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Electron Transport Phosphorylation Coupled to Fumarate Reduction by H_2- and Mg^{2+}-Dependent Adenosine Triphosphatase Activity in Extracts of the Rumen Anaerobe *Vibrio succinogenes*

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Vibrio succinogenes, an anaerobic bacterium, obtains its energy for growth from H_2 or formate oxidation coupled to the reduction of fumarate to succinate. Membrane preparations have been obtained from this organism that catalyze the synthesis of ATP during H_2 oxidation coupled to fumarate reduction. Esterification of orthophosphate is dependent on electron transfer, as evidenced by the requirement for both H_2 and fumarate. Phosphorylation is also dependent on ADP and is destroyed by boiling the membrane preparations. H_2 utilized for fumarate reduction and succinate formed are stoichiometric. The phosphorylation is markedly uncoupled by pentachlorophenol and gramicidin, but to a lesser extent by dinitrophenol and methyl viologen. 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide causes severe inhibition of H_2 oxidation as well as phosphorylation, but oligomycin or antimycin A has no demonstrable effect. Among several electron acceptors tested, significant phosphorylation is observed only with fumarate. A Mg^{2+}-dependent adenosine triphosphatase activity is present in both the membrane and soluble protein fractions. Highest activity is obtained with ATP as the substrate, and considerably less activity is obtained with other nucleoside triphosphates. The possibility that phosphorylation during "fumarate respiration" may play an important physiological role in the growth of many anaerobic and facultatively anaerobic bacteria is discussed.

Vibrio succinogenes is a gram-negative, anaerobic, curved rod-shaped bacterium, originally isolated from the bovine rumen by Wolin et al. (66). More recently, Smibert and Holdeman (53) reported isolation of a strain of this organism from a variety of human infections including jaw abscess, cheek abscess, and infections of the root canal. Wolin et al. (66) showed that this organism obtains energy for growth from the oxidation of H_2 or formate coupled to the reduction of fumarate to succinate according to the reaction: formate + fumarate + $H_2O \rightarrow$ succinate + HCO_3^-. The calculated free-energy change ($\Delta G'_0$) at pH 7.0 for this reaction is −20.4 kcal/mol (36), which is more than sufficient to account for the formation of at least one molecule of ATP. Malate, aspartate, or asparagine can substitute for fumarate in the above reaction, and it appears that these compounds are first converted to fumarate, which is then reduced to succinate (44, 66). Nitrate, which can substitute for fumarate in the above reaction, is predominantly reduced to nitrite in growing cultures but to ammonia by resting cells (66).

Jacobs and Wolin (29, 65) demonstrated *b*- and *c*-type cytochromes, hydrogenase, and fumarate reductase in this bacterium and suggested that cytochrome *b* is involved in the transfer of electrons from H_2 or formate to fumarate. More recent investigations (36, 37) showed that components of the formate fumarate electron transport system (ETS) in *V. succinogenes* include formate dehydrogenase (FD), flavin adenine dinucleotide, menaquinone (MQ), iron-sulfur protein (Fe-S), cytochromes *b* and *c*, and fumarate reductase (FR) and that these components were localized in the membrane fraction. Kröger and Innerhofer (37) presented evidence for the presence of two *b*-type cytochromes in *V. succinogenes* wich participate in the reduction of fumarate by formate; one cytochrome *b* (midpoint potential [E_m] = −200 mV) interacted between the FD and MQ, and a second cytochrome *b* (E_m =−20 mV) interacted between MQ and Fe-S. Based on these studies, Kröger and Innerhofer (37) proposed the following electron transport scheme:

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$$\text{FD} \rightarrow \text{cytochrome } b \rightarrow \underset{(E_m = -200\text{ mV})}{\text{MQ}} \rightarrow \text{cytochrome } b \rightarrow \text{Fe-S} \rightarrow \underset{(E_m = -20\text{ mV})}{\text{FR}}$$

Phosphorylation of endogenous AMP was demonstrated to occur in ampicillin spheroplasts of this organism during the oxidation of formate with fumarate (33); however, the effect of uncoupling agents on the phosphorylation in this system is not clear. Carbonylcyanide-*m*-chlorophenyl hydrazone (CCCP) was the only uncoupling agent studied and originally was stated to have no effect on the phosphorylation at 10 μmol per g of protein (34). More recently it has been briefly indicated by both Thauer et al. (55) and Kröger (34) that CCCP does uncouple the system, but no data are reported.

Based on the results of previous studies cited above, it appeared likely that *V. succinogenes* obtains ATP for growth by anaerobic oxidative phosphorylation. We present here for the first time direct evidence for the esterification of P_i into ATP which is coupled to the reduction of fumarate to succinate by H_2 by membrane preparations of this organism. We also provide evidence for the presence of a Mg^{2+}-adenosine triphosphatase (ATPase) activity in extracts of this organism.

