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Evidence for Cytochrome Involvement in Fumarate Reduction and Adenosine 5'-Triphosphate Synthesis by *Bacteroides fragilis* Grown in the Presence of Hemin

JOAN MACY,* IRMELIN PROBST, AND GERHARD GOTTSCHALK

Institut für Mikrobiologie der Universität Göttingen, and Gesellschaft für Strahlen und Umweltforschung mbH München in Göttingen, West Germany*

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Growth of *Bacteroides fragilis* subsp. *fragilis* on glucose was very much stimulated by the addition of hemin (2 mg/liter) to the medium. The generation time decreased from 8 to 2 h, and the molar growth yield increased from Y_M = 17.9 to Y_M = 47 g (dry weight) of cells per mol of glucose. In the absence of hemin, glucose was fermented to fumarate, lactate, and acetate. The cells did not contain detectable amounts of cytochromes or fumarate reductase. In the presence of hemin, the major products of fermentation were succinate, propionate, and acetate. A *b*-type cytochrome, possibly a *c*-type cytochrome, and a very active fumarate reductase were present in the cells. It is concluded from these results that hemin is required by *B. fragilis* to synthesize a functional fumarate reductase and that the hemin-dependent, enormous increase of the growth yield may be due to adenosine 5'-triphosphate production during reduction of fumarate to succinate.

Bacteroides fragilis is a strict anaerobe and the most predominant organism in the lower gut of man; normally 10^{10} to 10^{11} cells of this species are found per gram of feces (11). Such efficient growth, in what is frequently a famine-like environment, might indicate that this organism has developed a very effective system to gain adenosine 5'-triphosphate (ATP). In this connection two points are important.

(i) There is evidence now to show that certain anaerobes (*Clostridium formicoaceticum* and *C. thermoaceticum* [13], propionic acid bacteria [9, 10, 28], *Vibrio succinogenes* (18), *Desulfovibrio gigas* (3, 14), *Bacterioides* sp. (7, 25, 35; C. Reddy and M. P. Bryant, Bacteriol. Proc., p. 40, 1967), *Selenomonas ruminantium* [8], *Anaerovibrio lipolytica* [8], and *Veillonella alcalescens* [8] contain a primitive type of electron transport system with cytochrome *b* that is linked to the reduction of fumarate. There is also some evidence, from molar growth yields with propionic acid bacteria (10) and anaerobically grown *Proteus* (20), suggesting that this system may function to produce ATP. Direct phosphorylation of adenosine 5'-diphosphate in cell-free extracts has also been demonstrated in *D. gigas* (3).

(ii) Certain *Bacteroides* species have an absolute hemin (or related compound) requirement for growth (7, 25, 35), and the hemin is used for the synthesis of cytochromes (25, 35). *Bacteroides fragilis* subsp. *fragilis* can grow either in the presence or absence of this factor, but we found that they did so with markedly different growth rates. This "hemin effect" has also recently been shown by Varel and Bryant (33).

The following question then arose: Was the stimulatory effect of hemin due to its function as a precursor of cytochrome-containing enzyme systems which increased the ATP yield during fermentation? This was investigated in the present publication.

MATERIALS AND METHODS

Growth of bacteria. *B. fragilis* subsp. *fragilis* ATCC23745 was grown anaerobically (100% CO_2) in 5.5-liter bottles (containing 5 liters of medium) at 37 C. The medium, similar to Bryant-Robinson medium (6), had the following composition (grams per liter): glucose, 5.0; KH_2PO_4, 0.45; K_2HPO_4, 0.45; NaCl, 0.9; $CaCl_2$, 0.02; $MgCl_2 \cdot 6\,H_2O$, 0.02; $MnCl_2 \cdot 4\,H_2O$, 0.01; $CoCl_2 \cdot 6\,H_2O$, 0.01; $(NH_4)_2SO_4$, 0.9; $NaHCO_3$, 5.0; cysteine·HCl·H_2O, 0.3; vitamin B_{12}, 0.0001 (33); and hemin, 0.002 (when required). Glucose, $NaHCO_3$, cysteine, hemin (dissolved in 0.02% NaOH), and vitamin B_{12} were sterilized separately; B_{12} and hemin solutions were filter sterilized and the remainder was autoclaved. Medium preparation was carried out anaerobically under an atmosphere of 100% CO_2, except for glucose, cysteine, and hemin which were prepared under 100% N_2. All traces of oxy-

