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THE HYDROGEN-FUMARATE ELECTRON TRANSPORT SYSTEM IN THE OBLIGATE ANAEROBE BACTEROIDES FRAGILIS

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THE HYDROGEN-FUMARATE ELECTRON TRANSPORT SYSTEM IN THE OBLIGATE ANAEROBE *BACTEROIDES FRAGILIS*

By

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ABSTRACT

THE HYDROGEN-FUMARATE ELECTRON TRANSPORT SYSTEM IN THE OBLIGATE ANAEROBE *BACTEROIDES FRAGILIS*

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The hydrogen-fumarate electron transport system has been investigated in the anaerobic bacterium, *Bacteroides fragilis*. Hydrogenase activity can be detected with a variety of electron acceptors including methylene blue, benzyl viologen, flavin mononucleotide, and flavin adenine dinucleotide. Nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate are capable of serving as electron acceptors for hydrogen only in the presence of the low potential electron mediator benzyl viologen or ferredoxin from *Clostridium pasteurianum*. The hydrogenase activity is primarily associated with the soluble fraction of the cell. Fumarate reductase activity can be readily demonstrated in cell extracts of *B. fragilis*. Approximately 42% of the fumarate reductase activity is located in the particulate fraction of the cell. The coupled hydrogenase-fumarate reductase system in *B. fragilis* can be demonstrated only in the presence of a catalytic quantity of benzyl viologen, flavin mononucleotide, flavin dinucleotide or ferredoxin from

C. pasteurianum. There appears to be no sulfhydryl requirement for the hydrogen-fumarate coupling activity; however, mercapto-ethanol and dithiothreitol increase the specific activity by approximately 59% and 61%, respectively. Reduced nicotinamide adenine dinucleotide can substitute for H_2 as an electron donor for the reduction of fumarate. The electron transfer inhibitors acriflavin, rotenone, 2-hydroxyquinoline-N-oxide, and antimycin A inhibit fumarate reduction by H_2, suggesting the possible involvement of a flavoprotein, a quinone, and a b-type cytochrome in the reduction of fumarate. The involvement of a quinone in fumarate reduction is further evident from decreased fumarate reduction after exposing the cell extracts to ultraviolet light. In addition to a b-type cytochrome, previously detected in *B. fragilis*, a c-type cytochrome is also demonstrable in this organism. The role of the c-type cytochrome in fumarate reduction, if any, is not known. Based on molar growth yields from cells grown on limiting glucose and increasing fumarate concentrations, indirect evidence is shown for energy production during fumarate reduction by H_2. Attempts to obtain direct evidence for phosphorylation of adenosine-5' diphosphate to adenosine-5' triphosphate in fumarate reduction to succinate by H_2 using the 2-deoxy-D-glucose hexokinase trap and the luciferin-luciferase assay have been unsuccessful so far. From the data obtained, a tentative scheme is proposed for the transfer of electrons from hydrogen to fumarate in *B. fragilis*.

DEDICATION

To my family:

Reverend and Mrs. James Calvin Harris, Sr.

Deliah, Jeanette

Tyrone, James, Eric

and to the memory of Randy Keith

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INTRODUCTION

Bacteroides fragilis is a gram-negative, non-spore forming, non-motile, obligately anaerobic rod that is a normal inhabitant of the gastrointestinal tract of mammals (13,28). It is the most frequently isolated anaerobe from human pathological processes (22,28,54). A variety of carbohydrates including pentoses, hexoses, and disaccharides, are actively fermented by this organism (28,38). The major fermentation products from glucose catabolism are succinate and acetate with varying amounts of formate and propionate (28,38).

Evidence for fumarate reductase activity and the involvement of reduced nicotinamide adenine dinucleotide (NADH) as an electron donor in the reduction of fumarate to succinate has been obtained in cell extracts of *B. fragilis* by Macy, Probst, and Gottschalk (39). Naphthoquinone, a component of many bacterial electron transport systems (ETS) (35,67) has been isolated from *B. fragilis* (21), but the role of the naphthoquinone in the transfer of electrons from NADH to fumarate is not known. Cytochromes of the b and o types and a flavoprotein have been demonstrated spectrophotometrically in this organism (39, C. A. Reddy and M. P. Bryant, Bacteriol. Proc., p. 40, 1967). The addition of NADH or succinate to whole cells

of *B. fragilis* reduced cytochrome b and the addition of fumarate to endogenously reduced cytochrome b resulted in oxidation of the cytochrome (C. A. Reddy, Master's thesis, University of Illinois, Urbana, 1967). These preceding data suggested a cytochrome b-mediated reduction of fumarate to succinate by NADH in *B. fragilis*. Indirect evidence, based on molar growth yield studies obtained by Macy et al. (39) suggested that adenosine-5' triphosphate (ATP) is produced when fumarate is reduced to succinate in *B. fragilis*.

Bacteroides ruminicola, an obligate rumen anaerobe, is similar to *B. fragilis* in many biochemical characteristics (27,28,66). It contains cytochromes of the b and o types, produces fumarate reductase, and produces acetate and succinate as major acid end products of glucose catabolism (32,66). Evidence for ATP production in the reduction of fumarate to succinate by NADH in cell extracts of *B. ruminicola* has been obtained (C. A. Reddy, Rumen Function Conference, Chicago, 1973, unpublished).

Hydrogenase activity has not been previously demonstrated in *B. fragilis*. However, we recently observed that strains of this organism produce molecular H_2 when grown on carbohydrate-containing media. Previous studies with other cytochrome containing anaerobes (3,29,69, C. A. Reddy and H. D. Peck, Jr., Abst. Annu. Meet. Amer. Soc. Microbiol. 1973, 194) have shown that H_2 serves as an electron donor for the reduction of fumarate to succinate with a concomitant phosphorylation of adenosine

5' diphosphate (ADP) to ATP. C. A. Reddy and H. D. Peck, Jr. (Abst. Annu. Meet. Amer. Soc. Microbiol. 1973, 194) have demonstrated phosphorylation coupled to electron transfer between H_2 and fumarate in membrane preparations of *Vibrio succinogenes*, which is a non-carbohydrate fermenting anaerobe isolated from the rumen. This organism was shown to contain cytochromes similar to b and c (29,30,36,37). Phosphorylation coupled to the oxidation of H_2 with fumarate has been demonstrated by Barton, LeGall, and Peck (3,4) in *Desulfovibrio gigas*, a sulfate reducing anaerobe that contains b and c type cytochromes.

From the results of these earlier studies, it appeared that *Bacteroides* species and possibly some other anaerobes in the rumen and human intestine may obtain at least a portion of their energy by electron transport phosphorylation coupled to the reduction of fumarate to succinate by NADH and/or H_2. The basic objectives of this investigation were: (1) to study the hydrogenase activity in cell extracts; (2) to investigate the involvement of H_2 as an electron donor in fumarate reduction; and (3) to attempt to determine ATP generation in the reduction of fumarate to succinate by H_2 in cell extracts of *B. fragilis*.

REVIEW OF THE LITERATURE

General Characteristics of *Bacteroides fragilis*

Colonial and Cellular Morphology

Bacteroides fragilis, the type species of the genus *Bacteroides*, is an obligately anaerobic rod-shaped organism that produces small, circular, low convex, translucent colonies on blood agar (13,28). The cells are non-motile, non-spore forming, gram-negative, and usually stain deeply at both ends (28,54). The cells are usually 0.5 to 0.8 μm in diameter and 1.5 to 4.5 μm in length (54). Electron microscopic studies revealed that the ultrastructure of *B. fragilis* is similar to that of other gram-negative bacteria (14,53,54). The cell envelope in several unidentified *Bacteroides* species and in *B. convexus*, which has presently been reclassified as *B. fragilis* (28), consisted of external dense coat layer, an intermediate layer, and a three layered cytoplasmic membrane (6,62). Capsules and vacuoles are present in many strains of this organism and in the late logarithmic phases of growth, the cells become very pleomorphic (54).

Ecology

The ecological niche of *B. fragilis* is the lower intestinal tract of man and many animals (11,22,28,54).

Bacteroides species constitute a portion of the predominant flora in the lower gastrointestinal tract of mammals (11,22,28,54). Broido et al. (11) reported that *Bacteroides* species occur in population levels of 10^{11} organisms per gram in normal feces. *Bacteroides fragilis* is also present as normal flora in the oral and vaginal mucosa of humans but in much smaller numbers than that in the intestinal tract (22,28). Some *Bacteroides* species, including *B. fragilis*, may be found in other anaerobic habitats such as sewage (28).

Taxonomy

In 1919, Castellani and Chalmers (12) reclassified as *Bacillus fragilis* an organism first isolated by Veillon and Zuber (64). Later, in 1933, Eggerth and Gagnon (18) described a gram-negative, non-motile, non-spore forming rod isolated from human feces as *Bacteroides convexus*, which was later named *Pasteurella convexa* by Prevot (47). The organism described by Veillon and Zuber (64) has also been referred to at different times as *Pseudobacterium convexum*, *Ristella pseudoinsolita*, and *Eggerthella convexa* (28). Presently, however, the organism is classified as *Bacteroides fragilis* (28).

In 1970, Holdeman and Moore (26) subdivided *B. fragilis* into five subspecies, *ovatus*, *thetaiotaomicron*, *distasonis*, *vulgatus*, and *fragilis*. These five subspecies had previously been five distinct species within the genus *Bacteroides* (12,18). Eggerth and Gagnon (18) described

isolates from human feces as *B. ovatus*, *B. distasonis*, *B. vulgatus*, and *B. convexus*. Castellani and Chalmers (12) reclassified *Bacillus thetaiotaomicron*, which was isolated by Distaso (15,16), as *B. thetaiotaomicron*. As shown in Table 1, the cultural characteristics used to differentiate the subspecies were indole production and the fermentation of mannitol, raffinose, trehalose, and rhamnose (28,54). Varel and Bryant (63) found that *B. fragilis* subsp. *vulgatus* and *thetaiotaomicron* had nutritional requirements similar to those of *B. fragilis* subsp. *fragilis*. One strain of *B. fragilis* subsp. *distasonis* had an absolute requirement for methionine but not vitamin B_{12}, and *B. fragilis* subsp. *ovatus* required one or more amino acids and maybe vitamins (63). The most predominant subspecies present in normal human feces were *B. fragilis* subsp. *vulgatus* and *thetaiotaomicron* (54).

Recently, the validity of the five subspecies has been questioned. The data in Table 2 showed little homology among the subspecies (13,31). Therefore, Cato and Johnson (13) suggested that the five subspecies should be reclassified as five distinct species, *B. ovatus*, *B. thetaiotaomicron*, *B. distasonis*, *B. vulgatus*, and *B. fragilis*.

Nutritional Requirements

Tamimi, Hiltbrand, and Loercher (59) studied the nutritional requirements of seven strains of *B. fragilis*. The chemically defined medium used in this study contained

Table 1. Differentiation of the subspecies of *B. fragilis*[*]

Sub-species	Indole	Manni-tol	Raf-finose	Tre-halose	Rham-nose
ovatus	+	+	w	v	v
theta-iotaomicron	+	-	+	+	+
distasonis	-	-	+	w	v
vulgatus	-	-	+	-	+
fragilis	-	-	+	-	-

+ = positive; - = negative; w = weak; v = variable

[*]This table was taken from *The Pathogenic Anaerobic Bacteria* by Smith (54).

Table 2. The guanosine + cytosine (G + C) content and the interhomology of the five subspecies of *B. fragilis*[*]

Sub-species	ATCC Number	G+C Content	Interhomology of subspecies 25285	8503	8482	29184	8483
fragilis	25285	42 mol%	100	5	10	21	15
distasonis	8503	44 mol%	21	100	0	5	3
vulgatus	8482	41 mol%	24	9	100	9	8
theta-iotaomicron	29184	42 mol%	26	7	4	100	42
ovatus	8483	40 mol%	23	4	8	39	100

[*]From Johnson (31) and Cato and Johnson (13).

20 pure amino acids, vitamins, purines, pyrimidines, glucose and trace elements (59). The addition of sodium thioglycollate was required by the organism for growth in this defined medium while purines and pyrimidines were not essential for growth. Vitamins, on the other hand, were found to be either required or highly stimulatory since little or no growth occurred if they were deleted from the medium (59). The amino acid requirements were somewhat variable among the seven strains tested. Arginine, aspartic acid, glycine, histidine, hydroxyproline, leucine, isoleucine, lysine, serine, threonine, tryptophan, tyrosine, and valine were required by one or more of the strains (59).

A study, similar to that of Tamimi et al. (59), was conducted by Quinto (48). Five gram-negative anaerobic rods including three unidentified *Bacteroides* species, from clinical exudates, were examined for their nutritional requirements (48). Quinto found that sodium thioglycollate and erythrocyte extract stimulated the growth of all the anaerobes tested. All three *Bacteroides* strains required hemin but not vitamins, purines or pyrimidines, for growth (48). Quinto also studied the amino acid and vitamin requirements for several *Bacteroides* species in a later study (49). She reported that seven strains of *Ristella pseudoinsolita*, synonymous with *B. fragilis* (5), did not require amino acids or vitamins for growth, but that growth was stimulated by glycine, proline, histidine, alanine, and serine in different strains (49).

Recently, Varel and Bryant (63) did a comprehensive study of the minimal nutritional requirements of well characterized strains of *B. fragilis* subsp. *fragilis* (63). They reported that volatile fatty acids (VFA) were inhibitory and that hemin, casitone, and B vitamins were stimulatory but not essential for growth. If both casitone and the B vitamin solution were omitted from the medium, no growth occurred. Vitamin B_{12} was shown to be the essential component in the B vitamin solution (63). Methionine would substitute, however, for vitamin B_{12} or casitone. Ammonia was shown to be the primary nitrogen source. Urea, nitrate, and amino acids were not utilized as nitrogen sources. The best sulfur sources were cysteine and sulfide (63). This study, in agreement with that of Quinto (48,49), showed that purines and pyrimidines were not required and no B vitamins were essential when a complete amino acid mixture was added. Although Quinto (48) reported hemin to be essential for the growth of *B. fragilis*, Varel and Bryant found hemin to be very stimulatory but not required for growth (63). *Bacteroides fragilis* subsp. *fragilis* grows maximally in a chemically defined medium containing glucose, sulfide, vitamin B_{12}, carbon dioxide-bicarbonate buffer, hemin, NH_4^+, and minerals (63).

Biochemical Characteristics

A comparison of biochemical characteristics of *B. fragilis* ATCC 25285 with an original species is presented

in Table 3. *Bacteroides fragilis* is very active in fermenting a variety of carbohydrates (28,54). Esculin and starch are hydrolyzed and the addition of 20% bile stimulates growth (28). *Bacteroides fragilis* does not liquefy gelatin, digest cooked meat, or produce lipase or lecithinase (54).

The major fermentation products from glucose catabolism in a hemin containing medium are acetate and succinate with varying quantitiees of propionate and formate (28,39,42, 54). In the absence of hemin, Macy et al. (39) reported that the major products were lactate and fumarate with smaller quantities of acetate and formate. Mayhew et al. (42) studied the effects of five different types of media on volatile and non-volatile fatty acid production in *B. fragilis*. The three media that contained glucose were the best substrates for acetate and succinate production. Smaller quantities of propionate, isobutyric, and isovaleric acids were produced in the five media (42). Lactate was only produced in the chopped meat glucose medium. Elevated levels of propionate were produced in the peptone yeast medium. A pathway for glucose fermentation in the presence of hemin as proposed by Macy et al. (39) is shown in Figure 1. Although the exact pathways for the formation of the acid end products are not known, several key enzymes have been detected in *B. fragilis* (33,39). Keudell and Goldberg (33) have observed lactic, malic, and succinic dehydrogenases, and Macy et al. (39)

Table 3. Comparison of the biochemical characteristics of *B. fragilis* ATCC 25285 with the original species described by Eggerth and Gagnon (18)[a,b]

Reaction	*B. fragilis* Eggerth and Gagnon (18)	*B. fragilis* ATCC 25285
Acid from		
Amygdalin	+	-(+)
Arabinose	-	-
Cellobiose	+	-(w)
Dextrin	+	+
Dulcitol	-	-
Erythritol	-	-
Esculin	+	w
Fructose	+	+
Galactose	+	+
Glucose	+	+
Glycerol	-	-
Glycogen	+	+
Inositol	-	-
Inulin	+	+
Lactose	+	+
Maltose	+	+
Mannitol	-	-
Mannose	+	w(+)
Melezitose	-	-
Raffinose	+	+
Rhamnose	-	-
Salicin	-	-
Sorbitol	-	-
Starch	+	+
Sucrose	+	+
Trehalose	-	-
Xylose	+	+
Gas production	+	2
Milk	c	c
Gelatin digestion	slow	w
Indole production	-	-
H_2S production	+	+
Nitrate reduction	-	-

[a]Symbols: +, positive; -, negative; w, weak; c, curd, numbers (gas), amount on 1 to 4 scale. Where two reactions are given, the first is the more usual.

[b]From Cato and Johnson (13).

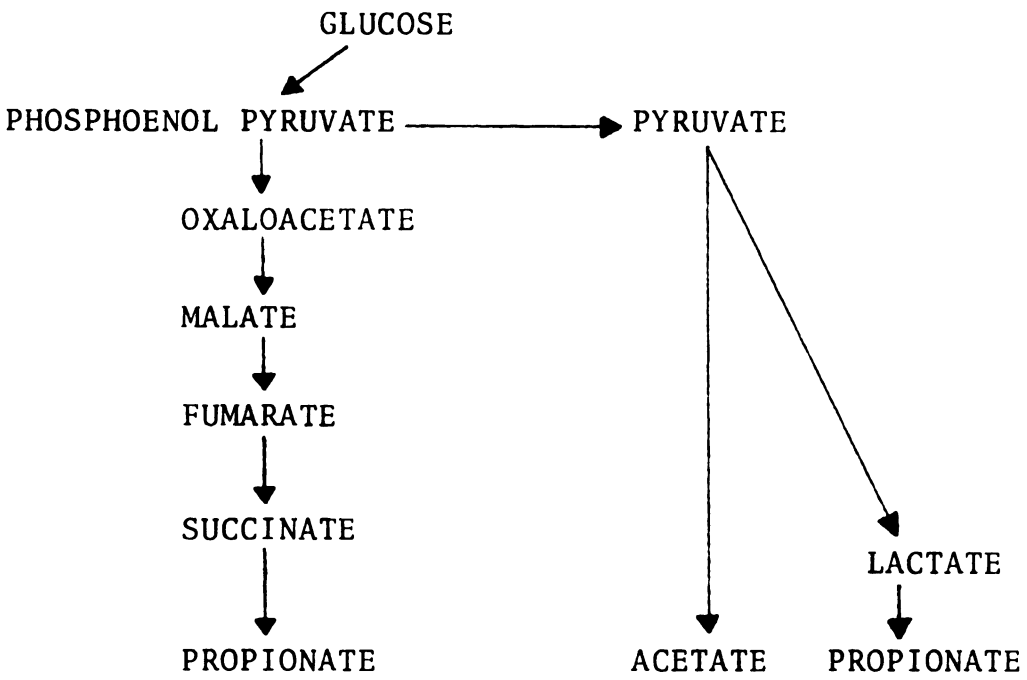

Figure 1. Proposed pathway for glucose catabolism in the presence of hemin. From Macy et al. (39).

have detected fumarate reductase, fumarase, and malate dehydrogenase activities in *B. fragilis*.

Pathogenic Nature of *Bacteroides fragilis*

Modes of Pathogenicity

At the present time, very little is known about the factors that are directly involved in the pathogenicity of many anaerobic bacteria (22,54). It has also been difficult to establish the pathogenicity of a single anaerobe in experimental animals. Frequently anaerobes are secondary invaders in infections and often they are associated with other organisms (22,24,54). Hill et al. (24) experimentally induced liver abscesses in mice by injecting several non-spore forming anaerobes, including *B. fragilis* and *Bacteroides melaninogenicus*. They observed that several species produced liver abscesses in the mice when injected singly; however, combinations of two or more species produced more severe progressive lesions (24). Hill et al. (24), however, did not suggest any factors that might have been involved in the pathogenicity of the anaerobes in the mouse model.

