ENZYME SYSTEMS IN THE MYCOBACTERIA

IV. THE PYRUVIC OXIDASE

DEXTER S. GOLDMAN*

Tuberculosis Research Laboratory, Veterans Administration Hospital, and the Institute for Enzyme Research, University of Wisconsin, Madison, Wis. (U.S.A.)

The oxidative decarboxylation of pyruvate to CO_2 and acetate has been shown to occur in cell-free extracts prepared from several microorganisms. Investigation of these reactions has revealed that several distinct metabolic pathways are involved in this decarboxylation. LIPMANN[1] demonstrated that cell-free extracts of *Lactobacillus delbrueckii* carry out a phosphoroclastic split of pyruvate to acetyl phosphate and CO_2. Acetyl CoA** was not an intermediate[2]. STUMPF[3] obtained a pyruvic oxidase system from *Proteus vulgaris* which catalyzed the oxidation of pyruvate to acetate; oxygen served as the ultimate electron acceptor. Neither acetyl phosphate[3] nor acetyl CoA[4] could be shown to be intermediates in this oxidation. MOYED AND O'KANE[5,6] have separated the *P. vulgaris* oxidase into two components: (a) a particulate cytochrome system and (b) a soluble pyruvic oxidase which requires DPT. No other coenzymes are required for the oxidation of pyruvate by this system; dyes could serve as electron acceptors.

Extracts of *Streptococcus faecalis* and *Escherichia coli* can catalyze the oxidation of pyruvate. These systems are quite distinct from that of *P. vulgaris*. The unfractionated crude extract of *E. coli* catalyzes a dismutation reaction in which acetyl phosphate, CO_2 and lactate are formed from pyruvate. Catalytic quantities of CoA and DPN are required[7,8]. The pyruvate oxidation system of *E. coli* has been resolved[8,9]. KORKES *et al.*[8] and GUNSALUS[9] purified extensively the *E. coli* enzymes and showed that one of the two fractions carries out the oxidation of pyruvate to acetate (ferricyanide as electron acceptor); DPT is required for this reaction. The second fraction contains the enzymic mechanisms for the conversion of the pyruvate oxidation product to acetyl CoA. This conversion requires CoA, DPN and lipoic acid.

Highly purified pyruvic oxidases have been isolated from animal tissues[10-12]. The preparation obtained from pig heart muscle by KORKES *et al.* is in many respects similar to that of *E. coli*. JAGANNATHAN and his coworkers obtained from pigeon breast muscle a high molecular weight pyruvic oxidase in homogeneous form which could not be resolved into component fractions. Subsequently LITTLEFIELD AND SANADI[13] demonstrated that the pigeon breast muscle pyruvic oxidase required both

* With the technical assistance of MRS. PATRICIA KOCH.

** The following abbreviations will be used: Coenzyme A (CoA), diphosphopyridine nucleotide (DPN), thiamine pyrophosphate (DPT), 2,6-dichlorophenolindophenol (PIP), ethylenediaminetetraacetic acid (EDTA).

References p. 512.

DPN and CoA and formed acetyl CoA as a product of the oxidation reaction. OCHOA[14] has recently reviewed the work on the mechanism of pyruvate oxidation.

The oxidation of pyruvate by two strains of *Mycobacterium tuberculosis* var. *hominis* was reported by MILLMAN and his coworkers[15-17]. These investigators prepared cell-free extracts of virulent (H37Rv) and avirulent (H37Ra) strains. These extracts take up oxygen in the presence of pyruvate; methylene blue is required for this uptake.

The purification and characterization of enzymes from cell-free extracts of the H37Ra strain of *M. tuberculosis* is being carried out in this laboratory as part of a study on the oxidative metabolism of the tubercle bacillus. The present report deals with a soluble pyruvic oxidase isolated from H37Ra. This oxidase shows characteristics of both the unresolved enzyme of pigeon breast muscle and the highly purified resolved components of the oxidase of *E.coli*. This pyruvic oxidase can use ferricyanide, PIP or oxygen as its electron acceptor; a subsequent paper[18] will describe the use of DPN as electron acceptor, the formation of acetyl CoA and the intermediation of thioctic acid. No evidence exists as yet that this enzyme or enzyme complex can be resolved into two or more fractions*.

