Decarboxylation of Pyruvate by Thiamine Analogue*

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(Received for publication, September 9, 1958)

Thiamine catalyzes the nonenzymic decarboxylation of pyruvate to $\alpha$-acetolactate and acetoin (1-4) in mildly basic aqueous solution. In order to study the mechanism of this reaction we have tested a number of thiamine analogues as catalysts. Breslow (5) has reported some very similar results which we confirm.

EXPERIMENTAL

Sources of Compounds Tested—Thiamine hydrochloride (Merck, U.S.P. grade), $\alpha$-acetylthiamine hydrochloride and oxythiamine dihydrochloride (California Foundation for Biochemical Research), pyriithiamine hydrobromide and 4-aminoo-5-aminomethyl-2-methyl pyrimidine dihydrochloride (Nutritional Biochemicals Corporation) were all used without further purification. For determination of pKₐ values, the 4-aminoo-5-aminomethyl-2-methyl pyrimidine dihydrochloride was recrystallized from ethanol and ether until it gave the theoretical end point when titrated with base.

5-(2-Hydroxyethyl)-4-methyl thiazole was redistilled from pooled commercial products of Merck and du Pont. The boiling point was 108°, 0.8 mm. Infrared inspection of the redistilled product showed the loss of a strong carbonyl peak which was originally present. The purified product was stored in a desiccator at −20° to hinder oxidation.

Salts of 5-(2-Hydroxyethyl)-4-methyl Thiazole—The method of Clarke (6) was employed to prepare the 3-methyl and 3-benzyl (7) and the 3-o-, m-, and p-nitrobenzyl (8) salts.

The 3-aminomethylbenzyl salt was prepared by dissolving 0.04 mole each of $\alpha$-bromoethyl benzene (Eastman, redistilled 89°, 15 mm.) and 5-(2-hydroxyethyl)-4-methyl thiazole in 4 ml. of thiophene-free anhydrous benzene in a stopped 18 x 150-mm. test tube. The solution was heated overnight at 55°. The $\alpha$-aminomethylbenzyl salt separates as a light yellow syrup. Attempts to crystallize this compound from a number of solvent systems were all unsuccessful. During crystallization attempts in hot absolute ethanol the salt was partially solvolyzed to the pyrimidylmethyl ethyl ether and 5-(2-hydroxyethyl)-4-methyl thiazole hydrobromide. The absorption spectra in both acid and base and the titration behavior very closely paralleled those of the 3-benzyl salt. These properties plus the method of preparation leave little doubt as to the character of the product.

The 3-cyanomethyl salt was prepared in a similar manner from chloroacetanitride (Eastman white label, redistilled 24°, 27 mm.) and 5-(2-hydroxyethyl)-4-methyl thiazole. The solution was heated at 80° for 3 days, the benzene supernatant was discarded, and the red resinous layer which remained was triturated twice with anhydrous ethyl ether to remove as much as possible of the starting materials and benzene solvent. The gummy mass remaining was dissolved in 25 ml. of absolute ethanol and was treated twice with about 0.5 gm. of charcoal (Darco). Anhydrous ethyl ether was added to the point of incipient turbidity and the solution cooled overnight at 2°C. The dense bright yellow crystals were collected and washed with cold ethanol and ethyl ether (50:50). The yield after recrystallization was 3.2 gm., m.p. 132-133°. Repeated recrystallization, charcoal treatment, and chromatography on Dowex 50-X4 exchange resin with 0.2 n HCl as the developing solvent failed to remove the yellow color. However, the ultraviolet absorption spectrum was typical of a thiazolium compound in both acid and basic solution and exhibited negligible absorption above 300 μm.

Surprisingly, the infrared spectrum does not contain a cyanide peak in the region of 4.5μ.

O- Heterothiamine (3-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride) was generously given to us by Dr. John F. Codington of the Sloan-Kettering Institute for Cancer Research.

5-(4-Aminomethyl-5-pyrimidinyl)-methyl-4-methyl Orazolium Bromide—4-Methyl oxazole was prepared by the method of Cornforth and Cornforth (9). 4-Methyl oxazole (0.02 mole) was dissolved in 3 ml. of freshly distilled n-butanol and heated to 95-100°. 4-Amino-5-bromomethyl-2-methyl pyrimidine dihydrobromide (0.007 mole, Merck, technical grade) was added in small portions and mixed well. The test tube was tightly stoppered and heated for 30 minutes more at 125° with shaking at 4 to 5 minute intervals. The solution was cooled and the crystals which formed were collected and washed with butanol and then ether. The crystals were dissolved in a minimal amount of water, 95 per cent ethanol was added to the point of incipient turbidity, and the solution was cooled to 2° for 2 days. The resultant crystals were collected, washed, and recrystallized to yield 0.23 gm. of small, light tan crystals which melted with decomposition at 235°.

