PYRUVIC ACID METABOLISM

II. An Acetoin-forming Enzyme System in Streptococcus faecalis[1]

M. I. DOLIN[2] and I. C. GUNSALUS[2]

Laboratory of Bacteriology, Indiana University, Bloomington, Indiana

Received for publication April 26, 1951

The significance of acetoin formation in biological systems and its relationship to other routes of pyruvate metabolism are as yet unknown. Present evidence indicates the existence of three mechanisms for acetoin formation: (1) the system in yeast described by Neuberg (see Gross and Werkman, 1947) for which one molecule of pyruvate and one of acetaldehyde are required as substrates; (2) the system present in various animal tissues as studied in pig heart extracts (Green *et al.*, 1942) for which one molecule of pyruvate and one of acetaldehyde or two molecules of acetaldehyde act as substrates; and (3) the system found in several bacterial species for which two molecules of pyruvate act as substrate without the intermediate occurrence of free acetaldehyde. The latter system has been studied in cell-free extracts of *Aerobacter aerogenes* (Silverman and Werkman, 1941; Juni, 1950), in dried cell preparations of *Leuconostoc mesenteroides* (Beckhorn, 1948), and as reported in the present paper in a strain of *Streptococcus faecalis*.

Watt and Krampitz (1947) reported the occurrence in *Staphylococcus aureus* of an enzyme which decarboxylates *alpha*-acetolactic acid to acetoin plus CO_2. More recently Juni (1950) made a notable advance in elucidating the mechanism of acetoin formation by resolving the acetoin-forming system of *Aerobacter aerogenes* into two components, one of which forms *alpha*-acetolactate and CO_2 from pyruvate and a second which decarboxylates *alpha*-acetolactate to acetoin and CO_2.

An effect of pantothenic acid on the balance between pyruvate oxidation and the condensation to acetoin was indicated in the work of McElroy and Dorfman (1948). The oxidation of pyruvate by *Proteus morganii* deficient in pantothenic acid was found to yield a high RQ with the accumulation of acetoin. Pantothenate-sufficient cells gave a lower RQ (approximately 2), and little, if any, acetoin accumulated. The present knowledge of the acetoin-forming reaction has recently been reviewed by Ochoa (1951).

Among the enterococci, acetoin formation has not been considered to involve a major portion of the substrate utilized. Davis *et al.* (1939) reported acetoin formation from glucose with "massive" cell suspensions of *Streptococcus faecalis*. Campbell and Gunsalus (1944) found traces of acetoin as a product of citrate fermentation by *S. faecalis*, strain 10C1, and O'Kane (1949, 1950), with the same organism, has shown that the oxidation of glucose by resting cells deficient

[1] This work supported in part by the Office of Naval Research.

[2] Present address: Department of Bacteriology, University of Illinois, Urbana, Illinois.

in the "pyruvate oxidase factor" (O'Kane and Gunsalus, 1948), leads to the accumulation of pyruvate, lactate, and acetoin.

In the present work, a potent acetoin-forming system was found in extracts of *S. faecalis*. Pyruvate metabolism by cell suspensions and vacuum-dried cells is predominantly oxidative, to yield aerobically acetate and CO_2, or anaerobically, by dismutation, lactate, acetate, and CO_2. Cell-free extracts, from either dried or resting cells, however, contained an active acetoin-forming system, which accounted for the bulk of the pyruvate decomposed, and interfered with attempts to study the components of the pyruvate dehydrogenase system in cell-free extracts.

In order to clarify the relationship of the acetoin system to the pyruvate dehydrogenase system, the properties of the former have been studied and are reported in this paper.

METHODS

Culture and growth: To obtain cells for enzyme studies, a transfer *S. faecalis*, strain 10C1, was grown in 10 ml of AC.3 medium (1 per cent tryptone, 1 per cent yeast extract, 0.5 per cent K_2HPO_4, 0.3 per cent cerelose). After 8 hours' growth at 37 C, 5 ml were used to inoculate 5 liters of the AC medium which had been modified by the addition of 0.08 per cent sodium acetate and 5 ml salts B per liter. The cells were grown for 12 hours at 37 C, harvested with a Sharples centrifuge, and further treated as described in the following paragraphs.

Cell preparations and cell-free enzyme: To obtain dried cell preparations, the packed cells were suspended in a small amount of distilled water, dispersed in a petri dish, and dried *in vacuo* over drierite in a 250 mm desiccator.

Cell-free extracts were prepared directly from the freshly harvested cells by washing them once with distilled water and suspending in M/10 K_2HPO_4 pH 8.2 (or occasionally in M/10 phosphate buffer at pH 6.5) at a concentration equivalent to about 30 mg dried cells per ml, and the suspension exposed to sonic oscillation for 3 hours in the Raytheon 9 KC, 50-watt oscillator at approximately 6 C. The cell debris was removed by centrifugation for 30 minutes at the top speed of the Servall Model SS1 centrifuge and the supernate adjusted to pH 6.5 with saturated KH_2PO_4. The cell-free carbinol-forming system and ammonium sulfate fractions thereof were stored in the deepfreeze without loss of activity.

Manometric and chemical: Oxygen consumption and CO_2 evolution were followed in the conventional Warburg apparatus at 37 C with air as the gas phase. When chemical balances were run, the retained CO_2 was determined by tipping in 0.3 ml of 3 N H_2SO_4.

Pyruvic acid was purified by vacuum distillation and neutralized to brom thymol blue before use. Methylacetoxyethyl acetoacetate was kindly supplied by Dr. D. J. O'Kane. Before use, it was hydrolyzed to *alpha*-acetolactic acid by the method of Krampitz (1948).