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MATERIALS AND METHODS

Bacteria and growth medium. The strain of *V. succinogenes* used in this study was the same as VPI strain 10659 (53) and was originally isolated from bovine rumen (66) and supplied to us by M. J. Wolin.

The organism was routinely maintained in a medium previously described by Wolin et al. (66) in 16- by 150-mm sterile, screw-cap tubes. About 0.1 ml of the culture was transferred once every 2 weeks to 10 ml of fresh medium; the inoculated tubes were incubated at 37°C for 24 h and stored at 4°C until the next transfer.

The medium used for growing the organism in 1- or 20-liter batches was the same as the maintenance medium except that $MgCl_2 \cdot 6H_2O$ and $FeSO_4$ were sterilized separately and added aseptically to cooled, autoclaved medium. For growing the organism in 20-liter batches, 10 ml of a 24-h culture was used to inoculate 1 liter of freshly prepared medium in a 2-liter rubber-stoppered flask containing an N_2 gas phase. After, incubation for 24 h, this flask culture was used to inoculate 19 liters of medium in a 20-liter rubber-stoppered glass carboy with an N_2 gas phase and was incubated for 24 h. All incubations were at 37°C.

Preparation of membrane fractions. Cells were harvested by centrifugation with a Sharples centrifuge and were suspended in 2 volumes of 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 6.5. The cells were broken in a French pressure cell at 5,000 lb/in^2, and a few crystals of deoxyribonuclease I (Sigma) were added to reduce the viscosity of the broken-cell preparation. After 10 min, the preparation was centrifuged at 36,400 × *g* for 20 min in a Sorvall RC-2 refrigerated centrifuge at 4°C. The pellet was discarded, and the supernatant was placed in Nalgene screw-cap centrifuge tubes and centrifuged at 134,200 × *g* for 30 min in a Spinco model L analytical ultracentrifuge. The supernatant (soluble protein fraction) was carefully decanted, and the reddish-brown membrane fraction was evenly suspended in 0.01 M HEPES buffer (pH 6.5) to give about 10 mg of protein per ml. This membrane preparation, used in various phosphorylation experiments and ATPase assays, was used immediately or stored under H_2 at −20°C for later use. The stored membrane fraction was found to be stable for up to 2 weeks. Soluble protein fraction, used for assay of ATPase activity, was freshly prepared.

Phosphorylation assays. All assays for determining the esterification of P_i coupled to fumarate reduction by molecular hydrogen were done in double side arm Warburg vessels. The reaction mixtures used in different experiments are described in Results. Bovine serum albumin (BSA) used in this study was treated with Norite as previously described by Chen (14). H_2 utilization for fumarate reduction was determined by standard manometric techniques at 30°C (57). At the end of incubation, all reactions were stopped by adding 1 ml of 10% trichloroacetic acid to 1 ml of reaction mixture and clarified by centrifugation, and the clarified supernatant was used for various analyses. Esterification of P_i refers to the difference between the initial phosphate concentration and that remaining after the completion of the reaction, as determined by isotopic and chemical procedures (16, 45). In general, there was good agreement in P_i esterification values determined by either procedure; however, the isotopic procedure (45) had greater sensitivity and less variability, and therefore most values for esterification reported in this paper were determined by this procedure. Also, except where mentioned otherwise, "P_i esterified" values given in Results represent esterification in an experimental cup minus esterification observed in an identical cup but with 3×10^{-4} M pentachlorophenol (PCP) present. Fumarate and succinate were separated by Celite column chromatography and were quantitated as previously described by Marvel and Rands (41). Protein in membrane fractions was determined by the procedure of Lowry et al. (38), with crystalline BSA as the standard.

ATPase assay. Reaction mixture for assaying ATPase (50) contained: tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0), 50 mM; $MgCl_2$, 4 mM; ethylenediaminetetraacetic acid, 2 mM; phosphoenolpyruvate, 5 mM; ATP, 5 mM; pyruvate kinase, 10 μg (5 U); and membrane protein, 1 to 3 mg, in a total reaction volume of 1 ml in Pyrex glass tubes (13 by 150 mm). All reactions were done at 37°C for 20 min and were stopped by the addition of 1 ml of 10% trichloroacetic acid. The reaction mixtures were clarified by centrifugation, and the concentration of inorganic phosphate in the supernatant was deter-

mined by the method of Fiske and Subbarow (16). The identification of various nucleotides and their sources were as previously described (18). Protein was estimated as described above.

