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REPLACEMENT OF THE COENZYME OF THIAMINASE BY CERTAIN NITROGEN COMPOUNDS *

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In previously published papers [1,2] it has been shown that thiaminase coenzyme is of widespread occurrence in animal tissues, quite apart from the question of whether or not these tissues exhibit any thiaminase activity. The considerable resistance of cothiaminase to severe hydrolysis by acids and alkalies testifies that it is a relatively simple substance; in the free state it was found to be easily soluble in water, alcohol and acetone, and insoluble in hydrocarbon solvents.

Further researches have established that there is apparently no phosphorus in the cothiaminase molecule, but that nitrogen enters into its composition. In agreement with this, cothiaminase was precipitated by phosphotungstic acid (though indeed incompletely) and in an electric field it moved toward the cathode.

The properties of cothiaminase as a nitrogenous base have been utilized by us for the purification of this substance. In conjunction therewith, we thought it expedient to take up the study of how apothiaminase was acted upon by various simple nitrogen compounds of biological origin. Our expectation was that the rôle of cothiaminase might be imitated by some nitrogenous substance of known structure.

As these studies were just beginning, a series of papers was published by Sealock et al. [3], in which it was reported that the ortho-, meta- and para-isomers of substituted synthetic aromatic amines of different degrees of complexity have an effect on thiaminase and moreover, the effect is not the same [in each of the three cases]. Thus the ortho-substituted isomers were inhibitors of thiaminase, the meta-isomers were activators, while the para-isomers either were neutral or slightly inhibited the enzyme. Using a relatively simple substance, meta-nitroaniline, Sealock and his associates showed that when thiaminase is split by thiaminase in the presence of an excess of the said substance, it enters into combination with the pyrimidine moiety

* Certain parts of this work were carried out with the participation of two graduate students of the Faculty of Animal Biochemistry, Moscow National University, namely Ye. V. Budilova and E. A. Pavlov.

of the thiamine by way of its amino group, while the thiazole is liberated in the free state [4].

In 1944 Krampitz and Woolley had pointed out that in the splitting of thiamine by thiaminase the pyrimidine residue of the vitamin initially unites with some not yet identified substance, and only in a second phase of the splitting does it appear in the free state. Sealock, on the basis of these experiments, suggests that the meta-nitroaniline used by himself had substituted in the role of the said non-identified natural substance, and that the latter substance was chemically of an amine nature.

There are well-known papers in the literature which describe attempts to isolate from the reaction-mixture the intermediate compound of the pyrimidine moiety with the unknown substance [6], but the structure of this substance has so far remained unestablished. It is however interesting that the investigators found it to contain oxygen, nitrogen and sulfur. The work was carried out not with carp thiaminase, but with that of a bivalve mollusk, the Latin name of which was not given.

As indicated above, the natural coenzyme of thiaminase, according to our findings, is a nitrogeneous substance. Therefore we cannot exclude the possibility that its participation in the enzymatic reaction involves precisely the formation of the intermediate compound with the pyrimidine moiety, according to the mechanism discovered by Krampitz and Wooley and confirmed by Sealock et al.

We first of all tried out nitrogen heterocyclic compounds substituted in the third position (with the N atom as the first position), and aliphatic β-amino compounds (β-alanine, β-phenylalanine), that is, in a certain sense the analogs of Sealock's meta-substituted amines. We also investigated compounds of other types (α-amino acids, dipeptides, nitrogen compounds simultaneously containing nitrogen both in the heterocycle and in the form of an amino group, and others).

It was found, as we shall explain below, that an activating effect, similar to the action of the thiaminase coenzyme, was exhibited by many nitrogenous substances of biological origin, substances moreover not of one but of several types of structure.

Table I

Action of thiaminase on amino acids and their derivatives. Abbreviations: ae = apoenzyme, he = holoenzyme, ce = boiled coenzyme extract.

