PREPARATION OF "ACTIVE GLYCOLIC ALDEHYDE" (2-(1,2-DIHYDROXYETHYL)-THIAMINE PYROPHOSPHATE) FROM HYDROXYPYRUVATE AND THIAMINE PYROPHOSPHATE WITH A PREPARATION OF PYRUVATE OXIDASE FROM PIG HEART MUSCLE[+)]

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In our first experiments to isolate "active acetaldehyde" (HETPP[+++)]) from enzymatic systems, we chromatographed short-time incubation mixtures of pyruvate decarboxylase from yeast and ^{14}C-pyruvate (Holzer and Beaucamp 1959; 1961). The yield of ^{14}C-HETPP with this method was small. Later we found that longtime incubation of ^{14}C-pyruvate with TPP and pyruvate oxidase from yeast mitochondria (Holzer et al. 1960) or pig heart muscle (Holzer et al. 1962a; Scriba and Holzer 1961) yielded large amounts of ^{14}C-HETPP which is formed according to the following equation:

(1) pyruvate + TPP ⟶ HETPP + CO_2.

Recently we could isolate ^{14}C-labelled "active glycolic aldehyde" from incubation mixtures of transketolase with ^{14}C-fructose-6-phosphate (Holzer 1961; Holzer et al. 1962b). The yield is small; it amounts to approx. 1 % with respect to the incubated fructose-6-phosphate. In this paper we show that by analogy with "active acetaldehyde" (cf. equation (1)) a good yield of "active glycolic aldehyde" can be obtained from hydroxypyruvate and TPP with

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+++) Abbreviations: DCP = 2,6-dichlorphenolindophenol; DETP = 2-(1,2-dihydroxyethyl)-thiamine monophosphate; DETPP = 2-(1,2-dihydroxyethyl)-thiamine pyrophosphate; HETPP = 2-(1-hydroxyethyl)-thiamine pyrophosphate; TP = thiamine monophosphate; TPP = thiamine pyrophosphate.

a preparation of pyruvate oxidase from pig heart muscle[+]. In the subsequent paper (Bock et al. 1962) we show that this "active glycolic aldehyde" is TPP whose position 2 of the thiazol ring is substituted by a 1,2-dihydroxyethyl group. Therefore the designation 2-(1,2-dihydroxyethyl)-TPP is used. The formation of this substance is formulated as follows:

(2) hydroxypyruvate + TPP ⟶ DETPP + CO_2.

First evidence for the accumulation of DETPP during incubation of hydroxypyruvate with pyruvate oxidase was obtained from the manometric experiments presented in fig. 1.

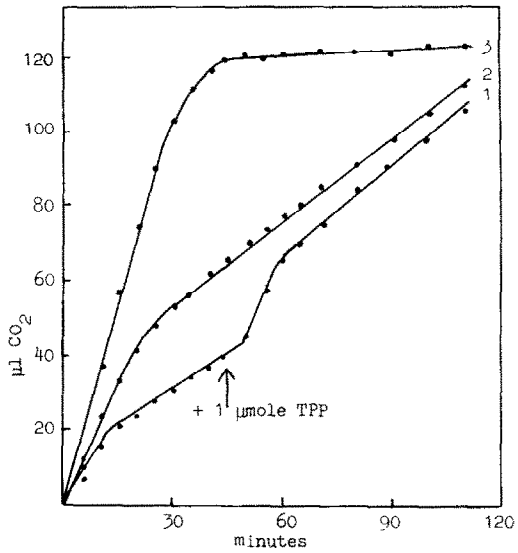

Figure 1: Decarboxylation of hydroxypyruvate with TPP and a preparation of pyruvate oxidase from pig heart muscle.
The Warburg vessels contained in 3.3 ml: 330 µmoles Sörensen-phosphate-buffer pH 5.9; 2 µmoles $MgSO_4$ and 28 mg of a preparation of pyruvate oxidase in a solution containing $(NH_4)_2SO_4$ (Scriba and Holzer 1961). 5.5 µmoles of lithium hydroxypyruvate (Dickens and Williamson 1958) were added at zero time. Vessels 1, 2 and 3 contained 1, 2 and 10 µmoles TPP respectively. 1 additional µmole of TPP from the second bulb was added to vessel 1 after 45 min. Temperature: 37°C; gas phase: H_2.