Dihydrothiamine and tetrahydrothiamine were prepared by the borohydride reductions of thiamine described by Bonvicino and Hennessy (10).
3-(3-Aminopropyl)-4-methyl thiazolium chloride and 3-(4-
aminobutyl)-4-methyl thiazolium chloride were from the col-
lection of the late R. R. Sealock and were prepared by Sarver (11)
using the method of Clarke (6).

Testing Procedure—The compounds were tested by incubation
with sodium pyruvate in evacuated Thunberg tubes at 50° in
borate or pyrophosphate buffers as previously described (4).
Acetoin was then measured, usually without acid treatment (i.e.
acetalactate present was not decarboxylated).

Accurate estimates of relative catalytic activity are especially
difficult with the poorer catalysts. Because of the pronounced
lag in acetoin production the amounts of acetoin measured are
very small. Fortunately the acetoin color test is very sensitive
though its accuracy is diminished by the presence of large
amounts of thiazolium compounds (4). More vexing is the prob-
lem of high blanks. Sodium pyruvate and thiamine itself both
cause a very small amount of color. The color from thiamine
may arise by hydrolytic breakdown to the ketol CH&OCHOH-
CH&CH&OH which would probably give the color test. The
related thiol is a known degradation product of thiamine (12) and
might also yield some color. The methyl thiazolium salt (3,4-di-
dimethyl-5-(2-hydroxyethyl) thiazolium chloride) appears to break
down in the same way. Since it is a much less active catalyst
than thiamine, the blanks become a serious problem. At pH 9
or below these blanks were negligible, but above 9 they increased
rapidly.

If the sulfur of thiamine is replaced by oxygen, the resulting
oxazolium salt (0-heterothiamine) decomposes gradually to a
color-yielding material. The formation of the previously men-
tioned ketol should occur readily in this compound. An accurate
estimate of its catalytic activity was therefore impossible. How-
ever, no such interference was observed with the 4-methyl ox-
azolium analogue of thiamine.

RESULTS AND DISCUSSION

One of the better catalysts studied is the benzyl analogue
of thiamine, 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium
chloride (VIII, Table I). As with thiamine (I) catalysis, a char-
acteristic lag in acetoin production is observed. Because of this
lag, the relative catalytic activities of two compounds cannot be
compared by simply measuring the amounts of acetoin produced
in a given length of time. Thus in 1 hour in borate buffer thia-
mamine catalysis produces 7 times as much acetoin as that of the
benzyl analogue at the same pH; in 3 hours it is only 3.5 times
as much. It is more proper to compare the lengths of time re-
quired for the production of a certain concentration of acetoin
under specified conditions. Thus with the benzyl analogue the
time required to produce any amount of acetoin in pH 8.8 borate
buffer at 50° is always 2.6 times that required with thiamine as
catalyst. If the amounts of acetoin produced by action of the
benzyl analogue are plotted against heating time divided by 2.6,
the points fall on the curve of acetoin versus time for thiamine
catalysis (Fig. 1). Thus we conclude that the benzyl analogue
is 1/2.6 times as active as thiamine at this pH.

Similarly we can conclude that the rate of production of acetoin
by thiamine at 50° is just 3 times as fast as at 40° (Fig. 1).
Apparent temperature and catalyst structure influence the
rate in similar ways.

The activities of a series of other catalysts have also been
measured. The activities relative to that of thiamine are sum-
marized in Table I.

The results show that in nonenzymic catalysis of acetoin for-
mation the thiazolium group of thiamine must be the site of inter-
action with pyruvate. The 3-methyl thiazolium analogue (X)
is slightly active and the 3-benzyl analogue (VIII) is 38 per cent
as active as thiamine. On the other hand, pyrithiamine (III) is
completely inactive despite the fact that the aminopyrimidine
group and the quaternary nitrogen are present as in thiamine.
These results are in agreement with those of Breslow (5, 13).
Another possible site of interaction with thiamine, the methylene
bridge, has been ruled out by work of Fry et al. (14) and Breslow
(13). Breslow has furthermore provided convincing evidence in
favor of the thiazolium dipolar ion XVIII

![Diagram](http://www.jbc.org/)

as an intermediate in the reaction with pyruvate (13, 15). Some
further support is lent to Breslow's suggestion by the inactivity
of 3-benzyl-, phenyl-, and allyl-2,4-dimethyl thiazolium salts
(Downes and Sykes, 16) and of 3-benzyl-2,4-dimethyl thiazolium
bromide in catalysis of the furoin condensation (Ugai et al., 17).
We have previously reported (18) that the 2,4-dimethyl thi-
azolium analogue of thiamine (V) is also inactive. However, we
have not obtained this compound in a satisfactory state of purity,
and this result must be regarded as tentative.

