THE OXIDATIVE DISSIMILATION OF SERINE BY PASTEURELLA PESTIS[1]

H. B. LEVINE,[2] R. WEIMBERG, J. H. DOWLING, MARGERY EVENSON,
M. ROCKENMACHER, AND H. WOLOCHOW

Department of Bacteriology, University of California, Berkeley, California

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A chemically defined culture medium which supported high viable cell yields of *Pasteurella pestis* was described in a recent communication (Rockenmacher *et al.*, 1952). While the amino acid serine was a component of this medium, further studies by one of us (Rockenmacher, 1952) have indicated that it is nonessential for the growth of 27 strains tested in several defined media less complex than the above. However, Rao (1940*a*,*b*) observed that a strain (no. 120/H) of *P. pestis* oxidized serine more rapidly than 15 other amino acids which were examined.

We have confirmed and extended Rao's observations, using an avirulent strain (no. A-1122) of *P. pestis*. The data presented in this report show that serine is oxidized completely by this organism through the intermediary steps of pyruvate and acetate. Attempts to demonstrate the intermediary formation of acetate were unsuccessful when washed resting cells were used. Cell-free extracts prepared by grinding the cells with powdered pyrex glass were enzymatically inactive on pyruvate, and poor activity was observed after grinding with levigated alumina (Hayaishi and Stanier, 1951).

A survey for other methods of lysis led us to the use of glycine as a lytic agent (Maculla and Cowles, 1948; Wolochow, 1950; Gordon *et al.*, 1951*a*,*b*). Using *P. pestis* as the experimental organism, we have repeated the findings of these workers that washed packed cells suspended in molar solutions of glycine underwent lysis. Preparations which were lysed extensively were found to be inactive enzymatically on pyruvate or acetate, but when the duration of exposure of the cells to glycine was shortened so that only a small amount of lysis occurred, the cells retained the capacity to oxidize pyruvate but lost completely their capacity to oxidize acetate. Under these conditions acetate accumulated from pyruvate in the presence of cells. Evidence is presented which indicates that acetate is formed by the oxidative decarboxylation of pyruvate.

EXPERIMENTAL METHODS

Washed cells of *P. pestis*, strain A-1122, harvested from heart infusion broth (Difco) after 20 to 22 hours of aerated growth at 30 C were used for manometric studies. The suspension was diluted with M/15 phosphate buffer at pH 6.8 to a predetermined turbidity on the Klett-Summerson photoelectric colorimeter (15 mm I.D. cuvette, 660 mμ filter) and stored in the refrigerator overnight prior to use.

Treatment with glycine was carried out by the general procedures described by Gordon *et al.* (1951*a*). The washed packed cell mass was weighed and suspended in 5 times its weight of 1 M glycine previously adjusted to pH 7.5 with 10 per cent K_2HPO_4. The suspension then was agitated slowly at room temperature for varying periods of time. Pyruvate oxidase activity appeared to be more consistent if the cells and glycine were chilled in the refrigerator overnight prior to mixing. In preliminary experiments the glycine-cell suspension was used directly for the measurement of oxidative activity. In later work, the glycine was removed from the cells by dialyzing approximately 15 ml of the suspension for 1½ hours at 0 to 5 C against a total of 6 L of flowing distilled water. After dialysis, the cell suspension was adjusted to pH 6.8 by the dropwise addition of 10 per cent KH_2PO_4.

Gas measurements were made using a Warburg respirometer according to the procedures described by Umbreit *et al.* (1949). Unless stated otherwise, measurements were made at pH 6.8. The pH was varied in studies on its effect by the addition of dilute NaOH or H_2SO_4 to the

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cell-substrate mixture. In studies on deaminase activity at pH 9.1, all components of the system were dissolved or suspended in M/15 K_2HPO_4. The pH values were measured with the glass electrode.

Inhibitors, when used in an experiment, usually were added to the cells from a second side arm of the Warburg vessel approximately 15 minutes before the addition of substrate. The concentration of inhibitor given is the final concentration after mixture with cells and substrate. 2,4-Dinitrophenol (DNP) was dissolved in phosphate buffer at pH 6.8. Arsenious trioxide was dissolved in a small amount of approximately M/10,000 KOH and adjusted to volume with phosphate buffer. The solution was prepared at 20 times the desired strength and was diluted further with buffer prior to use to facilitate solution and maintain the final pH at that of the diluent buffer.

