THE CARBOXYLASE ENZYME SYSTEM*

BY HENRY TAUBER

(From the Research Laboratory of the McLeod Infirmary, Florence, South Carolina)

(Received for publication, June 15, 1938)

Carboxylase was discovered by Neuberg and Hildesheimer in 1911 (1). It is a highly specific enzyme as it only attacks α-keto acids, such as pyruvic acid, converting them into the corresponding lower aldehydes; e.g., pyruvic acid into acetaldehyde. The coenzyme dependency of carboxylase, however, was only noticed 21 years later by Auhagen (2). This investigator has shown that when dry yeast was washed with Na2HPO4 it lost its power to decarboxylate pyruvic acid and on the addition of cocarboxylase, which he obtained in a semipure state, the enzyme became active again. To restore full activity, however, magnesium ions appeared to be necessary. This was known to be the case with zymase.

Recently Lohmann and Schuster (3) have isolated cocarboxylase from bottom yeast in a pure state and found that it is the pyrophosphoric acid ester of thiamine. In support of their work it had been shown that dry yeast (4), washed dry yeast (5), and duodenal phosphatase (5, 6) convert thiamine into cocarboxylase. Peters (7) found that "vitamin B1 is phosphorylated rapidly under the conditions of a catatorulin test." Stern and Hofer (8) attempted to convert thiamine into its pyrophosphoric acid ester by treatment with phosphorus oxychloride. While this reagent was very useful in the conversion of lactoflavin (vitamin B2) into flavin monophosphoric ester (9), which is one of the coenzymes of the yellow oxidation system of Warburg and Christian, only a small amount of the thiamine could be phosphorylated by POCl3. Apparently POCl3 is not an efficient reagent for the

* Preliminary reports of part of this paper have appeared (J. Am. Chem. Soc., 60, 730 (1938); Proc. Soc. Exp. Biol. and Med., 38, 888, 889 (1938)).
introduction of the pyrophosphate group. Nevertheless, Stern and Hofer have shown by cataphoretic tests that conversion of the vitamin to its diphosphoric ester took place.

In the present paper a synthesis will be described by which thiamine may be completely converted into cocarboxylase. The coenzyme has been obtained in crystalline form. A series of new activators of the carboxylase-cocarboxylase system has been found and a specific function of cocarboxylase has been observed. Experiments will be presented showing that, similar to thiamine, cocarboxylase is also a growth-promoting substance.

EXPERIMENTAL

Synthesis and Purification of Cocarboxylase—500 mg. of sodium pyrophosphate are placed in a Pyrex test-tube and heated until all of the water of crystallization is removed. 1 cc. of orthophosphoric acid (c.p. 85 per cent) is placed in another large Pyrex test-tube and heated until a slight amount of solid deposit forms on the side of the tube. Then the pyrophosphate is added and the mixture gently heated until solution takes place. After a few minutes of cooling 500 mg. of thiamine are added and the contents of the tube are well mixed. The tube is placed in an oil bath of 155°, kept there for 15 minutes, and constantly stirred. Then the tube is removed and, after cooling, the solid mass is dissolved in 10 cc. of cold water. Cold saturated Ba(OH)$_2$ solution is added until no more precipitate forms and the solution is just commencing to turn yellow. (The thiochrome compound of cocarboxylase is inactive. It becomes active again, however, on acidification.) The precipitate is centrifuged off and the supernatant fluid is decanted. The precipitate is extracted three times with 50 cc. of cold water. All four supernatant fluids are united and, after cooling, 3 per cent H$_2$SO$_4$ is added to slight blue reaction of Congo red paper. The BaSO$_4$ is centrifuged off and discarded. The Ba-free solution is concentrated to 30 cc. in a vacuum at 25°. It is cooled in ice water and 15 to 20 volumes of a mixture

1 In the purification and identification of synthetic cocarboxylase Mr. J. Weijlard of the Research Laboratory of Merck and Company, Inc., has collaborated. While this paper was in press the cocarboxylase was obtained in a 100 per cent pure state. Hydrolysis and cleavage products were analyzed. The results of this work will be published elsewhere.
of 1 part of absolute alcohol and 2 parts of ether is added which precipitates the thiamine pyrophosphate in the form of microscopic needles. Sometimes a gummy mass forms which will turn into long macroscopic needles on short standing in the cold. The product obtained on six recrystallizations from the alcohol-ether mixture, redissolved each time in 10 cc. of 0.1 N HCl and dried in a vacuum over H₂SO₄, is readily soluble in water and free of inorganic salts.

