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Kinetics of Dinitrophenylation of Amino Acids*

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ABSTRACT: The kinetics of reactions of 1-fluoro-2,4-dinitrobenzene (FDNB) with glycine, proline, and *N*-phenylglycine in water, of FDNB with glycine in acetonitrile–water and dimethyl sulfoxide–water mixtures, and of 2,4-dinitrophenyl phenyl ether (DNPE) with proline and pyrrolidine in 10% dioxane–90% water have been measured as a function of base concentration or solvent composition. Dinitrophenylation of glycine with FDNB is very much faster in dimethyl sulfoxide–water than in ethanol–water or acetonitrile–water mixtures; this may be of practical value in application of dinitrophenylation for analytical or peptide modification purposes. The pH dependence of rates of reactions of FDNB with amino acids is entirely accounted for by the effect of pH on the state of ionization of the amino acids; there is no evidence for base catalysis of the substitutions proper. The reaction of DNPE with proline anion is catalyzed by NaOH, but surprisingly the reaction of DNPE with pyrrolidine is insensitive to base catalysis.

Although the Sanger method of peptide end-group analysis and sequence determination through dinitrophenylation of amino groups with 1-fluoro-2,4-dinitrobenzene[1] (Sanger, 1945; Fraenkel-Conrat *et al.*, 1955) has to a large extent been supplanted by the Edman phenyl isothiocyanate degradation procedure (Schroeder, 1967; Konigsberg, 1967; Edman and Begg, 1967) and by dansylation with 1-dimethylaminonaphthalene-5-sulfonyl chloride (Gray, 1967a,b), dinitrophenylation with FDNB continues to be widely used for purposes of end-group analysis, modification of proteins, and identification of peptide fragments.

Despite the extensive use of this method in biochemical research, the kinetics of reactions of amino acids and peptides with FDNB have not received much attention. The rates of reaction of a few amino acids and peptides with FDNB in water solution were determined by Burchfield and Storrs (1957) and in water under heterogeneous conditions (FDNB was present as a separate liquid phase) by Brouwer *et al.* (1958). In both studies, some attention was given to variation of pH (the pH was varied by only one unit) but the influence of pH has otherwise been little investigated.

In view of the fact that reactions of FDNB with some amines in protic media are catalyzed by base (Bunnett and Randall, 1958; Beale, 1966) while reactions with other amines are not base catalyzed (Bunnett and Garst, 1965a), it was of interest to see if reactions of amino acids with FDNB might respond to catalysis by bases. Particularly if some reactions were base catalyzed and others not, variation in pH might enable selective dinitrophenylation of certain aminoacyl moieties. For this reason, the present investigation of the pH dependence of reaction rates in water solution was undertaken.

Inasmuch as the reactions of amines with nitro-activated aryl halides are much faster in Me_2SO and other dipolar aprotic solvents than in aqueous or alcoholic media (Suhr, 1963, 1964; Kingsbury, 1964), the influence of this factor

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[1] Abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; DNPE, 2,4-dinitrophenyl phenyl ether.

on rates of reactions of amino acids and peptides with FDNB was also investigated.

Materials

Amino Acids, Peptides, and Amines. Glycine and proline were commercial products repurified by recrystallization. *N*-Phenylglycine was synthesized by the reaction of aniline with chloroacetic acid (Hausdörfer, 1889) and obtained in a state of sufficient purity so that a few recrystallizations from ethanol under nitrogen gave a colorless compound of mp 127°, in agreement with the literature (Meyer, 1875). Because of its great sensitivity to oxidation, it must be stored under nitrogen. Pyrrolidine was purified by fractional distillation of the commercial product from sodium through a Vigreux column (bp 88.5–89°). Commercial FDNB was redistilled under vacuum. 2,4-Dinitrophenyl phenyl ether (mp 69°) was prepared after Raiford and Colbert (1926).

2,4-Dinitrophenyl (DNP) derivatives of amino acids were prepared after Rao and Sober (1954); DNP-glycine, mp 205°; DNP-L-proline, mp 138°; DNP-*N*-phenylglycine (from ethanol–water), mp 173° with decomposition, very sensitive to oxidation (*Anal.* Calcd for $C_{14}H_{11}N_3O_6$: C, 52.99; H, 3.47; N, 13.25. Found: C, 52.92; H, 3.50; N, 13.28; by Micro-Tech Laboratories, Skokie, Ill.); and DNP-pyrrolidine (from ethanol–water), mp 103° (*Anal.* Calcd for $C_{10}H_{11}N_3O_4$: C, 50.63; H, 4.64; N, 17.72. Found: C, 50.36; H, 4.73; N, 17.78; by Micro-Tech Laboratories).

