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Fermentative Metabolism of Pyruvate by *Rhodospirillum rubrum* After Anaerobic Growth in Darkness[1]

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Rhodospirillum rubrum grew anaerobically in darkness and fermented sodium pyruvate by a pyruvate formate-lyase reaction. During 30 min of anaerobic dark or light incubation with sodium pyruvate, crude extracts from fermentatively grown cells produced about 6 μmol of acetylphosphate and formate per mg of protein in reactions performed at pH 8.3. Cell extracts also catalyzed the exchange of sodium [^{14}C]formate into sodium pyruvate at an apparent pH optimum of 7.3 to 7.5, but only about 2.5 μmol of acetylphosphate was produced at this lower pH value. *R. rubrum* may also form pyruvate:ferredoxin oxidoreductase activity, as evidenced by low bicarbonate exchange activity. However, its participation in pyruvate metabolism in anaerobic dark-grown cells was not understood. During anaerobic, dark growth with pyruvate, formate was an intermediate in H_2 and CO_2 gas evolution. In contrast with H_2 production by a light-dependent H_2-nitrogenase system in photosynthetically grown cells, H_2 formation in fermenting *R. rubrum* occurred through a carbon monoxide-sensitive formic hydrogenlyase reaction not influenced by light.

Several species of *Rhodospirillaceae* (purple nonsulfur bacteria) have been reported to grow and ferment sodium pyruvate (34, 36) or respire anaerobically (10, 11, 27) in the absence of light. In addition, Krasil'nikov et al. (16) showed that *Thiocapsa roseopersicina*, a purple sulfur bacterium, also grew anaerobically in darkness. Consequently, it appears that anaerobic dark growth is a property shared by a variety of anoxygenic phototrophic bacteria.

It was observed (35) that when *Rhodospirillum rubrum* was grown first under O_2-free conditions in light and then placed in darkness, the pigment content of cells gradually decreased, during long-term anaerobic growth, until only a trace amount of bacteriochlorophyll *a* was produced in the cultures. Additional experiments indicated that, during anaerobic growth in darkness, some cells in the population continued to form significant amounts of bacteriochlorophyll *a* and carotenoids, whereas others did not. Subsequently, mutant phenotypes C and G1 were selected on the basis of pigment formation for further study (34, 35). Mutant C synthesized bacteriochlorophyll *a* and developed red-colored colonies on solid medium in the dark, whereas G1 produced only trace amounts of bacteriochlorophyll *a* and developed light-pink colonies. It was believed that the formation of pigments during anaerobic dark growth enabled C cells to grow equally well when exposed to light, whereas mutant G1 was light sensitive. However, regardless of these differences between mutants C and G1 in pigment production and growth properties, in the dark both mutant phenotypes fermented sodium pyruvate and produced acetate, formate, H_2, and CO_2 (34). From these data, it appeared that the energy-yielding substrate was degraded by a pyruvate formate-lyase reaction, with formate serving as an intermediate in gas formation. Alternatively, formate might be produced from H_2 and CO_2, by a reaction similar to those reported in clostridia (31). If this occurred, pyruvate:ferredoxin oxidoreductase (25) could operate to form H_2 and CO_2 gases as substrates for formate production. Evidence that such a pyruvate:ferredoxin oxidoreductase pathway might function in *R. rubum* was proposed earlier (34, 35), since cells accumulated poly-β-hydroxybutyrate during anaerobic, dark growth.

Results of experiments described in the present report indicate that suspensions of *R. rubum* mutants C and G1 and cell extracts from anaerobic, dark-grown G1 exhibited reactions characteristic of both pyruvate formate-lyase, evidenced by the exchange of [^{14}C]formate into pyruvate, and pyruvate:ferredoxin oxidoreductase, suggested by the exchange of [^{14}C]bi-

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carbonate into pyruvate. However, pyruvate formate-lyase appeared to be the important energy-yielding pathway in dark-grown cells. Results of initial studies on H_2 production from sodium formate, catalyzed by formic hydrogenlyase in anaerobic, dark-grown *R. rubrum*, are also presented.

MATERIALS AND METHODS

Organisms, growth medium, and growth conditions. Dark, fermentatively grown *R. rubrum* mutants C and G1 were used. Both mutant phenotypes were isolated from parent strain *R. rubrum* S_1 after long-term growth in anaerobic, dark conditions as described earlier (35).

Only anaerobic, dark-grown *R. rubrum* cells were used in experiments. Cells were grown in a complex medium containing yeast extract, peptone, and sodium pyruvate (34, 35). Batch cultures, used to prepare cell extracts, were grown in the dark under a stream of sterile O_2-free argon in 20-liter carboys. Liquid medium in carboys was prepared with sodium thioglycolate (0.02 g per 100 ml of distilled water) as a reducing agent. Sodium pyruvate solution was filter sterilized and added separately to sterile reduced medium to obtain a final concentration of about 0.5 g/100 ml of distilled water. The final pH of the growth medium was 7.0. The pyruvate medium was inoculated with 2.0 to 2.5 liters of a late-logarithmic culture of *R. rubrum* grown at 30°C in anaerobic, dark conditions. Sodium pyruvate, at the concentration added, allowed cells to grow to a density of 1×10^8 to 2×10^8 cells per ml in 24 h of incubation at 30°C. Once the cells reached this density, batch cultures were supplemented with additional sodium pyruvate to reestablish a substrate concentration approximately equal to 0.5 g/100 ml. This allowed cells to increase further to a density of 5×10^8 cells per ml and form about 0.7 g of wet cell material per liter of growth medium.

