activity. The diminished reactivity of benzenesulfonamide anion is reminiscent of discrepancies often observed in similar Brønsted-like plots for hydroxide ion as compared to alkoxide ions or of ammonia as compared to amines.

The autoprotolysis constant for methanol, as pK_s, is 16.9,[8] and pK_a by the usual arbitrary conversion is 18.3. Extrapolation of the line in Figure 2 would lead to a log k_N at pK_a 18.3 some 1.7 log units greater than observed for methoxide ion, equivalent to a 50-fold deficiency in reactivity for this oxyanion nucleophile.

Experimental Section

The several amides and imides were obtained from commercial sources or synthesized by familiar methods and purified by recrystallization to melting points in good agreement with literature values.

In reaction mixtures for kinetic runs, the initial concentration of methyl iodide was typically *ca.* 0.02 *M*, of the sodium derivative of the amide or imide *ca.* 0.06 *M*, and of the free amide or imide variously from *ca.* 0.02 to 0.14 *M*. Details for particular runs are given in the Ph.D. thesis of Beale.[1] Second-order rate coefficients, k_2, were reckoned by linear regression analysis from the expression, $[1/(a - b)] \ln [b(a - x)/a(b - x)] = k_2t$. In general, good second-order kinetics were observed. Enthalpies and entropies of activation were reckoned from standard expressions.[9]

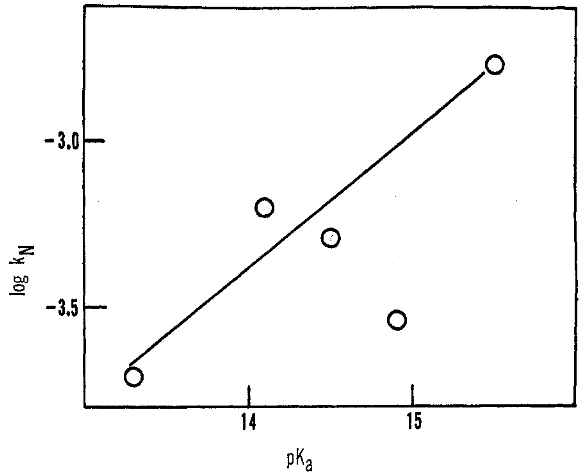

Figure 2.—Relationship of log k_N to pK_a in CH_3OH. From left to right, the points represent the anions of *N*-phenylbenzenesulfonamide, succinimide, phthalimide, benzenesulfonamide, and *N*-methylbenzenesulfonamide, respectively.

The expected *N*-methyl derivatives were isolated from reactions of succinimide, benzenesulfonamide, and *N*-methylbenzenesulfonamide under the conditions of rate measurement and were identified by melting point and/or infrared spectrum.

Registry No.—Methyl iodide, 74-88-4; $C_6H_5SO_2NCH_3^-$, 28627-66-9; succinimide anion, 28627-67-0; phthalimide anion, 28627-68-1; $C_6H_5SO_2NH^-$, 28627-69-2; $C_6H_5SO_2NC_6H_5^-$, 28627-70-5; $NaOCH_3$, 124-41-4.

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[8] R. G. Bates, "Solute-Solvent Interactions," J. F. Coetzee and C. D. Ritchie, Ed., Marcel Dekker, New York, N. Y., 1969, p 52.

[9] J. F. Bunnett, "Investigation of Rates and Mechanisms of Reactions," S. L. Friess, E. S. Lewis, and A. Weissberger, Ed., 2nd ed, Part I, Interscience, New York, N. Y., 1961, p 199.

Mercaptoethanol Catalysis for Hydrolysis of *N*-Benzyl-3-cyanopyridinium Bromide. A Model for the Nitrilase Reaction[1]

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The hydrolysis of *N*-benzyl-3-cyanopyridinium bromide is subject to marked catalysis by dilute aqueous solutions of mercaptoethanol. Under neutral or slightly acidic conditions, the predominant reaction product is the corresponding amide. Under conditions more acidic than pH 3, appreciable amounts of the corresponding acid are formed as well. First-order rate constants for the disappearance of nitrile at fixed concentrations of mercaptoethanol exhibit a bell-shaped dependence upon pH with a maximum near pH 7. First-order rate constants for the disappearance of nitrile at constant values of pH exhibit saturation with respect to mercaptoethanol concentration; under sufficiently basic conditions, excess mercaptoethanol actually causes inhibition of nitrile hydrolysis. These observations are interpreted in terms of (1) addition of mercaptoethanol to the nitrile function to form a thioimidate followed by hydrolysis of the thioimidate to yield the corresponding amide or acid, (2) addition of mercaptoethanol to both the nitrile and thioimidate to form 1,4 adducts which are kinetically inert, and (3) rate-determining formation of the thioimidate on the acidic side of the pH-rate maximum and rate-determining hydrolysis of this intermediate on the basic side.

Among functional groups at the acyl level of oxidation, nitriles are perhaps the most resistant to hydrolysis. The nitrile group is quite stable to both nucleophilic and electrophilic attack and, in consequence, strenuous conditions must usually be employed to effect hydrolysis under either basic or acidic conditions.[3-8] Nevertheless, enzymes have been identified in several sources which are capable of effecting rapid hydrolysis of nitriles at ambient temperatures in neutral aqueous solutions (see below). Our concern with these reactions centers on the elucidation of catalytic pathways through which these enzymes may function.

The basic hydrolysis of nitriles, including benzonitrile,[5,6] propionitrile,[7] and phenylacetonitriles,[9] is kinetically first order in both substrate and hydroxide

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[1] Supported by Grant GE 3277 from the National Science Foundation and by Grant AM 08232 from the National Institutes of Health.

[2] Career Development Awardee of the National Institutes of Health, Grant K3 GM 10248-03; Research Fellow of the Alfred P. Sloan Foundation.