Solvents. 1,4-Dioxane was purified after Fieser (1957) and stored under nitrogen. Commercial Me_2SO was purified by partial freezing and decantation of the unfrozen portion, melting the frozen portion, and repeating the cycle three times; it was stored under nitrogen. Acetonitrile was purified by a modification of the method of Donnell *et al.* (1965).

Procedures

Reactions in Water. In all cases, the amino acid was in large excess over FDNB, so as to afford pseudo-first-order kinetics. In almost all runs the concentration of FDNB was 10^{-4} M. The ionic strength, unless otherwise noted, was 0.25 M, maintained by compensation with NaCl. A standard solution of FDNB in 75% dioxane–25% water was prepared by diluting a solution of FDNB in three volumes of dioxane to four volumes by addition of water. Runs were initiated by pipetting 1 ml of this standard FDNB solution into a volumetric flask (100 or 250 ml) containing the amino acid or amine, buffer constituents, and NaCl as needed, diluted nearly to the mark with water, and thermostated. The flask was then quickly filled to the mark, shaken, and a portion of its contents transferred to the cell of a Gilford 2000 automated kinetics spectrophotometer with thermostated cell compartment, which recorded absorbance at λ_{max} for the DNP-amino acid anion (from 355 nm for glycine to 395 nm for *N*-phenylglycine) as a function of time. Pseudo-first-order rate coefficients, k_ψ, were reckoned either by the infinity method (Bunnett and Randall, 1958) or the Guggenheim method (Frost and Pearson, 1953). In all cases, clean first-order kinetics were observed. A sample of the "infinity" solution (from the main reaction flask, after 10 or more half-lives) was acidified with HCl and diluted in standard fashion, and its absorbance at the wavelength maximum of the DNP-amino acid was compared with that of a mock infinity solution (a solution of the DNP-amino acid at the concentration it would have had if formed quantitatively in the run) which had been acidified and diluted in the same way. Inasmuch as 2,4-dinitrophenol does not absorb appreciably in acidic solution at *ca.* 380 nm, the ratio of absorbances of the quenched infinity and mock infinity solutions gives the fractional yield of DNP-amino acid or DNP-amine formed in the run. Reactivity is also expressed by other sorts of rate coefficients, defined as follows.

k_A*: pseudo-first-order coefficient for formation of DNP-amino acid or DNP-amine, being k_ψ times fractional yield of that product.

k_A^{st}: second-order coefficient for reaction of FDNB with amino acid in all its ionized forms, being k_A*/$[A]_{st}$, where $[A]_{st}$ is the stoichiometric amino acid concentration.

k_A^-: second-order coefficient for reaction of FDNB with amino acid anion; see text for method of evaluation.

k_A: second-order coefficient for reaction of FDNP with neutral amino acid or amine; see text for method of evaluation.

pH as reported in tables was determined experimentally, the meter being calibrated against standard buffers.

Reactions in Me_2SO–Water and Acetonitrile–Water. The procedures used were basically similar to the above, but with some differences in the preparation of reaction solutions. To illustrate our designation of solvent compositions, 50% Me_2SO–50% water represents one volume of Me_2SO diluted to two volumes by addition of water as required. No effort was made to maintain constant ionic strength. The sodium salt of glycine was weighed out as such in setting up some runs.

Results

Reaction of FDNB with Glycine in Water. A series of runs over the pH range 7.3–13.0 is summarized in Table I. Below pH 11, DNP-glycine was formed quantitatively, but at higher pH values a side reaction of hydrolysis to 2,4-dinitrophenol became progressively more serious and at pH 12.6 and higher there was a further complication of partial decomposition of the DNP-glycine. For the latter reason, the rate coefficients from the last two runs are regarded as less accurate than the rest.

The apparent second-order rate coefficient for reaction of FDNB with glycine in all its ionized forms, which we symbolize k_A^{st}, rises with increasing pH until it levels off at about 3.2×10^{-1} l. mole^{-1} sec^{-1} at pH 11.5 and higher. This variation is entirely attributable to the effect of pH on the equilibrium between the zwitterion and anionic forms of glycine, and does not provide any indication of base catalysis of the substitution reaction proper. If the zwitterion is unreactive and k_A^- is the second-order rate coefficient for reaction of glycine anion with FDNB, the variation in k_A^{st} is given by eq 1 (Burchfield and Storrs, 1957). As is evident in Figure 1, k_A^{st} calculated by this expression on the basis of k_A^- being 0.320

$$k_A^{st} = \frac{K_a k_A^-}{K_a + [H^+]} \quad (1)$$

M^{-1} sec^{-1} (Table I) and pK_a being 9.65 (Owen, 1934) is in agreement with the experimental k_A^{st}. There is no need or justification to invoke any variation of k_A^- with pH.