Ammonia was determined colorimetrically by the method described by Johnson (1941). The colorimetric method of Friedemann and Haugen (1943) was used for the estimation of pyruvic acid. This compound was isolated as the 2,4-dinitrophenylhydrazone according to the procedures described by Chargaff and Sprinson (1943*a*). All colorimetric measurements were made with the Coleman spectrophotometer, Model 14, using matched square cuvettes with an internal light path of 13 mm.

Acetate was isolated by transferring a 2 ml aliquot of the reaction mixture from the Warburg vessel to the side bulb of a vacuum microlyophil apparatus. This was acidified by the addition of 0.3 ml of N/2 H_2SO_4 after which it was shell frozen at -78 C and distilled at 7 to 10 μ pressure for 5 hours. The volatile acid, trapped in the condensing portion of the apparatus (maintained at -78 C), was identified qualitatively by micro-Duclaux procedures. The distillate from the microlyophil was made to a total volume of 35 ml and transferred to a micro-still. Three successive 10 ml fractions were distilled and titrated against N/250 $Ba(OH)_2$. Controls on acetate in equivalent concentrations both in the presence and absence of the same concentration of cellular material were acidified, vacuum-distilled, and analyzed as above. The total amount of volatile acid formed after the oxidation of pyruvate was determined by microtitrimetric procedures.

All substrates except L-serine and sodium pyruvate were commercial preparations of high quality. L-Serine was obtained from Dr. D. M. Greenberg, University of California. Sodium pyruvate was prepared from a commercial source of pyruvic acid by the method of Robertson (1942).

RESULTS AND DISCUSSION

Resting cells of *P. pestis*, strain A-1122, were observed to oxidize serine rapidly (figure 1). The rate of oxidation was approximately the same when either 5 μmoles of L-serine or 10 μmoles of DL-serine were supplied as the substrate. It was observed also that the total amount of oxygen consumed during the oxidation of the 5 μmoles of the L-isomer was the same as during the oxidation of 10 μmoles of the DL-isomer. Thus, the oxidative reaction appeared to be specific for the natural stereoisomer of serine. No racemase activity was shown within the time limits of the experiment, and the D-isomer of serine did not affect the oxidation of the L-isomer.

Figure 1 illustrates further that the total oxygen consumption per unit weight of substrate utilized was increased in the presence of 2,4-dinitrophenol. The possible action of dinitrophenol as an assimilative inhibitor has been reported by Clifton (1937, 1946), Doudoroff (1940), and Bernstein (1944). While evidence for inhibition of assimilation by dinitrophenol was contradictory when different bacterial species and different substrates were examined (Clifton, 1951), we have noted, as will be described, that serine, pyruvate, and acetate were oxidized to completion by *P. pestis* in the presence of dinitrophenol.

The effect of dinitrophenol concentration on the oxygen consumption and carbon dioxide and ammonia production by the cells on serine is shown in table 1. Theoretical gas values for complete oxidation were obtained at approximately M/10,000 dinitrophenol concentration, and one mole of ammonia was formed per mole of L-serine oxidized over a wider range of the inhibitor. Subsequent studies (figure 2) showed that this amount of ammonia was produced even in the absence of dinitrophenol. Thus, in the presence of the inhibitor, L-serine was oxidized to completion in accordance with the equation:

$$CH_2OHCHNH_2COOH + 2.5O_2 \rightarrow 3CO_2 + NH_3 + 2H_2O$$

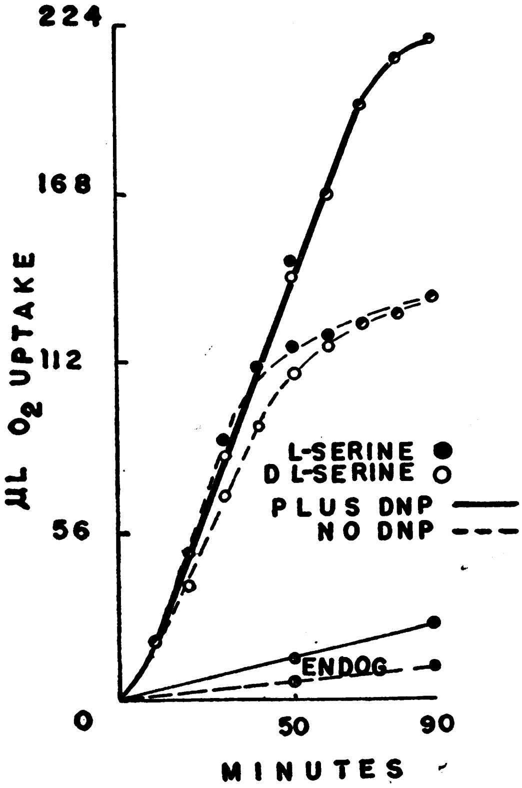

Figure 1. The oxidation of L-serine and DL-serine by *Pasteurella pestis*, strain A-1122, in the presence and absence of 2,4-dinitrophenol (DNP).

Vessels contained 2.0 ml cells (turbidity in flasks with inhibitor, 240; in flasks lacking inhibitor, 155), 0.5 ml of substrate or buffer (10 μmoles of DL-serine or 5 μmoles of L-serine), and 0.5 ml of M/1,000 DNP. Center cup contained 0.2 ml of 20 per cent KOH. Temperature 35 C, pH 6.8.

TABLE 1

Quantitative aspects of the oxidation of serine and pyruvate by Pasteurella pestis, strain A-1122, as a function of 2,4-dinitrophenol (DNP) concentration

SUBSTRATE	MOLARITY DNP ($\times 10^4$)	RATIO: MOLES GAS EXCHANGE PER MOLE SUBSTRATE		
		O_2 uptake	CO_2 evolved	NH_3 produced
Serine	0	2.1	—	—
	0.30	2.3	2.6	1.0
	1.00	2.4	3.0	0.9
	1.33	2.5	—	—
	1.66	2.0	1.9	1.1
	2.00	1.8	—	—
Pyruvate	0	1.6	—	—
	0.30	1.7	1.8	—
	1.66	2.5	2.6	—
	2.66	2.5	—	—
	3.03	2.5	3.0	—
	3.33	0.6	—	—

Vessels contained 1.5 ml cells (turbidity, 155), 1.0 ml DNP solution or buffer, 0.5 ml substrate (5.0 μmoles DL-serine or 2.5 μmoles sodium pyruvate). Center cup contained 0.2 ml 20 per cent KOH or water. Temperature 35 C, pH 6.8. Analytical ratios for serine based on L-isomer content of substrate. Different cell lots used for each substrate.

The concentration of dinitrophenol at which these values for oxygen consumption and carbon dioxide output were obtained experimentally was found to vary with different lots of cells. This unexplained variation occurred even when different lots were harvested at different times from aliquots of the same medium. The optimum level of dinitrophenol ranged between M/6,000 and M/14,000. Consequently, it was necessary to titrate the concentration of the inhibitor for each lot of cells. It was observed that the response to dinitrophenol of washed cells stored in the refrigerator overnight was somewhat more consistent, but the pretitration procedure was still necessary.

The above measurements on the serine oxidase of whole washed cells were made at pH 6.8. As shown in figure 3, the reaction proceeded most rapidly at this pH.

The relationship between some of the reaction components involved in serine oxidation in the absence of dinitrophenol was determined as a function of time (figure 2). It was observed that, as the oxidation proceeded, carbon dioxide and ammonia were liberated and a keto acid accumulated temporarily in the reaction vessel. In a flask-scale reproduction of this experiment, the keto acid was isolated as the 2,4-dinitrophenylhydrazone of pyruvic acid and identified by melting point determinations. The melting point of the derivative was 213 to 217 C (uncorrected). The melting point of a known 2,4-dinitrophenylhydrazine derivative of pyruvic acid was 216 to 218 C (uncorrected). A mixture of these derivatives melted at 213 to 217 C (uncorrected). Huntress and Mulliken (1941) state that this derivative of pyruvic acid melts at 218 C.