RESULTS

Requirements for phosphorylation. The requirements for phosphorylation coupled with fumarate reduction by molecular H_2 are presented in Table 1. The efficiency of phosphorylation is indicated in terms of H_2 oxidation as P/H_2. Routinely, P/H_2 ratios of 0.15 to 0.3 were obtained, although ratios as high as 0.54 have occasionally been observed. Fumarate, H_2, and membrane fraction were required for phosphorylation. There was no requirement for soluble protein fraction for phosphorylation, suggesting that all components required for the electron transport are membrane bound. ADP was required for phosphorylation but not for oxidation. There was no noticeable effect on H_2 oxidation but appreciable inhibition of phosphorylation when glucose plus hexokinase or BSA was deleted. In other experiments, single deletion of sodium fluoride had little effect on either H_2 oxidation or phosphorylation. The observed phosphorylation could not be from the metabolism of glucose, because *V. succinogenes* does not metabolize glucose (66) and phosphorylation was observed with ADP alone. The above results indicated that phosphorylation is dependent on electron transfer and that a system for accepting P_i is required.

Further studies (data not shown here) showed that HEPES buffer could be replaced by piperazine-*N,N'*-bis-2-ethanesulfonic acid (pH 6.5) or tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.0) without any noticeable change in either oxidation or phosphorylation. Membranes prepared from cells grown in either formate-fumarate (66) or formate-nitrate medium (64) gave similar values for H_2 oxidation and associated phosphorylation. In confirmation of the previous results obtained by Jacobs and Wolin (29), reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate could not replace H_2 as an alternate electron donor for fumarate reduction.

Effect of uncoupling agents on phosphorylation. PCP completely inhibited phosphorylation, but had a slight stimulatory effect upon the H_2 utilized (Table 2). There was considerable inhibition of phosphorylation by gramicidin and to a lesser extent by dinitrophenol (DNP). Methyl viologen appeared to act as an uncoupling agent, probably by circumventing the native ETS to which phosphorylation is linked, but the system was less sensitive to uncoupling by methyl viologen than is the phosphorylation

TABLE 1. *Requirements for phosphorylation coupled to fumarate reduction by H_2*

Reaction mixture[a]	H_2 oxidized (μmol)	P_i esterified[b] (μmol)	P/H_2[c]
Complete	20.1	3.8	0.19
Minus fumarate	0.0	0.0	
Minus ADP	21.3	0.38	
Minus hexokinase and glucose	21.2	2.30	0.11
Minus ADP, hexokinase, and glucose	20.4	0.55	
Minus BSA and NaF	20.0	2.24	0.11
Complete-N_2 gas	0.0	0.34	
Complete, except boiled membrane fraction	0.0	0.0	

[a] Complete reaction mixture (2.2 ml) contained in micromoles: HEPES, 40 (pH 6.5); magnesium acetate, 40; NaF, 50; $^{32}PO_4^{-3}$, 16 (5.9×10^5 cpm/μmol); glucose, 100; fumarate, 25; ADP, 1; BSA, 5 mg; hexokinase, 0.1 mg (6 U); and membrane protein, 4.4 mg. The gas phase was H_2 except where indicated otherwise. The incubation was at 30°C for 6 min.

[b] Values represent esterification observed in a given cup minus esterification observed in an identical cup but containing 3×10^{-4} M PCP. Since PCP was added in ethanol, the other flasks received an equivalent amount of ethanol (0.025 ml).

[c] Phosphate esterified + H_2 oxidized.

TABLE 2. *Effect of inhibitors and uncoupling agents on phosphorylation coupled to fumarate reduction by hydrogen[a]*

Addition[b]	H_2 utilized (μmol)	P_i esterified[c] (μmol)	P/H_2
None	15.8	4.0	0.25
PCP (3×10^{-4} M)	18.3	0.4	0.02
DNP (6×10^{-4} M)	16.5	2.0	0.12
Gramicidin (6×10^{-4} M)	17.0	1.1	0.05
Oligomycin (6×10^{-4} M)	16.7	4.0	0.24
Antimycin (6×10^{-4} M)	14.9	3.6	0.24
HQNO[d] (3×10^{-4} M)	5.2	0.5	0.09
Methyl viologen (4.5×10^{-4} M)	16.6	2.6	0.14

[a] Contents of the reaction mixture were as described in Table 1 with the following exceptions: $^{32}PO_4^{-3}$, 12 μmol (6.4×10^4 cpm/μmol); membrane protein, 3.0 mg; and uncoupling agents at the concentrations indicated. The incubation was at 30°C for 5 min.

[b] Gramicidin, PCP, and oligomycin were added in ethanol, and the other flasks also received an equivalent amount of ethanol (0.025 ml).

[c] P_i esterified represents the difference between identical flasks indicated under an atmosphere of H_2 and N_2.