According to the Breslow mechanism the α-methylbenzyl
analogue of thiamine (IX) might be anticipated to be an active
catalyst. There may be two reasons for its inactivity. First,
the addition of the methyl group on the methylene bridge may
facilitate the solvolysis of the compound at the quaternary
nitrogen so that the molecule splits before it has a chance to con-
dense with pyruvate. Secondly, the additional methyl group
may sterically hinder the attack of the dipolar ion on pyruvate,
although scale models seem to indicate that this hindrance should
not be great.

In addition, the series of o-, m-, and p-nitrobenzyl analogues

![Graph](http://www.jbc.org/)

FIG. 1. Acetoin production versus time for two catalysts. Py-
ruvate (0.2 M) plus catalyst (0.02 M) incubated at pH 8.8 to 9,
borate buffer. ○, Thiamine as catalyst, 50°; ○, thiamine 40°,
time divided by 3.0; △, 3-benzyl-5-(2-hydroxyethyl)-4 methyl
thiazolium chloride, 50°, time divided by 2.6.
Relative activities in catalysis of pyruvate decarboxylation to acetoin

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Relative activity pH 8.7-9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>100</td>
</tr>
<tr>
<td>Thiamine</td>
<td></td>
</tr>
<tr>
<td>II.</td>
<td>100</td>
</tr>
<tr>
<td>O-Acetylthiamine</td>
<td></td>
</tr>
<tr>
<td>III.</td>
<td>0</td>
</tr>
<tr>
<td>Pyriothiamine, 1-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-3-(2-hydroxyethyl)2-methyl pyridinium bromide</td>
<td>38</td>
</tr>
<tr>
<td>IV.</td>
<td>0</td>
</tr>
<tr>
<td>Oxythiamine, 3-[(4-hydroxy-2-methyl-5-pyrimidinyl)-methyl]-5-(2-hydroxyethyl)-4-methyl thiazolium chloride</td>
<td>2</td>
</tr>
<tr>
<td>V.</td>
<td>0</td>
</tr>
<tr>
<td>3-[(4-Amino-2-methyl-5-pyrimidinyl)-methyl]-2,4-dimethyl thiazolium bromide</td>
<td>5</td>
</tr>
<tr>
<td>VI.</td>
<td>0</td>
</tr>
<tr>
<td>O-Heterothiamine, 3-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-5-(2-hydroxyethyl)4-methyl oxazolium chloride</td>
<td>Trace (20 at pH 10.2)</td>
</tr>
<tr>
<td>VII.</td>
<td>0</td>
</tr>
<tr>
<td>3-[(4-Amino-2-methyl-5-pyrimidinyl)-methyl]4-methyl oxazolium bromide</td>
<td>5</td>
</tr>
<tr>
<td>VIII.</td>
<td>0</td>
</tr>
<tr>
<td>3-Benzyl-5-(2-hydroxyethyl)-4-methyl thiazolium chloride</td>
<td>Trace</td>
</tr>
<tr>
<td>IX.</td>
<td>0</td>
</tr>
<tr>
<td>3-α-Methylbenzyl-5-(2-hydroxyethyl)-4-methyl thiazolium chloride</td>
<td>0</td>
</tr>
<tr>
<td>X.</td>
<td>0</td>
</tr>
<tr>
<td>3,4-Dimethyl-5-(2-hydroxyethyl)thiazolium chloride</td>
<td>0</td>
</tr>
<tr>
<td>XI.</td>
<td>Trace</td>
</tr>
<tr>
<td>3-Cyanomethyl-5-(2-hydroxyethyl)-4-methyl thiazolium chloride</td>
<td>Trace (20 at pH 10.2)</td>
</tr>
<tr>
<td>XII.</td>
<td>0</td>
</tr>
<tr>
<td>3-α, m, p-Nitrobenzyl-5-(2-hydroxyethyl)-4-methyl thiazolium chlorides</td>
<td>0</td>
</tr>
<tr>
<td>XIII.</td>
<td>0</td>
</tr>
<tr>
<td>3-3-Aminopropyl)-4-methyl thiazolium chloride</td>
<td>0</td>
</tr>
<tr>
<td>XIV.</td>
<td>0</td>
</tr>
<tr>
<td>3-(4-Aminobutyl)-4-methyl thiazolium chloride</td>
<td>Trace</td>
</tr>
<tr>
<td>XV.</td>
<td>0</td>
</tr>
<tr>
<td>Dihydrothiamine</td>
<td>0</td>
</tr>
<tr>
<td>XVI.</td>
<td>0</td>
</tr>
<tr>
<td>Tetrahydrothiamine</td>
<td>0</td>
</tr>
<tr>
<td>XVII.</td>
<td>0</td>
</tr>
<tr>
<td>4-Amino-5-aminomethyl-2-methyl pyrimidine</td>
<td>0</td>
</tr>
</tbody>
</table>

(XII) and the γ- and δ-aminooalkyl analogues XIII and XIV theoretically should work as catalysts. However, it is apparent from the color of the reaction mixtures that these compounds are not stable under the test conditions and little can be said about their catalytic activities.

