The Natural Occurrence, Enzymatic Formation, and Biochemical Significance of a Hydroxyethyl Derivative of Thiamine Pyrophosphate*

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Thiamine pyrophosphate is required as a coenzyme for several enzymatic reactions which result in the decarboxylation of α-keto acids with the concomitant formation of corresponding aldehydes or their condensation products (α-keto alcohols). A number of investigators (1-3) have found that thiamine will catalyze the same reactions under mildly alkaline conditions in the absence of enzymes. This work has stimulated various workers to formulate mechanisms which might account for the action of thiamine in these systems. Theories have been proposed which suggest that the active portion of the thiamine molecule is the amino group on the pyrimidine ring (4), the sulfhydryl group resulting from the opening of the thiazole ring (5), and the methylene bridge carbon which connects the pyrimidine and thiazole portions of the compound (6). However, all of these theories have been discarded because none was supported by experimental evidence. In 1957, Breslow (7) discovered that deuterium ion rapidly exchanged with the hydrogen atom on position 2 of the thiazolium ring and, as a result of these theories have been discarded because none was supported by experimental evidence. In 1957, Breslow (7) discovered that deuterium ion rapidly exchanged with the hydrogen atom on position 2 of the thiazolium ring and, as a result of this finding, formulated (8) a mechanism for the action of thiamine in model systems which depends on the formation of a carbocation (due to the ionization of the hydrogen) at position 2 of the thiazole ring. This carbocation would then react with carbonyl carbonium ions to form hydroxyalkyl derivatives of thiamine, which upon decarboxylation would yield aldehyde adducts of thiamine. These adducts might be rendered stable by resonance and be capable of keto condensation reactions. In support of his formulation, Breslow prepared 2-(1-hydroxybenzyl)-3,4-dimethylthiazolium iodide and showed that it could be decomposed readily into benzaldehyde and 3,4-dimethylthiazolium iodide.

The hydroxyalkyl compound which would be formed in the manner described above by the decarboxylation of pyruvate would be 2-[2-(2-methyl 4-aminopyrimidyl)methyl] 2 (1-hydroxyethyl)-4-methyl-5-(2-hydroxyethyl) thiazole (shown in Fig. 1). In 1958, Kranepolz et al. (9) reported that this compound had been synthesized and was about 80% as active as thiamine in microbiological assays (i.e., it could be used effectively in place of thiamine as a growth factor) and, after enzymatic phosphorylation to give the pyrophosphate, could substitute in place of thiamine pyrophosphate as a coenzyme for yeast carboxylase. In a preliminary communication (10), we reported on the occurrence of this compound in bacteria and its enzymatic synthesis by wheat germ carboxylase. Holzer and Beauchamp (11) and other workers (12) have also briefly reported on its enzymatic formation with purified pyruvate decarboxylase from yeast.

The work to be presented in the present publication is an extension of that presented earlier (10) on the natural occurrence and enzymatic formation of hydroxyethyl thiamine pyrophosphate. Also evidence will be presented which indicates that hydroxyethyl thiamine pyrophosphate is either identical with or very closely related to "active acetaldehyde," i.e., the intermediate, formed by enzymatic decarboxylation of pyruvate which serves as a carboxyl donor in enzymatic reactions.

EXPERIMENTAL PROCEDURE

Materials—Thiamine and thiamine-P were purchased from Nutritional Biochemicals Company, thiamine-PP from Merck and Company, Inc., acetaldehyde, and DEAE-cellulose from Eastman Organic Chemicals, Dowex 50 from the Dow Chemical Company, and Taka-Diastase from Parke, Davis and Company. 4-Methyl-5-(2-hydroxyethyl)thiazole was kindly supplied by Dr. J. M. Sprague of Merck Sharp and Dohme Research Laboratories. Thiamine disulfide was prepared by the method of Zima et al. (13). Before use, acetaldehyde was converted to the crystalline dimer by the method of Kling (14) and then washed with cold ether. Acetaldehyde was redistilled, and solutions were prepared in cold deionized water. Inasmuch as pyruvic acid is known to undergo readily an acid- or base-catalyzed aldol condensation to yield 4-methyl-4-hydroxy-2-ketogluconate (15), pyruvate preparations used in the experiments to be described were examined for contamination with this dicarboxylic acid by the methods of Goldfine (15). Freshly prepared solutions of sodium pyruvate (Mann Research Laboratories, Lot 2001) and Research Quality sodium pyruvate-1

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The abbreviations used are: hydroxyethyl thiamine, 3-[2-(2-methyl 4-aminopyrimidyl)methyl]-2-(1-hydroxyethyl)-4-methyl 5-(2-hydroxyethyl) thiazole; thiazole, 4-methyl 5-(2-hydroxyethyl) thiazole.
The portion of pyruvate present in solutions as the dicarboxylic acid increased with increasing age of the solution; therefore, precautions were taken to use only freshly prepared solutions in experiments.

DEAE-cellulose was prepared for chromatography as described by Camiener and Brown (16). Dowex 50W-X4 ion exchange resin (50 to 100 mesh) was treated with N HCl, then with N NaOH at 60-80° and reconverted to the hydrogen form by washing with 5% KOH. Enzymatic activity was estimated by measuring acetoin production from pyruvate and acetaldehyde in the presence of added thiamine-PP (22). Two of the purified enzyme preparations contained substantial amounts of phosphatase activity which, in some of the experiments, interfered with quantitative measurements by degrading thiamine-PP and related pyrophosphate compounds to mixtures of the monophosphates and phosphate-free thiamine compounds. These difficulties were overcome by inhibiting the action of the phosphatase(s) by adding relatively large amounts of P1 and PP1 to the incubation mixtures.