TPP, added in low concentration to incubation mixtures of hydroxypyruvate with pyruvate oxidase, causes a rapid CO_2 formation which is stoichiometrical to the added TPP. By analogy with the formation of HETPP from pyruvate, it could be assumed that the added TPP is converted to a great extent to DETPP according to equation (2). The following slow phase of CO_2 formation is limited

[+] We have evidence that pyruvate oxidase and hydroxypyruvate oxidase are two different enzymes (Holzer and Heesen 1962).

by the rate of the liberation of TPP from DETPP. If excess TPP is added, only the first rapid phase of CO_2 formation takes place and lasts until all hydroxypyruvate is decarboxylated.

We have been able to isolate from such incubation mixtures about 2/3 of the added TPP as DETPP. DETPP can be satisfactorily separated from TPP by ion exchange chromatography (cf. fig. 2). The ^{14}C-DETPP, not contaminated with TPP (tubes no. 412-460), exhibits the same specific radioactivity as the used

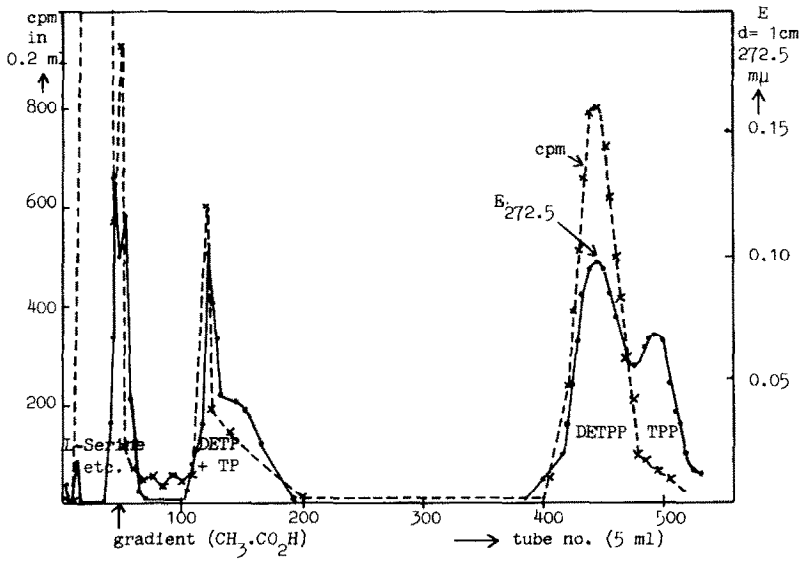

Figure 2: Ion exchange chromatography of an incubation of 3-^{14}C-hydroxypyruvate with TPP and a preparation of pyruvate oxidase from pig heart muscle. 3-^{14}C-hydroxypyruvate was obtained (cf. Batt et al. 1960) by incubating about 80 µmoles of 3-^{14}C-D,L-serine (32 · 10^6 cpm), D-aminoacid oxidase (Massey et al. 1961) and catalase at 37°C in a Warburg vessel with O_2 as gas phase. After termination of the O_2 uptake the resulting solution, containing 23 µmoles 3-^{14}C-hydroxypyruvate with 9 · 10^6 cpm was acidified with HCl to pH 5.9, reduced to 0.3 ml in a vacuum evaporator and incubated with 270 µmoles Sörensen-phosphate-buffer pH 5.9; 45 mg of a preparation of pyruvate oxidase in a solution containing $(NH_4)_2SO_4$ (Scriba and Holzer 1961); 4 µmoles $MgSO_4$ and 24 µmoles TPP in a total volume of 3.3 ml (cf. legend to fig. 1). After termination of the CO_2 formation it was deproteinized with the ninefold volume of 60°C methanol, centrifuged and the supernatant vacuum concentrated. It was extensively freed of salts by two extractions, each 4 ml of 75 % methanol in water. The extract was concentrated in a vacuum evaporator to a small volume and dissolved in water. 4/7 of the incubation mixture were separated by ion exchange chromatography on Dowex-2-acetate (X = 8; 200-400 mesh; 25 cm · 3.^{14} cm^2) at room temperature. After washing with 240 ml of H_2O, it was eluted by gradient elution (0.0167 M acetic acid flowing into a mixing chamber with 500 ml H_2O). 5 ml fractions were collected and the ultraviolet absorption at 272.5 mµ (water as blank) was measured. To determine the radioactivity, 0.2 ml samples were counted with the methane flow counter FH 407. 0.6 · 10^6 cpm ^{14}C-DETPP were found in the TPP-free fractions 412-460. This corresponds to a 6 % yield with respect to the used 3-^{14}C-hydroxypyruvate.