gen were removed from N_2 and CO_2 by passing them through a vertical Pyrex column packed with copper turnings heated electrically to approximately 350 C (17). Oxygen was removed from the distilled water used in preparing the medium and solutions by boiling; as the water cooled, either CO_2 or N_2 was bubbled through it. Cultures were maintained in the same medium in anaerobic roll tubes by the method of Hungate (17). The inoculum was 10 ml of organisms grown in the same medium but with 0.2% glucose. Bacteria used as inoculum for "-hemin" experiments were serially transfered, at least three times, into fresh medium (in roll tubes) without hemin. Growth was followed by measuring optical density of the cultures at 578 nm, with a Zeiss PM 4 spectrophotometer, in cuvettes with a 1-cm light path.

Determination of Y_M and assay of fermentation products. In late log phase, a portion of the culture was taken for dry weight determination, and the remainder was centrifuged for 30 min at 10,000 × *g* in a Christ Zeta 20 centrifuge at 4 C (Heraeus-Christ GmbH, Osterode/Harz). The supernatant was frozen and used later for analysis of glucose and fermentation products.

Dry weight was determined by the method of Stouthamer (29). The membrane filters used (Sartorius, Membrane-filter GmbH, 34 Göttingen, West-Germany) had a pore size of 0.2 μm.

Acetate, ethanol, and lactate were estimated enzymatically by the method of Bergmeyer (5). Succinate was determined as described by Kröger (20), with succinate dehydrogenase prepared by the method of Neufeld et al. (23). Measurement of fumarate was also according to Kröger (20). Propionic acid was analyzed by gas chromatography (16), using a Perkin-Elmer gas chromatograph (model 900) equipped with a hot wire detector, under the following conditions: stainless-steel column (¼ inch by 4 ft [ca. 0.63 by 121.9 cm]), Resovlex R23; injector, 150 C; manifold, 150 C; oven, 120 C; detector, 150 C, current, 325 mA; carrier gas, helium; flow rate, 120 ml/min.

Glucose was estimated by the blood sugar GOD-Perid method of Boehringer Mannheim GmbH.

Preparation of cell-free extracts. Buffer A, prepared anaerobically under 100% N_2 and used throughout extract preparation, contained: potassium phosphate (pH 7.0), 50 mM; $MgCl_2$, 5 mM; sucrose, 0.1 M; and sodium thioglycolate, 0.01 M. Cells in the log phase of growth were harvested by centrifugation for 30 min at 10,000 × *g* (4 C). The pellet was washed once with buffer A, resuspended in the same buffer (1 g/5 ml of buffer plus deoxyribonuclease [1 mg/10 ml]), and then passed through a French press (pressure, 66.6 to 80 kg/cm^2). The broken cells were collected at 0 C under 100% N_2. Unbroken cells were removed by centrifugation under N_2 for 20 minutes at 15,000 × *g*. The supernatant, kept at 0 C under N_2, was used immediately for measurement of fumarate reductase activity. Protein was estimated by the biuret method (12), with crystalline bovine serum albumin as standard.

Preparation of membranes. The cell-free extract was centrifuged for 4 h at 100,000 × *g* in a Beckman Spinco L 256 B ultracentrifuge. The pellet was washed twice in buffer A and finally resuspended in the same buffer with the aid of a homogenizer.

Assay of fumarate reductase. Manometric measurement of fumarate reductase activity in anaerobic cell-free extracts was done by the method of Peck at al. (24). However, since the *B. fragilis* enzyme appears to be oxygen sensitive, all solutions were prepared under N_2, and 0.01 M sodium thioglycolate was included in the reaction mixture. Presence of 5 mM $MgCl_2$ and 0.1 M sucrose in anaerobic buffer A, used for preparing extracts, was also necessary for enhancement of fumarate reductase activity.