Preparation of cell suspensions and cell extracts. Anaerobic, dark-grown *R. rubrum* was collected from late-logarithmic cultures while cells were actively motile. Bacteria for use in studies with whole-cell suspensions were harvested from growth medium under an O_2-free argon atmosphere with a refrigerated centrifuge (Sorvall RC2-B; 10,500 × *g*, 10 min, 4°C). Cells from batch cultures for preparation of protein extracts, however, were collected by continuous-flow centrifuging. To keep cells from clumping together during the harvesting period, culture fluid in carboys was maintained under a stream of O_2-free argon. Cell material collected in the continuous-flow centrifuge assembly, where it became exposed to air, was immediately removed after harvesting and placed under argon. All subsequent cell work was performed under strict anaerobic conditions. Before use in experiments, anaerobic, dark-grown *R. rubrum* mutant C and G1 cells were washed once at 4°C with an O_2-free solution containing: potassium phosphate buffer (pH 7.0), 50 mM; $MgCl_2$, 6 mM; and dithiothreitol, 1 mM. In experiments to determine the phosphate requirement of the pyruvate-degrading reaction(s) in cells, potassium phosphate buffer in the anaerobic washing solution was replaced with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) buffer, pH 7.0.

Suspensions of nongrowing *R. rubrum* were prepared under an argon atmosphere in serum bottles tightly sealed with a rubber sleeve serum bottle stopper. Bacteria were suspended in enough of the O_2-free buffered solution (the same as had been used to wash the cells) to produce a density of about 5×10^9 cells per ml (equivalent to 2 mg of cell protein per ml). To assure that no air was present, the cell suspension was evacuated and flushed with argon. This process was repeated five successive times.

Cell extracts were prepared from dark, fermentatively grown *R. rubrum* suspended in a buffered solution approximately equal to the volume of the packed cell material. The suspending solution contained: buffer, 100 or 150 mM; sodium pyruvate, 10 mM; $MgCl_2$, 6 mM; and dithiothreitol, 1 mM. When cell extracts were prepared for use in experiments performed at different pH values between 6.0 and 7.5, cells were suspended in a solution containing 150 mM potassium phosphate (pH 7.0) or 100 mM HEPES buffer. To prepare cell extract for use in experiments at a pH value greater than 7.5, potassium phosphate was replaced with either *N*-2-hydroxyethylpiperazine-*N'*-propanesulfonate (HEPS) or *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonate buffer at a concentration of 100 mM (pH 8.3). Failure to add sodium pyruvate to the suspending solution resulted in a rapid loss of pyruvate-degrading activity after cell breakage. Cells were disrupted by means of a French pressure cell at 18,000 lb/in^2, and cell debris was removed by centrifugation (41,000 × *g*, 30 min, 4°C). The soluble protein fraction was desalted at 4°C by means of a gel filtration chromatographic column prepared with coarse-grade Sephadex G-25. Before use, the chromatographic column (1.1 by 13 to 15 cm) was made anaerobic during equilibration with a solution containing $MgCl_2$, sodium pyruvate, and dithiothreitol, at concentrations given above. The amount of buffer in this solution, however, was reduced to 10 mM. Crude protein extract was eluted from the anaerobic column at a rate of about 0.3 ml/min and collected under a stream of O_2-free argon. Desalted crude protein extract was used immediately in experiments or distributed under argon gas into serum bottles sealed with rubber serum stoppers and stored in a liquid nitrogen refrigerator. After 4 to 5 weeks at liquid N_2 temperature, the activity in protein extracts decreased about 20 to 30%.

In studies indicated, crude, desalted protein extract received further treatment before use. In one experiment, crude protein extract was centrifuged for 1 h at 200,000 × *g* at 4°C to remove larger membrane fragments; in a second experiment, protein was treated with diethylaminoethyl-cellulose column chromatography to remove ferredoxin(s) (28).

Preparation of anaerobic solutions and reaction mixtures. Solutions used in experiments were put into bottles sealed with tight-fitting rubber sleeve serum bottle stoppers and made anaerobic by evacu-

ating and flushing the bottles with argon. The amount of oxygen in solutions of bicarbonate was decreased by bubbling with argon gas for 10 min at 4°C.

Reaction mixtures were prepared by transferring samples of anaerobic solutions or cell suspensions by means of a hypodermic syringe (preflushed with argon) into reaction vessels under a stream of argon. After preparing reaction mixtures, Warburg vessels used in manometric studies were immediately placed onto manometers, and gassing was continued an additional 15 min (37). In other experiments, reactions occurred in 5-ml Fernbach flasks sealed with rubber sleeve serum stoppers. By using stringent anaerobic techniques, reactions could be performed without reducing agents. Dithiothreitol, however, was added in most instances without affecting the reactions, whereas compounds such as sodium thioglycolate, mercaptoethanol, 3-mercapto-1,2-propanediol, or sodium dithionite caused a reduction in pyruvate-degrading activity.

Determination of cell numbers and protein. Direct cell counts were made by using a Petroff-Hausser bacterial cell counting chamber or estimated turbidimetrically in liquid medium, based on previously determined measurements of optical density at 660 nm versus cell number. For protein determinations, samples were digested with 1 M NaOH for 24 h at room temperature. In samples with HEPES or HEPS buffer, which interfered with the Folin protein assay (8), protein was precipitated by 10% (wt/vol) trichloroacetic acid. Precipitated protein was then washed with absolute ethanol and digested in alkali. Protein content in samples was estimated by the method of Lowry et al. (19), with bovine serum albumin as a standard.

Measurement of gas production. Conventional manometric techniques (37) were used to measure production of H_2 and/or CO_2 gases under an argon atmosphere. A Bronwell photosynthetic circular Warburg apparatus operating at 30°C in a room with subdued light illumination was used. Reaction mixtures contained: potassium phosphate buffer (pH 7.0), 50 mM; $MgCl_2$, 2 mM; sodium pyruvate or formate, 10 mM; and approximately 5×10^9 cells (equivalent to 2 mg of cell protein). Where indicated, sodium hypophosphite, 2 mM, was included in reaction mixtures. The final volume of the reaction mixture was 3.0 ml.

