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Thiamine Inactivation by the Chastek-Paralysis Factor. Inhibition[1] of Thiamine Inactivation

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Evidence of the enzymatic nature of the destruction of thiamine by the fish principle or Chastek-paralysis factor has been presented previously.[2] In an effort to obtain more conclusive evidence concerning the enzyme hypothesis, the effect of the usual enzyme inhibitors on the fish principle was studied. Included among the substances used were heavy metal ions, iodine, pyrophosphate, sulfite and cyanide. In the concentrations commonly employed with enzyme reactions several of these were found to interfere[3] with the determination of thiamine by the diazotized aminoacetophenone method of Melnick and Field.[4] Further analysis[3] of the interference indicated that only a few of the inhibitors might be used in the thiamine-fish principle experiments, and in some instances it is evident (Table I) that inhibition of thiamine destruction did occur. On the other hand, it was also apparent that the interference with the analytical method limited the usefulness of these reagents.

TABLE I

INHIBITION OF THIAMINE DESTRUCTION

Inhibitor	Concentration, moles/liter $\times 10^3$	Inhibition, %
Cupric sulfate	1.0	50.0
Zinc chloride	1.0	41.3
Ferric chloride	1.0	45.6
Potassium cyanide	1.0	23.8
Sodium fluoride	1.0	13.1
Iodoacetic acid	1.0	4.3
Cysteine	0.1	56.7
	.01	9.9
Sodium sulfite	.01	9.5

The inhibition of enzyme reactions, however, is not limited to reagents of this type for there are numerous classical examples of inhibition with compounds whose chemical structures are similar to those of the substrate molecules. The effect of various compounds, resembling the thiamine molecule in one or more features, on the destruction of thiamine by the fish principle was therefore investigated. In Table II in which the compounds are listed it is evident that certain ones

TABLE II

INHIBITION OF THIAMINE DESTRUCTION

Compound	Concentration, moles/liter $\times 10^4$	Inhibition, %
4-Methyl-thiazolium chloride derivatives		
3-*o*-Aminobenzyl-[a,b]	5.0	100
3-*o*-Nitrobenzyl-[a,b]	0.5	1.8
	5.0	0
	50.0	0
3-β-Aminoethyl-[a,b]	0.5	47.9
	5.0	56.4
	20.0	74.6
3-β-Phthalimidoethyl-[a,b]	0.5	5.7
	5.0	18.4
	20.0	30.2
3-Ethyl-[a,c]	10.0	9.3
3-Phenyl-[a,c]	5.0	2.3
	10.0	4.9
3-Ethyl-2-methyl-[a,c]	5.0	0
	10.0	0
	40.0	10.0
3-Phenyl-2-methyl-[a,c]	5.0	0
3-Methyl-5-β-hydroxyethyl-[c,d]	5.0	0
	15.0	0

(1) Presented in part before the Division of Biological Chemistry of the American Chemical Society at the annual fall meeting at Buffalo, N. Y., Sept. 10, 1942.

(2) Sealock, Livermore and Evans, THIS JOURNAL, **65**, 935 (1943).

(3) Sealock and Goodland, unpublished data.

(4) Melnick and Field, *J. Biol. Chem.*, **127**, 515 (1938).

TABLE II (*Concluded*)

Compound	Concentration, moles/liter × 10^4	Inhibition, %
2-Methyl-4-aminopyrimidine derivatives		
5-Bromomethyl-[d]	0.5	0
	5.0	34.8
	20.0	48.6
5-Methylenesulfonic acid	0.5	10.6
	10.0	19.7
	20.0	22.7
5-Ethoxymethyl-[d]	0.5	9.4
	5.0	13.2
	20.0	18.8

[a] These compounds were very kindly made available by Dr. H. T. Clarke. [b] Clarke, THIS JOURNAL, **66**, 652 (1944). [c] Clarke and Gurin, *ibid*, **57**, 1876 (1935). [d] Obtained through the courtesy of the Medical Department of Merck and Co., Inc. The authors are also indebted to this company for a generous supply of thiamine for these studies.

possess the property of greatly retarding the destruction of the vitamin.

