

THE BREAKDOWN OF PYRUVATE BY *ASTEROCOCCUS MYCOIDES*, THE ORGANISM OF BOVINE PLEUROPNEUMONIA

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Summary

The pathway for pyruvate breakdown was investigated. Evidence was obtained that α -acetolactic acid does not participate in the pyruvate metabolism of this organism.

The pyruvate oxidation and dismutation systems were sensitive to inhibition by arsenite. Inorganic phosphate, Mg^{++} , or Mn^{++} , coenzyme A, diphosphopyridine nucleotide, cocarboxylase, and cysteine or glutathione were all required for maximum activity in the dismutation system. Although it was not possible to show an accumulation of acetyl phosphate it was concluded that pyruvate breakdown proceeds with the intermediate formation of acetyl coenzyme A and acetyl phosphate.

I. INTRODUCTION

It was shown previously that cell suspensions of *Asterococcus mycoides* oxidized pyruvate to acetate and carbon dioxide, and catalysed the anaerobic dismutation reaction of pyruvate to lactate, acetate, and carbon dioxide. Small amounts of acetoin were also formed anaerobically (Rodwell and Rodwell 1954). The mechanism for pyruvate breakdown is considered in this paper.

The pyruvate metabolism of many species of bacteria and of animal tissues has now been clarified. Korkes *et al.* (1950) and Korkes *et al.* (1951) analysed the pyruvate dismutation systems of *Escherichia coli* and *Streptococcus faecalis*. The complete system required cocarboxylase, Mg^{++} or Mn^{++} , coenzyme A, DPN, lactic dehydrogenase, pyruvic oxidase, and an acetyl acceptor system, e.g. oxalacetate and condensing enzyme, or inorganic phosphate and trans-acetylase. The part played by coenzyme A in acetyl transfer reactions has been the subject of recent reviews (Ochoa and Stern 1952; Welch and Nichol 1952).

Pyruvic oxidase of pigeon breast muscle was purified by Jaganathan and Schweet (1952). With ferricyanide as electron acceptor, the system required only cocarboxylase and Mg^{++} for pyruvate oxidation (Schweet and Cheslock 1952). The pyruvic oxidase of *Proteus vulgaris* also required only cocarboxylase, Mg^{++} , and a particulate cytochrome-containing fraction for the oxidation of pyruvate (Moyed and O'Kane 1952*a*, 1952*b*). Both preparations would acetylate and carry out the pyruvate dismutation reaction in the presence of coenzyme A and the necessary accessory enzymes, coenzymes, and acceptors.

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Krampitz (1951) suggested that α -acetolactic acid, which had been shown to be an intermediate in the formation of acetoin by *Staphylococcus aureus* (Watt and Krampitz 1947) and *Aerobacter aerogenes* (Juni 1950), might undergo a hydrolytic or phosphorylytic split to form acetic acid (or acetyl phosphate) and lactic acid, and thus become an intermediate in the dismutation reaction. Juni (1952) investigated this possibility, but could find no evidence for the participation of α -acetolactic acid in the dismutation reaction.

The mechanism suggested by Krampitz could be extended to include pyruvate oxidation. In the presence of lactic dehydrogenase, and an aerobic mechanism for the oxidation of reduced diphosphopyridine nucleotide, lactate, formed by the splitting of α -acetolactic acid, would be oxidized to pyruvate, which could then condense with another molecule of pyruvate and thus establish a cycle.

II. METHODS

The organism, growth conditions, and method of preparation of cell suspensions were the same as previously described (Rodwell and Rodwell 1954).

Dialysed enzyme extract.—The enzyme extract used to determine the cofactor requirements of the pyruvate dismutation system was prepared from lyophilized cells. One gram of lyophilized cells was ground in a chilled mortar for 5 min with 10 g 600-mesh alumina grinding powder ("Alloxite"), and 5.0 ml ice-cold phosphate buffer (pH 7.4). The mixture was extracted with 15 ml ice-cold phosphate buffer (pH 7.4) and centrifuged at 15,000 r.p.m. for 1 hr at 0°C. The clear yellow top layer was dialysed with constant agitation for 2 hr in cold water. Solid ammonium sulphate was added slowly to the dialysed extract to saturation. After centrifugation, the precipitate was dissolved in 5 ml water and the solution dialysed with agitation for 18 hr in cold water. The final extract contained 18 mg protein per ml (Biuret reaction using bovine albumin as standard).