Similar experimental conditions were used during incubation of whole cells with sodium [1-^{14}C]pyruvate. In these studies, however, reaction mixtures contained 12 mM sodium [1-^{14}C]pyruvate with a specific activity equal to 163 cpm per μmol of sodium pyruvate and 6×10^9 cells (equivalent to 2.7 mg of cell protein). $^{14}CO_2$ produced from sodium [1-^{14}C]pyruvate was absorbed in 0.3 ml of methyl benzethonium hydroxide (1 M in methanol; Sigma Chemical Co., St. Louis, Mo.) placed in the center well of the reaction vessel. The concentration of $^{14}CO_2$ evolved under these conditions was estimated by measurement of H_2 gas production, since cells formed a H_2-CO_2 gas mixture (50:50, vol/vol) during incubation with sodium pyruvate (unpublished data). Gas chromatographic analysis established that only H_2 gas accumulated in the gas space when a CO_2 gas absorbent was present in the reaction vessel.

Exchange reactions. Enzyme-catalyzed exchange of sodium [^{14}C]bicarbonate or sodium [^{14}C]formate into sodium pyruvate was examined using whole cells and protein extract from anaerobic, dark-grown *R. rubrum*. Reaction mixtures contained: buffer, 100 mM; $MgCl_2$, 6 mM; dithiothreitol, 1 mM; sodium pyruvate, 30 mM; and sodium [^{14}C]bicarbonate or sodium [^{14}C]formate, 30 mM (specific activity equal to 3,080 or 3,800 cpm/μmol, respectively). Potassium phosphate buffer, pH 7.0, was used in experiments with whole-cell suspensions. The ability of protein extract to catalyze exchange reactions over a range of pH values was examined. In this case, different buffers were used: potassium phosphate was added to reactions performed at pH values between 6.0 and 7.5; HEPS was used between pH 7.5 and 8.7; and glycine-KOH buffer was added to reactions performed at pH values above 9.0. In the experiments indicated, 2 mM sodium hypophosphite, a formate analog (20, 32), was added to block the formate exchange reaction catalyzed by whole cells. Similar inhibition was obtained in reactions using protein extract when the concentration of sodium hypophosphite was increased to 9 mM. Exchange reactions were started by introducing 5×10^9 anaerobic, dark-grown cells (equivalent to 2 mg of protein) or protein extract into reaction vessels. Reactions with whole cells and cell extract were incubated in darkness at 30 and 37°C, respectively, and agitated in a Dubnoff metabolic incubator.

After 30 min, reactions were stopped with 0.1 ml of a 20% solution (vol/vol) of perchloric acid. Pyruvate was recovered as the 2,4-dinitrophenylhydrazone according to the method of Raeburn and Rabinowitz (25). Use of liquid chromatography and radioautographic techniques (1, 21) indicated that only [^{14}C]pyruvate was present in samples purified from reaction mixtures. This ^{14}C-labeled product and the 2,4-dinitrophenylhydrazone formed chemically from [1-^{14}C]pyruvic acid co-chromatographed in a solvent system (21) consisting of 1-butanol, water, and ethanol (5:4:1, vol/vol/vol). Purified 2,4-dinitrophenylhydrazone from the reaction was collected onto a dry, preweighed membrane filter (0.45-μm average pore diameter, Millipore Corp.) and dried at 50°C for 12 h, and the amount of pyruvate, as the 2,4-phenylhydrazone, was determined gravimetrically. Thereafter, the filter with dried sample was glued onto a planchet (9), and the amount of ^{14}C incorporated into the α-keto acid was determined.

Degradation of sodium pyruvate. Reaction mixtures were similar to those used in exchange studies described above, except the ^{14}C-labeled sodium bicarbonate or sodium formate was replaced by phosphotransacetylase (EC 2.3.1.8) (10 U) and 1 mM coenzyme A. In one experiment, described in Results, different types of ferredoxin were also present. The pH value of reactions performed between 6.5 and 7.5 was maintained, as before, using 150 mM potassium phosphate buffer. To support pyruvate-degrading activity at pH values greater than 7.5, a

similar concentration of potassium phosphate was included in reactions supplemented with 100 mM HEPS or glycine-KOH buffer. In certain other reactions, 100 mM imidazole sulfate instead of HEPS was used to buffer reaction mixtures at pH 8.3. The final volume of the reaction mixture was 1.0 ml. Incubation occurred at 37°C under conditions used during exchange studies.

Acyl phosphate(s) formed by cell extracts in reactions with sodium pyruvate was converted to their hydroxamic acid derivative with neutralized hydroxylamine by the method of Lovenberg et al. (18), except that 0.5 ml of 3 M HCl was added before reacting the hydroxylamine-treated solution with $FeCl_3$.

In one experiment, the hydroxamic acid derivative formed by protein extract was obtained from the reaction mixture and identified as acetylphosphate (Ac-P) by using ascending paper chromatography according to the method of Stadtman and Barker (30). Both acetylhydroxamic acid from Ac-P produced enzymically and that formed from chemically synthesized Ac-P (29) migrated with the same R_f value and co-chromatographed as a single spot. The amount of Ac-P formed in reaction mixtures was measured spectrophotometrically at 540 nm (18). Ac-P prepared chemically was used as a standard.

Production of formate during incubation of protein extract with sodium pyruvate was measured enzymically using tetrahydrofolate synthetase (EC 6.3.4.3) by the method of Rabinowitz (24). Samples were withdrawn from reaction mixtures, heated for 1 min at 100°C in a water bath, and immediately cooled at 4°C. The concentration of formate after formation of 5,10-methenyltetrahydrofolic acid was determined spectrophotometrically at 350 nm.