Substance	Substance was tested on:-	Interval of concentration tested, in moles	Activity-level of enzyme upon addition of activator	Threshold concentration of cysteine, moles	Concentration producing saturation in moles		Remarks
Monoamino-monocarboxylic acids.							
glycine	ae	$3\cdot10^{-2}$-$4\cdot10^{-3}$	-	-	-		Not active
α-alanine	ae, he	$2\cdot10^{-2}$-$7\cdot10^{-3}$	Low	$7\cdot10^{-3}$	-		
β-alanine	ae, he	$2\cdot10^{-2}$-$4\cdot10^{-3}$	Low	$7\cdot10^{-3}$	-		
Serine	ae	$2\cdot10^{-2}$-$7\cdot10^{-4}$	Low	$7\cdot10^{-3}$	-		
Cysteine*	ae, he	$4\cdot10^{-3}$-$1.4\cdot10^{-4}$	Very high	$1.4\cdot10^{-4}$	-	→	Insufficient substrate at high concentrations per liter.
Methionine	ae	$1.3\cdot10^{-2}$-$6.7\cdot10^{-4}$	High	$6.7\cdot10^{-4}$	-		
Tyrosine	ae	$1.3\cdot10^{-2}$-$4.6\cdot10^{-4}$	High	$6.7\cdot10^{-4}$	-		
Phenylalanine	ae, he	$1.8\cdot10^{-2}$-$3.3\cdot10^{-4}$	High	$3.3\cdot10^{-4}$	$5\cdot10^{-3}$	→	Saturation produced by decreasing the quantity of the enzyme-protein in the sample.
Hexane bases							
Lysine	ae	$1.3\cdot10^{-2}$-$2.2\cdot10^{-3}$	Low	$9\cdot10^{-3}$			
Histidine	ae, he	$2\cdot10^{-2}$-$2.5\cdot10^{-3}$	High	$2.5\cdot10^{-3}$	$2\cdot10^{-2}$		

Monoamino-dicarboxylic acids and amides.						
Aspargic acid	ae, he	$3.3 \cdot 10^{-2}$–$7 \cdot 10^{-4}$	–	–	–	Inactive
Glutamic acid	ae, he	$2.5 \cdot 10^{-2}$–$4 \cdot 10^{-4}$	–	–	–	
Aspargine	ae+ce	$1 \cdot 10^{-2}$–$1.25 \cdot 10^{-3}$	–	–	–	
Dipeptides and similar substances.						
Carnosine (β-alanyl-histidine	ae ae+ce	$2.2 \cdot 10^{-2}$–$1 \cdot 10^{-3}$	High	$1 \cdot 10^{-3}$	$2 \cdot 10^{-2}$	
p-amino-benzoyl-glutamic acid (folic acid residue)**	ae ae+ce	$3.3 \cdot 10^{-2}$–$7 \cdot 10^{-4}$	Depression	$2 \cdot 10^{-3}$	$7 \cdot 1)^{-3}$	Inhibits up to a certain limit.

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* Cysteine also showed a very strong activating effect on horothiaminase from the fresh-water mollusk Anodonta.

** We note with gratitud that the preparatio f p-amino-benzoyl-glutamic acid was supplied to us by A. V. Trufanov and the carnosine preparation by the Faculty of Biochemistry, Moscow National University (Director S. Ye. Severin).

EXPERIMENTAL PART

1. MATERIALS AND PROCEDURE

The enzyme was produced in the form of a salt-water extract from carp viscera, treated with acetone and dried in vacuo over P_2O_5 (for details, see [1]). Both non-dialysed and dialysed extracts were tested. The latter were produced by dialysis into twice-distilled ice-water for a period of 48 hours and were divided into small portions which were kept in the frozen state. A solution of the coenzyme was produced by heating a salt water extract of carp muscle over a boiling water bath. The sample mixtures were made up in the usual manner [1,2], and the dialysed and non-dialysed extracts were portioned out for each sample in such a way that the quantity corresponded to 8-10 mg of the initial dry visceral preparation. The quantity of thiamine in the sample was usually 0.125 mg. A borate buffer was used, of pH 7.4. Solutions of the test substances, taken in predetermined concentrations, were brought to pH ~7. The total volume of the sample varied in different tests from 5 to 7 ml. Incubation was at 37°C for 2 hours. The reaction was stopped by the addition of an equal volume of 10% CCl_3COOH.

Checks were maintained on the following: the fluorescence of the reagents; the fluorescence of the test substance; the influence of the test substance in thiamine determinations by the thiochrome method (verification of completeness of thiamine determination; quantity of potassium ferricyanide sufficient for complete oxidation).

The maximum amount of thiamine in 5 ml of solution at the analytic dilution was 2µg.

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2. EXPERIMENTS WITH SUBSTANCES CONTAINING THE PYRIDINE NUCLEUS

I. Nicotinic (pyridine-3-carboxylic) acid and nicotinamide.

Both substances were tested in final dilutions of $2 \cdot 10^{-2}$ to $5 \cdot 10^{-4}$ M. Both of them activated thiaminase, but the first one showed itself to be an incomparably stronger activator than the second (Fig.1). The nicotinic acid gave activation curves superficially quite similar to those obtained with the coenzyme extract. Saturation of the apoenzyme (a plateau on the activation curve) was produced by nicotinic acid at a final concentration

of about $5 \cdot 10^{-3}$ M of this substance. A distinct activation was already detected when the nicotinic acid concentration was at ~$5 \cdot 10^{-4}$ M (Fig.1).

II. Pyridoxine (2-methyl-3-hydroxy-4,5-
-dihydroxymethylpyridine).

This substance was tested in final dilutions of $1.7 \cdot 10^{-2}$ to $5.5 \cdot 10^{-4}$ M and was found to be inactive as regards both the apoenzyme and the holoenzyme.