The following colorimetric methods were used for substrates and products: pyruvic acid (Friedemann and Haugen, 1943); diacetyl (White, Krampitz, and

Werkman, 1946); lactic acid and acetaldehyde (Barker and Summerson, 1941); protein by quantitative biuret test (Robinson and Hogden, 1940); and orthophosphate by a modification of the Fiske and SubbaRow (1925) method. Acetoin was distilled from a slightly alkaline solution of the reaction mixture and measured by the authors' modification of a quantitative Voges-Proskauer reaction (Beckhorn, 1948).

EXPERIMENTAL RESULTS

Resting cell suspensions of *S. faecalis* grown under the optimum conditions for pyruvate dehydrogenase formation, as reported by Miller (1942), catalyze the oxidation of pyruvate according to the following equation:

$$\text{Pyruvate} + \tfrac{1}{2}O_2 \rightarrow \text{acetate} + CO_2 \qquad (1)$$

or anaerobically the dismutation as follows:

$$2 \text{ pyruvate} \rightarrow \text{lactate} + \text{acetate} + CO_2 \qquad (2)$$

O'Kane and Gunsalus (1948) have shown that the catalysis of both reactions (1) and (2) is dependent upon the presence in the growth medium of a nutrient of unknown composition which they have designated as "pyruvate oxidase factor" (POF). Cells harvested from a synthetic medium which supports optimum growth but lacks "pyruvate oxidase factor," do not oxidize pyruvate, but the oxidation may be activated without an appreciable lag period by the addition of "pyruvate oxidase factor." From this experiment a coenzyme function has been tentatively assigned to the factor. Natural materials have been subjected to fractionation, the partial purification of the pyruvate oxidase factor accomplished and shown to occur in several forms (Gunsalus, O'Kane, and Struglia, 1949).

In order to elucidate the steps in the pyruvate dehydrogenase reaction, the role of the "pyruvate oxidase factor" and the form in which it functions, it became essential to study pyruvate dehydrogenation in cell-free extracts. The extraction of cell suspensions, or dried cells, to prepare cell-free pyruvate dehydrogenase has revealed a third system which leads to acetoin formation, as follows:

$$2 \text{ pyruvate} \rightarrow 2\ CO_2 + \text{acetoin} \left(CH_3\overset{\overset{O}{\|}}{C}\text{—}\overset{\overset{OH}{|}}{\underset{\underset{H}{|}}{C}}\text{—}CH_3 \right) \qquad (3)$$

The acetoin-forming system in cell extracts prevents measuring the pyruvate dehydrogenase activity by the CO_2 released in dismutation (reaction 2). The route of anaerobic pyruvate breakdown can, however, be determined by measuring CO_2, lactate, and acetoin formation, or aerobically by measuring the O_2 taken up.

Table 1 shows the rate of oxygen uptake and percentage of substrate converted to each of the products as the preparations were simplified from a cell suspension, to dried cells and then to a cell-free extract, and ammonium sulfate frac-

tions of the extract. Briefly, under aerobic conditions, cell suspensions convert virtually all (86 per cent) of the pyruvate to acetate and CO_2 (reaction 1) even with high cell and substrate concentrations, which, as will be shown later, favors the acetoin system. Vacuum-dried cells still catalyzed predominantly, reaction 1, with acetoin formation accounting for one-fifth of the substrate (table 1, line 2). With a cell-free extract prepared by sonic disintegration of a cell suspension (see Methods) the products were predominantly acetoin, with about 30 per cent of the pyruvate undergoing oxidation (table 1, line 3). Ammonium sulfate precipitated extracts showed no oxygen uptake, and balances indicated nearly quantitative conversion of the substrate to acetoin (table 1,

TABLE 1

Pyruvate oxidation and acetoin formation by cells and extracts of Streptococcus faecalis

Per Warburg cup: Pyruvate, 50 to 100 μM
M/5 phosphate buffer, pH 6.5, 0.5 ml
Final volume, 2.5 ml; 37 C

1. Cell suspension, 1 ml; $MgSO_4$, 8 μM; $MnSO_4$, 0.45 μM.
2. Conditions as in 1.
3. Enzyme, 1 ml; glutathione 12.5 μM; cocarboxylase, 100 μg; FAD, 20 μg.
4. Enzyme, 0.5 ml (≅ 1.5 mg protein);† cocarboxylase, 20 μg; $MnSO_4$, 1 μM.

TYPE OF PREPARATION	Q_{CO_2}	MOLS/MOL OF PYRUVATE USED			PER CENT OF PYRUVATE	
		CO_2 formed	O_2 used	Acetoin formed	Oxidized	To acetoin
1. Resting cells, 43 mg	60	0.95	0.43*	0.035	86	7
2. Vacuum-dried cells, 50 mg	30	1.0	0.37	0.10	74	20
3. Cell-free extract, ≅ 30 mg cells	20	0.90	0.14	0.35	28	70
4. Cell-free enzyme, partially purified†	0	0.91	0	0.46	0	100

* Also 5 μM of lactate formed.

† Cell-free extract precipitated from 0.60 to 1 saturated ammonium sulfate, reprecipitated 2 × at 0.70 saturated ammonium sulfate, and dissolved in M/10 phosphate buffer, pH 6.5.

line 4). The high yield of acetoin per mole of pyruvate used shows that dismutation (reaction 2) had also been lost. Chemical analysis shows that this enzyme fraction does not form lactate, acetaldehyde, or diacetyl from pyruvate. More recently it has been found that extracts which no longer take up oxygen will catalyze the dismutation as indicated by lactate formation, or citrate accumulation under conditions which furnish a suitable acceptor for the acetyl generated in dismutation (Korkes, Stern, Gunsalus, and Ochoa, 1950).

The addition of glutathione partially reactivates the pyruvate dehydrogenase as measured by oxygen uptake (table 2). As indicated, glutathione at 5×10^{-3} molar will increase the respiration rate on pyruvate 4 to 5 fold, depending on the substrate concentration. It should also be noted that with low substrate concentration, 5 to 10 μM per cup (0.002 to 0.004 molar), the RQ approaches 2—characteristic of the oxidation of pyruvate. At increased substrate con-

centration, even in the presence of glutathione, the RQ is higher and acetoin accumulates whereas in the absence of glutathione, nearly all of the pyruvate is converted to acetoin.