The deamination of serine to pyruvate and ammonia by bacteria, yeasts, and mammalian tissues was reported by Chargaff and Sprinson (1943*a*,*b*), Binkley (1943), and Wood and Gunsalus (1949). An over-all equation for this reaction was formulated by Chargaff and Sprinson:

$$CH_2OHCHNH_2COOH \rightarrow CH_3COCOOH + NH_3$$

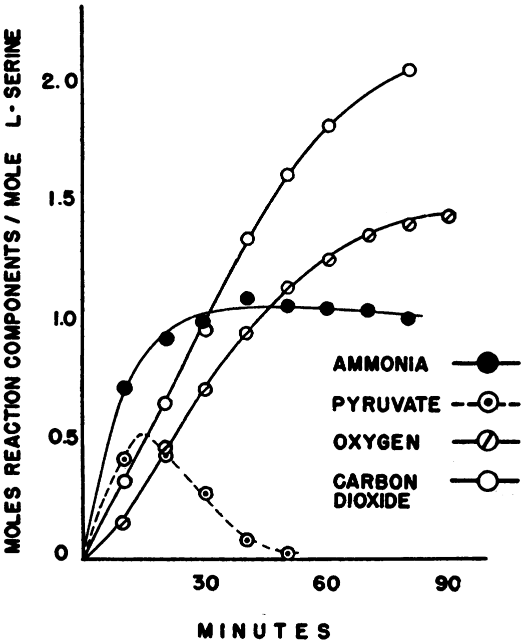

Figure 2. Correlation of CO_2, NH_3, and pyruvate production with O_2 uptake by *Pasteurella pestis*, strain A-1122, on serine substrate.

Vessels contained 0.5 ml cells (turbidity after 4-fold dilution, 130) in side arm 1, 0.5 ml of 4 N HCl (to arrest reaction) in side arm 2, 2.0 ml of substrate (10 µmoles DL-serine) in flask body. Center cup contained 0.2 ml of 20 per cent KOH or 4 N HCl. Temperature 35 C, pH 6.8.

The data in figure 2 illustrate that while theoretical amounts of ammonia were produced, low values were obtained for pyruvate. Since pyruvate, as will be shown, is itself a readily oxidizable substrate, low recovery values under the conditions described in figure 2 were expected. When conditions were made unfavorable for the oxidation reaction (figure 4) by adjusting the pH to 9.1 and incorporating arsenious trioxide into the system, one mole of ammonia and 0.85 moles of pyruvate were obtained per mole of serine supplied. Presumably, a quantitative recovery of pyruvate was not possible because the adverse conditions described in figure 4 were insufficient to suppress the oxidation of pyruvate completely. However, the data indicate that serine was deaminated essentially according to the equation outlined above.

Additional evidence on the possible key role of pyruvate was obtained by substituting this compound for serine as a substrate. As shown in table 1, pyruvate, like serine, was oxidized to completion in the presence of dinitrophenol. The concentration of dinitrophenol which inhibits assimilation again was found to vary with different lots of cells, and the effective concentration range of dinitrophenol was relatively small.

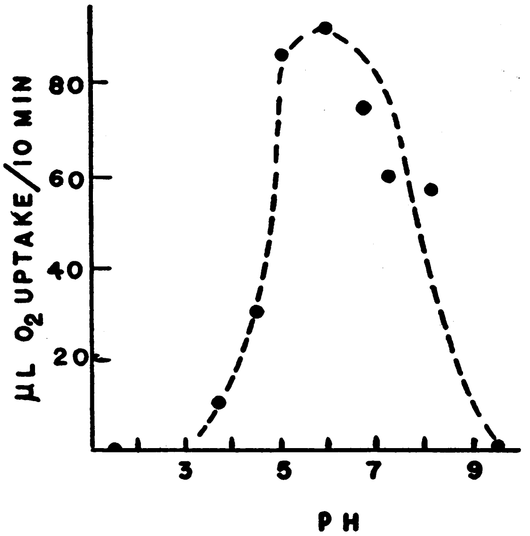

Figure 3. The rate of oxidation of serine by *Pasteurella pestis*, strain A-1122, as a function of pH.

Vessels contained 2.0 ml cells (turbidity, 135), 0.5 ml of acid or base, and 0.5 ml of substrate (20 µmoles of DL-serine). Center cup contained 0.2 ml of 20 per cent KOH. Temperature 35 C.