Endotoxic activity of the lipopolysaccharide (LPS) antigen in *B. fragilis* has been reported by Hofstad and Kristoffersen (25). The LPS was isolated by a phenol water extraction procedure (25). In order to determine the endotoxic potency of the LPS, they first injected rabbits intradermally on the shaved abdomen with serial dilutions of sterile LPS suspended in saline and 24 h

later they intravenously injected the rabbits with 400 μg of LPS (25). The rabbits were then examined for hemorrhagic lesions the next day (25). They observed that *B. fragilis* LPS did produce hemorrhagic lesions in the rabbits (25).

Various enzymes that may contribute to the pathogenicity of *B. fragilis* have been reported (20,43,44,65). The enzyme that degrades heparin, a naturally occurring mucopolysaccharide in connective tissue, was found in *Bacteroides* species isolated from stool samples by Gesner and Jenkin (20). They observed that heparinase was inducible because, in the absence of heparin, no heparinase activity was demonstrable (20). Gesner and Jenkin (20) suggested that the enzyme may be involved in the development of thrombophlebitis associated with anaerobic infections. Later Felner and Dowell (19) and Nobles (46) reported that heparinase activity by *Bacteroides* species, primarily *B. fragilis*, may have been involved in recurrent thrombophlebitis and pulmonary emboli in patients with various *Bacteroides* infections. Neuraminidase, which was shown to alter glycoproteins of human plasma, and deoxyribonuclease have also been found in *B. fragilis* (43,44,54,65).

Under certain conditions, *Bacteroides* species, which are normal flora in the gastrointestinal tract of man, become opportunists and serious disease conditions may occur in any human tissue organ (22,38,54).

Common Pathological Processes

Bacteroides fragilis is the most frequently isolated anaerobe from soft tissue infections (22,38,54). It comprises approximately 60% of all bacteroides isolated from blood, abscesses, and other infections (54).

In the 1930s, Thompson and Beaver (60) and Dixon and Deuterman (17) reported several cases of bacteroides bacteremia. Bacteremias due to anaerobic gram-negative, non-spore forming rods of the genus *Bacteroides* were seen in 39 patients over a 6 year period by Bodner et al. (7). From a total of 123 patients with positive blood cultures, Marcoux et al. (40) observed that, in 76.4% of the cases, *Bacteroides* were the only organism cultured from the blood and, in 29.2% of the patients, *Bacteroides* were found simultaneously in other sites in addition to the blood. Felner and Dowell (19) analyzed data from 250 patients with bacteremias due to anaerobic gram-negative bacilli. They reported that, in 93% of blood cultures, *B. fragilis* was isolated in pure culture and, in 7% of the positive blood cultures, *B. fragilis* was mixed with microaerophilic and anaerobic streptococci (19). Of 71 patients with positive blood cultures at the Mayo Clinic, Wilson et al. (68) reported that *Bacteroides* species were responsible for 78% of the bacteremias and clostridia and anaerobic cocci were causative agents in only 18% of the cases. Over an 18 month period, Nobles (46) observed that serious bacteroides infections occurred in 112 patients and, of the 43 cases of bacteroides septicemia,

B. fragilis was isolated from the blood in 33 of these cases. In the majority of the bacteroides bacteremia cases reported, intra-abdominal infections, infected surgical wounds, urinary tract infections, and gastro-intestinal infections were the major portals of entry and the most common primary disease conditions (7,19,40,68).

Invasion of blood by *Bacteroides* species is usually transient and a variety of disease complications may later develop. Felner and Dowell (19) reported that 20% of the patients with positive blood cultures later developed thrombophlebitis of the abdomino-pelvic veins. Thirty percent of these patients had pulmonary, liver, brain, kidney, or joint emboli (19). In 10 of the 43 patients with positive bacteroides blood cultures reported by Nobles, pulmonary emboli later occurred (46). Endocarditis later developed in 18 of the 250 patients that were reported by Felner and Dowell (19). Rotheram and Schick (50) observed that in 56 patients with septic abortions, bacteremias occurred in 34 patients. Principally anaerobic streptococci and *Bacteroides* species were isolated from 69 uterine exudates in the septic abortions (50).

Bacteroides species have also been isolated from abscesses in various organs in human (23,46,51). Generally these abscesses contain foul-smelling pus often with gas formation (23,46,51). From 69 soft tissue infections (46) caused by various *Bacteroides*, 49 cases were abscesses. Nobles isolated *B. fragilis* from 33 of these

69 soft tissue infections (46). From brain abscesses, Heineman and Braude (23) isolated *Bacteroides*, *Actinomyces*, and anaerobic streptococci. Sabbaj et al. (51) recovered *Bacteroides*, *Fusobacterium*, and *Actinomyces* from pyogenic liver abscesses.

In addition to the numerous clinical bacteroides infections reported, various *Bacteroides* species have also been recovered from different types of pleuropulmonary infections including pneumonitis, pulmonary abscesses, necrotizing pneumonia, multiple septic emboli, and empyema (2,55,61). Bartlett and Finegold (2) recovered *B. fragilis* from 26% of patients with various pleuropulmonary infections. Primarily bacteroides and anaerobic streptococci were detected by Sullivan et al. (55) in 19% of 226 empyemas. Bacteroides empyemas were also reported in 11 patients by Tillotson and Lerner (61).

Treatment

Surgical drainage in conjunction with appropriate antimicrobial therapy is necessary for effective treatment of many abscesses caused by *B. fragilis* and other anaerobes (22,54). Commonly used antibiotics for treatment of disease conditions caused by gram-negative rods are not effective against *B. fragilis*. Bodner et al. (8) observed that the *Bacteroides* species isolated from the blood of 39 patients were resistant to penicillin, cephalothin, and kanamycin, which are bactericidal antibiotics used for treating gram-negative rods (1,52).

Some of these isolates were resistant to tetracycline, but all were sensitive to chloramphenicol (8). In 1972, Bodner et al. (8) reported that 90% of 70 strains of *Bacteroides* were susceptible to clindamycin, chloramphenicol, carbenicillin, and lincomycin and only 40% were susceptible to tetracycline. Kislak (34) examined 40 clinical isolates of *B. fragilis* against 24 antibiotics. The cephalosporins and semi-synthetic penicillinase-resistant penicillins were not very effective, although some strains were inhibited by penicillin G, ampicillin, and carbenicillin (34). The polymyxins and aminoglycosides were not effective against *B. fragilis* and not very effective for treating anaerobic infections in general (34). More than one-half of the strains tested were resistant to tetracycline. The antibiotics rifampin, erythromycin, lincomycin, and chloramphenicol inhibited all isolates at concentration levels readily achieved in serum (34). Clindamycin, the most effective antibiotic tested, was 8 times more effective against *B. fragilis* than either erythromycin or lincomycin. The bactericidal activity of rifampin, clindamycin, vancomycin, and metronidazole was evaluated against *B. fragilis* by Nastro and Finegold (45). From their results, all strains examined were resistant to vancomycin. *Bacteroides fragilis* developed rapid resistance to rifampin (45). All strains used in the study were inhibited by clindamycin and metronidazole at concentration levels that are readily obtainable in serum (45). In 1971, Sutter and

Finegold found *B. fragilis* to be resistant to colistin, kanamycin, neomycin, and penicillin (56).

Variable results have been reported on the susceptibility of *B. fragilis* to lincomycin and erythromycin. Kislak (34) and Martin et al. (41) reported that both antimicrobial agents were very active against *B. fragilis*. Sutter et al. (58), however, found that only 7% of *B. fragilis* strains were sensitive to erythromycin and 13% to lincomycin.

Only one-third of the *B. fragilis* strains examined are presently sensitive to tetracycline, which was once the antibiotic of choice in treating bacteroides infections (8,41,57). Presently, chloramphenicol, clindamycin, and metronidazole are effective antimicrobial agents for the treatment of infections caused by *B. fragilis* (8,22, 41,45,54,58).

Evidence for Electron Transport Components and Energy Production in *Bacteroides fragilis*

The Involvement of NADH and H_2 in Fumarate Reduction

The electron donor NADH has been shown to be involved in the reduction of fumarate and other electron transport components in *B. fragilis*. Using spectral techniques, C. A. Reddy (M.S. thesis) showed that the addition of NADH to washed whole cells of *B. fragilis* resulted in reduction of the b-type cytochrome and the addition to fumarate to endogenously reduced cells oxidized the b-type cytochrome. The fact that oxaloacetate and malate also

oxidized the b-type cytochrome suggested that they may be precursors in the synthesis of fumarate which accepts the electrons from the b-type cytochrome. The addition of succinate, on the other hand, resulted in the reduction of the b-type cytochrome. Macy et al. (39) demonstrated fumarate reduction by NADH in cell extracts of *B. fragilis*. The oxidation of NADH was measured spectrophotometrically at 366 nm in an anaerobic cuvette under N_2 and the specific activity of the reaction was 0.13 μmol/min/mg of protein (39).

Macy et al. (39) reported fumarate reduction in extracts of *B. fragilis* with H_2 as the electron donor and *C. pasteurianum* extract as a source of hydrogenase and some other catalytic compounds. The artificial low potential electron carrier, methyl viologen, was also required for the reaction. Neither hydrogen gas production nor hydrogenase activity was detected in the strain of *B. fragilis* used in their investigation (39). A specific activity of 0.59 μmol/min/mg of protein was calculated for the reaction (39).

The Presence of a Flavoprotein

Flavoprotein, a component of most bacterial electron transport systems, has been detected in *B. fragilis*. Reddy (M.S. thesis) and Macy et al. (39) spectrophotometrically demonstrated the presence of a flavoprotein in whole cells and cell extracts of the organism as evidenced by a deep trough between 450-475 nm when NADH or succinate was

added to whole cells or cell extracts, respectively. C. A. Reddy (M.S. thesis) observed that more of the flavoprotein was reduced when sodium dithionite was added to cells already reduced by their endogenous metabolism.

Isolation of a Vitamin K Compound

Vitamin K compounds are widely distributed in electron transport systems in plants, animals, and microorganisms (9,10). These compounds were isolated from a number of obligately anaerobic chemoorganotrophic bacteria including *B. fragilis* by Gibbons and Engle (21). The purified lipid material had absorption maxima at 243, 248, 260, and 269 nm, which are characteristic of vitamin K compounds (21). Since *Bacteroides* species are one of the predominant genera in the intestinal tract, Gibbons and Engle proposed that *B. fragilis* may be an important producer of vitamin K in mammals (21).

Cytochrome-Linked Fermentation

Cytochromes were first observed spectrophotometrically in whole cells of *B. fragilis* by C. A. Reddy and M. P. Bryant (Bacteriol. Proc., p. 40, 1967). Absorption maxima which occurred at 560, 528, 427, and 571, 537.5, and 414 nm are characteristic of b and o type cytochromes, respectively. The o-type cytochrome was demonstrable only after exposing the sodium dithionite reduced cell suspension to carbon monoxide and taking a difference spectrum against sodium dithionite reduced cells. Various substrates were anaerobically added to cell suspensions

to determine if the b-type cytochrome would be oxidized or reduced. Fumarate, malate, and oxaloacetate oxidized the b-type cytochrome and NADH, pyruvate, and succinate reduced the b-type cytochrome in the strains of *B. fragilis* examined (C. A. Reddy, M.S. thesis, University of Illinois, 1967).

More recently, Macy et al. (39) have demonstrated the presence of b and o type cytochromes in cell extracts of another strain of *B. fragilis*. Difference spectra of *B. fragilis* at liquid nitrogen temperature by Macy et al. (39) showed absorption maxima at 565 and 554 nm, suggesting the presence of a b-type and possibly a c-type cytochrome. They observed that hemin is required for b-type cytochrome synthesis and that the b-type cytochrome appears to be involved in fumarate reduction to succinate. When hemin was not included in the growth medium for *B. fragilis*, cytochromes and fumarate reductase were not demonstrable (39). However, in the presence of hemin, cytochromes of the b and o types were detected spectrophotometrically and fumarate reductase activity was demonstrable manometrically (39).

Indirect Evidence for Additional ATP Synthesis Coupled to Fumarate Reduction

The growth of *B. fragilis* in a cultivation medium without hemin was very slow with a generation time of 8 h (39,63). However, the addition of 2 μg of hemin per

ml of medium stimulated the growth of the organism and decreased the generation time to 2 h (39,63).

Macy et al. (39) examined the molar growth yields (Ym, glucose) and acid end products in cultures of *B. fragilis* grown with and without hemin in the media. Lactate and fumarate were the major acid end products produced when hemin was not present (39). A small quantity of acetate was also produced when hemin was not included in the cultivation medium. The Ym value was 17.9 g of cells per mol of glucose utilized in the hemin-deficient medium. In the presence of hemin, however, they detected acetate, propionate, and succinate as the major acid end products and the Ym value was 47 g/mol of glucose (39). Macy et al. (39) calculated that 4.5 ATP/mol of glucose were made in the presence of hemin and only 1.7 ATP/mol of glucose were made in the absence of hemin. They stated that the larger quantity of acetate produced by substrate level phosphorylation in the hemin supplemented medium would not account for the increase in cellular growth that was observed on this medium as compared to the non-hemin medium. The results of Macy et al. (39) suggested that the synthesis of propionate and succinate may be directly coupled to ATP production via a primitive anaerobic electron transport system.

REFERENCES

REFERENCES

1. Anderson, J. D. and R. B. Sykes. 1973. Characterization of a β-lactamase obtained from a strain of *Bacteroides fragilis* resistant to β-lactam antibiotics. J. Med. Microbiol. 6:201-206.

2. Bartlett, J. G., and S. M. Finegold. 1972. Anaerobic pleuropulmonary infections. Medicine 51:413-450.

3. Barton, L. L., J. LeGall, and H. D. Peck, Jr. 1970. Phosphorylation coupled to oxidation of hydrogen with fumarate in extracts of the sulfate reducing bacterium, *Desulfovibrio gigas*. Biochem. Biophys. Res. Commun. 41:1036-1042.

4. Barton, L. L., J. LeGall, and H. D. Peck, Jr. 1972. Oxidative phosphorylation in the obligate anaerobe, *Desulfovibrio gigas*. p. 33-51. *Horizons of Bioenergetics*. Academic Press, Inc., New York and London.

5. Beerens, H. 1970. Report of the international committee on nomenclature of bacteria. Taxonomic Subcommittee for Gram-negative anaerobes rods. Int. J. Syst. Bacteriol. 20:297-300.

6. Bladen, H., and J. Waters. 1963. Electron microscopic study of some strains of *Bacteroides*. J. Bacteriol. 88:1309-1344.

7. Bodner, S. J., M. G. Koenig, and J. S. Goodman. 1970. Bacteremic bacteroides infections. Ann. Intern. Med. 73:537-544.

8. Bodner, S. J., M. G. Koenig, L. L. Trenor, and J. S. Goodman. 1972. Antibiotic susceptibility testing of *Bacteroides*. Antimicrob. Agents Chemother. 2: 57-60.

9. Brodie, A. F., M. M. Weber, and C. T. Gray. 1957. The role of a vitamin K_1 in coupled oxidative phosphorylation. Biochim. Biophys. Acta 25:448-449.

10. Brodie, A. F., and J. Ballantine. 1960. Oxidative phosphorylation in fractionated bacterial systems. II. The role of vitamin K. J. Biol. Chem. 235: 226-231.

11. Broido, P. W., S. L. Gorbach, and L. M. Nyhus. 1972. Microflora of the gastrointestinal tract and the surgical malabsorption syndromes. Surg. Gynecol. Obstet. 135:449-460.

12. Castellani, A., and A. J. Chalmers. 1919. *Manual of Tropical Medicine*. 3rd ed. Williams Wood and Co., New York.

13. Cato, E. P., and J. L. Johnson. 1976. Reinstatement of species rank for *Bacteroides fragilis*, *B. ovatus*, *B. distasonis*, *B. thetaiotaomicron*, and *B. vulgatus*: Designation of neotype strains of *Bacteroides fragilis* (Veillon and Zuber) Castellani and Chalmers and *Bacteroides thetaiotaomicron*, (Distaso) Castellani and Chalmers. Int. J. Syst. Bacteriol. 26:230-237.

14. Costerton, J., J. Ingram, and K. Cheng. 1974. Structure and function of the cell envelope of gram-negative bacteria. Bacteriol. Rev. 38:81-110.

15. Distaso, A. 1911. Sur les microbes proteolytiques de la flora intestinale de l'homme et des animaux. Zentrabl. Bakterol. Parasitenk. Infektionskr. Hyg. Abt. I. Orig. 59:97-103.

16. Distaso, A. 1912. Contribution a l'etude sur l'intoxication intestinale. Zentrabl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. I. Orig. 62: 433-468.

17. Dixon, C. F., and J. L. Deuterman. 1937. Postoperative bacteroides infection: Report of six cases. J. Am. Med. Assoc. 108:181-185.

18. Eggerth, A. H., and B. H. Gagnon. 1933. The bacteroides of the human feces. J. Bacteriol. 25:389-413.

19. Felner, J. M., and V. R. Dowell. 1971. Bacteroides bacteremia. Am. J. Med. 50:787-796.

20. Gesner, B. M., and S. R. Jenkin. 1961. Production of heparinase by bacteroides. J. Bacteriol. 81: 595-604.

21. Gibbons, R. J., and L. P. Engle. 1964. Vitamin K compounds in bacteria that are obligate anaerobes. Science 146:1307-1309.

22. Goodman, J. 1974. Infections due to anaerobic non-spore forming bacilli. p. 219-235. In A. Balows, R. DeHann, V. Dowell, and L. Guze (eds.). *Anaerobic Bacteria: Role in Disease.* Charles C. Thomas, Publisher, Springfield, Illinois.

23. Heineman, H. S., and A. I. Braude. 1963. Anaerobic infection of the brain. Am. J. Med. 35:682-696.

24. Hill, G., S. Osterhout, and P. Pratt. 1974. Liver abscess infection in mice produced by non-spore forming anaerobic bacteria. Infec. Immun. 9:599-603.

25. Hofstad, T., and T. Kristoffersen. 1970. Chemical characteristics of endotoxin from *Bacteroides fragilis* NCTC 9343. J. Gen. Microbiol. 61:15-19.

26. Holdeman, L. V., and W. E. C. Moore. 1970. *Bacteroides.* In E. P. Cato, C. S. Cummins, L. V. Holdeman, J. L. Johnson, W. E. C. Moore, R. M. Smibert, and L. D. S. Smith (eds.). *Outline of Clinical Methods in Anaerobic Bacteriology.* 2nd ed. Virginia Polytechnic Institute, Anaerobe Laboratory, Blacksburg, Virginia.

27. Holdeman, L. V., and W. E. C. Moore (eds.). 1973. *Anaerobe Laboratory Manual.* 2nd ed. VPI Anaerobe Laboratory, Blacksburg, Virginia.

28. Holdeman, L. V., and W. E. C. Moore. 1974. Gram-negative anaerobic bacteria. p. 384-391. In Buchanan, R., and N. Gibbons (eds.). *Bergy's Manual of Determinative Bacteriology.* 8th ed. Williams and Wilkins Co., Baltimore, Maryland.

29. Jacobs, N. J., and M. J. Wolin. 1963. Electron transport system of *Vibrio succinogenes.* I. Enzymes and cytochromes of the electron transport system. Biochim. Biophys. Acta 69:18-28.

30. Jacobs, N. J., and M. J. Wolin. 1963. Electron transport system of *Vibrio succinogenes.* II. Inhibition of electron transport by 2-heptyl-4-hydroxyquinoline-N-oxide. Biochim. Biophys. Acta 69:29-39.

31. Johnson, J. L. 1973. Use of nucleic acid homologies in taxonomy of anaerobic bacteria. Int. J. Syst. Bacteriol. 23:308-315.

32. Joyner, A., and R. Baldwin. 1966. Enzymatic studies of pure cultures of rumen microorganisms. J. Bacteriol. 92:1321-1330.

33. Keudell, K. C., and H. S. Goldberg. 1970. Dehydrogenase patterns in the study of *Bacteriodaceae*. Appl. Microbiol. 19:505-511.