Glutathione has been shown to stimulate the pyruvate dehydrogenase and not to inhibit the acetoin condensation. The rate of acetoin formation is unaffected by glutathione in systems lacking the pyruvate dehydrogenase.

Properties of the acetoin-forming system of S. faecalis: In addition to an interest in the occurrence of an active acetoin-forming system in extracts of *S. faecalis* cells, knowledge of its relationship to the pyruvate dehydrogenase system and the possibility of common steps in the two mechanisms require clarification. Therefore the properties, cofactor requirements, and possible intermediates in the acetoin-forming system have been examined.

TABLE 2

Effect of substrate concentration and of glutathione on pyruvate oxidation and acetoin condensation

Cell-free extract *S. faecalis*, strain 10Cl.
Per Warburg cup: Enzyme, 1 ml (≅ 30 mg cells)
M/5 phosphate buffer, pH 6.5, 0.5 ml
$MgSO_4$, 8 μM; $MnSO_4$, 0.45 μM
Pyruvate tipped from side arm
Final volume, 2.5 ml; 37 C.

ADDITIONS	INITIAL PYRUVATE	RQ	RATE O_2 UPTAKE
	μM		μl/hour
Cell-free enzyme	5	4.5	84
	10	3.9	108
	50	14	156
Cell-free enzyme + glutathione, 5×10^{-3} M	5	1.7	360
	10	1.8	360
	50	5.4	600

Cofactor requirements: The acetoin-forming system has been shown to require cocarboxylase and manganese as cofactors,—see experiments of Silverman and Werkman (1941) with extracts of *A. aerogenes*, and of Beckhorn (1948) with vacuum-dried cells of *Leuconostoc mesenteroides*. In the present experiments with extracts of *S. faecalis*, ammonium sulfate fractionation partially removed the cocarboxylase and metal components. The fraction precipitated between 0.6 to 1.0 saturated ammonium sulfate contained about 70 per cent of the acetoin-forming activity of the extracts and was activated about 2 fold by the addition of cocarboxylase (table 3, line 3). A second precipitation of this fraction to 0.7 ammonium sulfate saturation increased the cocarboxylase dependence and also rendered the preparation dependent upon the manganese for full activity. Dialysis of the twice ammonium sulfate precipitated fraction against M/10 citrate, pH 6.2 for two hours at 6 C, followed by dialysis against distilled H_2O to remove the citrate, gave a preparation almost devoid of activity unless both

manganese and cocarboxylase were added (figure 1). This fraction represented 40 per cent of the acetoin-forming activity of the cell extracts and showed a Q_{CO_2} (protein) of 530—about twice that of the initial extract. Near substrate saturation—above 0.35 molar pyruvate—this preparation shows a Q_{CO_2} protein of about 5,000. The curves for pyruvate, cocarboxylase, and manganese saturation of the over-all reaction as catalyzed by this enzyme preparation are shown in figures 2, 3, and 4, respectively. The half maximum saturation values for these preparations, calculated according to Lineweaver and Burk (1934), are for pyruvate 0.087 molar, for cocarboxylase 1×10^{-6} molar, and for manganese about 2×10^{-5} molar (the insolubility of manganese phosphate limited an

TABLE 3

Resolution of acetoin system for cocarboxylase and manganese

Per Warburg cup: Enzyme, 0.5 ml as indicated
M/5 phosphate buffer, pH 6.5, 0.5 ml
Cocarboxylase, 10 μg
$MnSO_4$, 1 μM
Pyruvate, 50 μM, tipped from side arm
Final volume, 2.5 ml; 37 C.

PREPARATION	SPECIFIC ACTIVITY*	ENZYME RECOVERY	RESOLUTION FOR:	
			Cocarboxylase	Mn
		%	%	%
1. Cell-free extract	255	100	46	4
2. Ppt. 0 to 0.6 saturated ammonium sulfate	142	34	—	—
3. Ppt. 0.6 to 1.0 sat. ammonium sulfate	640	67	61	7
4. #3, reppt to 0.7 sat. once	800	57	90	33
5. #4† dialyzed against citrate, then against distilled water	533	38	97	85

* μl CO_2 evolved per mg protein per hour (in this case = Q_{CO_2} based on protein).

† Dialyzed vs M/10 citrate pH 6.2 for 2 hours at 6 C then against distilled water at 6 C for 1 hour.

accurate determination of this value). The high pyruvate requirement agrees with the results of Werkman and Watt (1949) and of Juni (1951) for the *A. aerogenes* system and with Beckhorn's (1948) observations on the *Leuconostoc* system. The cocarboxylase dissociation is of the same order as that found by Lipmann (1939) for the pyruvate dehydrogenase of *Lactobacillus delbrueckii*.

Magnesium and cobaltous ions, when tested at a level of 8×10^{-4} molar, the concentration at which Mn gives its maximum activation, showed 65 and 20 per cent of the manganese activity, respectively. Other common divalent ions were virtually without activity. Concentration curves were not run for metals other than manganese.

It should be borne in mind that these preparations do not contain a single enzyme, but a system of enzymes, so that any calculations of dissociation of

the substrates or cofactors are at best approximations based on the assumption that later steps in the reaction chain are not rate limiting.

