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THE REACTION BETWEEN PYRIDINE-4-ALDOXIME AND METHIODIDE THIOESTERS: A MODEL FOR ENZYMIC TRANSACETYLATION*

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Abstract—Oximes are useful therapeutic adjuncts to atropine in the treatment of anticholinesterase poisoning, although their exact mechanism is not fully understood. Since it can be shown that oximes react rapidly and reversibly with thioesters *in vitro*, it is suggested that part of their therapeutic effect may be due to an interference with acetylcholine synthesis (a thioester-dependent reaction). Rate constants for the reactions of pyridine-4-aldoxime methiodide (4-PAM) with a series of thioesters (forward reactions) and of the acetate of pyridine-4-aldoxime methiodide (Ac-4-PAM) with the corresponding mercaptans (reverse reactions) are reported. The forward reactions are first-order with respect to the thioester and oximate ion concentration; the reverse reactions are first-order with respect to the aldoxime ester and mercaptide ion concentrations. Equilibrium constants calculated from kinetic data agree with those estimated from an analysis of reactants and products at steady state. Equations are given, relating the reactivity of a thioester with the acid dissociation constant of its corresponding mercaptan, which are useful for predicting the rate of a reaction of thioester with pyridine-4-aldoxime methiodide and in selecting a thioester for maximum reactivity at a given pH. At physiological pH (7·4), a thioester whose corresponding mercaptan has a pK_a of about 8·2 will have maximum reactivity with 4-pyridine aldoxime methiodide.

THE effectiveness of pyridine-2-aldoxime methiodide (2-PAM) and certain other oximes as adjuncts to atropine in the therapy of organophosphate poisoning[1] has aroused interest in the mechanism of action of these oximes. While it has been shown that these materials *in vitro* are reactivators of organophosphate-inhibited cholinesterase, and that part of their effectiveness *in vivo* can be ascribed to such action,[2, 3] still their beneficial effects in the treatment of intoxication by quaternary ammonium anticholinesterases[4] point to a more general mechanism of action. Furthermore, the observation that in treatment of organophosphate-poisoned patients with 2-PAM, restoration of neuromuscular activity is more rapid than regeneration of muscle cholinesterase, requires a mechanism not fully satisfied by cholinesterase reactivation.[4–6]

Cholinesterase regulates the level of acetylcholine, a chemical held vital for neuromuscular transmission,[7] which is formed by the choline acetylase (ChAc)-catalysed transacetylation reaction involving acetylated coenzyme A(CoA-S-Ac), a thioester, and choline.[8] In the presence of inactivated acetylcholinesterase, any action which might limit the production (or release) of acetylcholine would mimic the effect of a

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reactivator of inactivated enzyme. It had previously been observed that oximes (which do not react with acetylcholine) will react quite rapidly at physiological pH and temperature with the thioester, acetylthiocholine.[9] This suggested the possibility that oximes may act as competitive acceptors in transacetylation, and thus may interfere with the production of acetylcholine.

The present paper describes kinetic and equilibrium studies of the reaction between pyridine-4-aldoxime methiodide* (4-PAM) and various thioacetates and between the acetate of pyridine-4-aldoxime methiodide (Ac-4-PAM) and several mercaptans in dilute aqueous solution at 37 °C. The results of our studies indicate that a rapid reaction between oximes and thioesters may be expected to take place under physiological conditions.

EXPERIMENTAL

Oximes

Pyridine-4-aldoxime methiodide (m.p. 181 °C) and O-acetyl-pyridine-4-aldoxime methiodide (m.p. 158 °C) were prepared from pyridine-4-aldoxime (Aldrich Chemical Co) according to the method of Ginsburg and Wilson[10].

Thiols and thioesters

2-N-Diethylaminoethanethiol acetate hydrochloride was prepared from 2-N-diethylaminoethanethiol hydrochloride (96 per cent purity, Evans Chemetics, Inc.) by reaction at room temperature with redistilled acetic anhydride (10 per cent excess). After removal of unreacted anhydride and acetic acid, the ester hydrochloride was recrystallized from a benzene and ethanol mixture (m.p. 98–99·5 °C).