α -Acetolactic acid.—This was prepared by the method of Krampitz (1948). The methyl ethyl ester was hydrolysed just before use with the equivalent amount of sodium hydroxide. α -Acetolactic acid remaining in manometer vessels at the end of the experiments was estimated manometrically by aniline citrate decarboxylation (Edson 1935).

Coenzyme A.—A concentrate of coenzyme A was prepared from pig liver by the method of Buyske *et al.* (1951) and its coenzyme A content assayed by the method of Handschumacher, Mueller, and Strong (1951).

Diphosphopyridine nucleotide (DPN).—A concentrate of DPN was prepared from yeast by the method of Williamson and Green (1940).

Coccarboxylase.—This was synthesized by the method of Weijlard and Tauber (1938).

Acetyl phosphate.—Acetyl phosphate was estimated by the method of Lipmann and Tuttle (1945).

III. EXPERIMENTAL

(a) *Metabolism of α -Acetolactic Acid*

Intact cell suspensions and cell suspensions which had been frozen and thawed were found to have no action on α -acetolactic acid at pH 7.4. The concentration of α -acetolactic acid remained constant during incubation, no carbon dioxide was evolved from the substrate anaerobically, nor was oxygen absorbed in its presence.

TABLE 1

EFFECT OF INHIBITORS ON PYRUVATE OXIDATION AND DISMUTATION REACTIONS

Intact cell suspensions. Rate of dismutation reaction determined in bicarbonate buffer with 5 per cent. CO_2 in N_2 (v/v) as gas phase (pH 7.4). Rate of oxidation reaction by oxygen uptake in phosphate buffer with air as gas phase. Rates calculated from linear portions of progress curves. Results expressed as percentage inhibition of rate in absence of inhibitor

Reaction	Inhibitor (M concentration)		Inhibition
Pyruvate dismutation	Arsenite	0.0001	39
	Arsenite	0.001	77
Pyruvate oxidation	Arsenite	0.00005	16
	Arsenite	0.0001	31
	Arsenite	0.00025	47
	Arsenite	0.0005	76
	Arsenite	0.001	86
	2,4-Dinitrophenol	0.0001	12
Pyruvate oxidation	2,4-Dinitrophenol	0.001	23
	2,4-Dinitrophenol	0.01	96
Pyruvate oxidation	Azide	0.0001	0
	Azide	0.001	20
	Azide	0.01	63
Pyruvate dismutation	Fluoride	0.04	6
	Fluoride	0.06	30
	Fluoride	0.08	57
	Fluoride	0.1	63
Pyruvate oxidation	Fluoride	0.005	95
	Fluoride	0.0075	100
Glucose oxidation	Fluoride	0.005	77
	Fluoride	0.0075	92
	Fluoride	0.01	100

(b) *Action of Inhibitors on Pyruvate Oxidation and Dismutation Reactions*

The effects of some inhibitors on the rates of the pyruvate oxidation and dismutation reactions with intact cell suspensions are shown in Table 1. Both reactions were sensitive to inhibition by arsenite. Pyruvate oxidation was relatively insensitive to inhibition by azide and by 2,4-dinitrophenol. The dismutation reaction was insensitive to fluoride. Pyruvate oxidation, in the experiment reported, was strongly inhibited by 0.005M fluoride. With the particular

batch of suspension used in this experiment and in the absence of fluoride, there was a long lag period before oxygen uptake began, whereas the rate of the dismutation reaction was linear from the beginning. The same batch of suspension was used to determine the sensitivity of glucose oxidation to inhibition by fluoride. The lag period was shorter with glucose than with pyruvate. The results are included in Table 1 for comparison. Glucose oxidation was not as sensitive to fluoride inhibition as was pyruvate oxidation. With another batch of suspension which exhibited a shorter lag period, pyruvate oxidation was more resistant to fluoride; the rate was inhibited 76 per cent. by 0.02M fluoride and 92 per cent. by 0.04M fluoride. A possible reason for this variation in sensitivity is discussed later.