When the data for the saturation of the acetoin-forming system with pyruvate are analyzed according to the method of Lineweaver and Burk (1934), the reaction leading from pyruvate to acetoin shows first order kinetics, i.e., a plot of c/v against c gives a straight line (figure 2). It may be that both molecules

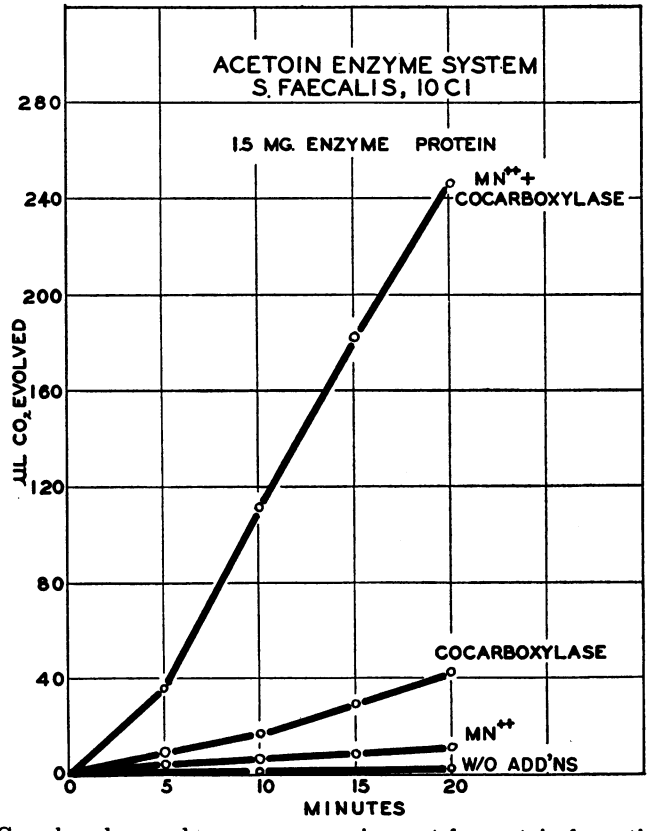

Figure 1. Cocarboxylase and manganese requirement for acetoin formation. Phosphate buffer, M/5, pH 6.5, 0.5 ml; enzyme, table 3, line 5, 1.5 mg protein; cocarboxylase, 10 μg; Mn, 1 μM; pyruvate, 50 μM tipped from side arm. Final volume, 2.5 ml. Temp 37 C.

of pyruvate do not have the same dissociation from the enzyme. If the dissociation constants differed greatly, the reaction rate would appear to be proportional to only one molecule of pyruvate. Since high pyruvate concentrations favor the acetoin system over pyruvate dehydrogenase, it seems probable that the condensation to *alpha*-acetolactate is the step with the high substrate saturation requirement.

No phosphate requirement could be shown for the acetoin system with a phosphate-free ammonium sulfate fractionated preparation—less than 1 μg of P per ml in final reaction mixture.

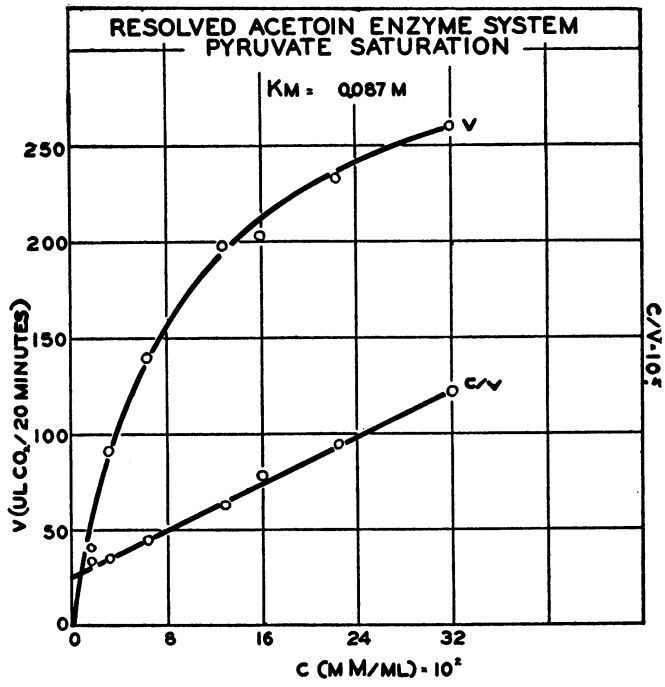

Figure 2. Pyruvate saturation curve for acetoin-forming system. Phosphate buffer, M/5, pH 6.0, 0.5 ml; enzyme, table 3, line 5, 300 μg protein; cocarboxylase, 20 μg; Mn, 2 μM; pyruvate tipped from side arm. Final volume, 2.5 ml. Temp 37 C.

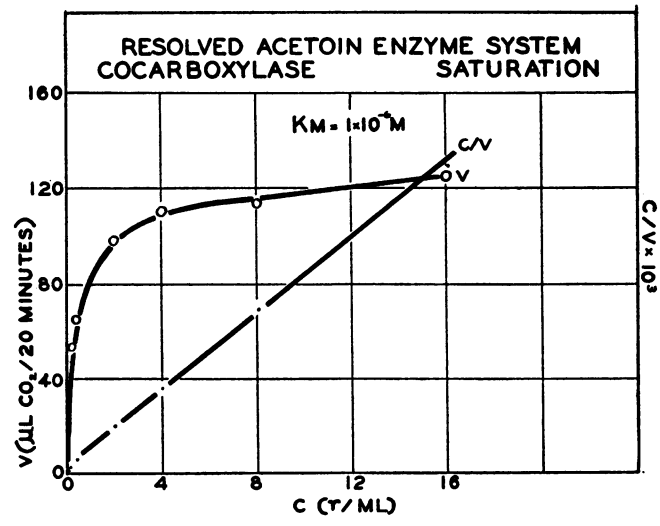

Figure 3. Cocarboxylase saturation curve for acetoin system. Phosphate buffer, M/5, pH 6.0, 0.5 ml; enzyme, table 3, line 5 (150 μg protein); cocarboxylase as indicated; $MnSO_4$, 2 μM; pyruvate, 560 μM, tipped from side arm. Final volume, 2.5 ml. Temp 37 C.