[3] J. D. McLean, B. S. Rabinovitch, and C. A. Winkler, *Can. J. Res., Sect. B*, **20**, 168 (1942).

[4] B. S. Rabinovitch, C. A. Winkler, and A. R. P. Stewart, *ibid.*, **20**, 121 (1942).

[5] K. B. Wiberg, *J. Amer. Chem. Soc.*, **77**, 2519 (1955).

[6] K. B. Wiberg, *ibid.*, **75**, 3961 (1953).

[7] B. S. Rabinovitch and C. A. Winkler, *Can. J. Res. Sect. B*, **20**, 185 (1942).

[8] R. Breslow, R. Fairweather, and J. Keana, *J. Amer. Chem. Soc.*, **89**, 2135 (1967).

[9] E. Angelescu, G. Vasiliu, and D. Zavoianu, *Rev. Chim., Acad. Repub. Pop. Roum.*, **7** (2), 655 (1962); *Chem. Abstr.*, **61**, 4168*d* (1962).

ion suggesting that the transition state involves the direct attack of hydroxide ion on the nitrile carbon atom; facile proton-transfer reactions complete amide formation. There appear to be two ways to promote amide formation from nitriles: increase in the electrophilicity of the nitrile carbon atom and utilization of nucleophilic reagents more reactive than hydroxide ion as nucleophilic catalysts.

That nitrile hydrolysis is subject to nucleophilic catalysis has been established by the observation that the addition of the hydroperoxide anion to nitriles is a facile reaction leading to the production of amide and molecular oxygen.[5,6] The kinetics of this reaction indicate that the rate-determining step is attack of the hydroperoxide anion on the nitrile followed by rapid decomposition of the resulting adduct to the indicated products. Since the hydroperoxide anion is less basic but more polarizable and more reactive toward nitriles than hydroxide ion, polarizability appears to be an important factor in determining nucleophilicity toward such substrates. This suggestion is supported by the observation that the weakly basic but highly polarizable hydrosulfide ion is also a good nucleophilic reagent against nitriles.[10,11]

The observation that nitriles are quite reactive toward sulfur nucleophiles is an interesting one biochemically since both ricinine nitrilase[12] and indolacetonitrile nitrilase[13] are subject to marked inhibition by reagents which bind thiol groups. Herein we present a detailed study of the catalysis of nitrile hydrolysis by mercaptoethanol. This system proves to have similarities with the enzymatic reactions involving similar substrates. A preliminary account of this work has been published.[14]

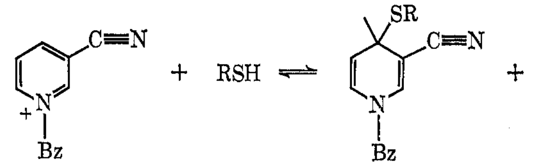
Experimental Section

Materials.—*N*-Benzyl-3-cyanopyridinium bromide was synthesized by refluxing a solution of 20 ml of α-bromotoluene and 20 g of 3-cyanopyridine in 150 ml of dry tetrahydrofuran for 20 min. The solution was cooled and concentrated under aspirator pressure and then under high vacuum until free of α-bromotoluene. The crystalline residue was recrystallized from *ca.* 500 ml of acetonitrile and dried under high vacuum, yield 35 g, mp 149°.[15]

Reagent grade mercaptoethanol (Matheson Coleman and Bell), ferric chloride (Fisher), hydroxylamine hydrochloride (Matheson Coleman and Bell), citric acid (Fisher), and tris-(hydroxymethyl)aminomethane (Sigma) were used without further purification. Imidazole was recrystallized twice from benzene and freed of solvent by prolonged drying under high vacuum. All solutions were prepared in water which had been glass distilled with a Corning AG 1a still. Solutions of sodium hydroxide and hydrochloric acid were employed to adjust solutions to desired values of pH. CM Sephadex C 50 cation exchanger (Pharmacia Fine Chemicals) was prepared for use according to the instructions of the manufacturer.

Kinetic Methods.—The progress of kinetic runs was monitored by one of the following two methods.

A. At values of pH higher than 7, the concentrations of substrate and nucleophilic reagent ordinarily employed in the kinetic experiments, 4.4×10^{-3} *M* *N*-benzyl-3-cyanopyridinium bromide and up to 0.45 *M* mercaptoethanol, resulted in the formation of equilibrium concentrations of a 1,4 adduct sufficient to quantitate by its ultraviolet absorption at 332 mμ[16] (eq 1). Since (1) formation of this adduct is rapid with respect to the rates of successive reactions (adduct formation is too fast to follow employing stopped-flow methods), (2) other intermediates and reaction products do not absorb appreciably at 332 mμ under the experimental conditions, and (3) the concentration of mercaptoethanol does not change appreciably during the course of the reaction, changes in the concentration of the substrate can be monitored by the optical density at this wavelength.[17]



(1)

B. At values of pH below 7 and within the range of concentrations normally employed for kinetic measurements (*vide infra*), insufficient 1,4 adduct is present at equilibrium in the reaction mixtures themselves to permit the reaction to be followed by observing its decay spectrophotometrically (eq 2). In these cases, changes in the concentration of substrate as a function of time were monitored by the periodic injection of 0.30-ml reaction solution aliquots into 2.7 ml of 0.1 *M* mercaptoethanol at pH 10.3 ± 0.2. The optical density of the adduct at 332 mμ was recorded as soon as possible.

Using either method, good first-order rate behavior is observed through at least three half-lives. First-order rate constants were, in all cases, obtained from the slopes of log $(\mathrm{OD}_t - \mathrm{OD}_\infty)$ against time plots employing a least-squares computer program and a CDC 3600/3400 computational facility. Derived rate and equilibrium constants were calculated from slopes and intercepts of double reciprocal plots of the observed rate constants against the concentrations of mercaptoethanol employing similar least-squares methods. Points obviously erratic were discarded. Reliable error estimates are not available.