TABLE I: Reaction of FDNB with Glycine in Water at 30.5°.

pH	[Glycine]$_{st}$ (M)	Buffer	$10^4 k_\psi$ (sec^{-1})	Yield[a] (%)	$10^4 k_A$* (sec^{-1})	$10^3 k_A^{st}$ (l. mole^{-1} sec^{-1})
7.34	0.05	$H_2PO_4^-$–HPO_4^{2-}	1.08	100	1.08	2.16
7.65	0.05	$H_2PO_4^-$–HPO_4^{2-}	2.21	100	2.21	4.42
7.95	0.05	$H_2PO_4^-$–HPO_4^{2-}	3.87	100	3.87	7.74
8.25	0.05	Barbiturate	7.64	100	7.64	15.3
8.42	0.05	Barbiturate	11.8	100	11.8	23.6
8.48	0.05	Barbiturate	14.3[b]	100	14.3	28.5
8.60	0.05	HCO_3^-–CO_3^{2-}	12.8[b]	100	12.8	25.6
8.88	0.05	HCO_3^-–CO_3^{2-}	18.7	100	18.7	37.4
8.96	0.05	HCO_3^-–CO_3^{2-}	25.8	100	25.8	51.6
9.26	0.05	HCO_3^-–CO_3^{2-}	44.0	100	44.0	88
9.55	0.05	Glycine	54.4[d]	100	54.4	109
9.73	0.05	Glycine	64.0	100	64.0	128
10.15	0.05	Glycine	127.0[c]	100	127	253
10.34	0.05	Glycine	114.4	100	114	229
10.46	0.05	Glycine	135.2	100	135	270
11.00	0.05	HPO_4^{2-}–PO_4^{3-}	163	93.8	153	305
11.35	0.05	NaOH	168	92.5	156	311
11.49	0.05	NaOH	176	90.5	159	318
12.04	0.05	NaOH	206	80.0	164	328
12.25	0.05	NaOH	218	74.4	162	325
12.62[e]	0.1	NaOH	531	67.5	360	360
13.01[e]	0.1	NaOH	806	47.3	380	380

[a] Yield of DNP-glycine, based on FDNB, determined photometrically. [b] Average of two runs. [c] Average of three runs. [d] Average of four runs. [e] Considerable decomposition of DNP-glycine occurred.

Reaction of FDNB with Proline in Water. It has been observed in several cases that reactions of FDNB and similar arylating agents with amines are subject to catalysis by bases when the amine is secondary but not when it is a closely related primary amine (Bunnett and Garst, 1965a; Kirby and Jencks, 1965; Bernasconi, 1967). Accordingly it was expected that if any of the common amino acids were to have its reaction with FDNB catalyzed by base, such behavior should be encountered with proline.

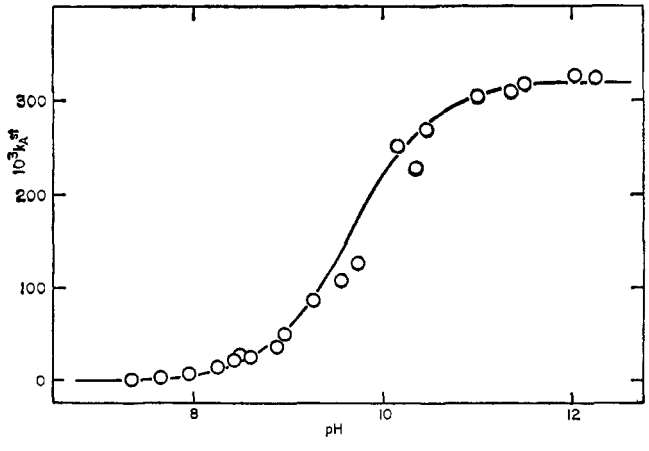


FIGURE 1: Apparent second-order rate coefficient for reaction of FDNB with glycine in water, as a function of pH. Data of Table I. The line drawn is calculated by means of eq 1; see text.

Our data concerning variation of rate with pH are summarized in Table II. The general form of behavior is similar to that observed with glycine: the apparent second-order rate coefficient, k_A^{st}, increases with pH but ultimately levels off to a plateau at high pH. Because of the high reactivity of proline with FDNB, it was inconvenient to observe the rate increase all the way to the plateau at 30.5°, but the full picture was observable at 10.0°. The 10° data were plotted similarly to Figure 1, together with a line calculated by eq 1 on the basis of k_A^- being 2.64 M^{-1} sec^{-1} (Table II) and pK_a being 11.20 (*cf.* Smith *et al.*, 1942). The plot (not shown) resembled that of Figure 1, and the agreement of experimental points with calculated curve was about as good, except for some scatter at higher pH values. Again the variation of rate with pH is adequately accounted for by the effect of pH on the ionization of proline, and there is no evidence for catalysis by base.