34. Kislak, J. W. 1972. The susceptibility of *Bacteroides fragilis* to 24 antibiotics. J. Infect. Dis. 125: 295-299.

35. Kroger, A., V. Dadak, M. Klingenberg, and F. Diemer. 1971. On the role of quinone in bacterial electron transport. Eur. J. Biochem. 21:322-333.

36. Kroger, A., and A. Innerhofer. 1976. The function of menaquinone, covalently-bound FAD and iron-sulfate protein in the electron transport from formate to fumarate of *Vibrio succinogenes*. Eur. J. Biochem. 69:487-495.

37. Kroger, A., and A. Innerhofer. 1976. The function of the b cytochrome in the electron transport from formate to fumarate of *Vibrio succinogenes*. Eur. J. Biochem. 69:497-506.

38. Loesche, W., S. Socransky, and R. J. Gibbons. 1974. *Bacteroides oralis*, proposed new species isolated from the oral cavity of man. J. Bacteriol. 88: 1329-1337.

39. Macy, J., I. Probst, and G. Gottschalk. 1975. Evidence for cytochrome involvement in fumarate reduction and adenosine 5'-triphosphate synthesis by *Bacteroides fragilis* grown in the presence of hemin. J. Bacteriol. 123:436-442.

40. Marcoux, J. A., R. J. Zabransky, J. A. Washington, W. E. Wellman, and W. J. Martin. 1970. Bacteroides bacteremia. Minn. Med. 53:1169-1176.

41. Martin, W. J., M. Gardner, and J. A. Washington. 1972. *In vitro* antimicrobial susceptibility of anaerobic bacteria isolated from clinical specimens. Antimicrob. Agents Chemother. 1:148-158.

42. Mayhew, J. M., A. B. Onderdonk, and S. L. Gorbach. 1975. Effects of time and growth media on short chain fatty acid production by *Bacteroides fragilis*. Appl. Microbiol. 29:472-475.

43. Muller, H. E., and H. Werner. 1970. Die Neuraminidase als pathogene tischer faktor bei cinem durch *Bacteroides fragilis* bedingten abscess. Z. Med. Mikerobiol. U. Immunol. 156:98-106.

44. Muller, H. E., and H. Werner. 1970. *In vitro* untersuchungen uber das vorkommen von neuraminidase bei *Bacteroides*-arten. Pathol. Microbiol. 36:135-152.

45. Nastro, L. J., and S. M. Finegold. 1972. Bactericidal activity of five antimicrobial agents against *Bacteroides fragilis*. J. Infect. Dis. 126:104-107.

46. Nobles, E. D. 1973. Bacteroides infections. Ann. Surg. 177:601-606.

47. Prevot, A. R. 1938. Etudes de systematique bacterienne. Ann. Inst. Pasteur (Paris) 60:285-307.

48. Quinto, G. 1962. Nutrition of five bacteroides strains. J. Bacteriol. 84:559-562.

49. Quinto, G. 1966. Amino acid and vitamin requirements of several bacteroides strains. Appl. Microbiol. 14:1022-1026.

50. Rotheram, E. B., Jr., and S. F. Schick. 1969. Nonclostridial anaerobic bacteria in septic abortion. Am. J. Med. 146:80-89.

51. Sabbaj, J., V. L. Sutter, and S. M. Finegold. 1972. Anaerobic pyogenic liver abscess. Ann. Intern. Med. 77:629-638.

52. Pinkus, G., G. Veto, and A. I. Braude. 1968. Bacteroides penicillinase. J. Bacteriol. 96:1437-1438.

53. Seryczynska, H., and F. Meisel-Mikolajezyk. 1972. Ultrastructures of *Sphaerophorus* and *Eggerthella* strains. Bull. Acad. Polonaise Sci. Ser. Sci. Biol. 6:369-371.

54. Smith, L. D. S. 1975. *The Pathogenic Anaerobic Bacteria*. Charles C. Thomas, Publishers, Springfield, Illinois.

55. Sullivan, K. M., R. D. O'Toole, R. H. Fisher, and K. N. Sullivan. 1973. Anaerobic empyema thoracis - the role of anaerobes in 226 cases of culture proven empyemas. Arch. Intern. Med. 131:521-527.

56. Sutter, V. L., and S. M. Finegold. 1971. Antibiotic disc susceptibility tests for rapid presumptive identification of gram-negative anaerobic bacilli. Appl. Microbiol. 21:13-20.

57. Sutter, V. L., Y. Y. Kwok, and S. M. Finegold. 1972. Standardized antimicrobial disc susceptibility of *Bacteroides fragilis* to tetracycline. Appl. Microbiol. 23:268-275.

58. Sutter, V. L., Y. Y. Kwok, and S. M. Finegold. 1973. Susceptibility of *Bacteroides fragilis* to six antibiotics determined by standardized antimicrobial disc susceptibility testing. Antimicrob. Agents Chemother. 3:188-193.

59. Tamimi, H., W. Hiltbrand, and H. Loercher. 1960. Some growth requirements of *Bacteroides fragilis*. J. Bacteriol. 80:472-476.

60. Thompson, L., and D. C. Beaver. 1932. Bacteremia due to anaerobic gram-negative organisms of the genus *Bacteroides*. Med. Clin. North Am. 15:1611-1626.

61. Tillotson, J. R., and A. M. Lerner. 1968. Bacteroides pneumonias. Ann. Intern. Med. 68:308-317.

62. Ushijima, T. 1969. Fine structure of strictly anaerobic, non-spore forming, gram-negative bacilli. II. Mesosomes and cell division of a strain of *Bacteroides convexus*. Japan J. Microbiol. 13: 51-63.

63. Varel, V., and M. P. Bryant. 1972. Nutritional features of *Bacteroides fragilis* subsp. *fragilis*. Appl. Microbiol. 18:251-257.

64. Veillon, A., and A. Zuber. 1898. Recherches sur quelques microbes strictement anaerobies et leur role en pathologie. Arch. Med. Exp. 10:517-545.

65. Werner, H., and G. Rintelen. 1968. Untersuchungen uber die konstanz der kohlenhydratspaltung bei intestinal *Bacteroides (Eggerthella)*-arten. Zen. Bat. Orig. 208:521-528.

66. White, D., M. P. Bryant, and D. Caldwell. 1962. Cytochrome linked fermentation in *Bacteroides ruminicola*. J. Bacteriol. 84:822-828.

67. White, D. C., and P. R. Sinclair. 1971. Branched electron transport systems in bacteria. p. 173-211. In A. H. Rose and J. F. Wilkinson (eds.). *Advances in Microbial Physiology*, vol. 5. Academic Press, Inc., New York.

68. Wilson, W. R., W. J. Martin, W. J. Wilkowske, and J. A. Washington. 1972. Anaerobic bacteremia. Mayo Clin. Proc. 47:639-646.

69. Wolin, M. J., E. A. Wolin, and N. J. Jacobs. 1961. Cytochrome producing anaerobic vibrio, *Vibrio succinogenes*. SP. N. J. Bacteriol. 81:911-917.

SECTION 1 (ARTICLE 1)

HYDROGENASE ACTIVITY AND THE H_2-FUMARATE ELECTRON TRANSPORT SYSTEM IN *BACTEROIDES FRAGILIS*

By

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Hydrogenase Activity and the H_2-Fumarate Electron Transport System in *Bacteroides fragilis*[1]

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Hydrogenase activity and the H_2-fumarate electron transport system in a carbohydrate-fermenting obligate anaerobe, *Bacteroides fragilis*, were investigated. In both whole cells and cell extracts, hydrogenase activity was demonstrated with methylene blue, benzyl viologen, flavin mononucleotide, or flavin adenine dinucleotide as the electron acceptor. A catalytic quantity of benzyl viologen or ferredoxin from *Clostridium pasteurianum* was required to reduce nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate with H_2. Much of the hydrogenase activity appeared to be associated with the soluble fraction of the cell. Fumarate reduction to succinate by H_2 was demonstrable in cell extracts only in the presence of a catalytic quantity of benzyl viologen, flavin mononucleotide, flavin adenine dinucleotide, or ferredoxin from *C. pasteurianum*. Sulfhydryl compounds were not required for fumarate reduction by H_2, but mercaptoethanol and dithiothreitol appeared to stimulate this activity by 59 and 61%, respectively. Inhibition of fumarate reduction by acriflavin, rotenone, 2-heptyl-4-hydroxyquinoline-*N*-oxide, and antimycin A suggest the involvement of a flavoprotein, a quinone, and cytochrome *b* in the reduction of fumarate to succinate. The involvement of a quinone in fumarate reduction is also apparent from the inhibition of fumarate reduction by H_2 when cell extracts were irradiated with ultraviolet light. Based on the evidence obtained, a possible scheme for the flow of electrons from H_2 to fumarate in *B. fragilis* is proposed.

Bacteroides fragilis is a gram-negative, nonmotile, obligately anaerobic rod that is a normal inhabitant of the human gastrointestinal tract and is the most frequently encountered anaerobe in clinical specimens (4, 13, 24, 29). It ferments a variety of carbohydrates and produces succinate and acetate as major acid end products (13, 20).

Evidence for fumarate reductase activity and the involvement of reduced nicotinamide adenine dinucleotide (NADH) as an electron donor in the reduction of fumarate to succinate in *B. fragilis* cell extracts has been obtained by Macy et al. (23). Naphthoquinone, a component of many bacterial electron transport systems (ETS) (19, 32), has been isolated from *B. fragilis* (9), but its role in the transfer of electrons from NADH to fumarate is not known. Cytochromes *b* and *o* and a flavoprotein have been demonstrated spectrophotometrically in this organism (23; C. A. Reddy and M. P. Bryant, Bacteriol. Proc., p. 40-41, 1967). The addition of NADH or succinate to whole cells of *B. fragilis* resulted in reduction of cytochrome *b*, and the addition of fumarate to endogenously reduced cytochrome *b* resulted in its oxidation (C. A. Reddy, M.S. thesis, University of Illinois, Urbana, 1967). These data suggest a cytochrome *b*-mediated reduction of fumarate to succinate by NADH in *B. fragilis*.

Hydrogenase activity has not been previously demonstrated in *B. fragilis*. We observed recently that some strains of this organism produce molecular H_2 when grown on carbohydrate-containing media. Previous studies with other cytochrome-containing anaerobes (5, 16, 33; C. A. Reddy and H. D. Peck, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P318, p. 194) have shown that H_2 serves as an electron donor for the reduction of fumarate to succinate, with a concomitant phosphorylation of adenosine diphosphate to adenosine 5'-triphosphate. The object of this investigation was to investigate the hydrogenase activity and the H_2-fumarate ETS in *B. fragilis* cell extracts.

MATERIALS AND METHODS

Cultivation of bacteria. *B. fragilis* subsp. *fragilis* ATCC 25285 was grown anaerobically under 100% CO_2 by using Hungate anaerobic techniques (14). The composition of the growth medium is given in

[1] Journal article 7914, Michigan Agricultural Experiment Station.

Table 1. The final pH of the medium was 6.5 to 6.7. Hemin and menadione was filter sterilized; glucose, cysteine hydrochloride, sodium carbonate, and 0.005% ferrous sulfate (when added) were sterilized separately, and aseptically and anaerobically added to the autoclaved and cooled medium.

B. fragilis cultures were maintained on agar slants of the above medium minus fumarate and stored at 4°C. The organism was transferred monthly with a sterile inoculating loop to a fresh agar slant, which was then incubated for 16 to 24 h at 37°C.

The inocula for large quantities of medium were prepared by transferring the organism with a sterile inoculating loop from a maintenance slant to 5 ml of growth medium and incubating the tube for 16 to 24 h at 37°C. Two milliliters of the above culture was used to inoculate 20 ml of medium, which, after the organism had grown to the late logarithmic phase, was used to inoculate 2 liters of the medium in a 3-liter Erlenmeyer flask. The latter was incubated for 22 h at 37°C. Bacterial growth was followed by measuring the absorbance at 600 nm (tubes, 18 by 150 mm) in a Bausch and Lomb Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Culture purity was checked by microscopic examination of a wet mount and a Gram-stained culture and by inoculation of an aerobic Trypticase soy agar (Baltimore Biological Laboratory, Cockeysville, Md.) slant with a loopful of culture. Strict anaerobes, such as *B. fragilis*, do not grow on this medium, whereas many common contaminants do.

Clostridium pasteurianum ATCC 6013, used as the source of hydrogenase and ferredoxin, was grown in 20-liter batches as described by Reddy et al. (27).

Preparation of cell extracts. *B. fragilis* cells were harvested in a refrigerated centrifuge (Sorvall RC-5, Ivan Sorvall, Inc., Norwalk, Conn.) with a continuous-flow rotor at 48,000 × *g* and were suspended in ca. 3 volumes of 50 mM PO_4 buffer (pH 7.5). The cells were disrupted in a French pressure cell at 15,000 lb/in^2 and centrifuged at 12,000 × *g* for 20 min to remove unbroken cells and large debris. The supernatant fraction (cell extract) was stored under H_2 at 4°C and used within a few hours for various enzymatic assays.

C. pasteurianum cells were harvested as described above, washed twice in 2 volumes of PO_4 buffer (pH 7.0), and centrifuged at 27,000 × *g* for 15 min. The cells were broken as described above, and the broken-cell suspension was centrifuged at 27,000 × *g* for 15 min. The pellet was washed in 2 volumes of PO_4 buffer and centrifuged at 27,000 × *g* for 10 min. The supernatant portions from the two centrifugation steps were combined.

Preparation of membrane fraction. *B. fragilis* cell extract was centrifuged at 104,000 × *g* for 1 h at 4°C in a Sorvall OTD ultracentrifuge. The supernatant fraction was decanted, and the pellet was suspended in 2 volumes of PO_4 buffer (pH 7.5). The supernatant and pellet fractions were stored under H_2 at 4°C and used for assaying various enzymatic activities within a few hours.

Enzyme assays. Standard Warburg manometric techniques (31) were used for assaying hydrogenase and coupled H_2-fumarate reductase activity. All gasses used were deoxygenated by passing them through a column of reduced copper wire that was heated to ca. 400°C (15). Distilled water, used for preparing reagents, was deoxygenated by boiling and gassing with N_2 for 30 min. Unless otherwise indicated, the Warburg flasks were gassed with H_2 for 20 min, and their contents were allowed to equilibrate for an additional 20 min. All assays were run at 37°C.

TABLE 1. *Composition of the B. fragilis growth medium*

Component	Concn (%)
Glucose	0.4
Sodium fumarate	0.2
Trypticase (BBL)	1.0
Yeast extract (Difco)	0.2
VFA solution[a]	1.0
Mineral solution I[b]	7.5
Mineral solution II[b]	7.5
Hemin	0.0004
Menadione	0.0004
Resazurin	0.0001
Cysteine $HCl \cdot H_2O$	0.05
Sodium carbonate	0.40
CO_2, gas phase	100.00

[a] Volatile fatty acid (VFA) solution contained 1 ml each of *n*-valeric, isobutyric, and DL-2-methyl-*n*-butyric acids and 1.6 ml of acetic acid per 100 ml of distilled water.

[b] Mineral solutions I and II were as described by Caldwell and Bryant (8).

Hydrogenase activity was measured by following H_2 uptake in the presence of various electron acceptors, including methylene blue (MB), benzyl viologen (BV), methyl viologen (MV), nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$), flavin mononucleotide (FMN^+), or flavin adenine dinucleotide (FAD^+). The electron acceptor was in the side arm and was tipped into the main vessel to start the reaction.

Hydrogen-fumarate coupling activity was measured by following hydrogen consumption in the presence of sodium fumarate, the electron acceptor. The main vessel of the Warburg flask contained potassium phosphate buffer, mercaptoethanol (reducing agent), *B. fragilis* extract, and BV, a required electron mediator. Sodium fumarate, in the side arm, was tipped into the main vessel to start the reaction. BV was deleted from experiments designed to determine the effect of electron transport inhibitors and ultraviolet (UV) irradiation on fumarate reduction. Crude *C. pasteurianum* extract was substituted for BV in these latter experiments since BV, an artificial electron carrier, might shunt electrons around the natural electron carriers involved in the H_2-fumarate ETS (23). *B. fragilis* and *C. pasteurianum* extracts were in the side arm and were tipped into the main vessel to start the reaction.

Irradiation with UV light. To determine the possible involvement of a quinone in fumarate reduc-

tion by H_2, *B. fragilis* cell extract was irradiated with UV light as described by Brodie et al. (6) and as modified by Prasad et al (26). A 250-ml beaker containing ca. 5 ml of extract was placed in an ice bath and was continuously gassed with H_2. The extract was then irradiated by placing a Burton UV lamp (366 nm) directly over the beaker for 40 min and assayed for H_2-fumarate coupling activity as previously described.

Protein determination. The biuret method (10) or the procedure of Lowry et al. (21) was used for protein estimation. Bovine serum albumin, fraction V, obtained from Sigma Chemical Co., St. Louis, Mo., was used as the standard.

Chemicals. *C. pasteurianum* ferredoxin, BV, MV, acriflavin, rotenone, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), antimycin A, and vitamins K_1, K_2, and K_3 were purchased from Sigma. MB was purchased from the Allied Chemical Co., New York, N.Y. All other chemicals were of reagent grade or higher quality.

RESULTS

Hydrogenase activity. Hydrogenase activity was readily demonstrable in cell extracts of *B. fragilis*, by using MB as the electron acceptor (Table 2). There was no detectable H_2 consumption when boiled extract was used or when the cell extract or MB was deleted from the reaction mixture. Activity was dependent upon H_2, since substituting N_2 for H_2 resulted in no gas consumption. The activity with BV and MV was ca. 50 and 10%, respectively, of that observed with MB.

Further studies showed that the specific activity of hydrogenase was much higher (1.54) in the 104,000 × *g* supernatant fraction as compared with that in 104,000 × *g* pellet fraction (0.22). In comparison, 12,000 × *g* supernatant fraction and whole cells had a specific activity of 1.2 and 1.54, respectively. Total hydrogenase activity in 104,000 × *g* pellet and supernatant fractions was, respectively, 2.9 and 37.9% of that observed with 12,000 × *g* supernatant. This suggests that much of the hydrogenase activity in this organism is associated with the soluble rather than the particulate fraction.

TABLE 2. *Hydrogenase activity in cell extracts*

Component[a]	Sp act[b]
Complete	1.2
Minus MB	0.0
Minus MB + BV	0.70
Minus MB + MV	0.15
Boiled extract	0.0
Minus extract	0.0
Minus H_2, N_2 added	0.0

[a] The complete system consisted of 150 μmol of potassium phosphate (pH 7.5) and cell extract (10 mg of protein) in a total volume of 2.8 ml. Where indicated, the extract was boiled for 15 min. The side arm contained 20 μmol of MB and, where indicated, 20 μmol of BV or MV.

[b] Micromoles of H_2 consumed per 10 min/mg of protein.

The data for hydrogenase activity with different biological electron acceptors are presented in Table 3. Cell extracts obtained from cells grown in the basal medium (Table 1) gave low activity with GMN^+ or FAD^+ as the electron acceptor; however, considerably higher activity was observed when a catalytic quantity of BV was present (Table 3, part A). There was no detectable H_2 consumption with either NAD^+ or $NADP^+$ as the electron acceptor, unless a catalytic quantity of BV was present.

Since an iron sulfide moiety is the electron-carrying prosthetic group of ferredoxin in bacteria (34), ferrous sulfate was added to the basal medium used for cultivating *B. fragilis*. The results of H_2 consumption with cell extracts obtained from cells grown in basal medium supplemented with 0.005% ferrous sulfate are presented in Table 3, part B. H_2 consumption with FMN^+ or FAD^+ was considerably higher with extracts prepared from cells grown in this medium than with extracts grown in a medium without ferrous sulfate. The addition of ferredoxin from *C. pasteurianum* to the cell extracts did not show any increase in hydrogenase activity with either FMN^+ or FAD^+. There was no detectable H_2 consumption with either NAD^+ or $NADP^+$ as the electron acceptor, even with extracts of cells grown in the high-iron medium. However, there was considerable H_2 consumption with NAD^+ when a catalytic quantity of ferredoxin from *C. pasteurianum* was present. Approximately the same amount of H_2 was consumed in the reaction with NAD^+ and a catalytic quantity of ferredoxin (Table 3, part B) as compared with the reaction containing NAD^+ and BV (Table 3, part A). There was no significant H_2 consumption with $NADP^+$ as the electron acceptor in the presence of a catalytic amount of ferredoxin. These results suggest that NAD^+ may be the natural electron carrier involved in fumarate reduction by H_2 in *B. fragilis*. Attempts to isolate ferredoxin from *B. fragilis* by the procedures of Buchanan et al. (7) were not successful.

