STUDIES ON THIAMINE ANALOGUES

III. EFFECTS ON ENZYME SYSTEMS*

BY STEPHEN EICH AND LEOPOLD R. CERECEDO

(From the Department of Biochemistry, Fordham University, New York, New York)

(Received for publication, August 27, 1953)

Several analogues of thiamine1 are known to compete with the vitamin when administered to animals. Thus, Woolley and White (1) have observed that pyrithiamine caused the manifestation of typical thiamine deficiency symptoms, which could be competitively overcome by the vitamin. The antivitamin effect of oxythiamine in mice has been reported by Soodak and Cerecedo (2). Cerecedo et al. (3, 4), comparing the effects of oxythiamine with those of neopyrithiamine in mice, have found the latter to be far more toxic.

The observation of Buchman et al. (5) that the enzymatic decarboxylation of pyruvate by dried brewers' yeast could be inhibited by thiazole pyrophosphate, and not by thiazole, suggested the possibility of a competition between cocarboxylase and thiazole pyrophosphate for the apoenzyme. This view has been further borne out by the results of Eusebi and Cerecedo (6, 7), demonstrating the inhibition of yeast carboxylase by oxythiamine diphosphate. A confirmation of this work has been reported by Velluz and Herbain (8), who have brought about inhibition of dried yeast carboxylase with oxythiamine triphosphate.

Woolley (9) has reported inhibition of yeast carboxylase with neopyrithiamine pyrophosphate and decreased formation of cocarboxylase from thiamine in chicken blood due to neopyrithiamine.

With the publication of methods of obtaining more satisfactory preparations of α-carboxylase by Singer and Pensky (10), and of thiamine phosphorylase by Leuthardt and Nielsen (11), it was deemed of interest to investigate further the mode of action of several thiamine analogues. The results of these investigations upon the two enzyme systems are reported in this study.

* Aided, in part, by contract No. AT(30-1)-1056 between the Atomic Energy Commission and Fordham University.

1 The following abbreviations are used: Bt, thiamine; OBI, oxythiamine; TDP, thiamine diphosphate (cocarboxylase); ODP, oxythiamine diphosphate; ATP, adenosinetriphosphate; AMC, acetylmethylcarbinol; NPT, neopyrithiamine.
EXPERIMENTAL

Materials

Sodium pyruvate was prepared from commercial pyruvic acid by the method of Robertson (12). Cocarboxylase and neopyrithiamine were generously supplied by Dr. Karl Folkers of Merck and Company, Inc. Oxythiamine was prepared according to the method of Rydon (13). This was converted to the diphosphate by the procedure of Cerecedo and Eusebi (14). Disodium adenosinetriphosphate was a commercial preparation obtained from the Pabst Laboratories, Milwaukee, Wisconsin; bovine serum albumin, Fraction V, was purchased from Armour and Company, Chicago, Illinois.

Acetaldehyde was prepared from paraldehyde immediately before use. The crystalline dimer of acetylmethylcarbinol was prepared, according to the method of Berl and Bueding (15), from acetylmethylcarbinol obtained from The Matheson Company, Inc., East Rutherford, New Jersey.

α-Carboxylase—Carboxylase was prepared from wheat germ according to the method of Singer and Pensky (10). This preparation involves defatting with acetone, extraction with water, isoelectric precipitation, alcohol fractionation, ammonium sulfate fractionation, and dialysis. The preparation obtained by these workers contains no cocarboxylase activity after final purification and was shown to be electrophoretically homogeneous. The enzyme was stored in the form of the alcohol-precipitated powder and was carried through the ammonium sulfate fractionation and dialysis when needed.

Thiamine Phosphorylase—The phosphorylase employed was the rat liver preparation of Leuthardt and Nielsen (11). For these experiments, the “precipitated HCl” fraction was most satisfactory, since it was free of cocarboxylase activity.

Methods

Pyruvate Decarboxylation Effects of inhibitors on decarboxylase activity were determined manometrically. The reaction mixtures contained 0.1 ml. of α-carboxylase preparation (stage (5) in the Singer and Pensky method), 1.0 ml. of 0.2 M succinate, pH 6.0, 0.1 ml. of 0.01 M MgSO₄, 1 mg. of serum albumin, and 0.6 ml. of a saturated aqueous solution of dimedon at pH 6.0. 0.2 ml. of 0.5 M sodium pyruvate was added to the reaction mixture after temperature equilibration. The thiamine analogues were added either to the reaction mixture immediately, or, after temperature equilibration, together with the substrate. The reaction temperature was 30.0°C.