Measurement of radioactivity. Samples of methyl benzethonium hydroxide containing absorbed $^{14}CO_2$ were removed from the center well of Warburg reaction vessels, and the center well was then washed twice with absolute methanol. The sample and methanol washings were placed in a glass vial with 5 ml of Bray solution (3), and radioactivity was determined at 4°C by use of a Tri-Carb liquid scintillation spectrometer (model 3320, Packard Instrument Co.). Measurements were corrected for background radiation and quenching. Radioactivity of dried samples of [^{14}C]pyruvate as the 2,4-dinitrophenylhydrazone was measured using a decade scaler (model 8703 Nuclear-Chicago Corp.). Determinations were corrected to infinite thinness.

Chemicals. Sodium pyruvate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and pyruvic acid was purchased from either Sigma Chemical Co., St. Louis, Mo., or Matheson, Coleman and Bell, Norwood, Ohio. Radioactive ^{14}C-labeled compounds were obtained from New England Nuclear, Boston, Mass. Tetrahydrofolic acid was purchased from Sigma Chemical Co. The dilithium salt of Ac-P was synthesized by the method of Stadtman and Barker (30). Ferredoxin from *Clostridium pasteurianum* was purchased from Worthington Biochemicals Corp., Freehold, N.J. Spinach ferredoxin and phosphotransacetylase purified from *Clostridium kluyveri* were obtained from Sigma Chemical Co. *Chromatium vinosum* D ferredoxin was purified by the method of Buchanan and Arnon (4). Tetrahydrofolate synthetase from *Clostrodium acidiurici* (132 IU per mg of protein) was a generous gift from J. C. Rabinowitz. N. Good kindly provided samples of buffering compounds synthesized in his laboratory (8) for testing in our system. Other commercially available chemicals and gas products were of reagent-grade quality.

RESULTS

Exchange experiments. The ability of resting suspensions of anaerobic, dark-grown *R. rubrum* mutant C or G1 cells to catalyze the exchange of sodium [1-^{14}C]formate or sodium [^{14}C]bicarbonate into pyruvate is shown in Table 1.

As observed, suspensions of fermentatively grown C or G1 both reacted with sodium pyruvate and either sodium [^{14}C]formate or sodium [^{14}C]bicarbonate to produce [^{14}C]pyruvate. These data suggested that both pyruvate formate-lyase (20, 23, 32) and pyruvate:ferredoxin oxidoreductase (25, 33) activities were present in cells. The amount of ^{14}C label exchanged into

TABLE 1. *Sodium [^{14}C]bicarbonate and sodium [^{14}C]formate exchanged into sodium pyruvate by cell suspensions[a] of anaerobic, dark-grown Rhodospirillum rubrum mutants C and G1*

Mutant	Reaction[b]	Sp act of pyruvate recovered (cpm × μmol^{-1})[c]	[^{14}C]pyruvate formed (μmol)
C	$^{14}CO_2$-pyruvate exchange	295	2.9
	Same, plus sodium hypophosphite	206	2.0
	H^{14}COOH-pyruvate exchange	1,255	9.9
	Same plus sodium hypophosphite	139	1.1
G1	$^{14}CO_2$-pyruvate exchange	230	2.3
	Same, plus sodium hypophosphite	142	1.4
	H^{14}COOH-pyruvate exchange	1,115	8.6
	Same, plus sodium hypophosphite	123	1.0

[a] In each experiment 5×10^9 cells were used. This number of C or G1 corresponded to 2.2 or 1.8 mg of protein, respectively.

[b] In reactions, 30 mM sodium pyruvate and an equal concentration of sodium [^{14}C]bicarbonate or sodium [^{14}C]formate were added to reaction mixtures to reach a specific activity of 3,080 and 3,800 cpm/μmol, respectively. Where indicated, sodium hypophosphite was added to reactions to obtain a concentration of 2 mM.

[c] Average values with a range of ±5% obtained after a 30-min reaction period from two experiments.

the α-keto acid, however, was different. When cells were placed with sodium [^{14}C]formate, the specific activity of [^{14}C]pyruvate was approximately 3.5 times greater than if sodium [^{14}C]bicarbonate was used (Table 1). In these experiments both exchange reactions occurred at a linear rate during a 30-min incubation period and were dependent upon the number of cells used. Exposure of anaerobic, dark-grown cells to light during the incubation period had no significant effect on exchange activity (data not shown).

To determine whether formate-pyruvate and bicarbonate-pyruvate specific exchange reactions were involved, sodium hypophosphite, a formate analog and potent inhibitor of pyruvate formate-lyase (20, 32), was added to reaction mixtures. As observed in Table 1, when the inhibitor was added to reactions with either mutant C or G1, pyruvate formate-lyase activity was inhibited and no [^{14}C]pyruvate was formed. In contrast, if sodium [^{14}C]formate was replaced with sodium [^{14}C]bicarbonate, sodium hypophosphite had no significant effect on the formation of [^{14}C]pyruvate. Consequently, these data were interpreted as supporting the idea that different exchange reactions were present. It appeared that formate or bicarbonate was exchanged directly into pyruvate to form the ^{14}C-labeled compound.

The extent of ^{14}C isotope exchanged into sodium pyruvate was calculated using data in Table 1. Under these conditions and after 30 min, the formate-pyruvate exchange reaction catalyzed by C and G1 whole cells reached approximately 66 and 57%, respectively, of the anticipated equilibrium value. The extent of the ^{14}C-labeled sodium bicarbonate-pyruvate exchange was much lower. It was noted, however, that whole cells used in these studies not only catalyzed the formation of [^{14}C]pyruvate, but also metabolized reactants involved in the different exchange reactions. Since changes in the concentration of pyruvate, formate, or bicarbonate could occur in studies with whole cells, assessment of the reactions based on theoretic equilibrium values was not completely reliable. Nevertheless, results of experiments with crude soluble protein extracts suggested that the relationship established with whole cells was qualitatively correct and that larger amounts of sodium [^{14}C]formate rather than sodium [^{14}C]bicarbonate were exchanged into pyruvate.