Values for k_A^{st} at 30.5°, calculated from eq 1 on the basis of k_A^- being 11.0 M^{-1} sec^{-1} and pK_a being 10.36, were in fair agreement with the experimental k_A^{st} values as recorded in Table II. This pK_a differs somewhat from the reported value (by interpolation, 10.52 at 30°; Smith *et al.*, 1942). If this k_A^- is correct, the nucleophilic reactivity of proline anion is about 35 times greater than that of glycine anion against FDNB at 30.5°.

No decomposition of DNP-proline was observed in alkaline solutions.

TABLE II: Reaction of FDNB with Proline in Water.

Temp (°C)	pH	$[Proline]_{st}$, M	Buffer	$10^3 k_\psi$ (sec^{-1})	Yield[a] (%)	$10^3 k_A^*$ (sec^{-1})	$10^2 k_A^{st}$ (l. $mole^{-1}$ sec^{-1})
30.5	7.98	0.05	$H_2PO_4^-$–HPO_4^{-2}	2.28	100	2.28	4.56
	8.48	0.05	Barbiturate	7.42	100	7.42	14.8
	9.20	0.05	HCO_3^-–CO_3^{-2}	31.0	100	31.0	62.1
	9.55	0.02	HCO_3^-–CO_3^{-2}	21.8	100	21.8	109
	10.04	0.02	HCO_3^-–CO_3^{-2}	54.6	100	54.6	273
10.0	8.91	0.05	$H_2PO_4^-$–HPO_4^{-2}	1.36	100	1.36	2.72
	9.44	0.05	HCO_3^-–CO_3^{-2}	3.81	100	3.81	7.62
	10.21	0.02	HPO_4^{-2}–PO_4^{-3}	4.90	100	4.90	24.5
	10.73	0.02	Proline	11.5	100	11.5	57.6
	11.55	0.02	HPO_4^{-2}–PO_4^{-3}	21.2	84.8	18.0	180
	11.84	0.01	HPO_4^{-2}–PO_4^{-3}	25.8	93.5	24.1	241
	11.91	0.01	NaOH	24.1	85.8	20.7	207
	12.61	0.01	NaOH	28.1	96.3	27.0	270[b]
	13.10	0.01	NaOH	32.4	88.5	28.7	287[b]
	13.09	0.01	NaOH	26.8	93.3	25.0	250[b]
	13.39	0.01	NaOH	28.2	89.1	25.1	251[b]

[a] Yield of DNP-proline, based on FDNB, determined photometrically. [b] Average k_A^{st} from the last four runs is 2.64 l. $mole^{-1}$ sec^{-1}.

Reaction of FDNB with N-Phenylglycine in Water. No evidence for base catalysis having been found for the reaction of FDNB with the most likely candidate among common amino acids, this phenomenon was sought with a nonbiological amino acid. *N*-Phenylglycine is structurally similar to *N*-methylaniline, the reaction of which with FDNB in ethanol or 60% dioxane–40% water is catalyzed by base (Bunnett and Randall, 1958), and therefore seemed a good candidate. However, kinetic investigation of its reaction with FDNB was greatly impeded by its great sensitivity to oxidation, especially at high pH where base catalysis would be manifest.

Phenylglycine, a white compound, turns yellow-brown on exposure to air. Solutions of it in water or especially in dilute NaOH solution assume, if exposed to oxygen, a deep purple color which sometimes changes to a dirty brown. Even in acidic solutions, it is oxidized with formation of a deep blue color. Reaction solutions were therefore prepared with great care to exclude traces of oxygen. Even so, acidic solutions were often faintly blue (λ_{max} 585 nm), but such solutions absorbed only slightly in the 400-nm region, and photometric determination of the rate of reaction with FDNB was therefore quite feasible. However, the intense colors of even the most carefully prepared solutions of pH >9 precluded accurate kinetic measurements.

Un-ionized phenylglycine exists to a considerable extent in the amino acid (rather than zwitterion) form (Bryson *et al.*, 1963), and the un-ionized substance has appreciable reactivity with FDNB. Equation 1 is therefore not applicable; one must instead use eq 2, in which k_A and k_A^- are second-order rate coefficients for reactions of the neutral and anionic forms, respectively. In eq 2, no differentiation is made between the amino acid and zwitterion forms, which are described collectively as "neutral" form.

$$k_A^{st} = k_A + \frac{K_a(k_A^- - k_A)}{K_a + [H^+]} \qquad (2)$$

Equation 2 implies that a plot of k_A^{st} *vs.* $1/(K_a + [H^+])$ should be linear with intercept k_A and slope $K_a(k_A^- - k_A)$. The plot (not shown), based on K_a being 3.9×10^{-5} (Bryson *et al.*, 1963), was basically linear though scattered; k_A was evaluated as 4.0×10^{-4} M^{-1} sec^{-1} and k_A^- as 1.37×10^{-3} M^{-1} sec^{-1}. Appropriately, the latter is approximately the average of the observed k_A^{st} values at higher pH values, at which phenylglycine is almost entirely present as its anion. The experimental k_A^{st} values and a curve calculated by means of eq 2 with use of k_A and k_A^- as evaluated above are plotted in Figure 2. Although the experimental points are rather scattered, probably due in part to the oxidation complication mentioned above, it is evident that they are adequately accounted for by eq 2, the derivation of which assumed the absence of base catalysis. On the other hand, inasmuch as reliable rate measurements could not be made above pH 8.9, these experiments do not adequately test the possibility of base catalysis.

