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Pyruvate Metabolism in *Sarcina maxima*

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The mechanisms of pyruvate cleavage and hydrogen production by *Sarcina maxima* were studied. It was found that a phosphoroclastic system for pyruvate oxidation, similar to that occurring in saccharolytic clostridia, is present in *S. maxima*. Cleavage of pyruvate by extracts of the latter organism resulted in the formation of acetyl phosphate, CO_2, and electrons which were transferred to ferredoxin. Formate was not an intermediate in this system. Pyruvate oxidation was coupled with ferredoxin-dependent nicotinamide adenine dinucleotide phosphate (NADP) reduction. A hydrogenase, active in particulate extracts of *S. maxima*, did not accept electrons from reduced ferredoxin. Formate was detected as a fermentation product when *S. maxima* was grown in media buffered with $CaCO_3$. Whole cells and extracts degraded formate to H_2 and CO_2. The evidence suggests that electrons generated by ferredoxin-linked pyruvate oxidation by *S. maxima* are not used for H_2 production, but that they serve for the reduction of NADP. Reduced NADP may be utilized by the organisms for synthesis of cell material. Production of H_2 by *S. maxima* may occur through a pyruvate clastic system similar to that present in coliform bacteria.

Sarcina maxima (*Zymosarcina maxima*), a large, strictly anaerobic bacterium, ferments carbohydrates with the formation of CO_2, H_2, and butyric, acetic, and lactic acids as main products (19). The fermentation pathways of *S. maxima* have not been investigated, probably because pure cultures of this organism are not readily available. The similarity between the fermentation products of this organism and those of saccharolytic clostridia (4) suggests a corresponding similarity in their fermentation mechanisms. However, as described later in this report, resting cells of *S. maxima* produce CO_2 and H_2 from either pyruvate or formate, a behavior which suggests the presence of a pyruvate clastic system such as that found in *Escherichia coli*. The primary purpose of these investigations was to study the mechanisms of pyruvate cleavage and H_2 production in *S. maxima*.

MATERIALS AND METHODS

Cultures. Sarcina maxima (strain 11) was received in lyophilized form from H. Knöll, Institut für Mikrobiologie und Experimentelle Therapie, Jena, Germany, and was maintained by transfer every 4 days in a medium (SM) including (grams/100 ml of distilled water): glucose and peptone, 1.0 each; yeast extract, 0.5; sodium thioglycolate, 0.1; agar, 0.1. Incubation temperature was 30 C. The organisms were also maintained in depression cultures (3) using medium SM with an agar content of 2 g/100 ml.

S. maxima 11 was grown in 14-liter fermentor vessels (NBS MicroFerm Fermentor, model MF-214) each containing 10 liters of medium SM (agar omitted) plus 0.005 g of $FeSO_4 \cdot 7H_2O$/100 ml, and with L-cysteine (0.05 g/100 ml) substituted for the thioglycolate. This medium yielded approximately 2.4 g of cells (wet weight) per liter. Except where indicated otherwise, cells were harvested by centrifugation during the exponential phase of growth.

Clostridium pasteurianum (ATCC 6013) was cultured at 30 C in 4- to 16-liter volumes of the following medium (grams/100 ml of tap water): glucose, 1.0; peptone and yeast extract, 0.5 each; K_2HPO_4, 0.25. The cells were harvested by centrifugation while actively producing gas.

Products of glucose fermentation. Gaseous products formed from glucose by growing cells were estimated by use of a fermentation train as described by Neish (13). Medium SM (minus agar) including 0.01 g of sodium thioglycolate/100 ml was used. The fermented medium was clarified (13) and the resulting solution was assayed for products. Volatile acids were isolated by steam distillation and assayed by column chromatography on acid-washed Celite 535 (13). Products volatile at neutral *p*H were distilled and estimated by dichromate oxidation followed by iodometric titration of the excess dichromate (13). Lactic acid was assayed by the method of Barker and Summerson (1). Thin-layer chromatography on Silica Gel G was employed for qualitative determination of fermentation acids. The following solvent systems were used: 95% ethyl alcohol-concentrated NH_4OH, 100:1, v/v (7) and *n*-propanol-concentrated NH_4OH, 7:3, v/v (6), singly or two-dimensionally. Bromocresol purple (17) was the indicator spray.