Anal. Calc. for $C_8H_{17}ONS.HCl$: C, 45·5; H, 8·0; N, 6·6. Found: C, 46·2; H, 8·0; N, 6·5.

S-Acetylthiophenol was prepared from redistilled thiophenol (b.p. 62·3°/15 mm) (Eastman Kodak Co) and freshly distilled acetyl chloride. After 16 hr at room temperature, the mixture was poured over cracked ice and extracted from sodium bicarbonate solution with diethyl ether and dried over sodium sulfate (anhydrous). After removal of ether at reduced pressure, the ester was distilled *in vacuo* (b.p. 78 °C;3 mm).

Anal. Calc. for C_8H_8OS: C, 63·2; H, 5·3. Found: C, 63·3; H, 5·4.

2-N-Di*iso*propylaminoethanethiol ($n_D^{32} = 1{\cdot}4654$), 2-N-di*iso*propylaminoethanethioacetate ($n_D^{23} = 1{\cdot}4732$) and allyl mercaptan were obtained from Dr. F. Hoffman, Chemical Research Division, Army Chemical Center, Md.

p-Methylthiophenol (m.p. 41·3 °C), *p*-chlorothiophenol (m.p. 51–3 °C) and thioglycolic acid (80 per cent in aqueous solution, analytical grade) were purchased from Eastman Kodak Co. Glutathione, S-acetylglutathione and l-cysteine hydrochloride were obtained from Nutritional Biochemicals Corp, and cysteine methyl ester hydrochloride (m.p. 145 °C) was prepared by Dr. R. E. Plapinger.[11] Acetylthiocholine bromide was purchased from LaWall-Harrisson Laboratories, Philadelphia, Pa.

Analytical methods

Iodometric titration. The mercaptan or thioester was weighed into a 100 ml volumetric flask containing KNO_3 (500 mg) and sequestrene-2 Na (Alrose Chemical Co)

* This oxime, although not as effective in therapy as the corresponding 2-substituted aldoxime, was chosen for these studies because of the greater stability of its derivatives. It is believed that the observations reported herein are, however, qualitatively applicable to 2-PAM and the trimethylene bis-4-pyridine aldoxime, TMB-4.

(200 mg), and brought to volume with 0·05 M acetic acid–sodium acetate buffer of the desired pH. Two 10 ml aliquots were withdrawn, acidified with 0·5 ml of 0·1 N HCl, and the solutions were titrated with standard iodine solution (5×10^{-3} N), using a 1 per cent starch (soluble) solution as indicator (19). In the presence of sequestrene-2 Na, oxygen and trace metals do not interfere with the analysis. In a separate flask, acetyl-4-PAM (612 mg) or 4-PAM (528 mg) and KNO_3 (500 mg) were dissolved in 50 ml of 0·05 M acetic acid–sodium acetate buffer of the same pH and placed in a water bath at 37·0 ± 0·1 °C. (The concentration of acetyl-4-PAM (or 4-PAM) was 2×10^{-2} M).

To 50 ml of the mercaptan or thioester solution was added, with mixing, 50 ml of the oxime solution. Aliquots (10 ml) were removed periodically, acidified with 0·5 ml of 0·1 N HCl and immediately titrated with the standard iodine solution.