Comparison of k_A^- values, at 30.5°, shows that phenylglycine anion reacts only about $^1/_{230}$ as fast with FDNB as does glycine anion.

In order to reckon the second-order rate coefficient for reaction of the amino acid form of phenylglycine with FDNB, one must divide the measured k_A (above) by the fraction of the phenylglycine which is present as the amino acid form (in equilibrium with the zwitterion form); the zwitterion form is assumed to be unreactive with FDNB. The fraction is 0.35 (Bryson *et al.*, 1963). Accordingly, the rate coefficient for reaction of the amino acid form with FDNB is 1.1×10^{-3} M^{-1} sec^{-1}. The reactivity of the amino function in $C_6H_5NHCH_2COOH$ is thus 82% of that of the amino function in $C_6H_5NHCH_2COO^-$.

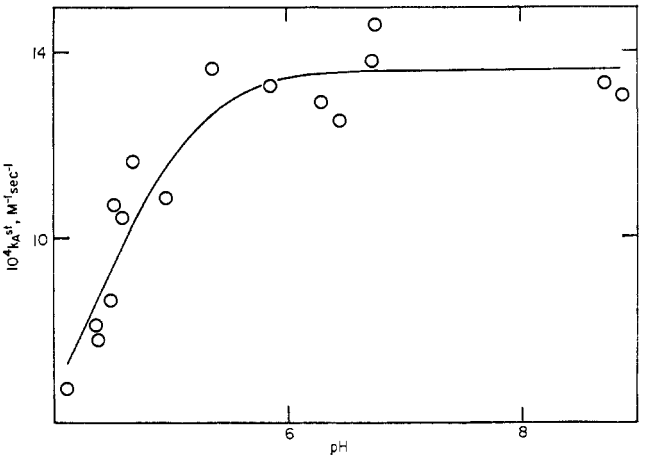


FIGURE 2: Apparent second-order rate coefficient for reaction of FDNB with *N*-phenylglycine in water at 30.5°. The line drawn is calculated by means of eq 2; see text. DNP-phenylglycine was formed quantitatively except in the two runs at pH >8, where the yield was 72%.

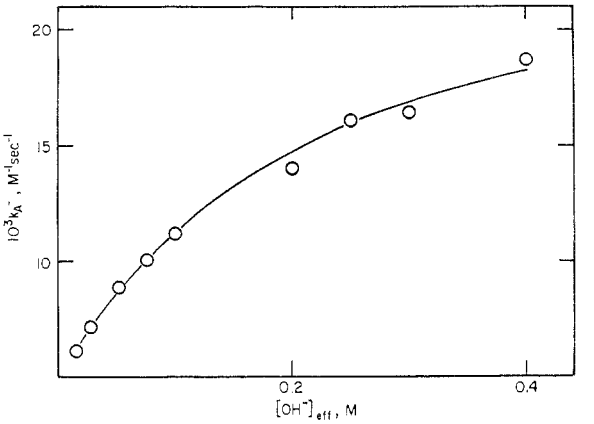
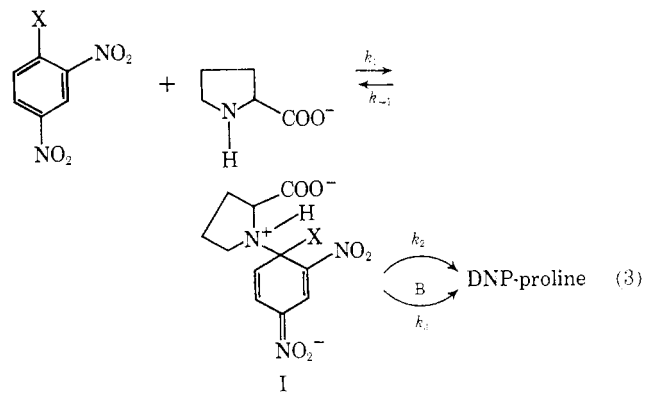


FIGURE 3: Second-order rate coefficient for reaction of FDNB with proline anion in 10% dioxane–90% water at 29.4°, as a function of effective hydroxide ion concentration. The line drawn is calculated by means of eq 4; see text.