Preparation of cell extracts. Cells were frozen immediately after harvest and stored at −20 C. Cell extracts were routinely prepared by grinding thawed cells with Alumina A-305 (Aluminum Company of America, Pittsburgh, Pa.) in a chilled mortar, and suspending the ground mixture in 0.01 M potassium phosphate buffer, *p*H 6.5, containing 1.5 μmoles of dithiothreitol/ml. Approximately 1 ml of buffer was added for each g (wet weight) of cells. The suspension was centrifuged for 10 min at 12,000 × *g*, and the resulting supernatant liquid was used in reaction mixtures. Cell extracts of *S. maxima* were always prepared immediately before use.

Ferredoxin purification. Ferredoxin was isolated from extracts of *S. maxima* by a modification of Mortenson's procedure (11), involving acetone fractionation, diethylaminoethane cellulose (DEAE) chromatography, and $(NH_4)_2SO_4$ fractionation. Fractions were assayed for ferredoxin by reconstitution of the pyruvate clastic system, employing DEAE-treated extracts of *C. pasteurianum*. Assay conditions were as described by Mortenson et al. (12), except that the amount of clostridial extract used was decreased by one-half (to 5.0 mg of protein/ml). Consequently, one unit of activity in this assay was defined as 0.5 μmole of acyl phosphate produced per 15 min, rather than 1.0 μmole per 15 min, as defined by Mortenson et al. Clostridial extracts for ferredoxin assay were prepared by disrupting cells (suspended in water) by the use of a French pressure cell. The mixture was centrifuged and the sediment was discarded. Ferredoxin was removed by fractionating the supernatant liquid with acetone, resuspending the 17 to 40% acetone precipitate in buffer (plus 0.3 μmole of dithiothreitol/ml), and passing this mixture through a DEAE column.

Enzyme assays. Products formed from pyruvate by cell extracts were determined by incubating the following mixture in Warburg vessels (in 3.0 ml total volume): potassium pyruvate, 20 μmoles; coenzyme A (HSCoA), 0.05 μmole; potassium phosphate buffer, *p*H 6.5, 300 μmoles; methyl viologen, 2.0 μmoles; cell extract protein, 16 mg. Incubation was for 20 min at 30 C under He. CO_2 and H_2 were determined manometrically, and acyl phosphate was assayed by the method of Lipmann and Tuttle (8). Stability to heating in acid solution was used as a means for distinguishing between acyl phosphate and acyl-CoA. The reaction mixtures were clarified by the addition of trichloroacetic acid to a final concentration of 1.5%. After centrifugation, the supernatant liquids were assayed for lactate (1), and for pyruvate by an adaptation of the procedure of Friedemann and Haugen (5). Acyl phosphates produced from pyruvate were identified as either acetyl or butyryl phosphate (20).

CO_2-pyruvate and formate-pyruvate exchange activities were determined (26). The reaction was carried out in Warburg flasks at 30 C under a N_2 atmosphere. The reaction mixture (3.2 ml) contained: potassium pyruvate, 100 μmoles; potassium phosphate buffer, *p*H 6.8, 100 μmoles; monosodium glutathione (reduced), 2 mg; cell extract protein, 9.0 mg; $NaH^{14}CO_3$ (CO_2 exchange only), 100 μmoles; KH_2PO_4 (CO_2 exchange only, to adjust the *p*H to 6.9 in the presence of $NaHCO_3$), 75 μmoles; $H^{14}COONa$ (formate exchange only), 102.5 μmoles. The initial *p*H of the reaction mixture containing formate was 7.1. The reaction was terminated after 30 min by the addition of H_2SO_4, and protein was removed by trichloroacetic acid precipitation. Pyruvate was collected as the 2,4-dinitrophenylhydrazone. Precipitates from CO_2 exchange reactions were washed once with hot 50% H_2SO_4, twice with distilled water, and twice with 95% ethyl alcohol. Those from formate exchange reactions were washed with hot 50% H_2SO_4, dissolved (in 2 M NaOH) and reprecipitated (by adding 0.5 M H_2SO_4) three times in the presence of 0.3 M Na formate, and finally washed with distilled water. Radioactivity was measured in a Nuclear-Chicago thin-window gas-flow Geiger counter. $NaH^{14}CO_3$ was counted as $BaCO_3$, and $H^{14}COONa$ was plated directly for standardization.