Pyruvate oxidase factor: As indicated in the introduction, O'Kane and Gunsalus (1948) have shown that pyruvate oxidase factor is necessary for the oxidative decarboxylation of pyruvate by cells of *S. faecalis*, but not for acetoin formation with glucose as the substrate (O'Kane, 1949). In order to investigate this point for the pyruvate to acetoin reaction, an extract was prepared in the usual manner from cells grown in the synthetic medium of O'Kane and Gunsalus (1948). The extract from these cells contained a highly active acetoin-forming system, and, further, the rate of the reaction was not altered in the presence of

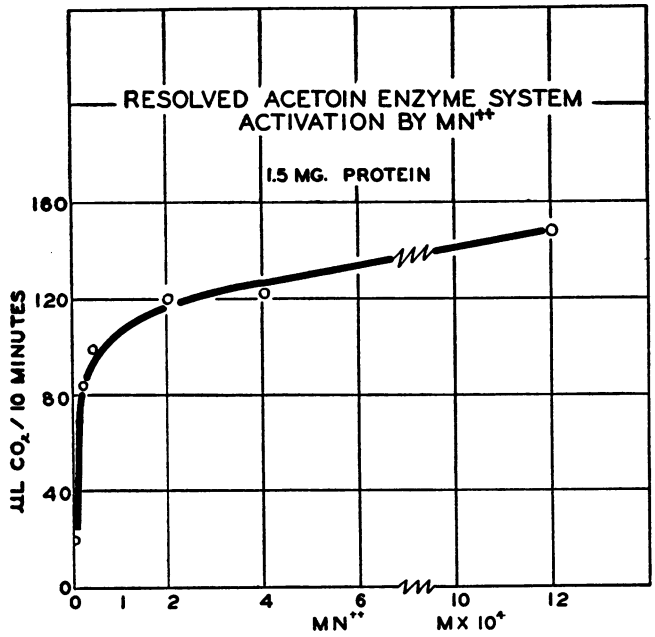

Figure 4. Manganese saturation for acetoin system. Phosphate buffer, M/5, pH 6.5; enzyme resolved as described in text, table 3, line 5, 1.5 mg protein; cocarboxylase, 10 μg; $MnSO_4$ as indicated; pyruvate, 50 μM, tipped from side arm. Final volume, 2.5 ml. Temp 37 C.

yeast extract. Thus, the pyruvate oxidase factor is not a component of the carbinol system.

pH optimum: The optimum pH for CO_2 release from pyruvate in the presence of 300 μg of the ammonium sulfate fractionated acetoin system (table 3, line 4) is shown in figure 5. Phosphate buffer was used for the experiments from pH 6.0 to 7.4, and below pH 6, a phosphate-acetate mixture. Correction was made for CO_2 retention by tipping in 3 N acid while the reaction was still on a linear portion of the curve. The optimum pH for the over-all reaction is between pH 5.5 and 6.0 (figure 5). Silverman and Werkman (1941) reported 5.6 to 6 to be optimum for the aerogenes enzyme, and Beckhorn (1948), 5.5 to 5.7 for the *L. mesenteroides* system.

Mechanism of Acetoin Formation

The mechanism of acetoin formation has been reviewed briefly in the introduction. In order to determine whether the enzyme from *S. faecalis* conforms to any of the known systems, several aspects of the reaction have been investigated, including tests for the participation of acetaldehyde as an intermediate and the presence of an *alpha*-acetolactic acid decarboxylase.

Acetaldehyde: As mentioned previously, acetaldehyde acts as substrate in the yeast and animal carbinol systems, but not in the bacterial systems so far studied. To determine if acetaldehyde functions in the carbinol system of *S. faecalis*, it was added to the test system and the balance of pyruvate used to CO_2, and acetoin formed measured. The ratio of CO_2 released to acetoin formed

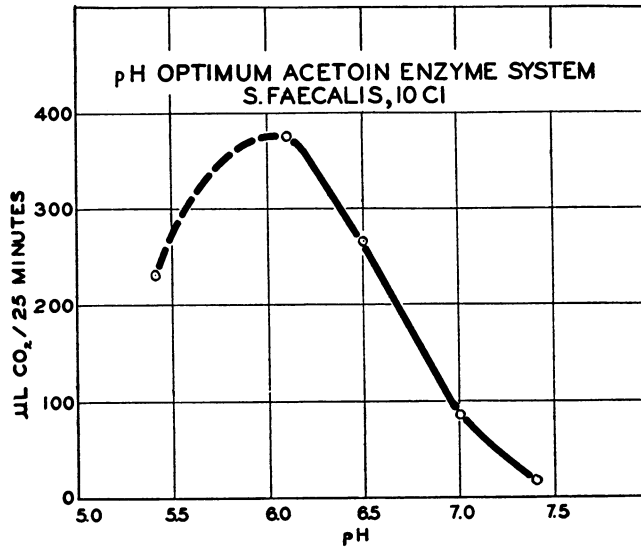

Figure 5. Influence of pH on rate of acetoin formation. Buffers, as indicated in text; enzyme, table 3, line 4 (300 μg protein); cocarboxylase, 20 μg; Mn, 2 μM; pyruvate, 100 μM tipped from side arm. Final volume, 2.5 ml. Temp 37 C.

should be 0, if two moles of aldehyde condense, 1, if one mole each of acetaldehyde and pyruvate condense, and 2, if two moles of pyruvate condense and acetaldehyde does not enter the reaction.

As shown in table 4, acetaldehyde as substrate did not yield acetoin. With pyruvate plus acetaldehyde, the ratio of pyruvate used to CO_2 and acetoin formed was 2:2:1—the same as that found with pyruvate as substrate. The rate of CO_2 evolution with the mixture of pyruvate and acetaldehyde was the same as that with pyruvate alone. Thus acetaldehyde did not alter the balance for acetoin formation from pyruvate, and as far as can be determined from an experiment of this type, acetaldehyde does not occur as an intermediate in acetoin formation by the *S. faecalis* system.