Recent studies on the metabolic relationship between pyruvate and acetate were reviewed by Ajl (1951). We have observed (table 2) that acetate was oxidized by *P. pestis* with the uptake of approximately two moles of oxygen and the production of two moles of carbon dioxide. Other possible intermediates in serine oxidation, such as formic acid, formaldehyde, ethanolamine, ethyl alcohol, methyl alcohol, and glycine, were not oxidized. However, attempts to trap and isolate acetate, the indicated intermediary product of pyruvate oxidation, were not successful using either washed cells or enzyme preparations prepared by the mechanical disruption of the cells.

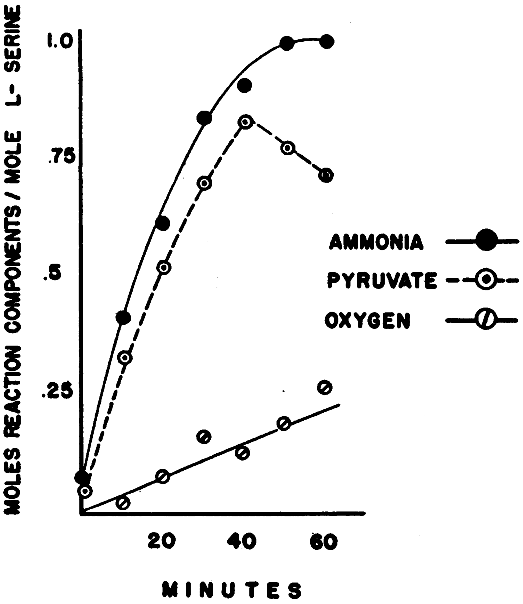

Figure 4. Correlation of CO_2 and pyruvate production with O_2 uptake by *Pasteurella pestis*, strain A-1122, in the presence of M/5,000 As_2O_3 at pH 9.1.

Vessels contained 2.0 ml cells (turbidity, 300) in flask body, 0.5 ml substrate (10 μmoles of DL-serine) in side arm 1, 0.5 ml of M/834 arsenite in side arm 2. Center cup contained 0.2 ml of 20 per cent KOH. Reaction was arrested by the addition of 3 ml of 1 N H_2SO_4 to the flask body.

TABLE 2

Quantitative aspects of the oxidation of acetate by Pasteurella pestis, strain A-1122, in the presence of 2,4-dinitrophenol

SUBSTRATE	EXPT NO.	MOLARITY DNP ($\times 10^4$)	μMOLES GAS EXCHANGE	
			O_2 uptake	CO_2 evolved
5 μmoles sodium acetate	1	0.56	9.2	No data
	2	0.60	9.2	9.6
	3	0.60	9.5	9.5

Vessels contained 2.0 or 2.5 ml cells, DNP and acetate as indicated. Center cup contained 0.2 ml 20 per cent KOH or water. Total fluid volume 3.2 ml. Temperature 35 C, pH 6.8.

Exposure of the cells to molar solutions of glycine was observed to render them oxidatively inactive on acetate as a substrate while not interfering appreciably with their capacity to oxidize pyruvate. This procedure was investigated as a means of determining the role of acetate in pyruvate oxidation. The effect of glycine treatment on the rate of pyruvate oxidation, the molar ratios of oxygen consumed to substrate added, and the viable content of the bacterial suspension are shown in table 3. While the oxidative rate remained relatively uniform after exposures ranging between 30 and 90 minutes, subsequent studies indicated that different cell lots responded to the action of glycine in a somewhat inconsistent manner. Some preparations were inactivated after a 90 minute exposure period, and others were not impaired after exposures of 150 minutes.

TABLE 3

The effect of glycine exposure on the viable count and oxidative activity of Pasteurella pestis, strain A-1122, on pyruvate substrate

ALIQUOT	DURATION EXPOSURE	VIABLE COUNT PER ML	μL O_2 UPTAKE PER 10 MINUTES	MOLES O_2 UPTAKE / MOLE SUBSTRATE
	min			
1	0	3×10^{12}	1,272*	1.9
2	30	No data	26.9	0.43
3	60	2×10^9	31.5	0.43
4	90	5×10^8	32.7	0.38

* Corrected value, cell suspension was diluted 8-fold.