To determine whether ferredoxin was reduced by extracts of *S. maxima* with pyruvate as electron donor, the following mixture (5 ml total volume) was incubated at 25 C under He in stoppered 15-ml colorimeter tubes: potassium pyruvate, 500 μmoles; HSCoA, 0.1 μmoles; potassium phosphate buffer, *p*H 6.5, 500 μmoles; crude extract protein, 6.0 mg; *S. maxima* ferredoxin (DEAE eluate), 0.56 mg, added as indicated in Fig. 2. Absorption was measured by use of a Klett-Summerson photoelectric colorimeter with a #42 filter.

Hydrogenase activity was measured by an assay involving H_2 production from reduced methyl viologen (15). Warburg flasks contained (3.0 ml total volume): $Na_2S_2O_4$, 20 μmoles (added as a solid); potassium phosphate buffer, *p*H 6.5, 100 μmoles; enzyme protein, 3.5 mg; methyl viologen, 12.8 μmoles; and *C. pasteurianum* ferredoxin, 0.5 mg, as indicated in Fig. 5. Enzyme preparations for this experiment were obtained by resuspending (in 0.01 M phosphate buffer, *p*H 6.5, plus 1.5 μmoles of dithiothreitol/ml) the upper layer of the pellet resulting from centrifugation of the ground cell-alumina-buffer mixture. Incubation was at 30 C under N_2.

Experiments involving pyridine nucleotide reduction with pyruvate as electron donor (23) were carried out in anaerobic cuvettes containing (2.5 ml reaction mixture): potassium pyruvate, 100 μmoles; HSCoA, 0.05 μmole; potassium phosphate buffer, *p*H 6.5, 250 μmoles; nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), ferredoxin, and cell extract, as stated in Fig. 3 and 4. Ferredoxin-free extracts were prepared by passing 5-ml amounts of cell extract through a DEAE column (50 × 11 mm). Incubation was at 22 C under He. Absorption at 340 mμ was measured on a Beckman DB spectrophotometer.

Butyrate kinase and acetate kinase activities were demonstrated in cell extracts of *S. maxima* by modifications of the methods of Rose et al. (18) and Twarog and Wolfe (21), respectively.

Other assays. Protein was determined by a modification of the method of Lowry et al. (9). Assays for formate produced from pyruvate were according to the procedure of Pickett et al. (16).

Chemicals. C. pasteurianum ferredoxin was obtained from Worthington Biochemical Corp., Freehold, N.J. Radioactive chemicals were purchased from Calbiochem, Los Angeles, Calif. Acetyl phosphate (Li salt) was donated by R. P. Mortlock.

RESULTS

Products of glucose fermentation. S. maxima 11 fermented glucose and formed the following products (micromoles/100 μmoles of glucose utilized): butyric acid, 77; acetic acid, 40; CO_2, 197; H_2, 223; neutral volatile products (calculated as butanol), 9. Formic acid, lactic acid, and acetoin were not detected. The *p*H of the fermented medium was 4.5. The amounts of fermentation products are similar to those reported by Smit (19) for *S. maxima* (except that Smit's strain produced lactic acid) and by Donker (4) for *Clostridium saccharobutyricum* (*C. butyricum*).

Products from pyruvate. Cell extracts of *S. maxima* incubated with pyruvate in the presence of methyl viologen reduced the dye and formed the following products (micromoles of product/18.6 μmoles of pyruvate utilized): acyl phosphate 12.2; CO_2, 14.7; H_2, 9.7. Formate and lactate were not present in measurable quantities. Part of the acyl phosphate formed from pyruvate was probably broken down during incubation of the reaction mixture, due to the presence of phosphatase activity. (Experiments in which extracts were incubated with acetyl phosphate indicated the presence of phosphatase activity in the mixture.) The acyl phosphate produced was identified as consisting mainly of acetyl phosphate and of a small amount of butyryl phosphate. Since the extracts exhibited butyrate kinase activity, the butyryl phosphate was probably formed from butyrate (present in crude extracts) and adenosine triphosphate (ATP). ATP, generated by the production of acetate from acetyl phosphate in the presence of acetate kinase (detected in the extracts), was available for this reaction. The amount (micromoles) of H_2 produced from pyruvate by cell-free extracts was smaller than the amount of CO_2 produced. This difference cannot be accounted for by the amount of methyl viologen reduced (reduction of all the dye present is assumed). If the dye was not added to the reaction mixture, no H_2 was evolved, and the other products were markedly decreased. Apparently methyl viologen was required as an electron carrier for H_2 production in these reaction mixtures.