MATERIALS AND METHODS

Cell-free extracts of H37Ra were prepared from washed 28-day-old surface cultures as described previously[20]. Protein was determined by the biuret method[21]; pyruvate as the 2,4-dinitrophenylhydrazone[22]. DPN, DPT, CoA and PIP were all obtained from commercial sources.

Enzyme assay (ferricyanide)

In bicarbonate medium the oxidation of pyruvate by ferricyanide, catalyzed by the pyruvic oxidase, produces 3 molecules of CO_2 for each molecule of pyruvate oxidized. Conventional Warburg manometric techniques were used to measure the rate of evolution of CO_2. The main compartment of the reaction vessel contained, in μmoles, $MgCl_2$ (20), DPT (0.50), $NaHCO_3$ (250), Li-pyruvate (50) and enzyme (10–30 mg). The total volume in the main compartment was 2.8 ml. The side-arm contained 100 μmoles (0.2 ml) of $K_3Fe(CN)_6$. The gas phase was CO_2, incubation temperature 38° and incubation time 60 min. A no-substrate blank was included in each experimental series. After equilibration of the flask and contents for 7 min at 38° the taps were closed and ferricyanide was added from the side-arm. The evolution of CO_2 proceeded in a linear fashion during the assay period.

Enzyme assay (PIP)

Into standard Coleman tubes were placed, in μmoles, phosphate buffer of pH 6.8 (200), $MgCl_2$ (30), DPT (0.025) and Li-pyruvate (15). The final volume was 5.85 ml. The blank tube contained no substrate. The tubes were incubated at 38° for 5 min and then removed from the water bath. To each tube was added 0.10 ml of 0.05% PIP and 0.05 ml of the enzyme (25–100 units). The contents of the tube were mixed and the optical density at 600 mμ read in the Coleman Junior spectrophotometer. The optical density of the blank was set to read 0.400. The tubes were returned to the water bath and incubated at 38° for 15 min. The optical densities at 600 mμ of both blank and experimental reactions were read, the blank having been set again to read 0.400. Under these conditions the rate of PIP reduction is linear with respect to both time and concentration of protein. One unit of enzymic activity causes a decrease in optical density at 600 mμ of 0.001 under the assay conditions. Specific activity refers to the PIP assay only.

Enzyme purification

All procedures were carried out at 1–3° C.

First ammonium sulfate (neutral). The crude cell-free extract is brought to 0.25 saturation by the addition of neutral, saturated ammonium sulfate. The resulting suspension is centrifuged at 20,000 × *g* for 1 h; the residue is discarded. The supernatant fluid is recovered and brought to 0.40 saturation by the addition of further neutral saturated ammonium sulfate. The suspension is centrifuged for 1.5 h at 5,000 × *g*. The residue (AS-1) is recovered, dissolved in water and dialyzed against 0.01 *M* phosphate buffer of pH 7.0.

Second ammonium sulfate (alkaline). To the dialyzed solution of AS-1 is added sufficient

* A preliminary account of this work has been reported (19).

References p. 512.

alkaline*, saturated ammonium sulfate to reach 0.26 saturation.The suspension is centrifuged (5,000 × *g*, 1 h) and the residue discarded. The supernatant fluid is then brought to 0.36 saturation by the addition of further alkaline, saturated ammonium sulfate. The precipitate (AS-2) is recovered by centrifugation at 5,000 × *g* for 1 h, dissolved in water and dialyzed against 0.01 *M* phosphate buffer of pH 7.0.

First gel adsorption. For each mg of protein in the dialyzed solution of AS-2 is added 3 mg of calcium phosphate gel[23]. The thick suspension is stirred and then centrifuged; the supernatant fluid is discarded. The gel is eluted 5 times with 0.05 *M* phosphate buffer of pH 7.0. The volume of each wash is about 1.5 times the volume of the original dialyzed AS-2 solution. These eluates are discarded. The gel is next eluted 5 times with similar volumes of 0.10 *M* phosphate buffer of pH 7.0, these eluates are retained and combined. The combined eluates (GE-1) are dialyzed against 0.005 *M* phosphate buffer of pH 7.0. The dialyzed solution of GE-1 is lyophilized to dryness, dissolved in a small amount of water and re-dialyzed against 0.01 *M* phosphate buffer of pH 7.0.