Microbiological Assays—Thiamine was determined by microbiological assay with Lactobacillus viridescens (ATCC No. 12706) by the methods previously described (23). In this assay, phosphorylated forms of thiamine are somewhat less active than thiamine (23) and synthetic hydroxyethyl thiamine is 79% as active as thiamine (9) (also confirmed in this laboratory). For quantitative determinations phosphates of thiamine and hydroxyethyl thiamine phosphates were first dephosphorylated by treatment with Taka-Diastase and then assayed for either thiamine (thiamine used to construct the standard curve) or hydroxyethyl thiamine (synthetic hydroxyethyl thiamine used for the standard curve). For dephosphorylation, the samples were incubated with Taka-Diastase (2 mg per ml) in 0.1 M acetate buffer (sodium salt), pH 4.5, at 47° for 2.5 to 3.0 hours.

Thiamine compounds were detected on bioautograms seeded with L. viridescens by previously described methods (23). When the amount of an active thiamine compound spotted on a chromatogram exceeded about 8 μg, the corresponding growth zone on the bioautogram contained an inner growth zone which appeared relatively clear when compared with the densely opaque outer zone. Because the margin between the inner and outer zones was sharply defined, accurate estimations of the areas of the inner zones became possible. A relation which was observed between the inner area of the growth zone and the amount of thiamine compound originally spotted permitted accurate estimations of a thiamine compound present in a growth zone by comparing the area of inner zone with the areas derived from standard amounts of thiamine compound spotted on the chromatogram.

For bioautograms prepared with the use of Kleoccera brevis (ATCC No. 9774), the growth medium of Hoff-Jørgensen and Hansen (24) was used. Cells for inocula for the bioautographic plates were taken from a fresh malt extract-agar subculture, washed once in sterile water, reuspended in sterile water (absorbancy of 0.2), and before pouring the bioautographic plates, 1 ml of the suspension was used to inoculate 150 ml of medium containing 2% agar. Incubation of the plates was for 24 hours at 30°. Bioautograms seeded with Escherichia coli mutant strain 26-43 (obtained from Dr. Bernard D. Davis) were prepared as described by Camiener and Brown (16). This mutant will utilize either thiamine or thiazole for growth.

RESULTS

Thiamine Compounds Present in Microorganisms—A 250 ml culture of E. coli B was grown for 20 hours with shaking at 30° on the following medium: K2HPO4, 14 g; KH2PO4, 6 g; (NH4)2SO4, 2 g; NaCl, 2 g; MgSO4.7H2O, 0.2 g; Fe(NH4)2(SO4)2-6H2O, 20 mg; and 0.5% mannitol, 10 g. A 27 ml aliquot of the culture was made 1 N with respect to HCl and

The composition of the medium was originally suggested by Dr. E. Lansford (personal communication) with the exception that mannitol was substituted for glucose.

![Hydroxyethyl Thiamine](http://example.com/hydroxyethyl.png)
heated at 100° (water bath) for 30 minutes. After cooling, the hydrolysate was adjusted to pH 4.5 (with 2.5 M sodium acetate), treated with Taka-Diastase, desalted with the phenol procedure of Iacono and Johnson (25), and chromatographed on paper. Other aliquots of the culture to which either thiamine, oxythiamine, or thiamine disulfide (3 μg of each were added per 27 ml) had been added were treated in the same way and also chromatographed. The results shown in Fig. 2 (bioautogram prepared from the chromatogram) indicate that the Taka-Diastase-treated acid extract contained a compound (RF 0.72) which was active as thiamine for L. viridescens and was neither identical with nor derived from (by the extraction procedure) any of the known compounds which were also chromatographed. Other experiments have revealed that this unknown compound is also present in Taka-Diastase-treated acid extracts of several other microorganisms. Rough estimations from sizes of growth zones on bioautograms (made with L. viridescens) suggest that in E. coli, Salmonella typhimurium, bakers' yeast, and Azotobacter vinelandii, the unknown compound accounts, respectively, for about 60, 50, 25, and less than 5% of the total thiamine present in the cells.

Properties of Unidentified Compound—In contrast to its stability in acid, the unknown compound was rapidly destroyed in 7N NaOH at 100° and gradually destroyed when heated at 100° at pH 8.0. The destruction at pH 8.0 (a preparation purified free of thiamine by repeated chromatography on paper was used) was accompanied by the formation of small amounts of a compound with the chromatographic characteristics of thiamine. These results when considered together with the fact that the unknown compound replaces thiamine as a growth factor for L. viridescens suggested that the unknown compound is structurally closely related to thiamine and very probably is a more complex chemical form of the vitamin.

Although the results of Fig. 2 show that the unknown compound is not thiamine disulfide, the possibility remained that it was a mixed disulfide formed from the thiol form of thiamine and another thiol. This possibility was discounted after it was found that heating preparations containing the unknown compound with large amounts of either l-cysteine or 2-mercaptoethanol in solutions buffered at pH 5.9 did not cause any variation of the chromatographic behavior of the compound. At this pH, a disulfide would have become reduced and the resulting thiol form of thiamine would immediately have undergone ring closure to the thiazolium form to yield thiamine; e.g. thiamine disulfide, when treated in this way, was converted quantitatively to thiamine.