H_2-fumarate coupling activity. Macy et al. (23) have shown the reduction of fumarate with H_2 in extracts of *B. fragilis* only when hydrogenase from *C. pasteurianum* was added. Since hydrogenase activity was observed in extracts of *B. fragilis* 25285, we designed experiments to determine whether fumarate could be reduced to succinate with H_2 as the electron donor, without any exogenously added hydrogenase.

TABLE 3. *Hydrogenase activity with different biological electron acceptors*[a]

Electron acceptor	Total activity[b]	Sp act[c]
A.		
FMN^+	1.54	0.03
FAD^+	1.13	0.02
NAD^+	0.0	0.0
$NADP^+$	0.0	0.0
FMN^+ + BV	7.20	0.25
FAD^+ + BV	5.40	0.25
NAD^+ + BV	3.5	0.15
$NADP^+$ + BV	4.2	0.16
B.		
FMN^+	11.30	0.68
FAD^+	2.60	0.58
NAD^+	0.0	0.0
$NADP^+$	0.0	0.0
FMN^+ + 0.1 mg of Fd[d]	13.10	0.68
FAD^+ + 0.1 mg of Fd	11.50	0.61
NAD^+ + 0.5 mg of Fd	4.37	0.15
$NADP^+$ + 0.5 mg of Fd	0.40	0.0

[a] Reaction conditions for (A) were the same as those given in Table 2 except that 28 μmol of mercaptoethanol, 10 μmol of FMN^+, FAD^+, NAD^+, or $NADP^+$, and 1 μmol of BV were present where indicated. Reaction conditions for (B) were the same as those in (A) except that 0.1 mg or 0.5 mg of ferredoxin from *C. pasteurianum* was also present where indicated. The cell extracts used in experiments in (B) were prepared from cells grown in basal medium (Table 1) plus 0.005% ferrous sulfate.

[b] Values represent micromoles of H_2 consumed in 60 min.

[c] Micromoles of H_2 consumed per 10 min/mg of protein.

[d] Fd, Ferredoxin.

The results showed that extracts of *B. fragilis* could indeed couple fumarate reduction to succinate with H_2 as the electron donor (Table 4). The highest activity was observed with the 12,000 × *g* supernatant fraction. Considerable activity was observed with the 104,000 × *g* supernatant fraction, but the activity with the 104,000 × *g* pellet fracton was only about 42% of that observed with the 104,000 × *g* supernatant fraction. The specific activity with the combined 104,000 × *g* supernatant and pellet fractions was slightly higher than that observed with the pellet fraction alone but less than that observed with the supernatant fraction. H_2 consumed during the reaction was stoichiometric, with the fumarate reduced to succinate (results not shown). No H_2 consumption occurred when sodium fumarate was deleted from the reaction, and no fumarate reduction occurred when H_2 was replaced by N_2. A catalytic quantity of BV present in all of the reactions listed in Table 4 was essential for H_2-fumarate coupling activity. This is supported by the results presented in Table 5. No H_2 consumption occurred in the absence of BV or when MV was substituted for BV. A catalytic quantity of FAD^+, FMN^+, or ferredoxin from *C. pasteurianum* was able to substitute for BV, although the activity was low. BV was consistently the most effective electron mediator examined, although, in one experiment, ferredoxin was equally as effective. There were also considerable differences in specific activity in experiments 1 and 2 with ferredoxin as the electron mediator. Two different batches of cells were used in these experiments, and appreciable variations were routinely observed in the H_2-fumarate coupling activity in different batches of cells. H_2-fumarate coupling activity could not be demonstrated in the absence of an electron mediator either with whole cells or with broken, uncentrifuged cells.

H_2-fumarate coupling activity was low (0.29)

TABLE 4. *H_2-fumarate coupling activity in different cell fractions*[a]

Cell fraction	Sp act[b]
A. 12,100 × *g* supernatant	0.71
Minus fumarate	0.00
Minus H_2, N_2 added	0.00
B. 104,000 × *g* supernatant	0.47
C. 104,000 × *g* pellet	0.20
D. B + C	0.36

[a] Reaction conditions were the same as those given in Table 2 except that MB was deleted and 28 μmol of mercaptoethanol, 1 μmol of BV, and 5 mg of the various cell fractions were added to each cup. The side arm contained 30 μmol of sodium fumarate (pH 6.5).

[b] Micromoles of H_2 consumed per 10 min/mg of protein.

TABLE 5. *Effect of various electron mediators on the H_2-fumarate coupling activity*[a]

Electron mediator	Sp act[b]	
	Expt 1	Expt 2
None	0.0	0.0
BV	0.52	0.61
MV	0.0	0.0
FAD^+	0.34	ND[c]
FMN^+	0.20	ND
Ferredoxin	0.20	0.59

[a] Reaction conditions were the same as those given in Table 4 except that 12,100 × *g* supernatant (5 mg of protein) was used and, where indicated, 1 μmol each of MV, BV, FAD^+, and FMN^+ or 0.5 mg of ferredoxin from *C. pasteurianum* was present.

[b] Micromoles of H_2 consumed per 10 min/mg of protein.

[c] ND, not determined.

in the absence of a sulfhydryl compound. Reduced glutathione appeared to inhibit the H_2-fumarate coupling activity (0.09). The specific activity with sodium thioglycolate was 0.44, which was ca. 34% higher than that observed with a negative control. Mercaptoethanol and dithiothreitol were the most effective, with specific activities of 0.71 and 0.74, respectively. This represents an increase in activity of 59 and 61%, respectively, as compared to that observed when no sulfhydryl agent was present. No fumarate reduction occurred when H_2 was replaced by N_2, suggesting that nonenzymatic reduction of fumarate by sulfhydryl compounds did not occur.

Effects of various electron transport inhibitors on fumarate reduction. The data on the effect of different electron transport compounds on fumarate reduction are shown in Table 6. The results show that acriflavin, rotenone, HOQNO, and antimycin A significantly inhibited electron transport between H_2 and fumarate. There was no inhibition of electron transport when an equal volume of the ethanol solution, used in preparing rotenone and antimycin A, was added to the reaction. No H_2 consumption occurred when *B. fragilis* extract was deleted from the reaction, indicating that *C. pasteurianum* extract did not have any detectable fumarate reductase activity.

Effect of UV light and vitamin K compounds on the reduction of fumarate. A specific activity of 2.3 was obtained with unirradiated cell extracts that were otherwise treated exactly as the irradiated extracts. The irradiation of cell extracts with UV light resulted in a 38% inhibition of H_2 uptake with fumarate as the electron acceptor. The addition of vitamins K_1 and K_2 to the irradiated extracts did not relieve this inhibition; however, the addition of vitamin K_3 restored much of the H_2-fumarate coupling activity lost on irradiation. The addition of the same volume of alcohol (used to suspend the vitamin compounds) to the extracts did not result in any inhibition of fumarate reduction.

TABLE 6. *Effect of electron transport inhibitors on H_2-fumarate coupling activity*[a]

Inhibition[b]	Concn (M)	Sp act[c]	Inhibition (%)
None[d]		2.51	0.0
Rotenone	1.7×10^{-4}	1.65	34.0
Acriflavin	3.6×10^{-3}	1.47	41.0
HOQNO	1.7×10^{-5}	1.40	44.0
Antimycin A	1.7×10^{-5}	1.36	46.0

[a] The reaction conditions were the same as those given in footnote *a* of Table 4, except that BV was deleted and 6 mg of *C. pasteurianum* extract was added. The cell extracts of *B. fragilis* and *C. pasteurianum* were added to the side arm and tipped into the main vessel to start the reaction.

[b] The inhibitors rotenone and antimycin A were prepared in a 10% ethanol solution. All other inhibitors were soluble in distilled water.

[c] Micromoles of H_2 consumed per 10 min/mg of protein.

[d] Ethanol equal in volume to that used in preparing rotenone and antimycin A was present in this cup.

DISCUSSION

The enzyme hydrogenase has been reported in a number of anaerobic bacteria (1, 11, 16, 25, 27, 28, 30). In *Vibrio succinogenes*, a succinic acid-producing anaerobe (3, 33), and in *Desulfovibrio gigas*, a sulfate-reducing anaerobe (11), hydrogenase activity was found primarily in the particulate fraction of the cells. On the other hand, the enzyme in *C. pasteurianum* and in *C. butylicum* (25) was located in the soluble fraction of the cells. In the present investigation, a majority of the hydrogenase activity was found in the soluble fraction of *B. fragilis*. MB was the preferred electron acceptor. This was also true for the hydrogenase in *V. succinogenes* (16). In contrast, low-potential electron acceptors MV and BV were the preferred electron acceptors for the hydrogenase activity in *C. pasteurianum* and *C. butylicum* (25, 28). FMN^+, FAD^+, and MV were the preferred electron acceptors for H_2 oxidation in the methane-producing anaerobe *Methanobacterium ruminantium* (30).

Molecular H_2 serves as an electron donor for the reduction of fumarate to succinate in several organisms (11, 16, 18, 22). H_2-fumarate coupling activity has been demonstrated in the anaerobically grown facultative anaerobes *Escherichia coli* (22) and *Proteus rettgeri* (18) and in the non-carbohydrate-fermenting anaerobes *V. succinogenes* (16) and *D. gigas* (11). Unlike *V. succinogenes* (16), which had most of the H_2-fumarate coupling activity in the particulate fraction, the activity in the soluble fraction of *B. fragilis* was approximately twice that in the particulate fraction. Furthermore, ferredoxin appears to be the primary acceptor of electrons from H_2 in *V. succinogenes* (C. A. Reddy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, I27, p. 121) and in *D. gigas* (11). The present results show that BV, FMN^+, FAD^+, or ferredoxin from *C. pasteurianum* effectively mediated the transfer of electrons from H_2 to fumarate in cell extracts of *B. fragilis*. This suggests that ferredoxin or another low-potential electron carrier serves as the primary acceptor of electrons from H_2. However, attempts

to isolate ferredoxin from *B. fragilis* by previously established procedures (7) have not been successful. The activity, if any, of other low-potential electron mediators, such as flavodoxin (34) or F_{420} (30), has not been tested. That we could not show H_2-fumarate coupling activity in the absence of an electron mediator with cell extracts suggests that the electron-mediating compound in the cell either was destroyed by the fractionation methods employed or was highly labile to oxygen.

Fumarate reduction by NADH has been demonstrated in cell extracts of *B. fragilis* by Macy et al. (23). They reported that H_2 could also serve as an electron donor but only in the presence of excess hydrogenase from *C. pasteurianum* (23). In *B. fragilis* 25285, used in the present study, there was no requirement for *C. pasteurianum* hydrogenase when a catalytic amount of BV was added. Furthermore, clostridial ferredoxin could effectively substitute for BV in catalyzing fumarate reduction by H_2. The present results also show that ferredoxin mediated the transfer of electrons from H_2 to NAD^+ but not to $NADP^+$, suggesting NAD^+ involvement as an electron mediator in the H_2-fumarate ETS in *B. fragilis*. The results show that rotenone and acriflavin inhibited fumarate reduction by H_2, suggesting the involvement of a flavoprotein in H_2-fumarate ETS (12). The decrease in fumarate reduction by HOQNO, a compound known to inhibit the flow of electrons between flavoprotein and cytochrome *b*, suggests the involvement of a quinone in this system (2, 11, 19). This possibility was further confirmed by the decrease in fumarate reduction when cell extracts were irradiated with UV light. The activity was partially restored when vitamin K_3 was added to the irradiated extracts. Strong inhibition of fumarate reduction upon addition of HOQNO was also reported in *V. succinogenes* (17) and *D. gigas* (11). It has previously been shown that antimycin A significantly decreases hydrogen-fumarate coupling activity in *D. gigas* by inhibiting the flow of electrons at the site of cytochrome *b* (11). Similar inhibition of fumarate reduction was observed when antimycin A was added to *B. fragilis* extracts.

Based on these results, a scheme for the ETS involved in fumarate reduction to succinate by H_2 is proposed (Fig. 1). Hydrogenase may be coupled to NAD^+ by a low-potential electron carrier represented by X. That such a compound probably mediates the transfer of electrons from H_2 to NAD^+ is indicated by the absolute requirements for BV or ferredoxin (from *C. pasteurianum*) even when crude cell extracts were used in the assay. However, due to the lack of direct evidence, the possibility that the low-potential electron acceptor compound may mediate the transfer of electrons directly from H_2 to the flavoprotein cannot be eliminated. Our present studies with different electron transport inhibitors and previous studies (22; Reddy, M.S. thesis) indicate the involvement of NADH, flavoprotein, quinone, and cytochrome *b* in fumarate reduction by H_2. The sequence of the ETS components presented is based on what is known of ETS systems in other bacteria (32). Also, the existence of branched electron transport pathways in this organism cannot be eliminated. Therefore, it is distinctly possible that later experiments may show that the sequence of participation of different ETS components may be somewhat different than that presented here.

Indirect evidence from molar growth yield studies obtained by Macy et al. (23) suggests that adenosine 5'-triphosphate is produced when fumarate is reduced to succinate in *B. fragilis*. Phosphorylation of adenosine 5'-diphosphate to adenosine 5'-triphosphate coupled to electron transfer between H_2 and fumarate has been demonstrated in *D. gigas* (5) and in *V. succinogenes* (Reddy and Peck, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P318, p. 194). Presently, studies are being conducted to determine whether there is production of adenosine 5'-triphosphate during fumarate reduction to succinate by H_2 in *B. fragilis*.

LITERATURE CITED

1. **Akagi, J. M.** 1967. Electron carriers for the phosphoroclastic reaction of *Desulfovibrio desulfuricans*. J.

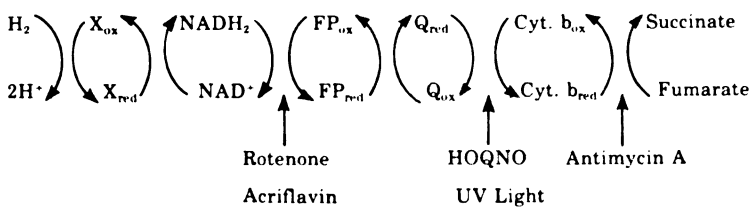

FIG. 1. *Proposed ETS in fumarate reduction to succinate by molecular hydrogen. Abbreviations: ox, oxidized; red, reduced; FP, flavoprotein; and Q, quinone.*

Biol. Chem. 242:2478–2483.
2. **Asano, A., and A. F. Brodie.** 1965. The properties of the non-phosphorylative electron transport bypass of *Mycobacterium phlei*. Biochem. Biophys. Res. Commun. 19:121–126.
3. **Aspen, A. J., and M. J. Wolin.** 1966. Solubilization and reconstitution of a particulate hydrogenase from *Vibrio succinogenes*. J. Biol. Chem. 241:4152–4156.
4. **Balows, A., R. DeHaan, V. Dowell, and L. Guze.** 1974. Anaerobic bacteria: role in disease. Charles C Thomas, Publishers, Springfield, Ill.
5. **Barton, L. L., J. LeGall, and H. D. Peck.** 1970. Phosphorylation coupled to oxidation of hydrogen with fumarate in extracts of the sulfate reducing bacterium, *Desulfovibrio gigas*. Biochem. Biophys. Res. Commun. 41:1036–1042.
6. **Brodie, A. F., M. M. Weber, and C. T. Gray.** 1957. The role of a vitamin K_1 in coupled oxidative phosphorylation. Biochim. Biophys. Acta 25:448–449.
7. **Buchanan, B. B., W. Lovenberg, and J. C. Rabinowitz.** 1963. A comparison of clostridial ferredoxins. Proc. Natl. Acad. Sci. U.S.A. 49:345–353.
8. **Caldwell, D., and M. P. Bryant.** 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. 14:794–801.
9. **Gibbons, R. J., and L. P. Engle.** 1964. Vitamin K compounds in bacteria that are obligate anaerobes. Science 146:1307–1309.
10. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum protein by means of the biuret reaction. J. Biol. Chem. 177:751–766.
11. **Hatchikian, E. C., and J. LeGall.** 1972. Evidence for the presence of a b-type cytochrome in the sulfate reducing bacterium *Desulfovibrio gigas*, and its role in the reduction of fumarate by molecular hydrogen. Biochim. Biophys. Acta 69:18–28.
12. **Hatefi, Y.** 1968. Flavoproteins of the electron transport system and the site of action of amytal, rotenone, and pericidin A. Proc. Natl. Acad. Sci. U.S.A. 60:733–740.
13. **Holdeman, L. V., and W. E. C. Moore.** 1974. Gram-negative anaerobic bacteria, p. 384–391. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergy's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
14. **Hungate, R. E.** 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. 14:1–49.
15. **Hungate, R. E.** 1970. A roll tube method for cultivation of strict anaerobes, p. 117–132. *In* J. R. Norris and D. W. Robbins (ed.), Methods in microbiology, vol. 3B. Academic Press Inc., New York.
16. **Jacobs, N. J., and M. J. Wolin.** 1963. Electron-transport system of *Vibrio succinogenes*. I. Enzymes and cytochromes of the electron-transport system. Biochim. Biophys. Acta 69:18–28.
17. **Jacobs, N. J., and M. J. Wolin.** 1963. Electron-transport system of *Vibrio succinogenes*. II. Inhibition of electron transport by 2-heptyl-4-hydroxyquinoline-N-oxide. Biochim. Biophys. Acta 69:29–39.
18. **Kroger, A.** 1974. Electron transport phosphorylation coupled to fumarate reduction in anaerobically grown *Proteus rettgeri*. Biochim. Biophys. Acta 347:273–289.
19. **Kroger, A., V. Dadak, M. Klingenberg, and F. Diemer.** 1971. On the role of quinone in bacterial electron transport. Eur. J. Biochem. 21:322–333.
20. **Loesche, W. J., S. S. Socransky, and R. J. Gibbons.** 1964. *Bacteroides oralis*, proposed new species isolated from the oral cavity of man. J. Bacteriol. 88:1329–1337.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and A. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
22. **Macy, J., H. Kulla, and G. Gottschalk.** 1976. H_2-dependent anaerobic growth of *Escherichia coli* on L-malate: succinate formation. J. Bacteriol. 125:423–428.
23. **Macy, J., I. Probst, and G. Gottschalk.** 1975. Evidence for cytochrome involvement in fumarate reduction and adenosine 5'-triphosphate synthesis by *Bacteroides fragilis* grown in the presence of hemin. J. Bacteriol. 123:436–442.
24. **Moore, W. E. C., E. P. Cato, and L. V. Holdeman.** 1969. Anaerobic bacteria of the gastrointestinal flora and their occurrences in clinical infections. J. Infect. Dis. 119:641–649.
25. **Peck, H. D., Jr., and H. Gest.** 1957. Hydrogenase of *Clostridium butylicum*. J. Bacteriol. 73:569–580.
26. **Prasad, R., V. K. Kalra, and A. F. Brodie.** 1975. Different mechanisms of energy coupling for transport of various amino acids in cells of *Mycobacterium phlei*. J. Biol. Chem. 251:2493–2498.
27. **Reddy, C. A., M. P. Bryant, and M. J. Wolin.** 1972. Ferredoxin-dependent conversion of acetaldehyde to acetate and H_2 in extracts of S organism. J. Bacteriol. 110:133–138.
28. **Shug, A. L., P. W. Wilson, D. E. Green, and H. R. Mahler.** 1954. The role of molybdenum and flavin in hydrogenase. J. Am. Chem. Soc. 76:3355–3356.
29. **Smith, L. D. S.** 1975. The pathogenic anaerobic bacteria, 2nd ed. Charles C Thomas, Publishers, Springfield, Ill.
30. **Tzeng, S. F., R. S. Wolfe, and M. P. Bryant.** 1975. Factor 420-dependent pyridine nucleotide-linked hydrogenase system of *Methanobacterium ruminantium*. J. Bacteriol. 121:184–191.
31. **Umbreit, W. W., R. H. Burris, and J. F. Stauffer.** 1964. Manometric techniques. Burgess Publishing Co., Minneapolis, Minn.
32. **White, D. C., and P. R. Sinclair.** 1971. Branched electron-transport systems in bacteria, p. 173–211. *In* A. H. Rose and J. F. Wilkinson (ed.), Advances in microbial physiology, vol. 5. Academic Press Inc., New York.
33. **Wolin, M. J., E. A. Wolin, and N. J. Jacobs.** 1961. Cytochrome-producing anaerobic vibrio, *Vibrio succinogenes*, sp. n. J. Bacteriol. 81:911–917.
34. **Yoch, D. C., and R. C. Valentine.** 1972. Ferredoxins and flavodoxins of bacteria. Annu. Rev. Microbiol. 26:139–162.