III. The alkaloids nicotine and anabasine (3-[N-methylpyrrolidyl-2]-pyridine and 3-[piperidyl-2-]
-pyridine)*.

In the anabasine molecule the nitrogen in the piperidine ring is in the form of an imine group; in the nicotine molecule the nitrogen in the pyrrolidine ring is methylated. These substances were tested in final dilutions of $3 \cdot 10^{-2}$ to $4 \cdot 10^{-3}$ M. Activation was detected even at the minimum experimental concentrations of both these alkaloids, with anabasine raising the activity of the system to a higher level than nicotine. Activation occurred both with the apoenzyme and the holoenzyme. It is characteristic that the activation curve shows a slump toward the horizontal axis with increase in the activator concentration (Fig.2).

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3. TESTS WITH AMINO ACIDS, DIPEPTIDES AND ACID AMIDES

As stated above, our initial experiments were made on the β-amino acids but later we also used the α-amino acids ... monoamino-monocarboxylic, monoamino-dicarboxylic and hexone bases (basic amino acids). Table 1 gives a summary of the results obtained. From examination of this Table it follows that the different amino acids are not equally effective in their action on thiamine. The simplest of the amino acids, glycine, showed itself to be practically inactive. Alanine (α- and β-), serine and lysine exhibited a small activity (Fig.3), but the amino acids containing ring structures in the β-position (phenylalanine, tyrosine, histidine) were incomparably more active. Cysteine stands by itself; it activates thiaminase more strongly

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* Pure preparations of these alkaloids were kindly supplied to us by G. S. Il'yin.

and in more minute concentrations than any other amino acid. Methionine, containing a methylated sulfur atom, corresponds in strength of activation to the aromatic amino acids.

The dicarboxylic acids ... glutamic and aspartic ... and the amide of one of them ... aspartic acid ... were found to have no effect; p-amino benzoyl-glutamic acid did indeed cause an inhibition, but not to the point of complete loss of activity. On the other hand, the dipeptide β-alanyl-histidine (carnosine) occupied an intermediate position as regards its effect, midway between its two component amino acids.

In the case of a number of the strongly activating substances, histidine, phenylalanine, carnosine, it was found possible to get activation curves with a plateau, like the curves obtained with the coenzyme extract. For this it was only necessary, occasionally, to reduce the concentration of the enzyme protein.

Beside the groups of compounds enumerated above, we also tested certain other substances: creatine, guanidine, and others (see Table 2). Among them not one was found which produced an activation of apothiaminase.

Table 2

Effect on thiamine of different biologically active nitrogen compounds. (Abbreviations as in Table 1.)

Substance	Tested on:	Range of concentrations studied in moles.	
Adenosine	ae	$5 \cdot 10^{-3}$-$1.1 \cdot 10^{-3}$	
Adenosine-triphosphoric acid	af, he	$1 \cdot 10^{-2}$-$8 \cdot 10^{-4}$	
Creatine	ae, he	$1 \cdot 10^{-2}$-$8 \cdot 10^{-4}$	No action detected
Creatinine	ae	$1.4 \cdot 10^{-2}$-$4.5 \cdot 10^{-4}$	
Guanidine	ae	$3.3 \cdot 10^{-2}$-$4 \cdot 10^{-3}$	
Urea	he	$1 \cdot 10^{-2}$-$1.25 \cdot 10^{-3}$	

4. EXISTENCE ESTABLISHED OF COMPETITION BETWEEN SUBSTITUTE ACTIVATORS (NITROGEN COMPOUNDS OF BIOLOGICAL ORIGIN) AND THE NATURAL COENZYME

It naturally occurred to us to enquire whether there is, for the enzyme protein, no difference between activation by the nitrogen compounds and the natural coenzyme. To settle this question, we conducted a series of experiments, in which the enzyme was activated by the coenzyme in conjunction with the "substitute" activators (α-alanine, β-alanine, β-phenylalanine, cysteine). What these experiments showed was that the activity of the apoenzyme + phenylalanine system was higher than the activity of the apoenzyme + phenylalanine + coenzyme system (the same picture was seen in the case of cysteine and the other activators), but the level of activity of the apoenzyme + coenzyme system was increased when activators were added (Fig.4, A and B).

To decide the question of which component (the natural coenzyme or the substitute activator) has the greatest activity with respect to the enzyme protein, we used, in a number of subsequent experiments, β-phenylalanine as substitute activator. For these tests, we took a mixture of the coenzyme and phenylalanine, prepared by dissolving the latter substance, in a concentration of $3.3 \cdot 10^{-2}$ M, in the boiled coenzyme extract. The result of one such test is shown in Fig.5.

