PYRUVATE SYNTHESIS BY A PARTIALLY PURIFIED ENZYME FROM *Clostridium acidi-urici*

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The enzymic activity present in extracts of *Clostridium acidi-urici* responsible for the exchange reaction between bicarbonate and the carboxyl group of pyruvate has been under investigation both in this laboratory (Rabinowitz, 1960; Rabinowitz and Allen, 1961) and by Sagers and Beck (1956). Mortlock, *et al.* (1959) examined the same reaction in fractionated extracts of *Clostridium butyricum*, and Mortlock and Wolfe (1959) were able to demonstrate the synthesis of pyruvate from acetyl phosphate and bicarbonate by such preparations in the presence of hydrosulfite. Bachofen, *et al.* (1964) and Buchanan, *et al.* (1964) have recently demonstrated the activity of reduced ferredoxin in synthesis of pyruvate from these substrates by extracts of *Clostridium pasteurianum* and *Chromatium*. We have partially purified the activity present in extracts of *C. acidi-urici* that catalyzes the exchange of bicarbonate and the carboxyl group of pyruvate. We wish to report here the activity of this purified enzyme in also catalyzing the synthesis of pyruvate from carbonate and acetyl phosphate or acetyl coenzyme A, the requirements for this reaction, and some properties of the enzyme.

Using the bicarbonate-pyruvate exchange activity assay described previously (Rabinowitz, 1960), a purification of approximately 50 fold of the activity in extracts of *C. acidi-urici* was achieved by means of ammonium sulfate fractionation, chromatography on Sephadex G-200, and column electrophoresis. The purified enzyme showed requirements for coenzyme A and 2-mercaptoethanol in the exchange reaction. The cobinamide requirement originally reported (Rabinowitz, 1960) could be replaced by anaerobiosis or certain transition state metal ions in the presence of 2-mercaptoethanol as suggested by Peel (1963), and no requirement for thiamine pyrophosphate could be demonstrated with the purified enzyme. In addition to the exchange activity, the purified enzyme catalyzes the oxidation of pyruvate in the presence of coenzyme A, 2-mercaptoethanol and ferredoxin or certain other electron acceptors such as FAD and 2,3,5-triphenyltetrazolium. Microbiological assays showed that the enzyme contains thiamine and trace amounts of flavin. It also contains a

non-flavin chromophore with an absorption maximum at 390 to 400 mμ, and iron and inorganic sulfide in approximately equimolar amounts. The most highly purified fraction contained three components by sedimentation analysis. The sedimentation coefficient of the component possessing both the exchange activity and the chromophore was calculated to be 9, and represented about 30% of the protein of this preparation.

The purified enzyme catalyzes the synthesis of pyruvate under conditions similar to those described by Bachofen, et al. (1964). Table I summarizes the requirements for this reaction by the purified enzyme.

Table I. Requirements for the Synthesis of Pyruvate by an Enzyme from C. acidi-urici

Conditions	Radioactivity of Pyruvate Phenylhydrazone cpm/mmole
Complete	3000
Complete minus dichlorophenolindophenol	2713
Complete minus 2-mercaptoethanol	1400
Complete in the dark	0
Complete in air	0
Complete minus C. acidi-urici enzyme	0
Complete minus phosphotransacetylase	0
Complete minus ferredoxin	0
Complete minus heated chloroplasts	0
Complete minus acetyl phosphate	0
Complete minus coenzyme A	0

The complete system contained 66 μg of protein from C. acidi-urici, 25 units of phosphotransacetylase (Stadtman, 1955) from C. butyricum, 120 μg of crystalline ferredoxin from C. acidi-urici (Lovenberg, Buchanan and Rabinowitz, 1963), heated chloroplasts containing 0.5 mg of chlorophyll prepared according to Whatley and Arnon (1963), 300 μmoles of potassium phosphate buffer, pH 7.3, 50 μmoles of dilithium acetyl phosphate, 0.5 μmole of coenzyme A, 75 μmoles of 2-mercaptoethanol, 10 μmoles of sodium bicarbonate containing 14,000 cpm, 0.2 μmole of dichlorophenolindophenol, and 20 μmoles of sodium ascorbate. The final volume was 3 ml, contained in a Warburg flask equipped with two side arms containing 0.2 ml each of 0.02 M potassium phosphate, pH 7. The C^{14}-bicarbonate was placed in one side-arm, and the enzymes were placed in the other. The remaining components were contained in the main compartment. The system was equilibrated with nitrogen for 10 min. at 37° with shaking. The reaction was started, after tipping in the contents of the side-arms, by turning on the light. The reaction mixture was incubated under nitrogen at 37° with a light intensity of 10,000 lux for 30 min. The reaction was stopped by turning off the lights and pouring the contents of the flasks into test tubes containing 0.1 ml of 70% perchloric acid. 50 μmoles of sodium pyruvate was added to 2 ml aliquots of the supernatant solutions obtained after centrifuging the acidified reaction mixture. The 2,4-dinitrophenylhydrazone derivative of pyruvate was prepared as previously described (Rabinowitz and Allen, 1961) and its radioactivity determined.