Second gel adsorption. The gel step is repeated. Elutions are carried out first with 0.05 *M* and then with 0.08 *M* phosphate buffer of pH 7.0. The former eluates are discarded, the latter retained. The 0.08 *M* eluate (GE-2) is dialyzed, lyophilized and re-dialyzed as described for the first gel step. Alternative to the second gel step, GE-1 may be refractionated with neutral, saturated ammonium sulfate. The fraction precipitating between the saturation limits of 0.27 and 0.31 is removed by centrifugation, dissolved in water and dialyzed against 0.01 *M* phosphate buffer of pH 7.0.

Solutions of the pyruvic oxidase at this level of purity are usually slightly yellow. No significant decrease in activity is noted after several months' storage at — 17°. A typical fractionation is shown in Table I.

TABLE I

PURIFICATION OF THE PYRUVIC OXIDASE OF H37Ra (PIP SYSTEM)

Fractionation step	*Protein*				*Activity*		
	Volume (ml)	*Mg/ml*	*Total mg*	*Per cent recovered*	*Specific activity**	*Total units*	*Per cent recovered*
Crude extract	895	14.6	13,050	100			
1st $(NH_4)_2SO_4$	88	25.4	2,235	17	25	56,200	100
2nd $(NH_4)_2SO_4$	42	23.6	991	7.6	42	41,600	74
CaP gel eluate	16	4.37	70	0.54	376	26,200	47
3rd $(NH_4)_2SO_4$	2.3	4.94	11	0.084	986	11,240	23

* Specific activity = (ΔD 600/15 min/mg protein) × 1,000.

The highest specific activity obtained by this procedure was 1,150. For most of the work on this enzyme the fractionation procedure was stopped at the GE-1 level where the specific activity is 300–400.

RESULTS

Characteristics of the assay systems

Ferricyanide, PIP and oxygen can serve as electron acceptors for the pyruvic oxidase of H37Ra. Table II gives the details of an assay system based on the ferricyanide reaction. A partial requirement for DPT exists at the AS-1 stage of purification. The addition of magnesium does not increase the rate of oxidation at this stage of purification of the enzyme. Although the oxidation of 1 molecule of pyruvate by this system should produce 3 molecules of CO_2, this is never achieved (Table III). CO_2 retention by the medium is probably the reason for this low yield of CO_2. As the amount of pyruvate oxidized is increased the CO_2/pyruvate ratio approaches 3.0.

The lack of sensitivity of this assay method was responsible for its elimination in favor of the PIP system. In the PIP assay as little as 0.10 mg of enzyme can be used; this is of considerable advantage when the yield of enzyme is small. Mg^{++} stimulates

* Prepared by the addition of 5.0 ml concentrated NH_4OH to 100 ml neutral, saturated ammonium sulfate. This solution was prepared as needed.

References p. 512.

TABLE II

FERRICYANIDE ASSAY SYSTEM FOR THE PYYRUVIC OXIDASE OF H37Ra

Description	*μM CO_2 produced**
Complete system	12.2
no Mg^{++}	11.7
no Cocarboxylase	7.3
2.0 μmoles Mn^{++} instead of 20 μmoles Mg^{++}	10.2

* Corrected for no-substrate blank. Incubation was carried out for 1.0 h.

TABLE III

STOICHIOMETRY OF PYRUVATE AND CO_2

(Ferricyanide System)

Each flask contained, in μmoles: $MgCl_2$ (20), DPT (0.50), $NaHCO_3$ (300), enzyme and Li-pyruvate as shown. The final volume was 3.0 ml. Gas phase was 100% CO_2. After 7 min equilibration at 38° $K_3Fe(CN)_6$ (100) was added from the sidearm. CO_2 evolution was followed until no further change was noted over a 30 min period. Values are corrected for no-substrate blanks.