Acetylmethylcarbinol Formation—Acetoin formation was followed colorimetrically by the method of Westerfeld (16). Each tube contained
0.1 ml. of α-carboxylase preparation, 1.0 ml. of 0.2 M succinate, pH 6.0, and 0.1 ml. of 0.03 M MgSO₄. The thiamine analogues were added either immediately to the reaction mixture, or, after a period of 20 minutes, along with the substrates. The reaction mixture was kept at 30.0°. When acetaldehyde and pyruvate were both added as substrates, 0.3 ml. of 0.5 M acetaldehyde and 0.3 ml. of 0.05 M pyruvate were employed. In the case in which acetaldehyde was the sole substrate, the addition was 0.6 ml. of 0.5 M acetaldehyde solution. Substrates were always added 15 minutes after the enzyme. Aliquots were removed immediately after introduction of the substrates to the reaction mixture and at the appropriate time intervals. The samples were deproteinized with zinc hydroxide by the method of Somogyi (17). The filtrate was employed for the colorimetric determination. The galvanometer readings of the initial aliquots were subtracted from the readings of the subsequent samples to correct for the color production due to acetaldehyde and pyruvate.

Thiamine Phosphorylation—For the phosphorylation, the following were used per tube: 0.5 ml. of enzyme preparation, 0.1 ml. of 1 M MgSO₄, 3 mg. of ATP, 2.0 ml. of 0.1 M phosphate, pH 7.4; total volume, 3.4 ml. Thiamine and its analogues were added either simultaneously with the above mixture or after an interval of 15 minutes. The total incubation time was usually 3 hours and 15 minutes, and the reaction temperature was 38°. At the conclusion of the incubation, the tubes were placed for 2 minutes in a boiling water bath, cooled, and centrifuged to remove protein. 1 ml. of the supernatant fluid was removed and tested for cocarboxylase activity.

For the determination of cocarboxylase activity we have employed the wheat germ carboxylase discussed above. It has been found that this preparation is more satisfactory than alkaline washed yeast as a source of apocarboxylase. Cocarboxylase standards were prepared by the addition of the coenzyme to the reaction mixture at the conclusion of incubation, thus compensating for any possible effects of the reaction mixture upon the cocarboxylase assay. These aliquots were transferred to Warburg vessels containing the mixtures described above under “Pyruvate decarboxylation.” The relationship of CO₂ evolution to TDP concentration is illustrated in Fig. 1. Thus, the method is seen to be satisfactory for the estimation of TDP for these purposes.

Results

Inhibition of Carboxylase Activity by Oxythiamine Diphosphate—The effects of cocarboxylase and oxythiamine diphosphate on the carboxylase system are determined in large part by the order of addition of the two to the reaction mixture (Fig. 2).

The compound added first was placed in the main compartment of the Warburg vessel in contact with the enzyme and the remainder of the re-
action mixture; the second compound was tipped in with the substrate after temperature equilibration. This allowed for approximately 20 minutes between additions of the two to the apoenzyme.

Curve 3 of Fig. 2 illustrates the lack of inhibition of the system by oxy-

FIG. 1. Relationship of CO₂ evolution to TDP concentration in phosphorylase reaction mixtures. 1 ml. was removed from the reaction mixture and used for assay.

FIG. 2. Effects of OB₁ and ODP on carboxylase activity. Curve 1, reaction mixture, no additions; Curve 2, 7.8 × 10⁻³ µM of TDP added with reaction mixture; Curve 3, 7.8 × 10⁻³ µM of TDP with reaction mixture and 3.88 × 10⁻¹ µM of ODP with substrate; Curve 4, 7.8 × 10⁻³ µM of TDP with the reaction mixture and 3.88 × 10⁻¹ µM of OB₁ with substrate; Curve 5, 7.8 × 10⁻³ µM of TDP with substrate; Curve 6, 7.8 × 10⁻³ µM of TDP and 3.88 × 10⁻¹ µM of OB₁ with substrate; Curve 7, 3.88 × 10⁻¹ µM of OB₁ with reaction mixture and 7.8 × 10⁻³ µM of TDP with substrate; Curve 8, 7.8 × 10⁻³ µM of TDP and 3.88 × 10⁻¹ µM of ODP with substrate; Curve 9, 3.88 × 10⁻¹ µM of ODP with reaction mixture and 7.8 × 10⁻³ µM of TDP with substrate; Curve 10, 3.88 × 10⁻¹ µM of ODP with reaction mixture and 1.95 × 10⁻¹ µM of TDP with substrate.

thiamine diphosphate when the analogue is introduced to the system after cocarboxylase, thus indicating a strong attachment of coenzyme to apoenzyme. Simultaneous addition of cocarboxylase and oxythiamine diphosphate to the system results in inhibition, as may be seen in Curve 8, while introduction of oxythiamine diphosphate before cocarboxylase (Curve 9) presents a still greater effect. Curve 10 indicates some displacement of oxythiamine diphosphate upon increasing the cocarboxylase level from 7.8 × 10⁻³ µM to 1.95 × 10⁻¹ µM. The difference between
Curves 2, 3, and 4 and Curves 5, 6, and 7 is attributed to the lag in time necessary for attachment of coenzyme to apoenzyme.