J. BACTERIOL.

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SECTION 2 (ARTICLE 2)

ADDITIONAL STUDIES ON HYDROGENASE ACTIVITY AND THE H_2-FUMARATE ELECTRON TRANSPORT SYSTEM IN *BACTEROIDES FRAGILIS*

By

Martha A. Harris and C. Adinarayana Reddy

INTRODUCTION

In the preceding section, hydrogenase activity and the H_2-fumarate coupling activities were demonstrated in the obligate anaerobe *Bacteroides fragilis*. Data obtained with a variety of electron transfer inhibitors suggested the involvement of a flavoprotein, quinone, and a b-type cytochrome in fumarate reduction with H_2 (Section 1).

Further studies on hydrogenase, fumarate reductase, and the H_2-fumarate coupling activities in *B. fragilis* are presented in this section. Evidence is obtained for the involvement of reduced nicotinamide adenine dinucleotide (NADH) as an electron donor in the reduction of fumarate to succinate. Cytochromes of the b and c types are demonstrated in cell extracts of *B. fragilis* ATCC 25285 used in the present investigation.

MATERIALS AND METHODS

Except as indicated below, methods for growth of bacteria, preparation of extracts, and enzymatic and chemical assays were as described previously (Section 1).

NADH oxidation with fumarate as the electron acceptor was followed by measuring a decrease in absorbance at 340 nm using a Varian Techtron spectrophotometer. The reaction mixture consisted of NADH, potassium phosphate (PO_4) buffer, mercapto-ethanol, and sodium fumarate (where indicated). All reactions were conducted at room temperature in Quarasil cuvettes with a 1.5 ml volume and a 1 cm

light path. The cuvettes were made anaerobic by gassing them with oxygen-free nitrogen through a tight-fitting serum stopper. The reaction was started by adding *B. fragilis* extract by a 1/2 cc syringe to cuvettes and was terminated after 6 min by the addition of 10% trichloroacetic acid (TCA).

Gas chromatographic analysis of organic acids

The stoichiometry of the H_2-fumarate coupling activity was determined by manometric and gas chromatographic techniques. H_2 consumption was estimated by manometry (Section 1). The fumarate and/or succinate in the samples were methylated, extracted into chloroform, and were estimated by a Dohrman gas chromatograph as per procedures described by Holdeman and Moore (11). The column packing was 15% CPE 2225 on Chromosorb W/AW45/60 mesh. A sample size of 14 µl was used for injection. The quantitation and identification of acids were based on the comparison of the peak heights and retention times with that of standard concentrations of acids. Each sample was injected at least twice in order to determine a mean peak height value and the reproducibility between injections was quite good.

Cytochrome spectra

Difference spectra methods described by Chance (3) were used to examine the cell extract for cytochromes. All analyses were done at room temperature with a Varian split beam recording spectrophotometer using Quarasil

cuvettes, with a 1.5 ml volume and a 1 cm light path. A few crystals of sodium dithionite were used to reduce the extract in the sample cuvette and air was used to oxidize the extract in the reference cuvette.

Extraction of hemes

The procedures described by Jacobs and Wolin (12) were used for the extraction and characterization of hemes of the b- and c-type cytochromes. The cell extract from cells grown in the basal medium (Section 1) was lyophilized. The lyophilized protein (approximately 200 mg) was mixed with 40 ml of a chloroform-methanol (2:1; vol/vol) solution and was centrifuged at 15,000 x g in a Sorvall centrifuge. The precipitated protein was washed in 40 ml of cold acetone and the protoheme was extracted by mixing the protein with 20 ml of cold acetone containing 1% 2 N HCl. This acid-acetone extraction was repeated once more. The fraction (20 ml volume) containing the protoheme was concentrated under vacuum to approximately 5 ml. The protoheme fraction was suspended in an equal volume of pyridine and 0.2 N KOH, and a difference spectrum was obtained. The residue remaining after the acid-acetone wash was suspended in an equal volume of pyridine and 0.2 N KOH and analyzed for a c-type cytochrome.

RESULTS

The effect of incubation time on hydrogenase activity

Experiments were designed to determine the effect, if any, of the age of cells at the time of harvesting on hydrogenase activity. As shown in Figure 1, the age of cells significantly influenced hydrogenase activity. There was a three-fold increase in the rate of hydrogenase activity in extracts from cells in the late stationary phase of growth as compared to that from cells in the early stationary phase of growth. Cells were therefore routinely harvested after 22 h of incubation at 37°C.

The effect of different buffers on hydrogenase activity

Results of the effect of different buffers on the hydrogenase activity are presented in Table 1. The hydrogenase activity was consistently higher in PO_4 buffer than in the other buffers examined. The activity of the enzyme was 49% and 78% lower in Tris (hydroxymethyl) aminomethane hydrochloride (Tris HCl) and N-2-hydroxy-ethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer, respectively, as compared to PO_4 buffer. It is possible that impurities in the PO_4 buffer such as iron may have indirectly contributed to the increase in hydrogenase activity with PO_4 buffer as compared to the activity with Tris HCl or HEPES buffer.

Figure 1. The effect of incubation time on hydrogenase activity in *B. fragilis*. Hydrogenase activity was measured manometrically. Each flask contained 150 µmoles of PO_4 buffer (pH 7.5), 10 µmoles of sodium thioglycollate, 20 µmoles of methylene blue (MB), and 10 mg of *B. fragilis* extract in a total volume of 2.8 ml. The reactions were done under H_2 gas at 30°C. The number of hours that cells were incubated in the basal medium (Section 1) at 37°C; 10 h (○); 14 h (□); 18 h (◇); and 22 h (△).

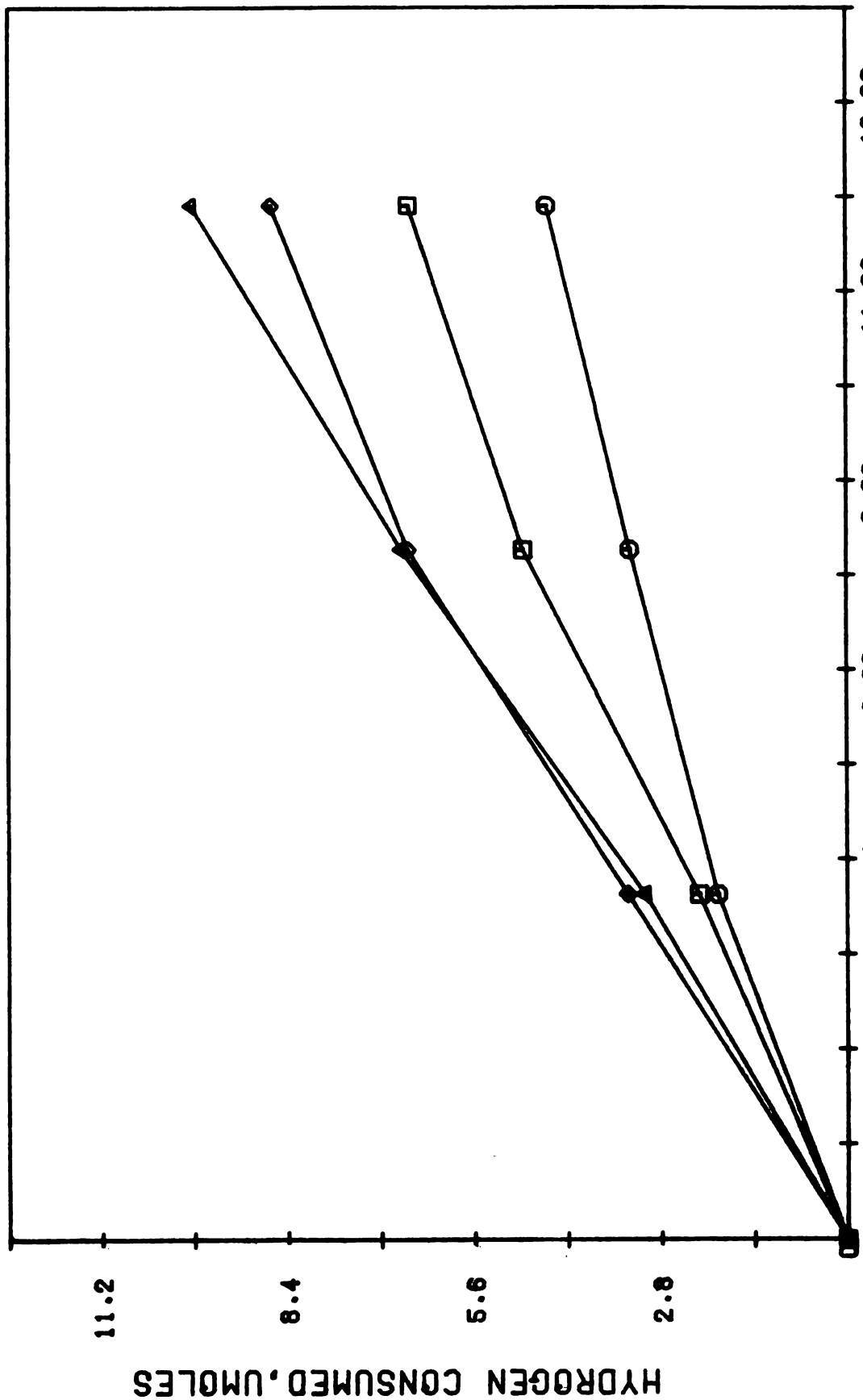
HYDROGEN CONSUMED,UMOLES
11.2
8.4
5.6
2.8
0
2.20
4.40
6.60
8.80
11.00
13.20
TIME(MIN)

Figure 1

Table 1. The effect of different buffers on hydrogenase activity[a]

Buffer	H_2 consumed (μmoles)
PO_4	8.27
HEPES	1.81
Tris HCl	4.22

[a]Each Warburg flask contained 150 μmoles of PO_4 (pH 7.5), HEPES (pH 7.6) or Tris HCl (pH 7.3), 20 μmoles of methylene blue (MB), and 10 mg of *B. fragilis* extract in a total volume of 2.8 ml. *Bacteroides fragilis* cells were suspended in the same buffer that was used to assay for hydrogenase activity. The reaction was done under H_2 gas phase at 37°C for 12 min.

The distribution of fumarate reductase activity

A high specific activity for fumarate reduction by H_2 was observed in the 12,100 x g supernatant fraction (Table 2). When that fraction was centrifuged at 104,000 x g, the fumarate reductase activity was about two times higher in the pellet (particulate) fraction as compared to that in the supernatant (soluble) fraction. It should be noted that the sum of the fumarate reductase activity in the 104,000 x g supernatant and pellet fractions was not equal to the 12,100 x g supernatant fraction. H_2 consumption did not occur in the absence of fumarate or cell extract.

The oxidation of H_2 with various carboxylic acids

The data on the ability of cell extracts to catalyze H_2 oxidation with different carboxylic acids are shown in Table 3. Appreciable H_2 consumption was detected only with fumarate or malate as the electron acceptors. No H_2 consumption occurred with succinate, oxaloacetate, or pyruvate.

The effect of different buffers on the H_2-fumarate coupling activity

The effect of different buffers on the H_2-fumarate coupling activity is presented in Table 4. The coupled hydrogenase-fumarate reductase activity was 47% and 71% lower in HEPES and Tris HCl buffers, respectively, as compared to PO_4 buffer. It is possible that impurities

Table 2. Distribution of the fumarate reductase activity in various cell fractions[a]

Cell fraction	Specific activity[b]	Total mg protein	Total activity	% Yield[c]
12,100 x g Supernatant	2.02	2000	4040	100
minus fumarate	0.00			
minus extract	0.00			
104,000 x g Supernatant	1.43	592	847	21
104,000 x g Pellet	3.27	518	1694	42

[a]The complete reaction contained 150 µmoles of PO_4 buffer (pH 7.5), 28 µmoles of mercapto-ethanol, 6 mg of *C. pasteurianum* extract, 5 mg of protein fraction in various cell fractions of *B. fragilis*, and 30 µmoles of sodium fumarate (pH 6.5), where indicated in a total volume of 2.8 ml. The cell extracts of *B. fragilis* and *C. pasteurianum* were added to the sidearm and tipped into the main vessel to start the reaction.

[b]µmoles of H_2 consumed/10 min/mg of protein.

[c] $\frac{\text{Total activity in a given fraction}}{\text{Total activity in 12,100 x g supernatant}} \times 100$

Table 3. Hydrogen oxidation with various carboxylic acids as electron acceptors

Substrate	Total activity[b]
Fumarate	18.5
Malate	15.1
Succinate	0.0
Oxaloacetate	0.8
Pyruvate	0.0
None	0.0

[a]The contents of the reactions were as described for the complete reaction in Table 2, except that 30 μmoles of the respective carboxylic acid was added. The 12,100 x g supernatant fraction (5 mg of protein) was added to each reaction.

[b]The μmoles of H_2 consumed/12 min.

Table 4. The effect of different buffers on the H_2-fumarate coupling activity[a]

Buffer	Total activity[b]
PO_4	10.64
HEPES	5.68
Tris HCl	3.05

[a]Reaction conditions were the same as those given in Table 2, except that 6 mg of *C. pasteurianum* extract was deleted and 1 μmole of benzyl viologen (BV) was added, and 150 μmoles of PO_4 (pH 7.5), HEPES (pH 7.6) or Tris HCl (pH 7.3) was present.

[b]μmoles of H_2 consumed/hr.

in the PO_4 buffer such as iron may have accounted for the higher H_2-fumarate coupling activity observed with PO_4 buffer as compared to the activity with Tris HCl or HEPES buffer.

Stoichiometry of the H_2-fumarate reaction

The partial stoichiometry of the H_2-fumarate reaction is presented in Table 5. Fumarate added at the beginning of the reaction was quantitatively recovered as fumarate and succinate at the end of the reaction. Carbon dioxide was not evolved during the reaction, which suggested that decarboxylation of succinate to propionate or dismutation of fumarate to succinate, acetate, and CO_2 did not occur.

The oxidation of NADH coupled to fumarate reduction

The oxidation of NADH by *B. fragilis* extract with fumarate as the electron acceptor was determined spectrally by observing a decrease in absorbance at 340 nm (Figure 2). All reaction components except fumarate and *B. fragilis* extract were added to the cuvettes and equilibrated for 2 min under N_2. When fumarate was added to the experimental cuvette, there was a slight decrease in absorbance which leveled off in 2 min. Subsequent addition of *B. fragilis* extract to this cuvette resulted in a sharp decrease in absorbance with a specific activity for NADH oxidation of 0.184 μmole/min/mg of protein. When *B. fragilis* extract was added to the control cuvette, there was an increase in absorbance which leveled off in 2 min

Table 5. Partial stoichiometry of the H_2-fumarate reaction[a]

Determination	Quantity (μmoles)
H_2 oxidized[b]	11.0
Fumarate added[c]	15.0
Fumarate remaining[c]	6.0
Succinate formed[c]	8.0
CO_2 produced[d]	0.0

[a]The reaction conditions were the same as those given in Table 4, except that 20 μmoles of fumarate was added.

[b]The μmoles of H_2 consumed/60 min (determined manometrically).

[c]Determined gas chromatographically.

[d]Determined manometrically by the difference in the amount of gas consumed between one vessel with 6 M KOH on a fluted filter paper in the centerwell and a second vessel which contained H_2O in place of KOH.

Figure 2. The oxidation of NADH coupled to fumarate reduction (□) and endogenous NADH oxidation in controls without fumarate (○) in extracts of *B. fragilis* were followed at 340 nm at room temperature. The anaerobic cuvettes contained 150 µmoles of PO_4 buffer, 15 µmoles of mercapto-ethanol, 0.4 µmoles of NADH, 5 mg of *B. fragilis* extract, and 30 µmoles of sodium fumarate (pH 6.5), where indicated, in a final volume of 1.5 ml. The addition of cell extract initially caused an increase in absorbance in the cuvettes with NADH and fumarate (□), but the absorbance decreased after approximately 20 sec. The gas phase was N_2.

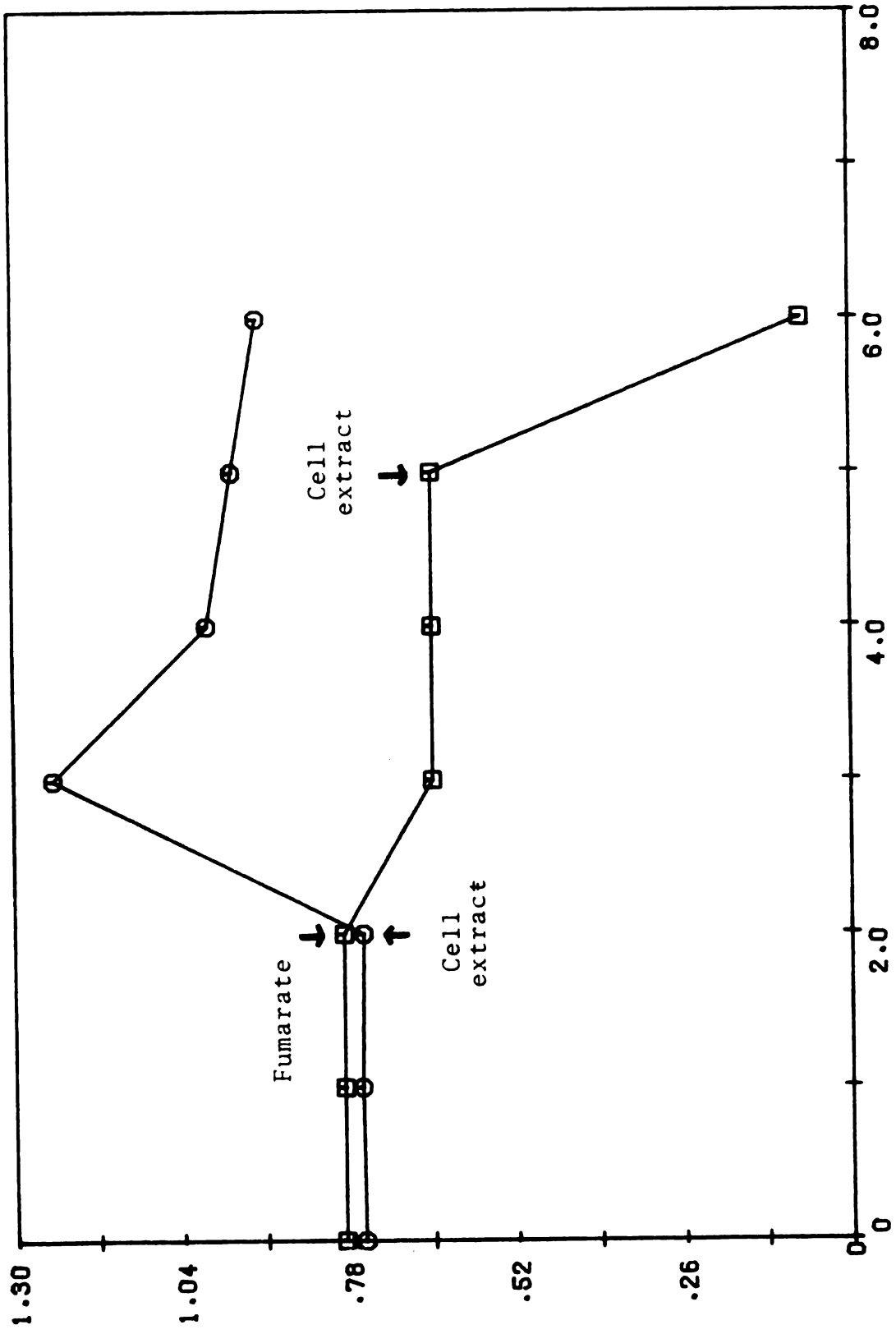
ABSORBANCE(340 NANOMETERS)
1.30
1.04
.78
.52
.26
0
2.0
4.0
6.0
8.0
TIME(MIN)
Fumarate
Cell extract
Cell extract

Figure 2

(Figure 2). Endogenous NADH oxidation in this control cuvette without fumarate occurred with a specific activity of 0.035 μmole/min/mg of protein. Therefore, the corrected specific activity for NADH oxidation coupled to fumarate reduction in the presence of *B. fragilis* was 0.149 μmole/min/mg of protein.