Reaction of DNPE with Proline in 10% Dioxane–90% Water. It seemed possible that the insensitivity of the FDNB–proline reaction to catalysis by bases might be due to a special intramolecular base catalysis effect (discussed below), which would make catalysis by external base unnecessary. Inasmuch as the reaction of 2,4-dinitrophenyl phenyl ether with piperidine is quite sensitive to catalysis by base (Bunnett and Garst, 1965b; Bunnett and Bernasconi, 1965), the reaction of DNPE with proline was investigated in order to test the hypothesis of intramolecular base catalysis. Because of the limited solubility of DNPE in water, rate studies were conducted in 10% dioxane–90% water and at 29.4°, conditions used by Bunnett and Bernasconi (1965).

The reaction was found to be accelerated by base. At ionic strength 0.2, k_A^- increased from 4.15×10^{-3} M^{-1} sec^{-1} in 1:1 proline buffer to 11.6×10^{-3} M^{-1} sec^{-1} with 0.1 M NaOH. A more extensive series of runs was conducted at ionic strength 0.6; as can be seen in Figure 3, k_A^- rises steadily with increasing OH^- concentration, but the rise is ever less steep as base concentration increases. It has been shown in earlier papers (Bunnett and Randall, 1958; Bunnett and Garst, 1965b; Bunnett and Bernasconi, 1965) that such a curvilinear response is intelligible in terms of a two-stage reaction sequence, as in eq 3. Formation of the σ complex intermediate



(3)

(I) is not base catalyzed but its progression to products is accelerated by base B (with rate coefficient k_3). If reversion of the intermediate to reactants is much faster than its progression to products, the overall second-order rate coefficient increases linearly with base concentration. However, if the rate of base-catalyzed progression to products approaches or exceeds that of regression to reactants, the acceleration is curvilinear in the fashion of Figure 3. Mathematically, the situation is represented by eq 4, in which k_2 refers not only to uncatalyzed or solvent-catalyzed transformation of intermediate to products, as implied in eq 3, but also that catalyzed

$$k_A^- = \frac{k_1k_2 + k_1k_3[B]}{k_{-1} + k_2 + k_3[B]} \quad (4)$$

by bases present in constant concentration throughout a series of runs (*e.g.*, proline anion).

The data of Figure 3 were plotted as an inversion plot ($1/k_A^-$ *vs.* $1/[OH^-]$), after Bunnett and Garst (1965b); only the four points at highest OH^- concentrations were linear, and k_1 was evaluated from the intercept as 0.026 M^{-1} sec^{-1}. Then eq 4 was rearranged into eq 5. The plot of $k_A^-/(k_1 - k_A^-)$

$$\frac{k_A^-}{k_1 - k_A^-} = \frac{k_2}{k_{-1}} + \frac{k_3[B]}{k_{-1}} \quad (5)$$

was linear; k_2/k_{-1} was evaluated as its intercept, 0.22, and k_3/k_{-1} as its slope, 5.36 M^{-1}. By means of eq 4 and these values of k_1, k_2/k_{-1} and k_3/k_{-1}, k_A^- were then calculated at various base concentrations; the calculated values are represented by the curved line drawn in Figure 3. It is evident that the data conform to eq 4.

Reaction of DNPE with Pyrrolidine in 10% Dioxane–90% Water. Base catalysis having been found in the reaction of DNPE with proline anion, its reaction with pyrrolidine, which differs from proline only in lacking a carboxyl group, was investigated. Eleven runs were made, at 29.4°, ionic strength 0.6, DNPE concentration 1×10^{-4} M, and pyrrolidine concentration 0.025 M or higher. Four runs involved 1:1 pyrrolidine–pyrrolidine hydrochloride buffers of varying

buffer concentration, and seven NaOH in concentration ranging from 0.002 to 0.25 M. DNP-pyrrolidine was formed quantitatively except in the run at 0.25 M NaOH, in which the yield was 87%. k_A values, reckoned as the pseudo-first-order rate coefficient for formation of DNP-pyrrolidine, k_A*, divided by the concentration of free pyrrolidine (after correction as necessary for its basic dissociation), varied only randomly among ten of the runs, the average value and average deviation being $4.57 \pm 0.14 \times 10^{-2}$ M^{-1} sec^{-1}. These runs provide no evidence of base catalysis. For the run in 0.25 M pyrrolidine buffer, k_A was 5.06×10^{-2} M^{-1} sec^{-1}; this may represent mild acceleration of uncertain character such as has been noted before (Bunnett and Garst, 1965a, and references cited therein).