[d] 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide.

system of *Desulfovibrio gigas* (5). There was hardly any inhibition of phosphorylation by oligomycin or antimycin A. 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide, which is known to inhibit electron transport at the quinone site (35, 36), inhibited H_2 oxidation and phosporylation.

Stoichiometry of the H_2/fumarate reaction. Results on the stoichiometry of the H_2/fumarate reaction and coupled phosphorylation are presented in Table 3. Fumarate added, fumarate remaining, succinate formed, and P_i esterified were determined both chemically and isotopically. H_2 utilized for fumarate reduction and the succinate formed were stoichiometric. Fumarate added was quantitatively recovered as fumarate and succinate at the end of the reaction. No CO_2 was evolved during the reaction from [1,4-^{14}C]fumarate. These results indicated that fumarate was reduced by molecular H_2 exclusively to succinate and that substrate phosphorylation involved in the metabolism of either fumarate or succinate could be ruled out as a possible source of the ATP.

Effect of membrane protein concentration on H_2 uptake and phosphorylation. Membrane protein concentration, H_2 oxidized, and P_i esterified appeared proportional (Fig. 1). P/H_2 ratios were similar at the three protein concentrations tested. Appreciable H_2 oxidation and phosphorylation with as little as 0.8 mg of membrane protein was a notable feature with the H_2/fumarate ETS in *V. succinogenes* as compared with the same system in *D. gigas*, a sulfate-reducing anaerobic bacterium, which requires much higher amounts of membrane protein (5, 6).

Effect of incubation temperature on phosphorylation. There was little difference in phosphorylation when the reactions were conducted between 20 and 37°C, but H_2 oxidation was consistently better at 37°C (Table 4). In a number of experiments, P/H_2 ratios were consistently higher at 20°C than at 37°C. These results were somewhat unexpected, since the organism grows optimally at 37°C and is not known to grow at 35°C or below (66).

TABLE 3. *Stoichiometry of the H_2 fumarate reaction and coupled phosphorylation*

Determination[a]	Quantity (μmol)
H_2 oxidized	15.5
P_i esterified	4.9
Fumarate added	20.4
Fumarate remaining	4.0
Succinate formed	16.25
CO_2 produced	0.0

Recovery of dicarboxylic acids 99%

[a] Contents of the reaction mixture were identical to those in the complete reaction mixture in Table 1 with the following exceptions: $^{32}PO_4^{-3}$, 12 μmol (6.6×10^5 cpm/μmol); fumarate, 20 μmol; membrane protein, 9.2 mg; and specific activity of [1,4-^{14}C]fumarate, 46,000 cpm/μmol. The center well contained 0.1 ml of NaOH and, after the reaction was terminated, portions were monitored for $^{14}CO_2$. The incubation was at 30°C for 7 min.

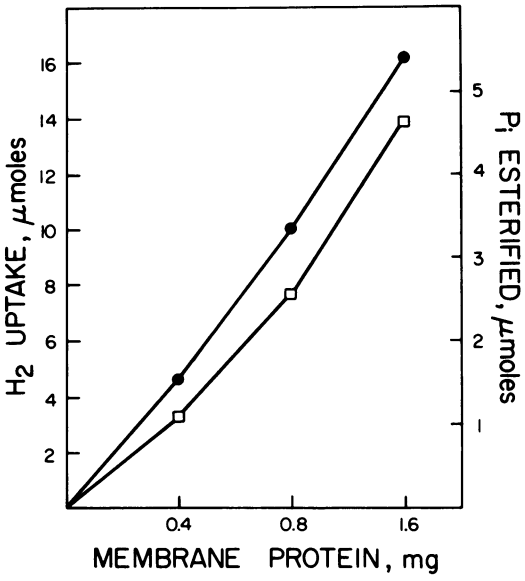

FIG. 1. *Effect of membrane protein concentration on H_2 uptake and phosphorylation. The contents of the reaction were as indicated in the complete reaction mixture of Table 1, with the following exceptions: $^{32}PO_4^{-3}$, 12 μmol (1.2×10^5 cpm/μmol) and membrane protein concentration as indicated. The incubation was at 30°C for 10 min. Symbols: (●) H_2 uptake; (□) P_i esterified.*

Phosphorylation with different electron acceptors. Among several electron acceptors tested, significant H_2 oxidation and coupled phosphorylation were observed only with fumarate (Table 5). Little oxidation or phosphorylation was obtained with malate, pyruvate, or thiosulfate. In another experiment (not shown here) in which nitrate instead of fumarate was used as the electron acceptor, there was no H_2 oxidation or phosphorylation. In a number of experiments, phosphorylation was observed when formate instead of H_2 was used as the electron donor, but the phosphorylation levels were quite low and the results were somewhat variable.