The activity of the unknown compound in replacing thiamine as a growth factor for microorganisms was tested by bioautography. In this way, it was shown that L. fermenti and K. brevis could utilize the unknown compound, but that E. coli mutant 26-43 could not. One explanation for the inactivity of the compound for the latter organism is that the unknown compound differs from thiamine in that it contains a modified thiazole moiety. To check this possibility, a thiamine-free preparation of the unknown compound was treated with sulfite as described by Williams et al. (26) and tested for the appearance of thiazole. Under these conditions, thiamine controls were cleaved to give a product that migrated on paper chromatograms identically with synthetic thiazole (located on chromatograms by bioautography with E. coli 26-43); however, the unknown compound yielded nothing that was active as thiazole for E. coli 26-43. Inasmuch as the treatment with sulfite destroyed the activity of the unknown compound as a growth factor for L. viridescens, it seemed likely that the compound was degraded by this treatment in a way similar to the manner in which thiamine is degraded; therefore, the tentative conclusion was drawn that the original compound differed from thiamine by containing a modified thiazole moiety.

Paper Chromatographic Identification of Unknown Compound as Hydroxethyl Thiamine—From the properties of the unknown compound, it seemed possible that it might be identical with hydroxethyl thiamine, the compound proposed by Breslow (8) as an intermediate in the decarboxylation of pyruvate. When synthetic hydroxethyl thiamine (9) became available, it was compared by paper chromatography with the unknown compound. The results summarized in Table I show that samples of the unknown compound prepared from E. coli migrated identically with hydroxethyl thiamine in five different solvent systems. Also, co-spotting both the unknown compound and synthetic hydroxethyl thiamine with 0.1 N HCl resulted in similar changes in the migration characteristics of the two materials in Solvent I so that the two again migrated identically, but with a lower Rf value (0.26) than that observed in the absence of acid. The observation that thiamine compounds migrate differently in the presence of acid was originally reported by Malnic, da Silva, and de Angelis (27) with thiamine.

The chromatographic evidence cited above, plus the additional finding that synthetic hydroxethyl thiamine, like the naturally occurring compound, is active for L. fermenti and K. brevis but will not replace thiamine as a growth factor for E. coli 26-43, indicated that the naturally occurring compound is identical with hydroxethyl thiamine.
Acid extracts of E. coli were prepared and treated with Taka-Diastase according to the directions given in the text. An amount of each material equivalent to about 50 mg of thiamine (measured by assay with L. viridescens) was spotted on Whatman No. 1 paper. After the chromatograms were developed, zones of migration were located by bioautography with L. viridescens as the test organism. Solvents used were the following (ratios are given in terms of volumes): Solvent I, n-butanol-ethylle Glycol 0.1 N HCl (4:1:1); Solvent II, isopropanol-6 N HCl (170:80); Solvent III, n-butanol-acetic acid-water (4:1:5, upper phase); Solvent IV, n-butanol-ethanol-water (4:1:1); Solvent V, pyridine-water (4:1).

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Preparation chromatographed</th>
<th>Thiamine</th>
<th>Hydroxyethyl thiamine</th>
<th>Extract of E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>R_f</td>
<td>0.60</td>
<td>0.74</td>
<td>0.60, 0.74</td>
</tr>
<tr>
<td>II</td>
<td>0.41</td>
<td>0.52</td>
<td>0.40, 0.52</td>
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</tr>
<tr>
<td>III</td>
<td>0.27</td>
<td>0.35</td>
<td>0.30, 0.38</td>
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</tr>
<tr>
<td>IV</td>
<td>0.34</td>
<td>0.48</td>
<td>0.34, 0.47</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>0.76</td>
<td>0.94</td>
<td>0.75, 0.94</td>
<td></td>
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</table>

Thiamine Compounds Formed Enzymatically by Carboxylase—
As the results presented above suggested that hydroxyethyl thiamine or phosphorylated forms of this compound occur in microorganisms, efforts were made to see whether or not hydroxyethyl thiamine-PP might be formed enzymatically. For this purpose, reaction mixtures containing purified wheat germ carboxylase, thiamine-PP, and either pyruvate or acetaldelyde were prepared (see Table II for details of preparation and incubation). After incubation, the thiamine compounds present in the reaction mixture were dephosphorylated and chromatographed on paper. The results presented in Table II show that a compound was formed enzymatically which, after dephosphorylation, was chromatographically indistinguishable from synthetic hydroxyethyl thiamine. The formation of this compound was dependent on the presence of substrate (either pyruvate or acetaldelyde), enzyme, and thiamine-PP. It was also found that neither thiamine nor thiamine-P could replace thiamine-PP and that acetoin could not be used in place of pyruvate or acetaldelyde. These facts indicated that hydroxyethyl thiamine PP was formed in the system and that this compound might be the product formed by the enzymatic decarboxylation of pyruvate.

It was estimated by comparison of the sizes of the growth zones on bioautograms with those given by standard amounts of synthetic hydroxyethyl thiamine that 10 to 15% of the added thiamine-PP was converted to the hydroxyethyl compound and, in rate studies, that under the conditions described in Table II, maximal yields from pyruvate resulted after 2 to 3 hours.