Vessels contained 2.1 ml untreated cells or treated cells, 0.2 ml of 0.1 per cent $MnSO_4$, 0.2 ml of 0.1 per cent cocarboxylase, and 20 μmoles of sodium pyruvate. Center cup contained 0.2 ml of 20 per cent KOH. Vessels were equilibrated for 30 minutes at 35 C prior to the addition of substrate to cells. Total fluid volume of vessels was 3.2 ml, pH 7.2.

The duration of the exposure was determined subsequently by noting the point of "minimal lysis" and then arresting the action of glycine by dialysis in the cold. This usually occurred between 30 and 50 minutes after the addition of glycine to the cells. At this point, the cell suspension appeared to "string-out" very slightly when poured from one beaker to another. Such preparations oxidized pyruvate but did not oxidize acetate. Undertreated preparations oxidized pyruvate rapidly and acetate slowly. When dialysis of the cell-glycine suspension was

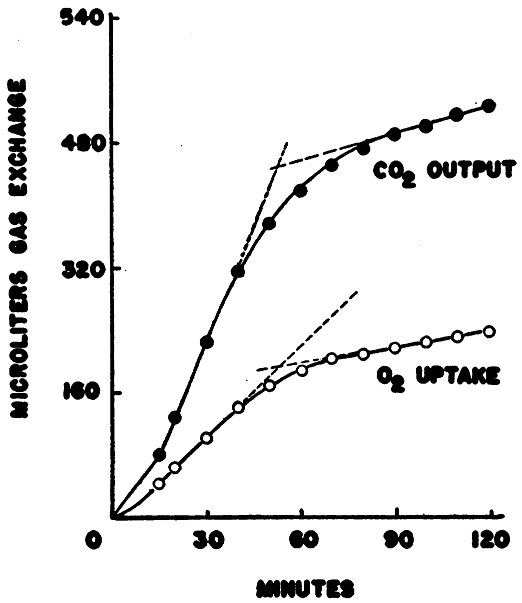

Figure 5. The oxidative decarboxylation of pyruvate by glycine treated cells of *Pasteurella pestis*, strain A-1122.

Vessels contained 2.1 ml of treated cells (1 hour at room temperature, dialyzed 1½ hours at 0 to 5 C against distilled water), 0.2 ml of 0.1 per cent of $MnSO_4$, 0.2 ml of 0.1 per cent cocarboxylase, and 20 μmoles of sodium pyruvate. Center cup contained 0.2 ml of 20 per cent KOH or water. Total fluid volume of vessels was 3.2 ml, pH 6.8, temperature 35 C.

performed at room temperature, or if the cells and glycine were not prechilled prior to mixing, pyruvate oxidase activity was slow.

The manometric measurements described above suggested the possibility that glycine treated cells oxidized pyruvate to acetate. The oxidative decarboxylation of pyruvate, previously demonstrated for several bacterial species (Still, 1941; Stumpf, 1945), followed the equation:

$$CH_3COCOOH + 0.5O_2 \rightarrow CH_3COOH + CO_2$$

Manometric studies on oxygen consumption and carbon dioxide production from pyruvate, using glycine treated cells, are shown in figure 5. Each vessel contained 20 μmoles of sodium pyruvate. The total oxygen uptake at the "break" in the curve was 200 μL, and 460 μL of carbon dioxide output were measured. Thus, 0.45 mole of oxygen was consumed, and 1.03 moles of carbon dioxide were produced per mole of pyruvate utilized.

TABLE 4

*Duclaux constants of volatile acid produced by Pasteurella pestis, strain A-1122, on pyruvate substrate**

FRACTION	DISTILLATION CONSTANTS		
	Exogenous	Acetate plus endogenous materials	Acetate alone
1	0.245	0.240	0.228
2	0.294	0.308	0.300
3	0.461	0.452	0.475

* Analyses made on vessel contents described in figure 5.