Activity and General Properties—This synthetic preparation is practically as active as natural cocarboxylase (very kindly furnished by Professor Lohmann). Phosphorus and thiamine content, however, indicates that my cocarboxylase still contains a small amount of impurities.

Similar to the natural cocarboxylase, the synthetic coenzyme becomes inactive on 15 minutes boiling with n HCl, and the total phosphorus is liberated only with difficulty in about 5 hours at 100°. Kidney phosphatase also hydrolyzes the synthetic coenzyme. In this respect it resembles the cocarboxylase obtained by enzymic synthesis (6).

Cocarboxylase forms the theoretical amount of thiamine when treated by an alkaline ferricyanide solution. The ferrocyanide formed during the reaction may be converted to Prussian blue and measured colorimetrically (10).

Cocarboxylase (10 micrograms) gives a yellow color with the formaldehyde-azo test (Kinnersley-Peters (11)). Thiamine gives a red color.

New Activators of the Carboxylase-Cocarboxylase System—Recently Lohmann and Schuster (3) have shown that manganese ions activate the carboxylase-cocarboxylase enzyme system better than magnesium ions. Copper, iron, bismuth, zinc, and cadmium salts as well as sodium flouride inhibit the activity of the enzyme carboxylase (12). It is well known that certain amylases (13) cannot hydrolyze starch in the absence of certain salts. Their activation, however, is not limited to a few ions. One might expect that with carboxylase similar conditions exist. My expectations were correct. Several neutral salts such as Na₂SO₄, NaCl, KCl, as well as NaCN, were found to activate the carboxylase-cocarboxylase system (see Table I). The fact that NaCN activates this system shows that carboxylase is not a heavy metal-
containing enzyme. It activates probably in part because it forms a more reactive enol compound (cyanohydrin) with pyruvic acid. NaCN is a good activator, since only one-tenth the concentration is required to obtain the activation similar to that shown by the neutral salts. While Mn ions activate best, the order of activation obtained by NaCN is close to the one obtained by MgCl₂. Li₂SO₄ activates slightly and in dilute concentration only. NaN₃ does not activate. As in the case of amylases, no explanation can be given of why these salts activate the carboxylase-cocarboxylase system.

**Table I**

*Activation of Carboxylase-Cocarboxylase System by Various Salts*

All samples contained 30 micrograms of cocarboxylase except in Experiment 10.

In all experiments described in this paper synthetic cocarboxylase and natural cocarboxylase were employed with identical results.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Salt employed</th>
<th>10 min.</th>
<th>20 min.</th>
<th>30 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MgCl₂ (0.1 mg. Mg)</td>
<td>53</td>
<td>88</td>
<td>112</td>
</tr>
<tr>
<td>2</td>
<td>MnSO₄ (10 micrograms Mn)</td>
<td>51</td>
<td>78</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>Na₂SO₄ (4 mg. Na)</td>
<td>38</td>
<td>64</td>
<td>81</td>
</tr>
<tr>
<td>4</td>
<td>NaCl (4 mg. Na)</td>
<td>37</td>
<td>62</td>
<td>79</td>
</tr>
<tr>
<td>5</td>
<td>KCl (4 mg. K)</td>
<td>36</td>
<td>62</td>
<td>77</td>
</tr>
<tr>
<td>6</td>
<td>NaCN (0.4 mg. Na)</td>
<td>38</td>
<td>63</td>
<td>78</td>
</tr>
<tr>
<td>7</td>
<td>Li₂SO₄ (0.4 “ Li)</td>
<td>35</td>
<td>44</td>
<td>59</td>
</tr>
<tr>
<td>8</td>
<td>“ (4 mg. Li)</td>
<td>25</td>
<td>44</td>
<td>59</td>
</tr>
<tr>
<td>9</td>
<td>No salt</td>
<td>20</td>
<td>35</td>
<td>47</td>
</tr>
<tr>
<td>10</td>
<td>“ “ no cocarboxylase</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

While it is not difficult to remove all of the cocarboxylase from the dry yeast (Table I, Experiment 10), it is not possible by the extensive washing applied to remove all of the other activators of carboxylase (Table I, Experiment 9), and some activation is apparently caused by the buffer (phosphate). In this experiment acid- and alkali-washed dry yeast was employed (see below).