Phosphotransacetylase activity (21) was detected in the extracts.

Exchange experiments. The presence of formic dehydrogenase in *S. maxima* complicated the interpretation of $^{14}CO_2$-pyruvate and H^{14}COOH-pyruvate exchange data. In $^{14}CO_2$-pyruvate exchange reaction mixtures, exchange of label between $^{14}CO_2$ and formate in the presence of formic dehydrogenase might result in H^{14}COOH-pyruvate exchange. Furthermore, in H^{14}COOH-pyruvate reaction mixtures, $^{14}CO_2$ produced from formate in the presence of formic dehydrogenase could exchange with pyruvate. However, it was found that if cells were harvested in the stationary phase of growth, after gas evolution had ceased, the extracts had no detectable formic dehydrogenase activity. These extracts catalyzed approximately 100% of theoretical exchange between $^{14}CO_2$ and pyruvate (Table 1, see Materials and Methods for conditions), whereas there was essentially no exchange between H^{14}COOH and pyruvate. This behavior is typical of organisms which possess a clostridial type of pyruvate cleavage (25).

Ferredoxin. Experiments were conducted to determine whether the electron carrier ferredoxin was present in *S. maxima* and whether it played a part in pyruvate oxidation by this organism. When *S. maxima* extracts, after acetone treatment (11), were passed through a DEAE column, a dark-brown band formed at the top of the column and a much larger yellow band appeared in a lower position. The latter band could be eluted with 0.05 to 0.2 M potassium phosphate buffer, *p*H 7.0, whereas the dark band was spread only slightly by this treatment. The dark band was batch-eluted with 1 M phsophate buffer (*p*H 7.0). After dialysis and $(NH_4)_2SO_4$ fractionation, a brown material, which exhibited typical ferredoxin absorption maxima (2) at 280 mμ and in the 390-mμ region (Fig. 1), was obtained. As occurs with clostridial ferredoxins, the maximum at 390 mμ disappeared when *Sarcina* ferredoxin was reduced by either pyruvate or $Na_2S_2O_4$.

Sarcina ferredoxin, prepared by this or similar procedures, replaced methyl viologen or *C. pasteurianum* ferredoxin in the reconstituted phos-

TABLE 1. *$^{14}CO_2$-pyruvate and H^{14}COOH-pyruvate exchange by extracts of Sarcina maxima*

Determination	Specific activity (counts per min per μmole)	
	$^{14}CO_2$-pyruvate exchange	H^{14}COOH-pyruvate exchange
$NaH^{14}CO_3$	147	—
H^{14}COOH	—	1,950
Recovered pyruvate	74.4	1.5
Percentage of theoretical exchange	101	0.1

phoroclastic system of the latter organism (Table 2). Assays for *S. maxima* ferredoxin were based on its ability to participate in the pyruvate clastic system of *C. pasteurianum*. As previously mentioned, this type of assay was used to determine units of ferredoxin activity in the purification of this protein from *S. maxima* (Table 3).

Sarcina ferredoxin accepted electrons from pyruvate in the presence of *S. maxima* extracts, as determined by the decrease in absorption of reaction mixtures at 420 mμ (Fig. 2). This indicated that ferredoxin participates in the pyruvate clastic reaction in *S. maxima*. Similar results were obtained by adding clostridial ferredoxin to crude extracts of *S. maxima*.

Participation of ferredoxin in pyridine nucleotide reduction and H_2 formation. The known properties of *S. maxima* suggested that ferredoxin might be involved in pyridine nucleotide reduction and hydrogen formation by this organism. Experiments were conducted to determine whether reduced ferredoxin donated electrons to NAD, NADP, and to the hydrogenase system.