Expt. No.	*mg protein*	*μmoles pryuvate present*	*μmoles CO_2 produced*	*Ratio*
1	24.6	5.0	11.2	2.24
2	24.6	10.0	24.0	2.40
3	24.6	20.0	52.0	2.60
4	49.2	20.0	39.4	1.97
5	49.2	30.0	65.9	2.20
6	49.2	40.0	96.5	2.40

this reaction slightly. Accordingly, Mg^{++} is always included in the assay. The requirement for DPT in the PIP assay is complete. This is shown in Fig. 1. Saturation is reached at a concentration of about 5 mμmoles/ml. The PIP system is strongly inhibited by EDTA; this inhibition can be partly reversed by the addition of Mg^{++}. Magnesium, even in large amounts, cannot completely reverse the EDTA inhibition as shown in Table IV.

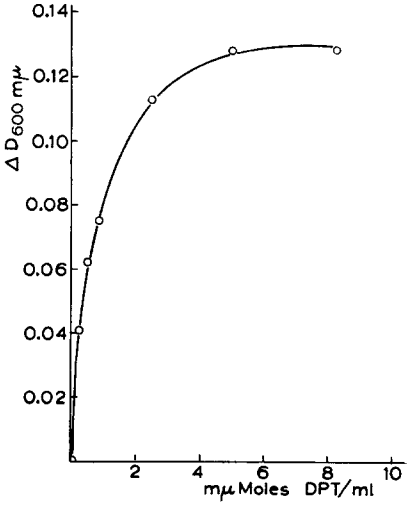

Fig. 1. Requirement for DPT in the PIP assay system. Conditions as described in the text.

The pyruvic oxidase of H37Ra was tested for its ability to react with oxygen as an electron acceptor. Table V shows an experiment in which a purified pyruvic oxidase preparation catalyzes the oxidation of pyruvate with oxygen as the electron acceptor. Neither CoA nor DPN nor a combination of the two had any effect on this rate. Addition of catalase and ethanol did not increase the rate of oxygen uptake.

The ratio of enzyme activities in the fer-

References p. 512.

ricyanide, PIP and oxygen systems are 2.5:1:3.0, expressed as μmoles substrate oxidized/mg protein/h at 38°.

TABLE IV

EFFECT OF EDTA ON THE PYRUVIC OXIDASE (PIP SYSTEM)

Standard PIP assay system except for the concentrations of $MgCl_2$ and EDTA as noted. Each tube contained 0.274 mg of enzyme of specific activity 605.

Expt. no.	*μmoles/6.0 ml*		*ΔD600*
	Mg++	*EDTA*	
1	30	0	0.166
2	0	0	0.110
3	0	0.30	0.005
4	0.20	0.30	0.035
5	0.40	0.30	0.053
6	1.0	0.30	0.083
7	0	0.50	0.003
8	0.20	0.50	0.014
9	0.40	0.50	0.044
10	0.60	0.50	0.046

TABLE V

EFFECT OF COENZYMES ON PYRUVATE OXIDATION (OXYGEN SYSTEM)

Additions	*μAtoms oxygen taken up*
none	9.7
+ 0.25 μmoles CoA	10.9
+ 2.5 μmoles DPN	10.1
+ CoA + DPN	8.1

Each reaction flask contained, in μmoles: phosphate buffer of pH 7.5 (100), $MgCl_2$ (20), DPT (0.5) and enzyme (4.6 mg; specific activity of 350). The center well contained 0.2 ml 6 *N* KOH. Total volume was 3.0 ml. Gas phase was oxygen. After 7 min equilibration at 38° the taps were closed and 40 μmoles Li-pyruvate were added from the side-arm. Reaction was followed for 2.0 h. Values given are corrected for a no-substrate blank.

Production of acetate from pyruvate

The formation of acetate as the oxidation product of pyruvate was demonstrated by three separate techniques: (a) diffusion and titration, (b) enzymic, and (c) chromatographic. The results are shown in Tables VI and VII*. At the end of the incubation period the reaction flasks were removed from the manometers, stoppered and placed in ice. 0.10 ml of 6 *N* KOH was added to each flask. After 20 minutes the contents of the flask were removed, the flasks were washed and the washings combined with the original reaction mixture. These solutions were lyophilized; the residues were dissolved in small amounts of water and diluted to 1 to 2 ml. Aliquots were taken for acetate assay.

Acetic acid was determined by the micro-diffusion method of BARTLEY[24]. The diffusion apparatus was coated with a silicone preparation** to avoid entrapment of volatile acids in micro-droplets adhering to the glass surface.

