IDENTIFICATION OF α-HYDROXYETHYL THIAMINE PYROPHOSPHATE ("ACTIVE ACETALDEHYDE") AS AN INTERMEDIATE IN THE OXIDATION OF PYRUVATE BY PYRUVIC OXIDASE FROM YEAST MITOCHONDRIA [+)]

H.Holzer, H.W.Goedde, K.-H.Göggel and B.Ulrich

Physiologisch-Chemisches Institut der Universität
Freiburg im Breisgau (Germany)

Received November 14, 1960

On the basis of model experiments, Breslow (1958) and Krampitz et al. (1958) suggested that "active acetaldehyde" has the structure of thiamine pyrophosphate (TPP), substituted in the 2 position of the thiazole ring by an α-hydroxyethyl group (α-hydroxyethyl thiamine pyrophosphate = HETPP). The enzymatic formation of HETPP has been demonstrated by Holzer and Beaucamp (1959; 1960) by identification of HETPP as an intermediate in the decarboxylation of pyruvate with pyruvic decarboxylase from yeast. It was to be expected that HETPP might also be an intermediate in the oxidation of pyruvate to acetyl-coenzyme A.

The present paper reports the purification of a radioactive TPP derivative, obtained after incubation of pyruvate-2-^{14}C with pyruvic oxidase from yeast mitochondria. This TPP derivate behaves paper-chromatographically in the same manner as the intermediate of pyruvate decarboxylation, which was earlier described as HETPP (Holzer and Beaucamp 1959; 1960). For the further identification of this derivative as HETPP, it was incubated with acid phosphatase. Samples were taken at various intervals and subjected to paper-chromatography. From the figure it is evident that the phosphate groups are successively split off following treatment with the phosphatase; i.e., HETPP is being converted via α-hydroxyethyl thiamine monophosphate to α-hydroxyethyl thiamine (HET). The identity of HET was confirmed by comparison with a synthetic preparation

[+)] This investigation was supported by the Deutsche Forschungsgemeinschaft and the Bundesministerium für Atomkernenergie und Wasserwirtschaft.