Values of optical density were obtained employing a Zeiss PMQII spectrophotometer equipped with a cell holder through which water from a thermostated bath was continuously circulated. Temperature was maintained at 24.9° throughout. Ionic strength of reaction solutions was maintained at 0.60 through addition of NaCl. Values of pH were obtained with a Radiometer PHM 4c pH meter equipped with a glass electrode.

In some experiments, the concentration of a transient intermediate, the thio ester formed from mercaptoethanol and *N*-benzylnicotinic acid, was followed as a function of time by use of the neutral hydroxylamine–ferric chloride method as described by Jencks, *et al.*[18]

Product Analysis.—Determination of the products of decomposition of *N*-benzyl-3-cyanopyridinium bromide in aqueous solutions of mercaptoethanol was accomplished through two preparative runs.

In the first, 1.0 g of *N*-benzyl-3-cyanopyridinium bromide was dissolved in 20 ml of 0.5 *M* mercaptoethanol and permitted to remain at room temperature for 18 hr. The pH of the reaction mixture was 4.5 and did not change in the course of the reaction. At the end of the incubation period, the reaction solution was washed five times with equal volumes of ether and then concentrated to one-third the original volume under aspirator pressure. Crystallization of a product occurred during volume reduction. These crystals were collected, recrystallized from ethanol, and dried under high vacuum to yield 0.85 g, mp 235° (lit.[19] 236°). Ir, uv, and pmr spectra were identical with those of authentic *N*-benzyl-3-carbamoylpyridinium bromide. The product migrated with *N*-benzyl-3-carbamoylpyridinium bromide in ascending paper chromatography in an ethyl acetate–methanol–water

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[10] K. Kindler, *Justus Liebigs Ann. Chem.*, **431**, 187 (1923).

[11] K. Kindler, *ibid.*, **450**, 1 (1926).

[12] W. G. Robinson and R. H. Hook, *J. Biol. Chem.*, **239**, 4257, 4263 (1964).

[13] K. V. Thimann and S. Mahadevan, *Arch. Biochem. Biophys.*, **105**, 133 (1964); **107**, 62 (1964).

[14] C. Zervos and E. H. Cordes, *J. Amer. Chem. Soc.*, **90**, 6892 (1968).

[15] G. Büchi, D. L. Coffen, K. Kocsis, P. E. Sonnet, and F. E. Ziegler, *ibid.*, **88**, 3099 (1966).

[16] J. van Eys and N. O. Kaplan, *J. Biol. Chem.*, **228**, 305 (1957).

[17] Note that the mercaptoethanol adduct of the thioimidate intermediate (Scheme I) will absorb at the wavelength employed to follow the reaction kinetics. This could introduce an uncertainty into the assay method only if this adduct accumulated under conditions in which the formation of the thioimidate is rate determining. This is the rate-determining step under relatively acidic conditions as developed below; however, reasonable estimates for the equilibrium constant for adduct formation based on that for adduct formation from the substrate (Scheme I) indicate that, under the experimental conditions, this situation does not arise.

[18] W. P. Jencks, S. Cordes, and J. Carriuolo, *J. Biol. Chem.*, **235**, 3608 (1960).

[19] P. Karrer and F. J. Stare, *Helv. Chim. Acta*, **20**, 418 (1937).

(5:4:1) solvent system on Whatman No. 1 paper. *Anal.* Calcd for *N*-benzyl-3-carbamoylpyridinium bromide: C, 53.30; H, 4.44; N, 9.56. Found: C, 53.24; H, 4.55; N, 9.36.

In the second, 5.0 g of *N*-benzyl-3-cyanopyridinium bromide was dissolved on 200 ml of 0.5 *M* mercaptoethanol and 0.25 *M* citrate, pH 2.18. The reaction mixture was permitted to remain at room temperature for 22 days at which time the appropriate assays indicated that little substrate or thio ester remained. The reaction mixture was washed five times with equal volumes of ether and then subjected to chromatography on a column of CM Sephadex C50 which was 5 cm in diameter and 90 cm high. The column was eluted with 0.015 *M* phosphate buffer, pH 7.10 with which it had been preequilibrated. Fractions of 15 ml were collected employing a Gilson Model LB1 fraction collector equipped with volumetric collecting unit Model LVM1, and their optical density at 260 mμ was determined. Fractions containing the first peak were pooled and lyophilized. Following desalting by passage of an aqueous solution through a column of Sephadex G15, 100 mg of crystals was obtained. This was determined to be *N*-benzylnicotinic acid through identity of the ir and pmr spectra with those of an authentic sample of this compound. A second peak was treated as described above. These fractions yielded 120 mg of *N*-benzyl-3-carbamoylpyridinium bromide identified by paper chromatography and pmr spectroscopy. Attempts to purify the small amounts of the material eluting from the column between the two major fractions were not successful.

The above procedure was modified to determine the effects of pH on the nature of the reaction products. Four reactions were set up (see Table I). At the conclusion of the runs it was established by use of assay II that no substrate remained unreacted. In the cases of reactions A and B, the time course of disappearance of the substrate and the time course for appearance and disappearance of the thio ester intermediate were followed by the appropriate assays for about four half-lives. Each of the reaction mixtures was thoroughly washed with ether and then chromatographed on columns of CM Sephadex C 50, 2.5 cm in diameter and 45 cm high. As before these were developed with 0.015 *M* phosphate buffer, pH 7.1. The elution patterns were similar to that observed before, the acid appearing early in the process and the amide late with a small amount of unidentified material in between.