Additional evidence which indicated that the enzymatically formed compound (after dephosphorylation) was, in fact, hydroxyethyl thiamine was provided with the observation that 1 mole of pyruvate-2-C\(^{14}\) was utilized for the formation of 1 mole of new radioactive thiamine compound. For this purpose, a reaction mixture (1.04 ml in volume) was prepared to contain the following components (amounts given in \(\mu\)moles): MgSO\(_4\) 2; potassium succinate buffer (pH 6.0), 84; thiamine-PP, 1.2; potassium pyruvate-2-C\(^{14}\) (29.6 \times 10^6 c.p.m.), 88; and 53 \(\mu\)g of purified carboxylase. After incubation at 25\(^\circ\) for 50 minutes, 3 ml of 0.1 N HCl were added, the mixture was heated for 2.5 minutes at 100\(^\circ\), and the resulting protein precipitate was removed by centrifugation. The precipitate was washed with water, and the washings were combined with the original supernatant liquid. The combined solution (12 ml) was adjusted to pH 2.5 with NaOH and, in order to separate the thiamine compounds from pyruvate, acetaldelyde, and acetoin (acetaldelyde and acetoin were used as products of the action of carboxylase), the solution was passed through a 0.4-\(\times\) 26-cm column of Dowex 50W-H\(^+\) ion exchange resin at a rate of 80 ml per minute. After the column was washed with 60 ml of water (until the radioactivity was reduced to 450 c.p.m. per ml), the thiamine compounds were eluted with 0.2 M (with respect to NH\(_4^+\)) ammonium acetate buffer, pH 6.0. Fractions of 2 to 3 ml were collected and stored at 4\(^\circ\). The radioactivity in the effluent liquid first reached a minimum of 185 c.p.m. per ml and then rose to a maximum of 2.9 \times 10^6 c.p.m. per ml after approximately 15 ml had been collected. Fractions containing large amounts of radioactivity were combined to give a total of 0.2 \times 10^6 c.p.m. in 4.5 ml, and this radioactive solution was adjusted to pH 4.5 and treated with Taka-Diastase to dephosphorylate the thiamine compounds. After heating briefly at 100\(^\circ\) followed by centrifugation to remove protein, the solution was placed on a Whatman No. 3MM paper sheet as a band 1 cm wide and 20 cm long and chromatographed with Solvent II (see Table I for composition). Bioautograms prepared from strips 0.2 cm wide gave a zone of radioactivity at an R\(_f\) of 0.75 corresponding to synthetic hydroxyethyl thiamine.
wide cut lengthwise from the chromatograms showed the presence of thiamine compounds which migrated with RF values of 0.40 and 0.55 (Table III, Step 1). The upper zone (RF 0.55), which corresponded in RF value to hydroxyethyl thiamine (see Table I), was eluted from the remainder of the paper sheet with 40 ml of water and the solution was reduced in volume (in a vacuum) to 0.4 ml. This material, which was acidic from the HCl present in Solvent II, was then rechromatographed in Solvent I (Table III, Step 2), and these steps were repeated with different solvents as described in Table III until a thiamine compound behaving chromatographically as a single component (with an RF value of hydroxyethyl thiamine) was obtained. Each of the last four of these seven successive chromatographic steps described in Table III yielded a single radioactive component which in each case was identical in RF value with hydroxyethyl thiamine. The specific radioactivity of the thiamine compound was calculated to be about 380 c.p.m. per mg-mole, a value which compares favorably with the theoretical value of 340 c.p.m. per mg-mole which would have resulted from the reaction of 1 mole of pyruvate with 1 mole of thiamine-PP to yield 1 mole of hydroxyethyl thiamine-PP.

α-Ketobutyrate has been reported to serve as substrate for wheat germ carboxylase to yield propionaldehyde or the corresponding acyloin formed by condensation of 2 moles of propionic acid (21). When α-ketobutyrate was tested as a substrate for the formation of a new thiamine compound, it was found that Taka-Diastase-treated reaction mixtures contained a compound active as a growth factor for E. coli (28), grown with aeration at 37°. This acidic suspension was heated in 50 ml amounts for 10 minutes at 100° and then centrifuged at 4° to remove insoluble material. The acid extract was adjusted to pH 6.0 with glacial acetic acid. The rate of flow through the column was about 65%. An aliquot of this preparation was eluted after about 1200 ml of buffer had passed through the column. The yield of total thiamine activity recovered from the column was about 60% to 90%.

Purification of Pyrophosphates of Thiamine Compounds

From E. coli—The method which was developed for the purification of pyrophosphate esters of thiamine compounds from E. coli is as follows. E. coli cells from 64 liters of the medium of Davis (28) (grown with aeration at 37°) were dispersed in 2 liters of 0.1 N HCl. This acidic suspension was heated in 50 ml amounts for 10 minutes at 100° and then centrifuged at 4° to remove insoluble material. The acid extract was carefully adjusted to pH 2.5 with NaOH and then placed on a 2.2-× 40-cm column of Dowex 50-W+. After the column was washed with 300 ml of water, the mono- and pyrophosphate esters of thiamine compounds were eluted as a group from the resin with a buffer of 0.1 N NH₄OH adjusted to pH 6.0 with glacial acetic acid. The rate of flow through the column was maintained at 2 ml per minute, and operations were carried out at 4° to minimize hydrolysis of pyrophosphates. Thiamine compounds (detected by assay with L. viridescens) were eluted after about 1200 ml of buffer had passed through the column. The yield of total thiamine activity recovered from such columns varied from 60 to 90%.