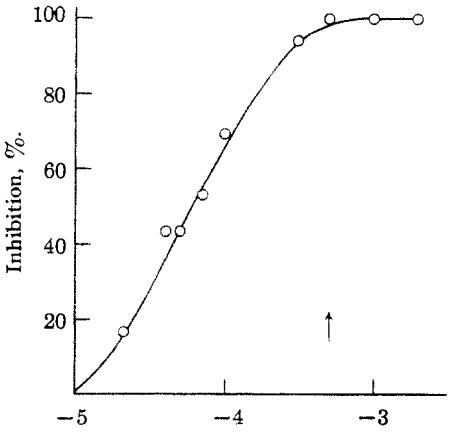
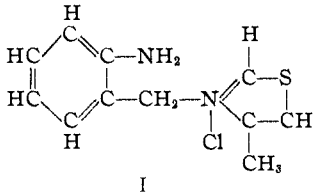


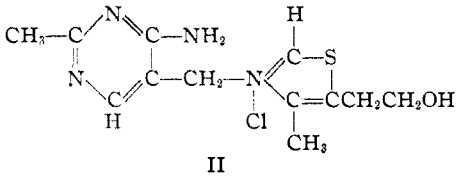
Fig. 1.—Inhibition of thiamine destruction: inhibitor, *o*-aminobenzyl-4-methylthiazolium chloride; the arrow indicates thiamine concentration (5×10^{-4} *M*).

Comparison of the results obtained with the aminobenzyl- or aminoethyl-thiazolium compounds and the corresponding nitro- and phthalimido- derivatives emphasizes the importance of the amino group even though it is attached to the thiazole ring by three carbons in the one case and by two in the other. That this group is not the sole decisive point in the inhibition is evidenced by the distinctly lessened effect obtained with the aminopyrimidines. It appears more likely from the data of Table II that the amine in combination with the thiazolium portion of the molecule furnishes the stronger inhibition. This point will be considered in more detail in a later communication.

Since the most pronounced inhibition occurs with *o*-aminobenzyl-4-methylthiazolium chloride (I) and because of the similarity of its structure to that of thiamine (II)[5] the effect of the former compound was investigated in greater detail.



I



II

Experiments with a wider range of concentrations yielded the results shown in Fig. 1, in which is plotted the per cent. inhibition of thiamine destruction against concentration of the aminobenzyl compound. The character of the curve obtained is suggestive of the fundamental relationship involved.

For the purpose of further experimentation the assumption was made that the disappearance of thiamine in the presence of the fish principle occurs by a mechanism similar to that adopted for the majority of enzyme reactions.[6] In these it is believed that the substrate (in this case thiamine) combines with the enzyme (fish principle) to form an active complex which decomposes to yield the products of the reaction and the regenerated enzyme, the velocity of the reaction being directly proportional to the concentration of the active complex. In addition, inhibitor compounds resembling the substrate molecule are believed to combine in the same way with the enzyme protein in the place of the substrate and thus competitively retard the enzyme reaction. It may also be assumed that a similar situation applies with the aminobenzyl derivative with the consequent decrease in thiamine destruction. The validity of these assumptions may be tested by the use of methods originally derived from the law of mass action by Michaelis and Menten.[7] Representation of the formation of the enzyme-substrate complex by the expression

$$E + S \rightleftarrows ES \qquad (1)$$

leads to the Michaelis–Menten equation

$$v = V(S)/(K_S + (S)) \qquad (2)$$

where v is the initial velocity of the reaction, V the maximal velocity, S the substrate concentration and K_S the dissociation constant. The last term (K_S) corresponds to the substrate concentration of half-maximal velocity and is commonly known as the Michaelis constant.

(5) The similarity in spatial arrangement is even more striking when the comparison is made with atomic models.

(6) Haldane, "Enzymes," Longmans, Green and Co., London 1930.

(7) Michaelis and Menten, *Biochem. Z.*, **49**, 333 (1913).

By inversion of the above equation Lineweaver and Burk[8] obtained the form

$$1/v = K_S/V(S) + 1/V \qquad (3)$$

with which, by plotting $1/v$ against $1/(S)$, the more exacting linear treatment of the data is possible. With the slope and intercept values so obtained, the Michaelis constant and maximal velocity are readily calculated.

The latter authors, representing the case with an inhibitor present by the expression

$$2E + S + I \rightleftharpoons EI \text{ (Inactive)} + ES \text{ (Active)} \qquad (4)$$

have derived a second equation similar to the first and also of linear form

$$1/v = \frac{1}{V}\left(K_S + \frac{K_S(I)}{K_I}\right)\frac{1}{(S)} + \frac{1}{V} \qquad (5)$$

Included are the additional terms for inhibitor concentration, (I), and the enzyme-inhibitor dissociation constant, K_I. It is evident from equation (4) that the enzyme of the inactive enzyme-inhibitor complex is no longer able to catalyze the reaction involving the substrate. Again, by plotting the values of $1/v$ and $1/(S)$ of the inhibitor experiment, one may obtain, as Lineweaver and Burk have shown, a straight line the intercept of which is identical with that of the experiment without the inhibitor, but the slope of which is increased by the term, $K_S(I)/VK_I$ of equation (5). In this case the inhibition may be considered competitive. On the other hand, if in the two cases the slope remains the same but the intercept is increased in the presence of the inhibitor then non-competitive inhibition is occurring.

