Preliminary Communications

The Natural Occurrence and Enzymatic Formation of a Hydroxyethyl Derivative of Thiamine Pyrophosphate*†

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Breslow (1) has formulated a mechanism of thiamine action in which the 2-position of the thiazolium ring of thiamine is postulated to be the reactive part of the molecule. Krampits et al. (2) have prepared thiamine with an α-hydroxyethyl group substituted on the 2-position and reported its probable identity as "active acetaldehyde."

Recent results from this laboratory indicate that the pyrophosphoric acid ester of α-hydroxyethyl thiamine† occurs naturally in microorganisms. Acid extracts of Escherichia coli cells, after dephosphorylation by treatment with Taka-Diastase, contained a compound which migrated on paper chromatograms identically with synthetic α-hydroxyethyl thiamine§ in six different solvent systems (Table I). Zones of migration were determined by the bioautographic technique (3) with Lactobacillus viridescens as the test organism. The relative sizes of the growth zones indicated that 60 to 75% and about 25% of the total thiamine present in E. coli and bakers' yeast, respectively, could be accounted for as phosphorylated α-hydroxyethyl thiamine.

Other evidence which indicates that the dephosphorylated product is identical with synthetic α-hydroxyethyl thiamine consists of the observations that both the natural and synthetic compounds behave in a similar manner in replacing thiamine as "active acetaldehyde."

It has been possible to form, enzymatically, the pyrophosphate ester of α-hydroxyethyl thiamine by incubating thiamine pyrophosphate with wheat germ carboxylase (purified to the alcohol powder stage (4)) and either pyruvate or acetaldehyde. After incubation, treatment of the reaction mixtures with Taka-Diastase followed by chromatography and bioautography revealed that 10 to 12% of the thiamine pyrophosphate had been converted to α-hydroxyethyl thiamine pyrophosphate (see Table I). The requirements for the production of the α-hydroxyethyl derivative are qualitatively identical with the requirements for acetaldehyde formation. Thiamine or thiamine monophosphate could not substitute for thiamine pyrophosphate. Boiled enzyme was inactive.

α-Ketobutyrate can also be decarboxylated by carboxylase. When this keto acid was used in place of pyruvate in the system described above, a new compound was formed which, after treatment with Taka-Diastase, exhibited migration characteristics on paper chromatograms which were different from those of α-hydroxyethyl thiamine (see Table I). Theoretical considerations suggest that this compound is α-hydroxypropyl thiamine.

Additional evidence, obtained from an incubation mixture (described in Table II) which contained pyruvate-2-C14, indicated that the enzymatic product was formed by reaction of 1 mole of pyruvate with 1 mole of thiamine pyrophosphate. After incubation and acidification, the phosphorylated forms of thiamine and α-hydroxyethyl thiamine were separated from the other components of the reaction mixture by chromatography on Dowex 50 W. The phosphorylated compounds eluted from the Dowex column were treated with Taka-Diastase and chromatographed on paper seven successive times, with the use of three different solvent systems. The radioactivity and thiamine activity (determined by assay with L. viridescens) of each portion of each chromatogram were determined. This procedure resulted in the separation of α-hydroxyethyl thiamine from the last traces of thiamine and radioactive contaminants, so that in each of the last four chromatographic steps, a single band of radioactive material which coincided in RF value with α-hydroxyethyl thiamine was obtained. As shown in Table II, the isolated product contained about 380 c.p.m. per m mole. This value compares with the theoretical value of 340 if 1 mole of C14-pyruvate had been used to form 1 mole of α-hydroxyethyl thiamine.

These data show that α-hydroxalkyl derivatives of thiamine pyrophosphate occur naturally and can be formed enzymatically, but, as yet, no good evidence has been presented to demonstrate unequivocally that these compounds are identical with so-called "active aldehyde" compounds. Until such evidence is forthcoming, the possibility must be considered that "active aldehyde" compounds are compounds which might be closely related to, but not identical with, the pyrophosphate esters of α-hydroxyalkyl thiamine compounds.

* These investigations were supported by a grant from the National Science Foundation.
‡ The abbreviation used is: α-hydroxyethyl thiamine, DL-3-[[2-methyl-4-amino-5-pyrimidyl]methyl]-2-(1-hydroxyethyl)-4-methyl-5-(2-hydroxyethyl)-thiazolium chloride hydrochloride.
§ The authors wish to thank Dr. J. M. Sprague of Merck, Sharp and Dohme Research Laboratories for a gift of synthetic α-hydroxyethyl thiamine.