Spectrophotometric determination of fumarate reductase activity, reduced the rate of nicotinamide adenine dinucleotide oxidation by fumarate, was done by the method of Kröger (21).

Measurement of spectra. Reduced-minus-oxidized difference spectra done at room temperature were recorded on a Zeiss DMR 21 spectrophotometer. Reduced-minus-oxidized difference spectra at −196 C were done with a Shimadzu MPS-50 L recording spectrophotometer. A few grains of solid dithionite were added to reduce the pigments; the reference cuvette was oxidized by ferricyanide or air (19).

RESULTS

Growth of B. fragilis subsp. fragilis. Varel and Bryant (33) have recently shown that *B. fragilis* subsp. *fragilis* can grow anaerobically in a simple defined medium. When we grew this organism in a similar medium, the results shown in Fig. 1 (curve a) were obtained. Growth under these conditions was very slow, with a generation time of 8 h. When 2 μg of hemin was added per ml of medium, growth was stimulated and the organism grew with a generation

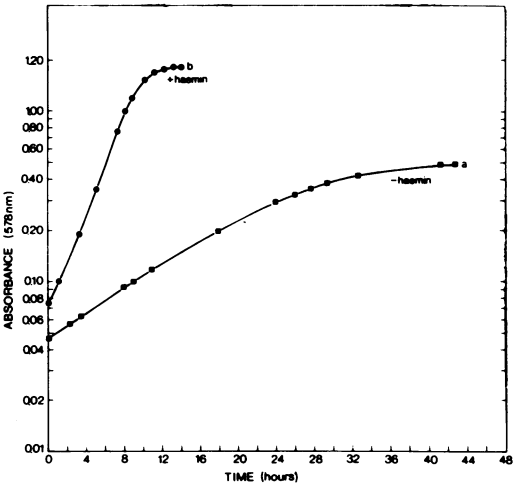

FIG. 1. *Growth of B. fragilis subsp. fragilis in minimal medium with glucose as the carbon source and in the presence and absence of hemin. Symbols:* ●, *2 μg of hemin present per ml;* ■, *no hemin present.*

time of 2 h (Fig. 1, curve b). Similar results were found by Varel and Bryant (33).

Analysis of fermentation products and determination of Y_m. Fermentation products and the molar growth yield (Y_m, glucose) were measured for cultures grown in the presence and absence of hemin to determine whether they might reflect a difference in ATP-synthesizing reactions. Table 1 shows the results of two experiments; because of their similarity and for simplicity, only experiment 1 will be referred to. When hemin was absent from the medium, lactate and fumarate were the predominant products, with some acetate being formed, and the Y_m was 17.9 g of cells per mol of glucose utilized. With hemin present in the medium acetate, succinate and propionate were the major products of the fermentation, and the molar yield of cells was 47 g/mol of glucose. Thus, almost three times as many cells were made from a mole of glucose when hemin was present in the medium. Although more acetate was made in the presence of hemin than when it was absent, the small amount of ATP gained during its formation would not account for the extra ATP required for the increased cellular synthesis of hemin-grown cultures. Calculation of the number of ATPs gained during the fermentation of glucose, from the determined Y_m values and a Y_{ATP} of 10.5 (4), indicated that in the presence of hemin 4.5 ATPs/mol of glucose were made as compared to 1.7 in its absence.

The fact that hemin-grown organisms formed succinate and propionate, which could not be accomplished in the absence of hemin, suggested that during their formation ATP might be generated. If this were the case during succinate synthesis, then when hemin was supplied to the medium an active fumarate reductase and cytochromes should have been present, whereas without hemin there should have been small amounts or no cytochromes, as well as lower or no fumarate reductase activity. Exactly how energy might be generated during propionate formation is not entirely clear. If propionate were synthesized via a pathway similar to that used by propionic acid bacteria, than a fumarate reductase and cytochromes should also be involved. If the "acrylate" pathway is present, it may be possible that one of the enzymatic steps in the pathway is cytochrome linked (thus making propionate formation hemin dependent).