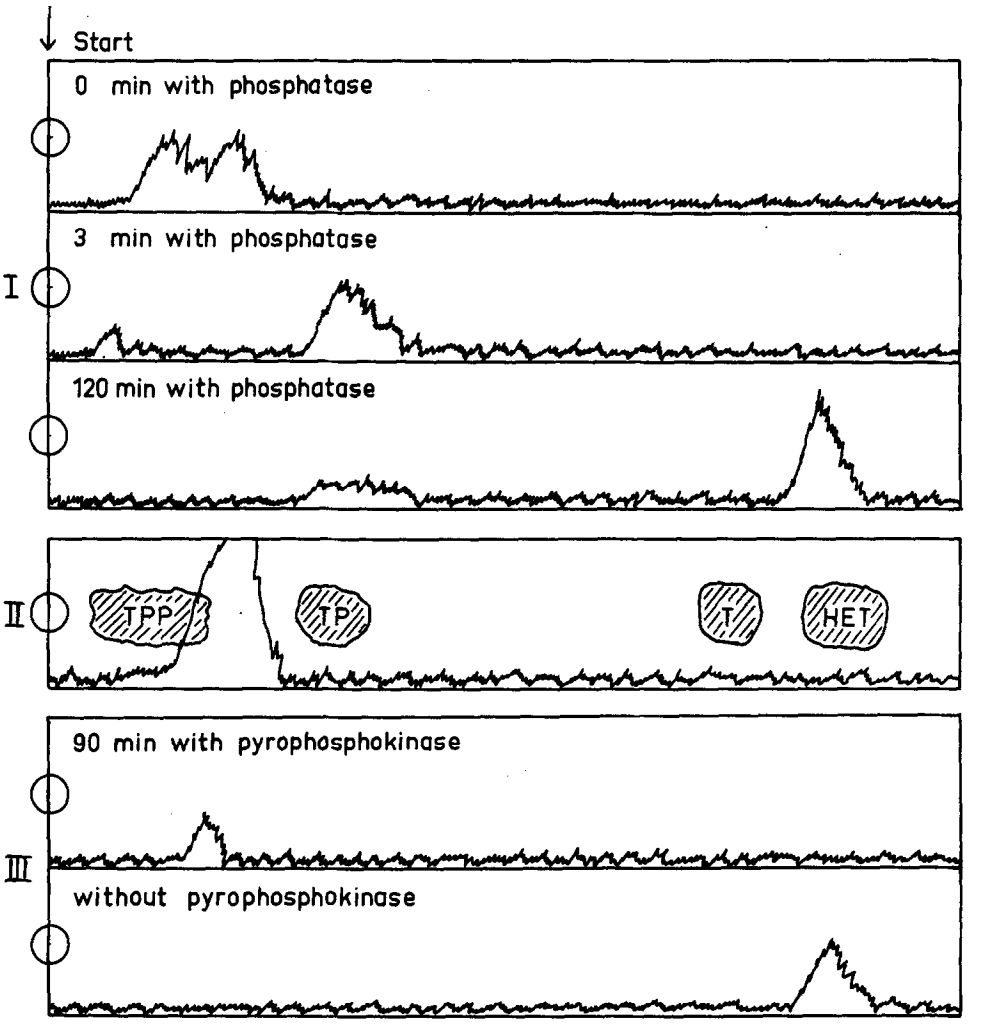

Figure 1: Dephosphorylation of ^{14}C-labeled HETPP by acid phosphatase and pyrophosphorylation of ^{14}C-labeled HET with ATP and thiamine pyrophosphokinase.
The radioactivities of paperchromatograms from ^{14}C-HETPP, incubated with acid phosphatase for 0, 3, and 120 minutes, are shown in part I. A radiochromatogram of untreated ^{14}C-HETPP, as well as a chromatogram of the reference substances TPP, thiamine monophosphate (TP), thiamine (T) and HET, located by thiochrom reaction, are illustrated in part II. In part III are shown the radiochromatograms of incubation mixtures from ^{14}C-HET with and without thiamine pyrophosphokinase.

(kindly supplied by Dr.J.H.Sprague of Merck, Sharp and Dohme,Rahway, New Jersey). With the same preparation, Carlson and Brown (1960) identified the "active acetaldehyde", which they had obtained as an intermediate in the decarboxylation of pyruvate by wheat germ

pyruvic decarboxylase. As can be seen from the figure, our compound has the same R_f value as Sprague's synthetic HET. The ^{14}C labeled HET was eluted from the paper and incubated with ATP and thiamine pyrophosphokinase. We obtained radioactive HETPP, which was recognized through its position on the paperchromatograms (see part III of the figure).

The experiments with phosphatase and thiamine pyrophosphokinase demonstrate the existance of HETPP as an intermediate in the oxidation of pyruvate to acetyl-coenzyme A. They provide further evidence for the identity of HETPP and "active acetaldehyde".

EXPERIMENTAL

Preparation of HETPP. 100 µg of 2-^{14}C-Na-pyruvate (2 µC ^{14}C) were incubated with 1-2 mg of purified pyruvate oxidase from mitochondria of baker's yeast (Holzer and Goedde 1957), 1 µmole TPP and 1.5 µmoles $MgSO_4$ in $2.8 \cdot 10^{-3}$ M tris(hydroxymethyl)aminomethane buffer, pH 7.4, at 22° C for 60 minutes. The reaction was stopped by addition of hot methanol as described by Holzer and Beaucamp (1960). The vacuum concentrated residue was dissolved in water, applied to a column of Dowex 2-Cl' X 10, 200-400 mesh, 15 cm · 1.13 cm^2, and fractioned by gradient elution with $2 \cdot 10^{-2}$ M HCl. The mixing chamber initially contained 250 ml H_2O. The HETPP-fraction, identified by radiopaper-chromatography of small samples from the different column-fractions, was paperchromatographed according to a procedure described by Holzer and Beaucamp (1960), eluted with water and used in the following experiments.

HETPP-hydrolysis with phosphatase. HETPP (22,100 c.p.m.) was incubated in aqueous solution with 5 mg acid phosphatase (4.7 mg protein) from wheat germ (Fluka AG, Switzerland) at pH 6.3 and 45° C (total volume 1 ml). The reaction was stopped by adding hot methanol as described by Holzer and Beaucamp (1960). The incubation times were: 0 min, 3 min, and 120 min (see part I of the figure).

Pyrophosphorylation of HET. HET (107,000 c.p.m.) was mixed with 6 µmoles ATP and 1.5 µmoles $MnCl_2$ in $6.9 \cdot 10^{-2}$ M tris(hydroxymethyl)-aminomethane buffer, pH 7.5. After addition of 3.4 mg thiamine pyrophosphokinase from dried baker's yeast (prepared according to Kaziro (1960) up to the column-chromatography step) the reaction

mixture (volume 1.15 ml) was incubated for 90 minutes at 30° C. The reaction was stopped by adding hot methanol according to Holzer and Beaucamp (1960). After paperchromatography the radioactivity was recorded (see part III of the figure).

The descending paperchromatography of the stopped, vacuum concentrated, reaction mixtures I and III was carried out with Schleicher and Schüll paper 2043 b Mgl, first in n-butanol : acetic acid : water (5 : 2 : 3) and subsequently in n-butanol : ethanol : water (4 : 1 : 1). The papers were run in each solvent system for 24 hours. The radioactivity of the dried chromatograms was recorded with radiopaperchromatograph FH 452 and methane-flow-counter FH 407 (Frieseke and Hoepfner, Erlangen/Germany). The positions of the thiamine derivatives were determined using the thiochrom-reaction according to Siliprandi (1954).

REFERENCES

Breslow,R., (1958) J.Am.Chem.Soc. 80, 3719.

Carlson,G.L., Brown,G.M., (1960) J.Biol.Chem. 235, PC 3.

Holzer,H., Beaucamp,K., (1959) Angew.Chem. 71, 776;
(1960) Biochim.Biophys.Acta, in press.

Holzer,H., Goedde,H.W. (1957) Biochem.Z. 329, 175.

Kaziro,Y., (1959) J.Biochem.Jap. 46, 1523.

Krampitz,L.O., Greull,G., Miller,C.S., Bicking,J.B., Skeggs,H.R., Sprague,J.M., (1958) J.Am.Chem.Soc. 80, 5893.

Siliprandi,D., Siliprandi,N., (1954) Biochim.Biophys.Acta 14, 52.