Acetolactic acid decarboxylase: Cell extracts and the fractions precipitated by

the 0 to 0.6 and the 0.6 to 1.0 saturated ammonium sulfate were found to decarboxylate synthetic DL-acetolactic acid (of about 90 per cent purity) to acetoin. As shown in table 5, only one of the isomers was decarboxylated as indicated by the liberation of 0.5 mole of CO_2 per mole of *alpha*-acetolactate added when the reaction was allowed to proceed to completion. The addition of H_2SO_4 de-

TABLE 4

Acetaldehyde and pyruvate as substrates for the acetoin system of Streptococcus faecalis

Per Warburg cup: Enzyme,* 0.5 ml (300 μg protein); cocarboxylase, 20 μg; $MnSO_4$, 2 μM
M/5 phosphate buffer, pH 6.0, 0.5 ml
Final volume, 2.5 ml; 37 C.

SUBSTRATE	PYRUVATE USED	CO_2 FORMED	ACETOIN FORMED	RATIO PYRUVATE/CO_2/ACETOIN
	μM	μM	μM	
1. Pyruvate, 48 μM	11	11	5.9	1.9/1.9/1
2. Acetaldehyde, 50 μM	—	0	0	—
3. (1) + (2) Exp. 1	12	14	6.5	1.9/2.2/1
Exp. 2	21	19	9	2.3/2.1/1

* 0.6 to 1.0 saturated ammonium sulfate precipitate reprecipitated to 0.70 saturation once and dissolved in M/10 phosphate buffer, pH 6.0.

TABLE 5

Decarboxylation of Acetolactic acid by Streptococcus faecalis, 10C1, enzyme

Per Warburg cup: Enzyme, 0.5 ml fraction 2, table 3
M/5 phosphate buffer, pH 6.0
≅ 30 μM acetolactate tipped from side arm
Final volume, 2.5 ml; 37 C.

CATALYST	PRODUCTS FORMED	
	CO_2	Acetoin
	μM	μM
Enzyme	17.4	14.5
Enzyme—followed by H_2SO_4*	33.5	28
H_2SO_4*	35	31

* 0.5 ml 3 N H_2SO_4.

carboxylated the isomer of acetolactate not attacked by the enzyme, or on a fresh sample of acetolactate caused the complete decarboxylation, i.e., 1 mole of CO_2 per mole of substrate. In these experiments, the distillation step was omitted in the acetoin determination, and, for the enzymatically decarboxylated acetolactate, the determination was performed on unacidified aliquots of the reaction mixture. Appropriate enzyme blanks were run to determine the recovery of acetoin standards.

The spontaneous rate of acetolactate decarboxylation by boiled enzyme is about one-tenth of the enzymatic rate. The specificity of the *alpha*-acetolactic acid decarboxylase agrees with the results of Juni (1950) for *A. aerogenes*.

Watt and Krampitz (1947) have reported the presence of an acetolactate decarboxylase in *Staphylococcus aureus*, which in common with other β-keto acid decarboxylases, requires manganous ions for activation. Surprisingly enough, the *S. faecalis* enzyme fractions, shown to require both manganous ions and cocarboxylase to form acetoin from pyruvate (table 3), decarboxylate acetolactate without added metals. The acetolactate was not contaminated with metals as indicated by its inability to activate the pyruvate to acetoin reaction

TABLE 6

Inhibition of acetoin formation from pyruvate and from acetolactate

Per Warburg cup: Enzyme, 0.5 ml, as indicated
M/5 phosphate buffer, pH 6.0, 0.5 ml
Inhibitors or inhibitor-metal mixtures incubated with enzyme 20 minutes before substrate tipped from side arm
Total volume, 2.5 ml; 37 C.

Pyruvate experiment: use 500 μg protein, enzyme fraction 5, table 3, plus cocarboxylase, 20 μg; $MnSO_4$, 2 μM.

Acetolactate experiment: cell-free enzyme from deficient cells (text), 0.5 ml ≅ 0.65 mg dried cells.

ADDITIONS	CO_2/35 MINUTES	PER CENT INHIBITION
	μl	
Pyruvate, 50 μM	146	—
Pyruvate + phenylpyruvate, 10^{-2} M	37	75
Pyruvate + phenylpyruvate, 10^{-3} M	127	13
Pyruvate + 8-hydroxyquinoline, 2×10^{-3} M	67	54
Pyruvate + TETDS,* 1 ml sat. aq. soln.	136	7
Acetolactate, 50 μM	310	—
Acetolactate + 8-hydroxyquinoline, 8.0×10^{-5} M	173	44
Acetolactate + 8-hydroxyquinoline, 2.4×10^{-4} M	94	70
Acetolactate + 8-hydroxyquinoline, 2.4×10^{-4} M + 1.2×10^{-3} M CO^{++}	320	0
Acetolactate + 8-hydroxyquinoline, 2.4×10^{-4} M + 1.2×10^{-3} M Zn^{++}	260	16
Acetolactate + 8-hydroxyquinoline, 2.4×10^{-4} M + 1.2×10^{-3} M Fe^{++}	191	38
Acetolactate + 8-hydroxyquinoline, 2.4×10^{-4} M + 1.2×10^{-3} M Mn^{++}	93	70
Acetolactate + 8-hydroxyquinoline, 2.4×10^{-4} M + 1.2×10^{-3} M Mg^{++}	93	70
Acetolactate + phenylpyruvate, 10^{-2} M	315	0

* Tetraethylthiuram disulfide ("antabuse").

of a manganous deficient preparation. Furthermore, treatment of the acetolactate with 8-hydroxyquinoline followed by chloroform extraction to remove metals did not influence the rate of decarboxylation.