It should be mentioned that estimates of the amount of CO_2 produced during the fermentation were not possible because the organism was grown under an atmosphere of 100% CO_2, and also the large amounts of dicarboxylic acids formed suggested that possibly CO_2 was being fixed during the catabolism of glucose (see "hemin" carbon balances, Table 1).

Fumarate reductase activity. Table 2 shows

TABLE 1. *Effect of hemin on the fermentation products formed and the Y_m (glucose) of B. fragilis subsp. fragilis*

Products	Products formed							
	Exp 1				Exp 2			
	− Hemin		+ Hemin		− Hemin		+ Hemin	
	mol/ 100 mol of glucose	mg atoms of C/600 mg atoms of glucose C	mol/ 100 mol of glucose	mg atoms of C/600 mg atoms of glucose C	mol/ 100 mol of glucose	mg atoms of C/600 mg atoms of glucose C	mol/ 100 mol of glucose	mg atoms of C/600 mg atoms of glucose C
Cells[a]		72.2		189.5		73.3		213.7
Lactate	68.6	205.8	7.0	14.0	72.0	216.0	5.5	16.5
Acetate	29.8	59.6	40.9	81.8	32.3	64.6	45.0	90.0
Ethanol	0.4	0.8	2.3	4.6	2.7	5.4	0.08	0.16
Fumarate	97.8	391.2	2.7	10.8	98.0	392.0	2.6	10.4
Succinate	6.0	24.0	36.0	144.0	3.4	13.6	27.5	110.0
Propionate	0.0	0.0	22.6	67.8	0.0	0.0	20.9	62.7
Formate[b]	29.8	29.8	43.2	43.2	35.0	35.0	45.0	45.0
Total (%)		130.6		92.6		133.0		91.4
Y_m[c]	17.9		47.0		18.2		53.0	

[a] Calculated from the determined Y_m and the assumed cell formula of $CH_2O_{0.5}N_{0.2}$ (R. E. Hungate, personal communication).

[b] Estimated from the amount of acetate and ethanol made.

[c] Grams (dry weight) per mole of glucose.

TABLE 2. *Specific activity of fumarate reductase in cell-free extracts from + and − hemin cultures of B. fragilis subsp. fragilis*

Exp	μmol/min per mg of protein[a]			μmol/min per mg of protein[b]	
	No extract or no fumarate	− Hemin	+ Hemin	No extract or no fumarate	+ Hemin
1	<0.02	<0.02	0.62	ND[d]	ND
2	<0.02	<0.02	0.53	ND	ND
3	<0.02	ND	0.63	0	0.13

[a] Manometric measurement of fumarate reductase activity, with H_2 as electron donor, was done by measuring uptake of hydrogen in the presence of excess hydrogenase (from *Clostridium pasteurianum*). Each flask contained: 0.05 M potassium phosphate buffer (pH 7.6), 0.033 M sodium fumarate (pH 7.6), 1 mM methyl viologen, 0.01 M sodium thioglycolate, hydrogenase (*C. pasteurianum* extract), and *B. fragilis* extract (0.1 to 0.5 mg of protein) in a total volume of 3 ml. The temperature was 30 C, and the gas phase was hydrogen. Double-side-arm Warburg vessels were used. The hydrogenase in one side arm was first tipped, and after 30 min the reaction was started by tipping the sodium fumarate in the second side arm.

[b] Spectrophotometric measurement of fumarate reductase, with $NADH_2$ as the electron donor, was done by measuring oxidation of $NADH_2$ in an anaerobic cuvettes under an atmosphere of N_2 at 366 nm. The reaction mixture contained: 0.05 M potassium phosphate buffer (pH 7.6), 1 mM sodium fumarate (pH 7.6), 0.01 M sodium thioglycolate, 0.2 mM $NADH_2$, and *B. fragilis* extract (0.1 to 0.5 mg of protein) in a total volume of 1 ml. The temperature was 25 C, and the gas phase was N_2. The reaction was started by adding *B. fragilis* extract.