Spectral studies. Changes in mercaptan concentration determined iodometrically were correlated with the formation or disappearance of Ac-4-PAM measured spectrophotometrically. Reaction mixtures containing equal concentrations ($2{\cdot}5 \times 10^{-3}$ M) of mercaptan (DET) and acetylated oxime (Ac-4-PAM) or of thioester (DETA) and oxime (4-PAM), were incubated at 37 °C in acetate buffer (0·05 M, pH = 5·0). At frequent intervals, 2 ml aliquots were removed and acidified with hydrochloric acid (0·1 N). One portion was assayed for mercaptan iodometrically and a second portion was diluted fifty-fold for spectral measurement. At this concentration of oxime (5×10^{-5} M), the solution had an absorption of less than 1·0 optical density units at the desired wavelength (250 mμ). The amount (X) of Ac-4-PAM (moles/l.) present in the mixture at any time was then calculated from the following relationship:

$$\text{Observed optical density at } 250\ m\mu = \text{O.D. (Ac-4-PAM)} + \text{O.D. (4-PAM)}$$

$$\text{where } e_{4\text{-PAM}} = 0{\cdot}53 \text{ O.D. units at } 5 \times 10^{-5} \text{ M}$$

$$e_{\text{Ac-4-PAM}} = 0{\cdot}81 \text{ O.D. units at } 5 \times 10^{-5} \text{ M}$$

$$\text{and O.O.D.} = \frac{0{\cdot}81X}{5 \times 10^{-5}\ \text{M}} + \frac{0{\cdot}53\ (5 \times 10^{-5}\ \text{M} - X).}{5 \times 10^{-5}\ \text{M}}$$

The initial changes in Ac-4-PAM concentration are compared with mercaptan values in Table 1.

TABLE 1. RSH + Ac-4-PAM $\underset{\text{REVERSE}}{\overset{\text{FORWARD}}{\rightleftharpoons}}$ RSAc + 4-PAM

Time (min)	Forward direction		Reverse direction	
	RSH (moles/l. × 10^3)	Ac-4-PAM (moles/l. × 10^3)	RSH (moles/l. × 10^3)	Ac-4-PAM (moles/l. × 10^3)
0	2·50	—	0·05	0·00
18	1·77	1·77	—	—
28	1·63	1·52	—	—
30	—	—	0·13	0·10
57	1·30	1·32	—	—
62	—	—	0·25	0·21
80	1·20	1·16	—	—
90	—	—	0·36	0·34
150	—	—	0·54	0·70
163	1·14	1·16	—	—

TABLE 2. RATE CONSTANTS FOR REACTION OF 4-PAM AND DETA AT 37 °C IN 0·05 M ACETATE BUFFER AT DIFFERENT pH LEVELS

No. of runs	pH	4-PAM ($M \times 10^2$)	DETA ($M \times 10^3$)	k_{obs} (corr.)*† $\times 10^3$ (min^{-1})	k'_{2f} (l. $mole^{-1}$ min^{-1})	$k_{2f} \times 10^{-2}$ (l. $mole^{-1}$ min^{-1})
2	5·0	2·0	0·625	6·50	0·325	10·3
4		1·0	1·25	3·16	0·316	10·0
2		2·0	1·25	7·20	0·360	11·4
2	5·5	2·0	0·625	23·66	1·18	11·8
2		2·0	1·25	21·4	1·07	10·7
2	6·0	2·0	0·625	80·66	4·03	12·8
1		2·0	1·25	62·26	3·11	9·9
						11·0 ± 0·7

* pH maintained constant by automatic addition of alkali.
† Corrections of first order rate constants ($\times 10^3$) for the spontaneous hydrolysis of DETA at pH 5·0, 5·5, 6·0 in 0·05 M acetate buffer are: 0·55, 0·54, and 1·14 min^{-1}.

TABLE 3. RATE-CONSTANTS FOR THE REACTION BETWEEN 4-PAMAc AND DET AT 37 °C IN 0·05 M ACETATE BUFFER AT DIFFERENT pH LEVELS

No. of runs	pH	4-PAMAc $\times 10^2$	DET $\times 10^3$	$k_{obs(corr.)}$* $\times 10^2$ (min^{-1})	k'_{2r}	$k_{2r} \times 10^{-3}$
1	5·0	1·0	0·625	3·82	3·82	2·71
2		1·0	1·25	3·50	3·50	2·48
2		2·0	1·25	8·82	4·41	3·13
2	6·0	1·0	0·625	36·5	36·5	2·61
						2·74 ± 0·20

* First-order rate-constants for the spontaneous hydrolysis of DETA at pH 5·0 and 6·0 in 0·05 M acetate buffer are: 0·55 and 1·14 $\times 10^{-3}$ min^{-1}, respectively.