We /now/ see that with a uniform increase in the concentration of both reagents the effect of the coenzyme becomes predominant. Our attention is drawn to the initial portions of the curves. The activity level in the beginning part of the coenzyme + phenylalanine curve is considerably higher than in the corresponding parts of the curves found for each component separately, at the same concentration of either of them. This may be understood on the basis of the assumption that at the start there is enzyme protein in excess; it is not saturated when the mixture of both activators is added to it in small amounts. The result of this is that at first there is an addition of the effects of both components of the mixture (compare with the dotted line, which is the calculated sum of the curves for the coenzyme and for the phenylalanine taken separately). However, with increase in the concentrations of the two activators added as a mixture, the phenylalanine is displaced by the coenzyme, and the effect of its presence becomes less and less perceptible. It is evident that the activators compete with one another for the protein, and that the affinity of the natural coenzyme for the protein, under the conditions of our experiments, is greater than the affinity of the substitute activator (phenylalanine) for the protein. This theory, it seems, also holds good in the case of the other amino acids. With respect to phenylalanine, which with the protein yields a more active complex than does the natural coenzyme, the said coenzyme acts as a kind of inhibitor.

From this point of view it is interesting to recall the activation curves obtained by us previously with thiaminase from a bivalve mollusc ... the marine scallop [Pecten] ... and the thiaminase from a coelenteratum ... the Actinia [2]. It is possible that the steep maximum of activation which is observed with gradually increasing additions of the coenzyme extracts is characteristic of these curves; it is connected with the presence in the system not of one but of several natural activators possessing unequal affinities for the enzyme protein and unequal activating capacities.

It is worthwhile to touch upon one more question. As we have already pointed out in a previous communication [1], we discovered cothiaminase activity universally present in different animal organs and tissues, but were unable to find it in baker's yeast. It has since become clear that our yeast extracts contained a substance or substances which exerted a limited inhibiting effect on thiaminase. This prevented us from finding the coenzyme in the yeasts.

The same type of effect on thiaminase is shown by extracts of the tissues of a marine mollusc, the Mytilus, after acid hydrolysis (Fig. 6, A and B). The initial parts of the activation curves obtained with these extracts clearly point to the presence of an activator in them. In the further course of the curves there is a fall in the level of activity below the starting level, apparently under the influence of a competing inhibitory principle. Concerning such inhibitors, one might say that they are activators which raise the activity of thiaminase to only a relatively low level, but which possess a great affinity for the enzyme protein.

The data obtained do not, generally speaking, indicate that coenzyme extracts obtained from different tissues of different living creatures contain one and the same active principle. Rather we should conclude that the opposite is more probable. Finding a precise answer to this question is a task for the future, but at the present moment, at any rate, we may say that there is a considerable similarity in the effects of the natural coenzyme and the substitute activators investigated by us.

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5. THE EFFECT OF THIAMINASE OF HEAVY METAL IONS AND THIOLIC POISONS [INHIBITORS]; THE ROLE OF SULFHYDRYL GROUPS IN THE PROCESS OF ACTIVATION OF APOTHIAMINASE AND ACTIVATION BY THE SUBSTITUTE ACTIVATORS

Thiaminase is highly sensitive to the heavy metals. As regards the strength of their action on thiaminase, the heavy metal ions may be arranged in the following series: $Hg^{++} > Cu^{++} > > Fe^{+++} > Fe^{++}$ (Table 3). - 7 -

Table 3
Action on thiaminase of heavy metal ions.

Ions	Threshhold concentration in moles	Concentration sufficient for full inhibition of the enzyme, in moles.	
Hg^{++}	$3 \cdot 10^{-8}$	$1 \cdot 10^{-7}$	
Cu^{++}	$1.2 \cdot 10^{-4}$	$4 \cdot 10^{-4}$	
Fe^{++}	$2 \cdot 10^{-4}$	$1 \cdot 10^{-3}$	
Fe^{+++}	$3 \cdot 10^{-}$	$1 \cdot 10^{-3}$	No full inhibition

The thiolic poisons likewise showed themselves to be efficient inhibitors of thiaminase. Iodoacetamide produced a noticeable inhibition at a concentration of 0.02 M; at 0.04 M there was a strong inhibition, but not yet a complete loss of the enzymatic activity. Much stronger is the action of mercury p-chlorobenzoate, which produced a marked inhibition even at a concentration less than $1 \cdot 10^{-}$ M and a complete inhibition at $1 \cdot 10^{-4}$ M.

In our endeavors to investigate the mechanism of thiaminasic action, we attempted to suppress the poisoning caused by mercury p-chlorobenzoate by use of the boiled coenzyme extract. We did indeed find that the addition of this extract weakened the action of the inhibitor, and that the removal of the inhibition was the more effective, the more coenzyme solution was added (Fig.7). But in view of the fact that the coenzyme extract used is a complex mixture of different substances, we employed, in subsequent experiments on the removal of the inhibition, a pure solution of β-phenylalanine, concerning which we already knew that it occupied the same areas on the enzyme protein /molecule/ as the coenzyme did, and that its affinity for the protein was less than that of the coenzyme.