* The CO_2: acetate (Table VI) and O: acetate (Table VII) ratios are, theoretically, 3:1 and 1:1, respectively. In our hands it has never been possible to quantitatively recover the acetic acid formed in the oxidative reaction. The discrepancies between the theoretical and observed ratios are probably due to this variable recovery of acetate. In a large series of experiments the observed ratios have never been in excess of the theoretical ratios and, in general, approximate those reported in the tables.

** "Desicote", Beckman Instruments.

References p. 512.

TABLE VI

PRODUCTION OF ACETATE FROM PYRUVATE (FERRICYANIDE SYSTEM)

Each reaction flask contained, in μmoles: $MgCl_2$ (20), DPT (0.50), $NaHCO_3$ (250), enzyme (3.86 mg; specific activity of 340) and Li-pyruvate as shown. The final volume was 3.0 ml. Gas phase was 100% CO_2. After 7 min equilibration at 38° the taps were closed and 100 μmoles $K_3Fe(CN)_6$ were added from the side-arm. Incubation carried out for 2.0 hours.

Expt. No.	*μmoles pyruvate present*	*μmoles CO_2 evolved*	*μmoles acetate produced*	*CO_2/acetate*
1	5	10.3	4.77	2.16
2	10	18.8	7.22	2.60
3	15	18.3	7.02	2.61
4	20	19.7	7.64	2.58

TABLE VII

BALANCE FOR PYRUVATE OXIDATION (OXYGEN AS ELECTRON ACCEPTOR)

Each reaction flask contained, in μmoles: phosphate buffer of pH 7.5 (100), $MgCl_2$ (20), DPT (0.50) and enzyme (2.74 mg; specific activity = 400). The center well contained a filter paper wick soaked in 0.2 ml 6 *N* KOH. Gas phase was oxygen. After 7 min equilibration the taps were closed and Li-pyruvate was added from the side-arm. The final volume was 3.0 ml. Incubation was carried out for 3.0 hours. Values are corrected for no-substrate blank.

Expt. No.	*μmoles pyruvate present*	*μatoms O taken up*	*μmoles acetate formed*
1	5	6.08	3.8
2	10	8.84	4.9
3	20	14.2	10.8

That the volatile acid so formed and titrated was indeed acetic acid was shown by use of the specific enzymic method of VON KORFF[25]. In this procedure acetate is converted to acetyl CoA by the acetate-activating enzyme, which is completely specific for acetate. The acetyl CoA so formed is condensed with oxalacetate to form citrate.

Finally, a sample of the volatile acid recovered from the diffusion apparatus was converted to the ammonium salt. Ascending chromatography of the ammonium salt on Whatman No. 1 filter paper with ethanol–conc. NH_4OH (100:1) as the solvent system[26] was carried out for 18 hours. The paper was dried and the spots were visualized after the paper was first sprayed with the formaldehyde–ethanol spray of REID AND LEDERER[27] and then exposed to NH_3 vapors. Acetate was identified as a yellow spot with an R_F of 0.25; known ammonium acetate had the same R_F.

The pyruvic oxidase of H37Ra is not sedimented by centrifugation for 120 min at 144,000 × *g*. Treatment of the oxidase with Dowex-1 leads to no loss in activity. Although versene, when added to the PIP assay system, strongly inhibits the oxidase, dialysis of the enzyme solution against versene followed by dialysis against trishydroxymethylaminomethane buffer did not lead to a decrease in enzymic activity.

DISCUSSION

The pyruvic oxidase of H37Ra has characteristics in common both with oxidases isolated from animal tissue and with those isolated from other bacterial cells. Like the oxidase of pigeon breast muscle, it has not been resolved into several components[12]. It requires DPT and is somewhat stimulated by Mg^{++}. Unlike the animal enzyme, oxygen and ferricyanide are equally effective electron acceptors. PIP can be used but is

References p. 512.

inferior to the other acceptors[11]. Again, like the animal enzyme but unlike the unresolved bacterial enzyme[8,13] the pyruvic oxidase of H37Ra can also use DPN in stoichiometric amounts as an electron acceptor. This will be discussed in the following paper[18].