**Specific Function of Cocarboxylase**—It is believed that cocarboxylase combines with the inactive enzyme carboxylase to form a new compound which in the presence of magnesium ions becomes
highly active. This compound, however, has not yet been isolated. Nor has the enzyme carboxylase been obtained in pure state. In any case nothing is known about the nature or mechanism of "activation" of carboxylase by cocarboxylase. The following experiments will show that one of the functions of cocarboxylase is to protect carboxylase, a very labile enzyme, from destruction.

Experiment 1 1 cc. of yeast suspension and 30 micrograms of cocarboxylase in 1 cc. of phosphate of pH 6.2 were placed in the main compartment of a Warburg vessel (17 cc. capacity and two side arms). In one side arm 0.5 cc. of sodium pyruvate (5 mg. of pyruvic acid containing 0.1 mg. of magnesium as MgCl₂) was placed. The vessel was connected with a Warburg-Barcroft respirometer and shaken for 105 minutes at 30°. Then the stopcock was closed and after 15 minutes of further shaking the pyruvate was washed in from the side arm.

Experiment 2—In another vessel the pyruvate was placed in one side arm and the cocarboxylase was placed in the second side arm, while the main compartment of the vessel contained 1 cc. of yeast suspension. This vessel also was shaken for 120 minutes and then the pyruvate and cocarboxylase were added. Cocarboxylase solutions are very stable between pH 4 and 10 at 30°.

Experiment 3—Here 30 micrograms of thiamine (Merck) in 0.1 cc. of phosphate of pH 6.2 and 1 cc. of yeast suspension were placed in the main compartment of the Warburg vessel. The cocarboxylase and pyruvate were added at the end of 120 minutes.

Experiment 4—The content of this vessel was similar to that

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Content of main compartment during first 120 min.</th>
<th>Carbon dioxide (after addition of contents in side arm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yeast suspension and cocarboxylase</td>
<td>102 c.mm.</td>
</tr>
<tr>
<td>2</td>
<td>&quot; &quot; alone</td>
<td>66 c.mm.</td>
</tr>
<tr>
<td>3</td>
<td>&quot; &quot; and thiamine</td>
<td>40 c.mm.</td>
</tr>
<tr>
<td>4</td>
<td>&quot; &quot; water</td>
<td>40 c.mm.</td>
</tr>
</tbody>
</table>

TABLE II

Showing Protective Function of Cocarboxylase
in Experiment 1. Cocarboxylase, however, was replaced by 1 cc. of H₂O.

It may be seen from Experiment 1, Table II, that when co-
carboxylase was immediately added to freshly washed yeast and
kept at 30° for 120 minutes in the presence of air CO₂ formed after
the addition of pyruvate very rapidly. If the washed yeast,
however, was kept for the same length of time without cocar-
boxylase, the enzyme carboxylase lost almost all of its activity
and there was hardly any CO₂ formed (Experiment 2). Thiamine
had no protective action on carboxylase and no cocarboxylase
was formed during the duration of the experiment from the vitamin
(Experiment 3). The yeast suspension without added cocarbox-
ylase did not form CO₂ from sodium pyruvate (Experiment 4).
Other experiments (not included in Table I) have shown that the
protective function cannot be replaced by Ba-adenosinetriphos-
phate, Mg-hexosediphosphate, or by reduced glutathione.

In these experiments brewers' yeast which had been washed
with alkaline phosphate was employed (see below).

Removal of Activators and Cocarboxylase from Dry Yeast. Al-
kaline Washing—For the cocarboxylase test and for the experi-
ments on the "Specific function of cocarboxylase" brewers' bot-
tom yeast² was extensively washed with water and dried at room
temperature with the aid of a fan. The alkaline washing was
carried out according to Lohmann and Schuster (3). To 2 gm.
of dry yeast in a 250 cc. centrifuge flask 100 cc. of 0.1 m Na₂HPO₄
at 30° was added and shaken in a shaking machine for 12 minutes.
Then the mixture was centrifuged. The supernatant fluid was
discarded. This procedure was repeated once more. The mate-
rial was washed for 3 minutes with 100 cc. of water at 30°. The
washed yeast was suspended in 20 cc. of phosphate of pH 6.2 and
was so used.