Cytochrome spectra

Previous studies by Reddy (M.S. thesis) and Macy et al. (15) showed that certain strains of *B. fragilis* contained cytochrome b, but it was not known whether or not the strain of *B. fragilis* used in this study contained cytochromes. The inhibition of fumarate reduction by the electron transfer inhibitor antimycin A suggested the involvement of a b-type cytochrome in the H_2-fumarate electron transport system in *B. fragilis* (Section 1). Experiments were done, therefore, to determine the presence of a b-type cytochrome in the strain of *B. fragilis* used in the present investigation. The dithionite-reduced versus air-oxidized difference spectra of the 12,100 x g supernatant, 104,000 x g supernatant, and 104,000 x g pellet fractions are shown in Figures 3, 4 and 5. All fractions gave absorption maxima at 560, 530, and 430 nm, which are indicative of a b-type cytochrome. No other cytochromes were detectable, but the presence of other cytochromes cannot be ruled out. A pyridine hemochromogen of the acid-acetone extract of *B. fragilis* had peaks at 556 and 524 nm in the visible region. This was indicative

Figure 3. Dithionite-reduced versus air oxidized difference spectrum of the 12,100 x g supernatant fraction (8 mg of protein per ml) of *B. fragilis* (ATCC 25285) (———); air oxidized versus air oxidized (- - - -).

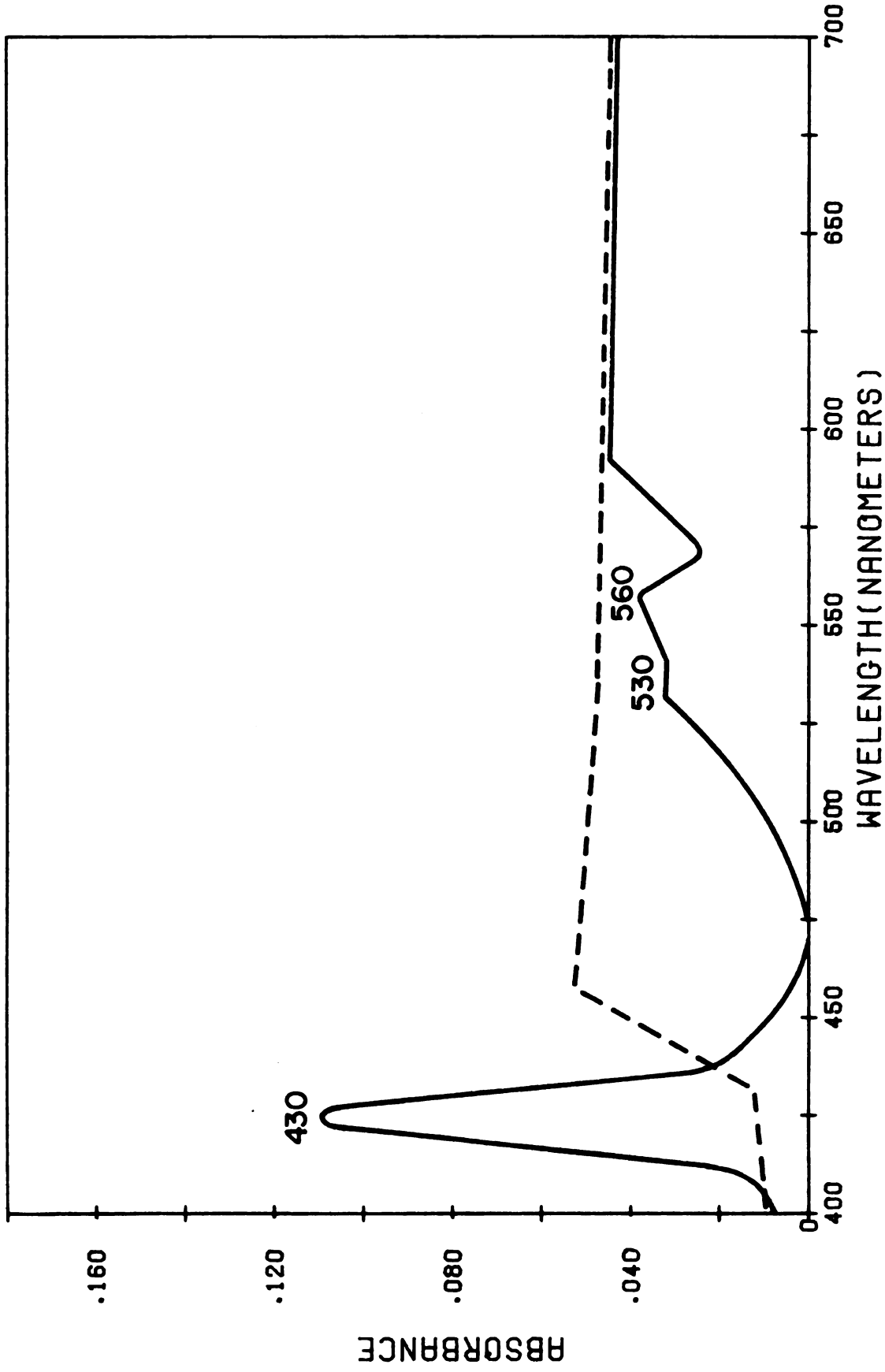
ABSORBANCE
.160
.120
.080
.040
0
400
450
500
550
600
650
700
WAVELENGTH(NANOMETERS)
430
530
560

Figure 3

Figure 4. Dithionite-reduced versus air oxidized difference spectrum of the 104,000 x g supernatant fraction (20 mg of protein per ml) of *B. fragilis* (———); air oxidized versus air oxidized (- - - -).

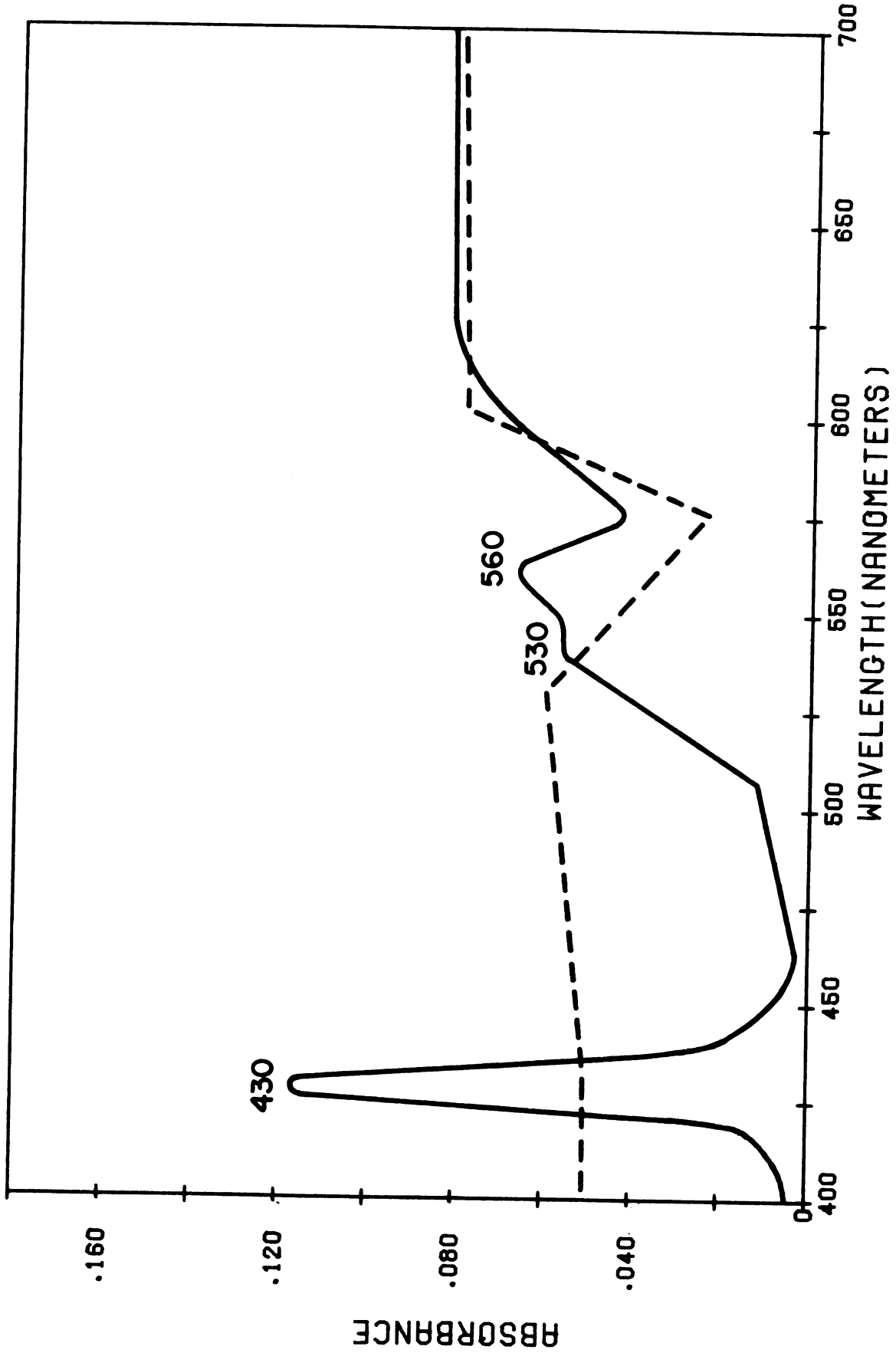
ABSORBANCE
.160
.120
.080
.040
0
400
450
500
550
600
650
700
WAVELENGTH(NANOMETERS)
430
530
560

Figure 4

Figure 5. Dithionite-reduced versus air oxidized difference spectrum of the 104,000 x g pellet fraction (3.8 mg of protein per ml) of *B. fragilis* (———); air oxidized versus air oxidized (– – – –).

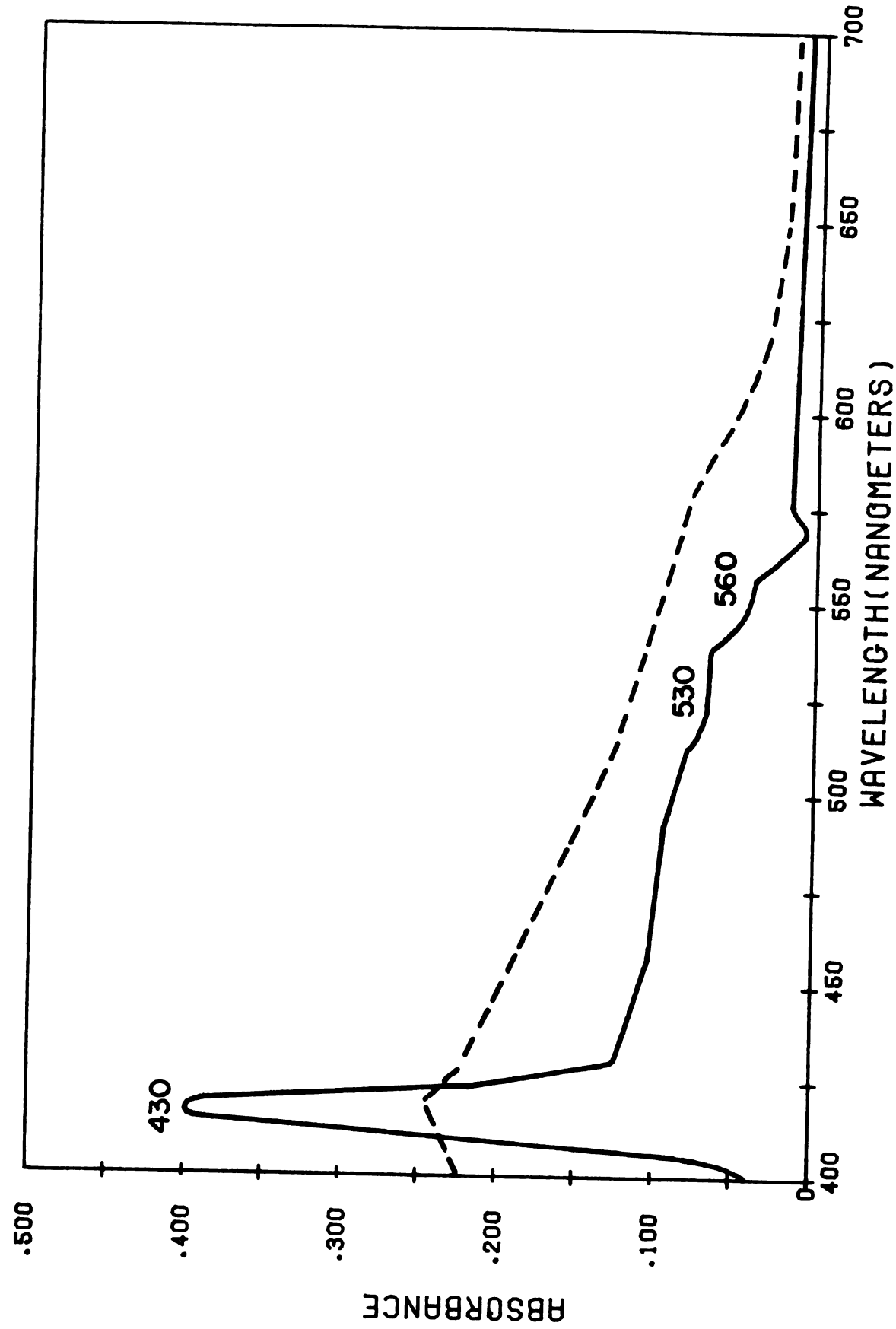
ABSORBANCE
.500
.400
.300
.200
.100
0
400
450
500
550
600
650
700
WAVELENGTH(NANOMETERS)
430
530
560

Figure 5

of a protoheme which is known to be the prosthetic group of the b-type cytochromes in general (12).

In studies by Macy et al. (15), a small absorption maxima was detected at 554 nm in cell extracts of *B. fragilis* when a liquid nitrogen difference spectrum was obtained. They suggested that a c-type cytochrome may be present in *B. fragilis* (15). In this study, there was no indication of a c-type cytochrome from the difference spectra at room temperature (Figures 3, 4 and 5). However, a pyridine hemochromogen was made of the residue remaining after the acid-acetone extraction of cell extracts (Figure 6). The dithionite-reduced versus air-oxidized difference spectrum of this residue had absorption maxima at 552, 520, and 414 nm, which are characteristic of a c-type cytochrome. Apparently the c-type cytochrome was completely masked by the b-type cytochrome in the different cell fractions.

DISCUSSION

Many chemoorganotrophic bacteria are thought to dispose of excess electrons by reducing protons to molecular hydrogen by the enzyme hydrogenase (8,9). Hydrogenase activity has been demonstrated in whole cells and cell extracts of *B. fragilis* (Section 1). Further results showed that hydrogenase activity was higher in cells in the late stationary phase of growth as compared to those in the early stationary phase of growth. Potassium phosphate buffer (pH 7.0-7.6), used by several previous

Figure 6. Dithionite-reduced versus air oxidized difference spectrum of a pyridine hemochrome from the residue of the acid-acetone extract of *B. fragilis*. The residue of 94 mg of HCl-acetone extracted cell-protein was suspended in 7 ml of pyridine-KOH (———); air oxidized versus air oxidized (----).

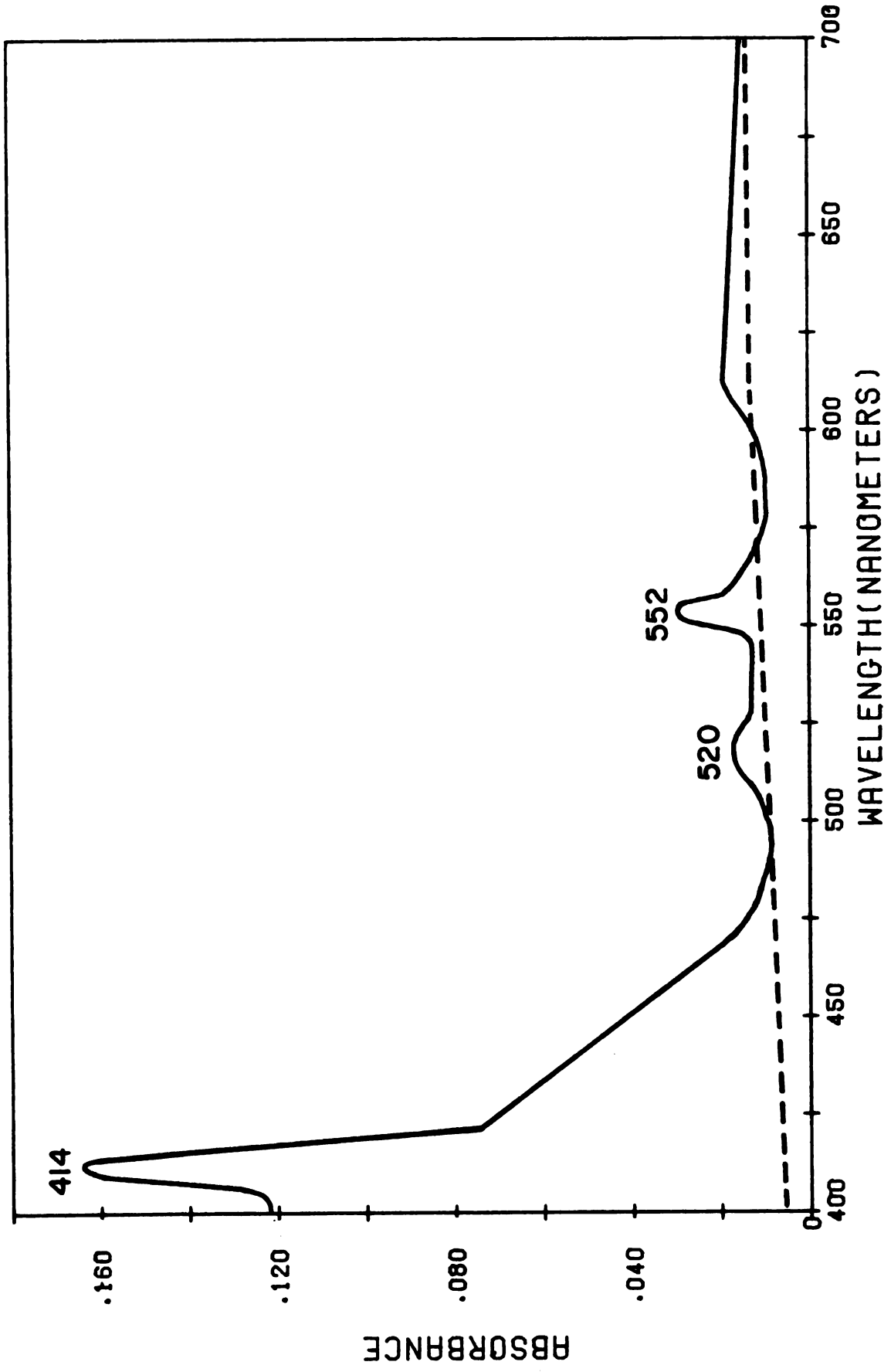
ABSORBANCE
.160
.120
.080
.040
0
400
450
500
550
600
650
700
WAVELENGTH(NANOMETERS)
414
520
552

Figure 6

investigators for demonstrating hydrogenase activity in several microorganisms (10,12,14,17), appeared to be better than Tris HCl or HEPES buffer for the hydrogenase activity. It is certainly possible that the impurities such as iron in the PO_4 buffer may have contributed to the increase in hydrogenase activity with PO_4 buffer as compared to the activity with Tris HCl or HEPES buffer. The slight variance in the pH values of the different buffers may have also been a factor in determining the rate of the hydrogenase activity.

