Effect of Various Compounds on the Rate of Thiamine Hydrolysis

By JOHN J. WINDHEUSER and TAKERU HIGUCHI

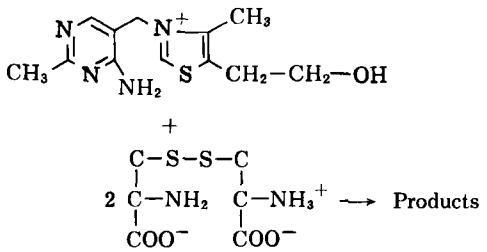
The catalytic effects of a variety of compounds on the rate of thiamine hydrolysis have been investigated. Although some catalysis was observed, thiamine degradation does not appear to be particularly sensitive to the presence of a number of nucleophilic agents other than sulfite and thiosulfate.

In our previous publication (1) dealing with the kinetics of thiamine hydrolysis, reference was made to the reports by others (2) indicating that certain compounds exhibited remarkable positive or negative catalytic effects on the rate of hydrolysis of this vitamin. Although investigation of some of these compounds under our experimental conditions failed to substantiate the reported dramatic effects, the possible theoretical and technical importance of finding catalytic agents has motivated further investigation in this area. The present communication is concerned with the effects of several amino acids and other compounds on the rate of thiamine cleavage at 96.4°.

RESULTS AND DISCUSSION

Amino Acids.—It has been reported in the literature (3) that the destruction of thiamine by shrimp thiaminase was retarded by the addition of cystine at pH 6.9. In order to determine whether the reported effect might be due to an interaction of cystine with thiamine resulting in stabilization or due to the amino acid lowering the catalyst activity, thiamine was subjected to hydrolytic conditions in the presence of various concentrations of cystine. Experiments were conducted at 96.4° under anaerobic conditions, at constant pH, ionic strength, and buffer concentration.

The results of studies at pH 6.3 are shown in Fig. 1. The increase of the reaction rate as a function of the cystine concentration indicates that rather than stabilizing thiamine, cystine actually acts to catalyze the degradation. The nonlinearity of the increase in reaction rate at rising amino acid titer suggests a higher order dependency of the reaction on cystine. Based on the observed pH dependence, the solid line on the graph was calculated on the assumption that the reaction could be represented as



From such a formulation it can be readily shown that

$$k_{obs} = k_o + k_{cy}\left[cy_t\left(\frac{K_a}{H^+ + K_a}\right)\right]^2$$

where k_{obs} is the observed pseudo first order rate constant, k_o is the first order rate constant at the experimental pH and buffer concentration, k_{cy} is the specific catalytic constant for cystine, cy_t represents the total cystine added to the system, and K_a denotes the acidic dissociation constant of the first amino group. The good agreement of the experimental points with the theoretical curve tends to support the proposed reaction dependency.

Figure 2 depicts the results obtained from the same reaction run at pH 6.69. The solid line again represents a theoretical curve calculated with the above equation utilizing the catalytic constant estimated from the reaction at pH 6.3.

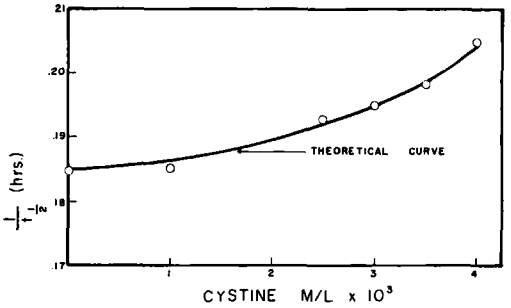


Fig. 1.—The effect of increasing cystine concentration on the rate of thiamine hydrolysis at pH 6.3, using 0.1 *M* phosphate buffer at an ionic strength of 0.5.

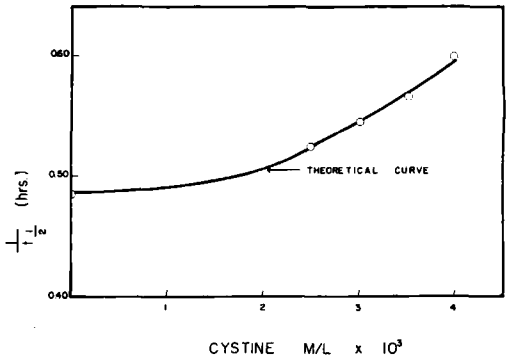


Fig. 2.—The effect of increasing cystine concentration on the rate of thiamine hydrolysis at pH 6.69 using 0.1 *M* phosphate buffer at an ionic strength of 0.5.