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Comparison of Rp values of thiamine and some thiamine derivatives

Solvent system I, n-butanol-ethylene glycol-0.1 N HCl (4:1:1, by volume); II, isopropyl alcohol-concentrated HCl-water (170:41:39); III, n-butanol-acetic acid-water (4:1:5, upper phase); IV, n-butanol-ethyl-H2O (4:1:1); V, pyridine-water (4:1); VI, isobutyric acid-concentrated ammonia-water (165:2:5:82.5).

The values are from paper chromatograms developed by the ascending technique.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Preparation chromatographed</th>
<th>Extraction of K. coli*</th>
<th>Carboxylase + pyruvate reaction mixture*</th>
<th>Carboxylase + α-ketobutyrate reaction mixture*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.60</td>
<td>0.74</td>
<td>0.60, 0.74</td>
<td>0.57, 0.72</td>
</tr>
<tr>
<td>II</td>
<td>0.41</td>
<td>0.52</td>
<td>0.40, 0.55</td>
<td>0.46, 0.59†</td>
</tr>
<tr>
<td>III</td>
<td>0.37</td>
<td>0.35</td>
<td>0.30, 0.38</td>
<td>0.36, 0.35†</td>
</tr>
<tr>
<td>IV</td>
<td>0.34</td>
<td>0.48</td>
<td>0.34, 0.47</td>
<td>0.29, 0.42†</td>
</tr>
<tr>
<td>V</td>
<td>0.76</td>
<td>0.94</td>
<td>0.75, 0.94</td>
<td>0.76, 0.94</td>
</tr>
<tr>
<td>VI</td>
<td>0.82</td>
<td>0.84</td>
<td>0.83</td>
<td>0.82</td>
</tr>
</tbody>
</table>

* Treated with Taka-Diastase before chromatography.
† Decomposition noted in this step may account for the high values shown for the preparation.

Incorporation of 2-C4-pyruvate into α-hydroxyethyl thiamine

The reaction mixture contained 2 μmoles of MgSO4, 84 μmoles of potassium succinate, pH 6.0, 1.2 μmoles of coenzyme A, 86 μmoles of 2-C4-potassium pyruvate (29.3 X 10⁵ c.p.m.), and 53 mg of wheat germ α-carboxylase alcohol powder in a total volume of 1.04 ml. After 80 minutes of incubation at 25°, 3 ml of 0.1 N HCl were added and the mixture was heated 2.5 minutes at 100°.

**REFERENCES**


### Dissociation of Hematin from Hemoproteins at Neutral pH

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The heme moiety in hemoproteins (hemoglobins and myoglobins) has until now been considered to be in water solution at neutral pH an immobile or fixed prosthetic group and no definite dissociation of the pigment has so far been recognized.

In this note we report the results of experiments which indicate that hematin does dissociate from myoglobins (Mb) and hemoglobins (Hb) in aqueous solution at neutral pH and room temperature, i.e., that, under such conditions, the following equilibrium exists:

Ferrihemoprotein ⇌ hematin + apohemoprotein

Present experiments will also show the different affinity of several apohemoproteins for protohemin and the different affinity of each apohemoprotein for hematin of different type.

The dissociation of hematin was shown by two different groups of experiments. The reactions observed were:

Ferrihemoprotein (x) + apohemoprotein (y) → apohemoprotein (x) + ferrihemoprotein (y)

Hematin (x) protein + hematin (y) → hematin (y) protein + hematin (x)

Reaction 1 was discovered in experiments with *Aplysi* (sea hare) Mb (1) and horse apoMb (2), taking advantage of the fact that the absorption spectrum of *Aplysi* FerriMb in the Soret region is very different from that of horse FerriMb (Fig. 1B). Fig. 1 shows that horse Mb is formed within few hours from *Aplysi* Mb and horse apoMb in 0.1 M phosphate buffer, pH 6.8. The presence of horse Mb in the solution was confirmed by paper electrophoresis, that of native *Aplysi* apoMb was shown by the formation of *Aplysi* Mb upon addition of hematin.

After this first observation, other "heme transfer" reactions of the same type could be shown in mixtures of different hemoproteins and globins. The exchange of the hematin from one hemoprotein to a different globin was demonstrated by spectrophotometry and paper electrophoresis of the solutions.

The following reactions were readily observed with equimolec-
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