Macy and her colleagues (15) demonstrated H_2-fumarate coupling activity in *B. fragilis* but only in the presence of *Clostridium pasteurianum* extract and methyl viologen (MV). *Clostridium pasteurianum* extract apparently served as a source of hydrogenase and other essential cofactors and MV served as a required electron mediator (15). In the present investigation, *C. pasteurianum* extract was not required for activity. In a few experiments, it was used to replace BV, FMN, or FAD, which are required for H_2-fumarate coupling activity (Section 1). Hydrogenase activity was readily demonstrated in the strain of *B. fragilis* used in this study, while Macy et al. (15) could not detect hydrogenase activity in their strain. The H_2-fumarate system in the study by Macy et al. (15) may therefore be considered artificial since they had to use *C. pasteurianum* extract and MV. In such an artificial system, MV may shunt the electrons around the natural carriers in the electron transport chain and the results

may not truly represent the events that occur in a natural electron flow.

The results of the present investigation indicated that a majority (42%) of the fumarate reductase activity in *B. fragilis* was located in the particulate fraction (104,000 x g pellet) of the cell. The 104,000 x g supernatant (soluble fraction) contained approximately 21% of the total fumarate reductase activity. Similar results were obtained by Jacobs and Wolin for the location of fumarate reductase in *Vibrio succinogenes* (12). They observed 3 times more fumarate reductase activity in the 144,000 x g pellet than the 144,000 x g supernatant fraction (12). The majority of the fumarate reductase activity in *Streptococcus faecalis* and *Desulfovibrio gigas* were generally associated with large particles also (1,2,7).

It is probable that multiple hydrogenases may be present in *B. fragilis*. From data previously shown (Section 1), the majority of the hydrogenase activity, 38%, was located in the soluble fraction of the cell. Only 2.9% of the hydrogenase activity was associated with the 104,000 x g pellet fraction. Yet, H_2-fumarate coupling activity was demonstrated in the 104,000 x g pellet fraction. It may be possible that one hydrogenase in *B. fragilis* is soluble and a second hydrogenase is located in the particulate fraction and is involved exclusively in the H_2-fumarate electron transport system.

Of the various carboxylic acids examined, H_2 consumption was observed only with fumarate and malate as

electron acceptors in the present study. H_2 oxidation with malate suggested that malate was initially metabolized to fumarate by the fumarase in cell extracts and fumarate then accepted the electrons from H_2. Fumarase activity has been detected in *B. fragilis* by Macy et al. (15). Similarly, C. A. Reddy (M.S. thesis, University of Illinois, 1967) showed that the addition of malate, fumarate, or oxaloacetate resulted in the oxidation of endogenously reduced cytochrome b in whole cells of *B. fragilis*. It was interesting that H_2 was not oxidized in the presence of oxaloacetate in the strain used in this study in view of the fact that Reddy (M.S. thesis) observed that oxaloacetate oxidized the reduced b-type cytochrome in other strains of *B. fragilis*. Malate dehydrogenase, the enzyme that reduces oxaloacetate to malate, may be subject to catabolite repression from the fumarate added to the growth medium.

In this study, fumarate was shown to be reduced to succinate in the presence of H_2. The fumarate added initially was quantitatively recovered as fumarate and succinate at the end of the reaction. There was not a perfect stoichiometry between the quantity of H_2 consumed and the quantity of succinate formed in the reaction. This discrepancy is probably due to the lack of sensitivity of the gas chromatograph to low levels of the acids.

The results of the present study in conjunction with those in Section 1 suggested that NAD^+ may be a component

in the H_2-fumarate electron transport system in *B. fragilis*. The results presented in the preceding section indicated that NAD^+ was reduced by H_2 in the presence of *B. fragilis* extract and a catalytic quantity of ferredoxin from *C. pasteurianum*, or BV. Contrary to findings concerning the H_2-fumarate complex in *B. fragilis*, Jacobs and Wolin (12) and Barton et al. (1,2) reported that pyridine nucleotides were not involved in the hydrogen-fumarate electron transport systems in *V. succinogenes* and *D. gigas*, respectively.

Cytochromes have been shown to be involved in the reduction of fumarate by H_2 or reduced pyridine nucleotides in many organisms (4,5,6,10,12,13,16,18,19); cytochrome-free *S. faecalis*, which reduces fumarate with NADH, is an exception, however (7). The involvement of the b-type cytochrome has been reported for fumarate reduction in *D. gigas* (10), *V. succinogenes* (13), *Escherichia coli* (16), *Selenomonas ruminantium* (6), *Anaerovibrio lipolytica* (6), *Propionibacterium freudenreichii* (5), and *Propionibacterium pentosaceum* (5). Cytochrome b has been demonstrated in several *Bacteroides* species including *B. ruminicola* (19), *B. oralis*, *B. succinogenes* (C. A. Reddy, M.S. thesis), and *B. fragilis* (C. A. Reddy, M.S. thesis; 15). A c-type cytochrome detected in *Bacteroides melaninogenicus* may be linked to fumarate reduction by NADH (18). Cytochromes of the b and c types were demonstrated in the strain of *B. fragilis* used in the present study. At the present time, the role

of the c-type cytochrome, if any, in fumarate reduction is not known.

The results in this study in conjunction with those in Section 1 suggested that fumarate is reduced to succinate in *B. fragilis* via an anaerobic electron transport system. The apparently oxygen labile low potential electron carrier in *B. fragilis* may be a protein with a flavin (FAD or FMN) prosthetic group. This hypothesis is supported by the observations that FAD or FMN accepted electrons from H_2 and transferred electrons from H_2 to fumarate in *B. fragilis* (Section 1). The oxidation-reduction potential of the low potential carrier may be similar to that of benzyl viologen (-350 millivolts), since benzyl viologen but not methyl viologen (-440 millivolts) could serve as an electron mediator in the H_2-fumarate electron transport system. These observations were again consistent with the idea that the low potential electron carrier may be a flavodoxin-like protein since the oxidation-reduction potential of flavodoxin from other anaerobic organisms has been similar to that of BV (20). The anaerobic electron transport system involved in the transfer of electrons from H_2 to fumarate in *B. fragilis* may consist of a low potential flavodoxin-like protein, NAD^+, flavoprotein, quinone, and cytochrome b (Section 1, Figure 1).

LITERATURE CITED

1. Barton, L. L., J. LeGall, and H. D. Peck, Jr. 1970. Phosphorylation coupled to oxidation of hydrogen with fumarate in extracts of the sulfate reducing bacterium *Desulfovibrio gigas*. Biochem. Biophys. Res. Commun. 41:1036-1042.

2. Barton, L. L., J. LeGall, and H. D. Peck, Jr. 1972. Oxidative phosphorylation in the obligate anaerobe, *Desulfovibrio gigas*. p. 33-51. *Horizons of Bioenergetics*.

3. Chance, B. 1954. Spectrophotometry of intracellular respiratory pigments. Science 120:767-776.

4. DerVartanian, D. V., and J. LeGall. 1974. A monomolecular electron transfer chain: Structure and function of cytochrome c_3. Biochim. Biophys. Acta 346:79-99.

5. DeVries, W., W. M. C. van Wijck-Kapteyn, and A. H. Stouthamer. 1973. Generation of ATP during cytochrome-linked anaerobic electron transport in propionic acid bacteria. J. Gen. Microbiol. 76:31-41.

6. DeVries, W., W. M. C. van Wijck-Kapteyn, and S. K. H. Oosterhuis. 1974. The presence and function of cytochromes in *Selenomonas ruminantium*, *Anaerovibrio lipolytica*, and *Veillonella alcalescens*. J. Gen. Microbiol. 81:69-78.

7. Faust, P. J., and P. J. Vandemark. 1970. Phosphorylation coupled to NADH oxidation with fumarate in *Streptococcus faecalis* 10C1. Arch. Biochem. Biophys. 137:392-398.

8. Gest, H. 1954. Oxidation and evolution of molecular hydrogen by microorganisms. Bacteriol. Rev. 18:43-73.

9. Gray, C. T. and H. Gest. 1965. Biological formation of molecular hydrogen. Science 148:186-192.

10. Hatchikian, E. C., and J. LeGall. 1972. Evidence for the presence of a b-type cytochrome in the sulfate reducing bacterium *Desulfovibrio gigas*, and its role in the reduction of fumarate by molecular hydrogen. Biochim. Biophys. Acta 267:479-484.

11. Holdeman, L. V., and W. E. C. Moore (eds.). 1973. *Anaerobe Laboratory Manual*, 2nd ed. VPI Anaerobe Laboratory, Blacksburg, Virginia.

12. Jacobs, N. J., and M. J. Wolin. 1963. Electron transport system of *Vibrio succinogenes*. I. Enzymes and cytochromes of the electron transport system. Biochim. Biophys. Acta 69:18-28.

13. Jacobs, N. J., and M. J. Wolin. 1963. Electron transport system of *Vibrio succinogenes*. II. Inhibition of electron transport by 2-heptyl-4-hydroxyquinoline-N-oxide. Biochim. Biophys. Acta 69:29-39.

14. Joyner, A. E., W. T. Winter, and D. M. Godbout. 1977. Studies on some characteristics of hydrogen production in cell-free extracts of rumen anaerobic bacteria. Can. J. Microbiol. 23:346-353.

15. Macy, J., I. Probst, and G. Gottschalk. 1975. Evidence for cytochrome involvement in fumarate reduction and adenosine 5'triphosphate synthesis by *Bacteroides fragilis* grown in the presence of hemin. J. Bacteriol. 123:436-442.

16. Macy, J., H. Kulla, and G. Gottschalk. 1976. H_2-dependent anaerobic growth of *Escherichia coli* on L-malate:succinate formation. J. Bacteriol. 125: 423-428.

17. Peck, H. D., Jr., and H. Gest. 1967. Hydrogenase of *Clostridium butylicum*. J. Bacteriol. 73:569-580.

18. Rizza, V., P. R. Sinclair, D. C. White, and P. R. Courant. 1968. Electron transport system of the protoheme-requiring anaerobe *Bacteroides melaninogenicus*. J. Bacteriol. 96:665-671.

19. White, D. C., M. P. Bryant, and D. Caldwell. 1962. Cytochrome linked fermentation in *Bacteroides ruminicola*. J. Bacteriol. 84:822-828.

20. Youch, D. C., and R. C. Valentine. 1972. Ferredoxins and flavodoxins of bacteria. Annu. Rev. Microbiol. 26:139-162.

SECTION 3 (ARTICLE 3)

ATTEMPTS TO DEMONSTRATE ADENOSINE 5'-TRIPHOSPHATE PRODUCTION COUPLED TO FUMARATE REDUCTION BY H_2 IN *BACTEROIDES FRAGILIS*

By

Martha A. Harris and C. Adinarayana Reddy

INTRODUCTION

Previous investigators have demonstrated phosphorylation of adenosine 5'-diphosphate (ADP) to adenosine 5'-triphosphate (ATP) in several organisms that reduce fumarate to succinate with either H_2 or reduced nicotinamide adenine dinucleotide (NADH) (1,2,11,21, C. A. Reddy and H. D. Peck, Jr. Abst. Annu. Meet. Amer. Soc. Microbiol., 1973, 194, C. A. Reddy, 1973, Rumen Function Conference, Chicago, unpublished). Many and her colleagues (21) have presented indirect evidence to suggest that additional ATP is produced when fumarate is reduced to succinate in *Bacteroides fragilis*. Recently, Harris and Reddy (15) have demonstrated a H_2-fumarate electron transport system in *B. fragilis*. Therefore, the primary objective of this investigation was to attempt to demonstrate more direct evidence for ATP production during fumarate reduction by H_2 in cell extracts of *B. fragilis*.

MATERIALS AND METHODS

Except as indicated below, methods for growth of bacteria, preparation of extracts, and enzymatic and chemical analysis were as described previously (15).

In all experiments designed to demonstrate ATP formation, the cells were grown in the basal medium (15) supplemented with 0.005% ferrous sulfate, 0.001% sodium molybdate, cobalt chloride, manganese chloride, and sodium selenate, and 0.5% of the B vitamin solution of

Varel and Bryant (31), which also contained 0.01% flavin mono- and adenine dinucleotides (FMN) and (FAD).

Molar growth yields were calculated from dry weight determinations of cells grown on media with varying glucose and fumarate concentrations. Specific components of each medium are given in the results section. Cells were grown in 400 ml of each medium in a 500 ml round bottom flask. The inoculum for each medium was prepared as previously described (15) and duplicate flasks of each medium were inoculated. The cells were harvested batch-wise in a Sorvall RC-5 refrigerated centrifuge and washed twice with distilled H_2O. The washed cells were dried to constant weight at 100°C. Unfermented glucose in the various media was determined as described by Dubois et al. (10) and Johnson et al. (19) as modified by Montgomery et al. (24).

Gas chromatographic analysis of organic acids and gas

Volatile and non-volatile acids were extracted and analyzed, from cells grown in Hungate anaerobic tubes containing 10 ml of medium for 22 h, in a Dohrmann gas chromatograph as described by Holdeman and Moore (17).

A Hewlett Packard 5700 A gas chromatograph was used to detect H_2 production. The gas in the head space of the tube, 0.2 cc, was used for injection. The oven and detector temperatures were 50°C and 100°C, respectively. The flow rate of the carrier gas (argon) was 60 cc/min.

The quantitation and identification of acids and gases were based on the comparison of peak heights and retention times with that of standard concentrations of acids and gases. Each sample was injected at least twice in order to determine a mean peak height value.

Adenosine triphosphatase (ATPase) assay

The procedures for assaying ATPase activity were similar to those described by Pullman et al. (25). The assay mixture contained 25 µmoles of N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES) buffer, pH 7.6, 1 µmole of magnesium chloride, 2 µmoles of ethylene diaminetetraacetic acid (EDTA), 7 µmoles of phosphoenol-pyruvate (PEP), 7 µmoles of ATP, 14 µg of pyruvate kinase, and 5 mg of *B. fragilis* extract in a volume of 2.8 ml. All reactions were done at 37°C for 20 min and stopped by adding 1 ml of 10% TCA. After centrifugation, the inorganic phosphate present was determined by the procedures of Fiske and Subbarow (12) as modified by Clark (6).

ATP determination by the hexokinase assay

The hexokinase assay (28) was one method employed to determine if phosphorylation of ADP to ATP is coupled to electron transfer from H_2 to fumarate in cell extracts of *B. fragilis*. Hexokinase catalyzes the production of D-glucose-6-phosphate and ADP from D-glucose and ATP (28).

The consumption of H_2 was measured by standard manometric techniques as described previously (30). Inorganic phosphate, ADP, and 2-deoxy-D-glucose were present in one

sidearm. An analog of glucose, 2-deoxy-D-glucose, was used in the assay since *B. fragilis* metabolizes glucose but not 2-deoxy-D-glucose. Fumarate and the uncoupler, pentachlorophenol (PCP) (suspended in ethanol), were present in the second sidearm, where indicated. *Bacteroides fragilis* and *Clostridium pasteurianum* extracts, HEPES buffer, FAD or FMN, magnesium chloride ($MgCl_2$), sodium fluoride (NaF), mercapto-ethanol, hexokinase, and bovine serum albumin (BSA). The BSA used in the assay was treated with norite by the method of Chen (5), for the purpose of removing fatty acid impurities. The center well contained 0.2 ml of freshly prepared 2-% KOH absorbed onto a fluted filter paper. The reaction was started by tipping the components from the sidearm into the main vessel.

The concentration of inorganic phosphate and that remaining after the completion of the reaction were determined in each cup (6,12). A decrease in the inorganic phosphate concentration after completion of the reaction in the experimental cup as compared to that in the control cup containing the uncoupler PCP or in the presence of a N_2 instead of a H_2 gas phase was used as an index of the Pi esterified. The concentration of inorganic phosphate in the sample was calculated by comparing the absorbance values to those of a standard containing 0.1-0.6 μmoles of inorganic phosphate treated the same way as the sample (6).

The formation of 2-deoxy-D-glucose-6-phosphate, a product of the hexokinase assay, was measured spectrophotometrically by taking a known volume of the completed reaction from the manometric assay and observing an increase in absorbance at 340 nm in the presence of $NADP^+$ and glucose-6-phosphate dehydrogenase (8).

ATP determination by the modified luciferin-luciferase assay

The modified luciferin-luciferase assay (7,23,26,27,28) was also used to determine if ATP is produced in fumarate reduction by H_2 in cell extracts of *B. fragilis*.

H_2 consumption was measured manometrically as described previously (15). The components of the reaction mixture for the luciferase assay were the same as those for the hexokinase assay except 2-deoxy-D-glucose and hexokinase were deleted and 2.5 µmoles of ADP and 10 µmoles of inorganic phosphate were added.

The perchloric acid method described by Cole et al. (7) and Roberton and Wolfe (26) was used to extract ATP from the unknown samples and the ATP standards. A 2-ml sample was mixed with 0.5 ml of cold 30% (v/v) perchloric acid (7). After standing for 3 h at 4°C, the suspension was neutralized with 4 N KOH and centrifuged in a clinical centrifuge (26). The supernatant was used immediately to determine the presence of ATP or stored at -20°C (26).

Firefly lantern extract in arsenate-magnesium buffer (Sigma Chemical Co., St. Louis, MO) was suspended in 5 ml of H_2O and activated by procedures of Kimmich et al. (20).

The suspension was mixed with 80 mg of calcium phosphate (tribasic) and kept at room temperature for 10 min. Following centrifugation at 400 x g for 2 min, the supernatant was treated with $Ca_3(PO_4)_2$ again and allowed to stand at room temperature for an additional 10 min. The suspension was then centrifuged at 18,000 x g for 10 min and the supernatant was used as the activated firefly lantern extract.

The ATP present in the ATP standards and unknown samples was assayed in a Searle Delta 300 liquid scintillation counter operated at ambient temperature. Standard scintillation vials contained 2.7 ml of 5 mM sodium arsenate, 4 mM $MgSO_4$, and 20 mM glycyl-glycine, pH 8.0, 0.15 ml of the sample, and 0.15 ml of the activated firefly lantern extract to the scintillation vials. Approximately 10 sec after adding and mixing the firefly lantern extract, the sample was counted for 4 consecutive periods of 30 sec each.

Chemicals and enzymes

The firefly lantern extract, BV, FMN, NAD^+, NADH, ATP, ADP, 2-deoxy-D-glucose, and 2-deoxy-D-glucose-6-phosphate were purchased from the Sigma Chemical Co. (St. Louis, MO). Glucose-6-phosphate dehydrogenase, hexokinase, and pyruvate kinase were purchased from the Sigma Chemical Co. (St. Louis, MO). Methylene blue (MB) was purchased from the Allied Chemical Co. (New York, NY). All other chemicals were of reagent grade or higher quality.

RESULTS

Molar growth yields as affected by various fumarate concentrations

From the data presented previously (15, Section 2), the H_2-fumarate coupling activity was demonstrated in cell extracts of *B. fragilis*. The calculated free energy change for the oxidation of H_2 with fumarate is more than sufficient to account for the formation of at least one ATP. If indeed fumarate is involved in energy generation in *B. fragilis*, then this should be reflected by an increase in cell yield of cells grown with fumarate present in the medium. Experiments were designed to determine if additional energy for growth is available when fumarate is reduced to succinate. Table 1 shows the effect of various fumarate concentrations on the growth of *B. fragilis*. The molar growth yields in Med. #1 and #2, which contained substrate quantities of glucose, were essentially the same regardless of the additional fumarate in Med. #1. Media #3, #4, and #5 had 0.05% glucose and various quantities of fumarate present. There was an increase of 25 g of cells per mole of glucose in Med. #3 as compared to Med. #5. These results suggested that fumarate reduction to succinate may be a source of additional ATP when the glucose concentration is limiting in the medium.