TABLE 4. RATE-CONSTANTS FOR THE REACTION BETWEEN 4-PAM (2×10^{-2} M) AND ACETYLGLUTATHIONE (Ac-GSH)* AT 37 °C IN 0·05 M ACETATE BUFFER

No. of runs	pH	Ac-GSH $\times 10^3$	k_{obs}* $\times 10^3$ (min^{-1})	k'_{2f} (l. $mole^{-1}$ min^{-1})	$k_{2f} \times 10^{-2}$ (l. $mole^{-1}$ min^{-1})
2	5·0	0·625	3·94	0·197	6·12
1		1·250	3·77	0·189	
1	5·96	0·625	35·7	1·79	6·23

* The spontaneous hydrolysis of Ac-GSH under these conditions was negligible.

TABLE 5. RATE-CONSTANTS FOR REACTION OF Ac-4-PAM (2×10^{-2} M) AND GLUTATHIONE (GSH) AT 37 °C IN 0·05 M ACETATE BUFFER

No. of runs	pH	(GSH) M $\times 10^3$	$k_{obs} \times 10^2$ (min^{-1})	k'_{2r} (l. $mole^{-1}$ min^{-1})	$k_{2r} \times 10^{-4}$ (l. $mole^{-1}$ min^{-1})
3	5·0	0·625	2·89	1·45	2·30
1		1·250	3·15	1·58	2·50
1	5·45	0·625	7·10	3·55	2·00
2	5·85	0·625	18·75	9·38	2·10
1	5·95	0·625	22·0	11·0	1·95
					2·17 ± 0·18

RESULTS AND DISCUSSION

In solutions containing approximately equimolar concentrations of 4-PAM and 2-diethylaminoethanethioacetate (DETA), or of 2-diethylaminoethanethiol (DET) and the acetate of 4-PAM (Ac-4-PAM) the reaction approached a steady state (Table 1). Kinetic data obtained at constant pH (in acetate buffer), over a pH range of from 5·0 to 6·5, are shown in Tables 2 and 3. Analysis of rate data of pH 5 by a differential method[12] indicates that the reaction is bimolecular, the rate of reaction

TABLE 6. RATES OF REACTION BETWEEN 4-PAMAc (2×10^{-2} M) AND VARIOUS MERCAPTANS AT pH 5·0 AND 37 °C IN 0·05 M ACETATE BUFFER

Compound	Mercaptan $\times 10^3$	$k_{obs} \times 10^2$ (min^{-1})	k'_{2r} (l. $mole^{-1} min^{-1}$)	$k_{2r} \times 10^{-3}$ (l. $mole^{-1} min^{-1}$)	pK_a of mercaptan (25 °C)
p-Chlorothiophenol	1·25	6·72*	3·36		
				0·0329	5·94†
	0·625	6·83*	3·42		
Thiophenol	1·25	7·5*	3·75	0·252	6·82†
p-Methylthiophenol	1·25	10·25*	5·13	0·377	6·86†
Cysteine methylester	1·25	4·34	2·17		
				0·602	7·45‡
	0·625	4·20	2·10		
2-Di*iso*propylamino-ethanethiol	0·625	10·4	5·20		
				2·60	7·70†
	1·25	10·3	5·15		
2-Diethylaminoethanethiol	—	—	—	2·74§	7·85
Glutathione	—	—	—	21·7§	9·20**
Thioglycolic acid	1·25	1·70	0·85	177·5	10·32**
	0·625	1·70	0·85		
Allylmercaptan	1·25	6·42	3·21	ca. 3×10^4	ca. 12††