The fractions (which contained thiamine activity) from two Dowex columns were combined, diluted so that the ionic strength was less than 0.1, and adjusted to pH 6.75 with NaOH. After the resulting solution (1300 ml) had been allowed to percolate through the column was washed with 300 ml of water, the mono- and pyrophosphate esters of thiamine-PP were eluted with 0.01 N HCl. Fractions which contained thiamine pyrophosphate compounds were combined and reduced in volume in a vacuum to 10 to 12 ml. The recovery of thiamine compounds from the DEAE-cellulose column was about 65%. An aliquot of this preparation was treated with Taka-Diastase, chromatographed (Solvent I, see Table I), and a bioautogram was prepared which showed that the purified preparation (after dephosphorylation) was a mixture (about 50% of each) of thiamine and hydroxyethyl thiamine. No further attempts were made to separate the thiamine-PP from the hydroxyethyl thiamine-PP (derived from E. coli) on a preparative scale although it seems likely that the ionophoretic method described in a following section could be utilized effectively for this purpose.

From Carboxylase Reaction Mixtures—A reaction mixture was prepared to contain per 0.94 ml (in moles): sodium succinate, 100; sodium pyruvate, 56.5; sodium phosphate, 22; sodium pyrophosphate, 10; MgSO₄, 2.4; thiamine-PP, 1; and 56 mg of purified wheat germ carboxylase. The F₄ and PP₄ salts were added to inhibit the action of phosphatase activity present as a contaminant in the carboxylase preparation. After incubation for 100 minutes at 25°, 2.8 ml of 0.1 N HCl were added, and the reaction mixture was heated for 1.5 minutes at 100°. The pH was then adjusted to 2.4, insoluble material was removed by

### Table III

<table>
<thead>
<tr>
<th>Solvents used in successive paper chromatographic purification steps</th>
<th>RF values</th>
<th>Specific activity of hydroxyethyl thiamine *&lt;sup&gt;c&lt;/sup&gt;</th>
<th>c.p.m./μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent I</td>
<td>0.35</td>
<td>Determined by radioactivity</td>
<td>250</td>
</tr>
<tr>
<td>Solvent I</td>
<td>0.50</td>
<td>Determined by radioactivity</td>
<td>300</td>
</tr>
<tr>
<td>Solvent I</td>
<td>0.55</td>
<td>Determined by radioactivity</td>
<td>372</td>
</tr>
<tr>
<td>Solvent I</td>
<td>0.60</td>
<td>Determined by radioactivity</td>
<td>438</td>
</tr>
<tr>
<td>Solvent I</td>
<td>0.74</td>
<td>Determined by radioactivity</td>
<td>500</td>
</tr>
<tr>
<td>Solvent II</td>
<td>0.46</td>
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<td>0.87</td>
</tr>
<tr>
<td>Solvent II</td>
<td>0.40</td>
<td>Determined by radioactivity</td>
<td>350</td>
</tr>
</tbody>
</table>

* Composition of solvent systems are given in Table I.

† Determined with compound of RF 0.74.

†• Decomposition with compound noted in this step (see RF values) may account for this high value.

1 Determined by bioautography with L. viridescens as the test organism.

* The amount of hydroxyethyl thiamine present after each step was determined by assay with L. viridescens against a standard of synthetic hydroxyethyl thiamine.

% Multiple growth zones were probably caused by an excess amount of acid in the preparation chromatographed. The zone of RF 0.74 was cut from the paper and used in further purification steps.

4 Determined with compound of RF 0.74.

1 Determined by ionophoresis with compound formed from a-ketobutyrate is hydroxypropyl thiamine when tested in Solvents I to V of Table I. The compound formed from a-ketobutyrate is hydroxypropyl thiamine. The specific radioactivity of the thiamine compound was calculated to be about 380 c.p.m. per mg-mole, a value which compares favorably with the theoretical value of 340 c.p.m. per mg-mole which would have resulted from the reaction of 1 mole of pyruvate with 1 mole of thiamine-PP to yield 1 mole of hydroxyethyl thiamine-PP.
centrifugation, and the supernatant solution was passed through a 0.4- x 18-cm column of Dowex 50W-H+; operations were carried out at 5º to avoid excessive decomposition of pyrophosphates of thiamine. The column was washed with 70 ml of water and the phosphorylated (mono- and pyrophosphates) forms of thiamine were eluted as a group by passing through the column a buffer consisting of 1% pyridine adjusted to pH 6.05 with glacial acetic acid. Phosphorylated thiamine compounds appeared as a sharp peak after about 18 ml of the effluent had been collected.