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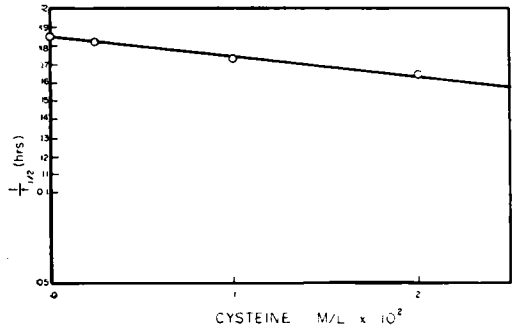


Fig. 3.—The effect of cysteine on the rate of hydrolysis at pH 6.3 using 0.1 *M* phosphate buffer at an ionic strength of 0.5.

TABLE I.—THE EFFECT OF VARIOUS AMINO ACIDS ON THE RATE OF THIAMINE HYDROLYSIS AT 96.4° IN THE PRESENCE OF 0.1 *M* PHOSPHATE BUFFER AT 0.5 IONIC STRENGTH

Compound	pH	Apparent Catalytic Constant, LM^{-1} $Hr.^{-1}$
Cystine	6.30	2.08×10^{6a}
Cystine	6.69	2.08×10^{6a}
Cysteine	6.30	-8.5×10^{-1b}
Tyrosine	6.30	...

[a] Calculated on the basis of Eq. 1 and using $pK_3 = 8.0$ for cystine. [b] Based on total cysteine concentration.

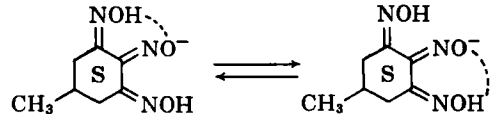
From the results of these experiments, which indicate that cystine *per se* catalyzes the decomposition of thiamine, it would appear probable that the retardation of the degradation of thiamine by thiaminase in the presence of cystine does not involve interaction of thiamine and cystine but rather an interaction of the amino acid with thiaminase.

The possible interconversion of cystine to cysteine under redox conditions led to the investigation of the behavior of cysteine. The results, as shown in Fig. 3, indicate that cysteine exhibits a definite inhibitory effect on the rate of cleavage. The apparent first-order dependence on cysteine and retardation of the rate of degradation, as compared to the results with cystine, would tend to suggest that the free sulfhydryl group is vital to the stabilizing mechanism. This is further substantiated when cysteine is compared to alanine which in our previous work (1) has been found to increase the rate of degradation. These findings would be in agreement with those of Watanabe (4) who reported that a number of sulfhydryl-containing compounds were found to prevent the formation of turbidity in thiamine solutions heated at 100° for three hours.

Although the structure of thiaminase has as yet not been elucidated, it has been shown that tyrosine is one of the amino acid components (5). Therefore experiments were carried out to determine if tyrosine might be one of the components of the active sites of this enzyme. The studies were conducted under the same conditions noted previously, and it was found that this compound exhibited no catalytic activity.

The results observed with the amino acids tested are summarized in Table I.

Carbonyl Derivatives.—Since it has been shown that thiamine hydrolysis is subject to general base catalysis, a number of ketones, ketoximes, and a triketo triazine were tested as each of these compounds is capable of acting as a nucleophilic catalyst, as has been shown by Russo (6) in his study of the hydrolysis of phosphate esters. Table II lists the compounds of this group that were tested and the catalytic constants observed. Figure 4 represents the kinetic dependency of the thiamine cleavage on the concentrations of cyclohexane oxime and 5-methyl-1,2,3-cyclohexanetrione-1,3-dioxime. In each case the reaction appears to be first order with respect to the catalytic agents. The higher catalytic constant of the dioxime may be due to a bifunctional attack of the weak nucleophilic and electrophilic centers in the molecule. The lack of activity of trioximes might be rationalized by the formation of intramolecularly bonded salts at this pH as follows



This supposition appears to be supported by the fact that the salicylate ion as well as the 2-hydroxy-1-naphthoate ion, both of which can form similar internal associations, exhibited no catalytic action.