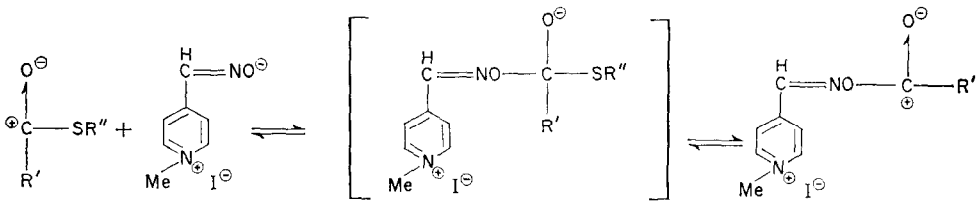
* Kinetic study in aqueous ethanol (20 per cent ethanol by volume) solution.
† Determined by potentiometric titration; thiophenols titrated in aqueous-acetone (20 per cent acetone by volume) solution.
‡ Based on value for cysteine ethylester given in footnote **.
§ Average value in 0·05 M Ac^- buffer, taken fom preceding tables.
** R. Benesch and R. Benesch, *J. Amer. Chem. Soc.* **77**, 5877 (1955).
†† Estimated from data given by H. C. Brown, D. H. McDaniel and O. Haflinger, *Determination of Organic Structures by Physical Methods*, Academic Press, New York (1955).

TABLE 7. RATES OF REACTION BETWEEN 4-PAM (2×10^{-2} M) AND VARIOUS THIOESTERS IN 0·05 M ACETATE BUFFER AT pH 5·0 AND 37 °C

Compound	Thioester conc. (moles/l. $\times 10^3$)	$k_{obs} \times 10^3$ (min^{-1})	k'_{2r} (l. $mole^{-1} min^{-1}$)
DETA			0·33*
Acetylglutathione	—	—	0·19†‡
Acetylthiocholine	0·625	8·5	0·425‡
Acetylthiophenol	0·55	10·0	0·50‡
DITA§	1·25	5·0	0·255‡

* Average value taken from data at pH 5·0, 0·05 M acetate buffer, 37 °C, given in Table 2.
† Average value taken from data at pH 5·0, 0·05 M acetate buffer, 37 °C, given in Table 4.
‡ No correction is made for the spontaneous hydrolysis of the esters. Under the conditions of the experiments, the spontaneous hydrolysis was negligibly small.
§ DITA = 2-Di*iso*propylaminoethane thioacetate.

being proportional to the first powers of the reactants. The constancy of the true bimolecular rate-constants (k_{2f} and k_{2r}), in contrast to the variability of pseudo-bimolecular rate-constants (k'_{2f} and k'_{2r}), the direct dependence of the rate in either direction upon the concentrations of reactants raised to the first powers, and the steady state analysis, suggest that the reaction may be depicted as:



A similar dependency of rate upon pH was found in studies of the reaction between glutathione and its ester with Ac-4-PAM and 4-PAM (Tables 4 and 5). Kinetic data of the reaction between 4-PAMAc and nine mercaptans, at pH 5·0 in 0·05 M acetate buffer, are shown in Table 6. Data on the reaction between 4-PAM and five thioesters are shown in Table 7.