In experiments with crude extracts, NADP was reduced in the presence of pyruvate (Fig. 3). NAD reduction was not apparent, possibly because of $NADH_2$ oxidizing activity, which was detected in the extract. Neither NAD nor NADP was reduced when formate was substituted for pyruvate as electron donor.

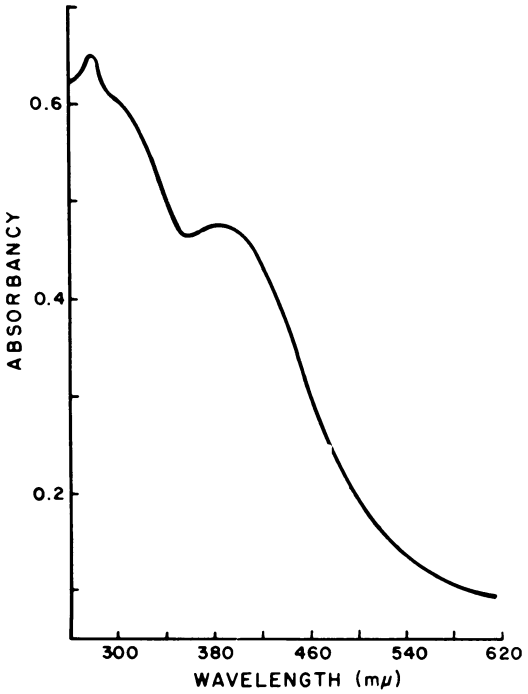

FIG. 1. *Absorption spectrum of oxidized ferredoxin from Sarcina maxima (60 to 90% saturated ammonium sulfate precipitate, resuspended in distilled water). Solution contained 0.1 mg of ferredoxin/ml. Model 2000 Gilford spectrophotometer.*

TABLE 2. *Participation of Sarcina maxima ferredoxin in the pyruvate clastic system of Clostridium pasteurianum*[a]

Addition	Acyl phosphate formed (μmoles)
None	0.4
S. maxima ferredoxin (0.22 mg)	3.8
C. pasteurianum ferredoxin (0.07 mg)	1.6
Methyl viologen (0.1 μmole)	1.7

[a] Reaction mixture and conditions were similar to those used in the assay for ferredoxin (see Materials and Methods).

TABLE 3. *Purification of ferredoxin from Sarcina maxima*

Fraction	Total units of activity	Units of activity/mg of protein	Fold purification
Crude extract	5,170	4.6	0
DEAE eluate	980	151[a]	33
$(NH_4)_2SO_4$, 65 to 75% saturated	302	210[a]	46

[a] In calculating total protein, allowance was made for the fact that ferredoxin has a more intense color with Folin reagent than does the albumin standard. A correction factor of 0.55 was used (11).

To determine whether ferredoxin participated in NADP reduction from pyruvate, DEAE-treated *Sarcina* extracts were incubated with substrate and NADP in the presence and absence of ferredoxin. Results indicated that ferredoxin was required for NADP reduction from pyruvate (Fig. 4).

Particulate extracts of *S. maxima*, prepared by suspending the upper layer of the pellet in buffer (see Materials and Methods), had a low ferredoxin content. These extracts catalyzed H_2 evolution from $Na_2S_2O_4$ in the presence of methyl viologen (Fig. 5). Unexpectedly, added ferredoxin did not replace methyl viologen, an indication that ferredoxin may not be involved in H_2 evolution by these extracts. Addition of both ferredoxin and methyl viologen resulted in a decrease in total H_2 evolved, possibly because electrons were shunted from reduced ferredoxin to an endogenous acceptor. Similar results were obtained

with the addition of either clostridial or *Sarcina* ferredoxin.

Participation of formate in S. maxima metabolism. Manometric experiments indicated that whole cells of *S. maxima* degraded formate to equimolar amounts of CO_2 and H_2. In experiments similar to those conducted to determine the products formed from pyruvate, cell extracts catalyzed evolution of CO_2 and H_2 from formate in the presence of methyl viologen. If the dye was omitted, a 15-min lag in H_2 production was observed, after which the reaction proceeded, but at less than one-third the rate noted in the presence of dye.