Requirements for ATPase activity. Association of ATPase with oxidative phosphorylation is well documented (2, 10, 25, 28, 40, 48). ATPase activity is considered to be an expression of the same enzyme which phosphorylates ADP with P_i to form ATP. Hence, phosphoryl-

ating membrane preparations of *V. succinogenes* were examined for ATPase activity. A membrane-bound ATPase activity was readily demonstrable (Table 6). There was absolute requirement for Mg^{2+}, ATP, and membranes for activity. Deletion of phosphoenolpyruvate or pyruvate kinase from the reaction resulted in about 30% inhibition of activity, probably due to accumulation of ADP, which is known to inhibit ATPase (50). Deletion of ethylenediaminetetraacetic acid did not result in any appreciable change in ATPase activity.

Nucleotide specificity of the ATPase activity. The activity of the ATPase was highest with ATP and showed decreasing activity with GTP, ITP, and UTP, in that order (Table 7). There was practically no activity with CTP.

Effect of divalent metals on ATPase activity. The enzyme appeared to be most active with Mg^{2+} and showed decreasing activity with Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, and Fe^{2+}, in that order (Table 8). There was a linear increase in ATPase activity with increasing Mg^{2+} concentration until the concentration of the Mg^{2+} was 4 mM (Fig. 2). Dependence on divalent cations for activity is a characteristic feature of ATPases associated with oxidative phosphorylation as opposed to ATPases that require monovalent cations for activity, which are primarily implicated in active transport. There was no detectable ATPase activity with Na^+ or K^+ as the cofactor (Table 8).

Distribution of ATPase activity in membrane and soluble protein fractions and the effect of DNP on activity. ATPase activity was found in both the soluble and membrane protein fractions of *V. succinogenes* (Table 9). DNP had no stimulatory effect on ATPase activity in either the membrane or soluble protein fraction. ATPase activity per milligram of protein was approximately equivalent in membrane and soluble fractions, and there was no apparent

TABLE 4. *Effect of incubation temperature on phosphorylation*[a]

Temp (°C)	H_2 oxidized (μmol)	P_i esterified (μmol)	P/H_2
37	20.3	2.1	0.10
30	15.2	2.0	0.13
20	15.4	2.2	0.14

[a] Components of the reaction mixture were identical to those in the complete reaction mixture in Table 1, with the following exceptions: $^{32}PO_4^{-3}$, 14 μmol (8.6×10^4 cpm/μmol); fumarate, 30 μmol; and membrane proteins, 1.5 mg. The incubation was for 8 min at the temperature specified.

TABLE 5. *H_2 oxidation and phosphorylation with different substrates*[a]

Electron acceptor	H_2 oxidized (μmol)	P_i esterified (μmol)
Fumarate	21.5	3.6
Malate	2.0	0.9
Pyruvate	1.0	0.5
Thiosulfate	0.1	0.1

[a] Contents of the reaction were as described for the complete reaction mixture in Table 1, with the following exceptions: $^{32}PO_4^{-3}$, 12 μmol (1.2×10^5 cpm/μmol); membrane proteins, 2.0 mg; and 20 μmol of an electron acceptor as indicated.

TABLE 6. *Requirements for ATPase activity*[a]

Reaction mixture	Activity[b]
Complete	3.4
Minus EDTA[c]	3.4
Minus pyruvate kinase	2.5
Minus PEP[d]	2.4
Minus membranes	0.2
Minus ATP	0.3
Minus Mg^{2+}	0.3

[a] Composition of the complete reaction mixture is given in Materials and Methods. Membrane protein concentration was 1 mg.

[b] Expressed as micromoles of P_i liberated per 20 min per milligram of membrane protein.

[c] Ethylenediaminetetraacetic acid.

[d] Phosphoenolpyruvate.

TABLE 7. *Nucleotide specificity of ATPase*[a]

Nucleotide	Activity[b]
ATP	3.05
GTP	2.1
ITP	1.75
UTP	0.60
CTP	0.10

[a] Experimental details were as described for Table 6, except that membrane protein was 2 mg and nucleoside triphosphate concentration was 5 mM.

[b] Expressed as in Table 6. All values were corrected for the basal level of P_i present in "minus ATP" and "minus membranes" reaction tubes.

TABLE 8. *Divalent metal requirement for ATPase activity*[a]

Metal	Activity[b]
None	0.2
Mg^{2+}	2.8
Mn^{2+}	2.3
Co^{2+}	1.7
Ca^{2+}	1.0
Zn^{2+}	1.0
Fe^{2+}	0.8
Na^+	0.0
K^+	0.0

[a] Components of the reaction were the same as in Table 6, with the following exceptions: membrane protein, 2.5 mg; and the divalent or monovalent cations as indicated, 4 mM.