[c] In controls, water replaced either fumarate or extract.

[d] Not determined.

fumarate reductase activity measured manometrically, with H_2 as the electron donor and methyl viologen as the artificial electron carrier. Specific activity in anaerobic cell-free extracts from organisms grown in the absence of hemin was less than 0.02 μmol/min per mg of protein, whereas that of hemin extracts was more than 25 times greater. When fumarate reductase was measured spectrophotometrically, with $NADH_2$ as the electron donor, it was found that in the presence of hemin extract fumarate was reduced with a specific activity of 0.13 μmol/min per mg of protein.

Cytochrome measurements. Membranes were isolated from the different cell-free extracts, washed, and then resuspended in buffer. Membranes from organisms grown in the presence of hemin were red-brown in color. The dithionite-reduced minus air-oxidized difference spectrum of membranes prepared from organisms grown in the absence of hemin is shown in Fig. 2. Although it was done at liquid nitrogen temperatures, no cytochromes could be seen. Spectra of unfractionated cell-free extracts also showed no cytochromes to be present. Room temperature and liquid nitrogen temperature reduced-minus-oxidized difference spectra of membranes from hemin-grown organisms are shown in Fig. 3a and b.

The room temperature difference spectrum (Fig. 3a) showed an unsymetrical α peak with a maximum at 562 nm (shoulder at 574 nm) and a soret peak at 428 nm, indicating the presence of a *b*-type cytochrome. The β-peak was somewhat masked because of much flavoprotein in the membrane.

When the difference spectrum was measured at −196 C (Fig. 3b), the α peak was split into two maxima at 565 and 554 nm, with a shoulder at 548 nm. Perhaps, along with cytochrome *b*, a *c*-type cytochrome might be present (19). However, the true number and nature of the cytochromes remain uncertain and need further elucidation. Reddy and Bryant have reported cytochromes *b* and *o* in *B. fragilis* (C. Reddy and M. P. Bryant, Bacteriol. Proc. p. 40, 1967); the presence of a CO-binding cytochrome was also confirmed in our work.

DISCUSSION

In anaerobically grown *Proteus* and a number of strict anaerobes, there is evidence for an anaerobic electron transport system involving

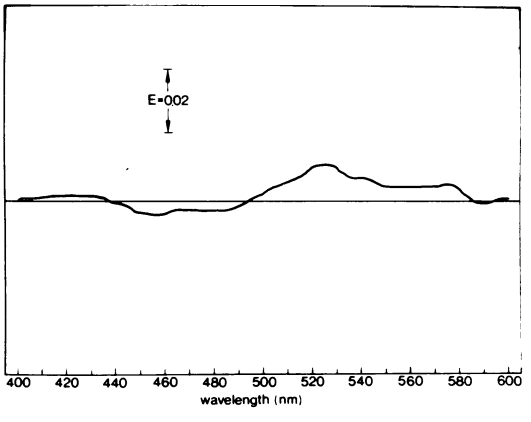

FIG. 2. *Dithionite-reduced minus air-oxidized difference spectrum (−196 C) of a washed-membrane suspension from organisms grown in the absence of hemin. The suspension contained 3.0 mg of protein per ml.*