The material from the Dowex column was transferred by repeated applications to a 2- x 11.5-cm band of Whatman No. 3MM paper. Between applications, the paper band was partially dried by a jet of air. The remainder of the original paper strip from which the band had been cut was soaked in buffer (10% pyridine adjusted to pH 6.4 with glacial acetic acid) and blotted to remove excess liquid. After all of the sample had been applied to the band, it was placed (while still moist) in its original position from which it had been cut and connecting bridges consisting of paper strips 0.5 cm wide moistened with the pyridine-acetate buffer were positioned to connect the band with the rest of the long paper strip. The preparation of the paper described above was carried out on a 30- x 40-cm polyethylene sheet (1/2 inch thick) placed on a special chromium-plated brass table equipped with a cooling coil on its under surface. A second polyethylene sheet was then used to cover the paper strip so that the paper was contained between two such sheets which served as insulating agents during ionophoresis. The top sheet was finally covered with a sponge rubber mat on which was placed a heavy glass plate. The ends of the 117-cm strip which protruded from either end of the two polyethylene sheets were doubled over on themselves and placed in dishes of the pyridine-acetate buffer described above. Two kilovolts were applied to electrodes placed in the buffer, and the current was regulated not to exceed 40 ma by intermittently adjusting the voltage during the 3½ hour ionophoretic run. During ionophoresis, tap water at 5-20º was passed through the coil under the paper as a cooling agent. After ionophoresis, zones of migration of thiamine compounds were determined by cutting a 0.2-cm strip lengthwise from the paper and using this narrow strip to prepare a bioautogram with L. viridescens. In this way, it was found that the paper contained three bands of thiamine compounds (see Fig. 3), one which moved only a short distance from the origin toward the anode (Zone 1) and which behaved in this way as thiamine-P, and two others which moved faster (Zones 2 and 3) and were incompletely separated from one another. It was considered likely that the faster moving compounds were thiamine-PP and hydroxyethyl thiamine-TP. The bands corresponding to zones of migration of each of these components were cut individually from the remainder of the ionogram and eluted from the paper with water. An aliquot of each was then treated with Taka-Diastase and chromatographed on paper (Solvent I, see Table I). Bioautograms prepared from these chromatograms showed that Zone 3 (shown in Fig. 3), which migrated at the fastest rate, yielded thiamine after dephosphorylation and that Zone 2 yielded largely hydroxyethyl thiamine, but still contained small amounts of thiamine.

In order to separate the hydroxyethyl thiamine-PP from the last traces of thiamine-PP, the material obtained from the ionophoretic step (Zone 2) was subjected to a second ionophoresis. For this purpose, the solution was first concentrated under reduced pressure to a volume of 0.2 ml and then placed as a narrow transverse band on a 12.7- x 117-cm Whatman No. 3MM paper strip. The pH 6.4 pyridine-acetate buffer described above was applied to the paper (by pipette) on both sides of the band and was allowed to absorb into the sample area evenly from both sides. Excess liquid was blotted from the other areas of the paper and ionophoresis was carried out as described above except that the voltage was applied for only 3 hours. The convenient method of sample application described in the second ionophoretic step resulted in good resolution of components which had already been through one ionophoretic purification step; however, this method of sample application could not be used successfully when applying material taken directly from the Dowex column, but instead, the more elaborate procedure described in the first ionophoretic step had to be followed to insure optimal results. It seems likely that the material from the Dowex column contained high enough concentrations of salts to interfere with ionophoresis if the salts are concentrated too heavily in one place. The procedure described in the first ionophoretic step would tend to result in less localization of salt in a narrow band than would the procedure used in the second step.

When zones of migration of thiamine compounds on the second ionogram were determined by bioautography as described for the first ionogram, it was found that a small amount of material migrating as thiamine-PP was present, but that the material consisted largely of a slower moving compound thought to be hydroxyethyl thiamine-PP. The material on the remaining portion of the ionogram was eluted with water from the zone of migration of the hydroxyethyl thiamine-PP and stored at -20º until use. The amount of material obtained in this way was about 30 nanomoles of thiamine compound.

**Chemical Nature of Purified Materials**—An aliquot of the material purified from carboxylase reaction mixtures was treated with Taka-Diastase and after chromatography on paper (Solvent I, Table I), a bioautogram was prepared with L. viridescens. The results showed that the dephosphorylated material consisted entirely of hydroxyethyl thiamine.

A second aliquot of the purified material was chromatographed on paper (without prior dephosphorylation) with the use

![Fig. 3. A bioautogram prepared from an ionogram, showing the ionophoretic behavior of thiamine compounds present in reaction mixtures in which pyruvate and thiamine-PP were incubated with carboxylase. See the text for a description of the material used and for details of ionophoresis.](http://www.jbc.org/content/236/7/2104/F3.large.jpg)
as solvent of isobutyric acid-concentrated NH₄OH-water (165:2.5:82.5, volume per volume), which is a solvent that separates thiamine-P from thiamine-PP. The resulting bio-autogram prepared from the chromatogram revealed that the purified material migrated as did thiamine-PP (presumably, this solvent does not distinguish between thiamine-PP and hydroxyethyl thiamine-PP). However, heating the purified material with N HCl at 100° for 15 minutes, but not with 0.1 N HCl, resulted in the conversion of the material to a product which migrated to the same zone as thiamine-P in the solvent mentioned above. Under these same conditions, authentic thiamine-PP was also stable to 0.1 N HCl but yielded thiamine-P when heated with N HCl. When all of these results were considered together, it was concluded that the purified material obtained from the second ionophoretic step consisted of hydroxyethyl thiamine-PP and that this material was free from other thiamine compounds.

Analytical determinations similar to those described above were also performed on the material purified from E. coli as described in an earlier section. This material, which had not been through any ionophoretic purification steps, was shown by ionophoresis to consist of two major components, which corresponded in migration characteristics to thiamine-PP and hydroxyethyl thiamine-PP, and a minor component (active as thiamine for L. viridescens) which moved faster than thiamine-PP on ionophoresis. The chemical nature of this minor component was not determined.