[b] Expressed as in Table 6.

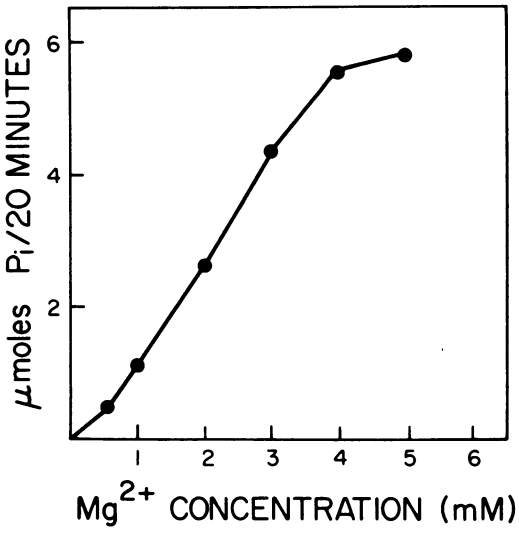

FIG. 2. *Effect of Mg^{2+} concentration on ATPase activity. Reaction conditions were the same as in Table 7.*

TABLE 9. *Effect of DNP on ATPase activity in membrane and soluble fractions of V. succinogenes*[a]

Protein fraction[b]	DNP	Activity[c]
Membrane	−	2.1
Membrane	+	2.2
Soluble	−	2.4
Soluble	+	2.4
Membrane + soluble	+	2.0
None	−	0.1

[a] Components of the reaction were the same as in Table 6, with the following exceptions: where indicated, membranes (2.8 mg) or soluble proteins (1.8 mg) and DNP (4 mM) were present.

[b] Membranes and soluble fraction represent 134,900 × *g* pellet and supernatant, respectively, as described in Materials and Methods.

[c] Expressed as in Table 6.

increase in activity when the membrane and soluble protein fractions were added together. In other experiments (not shown here), DNP did not have any stimulatory effect when Ca^{2+} instead of Mg^{2+} was used as the cofactor and the reactions were conducted at either pH 7.0 or 8.0. When PCP (3×10^{-4} M) was tried in place of DNP, there was 15 to 20% inhibition of ATPase activity.

Effect of membrane protein concentration and time on ATPase activity. A linear relationship between membrane or soluble protein concentration (0 to 1.0 mg/reaction) and the ATPase was observed (Fig. 3). ATPase activity was also linear with time in the range of 0 to 20 min (Fig. 4).

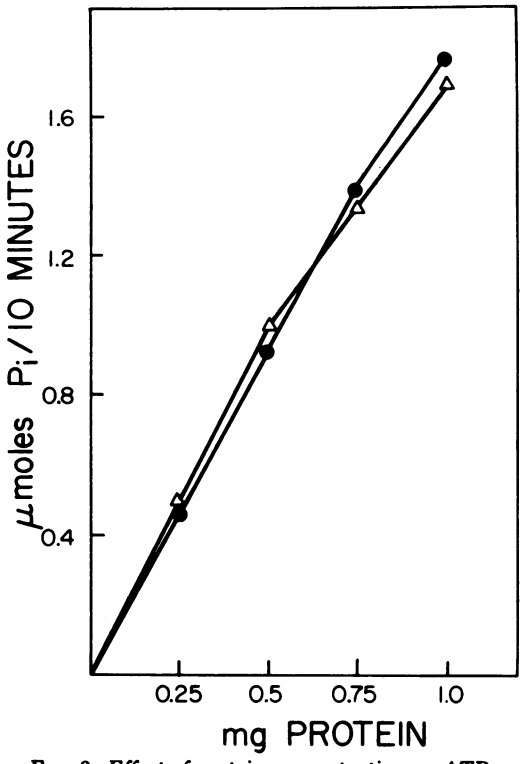

FIG. 3. *Effect of protein concentration on ATPase activity. Reaction conditions were as described in Table 7. Symbols: (●) Membrane protein; (△) soluble protein fraction.*

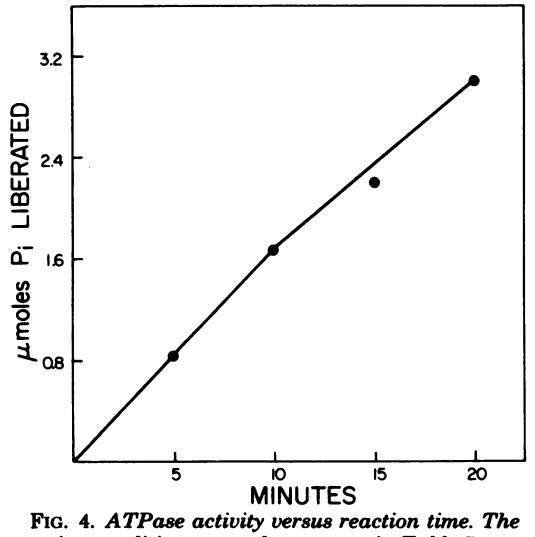

FIG. 4. *ATPase activity versus reaction time. The reaction conditions were the same as in Table 7.*