Failure of Inhibition of Carboxylase Activity by Oxythiamine—Oxythiamine added at the same molar levels and in the same manner as oxythiamine diphosphate fails to exert an effect on the carboxylase system (Fig. 2). Thus, whereas the addition of ODP before or simultaneously with TDP causes marked inhibition, equimolar quantities of OB$_1$ do not alter the rate of decarboxylation regardless of the order of addition.

**Fig. 3.** Effects of ODP and OB$_1$ on AMC formation from acetaldehyde and pyruvate. Curve 1, reaction mixture, no addition; Curve 2, $7.8 \times 10^{-3}$ $\mu$M of TDP added with reaction mixture; Curve 3, $7.8 \times 10^{-3}$ $\mu$M of TDP with reaction mixture and $7.8 \times 10^{-1}$ $\mu$M of OB$_1$ after 15 minutes with substrates; Curve 4, $7.8 \times 10^{-3}$ $\mu$M of TDP with reaction mixture and $7.8 \times 10^{-1}$ $\mu$M of ODP with substrates; Curve 5, $7.8 \times 10^{-3}$ $\mu$M of TDP with substrates; Curve 6, $7.8 \times 10^{-1}$ $\mu$M of OB$_1$ with reaction mixture, $7.8 \times 10^{-3}$ $\mu$M of TDP with substrates; Curve 7, $7.8 \times 10^{-3}$ $\mu$M of TDP and $7.8 \times 10^{-1}$ $\mu$M of OB$_1$ with substrates; Curve 8, $7.8 \times 10^{-3}$ $\mu$M of TDP and $7.8 \times 10^{-1}$ $\mu$M of ODP with substrates; Curve 9, $7.8 \times 10^{-1}$ $\mu$M of ODP with reaction mixture and $7.8 \times 10^{-3}$ $\mu$M of TDP with substrates.

Failure of Neopyrithiamine to Inhibit Carboxylase Activity—Neopyrithiamine, when employed at the same levels as oxythiamine diphosphate and oxythiamine, likewise fails to inhibit decarboxylation, curves identical with those shown for OB$_1$ in Fig. 2 being obtained.

In an effort to determine whether neopyrithiamine would act in the same manner as oxythiamine diphosphate if phosphorylated, a small amount of NPT was treated according to the method employed by Visconti et al. (18) for the preparation of thiamine triphosphate. An extremely hygroscopic crystalline material was obtained. No attempt at analysis was made, although paper chromatography indicated the NPT
to have been phosphorylated. This material inhibited the carboxylase in the same manner as oxythiamine diphosphate.

Inhibition of Acetymethylcarbinol Formation by Oxythiamine Diphosphate—Virtually the same inhibition is observed in the synthesis of AMC from acetaldehyde and pyruvate as in the carboxylase systems (Fig. 3).

**Table I**

Inhibition by Oxythiamine Diphosphate of Acetymethylcarbinol Formation from Acetaldehyde Alone

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Additions</th>
<th>AMC formed per tube after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 hr.</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>γ</td>
</tr>
<tr>
<td>2</td>
<td>8 γ TDP</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>8 γ &quot; &quot; 20 min. before 400 γ ODP</td>
<td>41</td>
</tr>
<tr>
<td>4</td>
<td>400 γ ODP, 20 min. before 8 γ TDP</td>
<td>8</td>
</tr>
</tbody>
</table>

**Table II**

Effects of Oxythiamine and Neopyrithiamine on Rat Liver Phosphorylase

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>TDP synthesized per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td>γ</td>
</tr>
<tr>
<td></td>
<td>10 γ B₁</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>10 γ &quot; &quot; 62.5 γ NPT after 15 min.</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>62.5 γ NPT; 10 γ B₁ &quot; 15 &quot;</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>50 γ B₁; 62.5 γ NPT &quot; 15 &quot;</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>62.5 γ NPT; 50 γ B₁ &quot; 15 &quot;</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>10 γ B₁</td>
<td>5.4</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>γ</td>
</tr>
<tr>
<td></td>
<td>1000 γ OB₁; 10 γ B₁ after 15 min.</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>5000 γ &quot; &quot; 10 γ &quot; &quot; &quot; 15 &quot;</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Again the inhibition due to oxythiamine diphosphate is dependent upon the order of addition with respect to cocarboxylase; when TDP is added 20 minutes before, a 100-fold quantity of ODP is ineffective. If, however, the two are added along with the substrate, there is pronounced inhibition, and almost total inhibition if the ODP is allowed to come in contact with the enzyme before TDP. In the case of oxythiamine, it is again seen that there is no inhibition. The same inhibition may be brought about with the use of acetaldehyde as the sole substrate (Table I).