Reaction of FDNB with Glycine in Me_2SO–Water and Acetonitrile–Water Mixtures. Reactions of the sodium salt of glycine with FDNB in several solvent mixtures were studied, as listed in Table III. It is evident that addition of acetonitrile causes only a slight increase in reaction rate, somewhat more than twofold when the solvent is 80% acetonitrile. On the other hand, addition of Me_2SO causes a great increase. With 50% Me_2SO, the reaction is accelerated about 15-fold, and with 80% Me_2SO the acceleration is about 150-fold.

The rate determinations reported in Table III were carried out with the sodium salt of glycine, introduced either as the analytically pure salt or formed *in situ* by combination of equivalent quantities of glycine and NaOH. Some determinations were performed under conditions more nearly resembling those commonly used in dinitrophenylation of peptides. In these, $NaHCO_3$ was used in 50% molar excess over glycine, which in turn was present in excess over FDNB (1×10^{-4} M); in 80% ethanol–20% water (glycine, 2×10^{-2} M), DNP-glycine was formed quantitatively and the apparent second-order rate coefficient (k_ψ/[glycine]$_{st}$) was 0.62 M^{-1} sec^{-1}; in 80% Me_2SO–20% water (glycine, 2×10^{-3} M), the yield of DNP-glycine was 91% and the apparent second-order rate coefficient (0.91 k_ψ/[glycine]$_{st}$) was 5.2 M^{-1} sec^{-1}. Thus under these conditions dinitrophenylation is much faster in Me_2SO–water than in ethanol–water.

TABLE III: Reaction of FDNB with Glycine in Me_2SO–Water and Acetonitrile–Water Mixtures.[a]

$[H_2NCH_2CO_2Na]$ (M)	Solvent Constituent	Temp (°C)	k_A^- (M^{-1} sec^{-1})
2.0×10^{-2}	Water, 100%	30.5	0.30
2.0×10^{-2}	CH_3CN, 50%	30.5	0.35
1.0×10^{-2}[b]	CH_3CN, 50%	30.5	0.35
2.0×10^{-2}	CH_3CN, 80%	30.5	0.66
1.9×10^{-3}	Me_2SO, 20%	25.0	0.44
2.2×10^{-3}	Me_2SO, 30%	25.0	0.65
2.0×10^{-3}	Me_2SO, 40%	25.0	1.1
4.0×10^{-3}	Me_2SO, 50%	25.0	3.3
2.1×10^{-3}	Me_2SO, 60%	25.0	4.7
1.9×10^{-3}	Me_2SO, 70%	25.0	13
5.5×10^{-4}	Me_2SO, 80%	25.0	33
3.9×10^{-4}	Me_2SO, 90%	25.0	68

[a] The yield of DNP-glycine was quantitative in water and acetonitrile–water, and *ca.* 88% in Me_2SO–water mixtures. [b] 1.0×10^{-2} M glycine also present.

Discussion

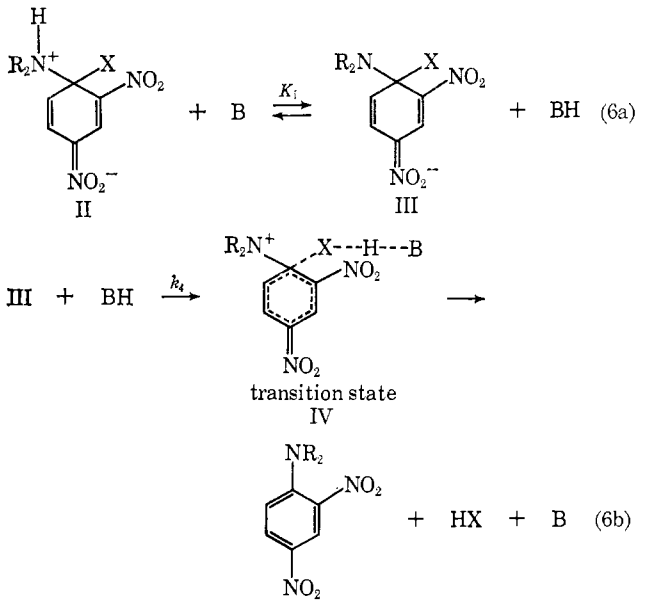
Our observations that the reactions of glycine anion and proline anion with FDNB are not detectably catalyzed by NaOH in concentrations as high as 0.1 M probably can be taken to indicate that the reaction of no common amino acid with FDNB is sensitive to such catalysis. It appears that the only effect of changing base concentration on the rate of reaction with FDNB is that having to do with partitioning of the amino acid between its neutral (mainly zwitterion) and anion forms.

To the biochemist, the most useful of our observations is perhaps the great acceleration of the reaction of FDNB with glycine which occurs when the solvent is changed from water or aqueous ethanol to Me_2SO–water. The reaction rate is more than one hundred times greater in 80% Me_2SO–20% water than in water, whereas the rates in 80% ethanol–20% water or 80% acetonitrile–20% water are only two or three times greater than in water.