TABLE I

	Reaction			
	A	B	C	D
pH	3.09	3.19	3.46	4.75
Citrate buffer, *M*	0.1	0.1	0.1	0.3
Mercaptoethanol, *M*	0.5	0.5	0.25	0.25
N-Benzyl-3-cyanopyridinium bromide, mg	250	250	200	200
Reaction volume, ml	10	10	40	40
Reaction time, hr	250	250	48	48

Ir spectroscopy was employed to follow the course of certain of the reactions of *N*-benzyl-3-cyanopyridinium bromide. The reaction solutions were prepared by dissolving 50 mg of *N*-benzyl-3-cyanopyridinium bromide in 1.0 ml of a 0.5 *M* solution of mercaptoethanol in deuterium oxide which had been adjusted to the desired value of pH with solutions of NaOD or DCl, as appropriate. The reaction solutions were then applied as a film to windows of IRTran-2 and assembled into cells. A Perkin-Elmer Model 621 infrared spectrometer was employed to record the spectra.

Results

Incubation of *N*-benzyl-3-cyanopyridinium bromide in aqueous solutions of mercaptoethanol in the pH range between 3 and 10 leads to a reasonably rapid disappearance of this material. As detailed in the Experimental Section, the predominant products of the reaction are the corresponding amide and acid, *N*-benzyl-3-carbamoylpyridinium bromide and *N*-benzylnicotinic acid. Under conditions more basic than pH 4, the amide is the only important product, while under more acidic conditions appreciable amounts of the acid are formed as well. At pH 2.1, the acid is the more important product quantitatively.

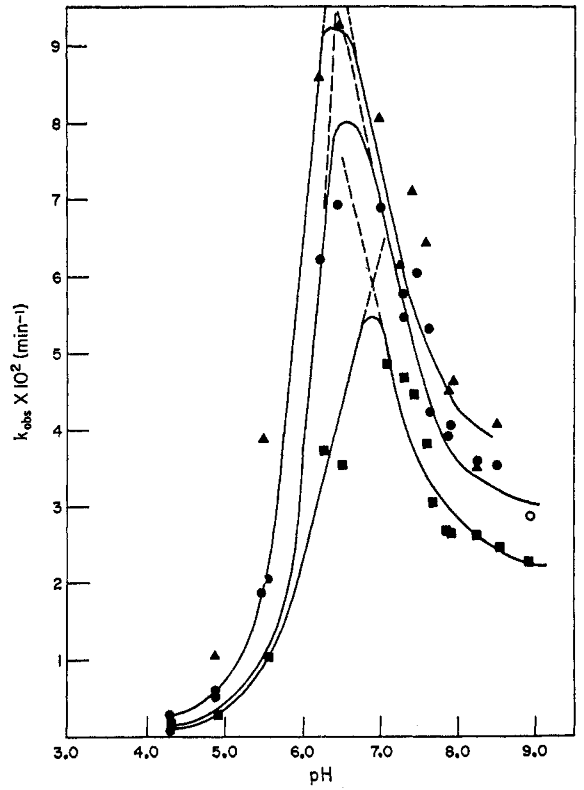


Figure 1.—First-order rate constants plotted against pH for the hydrolysis of *N*-benzyl-3-cyanopyridinium bromide in aqueous mercaptoethanol solutions. Experimental points (lines) refer to three mercaptoethanol concentrations, 0.04 *M* (closed squares), 0.08 *M* (closed circles), and 0.15 *M* (closed triangles).

In addition to the acid and amide, which are terminal products in the reaction process, evidence has been obtained to suggest that the thio ester between mercaptoethanol and *N*-benzylnicotinic acid is an intermediate. This suggestion is based on the observation of transient formation of a material which reacts with neutral hydroxylamine to yield a hydroxamic acid as judged from the nature of the color formed in the presence of acidic ferric chloride. This point is developed further below.

By employing either assay developed in the Experimental Section, the kinetics of disappearance of *N*-benzyl-3-cyanopyridinium bromide in aqueous solutions of mercaptoethanol at 25° were investigated. Pseudo-first-order rate behavior was observed throughout. In Figure 1, first-order rate constants for this reaction are exhibited as a function of the concentration of mercaptoethanol and pH.

In each case, the rate constants pass through a maximum as a function of pH. At low concentrations of mercaptoethanol, the maximum occurs near pH 7. As the thiol concentration is increased, the pH–rate maximum occurs at progressively more acidic values of pH, though not below pH 6 under any of the conditions examined in this study.

Saturation kinetics are observed with respect to mercaptoethanol concentration. That is, the apparent order of the reaction varies from unity to zero as the concentration of mercaptoethanol is increased. Such behavior is qualitatively accounted for in terms of

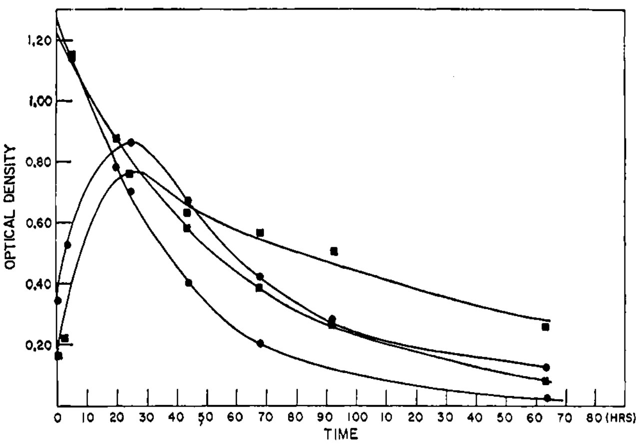


Figure 2.—Time course of disappearance (continuously declining curves) of the *N*-benzyl-3-cyanopyridinium ion and appearance of the neutral hydroxylamine-reactive intermediate (curves passing through a maximum), in aqueous mercaptoethanol solution at pH values below 3.5. Squares refer to reaction A and circles to reaction B (see Experimental Section).

the addition of mercaptoethanol to the substrate to form an unreactive complex (Experimental Section).