It was found that the system apoenzyme + phenylalanine was considerably more sensitive to mercury p-chlorobenzoate than the system apoenzyme + coenzyme extract. A concentration of $\sim 5 \cdot 10^{-6}$ M of the inhibitor was already sufficient to produce a distinct inhibiting effect. But still other differences are observed with phenylalanine. As is evident from Fig.8, a several-fold increase of the phenylalanine concentration does not produce any weakening of the effect of a thiolic poison ... in the presence of the latter, the activity of the system remains at just the same level at all our experimental points, that is, the curve of the activation of the enzyme by phenylalanine goes onto its plateau even at minimal doses of the activator. With

the same doses of the activator without the inhibitor, the activation curve will still rise, reaching its plateau only at maximum doses of phenylalanine.

Our findings may be interpreted as meaning that the inhibitor blocks those groups on the protein molecule with which phenylalanine reacts to form the active complex. Since in the experiment described the quantity of the inhibitor was the same in all the samples, so too the relative quantity of blocked and free groups remains the same in all cases. By reason of its relatively small affinity for the protein, phenylalanine in the concentrations used is not able to displace the inhibitor, but it is nevertheless capable of saturating areas /on the protein molecule/ which are free of the inhibitor already added in minimal amounts.

Since the inhibitor in question is a thiolic poison, it is possible that the blocking of SH groups hinders the formation of the active complex, and perhaps it is with these groups on the protein that the substitute activators and the coenzyme enter into reaction in the enzymatic splitting of thiamine.

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6. ACTION OF HYDROGEN SULFIDE ON THIAMINASE

As might be expected on the basis of the experiments with the heavy metal ions and the thiolic poisons, hydrogen sulfide exerts a strong activating effect on thiaminase. In this effect, certain features stand out. Thus different activation-effects were observed when water containing hydrogen sulfide was added to the dialysed enzyme and to the system saturated with the coenzyme. In the presence of the coenzyme, practically no activation occurred when the concentration of hydrogen sulfide was small. But here too it was possible, by increasing the concentration of H_2S, to heighten the activity somewhat. Control experiments showed that the activating capacity of the coenzyme extract was unchanged after a preliminary treatment with hydrogen sulfide. Thus we have to conclude that the action of H_2S is not on the activator solution, but on the enzyme protein. The observed relationships superficially resembled those found when the coenzyme and the substitute activators were simultaneously added to the enzyme protein ... the same competition between the two activating principles. The experiments carried out have once again emphasized the important part played by sulfhydryl groups in the enzymatic reaction here studied, and likewise that it is with these functional groups of the protein that the reaction between the protein and the activator takes place.

DISCUSSION OF THE RESULTS

From what has been said, it is seen that an activating effect on carp thiaminase is exhibited by many nitrogen compounds of biological origin, compounds very different in their chemical structure (these same substances also activate carp holothiaminase which, as was pointed out in a previously published article [2], is usually extracted from the tissues in a non-coenzyme-saturated state). We also find among the activators both cyclic and aliphatic structures; the nitrogen in them is contained either in the heterocycle or in the form of an amino group, or in both these forms simultaneously. Besides a polar group of basic character, all the activators contain at least one other polar group; as a rule, an acid group.

The importance of the acid group may be seen from the example of nicotinic acid versus nicotinamide (the action of the second is considerably inferior in comparison with the action of the first) and also of pyridoxine, which has no activating effect at all.

For there to be an activating effect, it is not necessary that the active groups should be exclusively in the m-or β-position relative to one another; this follows from the fact that the α-amino acids likewise will activate.

Complete inactivity relative to thiaminase was shown by many nitrogen compounds highly important in other connections: ATP, adenosine, creatine, creatinine, urea, guanidine; also by glycine, the simplest amino acid, and by the dicarboxylic amino acids.

In a class by themsleves are the experiments on the activation of thiaminase by the alkaloids, nicotine and anabasine. In spite of the fact that in their molecules there are no groups with acid properties, these substances showed themselves to have a distinct activating effect. Moreover, in contrast to the other activators, they gave curves with a pronounced maximum. It is not yet possible to say whether the mechanism is identical in the case of the alkaloids on the one hand and the other investigated substances on the other, but the activating effects of nicotine and anabasine serve to illustrate the routes whereby alkaloids introduced into the organism exert on it a specific pharmacological action: namely, by directly influencing this or that enzyme system.

As for the mechanism of action of the substitute activators, we should first of all point out that there are no grounds for assuming them to play a simple protective role.