The mechanism by which oxygen serves as an electron acceptor remains obscure. No evidence has as yet been obtained for the presence of an auto-oxidizable coenzyme component which would account for this activity. Further work on this point is in progress.

Although versene is a powerful inhibitor of the pyruvic oxidase of H37Ra, dialysis of the enzyme against versene did not reduce the activity of the enzyme. These results may be interpreted in the following manner: Mg^{++}, which is required for enzymic activity, is tightly bound when the enzyme is not oxidizing pyruvate. During oxidation of pyruvate, Mg^{++} is partially displaced from the enzyme and thus the formation of the versene-Mg^{++} complex is facilitated. Additional evidence for this hypothesis is needed.

SUMMARY

A soluble pyruvic oxidase has been isolated from cell-free extracts of the H37Ra strain of *Mycobacterium tuberculosis* var. *hominis*. The enzyme catalyzes the oxidation of pyruvate to acetate; DPT is required for this reaction. Magnesium stimulates the reaction slightly, versene is a strong inhibitor. Ferricyanide, PIP and oxygen can all act as electron acceptors in the activity ratio of 2.5:1:3. No evidence exists that this enzyme can be separated into more than one enzymically active component.

REFERENCES

[1] F. LIPMANN, *Cold Spring Harbor Symposia Quant. Biol.*, 7 (1939) 248.
[2] H. CHANTRENNE AND F. LIPMANN, *J. Biol. Chem.*, 187 (1950) 757.
[3] P. K. STUMPF, *J. Biol. Chem.*, 159 (1945) 529.
[4] H. S. MOYED AND D. J. O'KANE, *J. Biol. Chem.*, 195 (1952) 375.
[5] H. S. MOYED AND D. J. O'KANE, *Bacteriol. Proc.*, (1951) 124.
[6] H. S. MOYED AND D. J. O'KANE, *J. Biol. Chem.*, 218 (1956) 831.
[7] I. C. GUNSALUS, *J. Cellular Comp. Physiol.*, 41, Suppl. 1, (1952) 113.
[8] S. KORKES, A. DEL CAMPILLO, I. C. GUNSALUS AND S. OCHOA, *J. Biol. Chem.*, 193 (1951) 721.
[9] I. C. GUNSALUS, in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, The Johns Hopkins Press, Baltimore, Md., 1954, p. 545.
[10] S. KORKES, A. DEL CAMPILLO AND S. OCHOA, *J. Biol. Chem.*, 195 (1952) 541.
[11] R. S. SCHWEET, M. FULD, K. CHESLOCK AND M. H. PAUL, *Phosphorus Metabolism*, 1 (1951) 246.
[12] V. JAGANNATHAN AND R. SCHWEET, *J. Biol. Chem.*, 196 (1952) 551.
[13] J. W. LITTLEFIELD AND D. R. SANADI, *J. Biol. Chem.*, 199 (1952) 65.
[14] S. OCHOA, *Advances in Enzymol.*, 15 (1954) 183.
[15] I. MILLMAN AND G. P. YOUMANS, *J. Bacteriol.*, 68 (1954) 411.
[16] I. MILLMAN AND G. P. YOUMANS, *J. Bacteriol.*, 69 (1955) 320.
[17] I. MILLMAN AND R. W. DARTER, *Proc. Soc. Exptl. Biol. Med.*, 91 (1956) 271.
[18] D. S. GOLDMAN, *Biochim. Biophys. Acta*, 27 (1958) 513.
[19] D. S. GOLDMAN, *Federation Proc.*, 16 (1957) 187.
[20] D. S. GOLDMAN, *J. Bacteriol.*, 71 (1956) 732.
[21] A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
[22] T. E. FRIEDEMANN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.
[23] D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc. (London), B*, 124 (1938) 397.
[24] W. BARTLEY, *Biochem. J.*, 53 (1953) 305.
[25] R. VON KORFF, *J. Biol. Chem.*, 210 (1954) 539.
[26] E. P. KENNEDY AND H. A. BARKER, *Anal. Chem.*, 23 (1951) 1033.
[27] R. L. REID AND M. LEDERER, *Biochem. J.*, 50 (1951) 60.

Received August 19th, 1957