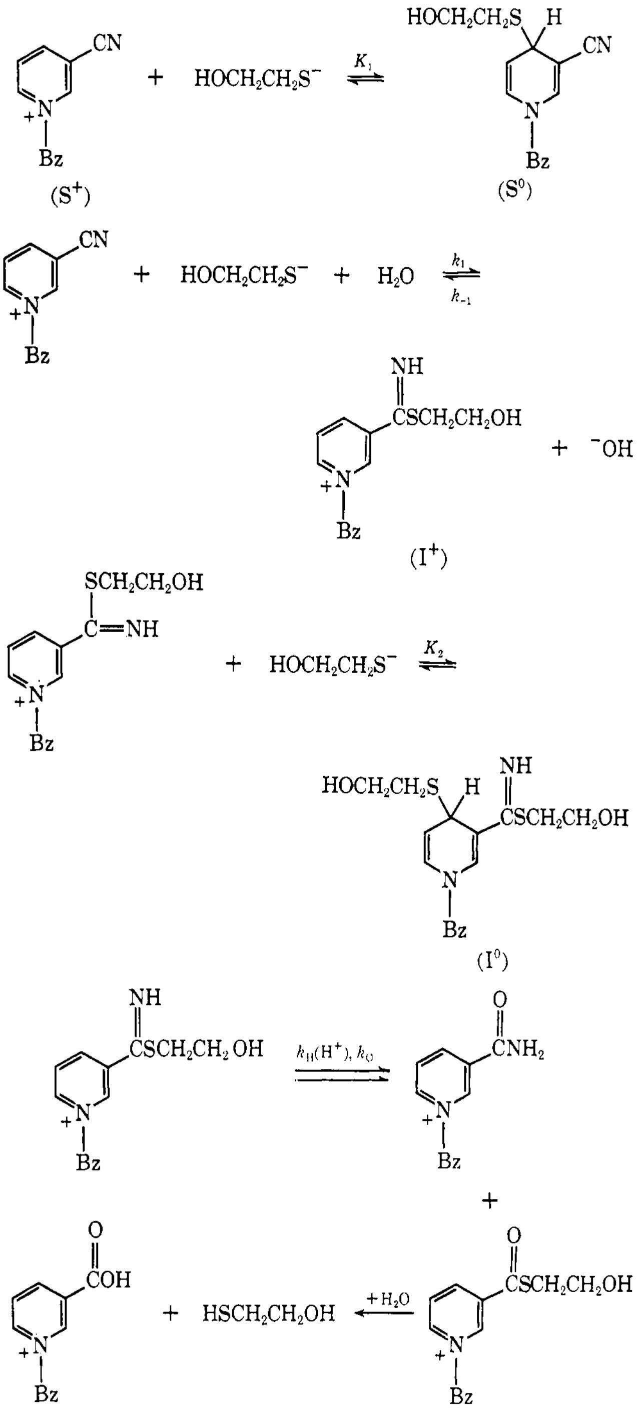
First-order rate constants for the disappearance of *N*-benzyl-3-cyanopyridinium bromide at pH 10.13 and ionic strength 0.20 decrease from 0.005 to 0.003 min^{-1} as the concentration of mercaptoethanol is increased from 0.02 to 0.20 *M*. This inhibition by thiol under basic conditions cannot be accounted for in terms of a single unreactive complex but requires, as is developed below, that a second unreactive complex be formed containing 2 molecules of thiol.

The time course of disappearance of *N*-benzyl-3-cyanopyridinium bromide and the time course of appearance and disappearance of a neutral hydroxylamine-reactive intermediate, presumably 2-hydroxyethyl-*N*-benzylthionicotinate, are shown in Figure 2. Reaction conditions for these kinetic studies have been described in the Experimental Section.

The time course of hydrolysis of *N*-benzyl-3-cyanopyridinium bromide to the corresponding amide on the alkaline side of the pH-rate maximum (Figure 1) was followed by ir spectroscopy as described in the Experimental Section. Under these conditions, one observes the immediate appearance of a new band at 1662 cm^{-1} which increases in intensity with time. In addition, one observes the immediate appearance of a new band at 1578 cm^{-1} the intensity of which decreases as a function of time. These observations contrast with those observed for the same reaction on the acid side of the pH-rate maximum. Here one finds only the gradual appearance of a new band at 1667 cm^{-1}, presumably due to the formation of the amide product.

In Figure 3, optical density values at 332 mμ are plotted against mercaptoethanol concentration for an aqueous solution of *N*-benzyl-3-cyanopyridinium bromide at pH 9.2, ionic strength 0.60, and 25.0°. The optical density values are assumed (*vide infra*) to be proportional to the concentration of the 1,4 adduct between *N*-benzyl-3-cyanopyridinium bromide and mercaptoethanol. They were obtained by extrapolating to "zero time" the semilogarithmic plots of a series of reactions run under these conditions. These conditions avoid the formation of large concentrations of the 1,4 adduct between the imidate intermediate and mercaptoethanol (Scheme I) for the reasons cited in the Discussion.

SCHEME I



Note that the midpoint of this curve, 0.006 *M* mercaptoethanol anion, is equal to the reciprocal of the sum of association constants for formation of the 1,4 adduct and thioimidate (Scheme I). Evaluation of these association constants independently is considered further in the Discussion.