At the temperature of 37°C, adopted by us in our determinations of thiaminase activity, this substance is quite stable, and is very little changed even after a four-hour incubation. The addition of phenylalanine to the thiaminase during this incu-

bation does not affect the preservation of the enzyme nor its ability to be activated by this same phenylalanine. So too we have no grounds for ascribing the effect of the activators to a counteracting of poisoning by the heavy metal ions. As we have already indicated, the added activators step up the activity not only of apothiaminase obtained by dialysis into distilled water, but as a rule they will also activate holothiaminase in that form in which it is found in the tissues. However, it is clear from our experiments with mercury p-chlorobenzoate and also with hydrogen sulfide that a whole extract from tissues is already protected against such small quantities of heavy metal ions as may be contained in it, and that the participation of phenylalanine, so strongly activating but yet at the same time so feeble a protector against a thiolic poison, has a quite different mechanism underlying it.

The differences in the degree of activation observed upon the addition of various amino acids, and particularly the absence of any activation when the dicarboxylic acids are used, likewise indicate a mechanism other than a protective effect.

On the other hand, the competitive relationships which we have shown to exist between the natural coenzyme and the substitute activators speak for a more profound participation of the latter substances in the enzymatic reaction, rather than a simple protective function exercised by them; indeed, they indicate a good deal of resemblence between the actions of the natural coenzyme and of the substitute activators. From the data obtained, it is clear that both the natural coenzyme and the substitute activators, to exert their activating effect, enter into reaction with the sulfhydryl groups of the enzyme protein.

There is real significance in the question of how we are to visualize this reaction between the activators of thiaminase and its sulfhydryl groups. Linderström-Lang {7} has indicated the probability of thiazolidine rings existing in proteins: labile rings capable of breaking up (for instance, under the action of ammonium salts) to form SH groups. It is interesting to compare this notion with the data obtained by us on the activation of Anodonta thiaminase by ammonium salts. In the presence of ammonium chloride the activity of this type of thiaminase markedly increased up to a concentration of 5-6%, after which it began to decline. A natural explanation of the phenomenon is just this assumption that free SH groups are formed, in accordance with the mechanism described by Linderström-Lang. In the case of carp thiaminase, however, ammonium salts showed themselves inactive. We are not in possession of any data re the effect of amino acids and similar substances on the thiazolidines. It is known that urea and guanidine are not capable of splitting these rings {8}; it is possible that the thiaminase activators too are incapable of bringing about such a reaction. But as follows from the findings of Pasynski and Cherniak {9}, the reactivity of SH groups is increased in a solution containing low-molecular amino compounds. Possibly the action of the nitrogen compounds which are activators of thiaminase depends on a similar effect.

There is now a question on which we should like to dwell: namely whether there is not a plurality of thiaminase activators, behaving like the natural coenzyme of this substance. There are some observations which may be explained as indirect evidence for the possibility of substitution of one substance for another in a prosthetic group of enzyme. Thus we know that in pellagra a therapeutic action has been shown for not only nicotinic acid, the lack of which is the direct cause of the ailment, but also for its analogs, quinoline-carboxylic acid, pyrazine-carboxylic acid and 2,3-pyrazine-dicarboxylic acid (quoted from [10]). The possibility is not excluded that the cothiaminasic activity of extracts from the organs and tissues of various living creatures likewise involves different, even though similar, substances. Here we should emphasize that the affinity of the natural enzyme from carp tissues for protein of carp thiaminase was found to be higher than the affinity, for this protein, of the substitute activators which we investigated. Consequently, the structure of the natural coenzyme must involve some important properties distinguishing it from the substitute activators. Preliminary experiments in which solutions of thiaminase coenzyme were treated with sodium nitrite have shown that the nitrogen in them is not in the form of an amino group. At this time we have at our disposal purified, but not yet pure, preparations of the coenzyme from carp tissues. The properties of these preparations favor the view that the coenzyme of thiaminase is very probably a simpler substance than the coenzymes of other enzymes.

Generally speaking, cases are known in which amino acids show themselves capable of substituting for a coenzyme. Thus phospho-mono-esterase (alkali phosphatase) is partially reactivated by the addition of hystidine and other amino acids. However, for complete restoration of the activity it is also necessary that magnesium ions be present [1]. But in the case of thiaminase the addition of certain amino acids can not only restore the initial activity, but sometimes it even yields an incomparably more active enzyme complex. It is interesting that we encounter such "primitiveness" in thiaminase, that "archaic" enzyme, found only in animal forms of a low level of organization and in archaic species of plant organisms (ferns, horsetails).

If some light has by now been shed on the question of what chemical properties thiaminase coenzyme must possess, and with what groups it reacts in the enzyme protein, it is nevertheless completely obscure how it behaves versus the substrate of the reaction ... thiamine ... and whether it really enters into an intermediate compound with the pyramidine moiety of thiamine.