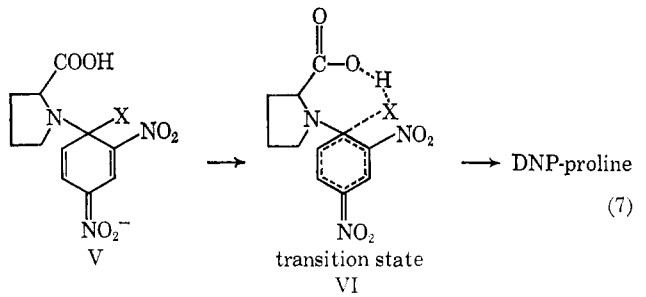
This suggests that in practical application of dinitrophenylation for purposes of the analysis or modification of peptides and proteins, important savings of time may be achieved by performing the reaction with FDNB in a medium containing a high percentage of Me_2SO as a solvent constituent. Roughly speaking, it should be possible to reduce the time required for dinitrophenylation from, say, 2 hr to 2 min. DNP derivatives of amino acids are easily retrieved from reaction mixtures by diluting the alkaline mixture with water, extracting with ether to remove unreacted FDNB, acidifying, and extracting with ether to remove the DNP-amino acid. Clearly, other procedures would need to be used to isolate DNP derivatives of complex peptides.

Use of 75% Me_2SO–25% water as a solvent for the ninhydrin reaction with amino acids has been advocated by Moore (1968).

Upon discovery that the reaction of proline anion with FDNB is not accelerated by base, we considered the possibility that intramolecular base catalysis by the COO^- function might be so fast as to surpass external catalysis by OH^-. It is believed that the slow step in general base-catalyzed aromatic nucleophilic substitutions involving amine nucleophiles is general acid catalyzed ejection of the leaving group from the conjugate base (III) of the initial covalent intermediate (II), as represented in eq 6 (Bunnett and Bernasconi, 1965; Bunnett and Garst, 1968). In terms of such a mechanism, the k_3 of eq 4 are replaced by K_1k_4, but otherwise eq 4 is undisturbed. Now, if the COO^- of intermediate I, eq 3 (X = F), were to act as the catalyzing base, the second intermediate (analogous to III in eq 6a) would have structure V, and intramolecular general acid catalysis *via* transition state VI (analogous to IV in eq 6b) would be conceivable. Acetate ion is known to be an effective catalyst of the reaction of *N*-methylaniline with FDNB (Bunnett and Randall, 1958), and transition state VI appears to be geometrically feasible. This mode of intramolecular base catalysis would call for the rate to be dependent only on the concentrations of FDNB and proline anion. If it were sufficiently rapid, catalysis by external OH^- would be undetectable.



However, the fact that the reaction of DNPE with proline anion *is* catalyzed by NaOH raises some doubts as to whether such intramolecular catalysis is really effective. Transition state VI would seem, from examination of models, to be equally feasible whether X were F or OC_6H_5. On the other hand, there is the possibility that the substantial magnitude of k_2 in the DNPE reaction (k_3/k_2 is 24 for the reaction of DNPE with proline anion and 2000 for its reaction with piperidine; Bunnett and Bernasconi, 1965) might represent this mode of intramolecular catalysis.



The surprising fact that the reaction of DNPE with pyrrolidine is insensitive to catalysis by NaOH casts a shadow of doubt on such an inference. The fact that introduction of a COO^- in the α position of the amine nucleophile causes the overall reaction to become sensitive to catalysis by base shows that it increases k_{-1} in eq 4 more than it does k_2, indeed, so much more as to cause k_{-1} to exceed $k_2 + k_3[B]$, whereas with pyrrolidine $k_2 + k_3[B]$ greatly exceed k_{-1}. In other cases, changes in the structure of amine nucleophiles so as to increase their steric requirements have shifted reactions from being insensitive to being sensitive to base catalysis, that is, they have increased the $k_{-1}/(k_2 + k_3[B])$ ratio from much less than to much greater than unity (Bunnett and Garst, 1965a; Beale, 1967). Apparently, the increased steric requirements of the COO^- group are also responsible in the present instance.

The remarkable fact that the reactions of DNPE with piperidine and pyrrolidine are, respectively, sensitive and insensitive to catalysis by base seems also to require interpretation in steric terms. Piperidine differs from pyrrolidine only in having an additional methylene group. It does, therefore, occupy a greater volume, but the increase in bulk is rather far from the nucleophilic center. The change from a five- to a six-membered ring also changes the conformational situation. We believe that a proper interpretation must take account of the detailed conformation of the intermediate as a whole, but our understanding is not yet sufficient to enable a full interpretation to be offered.

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