TABLE 5

Analysis of reaction components involved in the oxidation of pyruvate by glycine treated cells of Pasteurella pestis, strain A-1122

SUBSTRATE	FINAL CONC DNP	MICROMOLES			
		Substrate disappeared	O_2 uptake	CO_2 output	Volatile acid recovered
20 μmoles Na pyruvate	0	18.5	10.0	22.0	14.5
20 μmoles Na acetate	0	—	1.8	—	19.0
Endogenous	0	—	1.8	—	3.9
20 μmoles Na pyruvate	M/750	19.1	10.0	18.0	17.3
20 μmoles Na acetate	M/750	—	2.4	—	19.2
Endogenous	M/750	—	2.4	—	2.2

Vessels contained 2.0 ml treated cells (40 minutes at room temperature, dialyzed 1½ hours at 0 to 5 C against distilled water), 0.1 ml of buffer or DNP solution, 0.2 ml of 0.1 per cent cocarboxylase, 0.2 ml of 0.1 per cent $MnSO_4$, and 0.5 ml of substrate or buffer as indicated. Center cup contained 0.2 ml of 20 per cent KOH or water. Total fluid volume 3.2 ml, pH 6.8, temperature 35 C.

The Duclaux distillation data, shown in table 4, indicated that acetate was formed from pyruvate at the termination of the oxidative process. Some variation between the distillation constants for pure acetate and acetate formed from pyruvate or distilled in the presence of endogenous material was expected since small amounts of other volatile acids were produced endogenously (table 5).

The relationship between pyruvate utilization and acetate production is shown in table 5. After the termination of the oxidative reaction, more than 92 per cent of the pyruvate had disappeared from the reaction mixture. This was correlated with the uptake of one atom of oxygen and the output of one mole of carbon dioxide per mole of substrate. However, only 73 per cent of expected acetate was recovered.

When a high level of dinitrophenol (M/750) was added to the cells, acetate recovery approaching theoretical amounts was obtained (table 5). Of 20 μmoles pyruvate supplied, 19.1 μmoles disappeared at the end of the reaction. This was correlated with the formation of 17.3 μmoles of acetate, indicating a 91 per cent conversion of pyruvate to acetate.

The low value obtained for acetate formation in the absence of dinitrophenol is unexplained but may again reflect the assimilation of substrate although the manometric data do not agree completely with this interpretation. However, the gas values were calculated without correction for endogenous activity. It seemed advisable not to subtract the endogenous value in these experiments because (1) the oxygen and carbon dioxide curves "broke" consistently at the expected theoretical values and (2) in the presence of dinitrophenol (table 5) where endogenous activity was unusually high and where there was almost quantitative conversion of pyruvate to acetate, the oxygen uptake value again agreed with our considerations.

The role of added Mn^{++} and cocarboxylase on the oxidative activity of glycine treated cells was not determined precisely. Still (1941) and Stumpf (1945) have reported the essentiality of these materials for the activity of cell-free pyruvate oxidases derived from several bacterial species. The glycine treated preparation which we have used consisted of whole cells and lysed material. Prolonged dialysis, which was considered to be necessary in studies on the effect of trace materials, resulted in the inactivation of the preparation. After a 90 minute dialysis period, added Mn^{++} had no stimulatory effect on the oxidative rate. The addition of cocarboxylase accelerated the oxidative rate by approximately 22 per cent in one experiment.

The data obtained in these studies suggest that resting cells of *P. pestis* dissimilate serine in accordance with the following scheme:

$$
\begin{array}{c}
CH_2OHCHNH_2COOH + 2.5O_2 \rightarrow 3CO_2 + NH_3 + 2H_2O \\
\downarrow \\
CH_3COCOOH + NH_3 \\
CH_3COCOOH + 2.5O_2 \rightarrow 3CO_2 + 2H_2O \\
\downarrow + 0.5O_2 \\
CH_3COOH + CO_2 \\
CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O
\end{array}
$$

SUMMARY

Serine was oxidized rapidly at pH 6.8 by resting cells of *Pasteurella pestis*, strain A-1122. The oxidation was specific for the L-isomer of serine. The oxidation of serine to completion was obtained in the presence of 2,4-dinitrophenol. Pyruvate and acetate were identified as intermediary compounds of serine metabolism. Accumulation of acetate was favored by pretreatment of the cells with glycine. The complete oxidation of these compounds to carbon dioxide was demonstrated.

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