3-^{14}C-hydroxypyruvate, if the molarity of the preparation is determined by the thiochrome test or by the ultraviolet absorption at 272.5 mμ (Goedde et al. 1962a). The molar ratio of glycolic aldehyde to TPP is therefore 1 : 1. When submitted to paper chromatography and electrophoresis, DETPP behaves like "active glycolic aldehyde" previously isolated from transketolase incubations (Holzer et al. 1962b). ^{14}C-DETPP obtained from ^{14}C-hydroxypyruvate yields ^{14}C-erythrulose just like the preparation obtained from transketolase incubations, when incubated with free glycolic aldehyde and transketolase (Kattermann et al. 1962). When incubated with ribose-5-phosphate and transketolase, it yields ^{14}C-sedoheptulose-7-phosphate (Prochoroff et al. 1962). Therefore it is very probable that the DETPP described in this paper is identical with the one obtained from transketolase incubations, as previously described. Krampitz et al. (1961a; 1961b) have reported on an "active glycolic aldehyde" synthesized from formaldehyde and TPP. Whether this substance is identical with the "active glycolic aldehyde" described here and in the following two papers can be determined only after the experimental details about the work of Krampitz et al. are available.

For further characterization, ^{14}C-DETPP obtained from 3-^{14}C-hydroxypyruvate was oxidized with pyruvate oxidase and 2,6-dichlorphenolindophenol. Fig. 3 shows that a radioactive compound is formed, which has the same Rf-value as authentic glycolic acid. The yield of ^{14}C-glycolic acid was about equimolar to

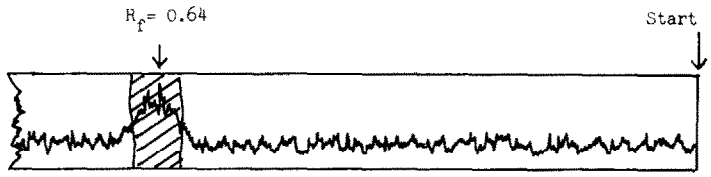

Figure 3: Detection of glycolic acid in an incubation mixture of ^{14}C-DETPP with a preparation of pyruvate oxidase and DCP by paper chromatography. 200 μmoles Sörensen-phosphate-buffer pH 6.2; 1.72 μmoles ^{14}C-DETPP ($0.344 \cdot 10^6$ cpm); 0.05 mg DCP and 2.8 mg of a preparation of pyruvate oxidase (Scriba and Holzer 1961) were incubated in a cuvette in a total volume of 2 ml. The decrease of the extinction was observed at 578 mμ against a control cuvette without DETPP. After 2 hours it was deproteinized with the ninefold volume of 60°C methanol, concentrated to dryness, dissolved in 5 ml 1 N H_2SO_4 and perforated with ether for 4 hours. After evaporating the ether it was submitted together with authentic glycolic acid to descending paper chromatography for 17 hours on Schleicher-and-Schüll-paper 2043b Mgl in the solvent system butanol/glacial acetic acid/water = 5 : 2 : 3. Glycolic acid (cross-lined area) was located with bromphenol blue / bromcresol green according to Paskova and Munk (1960). The decrease of the extinction of DCP at 578 mμ corresponds to 1.6 % of the incubated ^{14}C-DETPP; the radioactivity of the ^{14}C-glycolic acid formed, corresponds to 1.2 % of the incubated ^{14}C-DETPP.

the reduced dichlorphenolindophenol. It is very probable that glycolic acid is formed according to the reaction sequence:

$$(3)\quad \text{DETPP} \xrightarrow{(-\,2H)} \text{glycolyl TPP} \xrightarrow{(+\,H_2O)} \text{glycolic acid} + \text{TPP}.$$

In a completely analogical reaction sequence HETPP is oxidized to acetic acid with dichlorphenolindophenol and pyruvate oxidase (Goedde et al. 1962b).

SUMMARY

^{14}C-labelled "active glycolic aldehyde" prepared by incubating 3-^{14}C-hydroxypyruvate with thiamine pyrophosphate and a preparation of pyruvate oxidase from pig heart muscle was isolated by ion exchange chromatography. The substance contains thiamine and ^{14}C in the ratio of 1 : 1. It yields glycolic acid by enzymatic oxidation with 2,6-dichlorphenolindophenol. By analogy with "active acetaldehyde" it is proposed to be 2-(1,2-dihydroxyethyl)-thiamine pyrophosphate.

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