TABLE II.—EFFECT OF VARIOUS COMPOUNDS ON THE RATE OF THIAMINE HYDROLYSIS AT PH 6.30 AND 96.4° IN THE PRESENCE OF 0.1 *M* PHOSPHATE BUFFER AND 0.5 IONIC STRENGTH

Compound	Apparent Catalytic Constant, LM^{-1} $Hr.^{-1}$	Max. Concn. *M*/L.
Cyclohexanone	No activity	1×10^{-2}
Cyclohexanone oxime	1.18	5×10^{-2}
1,2,3-Cyclohexanetrione trioxime	No activity	1×10^{-2}
5-Methyl-1,2,3-cyclohexanetrione 1,3 dioxime	2.46	3.5×10^{-2}
5-Methyl-1,2,3-cyclohexanetrione trioxime	No activity	1×10^{-2}
Trimethyl isocyanuric ester	No activity	1×10^{-2}

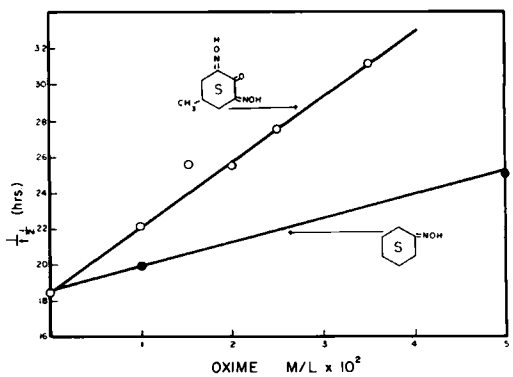


Fig. 4.—The effect of cyclohexanoxime and 5-methyl-1,2,3-cyclohexanetrione 1,3-dioxime on the rate of thiamine hydrolysis at pH 6.3 using 0.1 *M* phosphate buffer at an ionic strength of 0.5.

Other Compounds.—In addition to the amino acids and the agents tabulated in Table II, catechol, aniline, and 2-hydroxy-1-naphthoic acid were evaluated. None of these compounds altered the rate of the reaction.

EXPERIMENTAL

Reagents.—All chemicals used in the preparation of the buffers and the kinetic solutions were analytical reagent grade with the exception of the catalytic agents tested. These compounds were all purified by recrystallization from suitable solvents prior to use.

Analytical Procedure.—Residual thiamine was determined by the U.S.P. XVI (7) thiochrome method. All substances added to the system were checked for interference with the assay method.

Kinetic Procedure.—The procedure for the kinetic runs was identical with that previously described (1).

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Kinetics of Air Oxidation of Sulfurous Acid Salts

By LOUIS C. SCHROETER

Sulfurous acid salts in common with many pharmaceutical antioxidants and oxygen-sensitive drugs undergo oxidation by a radical process. This has important implications in the testing and evaluation of oxidative tendencies of formulations since radical processes are inordinately sensitive to slight amounts (10^{-6} *M*) of compounds acting as radical inhibitors or initiators. Measurable inhibition of the rate of sulfite oxidation occurred in the presence of 10^{-6} *M* DMA or DMF; the inhibition is described by an empirical equation. Effect of hydrogen ion concentration on the oxidation of sulfurous acid salts was studied in detail and the results described in terms of a theoretically derived equation. Experimental data are presented in corroboration of the Abel theoretical equations and radical mechanism for sulfite oxidation.

SULFUROUS ACID salts in aqueous solution undergo oxidation in the presence of molecular oxygen by a radical process (1). Radical initiation very likely occurs through interaction of oxygen with an anion to produce free radicals

$$(I)\ O_2 + OH^- \rightleftharpoons OH + O_2^-(O_3H^-)$$

which in turn react with hydrogen sulfite species to produce a highly reactive radical.

$$(II)\ HSO_3^- + OH \rightarrow HSO_3 + OH^-$$

The product of the oxidation, sulfate ion (SO_4^{2-}), may be formed by the interaction of HSO_3 and OH radicals

$$(III)\ OH + HSO_3 \rightarrow SO_4^{2-} + 2H^+$$

Other mechanistic interpretations consistent with the concept of a radical process and conforming to the energetics of the system may adequately explain the experimental data. Consideration of plausible radical mechanism is aided by reviewing treatment accorded to other radical systems (2) and especially to free radical reactions occurring in solution (3).

Abel (4, 5) has proposed the following scheme for generalized anionic auto-oxidation

$$O_2 + A^- \rightarrow AOO^-$$
$$AOO^- + X \rightarrow 2O^- + X^+ + A$$
$$A + X \rightarrow X^+ + A^-$$

in which the hydroxyl ion appears to be the preferred electron donor, A^-. In this scheme X would represent hydrogen sulfite species, HSO_3^-.

Radical processes are extraordinarily sensitive to small amounts of compounds which may act to inhibit or to catalyze the reaction. Addition of sulfurous acid salts to pharmaceutical formulations generally results in rather complex systems from the standpoint of free radical chemistry. Drug molecules or other additives in the formulation may act as initiators, inhibitors, or catalysts of the oxidative process. Systems which decrease the efficiency of the antioxidant ultimately result in poor stability of the drug since failure of the antioxidant is generally followed by oxidative attack on the drug.

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