The demonstration of an active hydrogenlyase system suggested that a coliform-type cleavage of pyruvate was present in *S. maxima*, along with the clostridial clastic mechanism. It was not possible, however, to demonstrate a formate-pyruvate exchange system, either in extracts or in whole cells. This system, which is closely associated with the pyruvate clastic reactions in *E. coli* and in other bacteria, is unusually sensitive to O_2 (27). Even though reductants were added to extracts or to freshly harvested cells of *S. maxima*, activity may have been lost before exchange could occur. It is possible that formate-pyruvate exchange activity may be demonstrated in *S. maxima* by using assay conditions (e.g., high phosphate concentration and alkaline *p*H) optimal for certain described exchange systems (10, 14).

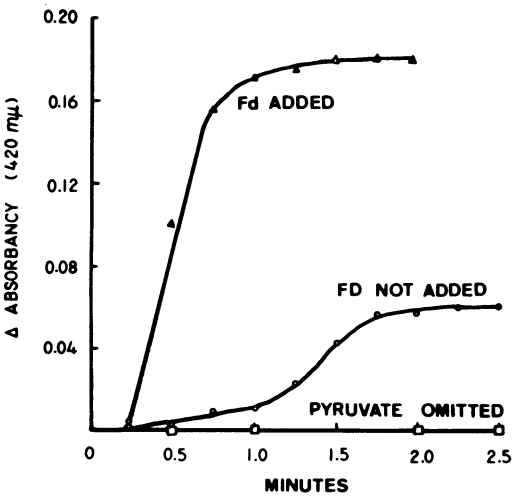

FIG. 2. *Reduction of Sarcina ferredoxin (Fd) by an extract of S. maxima with pyruvate serving as the electron donor. The reaction mixture from which pyruvate was omitted included added ferredoxin.*

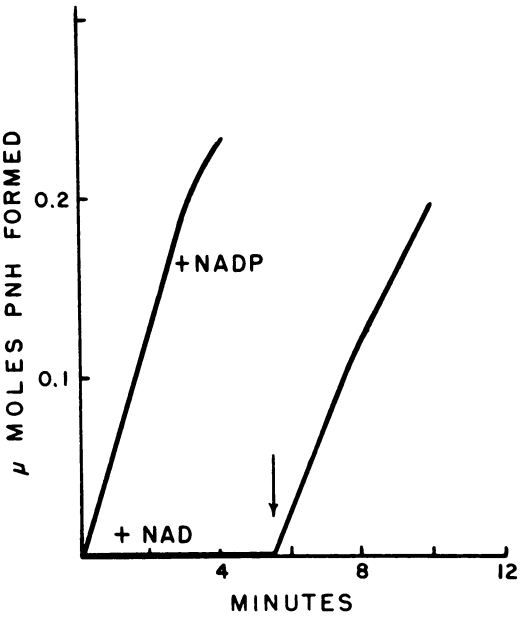

FIG. 3. *Reduction of pyridine nucleotide by an extract of Sarcina maxima with pyruvate serving as the electron donor. Crude extract, 4.3 mg; NAD or NADP, 2.0 μmoles, as indicated. At arrow, 1.0 μmole of NADP was added to the NAD-containing mixture. See Materials and Methods for other details. PNH = reduced pyridine nucleotide.*

Whole cells or extracts oxidizing pyruvate did not accumulate formate in detectable amounts. This is not surprising because hydrogenlyase activity was present in these systems. As previously mentioned, formate was not accumulated by growing cells fermenting glucose. However, if medium SM (minus agar) was buffered at a *p*H of approximately 6.7 by the addition of 0.5 g of $CaCO_3$/100 ml, formate, as well as acetate and butyrate, accumulated during fermentation (in a final molar ratio of 0.6:2:1). This observation is in agreement with data reported by Smit (19), who found formate as a minor product of glucose fermentation by *S. maxima*.

Formate production from glucose and, more indirectly, the occurrence of an active hydrogenlyase system in *S. maxima*, constitute presumptive evidence for the presence of a coli-type cleavage of pyruvate in this organism.

