PURIFICATION OF SYNTHETIC ^{14}C-D,L-α-HYDROXYETHYL-2-THIAMINE PYROPHOSPHATE

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"Active acetaldehyde", which is supposed to be an intermediate in the decarboxylation and oxidation of pyruvate, was suggested by Breslow (1957; 1958), on the basis of model experiments, to have the structure of thiamine pyrophosphate substituted in the 2-position of the thiazole ring by an α-hydroxyethyl group. Experiments by Krampitz et al. (1958) showed that HETPP+) was probably an intermediate in the decarboxylation of pyruvate in yeast. Holzer et al. isolated HETPP as an intermediate in the decarboxylation of pyruvate with pyruvic decarboxylase from brewer's yeast (Holzer and Beaucamp 1959; 1961) and in the oxidation of pyruvate with pyruvic oxidase from baker's yeast mitochondria (Holzer et al. 1960) and pig heart muscle (Scriba and Holzer 1961). In a preliminary communication Carlson and Brown (1960) reported on the identification of HETPP as an intermediate in the decarboxylation of pyruvate with pyruvic decarboxylase from wheat germ. HETPP could be converted to free acetaldehyde or acetoin by pyruvic decarboxylase from yeast (Holzer and Beaucamp 1959; 1961). Acetolactate is formed in addition to acetoin with pyruvic oxidase from pig heart muscle (Holzer and Kohlhaw 1961). With pyruvic oxidase from yeast mitochondria the α-hydroxyethyl group of HETPP could be transferred to coenzym A to yield acetyl-coenzym A (Goedde, Inouye and Holzer 1961). In all these enzymatic reactions HETPP

+) Abbreviations: HETPP = D,L-α-hydroxyethyl-2-thiamine pyrophosphate; HET = D,L-α-hydroxyethyl-2-thiamine; TPP = thiamine pyrophosphate; T = thiamine; DPNH = reduced diphosphopyridine nucleotide; AMC = acetyl-methyl-carbinol = acetoin.

is converted only very slowly. Studying the liberation of acetaldehyde from HETPP with pyruvic decarboxylase, Scriba et al. (1961) showed this to be caused by TPP competitively inhibiting the conversion of HETPP. To eliminate this interference it was necessary to prepare HETPP free of TPP. In the present paper we describe the preparation of highly purified HETPP.

For the preparation of HETPP, 1,2-^{14}C-labelled acetaldehyde was incubated with TPP at pH 8.7 according to Sprague (1960). After ether extraction of excess acetaldehyde the reaction mixture was fractioned by ion exchange chromatography with Dowex 2-formate.

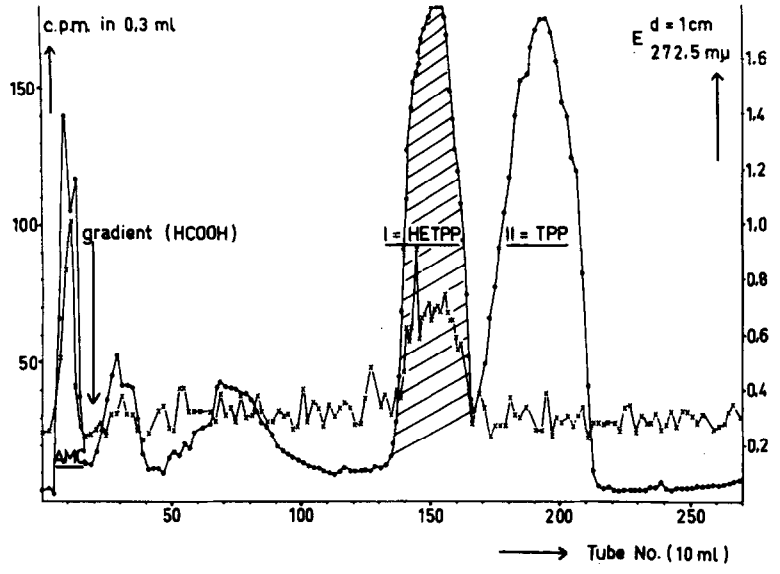

Figure 1: Purification of synthetic ^{14}C-D,L-HETPP by ion exchange chromatography.
For synthesis of ^{14}C-D,L-HETPP and details of the chromatographic separation see EXPERIMENTAL.