Indirect evidence for a metal requirement of the *alpha*-acetolactate decarboxylase of *S. faecalis* was obtained by treating the enzyme with 8-hydroxyquinoline. As shown in table 6, 2.4×10^{-4} molar 8-hydroxyquinoline inhibited the rate of acetolactate decarboxylation 70 per cent. The addition of 10 equivalents of cobaltous ions completely restored the activity—zinc and ferrous ions

were also partially effective. Manganous ions, however, did not restore decarboxylase activity. It is perhaps significant that the order of effectiveness of the ions coincides with the order of stability of their complexes with 8-hydroxyquinoline (Mellor and Maley, 1948). The lack of activity of manganese in relieving the inhibitory effect of 8-hydroxyquinoline on decarboxylation is not readily explained unless the enzyme may contain bound manganese which complexes with the 8-hydroxyquinoline and the complex is not reversed by added manganous ions. This point will require further elucidation.

Phenylpyruvic acid, reported by Werkman and Watt (1949) to inhibit acetoin formation from pyruvate, and tetraethylthiuram disulfide (TETDS), an aldehyde oxidase inhibitor (Kjeldgaard, 1949), were tested as possible inhibitors of pyruvate dehydrogenation and of acetoin formation from both acetolactate and pyruvate. Phenylpyruvic acid inhibited the acetoin-forming system about 75 per cent at 10^{-2} molar and about 15 per cent at 10^{-3} molar (table 6). In an extract which contained the pyruvate oxidase, the oxidase and acetoin systems were inhibited to approximately the same degree. Neither the pyruvate dehydrogenase nor the acetoin-forming system was inhibited by TETDS. The rate of acetolactate decarboxylation was not affected by either the TETDS or by phenylpyruvic acid.

As mentioned in the introduction, Juni (1950) was able to separate the pyruvate to acetoin system of *A. aerogenes* into 2 fractions one of which forms acetolactic acid from pyruvate and the second of which decarboxylates acetolactic acid. In the present study the separation of the acetoin-forming system of *S. faecalis* was not attempted. However, the 2 ammonium sulfate fractions (table 3, lines 2 and 3) show significantly different rates of CO_2 release from pyruvate and acetolactate.

The 0 to 0.6 saturated ammonium sulfate fraction released CO_2 from acetolactate 8.5 times as fast as from pyruvate (i.e., at equal substrate concentration the ratio of the rate of CO_2 release from acetolactate and from pyruvate is 8.5). The fraction precipitated by 0.6 to 1.0 saturation gave a ratio of 3.8, thus one would assume that a greater excess of acetolactate decarboxylase exists in the 0 to 0.6 saturated ammonium sulfate precipitate. The substrate concentration required for one-half saturation of the acetolactate decarboxylase is lower (3×10^{-3} molar) than that required for the whole system of enzymes from pyruvate (9×10^{-2} molar). Thus in the over-all reaction, the initial enzymes, either for pyruvate decarboxylation or for condensation to yield acetolactate, may not be saturated with substrate at the levels used, and therefore the rates as measured may be fictitiously low. In any event, under most circumstances it would seem that the earlier enzymes in the system are the rate-limiting ones.

DISCUSSION

An interesting feature of the acetoin-forming system of *S. faecalis* is that this reaction is practically undetectable in resting cell suspensions. With these suspensions the pyruvate metabolism consists, aerobically, of an oxidation to acetate and CO_2 and, anaerobically, of a Krebs dismutation, to acetate, CO_2

and lactate in equimolar amounts. The same reactions occur with "deficient" cells (harvested from synthetic medium) which require the "pyruvate oxidase factor" for both the oxidation and dismutation. With dried cells from either complete or synthetic medium, part of the substrate enters the acetoin system and with cell-free extracts, practically the entire reaction consists of acetoin formation. These results may arise from a low permeability of intact cells for pyruvate, coupled with the higher substrate saturation level of the acetoin-forming system. Somewhat the same situation exists with regard to the decarboxylation of *m*-hydroxyphenylalanine by *S. faecalis*, R (Sloane-Stanley, 1949), in which the reaction can be detected in acetone-dried cells, but not in living cell suspensions.

McElroy and Dorfman (1948) have suggested that pantothenate is concerned with the further metabolism of acetoin or some closely related compound since acetoin accumulates during pyruvate oxidation by pantothenate deficient *Proteus morganii*. With cell-free extracts from *S. faecalis*, the evidence seems to indicate that carbinol condensation and pyruvate oxidation are competing reactions controlled in part by the substrate concentration. It may be, however, that the initial steps in the two pathways are common.

The acetoin-forming system of *S. faecalis* resembles the other bacterial systems so far described in its requirement for cocarboxylase and manganese, its high pyruvate requirement, and the nonparticipation of acetaldehyde as an intermediate. There is no requirement for phosphate. The lack of activity of the "pyruvate oxidase factor" in the acetoin system emphasizes the observations that this factor is specific for pyruvate oxidation. If the initial step in the 2 routes is common, the pyruvate oxidase factor must function after the branching of the pathways. Acetolactic acid appears to be an intermediate in acetoin formation by *S. faecalis* as shown for *A. aerogenes* by Juni (1950). Carbon dioxide and acetoin analyses suggest that only one isomer of the synthetic DL-acetolactate is attacked. Inhibitor studies with 8-hydroxyquinoline indicate a possible requirement for a divalent ion for *alpha*-acetolactate decarboxylase, but resolution of a metal from the enzyme by direct means has not been accomplished.

Although no attempt was made to isolate an acetolactate-forming system from *S. faecalis*, the assumption has been made throughout this paper that the mechanism of acetoin formation follows the pattern found in *A. aerogenes*. The similarity of the bacterial acetoin systems studied suggests that the same mechanism holds for all. Juni (1950) has shown that in all the bacterial species he examined, those which form acetoin, also decarboxylate acetolactate and conversely that nonacetoin-forming strains do not decarboxylate acetolactate.