In order to determine whether the decreased thiamine loss in the presence of the aminobenzyl thiazole was due to competition between the former and the latter for the fish principle molecule, an experiment was designed so that the analytical treatment of Lineweaver and Burk might be applied. From solutions containing five different concentrations of thiamine and the same amount of fish principle, aliquots were removed at intervals for thiamine analysis. Similar solutions to which the inhibitor had been added were also analyzed, the over-all time in both cases being sufficiently short so that a plot of the thiamine destruction yields a straight line. From the amounts of thiamine destroyed at the different times the initial velocity of destruction (slope of best straight line through each set of values) in each solution was calculated using the method of least squares. Comparison of the initial velocities recorded in Table III again demonstrates the decreased activity of the fish principle in the presence of the aminobenzyl compound. Also recorded are the values for the amount of thiamine destroyed in each solution at sixty minutes. These may be compared to the values for the same time obtained by calculation. The decreased inhibition (increased destruction) with decreasing ratio of inhibitor to thiamine concentration apparent in Table III further confirms the fundamental relationship suggested by the results shown in Fig. 1.

(8) Lineweaver and Burk, THIS JOURNAL, 56, 658 (1934).

TABLE III

INHIBITION OF THIAMINE DESTRUCTION

Thiamine concentration, moles/liter × 10^4	Destruction: Initial velocity, v, moles/liter/min. × 10^6	Destruction: At 60 minutes, moles/liter × 10^6 Obs.	Calcd.
Without inhibitor			
1.0	0.746	42.8	44.8
2.5	.992	56.6	59.5
5.0	1.12	68.0	67.4
10.0	1.26	74.0	75.6
20.0	1.36	70.0	81.6
With 0.2 × 10^{-4} *M* inhibitor			
1.0	0.136	9.2	8.2
2.5	.285	18.0	17.1
5.0	.618	44.0	37.1
10.0	.758	46.0	45.5
20.0	1.29	76.0	77.4

The reciprocals of the initial velocities and substrate concentrations were plotted as shown in Fig. 2, the best straight lines being calculated by the method of least squares. From the calculation, the values of slopes and intercepts recorded in Table IV were obtained. Since the intercepts of the two straight lines coincide and the slopes are widely divergent, we may consider in the terms of Lineweaver and Burk that the inhibitory action of the aminobenzylthiazole is specific and competitive rather than general and non-specific. In addition, it is evident that the reaction involving thiamine and the fish principle exhibits features previously encountered only in the case of enzymatic reactions.

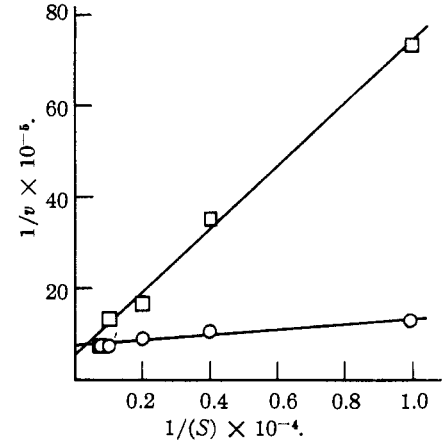


Fig. 2.—Inhibition of thiamine destruction: v, initial velocity, moles/l./minute; (S), substrate (thiamine) concentration, moles/l.; O, no inhibitor; □, with 0.2 × 10^{-4} *M* inhibitor (*o*-aminobenzyl-4-methylthiazolium chloride).

From the above slope and intercept values the maximal velocity, the Michaelis constant for the thiamine-fish principle complex and the corre-

TABLE IV

SUMMARY OF CONSTANTS

	Inhibitor	
	Absent	Present
Slope	62.0	693
Increase in slope, $K_S(I)/VK_I$		631
Intercept	7.47×10^5	4.90×10^5
Maximal velocity, V[a]	0.134×10^{-5}	0.204×10^{-5}
Michaelis constant, K_S[b]	0.831×10^{-4}	
Inhibitor constant, K_I[b]		0.0197×10^{-4}
Affinity constants		
$1/K_S$	1.20×10^4	
$1/K_I$	50.7×10^4	

[a] Moles/liter/minute. [b] Moles/liter.

sponding constant for the inhibitor-fish principle complex have been calculated (Table IV). The reciprocals of the latter may be regarded as affinity constants. The ratio of $1/K_I$ to $1/K_S$ is 42.2, from which it is reasonable to believe that the inhibitor compound combines somewhat more firmly with the fish principle than does thiamine.