From the figure it can be seen that two completely separated UV peaks are found, both of them giving a positive thiochrome test (Bessey et.al. 1952). Radioactivity was only found in peak I, which proved to be ^{14}C-HETPP, whereas peak II contained the separated TPP. The HETPP thus obtained showed, when submitted to paper chromatography, the same R_f-value as did former enzymatic preparations (Holzer and Beaucamp 1961). When incubated with pyruvic decarboxylase from yeast, acetaldehyde was liberated and acetoin was formed (Goedde, Blume and Holzer 1961). The enzymatic liberation of acetaldehyde by pyruvic decarboxylase may be used to assay HETPP spectrophotometrically according to Warburg (1948), when the reaction is performed with alcohol dehydrogenase and DPNH. TABLE I shows that

41-43 per cent of the HETPP used (determined by the thiochrome test) are recovered as acetaldehyde. Since neither a prolongation of incubation nor an increase in the concentration of pyruvic decarboxylase raises the yield in acetaldehyde over 50 per cent (Goedde 1961), we conclude from this experiment that only one antipode produced in the chemical synthesis reacts enzymatically. At present, we are employed in separating the stereoisomers.

TABLE I DETERMINATION OF HETPP IN THE SPECTROPHOTOMETRIC ASSAY ACCORDING TO WARBURG (1948).

For preparation of the HETPP samples as well as incubation to liberate and determine acetaldehyde see EXPERIMENTAL.

	Synthetic D,L-HETPP				Pyruvate
	Preparation I		Preparation II		
µmoles of HETPP and pyruvate, resp.	0,150	0,300	0,150	0,300	0,240
µmoles Acetaldehyde	0,065	0,122	0,054	0,115	0,202
Acetaldehyde as a percentage of the initial amount of HETPP used	43	41	36	38	84

EXPERIMENTAL

Preparation of ^{14}C-D,L-HETPP from 1,2-^{14}C-acetaldehyde and TPP.

115 mg. TPP and 220 mg. freshly distilled acetaldehyde (containing 38 µC 1,2-^{14}C-acetaldehyde) were dissolved in 1 ml water, and the pH was adjusted to 8.7 with 2 N NaOH. After 4 hours' incubation at 45° C the pH was adjusted to 3.4 with 2 N HCl and continously extracted with ether for 90 minutes. After the ether had been removed by distillation and the pH adjusted to 6.5, the aqueous solution was evaporated to 1 ml in vacuo.

Separation of ^{14}C-D,L-HETPP and TPP by column chromatography (cf.fig.1)

To separate ^{14}C-HETPP from TPP the concentrated preparation was applied to a column of Dowex 2-formate, x 8, 200-400 mesh (40 cm x 3.14 cm^2). After elution with 200 ml water, it was fractioned by gradient elution with 5 x 10^{-3} M HCOOH. The mixing chamber initially contained 1000 ml

water. 10 ml fractions were collected. The absorption of each fraction was measured and 0.3 ml samples were taken to determine ^{14}C-radioactivity, which was detected by the methane flow counter FH 407 (Frieseke and Hoepfner, Erlangen). The quantitative determination of HETPP and TPP was performed by dephosphorylation to HET and T by means of phosphatase and paper chromatographic separation of the latter (Holzer et al. 1960), followed by elution and quantitative determination by means of the thiochrome test (Goedde, Blume and Holzer 1961). 93-97 per cent HETPP was found in the fraction of peak I, whereas peak II contained only TPP. After adjustment to pH 6.0, the HETPP fraction was evaporated in vacuo to ca. 0.5 ml. The yield of HETPP from such preparations was 18-38 per cent with respect to the initial amount of TPP.

Incubation of ^{14}C-D,L-HETPP with apo-pyruvate decarboxylase (cf. TABLE I) D,L-HETPP and pyruvate respectively, were incubated in stoppered tubes at 23-24° C for 60 minutes in 1.4 ml 2.6×10^{-2} M citrate buffer pH 6.0 containing 4.3×10^{-3} M $MgSO_4$ and 32 mg. apo-pyruvate decarboxylase (Holzer and Goedde 1957). The pyruvate sample additionally contained 1.6×10^{-6} M TPP. After the addition of 0.35 ml 6 per cent $HClO_4$ (w/v) the incubation mixture was centrifuged. The supernatant was neutralized with 2 N KOH, maintained at 0° C for 40 minutes, and the $KClO_4$ precipitate was centrifuged. The supernatant was used to determine acetaldehyde with alcohol dehydrogenase (Boehringer) (Holzer et al. 1956).

SUMMARY

The purification of ^{14}C-D,L-HETPP obtained from non-enzymatic reaction mixtures of TPP and acetaldehyde by ion exchange chromatography is described. A spectrophotometric determination of HETPP with apo-pyruvate decarboxylase and acetaldehyde dehydrogenase is communicated.

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