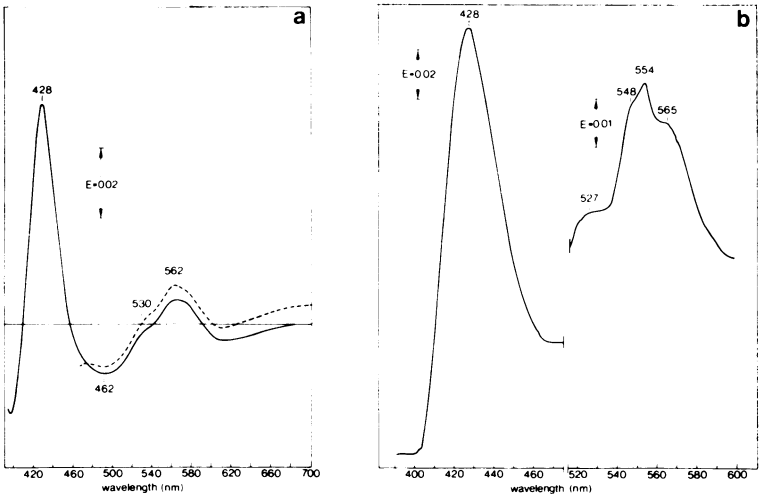

FIG. 3. *Dithionite-reduced minus air-oxidized difference spectra of a washed-membrane suspension from organisms grown in the presence of hemin. (a) Spectrum at room temperature; suspension contained 0.19 mg of protein per ml. (b) Spectrum at −196 C; suspension contained 1.62 mg of protein per ml. (----) Dithionite reduced versus* $Fe(CN)_6^{3-}$ *oxidized; (——) dithionite reduced versus air oxidized.*

cytochrome *b* and with fumarate as the terminal electron acceptor (7–10, 13, 18, 25, 28, 35; C. Reddy and M. P. Bryant, Bacteriol. Proc., p. 40, 1967). Phosphorylation of adenosine 5′-diphosphate coupled to reduction of fumarate has been demonstrated in *D. gigas* (3), whereas determination of growth yields for propionic acid bacteria (10), anaerobically grown *Proteus* (20), and *Selenomonas ruminantium* (15) has strongly suggested that these organisms generate ATP by such a system.

In the present study we have used *B. fragilis* subsp. *fragilis* to determine whether the primitive anaerobic electron transport system described above might possibly be functioning to generate energy during growth of this organism. Unlike related species such as *B. ruminicola* or *B. melaninogenicus*, *B. fragilis* subsp. *fragilis* grew without hemin being present in the medium; however, the growth rate was very slow (8 h), Y_m was 17.9, it was unable to synthesize cytochromes and a functional fumarate reductase and, instead of succinate or propionate being formed, fumarate was made. By simple addition of hemin to the medium, the growth rate quadrupled (2 h), Y_m increased to 47, succinate and propionate were end products, and cytochromes and fumarate reductase were synthesized. Therefore, it appears that only the omission of hemin from the medium prevented these biochemical changes and the possible ATP formation via anaerobic electron transport phosphorylation during the reduction of fumarate to form succinate. Again it should be mentioned that it is not presently known whether the pathway synthesizing propionate involves fumarate reductase; yet its formation is definitely hemin dependent.

Based upon a Y_{ATP} of 10.5 (4) and the determined Y_m values, the amount of ATP produced by cultures fermenting glucose in the absence of hemin was 1.7 ATPs/mol of glucose and for the hemin culture 4.5 ATPs/mol of glucose. The greater amount of ATP made in the presence of hemin could not be accounted for by the change of fermentation products and the concomitant increase in substrate-level phosphorylation reactions. As is evident from Fig. 4a and b, there was some additional ATP formation resulting from the increase of acetate synthesis in the cultures grown with hemin. However, much of the increase in ATP yield must have been due to a linkage between the reduction of fumarate to succinate (or possibly acrylyl-coenzyme A to propionyl-coenzyme A) and the phosphorylation of adenosine 5′-diphosphate. Relative to the amount of ATP an aerobically growing *E. coli* can gain from a glucose molecule, 4.5 ATPs/mol of glucose is perhaps not large. However, anaerobically the maximum theoretical number of ATPs *E. coli* can make is only 2.5/mol of glucose.