(c) Formation of Acetyl Phosphate

Several attempts were made to detect the formation of acetyl phosphate during pyruvate oxidation, and in the anaerobic dismutation reaction. Both intact cell suspensions and frozen and thawed suspensions were used. Fluoride, in concentrations ranging from 0.02 to 0.1M, was included as an inhibitor of phosphorylases. In none of the experiments was any acetyl phosphate detected.

(d) Cofactor Requirements for the Dismutation Reaction

The effect of various cofactor additions on the rate of the dismutation reaction was tested with the ammonium sulphate precipitated and dialysed enzyme extract. The results are set out in Table 2. It was not possible to measure a true rate in this experiment because of carbon dioxide retention. Bound carbon dioxide was liberated when the rate of the reaction appeared to be decreasing and the results are expressed as the amount of carbon dioxide evolved during a fixed interval (90 min). Nevertheless the figures may be accepted as an approximation to the rates. It may be seen that replacement of phosphate by veronal buffer, omission of Mg^{++} and Mn^{++} , or of the coenzyme A preparation all decreased the reaction appreciably. Omission of DPN and cocarboxylase as well as coenzyme A resulted in a further decrease. Omission of all the cofactor components and cysteine and glutathione reduced the amount of carbon dioxide to that obtained in the complete system in the absence of pyruvate.

IV. DISCUSSION

It is concluded that the pyruvate metabolism of *A. mycoides* proceeds with the intermediate formation of acetyl coenzyme A. Although the coenzyme preparations used were very impure, the presence of Mg^{++} or Mn^{++} , coenzyme A concentrate, DPN concentrate, cocarboxylase, and cysteine and glutathione were all required for maximum activity. Greater activity was found in phosphate buffer than in veronal buffer. It is considered unlikely that this effect was due to an inhibition of the reaction by veronal. In comparative experiments with undialysed extracts, no difference in the rate of the dismutation reaction was found with phosphate and veronal buffers.

No attempt was made to determine whether coenzyme A and DPN were required for pyruvate oxidation, but it is logical to consider that, in the intact cell at least, the only difference in the oxidation and dismutation systems lies in the manner in which reduced DPN is re-oxidized. The pyruvate metabolism of *A. mycoides* would then resemble that of *Strep. faecalis* as described by Korkes *et al.* (1951).

TABLE 2
COFACTOR REQUIREMENTS FOR PYRUVATE DISMUTATION SYSTEM

Complete system: enzyme extract ($\equiv$ 18 mg protein); phosphate (pH 7.4) $200\mu\text{M}$; MgCl_2 $4\mu\text{M}$; MnCl_2 $2.5\mu\text{M}$; coenzyme A concentrate (CoA) $200\mu\text{g}$; cysteine $20\mu\text{M}$; glutathione (GSH) $10\mu\text{M}$; pyruvate $20\mu\text{M}$. Vol. 3.0 ml; gas phase, O_2 -free N_2

Components	CO_2 (μl)
Complete system	181
Without—phosphate*	61
Without— Mg^{++} , Mn^{++}	156
Without—coenzyme A†	92
Without—CoA, DPN, cocarboxylase	80
Without— Mn^{++} , Mg^{++} , CoA, DPN, cocarboxylase, cysteine, GSH	13
Without—pyruvate	12

* Phosphate buffer replaced by veronal buffer ($200\mu\text{M}$).

† Coenzyme A concentrate replaced by DPN concentrate ($= 0.04\mu\text{M}$ DPN) and cocarboxylase (approx. $0.1\mu\text{M}$).

The sensitivity of both the oxidation and dismutation systems to inhibition by arsenite is consistent with a requirement for α -lipoic acid (Reed *et al.* 1951; Moyed and O'Kane 1952*b*).

It is considered unlikely that fluoride inhibits the oxidation system itself. Since the sensitivity was found to vary with the duration of the lag period, and since glucose oxidation was less sensitive than with pyruvate, it is probable that fluoride exerted its effect by inhibiting the biosynthesis of some components of the oxidizing system, e.g. a flavine nucleotide.

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