Table 1. Molar growth yields as affected by fumarate concentration in the medium

Medium[a]	% Glucose	% Fumarate	Dry wt (g)	Ym[b]
1	0.400	0.20	0.380	44.19
2	0.400	0.00	0.399	46.39
3	0.050	0.20	0.111	123.30
4	0.050	0.05	0.105	116.10
5	0.050	0.00	0.089	98.80

[a]Med. #1 is the basal medium described previously (15). Media 2-5 are the same as Med. #1 except for the varying glucose and fumarate concentrations.

[b]Ym = grams dry weight/mole of glucose.

The effect of added fumarate on acids and gas production

From the results shown in Table 1, the molar growth yields from cells grown with substrate quantities of glucose (Med. #1 and #2) were essentially the same regardless of the additional fumarate in Med. #1. Experiments were then designed to determine the effect, if any, of added fumarate on acid end products produced by cells on a medium that contained fumarate (Med. #1) and a medium that contained no fumarate (Med. #2). The results (Table 2) showed that in Med. #1, approximately equal quantities of acetate and succinate were produced while in Med. #2, there was a four-fold increase in succinate as compared to acetate production. The lower quantities of H_2 detected in Med. #1 as compared to that in Med. #2 may be due to the use of H_2 in Med. #1 for the reduction of additional fumarate to succinate.

ATPase activity

ATPase activity was examined in cell extracts of *B. fragilis* since it is usually considered to be an expression of the same enzyme that is involved in the production of ATP. As shown in Table 3, A Mg^{2+}-stimulated ATPase activity was demonstrated. The addition of NaF and the deletions of PEP and pyruvate kinase caused a 30% and 45% decrease in the ATPase activity, respectively. EDTA had no apparent effect on the enzymatic activity. Cell membrane, ATP, and Mg^{++} were essential components

Table 2. The effects of exogenous fumarate on hydrogen, acetate, and succinate production in *B. fragilis*

Medium[a]	H_2 µmoles[b]	Acetate µmoles[c]	Succinate µmoles[c]
Med. #1	2.90	127	155
Med. #2	6.34	25	102

[a]Med. #1 is the same as the basal medium described previously (15). Med. #2 is the same as Med. #1 except that fumarate has been deleted.

[b]H_2 production was measured by gas chromatographic techniques described in the Materials and Methods section. The numbers represent the total amount of H_2 produced per tube.

[c]Acetate and succinate production was measured by gas chromatographic techniques described in the Materials and Methods section. The numbers represent the amount per tube.

Table 3. Requirements for ATPase activity[a]

Reaction mixture	Activity[b]
Complete	1.52
plus NaF	1.06
minus EDTA	1.67
minus pyruvate kinase	0.84
minus PEP	1.06
minus membrane	0.23
minus ATP	0.68
minus Mg^{++}	0.61
minus Mg^{++}, plus Na^{+}	0.76
minus Mg^{++}, plus K^{+}	0.84

[a]The composition of the complete reaction mixture is given in the Materials and Methods section. The membrane protein concentration was 5 mg.

[b]Activity expressed as μmoles of Pi liberated per 20 min per mg of membrane protein.

to demonstrate ATPase activity. No appreciable ATPase activity was observed on substitution of Na^+ and K^+ for Mg^{++}.

Attempts to demonstrate phosphorylation coupled to fumarate reduction by H_2

Experiments were designed to determine directly if phosphorylation of ADP to ATP occurs during fumarate reduction to succinate by H_2 in cell extracts of *B. fragilis*. Results obtained with the 2-deoxy-D-glucose hexokinase trap are presented in Table 4. In the complete assay and likewise in the complete assay without ADP, PO_4, and ADP and hexokinase, hydrogen consumption was readily detected in the presence of fumarate. Minimal levels of hydrogen were consumed when FAD, *C. pasteurianum* extract, or *B. fragilis* extract was deleted from the complete assay. Deletion of fumarate or substitution of N_2 for H_2 resulted in no hydrogen consumption. There was, however, no detection of esterification of orthophosphate or 2-deoxy-D-glucose-6-phosphate, a product of the 2-deoxy-D-glucose hexokinase trap, in the complete reaction. As shown in Table 5, attempts to demonstrate phosphorylation of ADP to ATP coupled to fumarate reduction with the particulate or soluble fraction were unsuccessful.

Attempts to demonstrate ATP production during fumarate reduction with NADH as the electron donor were done using the 2-deoxy-D-glucose-hexokinase trap. The calculated free energy change for fumarate reduction by NADH

Table 4. Attempt to demonstrate phosphorylation coupled to fumarate reduction by H_2 using the hexokinase assay

Reaction mixture[a]	H_2 oxidized (μmoles)	Pi esterified (μmoles)[b]	2-deoxy-D-glucose 6-phosphate (μmoles)[c]
Complete	16.50	0.0	0.0
minus fumarate	0.0		
minus ADP	17.60		
minus PO_4	14.30		
minus ADP, hexokinase	16.80		
minus FAD	1.86		
minus *C. pasteurianum* extract	0.98		
minus *B. fragilis* extract	1.30		
Complete--N_2 gas	0.0		

[a]The complete reaction contained 50 μmoles of HEPES buffer (pH 7.6), 40 μmoles of $MgCl_2$, 50 μmoles of NaF, 20 μmoles of PO_4 (pH 7.0), 1 μmole of FAD, 1 μmole of ADP, 100 μmoles of 2-deoxy-D-glucose, 30 μmoles of sodium fumarate (pH 6.5), 5 mg of BSA, 0.1 mg of hexokinase, 3 mg of *C. pasteurianum* extract, and 5 mg of *B. fragilis* extract in a total volume of 2.8 ml. The reaction was done at 37°C for 20 min with a H_2 gas phase, unless otherwise specified.

[b]Calculated from the difference in esterification between each reaction and the one with the uncoupler pentachlorophenol (6×10^{-4} M) present.

[c]Determined spectrophotometrically.

Table 5. Attempt to demonstrate phosphorylation coupled to fumarate reduction by H_2 with various cell fractions[a]

Cell fraction	H_2 oxidized (μmoles)	Pi esterified (μmoles)
A. 12,100 x g Supernatant	12.50	0.0
B. 104,000 x g Supernatant	2.98	0.0
C. 104,000 x g Pellet	19.98	0.0
D. B + C	16.3	0.0

[a]Reaction conditions were the same as those given in Table 4, except that 10 mg of the various cell fractions were present. The reaction was done for 10 min.

is more than sufficient to account for the formation of at least one ATP. In these experiments *C. pasteurianum* extract and FAD or FMN, which substituted for the oxygen labile electron carrier in *B. fragilis*, were deleted. The primary objective of this experiment was to avoid the site of the oxygen labile, low potential electron carrier that is apparently involved in the H_2-fumarate electron transport system. *Bacteroides fragilis* can reduce fumarate by NADH directly without any additional electron mediators (Section 2). Direct evidence for ATP production during fumarate reduction by NADH, however, was not detected.

The luciferin-luciferase assay was a second independent method used to attempt to demonstrate direct phosphorylation of ADP to ATP. There was no detection of ATP in the complete assay with cell extracts of *B. fragilis* (Table 6).

DISCUSSION

Indirect evidence for phosphorylation coupled to fumarate reduction has been obtained in several organisms from molar growth yield studies (16,18,21,22). Anomalous growth yields have been reported in the facultative anaerobe *Proteus rettgeri* (21), in the propionic acid bacteria *Propionibacterium freudenreichii* and *Propionibacterium pentaosaceum* (9), in the rumen anaerobes *Selenomonas ruminantium* and *Anaerovibrio lipolytica* (16,18), and the human *Bacteroides* species *B. fragilis* (22).

Table 6. Attempt to demonstrate phosphorylation coupled to fumarate reduction by H_2 using the luciferin-luciferase assay

Reaction mixture[a]	H_2 oxidized (μmoles)	ATP formed (μmoles)
Complete	8.13	0.0
minus fumarate	0.33	
minus ADP	9.20	
minus PO_4	8.43	
minus FMN	2.13	
minus *C. pasteurianum* extract	1.14	
minus *B. fragilis* extract	0.27	
Complete--N_2 gas	0.0	

[a]The reaction conditions were the same as those given in Table 4, except that 2-deoxy-D-glucose and hexokinase were deleted and 10 μmoles of PO_4 and 2.5 μmoles of ADP were added. The samples were extracted with perchloric acid (7) and assayed in a Searle Delta 300 scintillation counter.

Macy and her colleagues (22) calculated that 4.5 ATPs per mole of glucose were produced by *B. fragilis* on a hemin medium as compared to 1.7 ATPs per mole of glucose on a hemin deficient medium. The major acid end products produced by the cells on the hemin medium were acetate, propionate, and succinate as compared to acetate, lactate, and fumarate on a hemin-deficient medium (22). Macy et al. (22) interpreted these results to suggest that propionate and succinate production may be involved in additional energy production in *B. fragilis*. In the present study, more direct evidence for the involvement of fumarate in additional energy production was presented. From a molar growth yield value of 44.19 g/mole of glucose (Table 1) and a Y_{ATP} of 10.5 (3), 4.2 ATPs/mole of glucose were made by *B. fragilis*. Higher molar growth yields obtained on a hemin medium supplemented with fumarate indicated that additional energy is probably produced when fumarate is reduced to succinate in *B. fragilis*.

In the present investigation, the addition of fumarate to modifications of the basal medium was found to affect the molar growth yields. When substrate quantities of glucose (Med. #1 and #2, Table 1) were present, the molar growth yields were approximately the same regardless of the fumarate concentration. However, different quantities of the acids and hydrogen gas were detected in the media. If additional energy were produced from fumarate reduction in Med. #1, it may not have been

reflected in an increase in cell yield, but rather in the increased quantities of acetate and succinate produced by the cells on Med. #1 as compared to Med. #2. The lower quantity of H_2 detected in Med. #1 as compared to Med. #2 may have been due to the use of H_2 in the reduction of the exogenously added fumarate to succinate. When cells were grown on media with limiting glucose and high fumarate concentrations (Med. #3, Table 1), a 20% increase was observed in the cell yield when compared to growth of the cells on media with limiting glucose and lower fumarate concentrations (Med. #4 and #5, Table 1). Again, these data implied that additional ATP is generated from fumarate reduction. These observations are consistent with the calculated free energy change (-20.4 Kcal/mole) for H_2 oxidation with fumarate, which should be sufficient to allow for the formation of at least one ATP in *B. fragilis*. ATP production from the transfer of electrons from H_2 to fumarate may occur at the flavoprotein and/or cytochrome b site(s).

Divalent cation stimulated ATPases, particularly Mg^{++} or Ca^{++}, have been shown to be generally associated with phosphorylation of ADP to ATP (1,2,4,13,14). ATPases stimulated by monovalent cations such as Na^{+} or K^{+} have been usually associated with active transport (4,13,14). Mg^{++} or Ca^{++} stimulated ATPase activity associated with phosphorylation during fumarate reduction has been demonstrated in *Desulfovibrio gigas* (1,2,13), *Escherichia coli* (4,14), and *Vibrio succinogenes* (C. A. Reddy and H. D.

Peck, Jr., Abst. Annu. Meet. Amer. Soc. Microbiol., 1973, 194). In the present investigation, ATPase activity was detected in cell extracts of *B. fragilis*. The monovalent cations Na^+ or K^+ did not stimulate the ATPase activity, which suggested that the ATPase is not involved in active transport in *B. fragilis*.

Direct evidence for electron transport phosphorylation coupled to fumarate reduction has been demonstrated in several organisms that reduce fumarate to succinate with either H_2 or NADH (1,2,11, C. A. Reddy and H. D. Peck, Jr., Abst. Annu. Meet. Amer. Soc. Microbiol., 1973, 194; C. A. Reddy, Rumen Function Conference, Chicago, 1973, unpublished). Barton et al. (1,2) have demonstrated phosphorylation of ADP to ATP when fumarate is reduced to succinate by H_2 in cell extracts of *D. gigas*. Similar results have been obtained by C. A. Reddy and H. D. Peck, Jr. (Abst. Annu. Meet. Amer. Soc. Microbiol., 1973, 194) in *V. succinogenes*. Direct evidence for ATP formation coupled to fumarate reduction by NADH in cell extracts of *Streptococcus faecalis* and *Bacteroides ruminicola* has been presented by Faust and Vandemark (11) and C. A. Reddy (1973, Rumen Function Conference, Chicago, unpublished), respectively.

Efforts to demonstrate direct evidence for ATP production during fumarate reduction in cell extracts of *B. fragilis* have been unsuccessful to date in the present investigation. Barton et al. (1,2) and C. A. Reddy and H. D. Peck, Jr. (Abst. Annu. Meet. Amer. Soc. Microbiol.,

1973, 194) employed isotopic and chemical procedures of the glucose-hexokinase trap to demonstrate esterification of orthophosphate during fumarate reduction in *D. gigas* and *V. succinogenes*. Using chemical procedures of the 2-deoxy-D-glucose hexokinase trap in the present study, phosphorylation during fumarate was not detected in *B. fragilis*. Attempts to show ATP formation coupled to fumarate reduction by the luciferin-luciferase assay were also unsuccessful. The luciferin-luciferase assay was chosen as a second independent means to attempt to demonstrate ATP production from fumarate reduction because of its extreme sensitivity (7,23,26,27,28). The assay can detect nanomole or picomole quantities of ATP (7,23,26, 27,28).

Direct evidence for phosphorylation of ADP to ATP during fumarate reduction in *B. fragilis* was not obtained, probably due to the lack of coupling factors or some essential coupling proteins which may be extremely sensitive to oxygen and therefore destroyed during the fractionation procedures. The lack of an essential trace element may also have been a factor in being unable to demonstrate ATP formation during fumarate reduction. Turner and Stadman (29) have shown the requirement of selenium for a protein that is involved in ATP production in the glycine reductase system of *Clostridium sticklandii*. In this study, however, trace quantities of several compounds including selenium and molybdenum were added to

basal medium in all experiments designed to demonstrate phosphorylation in *B. fragilis*.

In the present investigation, the assays employed would have only detected phosphorylated compounds as an index of energy production from fumarate reduction in cell extracts of *B. fragilis*. It may be possible that the high energy compound is a non-phosphorylated compound such as an acyl thioester and therefore it would not have been demonstrated in the assay procedures used. It is also possible that pyrophosphate (PPi) may actually have been produced instead of ATP from fumarate reduction and therefore it would not have been detected in the assays. Additional energy production from fumarate reduction in *B. fragilis* may be utilized for a favorable conformational change in the membrane and not for the synthesis of a high energy compound. Additional studies, of course, are needed to investigate these possibilities.

LITERATURE CITED

1. Barton, L. L., J. LeGall, and H. D. Peck, Jr. 1970. Phosphorylation coupled to oxidation of hydrogen with fumarate in extracts of the sulfate reducing bacterium *Desulfovibrio gigas*. Biochem. Biophys. Res. Commun. 41:1036-1042.

2. Barton, L. L., J. LeGall, and H. D. Peck, Jr. 1972. Oxidative phosphorylation in the obligate anaerobe, *Desulfovibrio gigas*. p. 31-51. *Horizons of Bioenergetics*. Academic Press, Inc. New York and London.

3. Bauchop, T., and S. R. Elsden. 1960. The growth of microorganisms in relation to their energy supply. J. Gen. Microbiol. 23:457-469.

4. Butlin, J. D., G. B. Cox, and F. Gibson. 1971. Oxidative phosphorylation in *E. coli* K-12. Mutation affecting magnesium ion- or calcium ion-stimulated adenosine triphosphatase. Biochem. J. 124:75-81.

5. Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. J. Biol. Chem. 242:173-181.

6. Clark, J. M. (ed.). 1964. *Experimental Biochemistry*. W. H. Freeman and Co., San Francisco, California.

7. Cole, H. A., J. W. Wimpenny, and D. E. Hughes. 1967. The ATP pool in *Escherichia coli*. I. Measurement of the pool using a modified luciferase assay. Biochim. Biophys. Acta 143:445-453.

8. DeMoss, R. D. 1955. Glucose-6-phosphate and 6-phosphogluconic dehydrogenase from *Leuconostic mesenteroides*. p. 328-329. In S. P. Colowick and N. O. Kaplan (eds.), *Methods in Enzymology*, Vol. 1. Academic Press, Inc., New York.

9. DeVries, W., W. M. C. van Wijck-Kapteyn, and A. H. Stouthamer. 1973. Generation of ATP during cytochrome-linked anaerobic electron transport in propionic acid bacteria. J. Gen. Microbiol. 76: 31-41.

10. Dubois, M., K. A. Giles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

11. Faust, P. J., and P. J. Vandemark. 1970. Phosphorylation coupled to NADH oxidation with fumarate in *Streptococcus faecalis* 10C1. Arch. Biochem. Biophys. 137:392-398.

12. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66:375-400.

13. Guarraia, L. J., and H. D. Peck. 1971. Dinitrophenol-stimulated adenosine triphosphatase activity in extracts of *Desulfovibrio gigas*. J. Bacteriol. 106: 890-895.

14. Hanson, L. R., and E. P. Kennedy. 1973. Energy transducing adenosine triphosphatase from *Escherichia coli*: Purification, properties, and inhibition by antibody. J. Bacteriol. 114:772-781.

15. Harris, M. A., and C. A. Reddy. 1977. Hydrogenase activity and the H_2-fumarate electron transport system in *Bacteroides fragilis*. J. Bacteriol. 131: 922-928.

16. Hobson, P. N., and R. Summers. 1972. ATP pool and growth yield in *Selenomonas ruminantium*. J. Gen. Microbiol. 70:351-360.

17. Holdeman, L. V., and W. E. C. Moore (eds.). 1973. *Anaerobe Laboratory Manual,* 2nd Edition. VPI Anaerobe Laboratory, Blacksburg, Virginia.

18. Hungate, R. E. 1966. *The Rumen and Its Microbes*. Academic Press, New York and London.

19. Johnson, R. R., T. C. Balwani, L. J. Johnson, K. E. McClure, and B. A. Dehority. Corn plant maturity. II. Effect on *in vitro* cellulose carbohydrate content. J. Animal Sci. 25:617-623.

20. Kimmich, G. A., J. Randles, and J. S. Brand. 1975. Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. Anal. Biochem. 69:187-206.

21. Kroger, A. 1974. Electron transport phosphorylation coupled to fumarate reduction in anaerobically grown *Proteus rettgeri*. Biochim. Biophys. Acta 347:273-289.

22. Macy, J., I. Probst, and G. Gottschalk. 1975. Evidence for cytochrome involvement in fumarate reduction and adenosine 5' triphosphate synthesis by *Bacteroides fragilis* grown in the presence of hemin. J. Bacteriol. 123:436-442.

23. McElroy, W. D. 1947. The energy source for bioluminescence in an isolated system. Proc. Natl. Acad. Sci. USA 33:342-345.

24. Montgomery, R. 1961. Further studies of the phenol-sulfuric acid reagent for carbohydrate. Biochim. Biophys. Acta 48:591-593.

25. Pullman, M. E., H. S. Penefsky, A. Datta, and E. Racker. 1960. Partial resolution of the enzymes catalyzing oxidative phosphorylation. I. Purification and properties of soluble, dinitrophenol-stimulated adenosine triphosphatase. J. Biol. Chem. 235:3322-3329.

26. Roberton, A. M., and R. S. Wolfe. 1970. Adenosine triphosphate pools in *Methanobacterium*. J. Bacteriol. 102:43-51.

27. Strehler, B. L., and J. R. Totter. 1952. Firefly luminescence in the study of energy transfer mechanisms. I. Substrate and enzyme determination. Arch. Biochem. Biophys. 40:28-41.

28. Strehler, B. L., and J. R. Totter. 1954. Determination of ATP and related compounds: Firefly luminescence and other methods. In *Methods of Biochemical Analysis*, Vol. 1. D. Glick (ed.). Interscience Publishers, New York, p. 341-356.

29. Turner, D. C., and T. C. Stadtman. 1973. Purification of protein components of the clostridial glycine reductase system and characterization of protein A as a selenprotein. Arch. Biochem. Biophys. 154: 366-381.

30. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. *Manometric Techniques*. Burgess Publishing Co., Minneapolis, Minnesota.

31. Varel, V., and M. P. Bryant. 1972. Nutritional features of *Bacteroides fragilis* subsp. *fragilis*. Appl. Microbiol. 18:251-257.