 (Table 4), indicating that the assumption that the reactive species of the amino acid is the unprotonated amino group is correct.

The spectrophotometric methods for the study of the kinetics of the reaction between TNBS and amino acids suffer from several serious drawbacks. If the reaction is carried out without the addition of sulfite and the absorbance of the product TNP–NH–R is followed at 345 nm, then the use of high TNBS concentrations is not possible since TNBS absorbs strongly at this wavelength. Also, during the course of the reaction, two distinct phenomena are taking place. The increase in absorbance is due to the TNP–NH–R produced, and a decrease in absorbance is due to the consumption of TNBS in the reaction. So, the calculations become complicated (3). Addition of sulfite in the reaction mixture is claimed not to affect the rate of the reaction (4) and to shift the absorbance maximum to 420 nm, where TNBS does not absorb, and thus high concentrations of TNBS may be used. This alternative has the drawback of introducing an additional reagent in the reaction mixture.

The reaction cannot be followed spectrophotometrically at pH values greater than 9.5 since TNBS forms complexes with hydroxide which absorb strongly at 420 nm. The absorbance of these complexes is stimulated by light (4).

The TNBS electrode has the great advantage of continuous monitoring of TNBS concentration in the reaction mixture. It can be used in concentrated (e.g., 10^{-2} M) as well as in dilute solutions in the pH range 3–12. Addition of sulfite to the reacton mixture is not needed and the formation of TNBS–hydroxide complexes does not interfere with the kinetic study. This happens because even if the initial TNBS concentration $[TNBS]_o$ changes in the presence of hydroxide, the calculated k_{obs} from Eq. [5] is independent of initial TNBS concentration (Eq. [4]) if the reaction is first-order with respect to TNBS concentration.

In conclusion, we feel that the TNBS electrode can be used successfully in kinetic and equilibrium studies involving the reaction of TNBS with amino acids, and possibly with amines, peptides, and proteins because the potentiometric technique has several advantages over the classical spectrophotometric techniques.

TABLE 5

VALUES OF THE SECOND-ORDER REACTION RATE CONSTANT k AT VARIOUS pH LEVELS FOR THE AMINO ACIDS GLYCINE AND ALANINE[a]

	k (M^{-1} s^{-1})	
pH	Glycine	Alanine
8.0	8.5	4.2
9.0	9.2	3.9
9.5	9.8	4.0
10.5	9.0	4.1
11.0	9.4	4.6
	Av. 9.18	Av. 4.16

[a] Only the unprotonated concentration of the amino acid is taken into account. For details, see text.

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