It may be further concluded from the above results that *o*-aminobenzyl-4-methylthiazolium chloride possesses the functional groups which allow its combination with the fish principle. There is considerable probability that these are the same as those responsible for the combination of thiamine with the fish principle. Of interest in this connection is the absence of the hydroxyethyl- side chain from the inhibitor molecule. From this it may be argued that it is unimportant in the combination process. This point is also supported by comparison of the results contained in Table II.

The term inhibition has assumed in recent times considerable importance in connection with the water soluble vitamins. The elucidation of the antagonism between *p*-aminobenzoic acid and the sulfa drugs in bacterial metabolism has stimulated numerous investigations involving analogs of other members of the vitamin-B complex. In general, the antagonisms encountered involve competition with or inhibition of the positive physiological functioning of the vitamin in question. In contrast, the above experiments deal with the inhibition of vitamin destruction, a destruction which results from the action of a substance of biological origin. This substance, the fish principle, may be an important metabolic factor in the fish organism; however, with our present knowledge it can hardly be regarded as a positive agent engaged in promoting the physiological action of thiamine. It may even be suggested that similar destructive agents affecting other vitamins may occur in nature, in which case the search for specific inhibitor analogs should prove profitable.

Experimental

The fish principle used in these experiments was prepared from acetone desiccated powder of carp viscera[1] by four or five extractions with 10% sodium chloride in 0.2 *M* phosphate buffer of *p*H 7.4. The combined supernatants obtained by centrifuging were diluted with the same solvent so that 2 ml. contained the activity of 100 mg. of original powder and were adjusted to *p*H 7.4 before being used. In the inhibition tests 2 ml. of the extract plus the desired volume of inhibitor and water to make a total volume of 4 ml. were placed in test-tubes. One ml. of 25×10^{-4} *M* thiamine was added and the tubes were incubated for two hours at 37.5°. The reaction was stopped by the addition of 5 ml. of 20% trichloroacetic acid. After thirty minutes the flocculated protein was removed by centrifugation and 2 ml. was analyzed for thiamine,[4] comparison being made with an unincubated control to which the trichloroacetic acid had been added immediately following the thiamine. The per cent. inhibition was calculated by comparison with incubated tubes containing no inhibitor.

For the experiment involving the Lineweaver-Burk treatment 28 ml. of extract of the fish principle equivalent to 700 mg. of desiccated powder was placed in one flask and 28 ml. of extract and inhibitor in a second flask. After warming to 37.5°, 7 ml. of the proper concentration of thiamine also at 37.5°, was added to both flasks. Five ml. was removed at intervals for determination of the thiamine remaining, the reaction being stopped as described above. In all, five aliquots were removed from each flask, those from the two lower concentrations of thiamine at fifteen-minute and the others at twenty-minute intervals.

Summary

The destruction of thiamine by the Chastek-paralysis factor of fish tissues could be markedly decreased by the addition of cupric, zinc and ferric ions. Cyanide, fluoride, iodoacetate, sulfhydryl and sulfite were less effective. Other common enzyme inhibitors could not be tested since they interfered with the determination of thiamine by the diazotization method.

Compounds resembling the thiamine molecule in one or more features were also tested for inhibitory action. Among these, *o*-aminobenzyl- and *β*-aminoethyl-4-methylthiazolium chloride were found to be strongly inhibitory. Substitution of the amino group of these two by the nitro and phthalimido groups, respectively, abolished the inhibition in the case of the former and greatly reduced it in the case of the latter.

Other thiazoles and various 4-aminopyrimidines were either without effect or exhibited considerably lessened inhibition.

In the case of the aminobenzyl-methylthiazole, the extent of the inhibition proved to be dependent upon the ratio of its concentration to that of thiamine.

Use of the Lineweaver-Burk method showed that this compound specifically inhibited thiamine destruction by competing with the vitamin for the fish principle. The same method yielded a Michaelis constant of 0.831×10^{-4} mole per liter for thiamine and a similar constant of 0.0197×10^{-4} for the inhibitor.

The data presented show that the fish principle behaves with inhibitor substances as do many enzymes. This finding may be interpreted as additional evidence of the enzymatic nature of the fish principle.

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