Discussion

The results cited above indicate that mercaptoethanol is an effective catalyst for the hydrolysis of *N*-benzyl-3-cyanopyridinium bromide. There is no simple way to express the magnitude of the catalysis since the uncatalyzed hydrolysis is simply first order in hydroxide ion under the conditions investigated here, while the catalyzed reaction exhibits a complex order with respect to mercaptoethanol and exhibits a pH–rate maximum as well. Kosower and Patton have measured a second-order rate constant of 0.28 M^{-1} sec^{-1} for the attack of hydroxide ion on the *N*-methyl-3-cyanopyridinium ion under comparable conditions with those employed in this study.[20] Comparing the corresponding first-order rate constant for that reaction at pH 7 with our observed maximal velocity at the same pH, a rate accentuation of about 10^3 is obtained. This value is about one order of magnitude smaller than one obtained in a similar study from this laboratory employing the *N*-benzyl substrate (unpublished observations).

The various characteristics of the mercaptoethanol-catalyzed hydrolysis of *N*-benzyl-3-cyanopyridinium bromide developed above suffice to indicate that the reaction is a complex one. The simplest reaction pathway that can account for the data is that shown in Scheme I. This reaction pathway includes (1) addition of thiol to the pyridinium ion at the 4 position to form an unreactive complex (S^0); (2) attack of the thiol, as the anion, on the cyano function of the substrate with formation of the corresponding thioimidate (I^+); (3) addition of the thiol to the thioimidate at the 4 position with formation of a second unreactive complex (I^0); and (4) hydrolysis of the thioimidate either to generate the amide directly or to generate the thio ester which may then hydrolyze to the acid.

The pathway indicated in Scheme I qualitatively accounts for the characteristics of the reaction in the following ways.

First, the saturation kinetics observed with respect to mercaptoethanol can be understood on the basis of the addition of the anion of mercaptoethanol to the substrate to form the 1,4 adduct which is unreactive toward attack by the thiol at the cyano function. That the complex should be much less reactive toward nucleophilic attack at the cyano group is entirely reasonable since (1) the activating electron-withdrawing effect of the cationic pyridinium nitrogen atom has been lost, and (2) electron donation by resonance from the ring nitrogen atom to the cyano function has been introduced. Overall, addition of the thiol to the ring system has transformed a strongly electron-withdrawing site into a strongly electron-donating one. A diminution of reactivity toward nucleophilic attack of 10^6 might well be expected to result. Furthermore, the formation of an adduct can be directly demonstrated spectrophotometrically and, in fact, serves as the basis for the rate measurements reported in this study. The observation of an absorption maximum for the adduct at 332 mμ is entirely consistent with the assignment of position 4 as the site of addition of the thiol although it does not rigorously exclude addition at sites 2 or 6. The assignment is further supported by observations that indicate that nucleophilic addition to 3-carbamoylpyridinium ions ordinarily is favored kinetically at the 4 position.[21] Since formation of the adduct at concentrations of mercaptoethanol employed in these kinetic studies is too fast to be measurable employing stopped-flow methods, it follows that adduct formation may be considered to be an equilibrium process, as indicated in Scheme I.

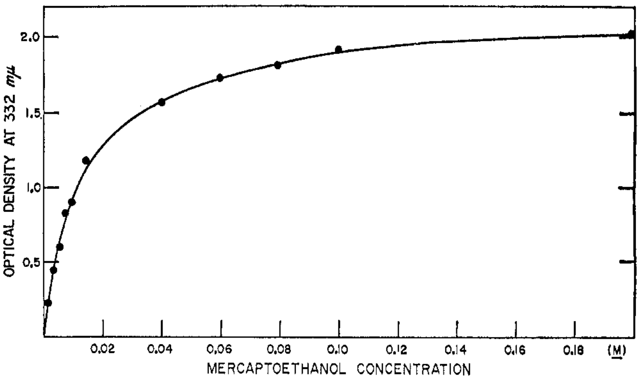


Figure 3.—Titration curve of *N*-benzyl-3-cyanopyridinium bromide with mercaptoethanol at pH 9.2, ionic strength 0.6, and 24.9°. The "zero time" optical density at 332 mμ (see text for details), which is proportional to the concentration of the 1,4 adduct between the substrate and mercaptoethanol anion, is plotted against the total mercaptoethanol concentration.

Second, the inhibition of the reaction by high concentrations of mercaptoethanol under relatively basic conditions (see Results) is accounted for in terms of the formation of an unreactive adduct between the thioimidate and a second molecule of mercaptoethanol. This intermediate, for which no direct spectrophotometric evidence is available, is expected to be unreactive toward nucleophilic attack by water compared to the corresponding pyridinium ion for the reasons developed just above. Assignment of the structure of the 4 adduct is arbitrary but reflects the kinetic reactivity of this position in relation to the 2 and 6 positions as previously pointed out. Accumulation of the thioimidate–mercaptoethanol adduct is expected to be important only under the conditions of high basicity since (1) the thioimidate is considered to accumulate only under these conditions (see below) and (2) adduct formation involves the thiolate ion, not the free thiol. Adduct formation is regarded as an equilibrium process in analogy with the behavior observed for adduct formation from the nitrile substrate.

Third, the pH–rate maximum observed for mercaptoethanol-catalyzed hydrolysis of *N*-benzyl-3-cyanopyridinium bromide (Figure 1) is interpreted as reflecting a transition from rate-determining formation of the thioimidate under acidic conditions to rate-determining hydrolysis of this intermediate under basic conditions. Such a transition in the rate-determining step is reasonable since formation of the adduct depends kinetically on the basic form of the nucleophilic reagent and, hence, the rate will increase with increasing pH below pH 9 and become pH independent above this value. In contrast, hydrolysis of the thioimidate very likely proceeds through acid-catalyzed and pH-independent reaction pathways reflecting, respectively, attack of water and hydroxide ion on the protonated substrates. These considerations are expressed diagrammatically in Figure 1. Related transitions in the rate-determining step have been observed for a number of reactions

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[20] E. M. Kosower and J. W. Patton, *Tetrahedron*, **22**, 2081 (1966).

[21] R. N. Lindquist and E. H. Cordes, *J. Amer. Chem. Soc.*, **90**, 1269 (1968).