The amount of pyruvate synthesized is proportional to the amount of enzyme in the range of 0.033 to 0.330 mg of protein. The average specific activity of the pyruvate formed after 30 min. incubation in the complete system was 48,700 cpm per mmole of phenylhydrazone per mg of protein. This is equivalent to the synthesis of 2.55 μmoles of pyruvate per 30 min. per mg. protein, and represents the fixation of 1.7% of the added bicarbonate per 30 minutes. The reaction was linear with time over the 60 minute incubation period.

The product of the reaction was identified as pyruvate and was differentiated from oxaloacetate by paper chromatography and radioautography of the 2,4-dinitrophenylhydrazones in iso-amyl alcohol, ethanol, and water (5:1:4) in the presence of ammonia vapor, and n-butanol, ethanol, 0.5 N NH_4OH (7:1:2). The phenylhydrazone derivative of pyruvate obtained as the product of the enzymic reaction was reduced in the presence of platinum oxide (Meister and Abendschein, 1956), and the product of the reduction was identified as alanine-C^{14} by chromatography in methanol, water, pyridine (80:20:4), and pyridine, acetic acid, water (50:35:15) followed by radioautography. Alanine was readily separated from aspartate, the reduction product of oxaloacetate, in both solvent systems.

The dependence of pyruvate synthesis on the concentration of ferredoxin was determined under the conditions of the complete reaction described in Table I. Using from 0.12 to 62 μg of crystalline C. acidiurici ferredoxin, the K_m value of ferredoxin calculated from the Lineweaver-Burk plot was 1.7 μg per ml, or 3×10^{-7} M, using a value of 6000 for the molecular weight of ferredoxin (Lovenberg, Buchanan and Rabinowitz, 1963).

The requirement for acetyl phosphate, coenzyme A and phosphotransacetylase could be replaced by acetyl coenzyme A, as shown in Table II.

Avidin had no effect on the reaction catalyzed by this enzyme under the conditions used and described in Table III. Shuster and Lynen (1960) found that 0.06 units of avidin completely inactivated the carbonate-pyruvate exchange reaction catalyzed by 1 mg of protein from a C. kluyveri fraction. Kaziro, et al. (1960) reported a complete inhibition of 40 μg of 80%-pure propionyl carboxylase by 0.075 units of avidin.

Since the enzyme exhibits little chemical specificity with respect to the electron acceptor in the oxidation of pyruvate, it was of interest to determine if other dyes could replace ferredoxin in the reductive synthesis of pyruvate. The same amount of pyruvate was formed under the conditions described in Table I using 20 mμmoles of methyl viologen in

Table II. Activity of Acetyl Coenzyme A in Pyruvate Synthesis

Added Acetyl Coenzyme A μmole	Added Acetyl Phosphate μmole	Radioactivity of Pyruvate cpm/mmole
0.0	0.0	0
2.5	0.0	23,880
5.0	0.0	34,444
0.0	5.0	6,944

The system was the same as that described in Table I except for the following: 100 μmoles of sodium bicarbonate containing 140,000 cpm, 132 μg of the C. acidi-urici enzyme, and no 2-mercaptoethanol. The reaction mixture was incubated for 2 hours. When acetyl coenzyme A was used, acetyl phosphate, coenzyme A, and phosphotransacetylase were omitted.

Table III. Effect of Avidin on Pyruvate Synthesis

Avidin units	Radioactivity of Pyruvate cpm/mmole
0.00	3183
0.05	3514
0.10	3422
0.50	3333
1.00	3292

The enzyme, 66 μg, was preincubated with varying amounts of a freshly prepared solution of avidin (25 units/10 mg) for 30 min. at 20°. The reaction mixture was then incubated under the conditions described in Table I for one hour at 37°.

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place of an equimolar amount of ferredoxin. No pyruvate could be detected when equimolar amounts of FAD or menadione replaced the ferredoxin. The oxidation-reduction potentials at pH 7 for clostridial ferredoxin (Tagawa and Arnon, 1962), methyl viologen, FAD, and menadione are - 0.418 v, - 0.432 v, - 0.219 v, and 0.002 v, respectively. It therefore appears that the requirement for the electron donor in the synthesis of pyruvate by this enzyme is determined primarily by a sufficiently low oxidation-reduction potential, and not by a specific chemical structure.

The system responsible for pyruvate synthesis, as described here, utilizes photo-reduced ferredoxin. Clostridium acidi-urici is unable to reduce ferredoxin in this way; however, a number of enzymic reactions resulting in ferredoxin reduction have been demonstrated in the purine fermenting clostridia. These include formic dehydrogenase (Bradshaw and Reeder, 1964), xanthine oxidase (Bradshaw and Reeder, 1964) and DPN reductase (Valentine, Brill and Sagers, 1963). Until further in vivo

studies are undertaken, we cannot ascribe any physiological significance to the reductive synthesis of pyruvate and the accompanying CO_2 fixation in these organisms. However, the evidence presented here does illustrate the reversibility of pyruvate oxidation in C. acidi-urici.

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