The compound formed by carboxylase from thiamine-PP and α-ketobutyrate, which is considered to be hydroxypropyl thiamine-PP, was purified by the same methods which were used for purification of enzymatically formed hydroxyethyl thiamine-PP. The hydroxypropyl compound also migrated on ionophoresis somewhat more slowly than did thiamine-PP. The purified material was analyzed by the methods described for the analysis of hydroxyethyl thiamine-PP and found to be free from thiamine-PP. Enough of the monophosphate ester (made by heating the pyrophosphate ester in N HCl) of this material was prepared so that ultraviolet absorption spectra could be obtained. These spectra are compared with those of thiamine and synthetic hydroxyethyl thiamine in Fig. 4. The shifts in absorption of the hydroxypropyl thiamine-P that accompany changes in pH (Fig. 4) are typical of a compound containing the pyrimidine and thiazole ring structures found in thiamine.

Behavior of Hydroxyalkyl Thiamine Pyrophosphates in Enzyme Systems

The abilities of purified hydroxyalkyl thiamine-PP compounds to act coenzymatically in place of thiamine-PP to activate aperoxycarboxylase were determined. A comparison of the effectiveness of hydroxypropyl thiamine-PP and thiamine-PP as coenzymes for acetoin production by carboxylase is given in Fig. 5. From these data it can be calculated that hydroxypropyl thiamine-PP was about 80% as effective as thiamine-PP. Neither thiamine nor thiamine-P showed any activity in this system. Experiments similar to the one described in Fig. 5 have also been performed with hydroxyethyl thiamine-PP, and this compound was also observed to be active as a coenzyme in place of thiamine-PP.

If hydroxyalkyl thiamine-PP compounds serve as intermediates in enzymatic decarboxylations of α-keto acids, then it might be possible to show that in the absence of other substrates the hydroxyalkyl thiamine-PP is converted by aperoxycarboxylase to thiamine-PP as one product. In an experiment designed to check this possibility, reactions were prepared (see Fig. 6) which contained only buffer, purified hydroxyalkyl thiamine-PP compounds, and carboxylase. After incubation, the reaction mix-
RF 2106 spotted on Whatman No. 1 paper and the chromatogram was de-
migration were located by bioautography with at 100° for 15 minutes to hydrolyze pyrophosphate esters to the
30 ml of 2 N HCl were added to each vessel and each was then heated
after incubation,
by the presence of substantial amounts of thiamine-
P. The interpretation of these results was complicated
data summarized in Fig. 6 indicate that hydroxypropyl thia-
molecules (in pmoles) : sodium succinate (pH 6.0), 3.0; sodium phosphate (pH 6.0), 0.3; MgSO4, 0.125; either hydroxy-
ethyl thiamine-PP (0.41 mpmole) or hydroxypropyl thiamine-PP (0.52 mpmole); and 237 μg of purified carboxylase (alcohol pow-
der). Incubation was for 23 hours at 30°. After incubation,
controls were prepared by heating the pyrophosphate esters in HCl as described
above. The control hydroxyalkyl thiamine-P compounds which were chromatographed were prepared by heating the pyrophosphate esters in HCl as described above.

Enzymatic Utilization of Hydroxyethyl Group for Formation of Acetoin—The foregoing results have suggested that hydroxy-
thiamine-PP is an intermediate in the enzymatic reaction by which pyruvate is decarboxylated and converted to acetoin. Support for this suggestion was sought with the use of a radio-
active compound. C14-Hydroxyethyl thiamine-PP was formed by incubation of 86.5 μmoles of sodium pyruvate-2-C14 (specific
activity, 355 c.p.m. per mpmole) with 1 μmole of thiamine-PP in the presence of 56 mg of the purified wheat germ carboxylase, as described in a previous section. The resulting radioactive hydroxyethyl thiamine-PP was purified by the methods de-
scribed earlier to yield finally 30 mpmoles of hydroxyethyl thiamine-PP with a specific activity of 462 c.p.m. per mpmole. The preparation was found to be free from thiamine-PP and radioactive pyruvate. This was determined by mixing an aliquot along with carrier pyruvate with 2,4-dinitrophenylhydra-
zine and isolating and determining the radioactivity of the re-
sulting 2,4-dinitrophenylhydrazone.

The C14-hydroxyethyl thiamine-PP was used in enzymatic experiments designed to show whether or not the radioactive hydroxyethyl group was used in the formation of acetoin. Reaction mixtures were prepared in duplicate as described in Table IV. The general plan of this experiment was to incubate C14-hydroxyethyl thiamine-PP with carboxylase and nonradio-
active acetaldehyde and pyruvate as substrates for acetoin formation. To one set of reaction mixtures thiamine-PP was also added at 0 time (Table IV, reaction mixtures 3 and 4); to a second set thiamine-PP was added after a 90-minute incubation period (Table IV, reaction mixtures 5 and 6). All reaction mixtures were incubated for a total of 240 minutes and the conditions were chosen so that in those vessels which contained active enzyme the major portion of the pyruvate and acetalde-
hyde were converted to acetoin. In those vessels to which thiamine-PP was added (at approximately 250 times the molar excess of the amount of hydroxyethyl thiamine-PP which was