In conclusion, we must note the following. The strong activation of carp thiaminase by amino acids probably means an intensification of its anti-thiaminic action. We may believe that when animals are fed with raw fish of the carp family, the decomposition-products of the proteins in the intestines will accelerate the splitting of vitamin B_1 by thiaminase. It is possible that the study, under natural conditions, of predatory fish feeding on the carp tribe will help us to discover the pro-

tec' ve mechanisms which counteract the harmful effect of exogenic thiaminase.

We express our profound gratitude to V. A. Engel'hardt for his unfailing attention and valuable advice in the carrying out of this work.

CONCLUSIONS

Many low-molecular nitrogenous substances of biological origin are activators of thiaminase; both cyclic and aliphatic substances. Besides a polar grouping of basic character (nitrogen in one form or another), all the activators contain in addition one other polar grouping, of acid character as a rule.

Of the monocarboxylic amino acids studied, only glycine was found to be inactive. A particularly strong activating effect was shown by amino acids containing cyclic structures and sulfur; also by the dipeptide carnosine. The dicarboxylic amino acids were not activators.

For the activating effect to exist, it is not necessary that the active groupings of the nitrogen compound should be exclusively in the beta-and meta-positions relative to one another (the α-amino acids also activate).

The alkaloids nicotine and anabasine also activate thiaminase, although somewhat differently from the other activators.

The substitute activators, according to our experiments with amino acids, compete with the coenzyme for the proteins, the affinity of the coenzyme for the protein being greater than the affinity of the substitute activators.

The formation of the active apoenzyme + coenzyme or apoenzyme + substitute activator complex takes place by way of a reaction between sulfhydryl groups of the protein and the activating agent.

The activity of the apoenzyme + substitute activator complex is sometimes found to be higher than the activity of the apoenzyme + coenzyme complex.

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24 June, 1952.

NOTE:-

When the present paper was in the press, there appeared a number of articles dealing with thiaminase (A. Fujita et al, J. Biol. Chem. 196, 289, 297, 305, 313, 1952). These authors have tested the effect of different nitrogenous compounds, synthetic for the most part, on the splitting of thiamine by thiaminase of different origins. It was discovered that the splitting of thiamine by the enzyme from a bivalve mollusc and from a fish of the carp family was accelerated only by synthetic nitrogen compounds, while the natural substances, such as α-and β-alanine, phenylalanine, histidine, nicotinic acid and nicotinamide had no effect on the reaction.

The reason for the divergences between the Japanese authors' findings and ours is not yet clear. Possibly it has something to do with different sources of the enzymes. Besides, it is incorrect procedure for the authors to investigate the action of the said compounds on the holoenzyme only. It follows from our results that between the natural coenzyme and the substitute activators there exists a competitive relationship, and in the presence of the coenzyme the effect of the latter substances is little felt. This applies particularly to such activators as α-and β-alanine, which increase the activity of the enzyme to approximately the same level as does the natural coenzyme.

We should mention that the Japanese authors, speaking of the mutual substituability of coenzymes from different sources, do not refer to our similar findings published in the Journal "Biokhimiya", 16, 305, 1951.

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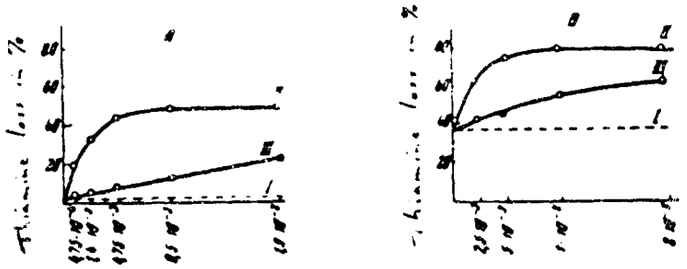

Fig. 1. Activation of thiaminase by nicotinic acid and nicotinamide.

Graph A.

I - Activity level of apoenzyme.
II - Activation of apoenzyme by increasing amounts of nicotinic acid.
III - Activation of apoenzyme by increasing amounts of nicotinamide.

Graph B.

I - Activity level of holoenzyme.
II - Activation of holoenzyme by increasing amounts of nicotinic acid.
III - Activation of holoenzyme by increasing amounts of nicotinamide.

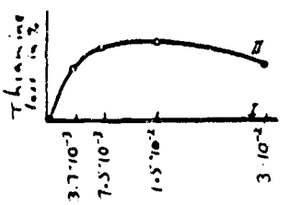

Fig. 2. Activation of thiaminase by anabasine.

I - Activity level of apoenzyme.
II - Activation of apoenzyme by increasing amounts of anabasine.

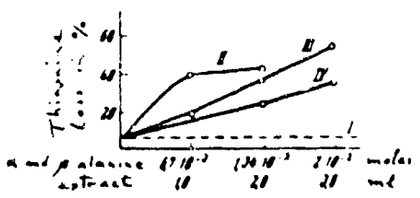

Fig. 3. Activation by α- and β-alanine.