We have also found, in agreement with the observation of Singer and...
Pensky (19), that synthesis of AMC is greater from acetaldehyde plus pyruvate than from acetaldehyde alone.

Effects of Oxythiamine and Neopyrithiamine on Rat Liver Thiamine Phosphorylase—Experiments on the rat liver phosphorylase indicate an interesting difference between the actions of oxythiamine and neopyrithiamine on the enzyme (Table II). Whereas the presence of neopyrithiamine at a molecular ratio to thiamine of only 5:1 results in significant inhibition, oxythiamine at levels 100 times those of thiamine is found to be ineffective, and only partially effective at levels of 500:1. The inhibition due to NPT can be overcome by the addition of larger amounts of thiamine, indicating the mode of action to be competitive. In contrast to the inhibition involving carboxylase, however, the order of addition of substrate and inhibitor in this case is found to be immaterial. Thus, the amount of TDP synthesis remains the same whether NPT is added 15 minutes before or after the addition of B1.

DISCUSSION

As a result of these data, we have been led to the following considerations in an attempt to clarify the mode of action of these analogues.

Since no enzyme system containing thiamine diphosphate has as yet been discovered which is inhibited by either neopyrithiamine or oxythiamine, it would seem unlikely that the action of these two compounds is directly upon such a system. From this assumption, we may consider the following three courses. (1) The inhibitor may block the synthesis of thiamine diphosphate from thiamine. (2) The inhibitor may itself be phosphorylated, and the resulting compound could in turn compete with TDP for the apoenzyme. (3) The inhibitor may in some manner cause the displacement of thiamine from the tissue of the organism.

Inhibition of the phosphorylation of thiamine has been definitely shown in this study to be the case with NPT. However, there is no action of oxythiamine upon the rat liver phosphorylase except at extremely high levels.

Such a difference between the two compounds could possibly explain differences found in vivo with mice (3, 4) and with microorganisms (20).

It is of interest in this respect that Eusebi and Cerecedo (7) report inhibition of TDP synthesis from thiamine by oxythiamine with a purified yeast preparation and also with Propionibacterium pentosaceum. Eusebi and Cerecedo2 have also found no inhibition of phosphorylation of the yeast system by NPT. Thus the results obtained with the rat liver preparation are diametrically opposed to those found with the yeast system.

The lack of effect of oxythiamine on liver phosphorylase is especially

2 Unpublished work.
difficult to explain in view of the effects of chlorooxythiamine and bromooxythiamine *in vivo* (4). These compounds, differing from OB₁ by replacement of the thiazole alcoholic hydroxyl group with halogen, have been shown to be free of antithiamine activity. Thus, the need for the hydroxyl group for inhibition *in vivo* is demonstrated, suggesting the possible involvement of the compound in a phosphorylation mechanism.

The possibility of phosphorylation of the analogues is attractive in the light of the inhibitory properties of the synthetically prepared phosphate derivatives. Whether this is the actual case, however, cannot be stated definitely. From the data presented, this might be expected, at least with NPT. If such were the case, phosphorylated NPT would be capable of competing with TDP enzymes.

Elimination of thiamine in the urine of rats injected with oxythiamine has been observed by Frohman and Day (21). The authors postulate a displacement of thiamine by OB₁ from enzymes and related proteins with which they are reversibly combined. This hypothesis, although inconsistent with the results obtained with rat liver thiamine phosphorylase, could possibly explain the toxic properties of oxythiamine *in vivo* if we assume thiamine to be stored in the organism by combination with a protein.

Of course, the possibility of phosphorylation of thiamine by a system other than that studied here might be influenced by oxythiamine. By the same reasoning, phosphorylation of oxythiamine itself is not entirely ruled out.

**SUMMARY**

1. The decarboxylation of pyruvate and the formation of acetylmethylcarbinol by wheat germ carboxylase are inhibited by oxythiamine diphosphate.

2. The inhibition is largely dependent upon the order of addition of the inhibitor and thiamine diphosphate to the enzyme.

3. Oxythiamine and neopyrithiamine have no effect upon pyruvate decarboxylation. Acetylmethylcarbinol formation is not influenced by the addition of oxythiamine.

4. Neopyrithiamine, but not oxythiamine, is a very potent inhibitor of rat liver thiamine phosphorylase. In this case, order of addition of substrate and inhibitor is immaterial.

5. The significance of these findings is discussed.

**BIBLIOGRAPHY**


STUDIES ON THIAMINE ANALOGUES:
III. EFFECTS ON ENZYME SYSTEMS
Stephen Eich and Leopold R. Cerecedo


Access the most updated version of this article at http://www.jbc.org/content/207/1/295.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/207/1/295.citation.full.html#ref-list-1