**TABLE IV**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Time of thiamine-PP addition</th>
<th>Specific activity of nickel dimethylglyoximea</th>
<th>Acetoin formed</th>
<th>Specific activity of acetoin formed</th>
<th>Total C14 added which was incorporated into acetoin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>min</td>
<td>0 c.p.m./μg</td>
<td>0 mpmole</td>
<td>0 c.p.m./μmole</td>
<td>0 %</td>
</tr>
<tr>
<td>2</td>
<td>240</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>240</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>5.13</td>
<td>31.8</td>
<td>8.75</td>
<td>16.7</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>18.5</td>
<td>27.1</td>
<td>36.6</td>
<td>59.7</td>
</tr>
<tr>
<td>6</td>
<td>90</td>
<td>17.0</td>
<td>27.2</td>
<td>34.6</td>
<td>57.8</td>
</tr>
</tbody>
</table>

a Corrected for self-absorption.

b Contained boiled enzyme; all others contained active enzyme.
added) at 0 time, any radioactive acetoin which was produced could have resulted from (a) C4-hydroxyethyl thiamine-PP which would be competing with thiamine PP for the active region of the enzyme, or (b) radioactive contaminants of an undetermined nature which might have been present in the preparation of hydroxyethyl thiamine-PP. Thus the C4-acetoin produced in these reaction mixtures functioned to set a maximal limit to the amount of C4-acetoin which could have been formed from radioactive contaminants. In reaction mixtures 5 and 6 (Table IV), the thiamine PP was withheld for the first 90 minutes of incubation to give the C4-hydroxyethyl thiamine-PP a chance to react in the system to form C4-acetoin; then thiamine-PP was added in excess in order to promote maximal conversion of the pyruvate and acetalddehyde substrates to acetoin during the remaining 150 minutes of incubation. After incubation and after an aliquot had been removed from each reaction mixture to determine the amount of acetoin which had been produced, carrier acetoin was added to each reaction mixture and the acetoin was oxidized to diacetyl which in turn was converted to the nickel dimethylglyoxime derivative. Table IV gives data on the radioactivity of the dimethylglyoxime compound formed in the different reaction mixtures. Clearly, more radioactivity was incorporated into acetoin in the reaction mixtures (Table IV, 5 and 6) from which thiamine-PP was withheld for the first 90 minutes than was incorporated in the reaction mixtures (Table IV, 3 and 4) which contained thiamine-PP throughout the total incubation period. Reaction mixtures prepared with boiled enzyme resulted in no incorporation, a result which indicates that the incorporation observed in other reaction mixtures resulted from an enzymatic reaction and also that the radioactive hydroxyethyl thiamine-PP used in the experiment was free from radioactive acetoin. The data presented in Table IV therefore conclusively show that the hydroxyethyl group of hydroxyethyl thiamine-PP was used for the enzymatic synthesis of acetoin.

**DISCUSSION**

The results of the present communication show that hydroxyethyl thiamine-PP is a naturally occurring material and provide strong evidence in support of the hypothesis that this compound is identical with “active acetalddehyde.” This evidence is (a) that hydroxyethyl thiamine-PP can be formed enzymatically from pyruvate and (b) that it can be utilized as an acetaldheyde donor for the enzymatic formation of acetoin. Other workers have obtained evidence which also supports this view. Holzer and Beaucamp (11) and others (12) have reported that hydroxyethyl thiamine-PP is formed when a-ketobutyrate was substituted for pyruvate.

Theoretical considerations suggest that this compound is hydroxyethyl thiamine pyrophosphate. A compound chromatographically different from hydroxyethyl thiamine-PP was formed when α-ketobutyrate was substituted for pyruvate. Theoretical considerations suggest that this compound is hydroxypropyl thiamine pyrophosphate.

**SUMMARY**

1. Hydroxyethyl thiamine pyrophosphate has been identified by paper chromatographic and bioautographic methods as a compound that occurs naturally in microorganisms and also as a product formed from thiamine pyrophosphate and either pyruvate or acetalddehyde in the presence of relatively large amounts of purified wheat germ carboxylase. A compound chromatographically different from hydroxyethyl thiamine-PP was formed when α-ketobutyrate was substituted for pyruvate.

2. Procedures were devised for the purification of hydroxyethyl thiamine pyrophosphate from microorganisms or from carboxylase reaction mixtures. The purification steps include chromatography on Dowex 50 and on N,N-diethylaminoethy cellulose and paper ionophoresis.

3. Experimental data which were presented which are pertinent to a consideration of the biochemical importance of hydroxyethyl thiamine pyrophosphate include: (a) the compound can be used in place of thiamine pyrophosphate as a coenzyme for wheat germ carboxylase; (b) incubation of the compound with wheat germ carboxylase in the absence of other substrates yields, as one product, thiamine pyrophosphate; and (c) C4-labeled 1-hydroxyethyl group of hydroxyethyl thiamine pyrophos-
phate (prepared enzymatically from pyruvate-2-C\textsuperscript{14}O) was shown to be utilized enzymatically by wheat germ carboxylase to form radioactive acetoin. These results are discussed with reference to whether or not hydroxyethyl thiamine pyrophosphate is identical with "active acetaldehyde."

Addendum—After this paper was submitted for publication, a paper appeared by Holaer and Beaucamp (38) in which evidence was presented that hydroxyethyl thiamine-PP serves as an acetaldehyde donor for the formation of acetoin by yeast carboxylase.

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The Natural Occurrence, Enzymatic Formation, and Biochemical Significance of a Hydroxyethyl Derivative of Thiamine Pyrophosphate

Gerald L. Carlson and Gene M. Brown


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