ENZYMATIC FORMATION OF α-ACETOLACTATE FROM α-HYDROXYETHYL-2-THIAMINE PYROPHOSPHATE ("ACTIVE ACETALDEHYDE") AND PYRUVATE +)

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The formation of α-acetolactate from pyruvate in biological systems has been first postulated by Watt and Krampitz (1947). Krampitz (1948) synthesized acetolactate and showed it to be decarboxylated to acetoin in extracts from Staphylococcus aureus. In further publications the formation of acetolactate from pyruvate was demonstrated in extracts from A.aerogenes (Juni 1950; 1952; Halpern and Umbarger 1959), E.coli (Umbarger et al. 1957; Umbarger and Brown 1958; Lewis and Weinhouse 1958), baker's yeast (Lewis and Weinhouse 1958) and pigeon breast muscle (Juni and Heym 1956). It was first shown by Strassman, Thomas and Weinhouse (1953; 1955) that an essential rôle is due to acetolactate in the metabolism of valine-autotrophic organisms: in isotope experiments with yeast they rendered probable that acetolactate is an intermediate in the biosynthesis of valine from pyruvate.

Ochoa (1951) supposed a reaction between pyruvate and "active acetaldehyde" to take part in the biosynthesis of acetolactate. Since "active acetaldehyde" (α-hydroxyethyl-2-thiamine pyrophosphate = HETPP) was available from reaction mixtures of pyruvate with pyruvic decarboxylase (Holzer and Beaucamp 1959; 1961) and pyruvic oxidase, respectively (Holzer et al. 1960; Scriba and Holzer 1961), we could study the mechanism suggested by Ochoa.

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We used pyruvic oxidase from pig heart muscle as acetolactate-forming enzyme. The reaction mixture contained as substrates unlabelled pyruvate and HETPP which was ^{14}C-labelled in the α-hydroxyethyl-group. The latter had been obtained by incubating pyruvic oxidase with 2-^{14}C-pyruvate and thiamine pyrophosphate (TPP) according to Scriba and Holzer (1961). After incubation at 30° C for 3 1/2 hours, the acetolactate-forming reaction was stopped by adding ethanol. The deproteinized and vacuum-concentrated extract was submitted to ion exchange chromatography in order to separate the radioactive products.

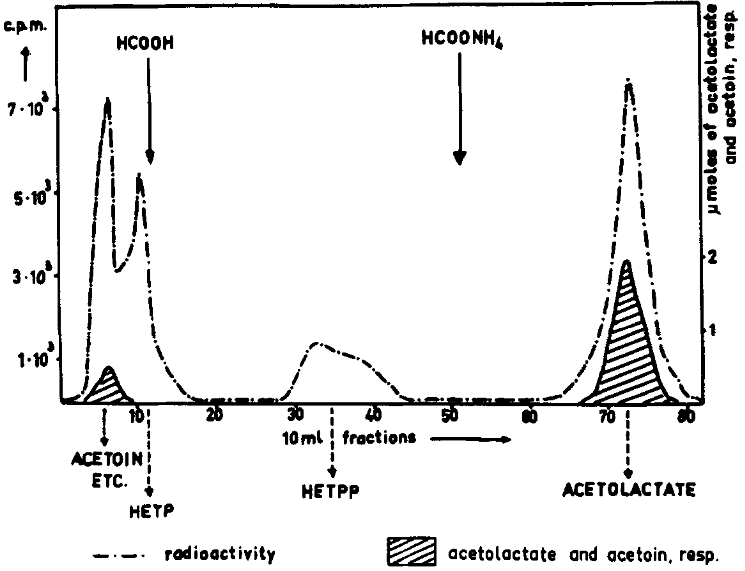

Figure 1: Ion exchange chromatography with Dowex 2-formate, to isolate ^{14}C-labelled acetolactate.
Fractions 1-10 were obtained by elution with water, fractions 11-50 by elution with increasing concentrations of formic acid (mixing chamber 250 ml H_2O, reservoir 0.02 N formic acid), fractions 51-85 by elution with 0.5 M $HCOONH_4$.

From the figure it is obvious that there is a radioactivity-containing peak exactly at the position of the acetolactate peak. Control experiments with authentic acetolactate, synthesized according to Krampitz (1948), also resulted in an exact accordance with the position of this radioactivity-containing peak. For further identification part of the radioactive acetolactate was decarboxylated. Following Happold and Spencer (1952), the formed acetoin was oxidized with ferric chloride to diacetyl, and from this latter the (bis)-2,4-dinitrophenylhydrazone was prepared. It contained more than 90 per cent of the radioactivity employed. Finally. the identity of the radioactivity-containing peak with acetolactate was studied by means of high voltage electrophoresis with the apparatus according to Wieland and Pfleiderer (1955). Again, the labelled substance behaved in the same way as did synthetic acetolactate.