In these in vitro experiments, protein was obtained from *R. rubrum* mutant G1, since cells grew somewhat faster (34) and did not produce the extensive internal membranes present in mutant C (35). Nevertheless, reactions detected in G1 were also catalyzed by protein from mutant C. No significant difference in the metabolism of sodium pyruvate by protein from either anaerobic, dark-grown C or G1 was identified.

In cell-free studies with G1 protein extract, as in experiments using whole cells, the amount of [^{14}C]pyruvate formed in reaction mixtures increased at a linear rate during 30 min of incubation and was dependent on the amount of protein used in reactions at concentrations up to 5 to 7 mg of protein per ml. When a larger amount of cell extract was added to reaction solutions, material(s) in the crude protein solution interfered with formation of the bright-yellow-colored phenylhydrazone of pyruvate and resulted in production of an orange-yellow flocculant precipitate. Chromatographic analysis suggested that the orange-yellow product was a mixture of the phenylhydrazone of pyruvate and an orange-colored material that failed to migrate on thin-layer chromatographic plates (see Materials and Methods). Although the chemical nature of the orange-colored compound(s) was not determined, thin-layer chromatographic and radioautographic techniques indicated that ^{14}C radioactivity was only located in the phenylhydrazone of pyruvate purified from reaction mixtures. No evidence for other phenylhydrazones or ^{14}C-labeled material that co-purified with the phenylhydrazone of pyruvate (e.g., the orange-colored product described above) was detected. To minimize error in measuring the amount of pyruvate purified from reaction mixtures by gravimetric methods, experiments were performed with a concentration of protein such that formation of the orange-colored material was avoided.

The effect of pH on sodium formate and sodium bicarbonate exchange activity in reaction mixtures with protein extract from G1 is presented in Fig. 1. When experiments were performed at different pH values ranging from 6.0 to 9.4, the largest amounts of sodium [^{14}C]formate were exchanged into pyruvate in reactions performed between pH 7.3 and 7.5. Since the concentration of reactants did not change significantly during the incubation period, it was determined that approximately 4.5 μmol of sodium [^{14}C]formate was exchanged into pyruvate after 30 min. Likewise, protein extracts reacted with sodium [^{14}C]bicarbonate and pyruvate under similar conditions, but only about 0.3 μmol was exchanged in reactions performed at pH between 6.7 and 7.1 (Fig. 1). Calculations based on these data, taking into account the different amount of protein used in each reaction series (Fig. 1), indicated that at

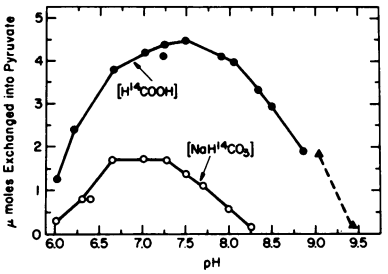

FIG. 1. *Exchange of sodium [^{14}C]formate or sodium [^{14}C]bicarbonate into sodium pyruvate at different pH values by crude protein extract from anaerobic, dark-grown Rhodospirillum rubrum mutant G1 cells. Measurement of [^{14}C]formate exchange activity after 30 min of incubation at 37°C was performed with reactions containing 2.9 mg of protein; 5.9 mg of protein was used in reactions with [^{14}C]bicarbonate. Potassium phosphate was added to reactions at pH values between 6.0 and 7.5, HEPS was used between pH 7.5 and 8.7, and glycine-KOH buffer was added to reactions performed at pH 9.0 or higher* (▲).

respective optimum pH values, pyruvate formate-lyase activity was about fivefold greater than that of pyruvate:ferredoxin oxidoreductase. Nevertheless, unequal activity and the slight difference in apparent pH optima by no means established that separate enzyme systems were involved. To examine this question, reactions were performed in the presence of sodium hypophosphite or sodium glyoxylate, a reported inhibitor of pyruvate:ferredoxin oxidoreductase (33).

In these experiments, addition of 9 mM sodium hypophosphite to reaction mixtures containing sodium [^{14}C]formate and pyruvate inhibited the exchange reaction. Under similar conditions, with sodium [^{14}C]bicarbonate instead of sodium [^{14}C]formate, hypophosphite had no effect. The amount of sodium [^{14}C]bicarbonate exchanged into pyruvate in reactions with and without the inhibitor was 0.41 and 0.39 μmol/mg of protein after 30 min, respectively. In the presence of 9 mM sodium glyoxylate, instead of sodium hypophosphite, the amount of sodium [^{14}C]bicarbonate exchanged into pyruvate after 30 min decreased from 0.30 to 0.02 μmol/mg of protein. Conversely, when sodium glyoxylate was included in reactions with sodium [^{14}C]formate, the amount of [^{14}C]pyruvate formed decreased about 50%. The partial inhibition of the formate exchange reaction by sodium glyoxylate, however, was not understood. From the preceding data, it appeared that crude protein extract from anaerobic, dark-grown *R. rubrum* contained both formate- and bicarbonate-specific exchange reactions. Further substantiation of this notion, however, will require enzyme purification. Nevertheless, since larger amounts of formate were exchanged into pyruvate, it was concluded from these data that pyruvate formate-lyase was the quantitatively important substrate-degrading enzyme involved in the reaction in anaerobic dark-grown cells.

Additional experiments were performed to determine the effect of different buffering compounds on the formate exchange reaction. Results indicated that, at pH 7.5, either potassium phosphate, HEPS, or tris(hydroxymethyl)methylaminopropanesulfonate buffer could be used at concentrations of up to 150 mM without any measurable decrease in pyruvate formate-lyase activity. Imidazole sulfate or imidazole acetate at 100 mM decreased activity by about 15%.