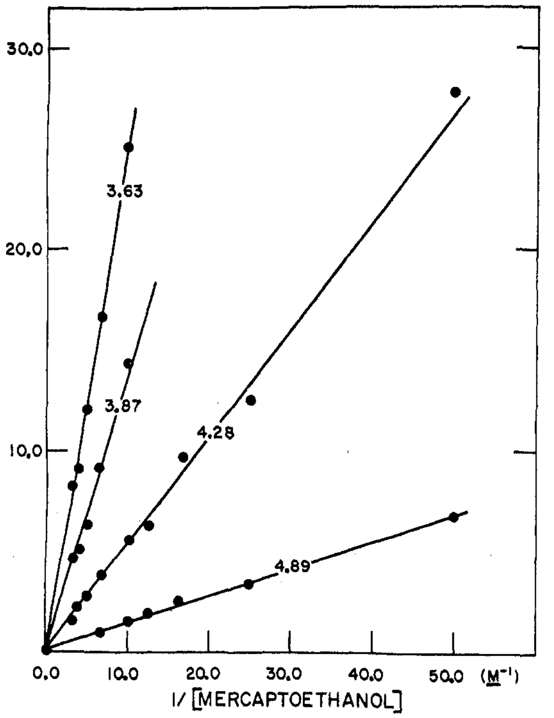

Figure 4.—Selected double reciprocal plots of k_{obsd} *vs.* mercaptoethanol concentration for the hydrolysis of *N*-benzyl-3-cyanopyridinium bromide in aqueous mercaptoethanol solutions on the acidic side of the pH–rate maximum, 25°, and ionic strength 0.60. The pH values of the reaction solutions are shown on the graph.

including carbonyl addition reactions,[22,23] Schiff base hydrolysis,[24] ester aminolysis,[25,26] thio ester hydrolysis,[27] and others.

Fourth, the course of the reaction as revealed by infrared spectroscopy is consistent with Scheme I (see Results). On the alkaline side of the pH–rate maximum, in which decomposition of the thioimidate is thought to be rate determining, the immediate appearance of the new band at 1662 cm^{-1} must reflect the rapid formation of equilibrium concentrations of the thioimidate. Increase in the intensity of this band reflects formation of the amide product whose infrared spectrum in this region is difficult to distinguish from that of the thioimidate. The new band at 1578 cm^{-1}, which appears immediately and decreases in intensity with time, is reasonably attributed to the thioimidate alone. On the acid side of the pH–rate maximum, in which formation of the thioimidate is considered to be rate determining, only the gradual appearance of a new band at 1666 cm^{-1} is observed, reflecting the gradual formation of the amide product.

Fifth, the pH dependence of the product distribution is accounted for in terms of the modes of partitioning of the thioimidate intermediate. Chaturvedi, MacMahon, and Schmir[25,28,29] have specifically investigated the decomposition of tetrahedral intermediates formed from the addition of water to thioimidates and have observed a transition from the expulsion of thiol with formation of amide to dominant expulsion of the amine with formation of the thio ester as the pH of the solution is lowered (Scheme II). Such behavior is completely consistent with that observed in our study and constitutes a strong line of evidence favoring the intermediate formation of the thioimidate.

Finally, the transient appearance of a material reactive toward neutral hydroxylamine suggests the presence of the thio ester intermediate (Figure 2). The kinetics of appearance and disappearance of this material is consistent with its participation as a reaction intermediate. Furthermore, under the conditions of these experiments, the maximum amount of thio ester formed is never more than 5% of the total amount of substrate utilized, consistent with the observation that the amide is the major reaction product.

Taken together, these lines of evidence provide a strong case for the reaction pathway of Scheme I. It is, of course, possible that the pathway is actually somewhat more complicated than this. Nevertheless, all of the features of Scheme I do seem to be required by the data which is accounted for within the limits of accuracy by it. We turn now to a consideration of the quantitative aspects of this reaction pathway and to the establishment of the rate and equilibrium constants which characterize it.

The complexity of the reaction pattern (Scheme I) precludes the derivation of a tractable rate law applicable to the total span of pH values for the kinetic experiments. No steady-state assumption valid over the whole pH range can be made for the concentration of the thioimidate involved in the reaction pathway. The derivation of two rate equations, one for each limb of the pH–rate profile, thus becomes necessary.

To the left of the pH–rate maximum, where the rate-determining step is the attack of the mercaptoethanol anion on the substrate, kinetically pertinent equations are depicted in the first two equations of Scheme I. The appropriate rate law is $[K_n \ll (\mathrm{H}^+)]$

$$k_{\text{obsd}} = \frac{k_1(\mathrm{RSH})_{\mathrm{T}}K_n}{(\mathrm{H}^+) + K_1K_n(\mathrm{RSH})_{\mathrm{T}}} \tag{2a}$$

or

$$1/k_{\text{obsd}} = \frac{(\mathrm{H}^+)}{k_1K_n}\frac{1}{(\mathrm{RSH})_{\mathrm{T}}} + \frac{K_1}{k_1} \tag{2b}$$

Equation 2b predicts that a double reciprocal plot of k_{obsd} *vs.* $(\mathrm{RSH})_{\mathrm{T}}$ ought to be a straight line with the slope equal to $(\mathrm{H}^+)/k_1K_n$ and the intercept equal to K_1/k_1. A series of such plots for several values of pH are collected in Figure 4. A secondary plot of slope against (H^+) (not shown) yields a straight line with the slope equal to $1/k_1K_n$. The value of K_n was obtained by independent titration under the reaction conditions of temperature and ionic strength and found identical within experimental error with the one appearing in the literature,[30] $K_n = 3.16 \times 10^{-10}$ *M*. This value of K_n and the secondary-plot slope suffice to determine k_1 ($k_1 = 1.5 \times 10^{-3}$ M^{-1} min^{-1}). This value of k_1 together with the intercepts of the primary plots (Figure 4) should suffice to establish K_1. These intercepts, however, are small and

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[22] W. P. Jencks, *J. Amer. Chem. Soc.*, **81**, 475 (1959).