As would be expected from Breslow's mechanism, reduction of the carbon-nitrogen double bond of the thiazolium ring causes complete loss of activity (dihydro- and tetrahydrothiamine). Two compounds in which the sulfur of the thiazolium ring is replaced by oxygen (oxazolium salts) were tested. One of these (O-heterothiamine) is otherwise identical with thiamine; the second (VII) lacks a group on C-5 of the thiazolium ring. Both compounds appear to be completely inactive as catalysts although the result with O-heterothiamine was obscured by its decomposition (see section on testing procedure). The inactivity of oxazolium salts probably results from their reaction with base to form inactive pseudo bases. Titrations indicate that this occurs at a low pH with a pKₐ of about 5.8 for both of the oxazolium compounds (K = [H⁺][pseudo base]/[oxazolium ion]).

The large difference in activity between the 3-methyl and 3-benzyl salts (X and VIII) is surprising. Breslow (13) suggests that the difference in inductive effects of the methyl and benzyl groups is responsible. As evidence Breslow compares the pKₐ of methyl amine (10.6) with that of benzyl amine (9.3). We find the pKₐ of the aminomethyl group of 4-amino-5-aminomethyl-2-methyl pyrimidine (XVII) to be 8.4. Thus the inductive effect of the aminopyrimidine group in lowering the basicity of the aminomethyl group is even greater than that of a benzene ring, and thiamine is correspondingly more reactive than the benzyl analogue. However, the relationship is not really quantitative.

If the rate of reaction is governed by the extent of formation of the dipolar ion XVIII, the rate should be a maximum at the pK (average) of the thiazolium ring-opening reaction. This has been shown to be the case with thiamine and with the benzyl analogue (VIII) (4). The methyl thiazolium salt (X) could not be tested at its high pK value because of decomposition. The pK values for the ring-opening reactions of the three compounds, thiamine, the benzyl (VIII), and methyl (X) thiazolium salts at 25° are 9.25, 9.9, and 10.2, respectively. Thus, the effect of placing a hydrogen on the N-methyl group of compound X with a phenyl or aminopyrimidyl group is to shift the optimal pH for reaction to a lower value.

At pH 8.7 to 9.0 the benzyl and methyl compounds exist almost completely in the thiazolium form but thiamine has been half converted to the inactive thiol form. A truer comparison of activities could be made at a pH of 8 or below where thiamine is over 99 per cent in the thiazolium form. We would expect thiamine to be about 5 times as active as the benzyl compound under these conditions. Thus one function of the aminopyrimidine group of thiamine may be to permit efficient catalysis in the physiological pH range.

Oxythiamine (IV), in which the amino group of thiamine has been replaced by a hydroxyl, is almost inactive at pH 8.4 to 8.9 as reported by Breslow (13). However, at pH 10.2 it has 20 per cent of the activity of thiamine. The pH for the ring-opening reaction is about 10.6. Titration of oxythiamine indicates that the oxypyrimidine group dissociates with a pK of about 8.2. Thus, under our test conditions the pyrimidine ring bears a negative charge and we would anticipate a marked decrease in reactivity at C-2 of the thiazolium group and the observed increase in the pK of the ring-opening reaction. Downes and Sykes (16) have attributed the low activity of oxythiamine to an actual hydrogen bond between the oxygen on the pyrimidine ring and C-2 of the thiazolium ring. However, it is unnecessary to postulate this unique hydrogen bond. There is also a possibility that the oxygen adds intramolecularly to the thiazolium ring as does the amino group of thiamine at a higher pH (7).

**SUMMARY**

1. A number of thiamine analogues have been synthesized and tested for catalytic activity in converting pyruvate to acetoin. The thiazolium ring is necessary for catalytic activity, but the pyrimidine ring is not.
2. Oxazolium salts, 2,4-dimethyl thiazolium analogue of thiamine.
3. The pKₐ value for the amino group attached directly to the ring in position 4 is 5.4. The change in the ultraviolet spectrum accompanying this first dissociation confirms that the 4-amino group is involved.
thiamine, pyrithiamine, dihydrothiamine, and tetrahydrothiamine are inactive.

3. The relative catalytic activities and pH optima of several thiazolium salts are discussed. The results are in harmony with the theory of Breslow in which a thiazolium dipolar ion is an intermediate.

REFERENCES

Decarboxylation of Pyruvate by Thiamine Analogues
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