Production of Ac-P from pyruvate. When cell extract from fermentatively grown G1 was incubated anaerobically with sodium pyruvate in reactions containing coenzyme A and potassium phosphate, pyruvate was degraded with production of Ac-P and formate (Fig. 2; production of formate, also shown, is discussed in a separate section). Pyruvate-degrading activity was associated with the soluble protein fraction. No activity was detected with particulate material obtained after ultracentrifugation of crude protein extract (see Materials and Methods).

The effect of pH on Ac-P formation from pyruvate by the crude soluble protein fraction is presented in Fig. 2. Maximum amounts of Ac-P

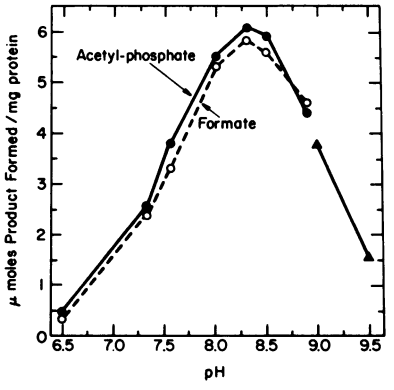

FIG. 2. *Production of Ac-P (solid line) and formate (broken line) from sodium pyruvate after 30 min of incubation at 37°C. Reactions contained 1 mg of protein extract from anaerobic, dark-grown Rhodospirillum rubrum mutant G1 cells. Potassium phosphate (150 mM) was present in all reactions, and different pH values were obtained with buffers described in Fig. 1.*

were produced during a 30-min incubation in reactions performed at pH values between 8.3 and 8.5, with 42% activity at about 7.3 and 9.2. From these data, it appeared that the direction of pyruvate formate-lyase activity in *R. rubrum* was influenced by hydrogen ion concentration, as has also been suggested for the analogous enzymic reaction in *Escherichia coli* (14). In *R. rubrum*, the enzyme appeared to catalyze the exchange in the forward and reverse directions (Fig. 1) at pH 7.3 to 7.5 and exchanged larger amounts of sodium [^{14}C]formate into pyruvate; at pH 8.3 to 8.5, smaller amounts of sodium [^{14}C]formate were exchanged, but sodium pyruvate was degraded more extensively to Ac-P and formate (Fig. 2). The alternative suggestion, that separate pyruvate-formate-specific reactions are involved, however, is under study.

Additional experiments were performed at pH 8.3 to determine which reaction conditions best supported Ac-P production. Anaerobic degradation of sodium pyruvate with production of Ac-P occurred in a linear manner during the 30-min incubation with respect to the amount of substrate and potassium phosphate present in reaction mixtures. With excess phosphate (150 mM) and 1 mM coenzyme A, maximum production of Ac-P was achieved in reactions supplemented with sodium pyruvate at concentrations ranging from 20 to 40 mM. The amount of Ac-P formed decreased when larger amounts of sodium pyruvate were used. Pyruvate formate-lyase activity required both phosphate and coenzyme A.

In experiments performed at pH 8.3 with imidazole sulfate buffer, concentrations of 1 mM coenzyme A and 100 mM potassium phosphate supported virtually maximal activity (Fig. 3). Further addition of coenzyme A to reach a final concentration of 1.5 mM (Fig. 3B) did not appreciably influence the reaction, but enzyme activity decreased when the concentration of potassium phosphate exceeded 180 mM. Phosphotransacetylase (10 U) was also added to reactions and resulted in a twofold increase in Ac-P formation (data not shown). In reactions with cell extract, the largest amounts of Ac-P were formed when HEPS buffer (pH 8.3) was used (Fig. 2) and with imidazole sulfate; the amount of pyruvate degraded was reduced by 61% (Fig. 3). (As indicated earlier, imidazole buffers had a smaller inhibitory effect on formate exchange activity, but the reason for this was not clear.)

In experiments performed in HEPS buffer (pH 8.3), formation of Ac-P was directly related to the amount of protein extract used, up to a final concentration of about 3 mg of protein per ml of reaction mixture, and increased in a linear fashion during the incubation period. Illumination of reaction mixtures with 9,600 lx of white light did not affect Ac-P formation, but the presence of air completely inactivated the enzymic reaction. Likewise, under argon gas, no Ac-P was produced from pyruvate when boiled extract was used.

Production of formate. The demonstration of an active formate-pyruvate exchange reaction in whole cells and in protein extract suggested that pyruvate formate-lyase was the important substrate-degrading reaction in anaerobic, dark-grown *R. rubrum*. In accord with this idea, cell extracts incubated with sodium pyruvate produced formate and Ac-P at similar rates (data not shown) and, after 30 min of incubation, approximately equal amounts of these compounds were present in reactions performed over a range of pH values (Fig. 2). Under these experimental conditions, performed without adding adenosine 5'-diphosphate, acetyl kinase in cell extracts did not degrade significant amounts of Ac-P to acetate and inorganic phosphate, and formate was not oxidized by formic hydrogenylase to H_2 and CO_2 (unpublished data).

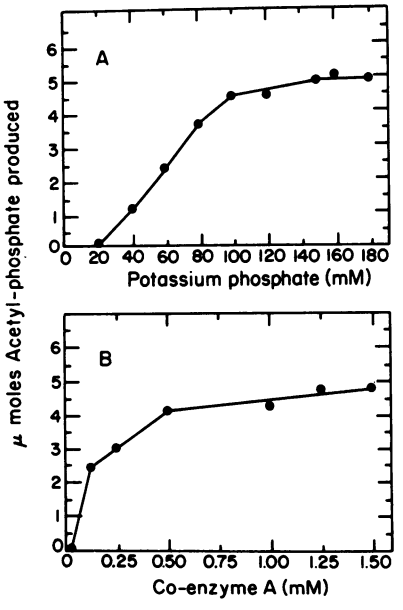

FIG. 3. *Formation of Ac-P from sodium pyruvate (30 mM) by protein extract from anaerobic, dark-grown Rhodospirillum rubrum in reactions with (A) coenzyme A (1 mM) and different concentrations of potassium phosphate and (B) potassium phosphate (150 mM) with different amounts of coenzyme A. Reactions were incubated for 30 min at 37°C with 100 mM imidazole sulfate buffer (pH 8.3) and contained 2.4 mg of protein.*