DISCUSSION

The data conclusively establish the formation of ATP from ADP and P_i coupled to the oxida-

tion of H_2 and concomitant reduction of fumarate to succinate by membrane preparations of *V. succinogenes*. ATP generation appears to result from electron transfer-coupled proton translocation, since phosphorylation is completely dependent on electron transfer and inhibited by classical uncoupling agents for oxidative phosphorylation. In addition, the membrane preparations exhibit ATPase activity, and the stoichiometric reduction of fumarate to succinate eliminates substrate phosphorylation as the source of the observed ATP synthesis. The observed P/H_2 ratios were variable, ranging from 0.15 to 0.5, but reasonable when compared with the maximum P/H_2 ratio of 1.0 theoretically expected from electron transfer-coupled phosphorylation with this system (55).

An electron transfer chain functioning between formate and fumarate has been demonstrated in isolated membranes of this anaerobic bacterium consisting of a cytochrome *b* (−20 mV), MQ, cytochrome *b* (−20 mV), and a nonheme iron protein in ratios of 1:16:1:10 (33). The electron transfer chain functioning between H_2 and fumarate has not been investigated, but it is quite likely that all or a portion of the electron transfer components of the formate/fumarate system, and possibly additional components are involved in the H_2/fumarate couple. It has been suggested that proton translocation in the formate/fumarate system is specifically coupled to the reduction and oxidation of MQ (34). Alternatively, it has been proposed that proton translocation can result directly from the oxidation of formate (or H_2) without proton translocation being specifically coupled with the electron transfer chain (55). Thus, with formate dehydrogenase localized on the outside of the membrane and fumarate reductase on the inside (33), the formate (or H_2)/fumarate couple can effect a "substrate-level" proton translocation with only the transfer of electrons between the two enzymes. Whether the formation of ATP is specifically coupled to either system or to both systems cannot be decided on the basis of available information; however, in the latter instance, the generation of more than one ATP per electron pair in these coupled systems becomes a possibility.

Our results on the effects of uncoupling agents are in general agreement with those of Peck (46) and Barton et al. (5), obtained with the H_2/fumarate and H_2/sulfite oxidative phosphorylation systems in *D. gigas*, respectively. PCP effectively uncoupled phosphorylation from H_2 oxidation in membrane preparations from both of these organisms. Failure of DNP to effectively uncouple phosphorylation in *V. succinogenes* is in general agreement with the results obtained by a number of previous investigators with phosphorylation systems in other bacteria (1, 10, 12, 28, 30, 49, 56). Inhibition of electron transport and phosphorylation by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide reported here is in agreement with the previous results obtained by Kröger and Innerhofer (36, 37) and Wolin and Jacobs (65) with this organism and with the results obtained for H_2/fumarate ETS in other organisms as well (6, 21, 35, 51, 59, 60, 61).

Electron transport phosphorylation coupled with the transfer of electrons from a reduced organic or inorganic electron donor to fumarate may be more widespread in anaerobic and facultative anaerobic bacteria than hitherto believed (6). Barton et al. (5, 6) reported ATP generation coupled to the H_2/fumarate ETS (21) in extracts of a sulfate-reducing anaerobe, *D. gigas*. Phosphorylation in lactate oxidation coupled to the reduction of fumarate has also been demonstrated in this organism (L. L. Barton and H. C. Peck, Bacteriol. Proc., p. 155, 1971). Both hydrogenase and fumarate reductase are widely distributed in a number of anaerobic and facultatively anaerobic bacteria (15, 17, 39, 42, 47, 51, 59–62).

Molar growth yields of a number of anaerobes have been shown to be greater than expected on the basis of known substrate phosphorylations (7, 22, 32, 39). Propionic acid bacteria are a case in point. These organisms have cytochromes (13, 54, 57–60), fumarate reductase (60), and an anomalous growth yield (7), and deVries et al. (60) recently obtained evidence which suggested the generation of 2 mol of ATP in cytochrome-mediated electron transfer from NADH to fumarate in *Propionibacterium freudenreichii* and *P. pentosaceum*. A number of rumen anaerobic bacteria produce succinate as a major product of carbohydrate fermentation (24) and contain cytochromes (61, 62) and fumarate reductase (30); some have been shown to have anomalous growth yields (22, 24), and there is a good possibility that these bacteria obtain a portion of the energy required for growth from anaerobic oxidative phosphorylation coupled to fumarate reduction. The rumen anaerobes *Selenomonas ruminantium* and *Anaerovibrio lipolytica* are similar to propionic acid bacteria in forming propionic acid, via the succinate pathway, from carbohydrate fermentation (22, 24). Hobson and Summers (22) reported anomalous growth yields for *S. ruminantium* growing on glucose and *A. lipolytica* growing with glycerol or fructose in substrate-limited continuous cultures; de Vries et al. (61) demonstrated a membrane-bound, cytochrome-linked ETS capable of using fuma-