It is important to note that there is no direct proportionality
between cocarboxylase concentration (activity) and CO₂ formation
from pyruvate. For instance, if the cocarboxylase concentration
is increased 3 times, CO₂ formation is only doubled after the first
15 minutes of the experiment (3, 6, 8). It should also be noted

² I am indebted to the Jacob Ruppert Brewery, through the kindness
of Mr. E. Muhlhausen, for furnishing the bottom yeast.
that the carboxylase content of various yeasts differs greatly, and for this reason it is difficult to make exact comparisons of cocarboxylase activities by the enzymic test.

Acid and Alkaline Washing—For the salt activation experiments the dry yeast was washed with acid and alkaline phosphate according to Lohmann and Schuster (Table VII (3)). 2 gm. of dry yeast were washed by shaking in a machine for 10 minutes at room temperature, three times with 100 cc. portions of 0.1 M KH$_2$PO$_4$, once with 100 cc. of H$_2$O, twice with 100 cc. portions of 0.1 M Na$_2$HPO$_4$, and once with 100 cc. of H$_2$O. The washed yeast was suspended in 20 cc. of phosphate, pH 6.2. A solution of sodium pyruvate containing 10 mg. of pyruvic acid per cc. was prepared and adjusted to pH 6.2 with NaOH. No mineral acids were introduced. 0.5 cc. of sodium pyruvate (5 mg.) was placed in the side arm of the Warburg vessels. 1 cc. of washed yeast suspension, 0.5 cc. of cocarboxylase, 0.5 cc. of phosphate of pH 6.2, and 0.5 cc. of salt solution were placed in the main compartment; total volume, 3 cc. The temperature was 28°.

Cocarboxylase Growth Substance—Thiamine has an accelerating action on the growth of yeast. With cocarboxylase acceleration is more pronounced.

In the presence of a proteose-peptone-sucrose-salt medium 1 microgram of the pyrophosphate per cc. increases the growth considerably in 20 hours at 25°. In the following a set of typical experiments is described. To the medium (pH 5.6) containing 5 gm. of sucrose, 1 gm. of proteose-peptone (Difco), 0.3 gm. of KH$_2$PO$_4$, and 0.3 gm. of MgSO$_4$·7H$_2$O in 100 cc. of distilled water, 0.1 mg. of thiamine or 0.1 mg. of thiamine pyrophosphate (in 0.2 cc. of water) was added. The medium was made up fresh every day and was boiled for 10 minutes before use. Controls were run without the vitamin or ester. 100 cc. samples of the medium were placed in large culture dishes and to each 1 cc. of a 0.1 per cent suspension of commercial bakers' yeast (Fleischmann) was added. After 20 hours three of each of the samples were united, centrifuged, and weighed. Those with added thiamine pyrophosphate weighed 5.1 gm.; those with thiamine weighed 4.6 gm.; whereas without the vitamin or coenzyme samples weighed 2.7 gm.
SUMMARY

The synthesis of cocarboxylase (pyrophosphoric acid ester of thiamine) from synthetic thiamine has been described. The synthetic, crystalline coenzyme contains a small amount of impurities. It is, however, as active as the pure natural preparation.

Several salts have been found to be activators of the carboxylase-cocarboxylase system, one of which was NaCN. The fact that NaCN activates the system proves that heavy metals are not a part of this enzyme system. The activation by NaCN is probably due to the formation of a more reactive addition (enol) compound, cyanohydrin, with pyruvic acid.

Experiments have been described which show that cocarboxylase has a specific protective action on carboxylase. The enzyme freed of cocarboxylase is very labile. Cocarboxylase prevents it from rapid inactivation. Adenosinetriphosphate, hexosediphosphate, or reduced glutathione cannot replace cocarboxylase in its protective function.

While in plants thiamine pyrophosphate functions as an indispensable specific coenzyme for carboxylase, in mammalian metabolism it acts as a coenzyme for a pyruvic acid dehydrogenase (14).

Experiments with bakers' yeast show that, similar to thiamine, cocarboxylase also functions as a growth substance, and it appears that the growth-promoting action of thiamine is based on its conversion to cocarboxylase by the living cell. After thiamine has been phosphorylated, it acts as an accelerator of carbohydrate metabolism.

I am grateful to Merck and Company, Inc., and to the Winthrop Chemical Company, Inc., for generous gifts of thiamine, and to Professor Lohmann for a sample of pure (natural) cocarboxylase.

BIBLIOGRAPHY

THE CARBOXYLASE ENZYME SYSTEM
Henry Tauber


Access the most updated version of this article at http://www.jbc.org/content/125/1/191.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/125/1/191.citation.full.html#ref-list-1