Already in former experiments HETPP had been shown to be an intermediate in the conversion of pyruvate to acetaldehyde and acetoin (Holzer and Beaucamp 1959; 1961) as well as to acetyl-coenzyme A (Goedde, Inouye and Holzer 1961). The present experiments demonstrate that HETPP is also an intermediate in the formation of α-acetolactate from pyruvate. For this reaction the equation is as follows:

$$\text{HETPP} + \text{pyruvate} \longrightarrow \alpha\text{-acetolactate} + \text{TPP}.$$

EXPERIMENTAL

Preparation of HETPP. 1 mg. of 2-^{14}C-sodium pyruvate (20 µC ^{14}C) was incubated at 25° C for 150 minutes with 71 mg. of purified pyruvic oxidase from pig heart muscle, 8 µmoles TPP, 7.5 µmoles $MgSO_4$ and 40 µmoles KH_2PO_4. The pH was adjusted to 5.0 with 0.1 N H_2SO_4 (cf. Scriba and Holzer 1961). The reaction was stopped by addition of hot methanol. The vacuum-concentrated preparation was applied to a Dowex 2-acetate column (x 10, 200-400 mesh, volume 24 cm x 3.14 cm^2) and fractioned by gradient elution with 1.7×10^{-2} M acetic acid. The mixing chamber initially contained 250 ml H_2O. By this procedure a complete separation of HETPP from TPP was achieved. The HETPP fractions identified by paper chromatography (Holzer and Beaucamp 1961) were concentrated, neutralized, and employed in the following experiments.

Formation and identification of radioactive acetolactate.

Two Warburg vessels were used, each containing the following incubation mixture: 38 mg. protein of the ethanol precipitate of pyruvic oxidase purified from pig heart muscle (Scriba and Holzer 1961), 0.18 µmoles ^{14}C-HETPP with 250,000 c.p.m. (specific activity 1.4×10^6 c.p.m. per µmole), 2.5 µmoles $MgSO_4$, 640 µmoles sodium pyruvate, and 0.25 ml 0.5 M phosphate buffer, pH 6,5. Total volume 3.2 ml. Gas phase: N_2. Incubation time 3 1/2 hours at 30° C. The reaction was stopped by the addition of 9.6 ml ethanol. The reaction mixtures were then centrifuged, the sediments stirred with little water and recentrifuged after the addition of the threefold volume of ethanol. The combined supernatants of the two preparations were vacuum-concentrated, applied to an ion exchange column (Dowex 2-formate, x 10, 200-400 mesh, 24 cm x 3.14 cm^2), and eluted at +4° C with water, then with increasing concentrations of HCOOH (mixing chamber 250 ml H_2O, reservoir 2×10^{-2} N HCOOH), and finally with 0.5 M ammonium formate. 10 ml fractions were collected (cf. figure). By this procedure a decarboxylation of acetolactate was prevented, which readily takes place below pH 3 and at raised temperatures. The acetolactate-containing fractions were identified by submitting an aliquot, after acid decarboxylation at 100° C, to the acetoin test according to Wersterfeld (1945). The acetolactate formed amounted to 8 µmoles . Radioactivity was measured in the methane flow counter FH 407 (Frieseke and Hoepfner, Erlangen) after plating an aliquot. Altogether 38,500 c.p.m. were contained in the acetolactate fraction, the yield thus amounting to 7.7 per cent with respect to the HETPP employed (2 x 250,000 c.p.m.). As can be calculatedfrom the above results, the specific activity of the acetolactate was 4.8×10^3 c.p.m. per µmole, i.e. 1/290 of the specific activity of the HETPP employed. This decrease in the specific activity is explained by the formation of unlabelled HETPP from unlabelled pyruvate added in large excess; the non-labelled HETPP competes with the added ^{14}C-HETPP in the formation of acetolactate.

Part of the acetolactate (0.86 µmoles with 4,150 c.p.m.) was oxidized by ferric chloride after acid decarboxylation at 100° C. The produced diacetyl was distilled into a solution of 2,4-dinitrophenylhydrazine in 2 N HCl. The precipitate of diacetyl-(bis)-2,4-dinitrophenylhydrazone was dried, dissolved in a small quantity of dimethyl formamide, plated, and counted. It contained 3,800 c.p.m., i.e., 92 per cent of the radioactivity employed in this experiment. A further portion of the radioactive acetolactate was submitted to high voltage electrophoresis (0.1 M phosphate buffer pH 7.5; 40 V/cm; 90 mA; 80 min.). Acetolactate was identified by spraying with the reagents according to Westerfeld (1945) and subsequent warming; the substance migrated 10 cm towards the anode. The position of the radioactivity was discovered with the radiopaperchromatograph FH 452 (Frieseke and Hoepfner, Erlangen).

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