SUMMARY

Cell suspensions and vacuum-dried cells of *Streptococcus faecalis*, strain 10C1, oxidize pyruvate to acetate and CO_2, but cell-free extracts from these cells catalyze the breakdown of pyruvate predominantly to acetoin and CO_2. Lower substrate concentration and the addition of glutathione shift the balance, with extracts, to favor pyruvate oxidation.

By ammonium sulfate precipitation and dialysis against citrate buffer, the acetoin system has been rendered almost completely inactive in the absence of cocarboxylase and manganous ions. In the presence of these cofactors, the reaction is:

$$2 \text{ pyruvate} \rightarrow \text{acetoin} + 2CO_2$$

The extracts contain an active *alpha*-acetolactic acid decarboxylase. Acetaldehyde does not serve as an intermediate in acetoin formation. Neither phosphate nor "pyruvate oxidase factor" is required for acetoin formation. Indirect evidence has been obtained for the function of divalent ions in the *alpha*-acetolactate decarboxylase.

Saturation requirements have been determined for the components of the system. Expressed as the molar concentration necessary for half maximum activation, they are: pyruvate, 9×10^{-2} M; acetolactate, 3×10^{-3} M; cocarboxylase, 1×10^{-6} M; manganese, approximately 2×10^{-5} M.

REFERENCES

BARKER, S. B., AND SUMMERSON, W. H. 1941 The colorimetric determination of lactic acid in biological material. J. Biol. Chem., **138**, 535–554.

BECKHORN, E. J. 1948 Dissertation. Cornell University.

CAMPBELL, J. J. R., AND GUNSALUS, I. C. 1944 Citric acid fermentation by Streptococci and Lactobacilli. J. Bact., **48**, 71–76.

DAVIS, J. G., ROGERS, H. J., AND THIEL, C. C. 1939 Production of diacetyl by faecal streptococci. Nature, **143**, 558.

FISKE, C. H., AND SUBBAROW, Y. 1925 The colorimetric determination of phosphorus. J. Biol. Chem., **66**, 375–400.

FRIEDEMANN, T. E., AND HAUGEN, G. E. 1943 Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem., **147**, 415–442.

GREEN, D. E., WESTERFELD, W. W., VENNESLAND, B., AND KNOX, W. E. 1942 Carboxylases of animal tissues. J. Biol. Chem., **145**, 69–84.

GROSS, N. H., AND WERKMAN, C. H. 1947 Isotopic composition of acetylmethylcarbinol formed by yeast juice. Arch. Biochem., **15**, 125–131.

GUNSALUS, I. C., O'KANE, D. J., AND STRUGLIA, L. A. 1949 Unpublished data.

JUNI, E. 1950 Alpha acetolactic acid, an intermediate in acetylmethylcarbinol formation. Federation Proc., **9**, 396.

JUNI, E. 1951 Personal communication.

KJELDGAARD, N. O. 1949 Inhibition of aldehyde oxidase from liver by tetraethylthiuram disulfide (antabuse). Cited in Chem. Abstracts, **44**, 6450.

KORKES, S., STERN, J. R., GUNSALUS, I. C., AND OCHOA, S. 1950 Enzymatic synthesis of citrate from pyruvate and oxalacetate. Nature, **166**, 439.

KRAMPITZ, L. O. 1948 Synthesis of alpha acetolactic acid. Arch. Biochem., **17**, 81–85.

LINEWEAVER, H., AND BURK, D. 1934 The determination of enzyme dissociation constants. J. Am. Chem. Soc., **56**, 658–666.

LIPMANN, F. 1939 An analysis of the pyruvic acid oxidation system. Cold Spring Harbor Symposia Quant. Biol., **7**, 249–259.

MCELROY, O. E., AND DORFMAN, A. 1948 The role of pantothenic acid in the metabolism of pyruvate by *Proteus morganii*. J. Biol. Chem., **173**, 805–806.

MELLOR, D. P., AND MALEY, L. 1948 Order of stability of metal complexes. Nature, **161**, 436–437.

MILLER, A. K. 1942 Dissertation. Cornell University.

OCHOA, S. 1951 Biological mechanisms of carboxylation and decarboxylation. Physiol. Revs., **31**, 56–106.

O'KANE, D. J., AND GUNSALUS, I. C. 1948 Pyruvic acid metabolism. A factor required for oxidation by *Streptococcus faecalis*. J. Bact., **56**, 499–506.

O'KANE, D. J. 1949 Oxidation of glucose by Streptococci unable to oxidize pyruvate. Proc. 49th Annual Meeting Soc. of Am. Bact. 43–44.

O'KANE, D. J. 1950 Influence of the pyruvate oxidation factor on the oxidative metabolism of glucose by *Streptococcus faecalis*. J. Bact., **60**, 449–458.

ROBINSON, H. W., AND HOGDEN, C. G. 1940 The biuret reaction in the determination of serum proteins. J. Biol. Chem., **135**, 707–725.

SILVERMAN, M., AND WERKMAN, C. H. 1941 The formation of acetylmethylcarbinol from pyruvic acid by a bacterial enzyme preparation. J. Biol. Chem., **138**, 35–48.

SLOANE-STANLEY, G. H. 1949 The substrate specificity of the tyrosine decarboxylase of *Streptococcus faecalis*. J. Bact., **44**, 373–377.

WATT, D., AND KRAMPITZ, L. O. 1947 Alpha acetolactic acid, an intermediate in acetylmethylcarbinol formation. Federation Proc., **6**, 301–302.

WERKMAN, C. H., AND WATT, D. 1949 Personal communication.

WHITE, A. G. C., KRAMPITZ, L. O., AND WERKMAN, C. H. 1946 Method for the direct determination of diacetyl in tissue and bacterial filtrates. Arch. Biochem., **9**, 229–234.