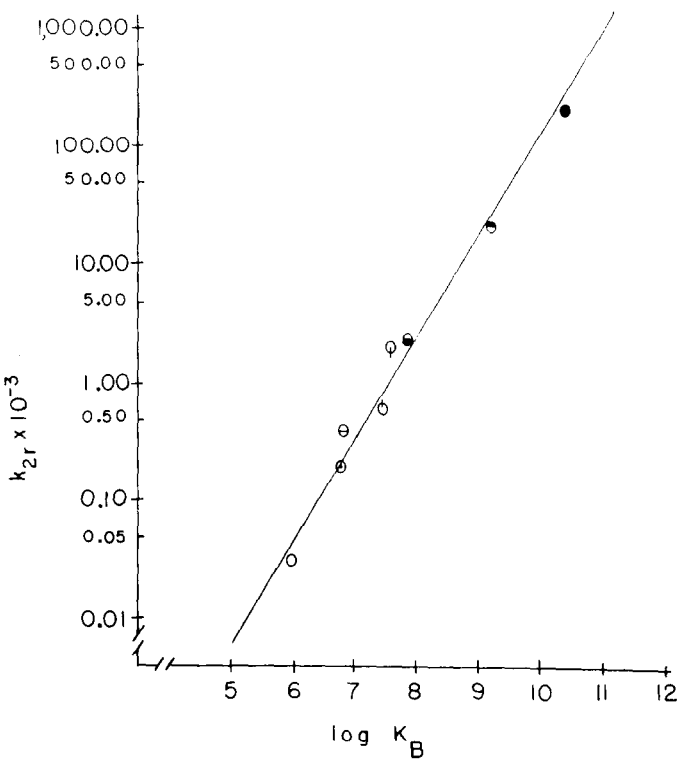


FIG. 1. Bronsted relationship between the true bimolecular rate constant (k_{2r}) and the basicity of several mercaptans reacting at pH 5·0 with acetyl-4-PAM: *p*-Chlorothiophenol, ○; thiophenol, ⊙; and *p*-methylthiophenol, ⊖; cysteine methylester, ◑; 2-N-di*iso*propylaminoethanethiol, ♀; 2-N-diethylaminoethanethiol, ◒; glutathione, ◓; and thioglycolic acid, ●.

Although the basicities of the mercaptide ions (Table 6) vary by as much as four powers of ten, the observed first-order constants (in the pH region in which a linear relationship exists between the ion concentration and pH) are all of the same order of magnitude. This implies that a Bronsted type of relationship exists between the true bimolecular rate constants and the basicities of the nucleophile (Fig. 1). The equation relating the bimolecular rate constant and the basicity of the anion, calculated by the method of least squares, is:

$$k_{2r} = 3{\cdot}39 \times 10^{-4} (1/K_A)^{0{\cdot}864}$$

where K_A is the acid dissociation constant. For the true bimolecular rate constant (k_{2r}), in the equation showing the relationship between this constant and the basicity of the mercaptide ion, is substituted its equivalent k'_{2r} ($H^+/K_A + 1$):

$$k'_{2r} = \frac{3{\cdot}39 \times 10^{-4} (1/K_A)^{0{\cdot}864}}{(H^+/K_A + 1)}$$

By differentiating k'_{2r} with respect to $1/K_A$, and setting the differential equal to zero, one obtains the relationship:

$$1/K_A = \frac{6{\cdot}35}{[H^+]}$$

from which at any pH, a value of K_A of the mercaptan of maximum reactivity may be calculated. At pH 7·4, and $K_A = 6{\cdot}25 \times 10^{-9}$, the bimolecular rate constant for a mercaptan with such a K_A can be calculated from the relationship:

$$k_{2r} = 3{\cdot}39 \times 10^{-4} (1/K_A)^{0{\cdot}864}$$

and, using the equation:

$$K_{eq} = \frac{[k_{2f} K_A/(H^+ + K'_A)]}{k_{2r} [K'_A/(H^+ + K'_A)]}$$

where K_A and K_A' are the acid dissociation constants of the 4-PAM and mercaptan, respectively, one can calculate k_{2f}. Equilibrium constants of several systems calculated from the rate data and determined experimentally are shown in Table 8.