It must also be added that the Y_{ATP} value may vary depending upon growth conditions. Not only has Stouthamer found that theoretically Y_{ATP} can be as great as 28.8 in minimal medium (30), but he has also found that Y_{ATP} in

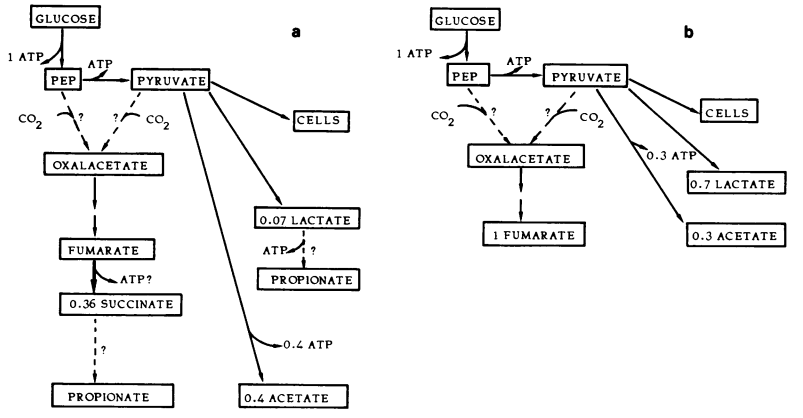

FIG. 4. *Possible pathway used by B. fragilis subsp. fragilis for the fermentation of glucose (a) in the presence of hemin and (b) in the absence of hemin.*

batch cultures is a function of maintenance energy and growth rate; at slower growth rates a larger part of the energy source is used to satisfy the maintenance requirements.

The maximum amount of ATP that could be made during the oxidation of one $NADH_2$ by fumarate is 2, based upon an oxidation reduction potential difference of $\Delta E_0 = 0.35$ (22). However, from the fermentation balance it is not possible to calculate the actual amount of ATP that might have been formed in this reaction because it is not yet known how propionate is formed or what reaction *B. fragilis* uses to form oxaloacetate (malate dehydrogenase and fumarase are present, [unpublished data]). The carboxylation reaction could be carried out either with pyruvate carboxylase or phosphoenolpyruvate carboxylase, both of which result in the loss or use of ATP (27, 32). Also possible are the energy-conserving reactions of phosphoenolpyruvate carboxykinase (32) or transcarboxylase (36). The fermentation balance of the culture grown in the absence of hemin suggest that one carboxylating enzyme may be present that is not a transcarboxylase, since fumarate was accumulated but no propionate was made. The carboxylating enzyme found in *B. ruminicola* was an adenosine 5'-diphosphate- and Co^{+2}-dependent phosphoenolpyruvate carboxykinase (26), suggesting that, because of the similarity of the two species, *B. fragilis* might also have such an enzyme.

With regard to hemin-dependent propionate formation, the pathway employed is as yet unknown. If a pathway similar to that found in propionic acid bacteria is present, fumarate reductase would be involved and ATP might be generated with concomitant reduction of fumarate. However, as in *B. ruminicola* (34), the "acrylate" route may be present, but then the question arises as to why the presence of hemin is required. It is, of course, possible to postulate ATP synthesis via a cytochrome-linked reduction of acrylyl-coenzyme A (with acrylyl-coenzyme A reductase). In fact, electron transport-linked phosphorylation has been postulated to occur during reduction of acrylyl-coenzyme A in *Peptostreptococcus elsdenii* (1, 2).

Based then upon our present knowledge, the possible pathways for metabolism of glucose by *B. fragilis* subsp. *fragilis* in the absence and presence of hemin are shown in Fig. 4a and b. The difference in these two fermentations suggests that the success of *B. fragilis* in its anaerobic competitive habitat lies partly in the fact that it has "learned" through evolution to synthesize cytochromes from hemin (or a similar compound) and fumarate reductase; both of which appear to be involved in a system that synthesizes ATP during the reduction of fumarate. A source of heme compounds for *B. fragilis*, in its natural habitat of the gut, is probably no difficulty; bile is always present as well as autolyzing epithelial and bacterial cells.

The evidence indicating ATP synthesis in cytochrome-mediated electron transport is indirect. Direct demonstration of adenosine 5'-diphosphate phosphorylation coupled to fumarate or acrylyl-coenzyme A reduction is presently under study.

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