rate as an electron acceptor and NADH as an electron donor in both of these organisms. Furthermore, evidence for electron transport phosphorylation coupled to fumarate reduction by NADH (or formate) had been obtained for anaerobically grown *Proteus rettgeri* (32) and for *Streptococcus faecalis* (15). The preceding data strongly support the hypothesis that reduction of fumarate by molecular H_2, reduced pyridine nucleotides, or some other electron donor may serve as an additional source of ATP for growth in a number of anaerobic and facultative anaerobic bacteria.

Synthesis of ATP coupled to fumarate reduction to succinate appears to be important, under anaerobic conditions, in higher organisms also. Obligate helminth anaerobes such as *Ascaris* as well as facultative anaerobes such as oysters obtain part of their metabolic energy, under anaerobic conditions, from ATP synthesis coupled to the reduction of fumarate to succinate by NADH (23). Under anoxic conditions, ATP synthesis coupled to the reduction of fumarate has also been demonstrated in heart sub-mitochondrial particles as well as in the liver and gastrocnemius muscle of rats (63). Succinate was recovered as the reaction product and was stoichiometric with the NADH oxidized. These results can be taken as further evidence that H_2/fumarate oxidative phosphorylation plays an important role in energy generation in a wide variety of biological systems.

A number of investigators have shown that divalent cation-dependent ATPase activity is associated with oxidative phosphorylation and photophosphorylation in bacterial extracts and that bacterial mutants lacking ATPase are unable to carry out oxidative phosphorylation (4, 11, 12, 18, 19, 25, 28, 40, 56, 58). The demonstration of a Mg^{2+}-dependent ATPase activity in *V. succinogenes* is consistent with the presence of anaerobic oxidative phosphorylation in this strict anaerobe and suggests that production of high-energy phosphate in this organism probably proceeds through energy transfer steps analogous to those in other phosphorylation systems. The ATPase activity observed with the soluble protein fractions of *V. succinogenes* was similar to the membrane-bound ATPase activity in this organism in many respects and may have resulted from a partial solubilization of the membrane-bound activity during disruption of cells. However, preliminary attempts to solubilize the membrane-bound ATPase activity by alkali treatment, as previously described by Aspen and Wolin (3), have not been successful. The ATPase activity in *V. succinogenes* is similar to that reported in a number of other bacterial systems in not showing stimulation by DNP (11, 25, 28, 52), but is different from the enzymes in *D. gigas* (18), photosynthetic bacteria (4), baker's yeast (8), and mitochondria (31), which show considerable stimulation by DNP.

There appears to be a good deal of variation between membrane-bound ATPases in different organisms in their requirement for divalent cations and in their nucleotide specificities. Some ATPases, similar to the enzyme in *V. succinogenes*, were reported to be Mg^{2+} dependent (8, 9, 18, 20, 52), some were dependent on Ca^{2+} (18, 20, 27, 43), and in a few cases the enzyme was active with both the cations (18, 20). The ATPase activity in *Rhodospirillum rubrum* (9) appeared different from others in being most active with Mn^{2+}. ATPase in baker's yeast (8) was most active with Mg^{2+} or Mn^{2+} but was inactive with Ca^{2+}. ATPase activity in *V. succinogenes* appears to be very similar to that in *Trypanasoma cruzi* (52) in its specific activity and divalent cation requirement. ATP is the preferred substrate for most ATPases, including the ATPase in *V. succinogenes*, but GTP gave values ranging between 20 and 70% of the specific activity observed with ATP in different organisms (18, 20, 51). The enzyme from baker's yeast appears to be different from others in being more active with GTP (8).

ATPases requiring Mg^{2+} or Ca^{2+} are usually known to function as coupling factors (or associated with coupling factor activity) for oxidative phosphorylation in higher organisms, bacteria, and plants (4, 11, 26, 28, 48, 58). Whether ATPase activity in *V. succinogenes* serves as a coupling factor for the electron transport phosphorylation in this organism has not been investigated. However, soluble protein fraction from *V. succinogenes* serves as a coupling factor for anaerobic oxidative phosphorylation coupled to fumarate reduction by H_2 in extracts of *D. gigas* (C. A. Reddy, unpublished data).

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