For the thioester derived from a mercaptan of $K_A = 6{\cdot}25 \times 10^{-9}$, it can be estimated that the observed bimolecular rate constant (k'_{2f}) of the reaction between this

TABLE 8. EQUILIBRIUM CONSTANTS FOR REACTION:
RSAc + 4-PAM $\rightleftharpoons$ 4-PAMAc + RSH

RSAc	k'_{2f} (l. mole^{-1} min^{-1})	k'_{2r} (l. mole^{-1} min^{-1})	$K_{eq} = k'_{2f}/k'_{2r}$	K_{eq} exp $= \frac{\text{(products)}}{\text{(reactants)}}$
DETA	0·33	3·91	0·085	0·128†
Acetylglutathione	0·19	1·51	0·126	0·136‡
Acetylthiophenol	0·50	3·75	0·133	0·126‡
DITA	0·255	6·55	0·039	0·038§

† Average of two determinations.
‡ Average of three determinations.
§ Average of three determinations.

thioester and 4-PAM will be of the order of 50 l. mole^{-1} min^{-1}. Thus, with concentrations of the oxime as low as 10^{-3} M, the thioester might be expected to be deacylated at a rate of 5 per cent per min. Although this paper describes an *in vitro* study and no *in vivo* experiments have been performed to show that the antidotal properties of oximes are actually due, in part, to an interference with acetylcholine synthesis, these calculations would indicate that such an occurrence is indeed plausible.

That oximes may exert a significant therapeutic action by interference with acetylcholine production (synthesis or release) rests with the acceptance of two premises: (a) oximes reach the site of acetylcholine synthesis; and (b) the oxime is of sufficient reactivity to compete with acetyl acceptors normally involved. Premise (a) appears reasonable if one assumes that cholinesterase and the enzymes associated with acetylcholine formation are close to one another in space, since *in vivo* the oximes have been shown to reactivate inhibited cholinesterase.

Premise (b) is more difficult to substantiate since the rate of acetylcholine formation *in vivo* is difficult to assess.* It can be stated, however, that reactions involving 4-PAM and a thioester, whose corresponding mercaptan has a pK_a of about 8·2, are relatively rapid. While the reaction rate at physiological pH will be a maximum for thioesters of such a mercaptan, nevertheless thioesters of mercaptans having values for pK in the range 7·5–9·2 will react at 80 per cent of this rate. The likelihood of mercaptans having such a pK range *in vivo* is tenable, in view of the known dissociation constants of mercaptans, e.g. pantetheine† (pK_a = 9·25), mercaptoethylamine (p$K_{1,2}$ = 8·35),[13] glutathione (pK_a = 9·2), 2-N-diethylaminoethanethiol (pK_a = 7·85).

The rate data given in Table 7 indicate that there is no marked effect of thioacetate structure upon its reactivity with 4-PAM. This is in agreement with observations by Tarbell *et al.*[14] on the rates of ammonolysis and of hydrolysis of various thioacetates and in contrast to the observations of Noda *et al.*,[15] who found appreciable effects of structure on reactivity of thioacetates in reactions with hydroxylamine.

The importance of the mercaptide ion (rather than the undissociated mercaptan) in biological reactions has been pointed out by Benesch and Benesch.[13] In biochemical reactions involving a series of mercaptans having varying dissociation constants, the treatment of the data by the method shown herein, in order to predict at a given pH level, the mercaptan of maximum reactivity should prove useful in helping to explain the observed biological variations of drug potency.

* That oximes interfere with sulfanilamide acetylation catalysed by pigeon liver enzyme, a reaction involving acetylated co-enzyme-A, has been demonstrated since this study was completed (T. Wagner-Jauregg and H. Saner, *Arznei-Forsch.* **9**, 579 (1959).

† While pantetheine is most closely related structurally to co-enzyme-A, the pK_a of co-enzyme-A may actually be lower since it contains an amino group. That a neighboring group can influence sulfhydryl dissociation has been pointed out in the case of cysteine (pK_{12} = 8·53) and its ethyl ester (p$K_{1,2}$ = 7·45) by J. T. Edsall and J. Wyman, in *Biophysical Chemistry*, Academic Press, New York (1958), p. 502. Pantetheine was prepared from pantethine (Calif. Corp. for Biochem. Research) by reduction over zinc and hydrochloric acid. Spectral absorption (x = 234 mμ) was measured at pH = 1·0, 7·89 and 9·29, and the pK_a was estimated graphically by the method of Marsh and Saville (personal communication).

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