Additional evidence that pyruvate formate-lyase was the major energy-yielding reaction in anaerobic, dark-grown *R. rubrum* was provided by the following observations. When sodium hypophosphite was present with sodium pyruvate in experiments performed at the different pH values indicated in Fig. 1 and 2, even though bicarbonate exchange activity occurred, no Ac-P was detected in reactions after 30 min of incubation. Second, in studies without sodium hypophosphite, the amount of Ac-P produced from pyruvate per milligram of protein was not significantly influenced after cell extracts were treated with diethylaminoethyl-cellulose to remove ferredoxins (28). In this regard, addition of 50 μg of ferredoxin purified from spinach, *C. pasteurianum*, or *C. vinosum* D to reactions had no effect on increasing the amount of Ac-P formed from sodium pyruvate. Although these experiments suggested that pyruvate formate-lyase alone generated Ac-P for the production of adenosine 5'-triphosphate in cells during dark growth, results of recent studies using radioisotope techniques suggested that an additional reaction, which was not influenced by hypophosphite, functioned at a very low level in protein extract to produce $^{14}CO_2$ from sodium [1-^{14}C]pyruvate. Studies are under way to identify the reaction, presumably pyruvate:ferredoxin oxidoreductase (35), and determine its role in anaerobic, dark-grown *R. rubrum*.

Gas production from formate. During anaerobic, dark growth, *R. rubrum* fermented sodium pyruvate and produced formate and approximately equal amounts of H_2 and CO_2 gases (34). Moreover, with pyruvate formate-lyase as the important pyruvate-degrading reaction in anaerobic, dark-grown cells, it followed that formate served as an intermediate during gas production. Results of experiments supporting this idea are presented in Fig. 4. As observed, when G1 was suspended in solution with sodium formate or sodium pyruvate, cells produced approximately equal amounts of H_2 gas from either substrate at a rate linear with time. In separate manometric experiments, it was determined that when KOH was not included in the center well of reaction vessels, as in Fig. 4, a gas mixture composed of equimolar amounts of H_2 and CO_2 was formed.

Additional evidence that formate was the important intermediate in gas formation from sodium pyruvate was obtained in experiments using sodium hypophosphite and sodium [1-^{14}C]pyruvate. As observed in Fig. 4, when cells were exposed to sodium pyruvate and 2 mM sodium hypophosphite, the latter a potent inhibitor of pyruvate formate-lyase (Table 1), no H_2 gas production was detected. In addition, in separate experiments, when G1 was allowed to metabolize sodium [1-^{14}C]pyruvate, cells evolved $^{14}CO_2$ at a linear rate (Fig. 5). As expected, with formate as an intermediate in gas evolution, when unlabeled sodium formate was introduced into the reaction, the concentration of $^{14}CO_2$ produced in the gas atmosphere was rapidly diluted. From these data, it seemed evident that during anaerobic, dark development, *R. rubrum* produced formate from sodium pyruvate, which was then oxidized to H_2 and CO_2. The formic hydrogenlyase reaction operated unaffected in either anaerobic, light, or dark conditions, produced equimolar amounts of H_2 and CO_2 with formate, and was strongly inhibited by carbon monoxide (22).

DISCUSSION

R. rubrum grew under strict anaerobic conditions in darkness and fermented sodium pyruvate. During anaerobic dark growth, cells obtained about 1 mol of ATP per mol of sodium pyruvate and producted acetate, formate, H_2, and CO_2 (34). In accord with these observations, pyruvate formate-lyase appeared to be the quantitatively important substrate-degrading enzyme. Formate produced by pyruvate formate-lyase was subsequently oxidized to H_2 and CO_2 by a formic hydrogenlyase reaction. The proposed pathway for fermentative metab-

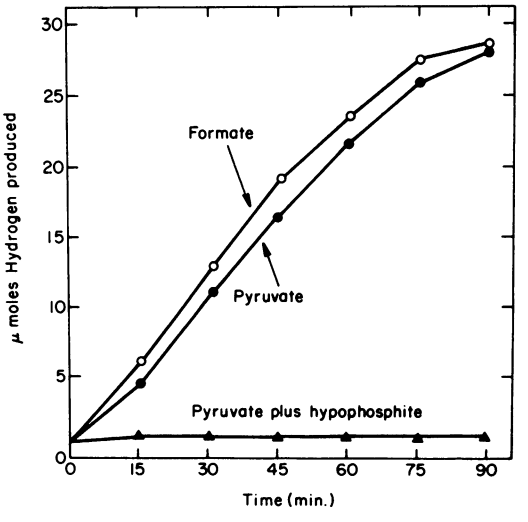

FIG. 4. *Evolution of hydrogen gas by whole-cell suspensions of anaerobic dark-grown Rhodospirillum rubrum during incubation at 30°C with sodium-formate (○); sodium pyruvate (●); and sodium pyruvate plus sodium hypophosphite (▲). Reactions contained approximately 5 × 10⁹ cells, which corresponded to 2 mg of protein.*

olism of sodium pyruvate by anaerobic dark-grown *R. rubrum* is shown in Fig. 6.