I - Activity level of apoenzyme.
II - Activation of apoenzyme by increasing amounts of boiled coenzyme extract.
III - Activation of apoenzyme by increasing amounts of α-alanine.
IV - Ditto, β-alanine.

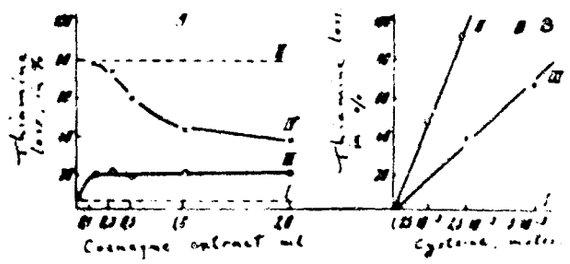

Fig. 4. Depression of coenzymatic action by addition of activators.

Graph A.

I - Activity level of apoenzyme.
II - Ditto, in presence of 0.01 M of β-phenylalanine.
III - Activation of apoenzyme by increasing amounts of boiled coenzyme extract.
IV - Apoenzyme with increasing amounts of boiled coenzyme extract added, in presence of 0.01 M of β-phenylalanine.

Graph B.

I - Activity level of apoenzyme.
II - Activation of apoenzyme by increasing amounts of cysteine.
III - Ditto, in presence of 0.5 ml of boiled coenzyme extract.

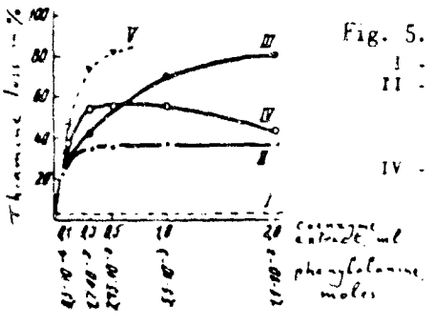

Fig. 5. Competition between coenzyme and β-phenylalanine.
I - Activity level of apoenzyme.
II - III - IV - Activation of apoenzyme by increasing amounts of boiled coenzyme extract (II); β-phenylalanine (III), mixture of boiled coenzyme extract and $3.3 \cdot 10^{-4}$ M of β-phenylalanine (IV).
IV - Sum of curves II and III.

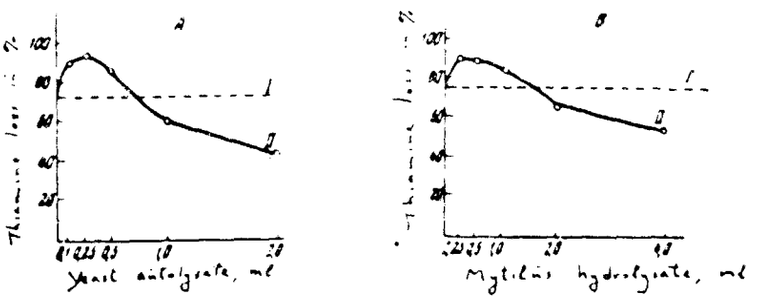

Fig. 6. Change of activating effect to inhibiting effect when autolysate of yeast and acid hydrolysate of Mytilus tissue are added to carp thiaminase.
Graphs A and B: I - Activity level of holoenzyme.
II - Change in holoenzyme activity upon addition of increasing amounts of: yeast autolysate (II A) and acid hydrolysate of Mytilus tissue (II B).

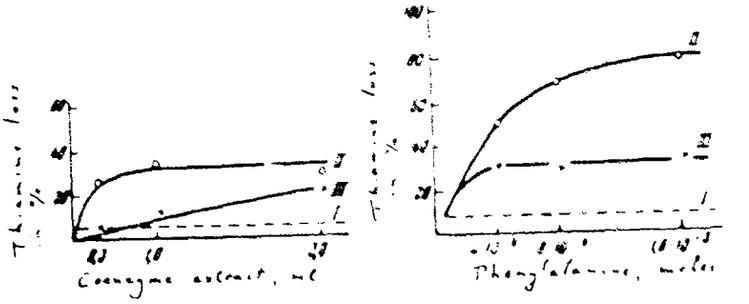

Fig. 7. Suppression of inhibiting effect of mercury p-chlorobenzoate.
I - Activity level of apoenzyme.
II - Activation of apoenzyme by increasing amounts of boiled coenzyme extract.
III - Same as II, in presence of $5 \cdot 10^{-5}$ M of mercury p-chlorobenzoate.

Fig. 8. Competition between β-phenylalanine and mercury p-chlorobenzoate.
I - Activity level of apoenzyme.
II - Activation of apoenzyme by increasing amounts of β-phenylalanine.
III - Same as in II, but in presence of $5 \cdot 10^{-4}$ M of mercury para-chlorobenzoate.