[23] E. H. Cordes and W. P. Jencks, *ibid.*, **84**, 4319 (1962).

[24] E. H. Cordes and W. P. Jencks, *ibid.*, **85**, 2843 (1963).

[25] G. L. Schmir, *ibid.*, **90**, 3478 (1968).

[26] E. S. Hand and W. P. Jencks, *ibid.*, **84**, 3505 (1962).

[27] L. R. Fedor and T. C. Bruice, *ibid.*, **87**, 4138 (1965).

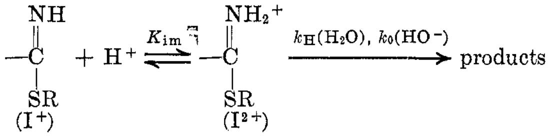
[28] R. K. Chaturvedi, A. E. McMahon, and G. L. Schmir, *ibid.*, **89**, 6984 (1967).

[29] R. K. Chaturvedi and G. L. Schmir, *ibid.*, **91**, 737 (1969).

[30] J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic Press, New York, N. Y., 1958.

scatter widely precluding such calculation without large uncertainty. However, the reciprocal of the concentration of mercaptoethanol required to reach one-half of the maximal concentration of the adduct (Figure 3), 165 M^{-1}, is just the sum of the equilibrium constants for the adduct and thioimidate formation. Since that for the former is likely to be the greater of the two, this value may approximate K_1.

The rate law applicable to values of pH to the right of the pH–rate maximum may be derived on the basis of Scheme I assuming explicitly that hydrolysis of thioimidate is rate determining. Thioimidate hydrolysis occurs *via* attack of water and hydroxide ion on the protonated substrate.



$$-\overset{\overset{\displaystyle NH}{\|}}{\underset{\underset{\displaystyle SR}{|}}{C}} + H^+ \underset{}{\overset{K_{im}}{\rightleftharpoons}} -\overset{\overset{\displaystyle NH_2^+}{\|}}{\underset{\underset{\displaystyle SR}{|}}{C}} \xrightarrow{k_H(H_2O),\ k_0(HO^-)} \text{products} \quad (3)$$

(I^+) (I^{2+})

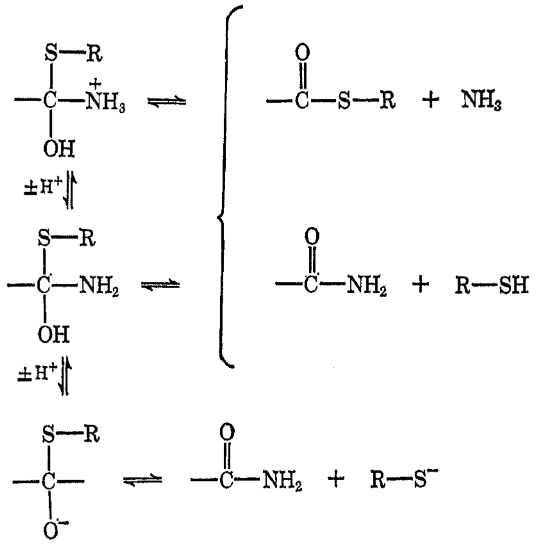
Letting $K_1' = k_1/k_{-1}$, and recognizing that $K_n < (H^+) < K_{im}$, one obtains

$$k_{obsd} = \frac{[k_H + k_0(OH^-)][(H^+)/K_{im}]}{1 + \frac{K_2K_n[RSH]_T}{(H^+)} + \frac{(OH^-)}{K_1'(RSH)_T} + \frac{K_w}{K_1'K_n(RSH)_T} + \frac{K_1(OH^-)}{K_1'}} \quad (4)$$

Although qualitative inspection of this rate law reveals that it is adequate to account for the experimental information, it is too complex to permit reliable derivation of values for individual rate constants.

There are three basic similarities between the catalysis of nitrile hydrolysis by mercaptoethanol described herein and that catalyzed by enzymes.[12,13] First, both the enzymatic and nonenzymatic processes result in marked catalysis; the enzymatic catalysis is, as usual, the more efficient of the two. Second, the enzymes, like mercaptoethanol, appear to be sulfhydryl reagents. Third, both the enzymatic and nonenzymatic reactions exhibit pH–rate maxima and the maxima occur at similar values of pH. It is true, however, that the pH–rate profiles are not identical in the various cases. This might reflect differences in the nature of thioimidates formed as reaction intermediates. If these similarities reflect more than coincidence, they suggest that the enzyme may function as a nucleophilic catalyst for nitrile hydrolysis in accord with the suggestion of Robinson, *et al.*[12] Moreover, the similarities suggest that an enzyme-bound thioimidate may be an intermediate and that decomposition of this intermediate is the rate-determining step on the alkaline side on the pH–rate maximum and, therefore, that this intermediate ought to be isolable and capable of characterization by the usual methods.

SCHEME II



The central distinction between the enzymatic and nonenzymatic reactions is the observation that the former yield the acid almost exclusively as product[12,13] while the latter yields principally the amide except under quite acidic conditions. Schmir and his associates have demonstrated that the pathway for decomposition of imidates and thioimidates is subject to influence by bifunctional catalysts such as bicarbonate and the phosphate dianion.[25,28] Thus, were the nitrilases to employ a similar mode of catalysis for decomposition of the thioimidate intermediates, this difference would be accounted for in a natural manner.

Registry No.—*N*-Benzyl-3-cyanopyridinium bromide, 6516-53-6; mercaptoethanol, 75-08-1.