DISCUSSION

The data reported in the preceding section indicate that a phosphoroclastic system for pyruvate oxidation, similar to that found in saccharolytic clostridia, is present in *S. maxima*. The cleavage

of pyruvate by extracts of *S. maxima* results in the formation of acetyl phosphate, CO_2, and electrons, which are transferred to ferredoxin. As shown by exchange experiments, formate is not an intermediate in this system. In contrast to results obtained with other organisms possessing the clostridial pyruvate clastic system (22, 24), the hydrogenase active in extracts of *S. maxima* did not accept electrons from reduced ferredoxin. However, in the presence of the same extracts, reduced methyl viologen transferred electrons to the hydrogenase system. If a ferredoxin-linked hydrogenase is present in *S. maxima*, this enzyme system may be labile to the extent that, in reaction mixtures it no longer reacts with the natural electron carrier ferredoxin, but accepts electrons from the less specific methyl viologen.

Pyruvate oxidation in *S. maxima* results in ferredoxin-dependent NADP reduction. Although the absence of ferredoxin-linked hydrogenase activity in the extracts may be due to lability of this enzyme, it is possible that electrons generated by ferredoxin-linked pyruvate oxidation in *S. maxima* are not used in H_2 production, but that they serve mainly to reduce NADP, which is then utilized for synthesis of cell material. Production of H_2 by *S. maxima* may occur through a pyruvate clastic system similar to that present in coliform bacteria and involving formate. This system may play a major role in pyruvate cleavage and serve as the main source of acetyl-CoA for butyrate and acetate production.

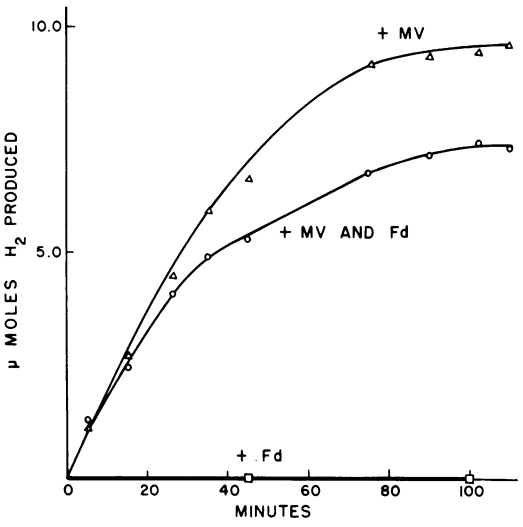

FIG. 5. *Hydrogen production by extracts of Sarcina maxima. MV = methyl viologen; Fd = Clostridium pasteurianum ferredoxin. See Materials and Methods.*

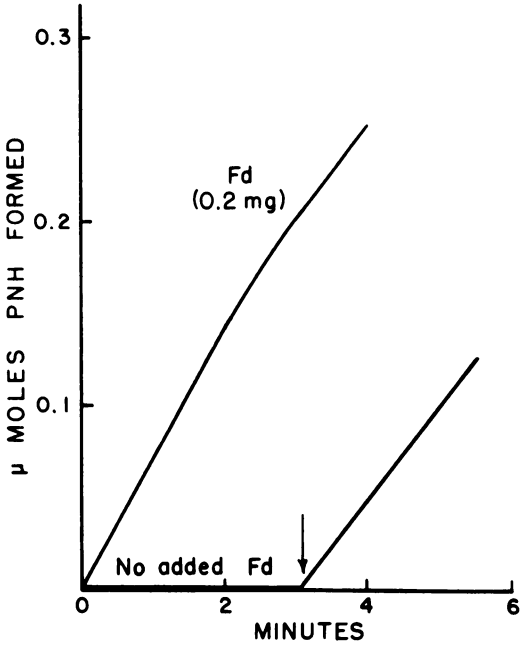

FIG. 4. *Ferredoxin requirement for NADP reduction with pyruvate serving as the electron donor. DEAE-treated sarcina extract, 5.1 mg; NADP, 0.5 μmole. C. pasteurianum ferredoxin (Fd), as indicated. At arrow, 0.1 mg of ferredoxin was added. See Materials and Methods for other details. PNH = reduced pyridine nucleotide.*

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