In *R. rubrum*, pyruvate-degrading reactions appeared to function at widely separated pH values. In in vitro studies, the largest amounts of Ac-P and formate were produced from sodium pyruvate in reactions at pH 8.3 to 8.5 (Fig. 2 and 6). In contrast, gas production from formate, catalyzed by crude protein extract, occurred best at pH 6.5 to 6.7 (unpublished data). These observations suggested that pH changes inside cells during anaerobic, dark development might occur to regulate the degradation of the energy-yielding substrate. At low pH values, formic hydrogenlyase should rapidly degrade formate to H_2 and CO_2. Oxidation of the acid product in this manner might result in an increase in the pH value inside the cell and stimulate pyruvate formate-lyase activity. Composite results from other laboratories (14, 23) have suggested that a similar regulatory mechanism occurs in *E. coli*, which also degrades sodium pyruvate by the pyruvate formate-lyase and formic hydrogenlyase pathways during anaerobic growth. However, since experiments with *E. coli* were performed under a variety of different reaction conditions, further study is warranted before a direct comparison can be made with the system in *R. rubrum*. A similar regulatory influence of pH on pyruvate metabolism was suggested by McCormick et al. (20) in studies using *Veillonella alcalescens* (formerly *Micrococcus lactilyticus*; see reference 5). In *V. alcalescens*, however, pH appeared to influence the activity of two different energy-yielding reactions inside cells. When cells were suspended in solution at acid pH, pyruvate was degraded via a pyruvate:ferredoxin oxidoreductase pathway; at alkaline pH, the α-keto acid appeared to be degraded by pyruvate formate-lyase.

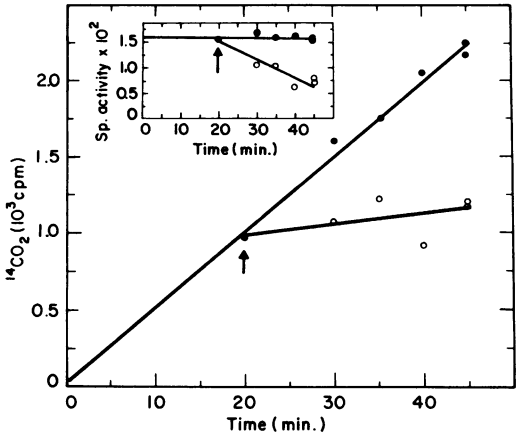

FIG. 5. *Production of [^{14}C]CO_2 from sodium [1-^{14}C]pyruvate by suspensions of Rhodospirillum rubrum mutant G1 cells after growth in anaerobic, dark conditions. At the beginning of the experiment, reaction vessels contained 36 μmol of sodium [1-^{14}C]pyruvate (specific activity equal to 163 cpm/μmol) and 6.9 × 10^9 cells, which corresponded to 2.7 mg of protein. After 20 min of anaerobic, dark incubation at 30°C, when approximately 30 μmol of sodium [1-^{14}C]pyruvate remained, an equal amount of sodium formate was added (arrow) to certain reaction vessels. Measurement of ^{14}C radioactivity (counts per minute) formed in reactions with (○) or without (●) sodium formate added are indicated. (Insert) Specific activity of [^{14}C]CO_2 (counts per minute per micromole) produced from sodium [1-^{14}C]pyruvate in reactions with (○) and without (●) sodium formate.*

The role of formic hydrogenlyase in fermentatively grown *R. rubrum* is a topic of continuing study. As suggested, the enzyme may play a role in regulating pyruvate formate-lyase during generation of energy from sodium pyruvate. In addition, formate oxidation might also occur with the reduction of nicotinamide adenine dinucleotides, as in other microbial systems (17, 26, 39). Of these, the report of formate dehydrogenase-linked NAD^+ reduction in anaerobic, light-grown *Rhodopseudomonas palustris* (39), an anoxygenic phototrophic microbe, was of particular interest. However, repeated attempts to demonstrate similar activity with crude protein extract from fermentatively grown *R. rubrum* were unsuccessful. In view of this, an alternate pyruvate-degrading reaction with low activity could function in cells to provide reduced nicotinamide adenine dinucleotide (phosphate) [$NAD(P)H_2$] required during growth. One such pathway involves pyruvate:ferredoxin oxidoreductase, the participation of which was indicated by the presence of pyruvate-bicarbonate exchange activity (Table 1 and Fig. 1) and by the observation that cell extracts from anaerobic, dark-grown *R. rubrum* catalyzed the production of small amounts of CO_2, but not H_2, from sodium pyruvate even during incubation with sodium hypophosphite (unpublished data). In addition to $NAD(P)^+$ re-

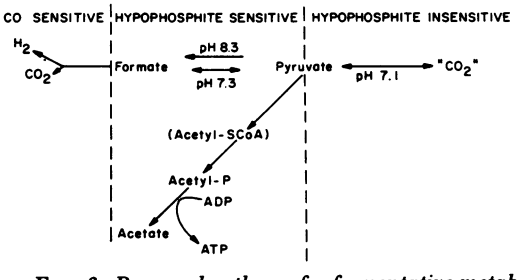

FIG. 6. *Proposed pathway for fermentative metabolism of sodium pyruvate by anaerobic, dark-grown Rhodospirillum rubrum.*

duction, the pyruvate:ferredoxin oxidoreductase pathway could operate, as in light-brown *R. rubrum* (2), and help explain the accumulation of poly-β-hydroxybutyrate in cells during anaerobic dark development (34, 35).

Results indicated that during anaerobic, dark growth, *R. rubrum* developed sodium pyruvate-degrading reactions that could function to produce energy and support cell growth, as in other anaerobic heterotrophic microorganisms. Accordingly, fermentative reactions in dark-grown cells involving adenosine 5'-triphosphate production, H_2 evolution, and, presumably, $NAD(P)^+$ reduction did not appear to be influenced by light. In contrast, the analogous activities in photosynthetically grown cells operated only in light (6, 7, 12, 13, 15, 38) where both light- (or adenosine 5'-triphosphate-) dependent H_2 evolution and NADP reduction were believed to play a role in the regulation of photosynthetic metabolism and cell growth (6). With knowledge about fermentative reactions in *R. rubrum*, it is possible to study this question further and determine how light-dependent H_2 production and $NAD(P)^+$ reduction reactions, as well as fermentative activities, are regulated by and participate in the development of photosynthetic competence when anaerobic, dark-grown cells are exposed to light.

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