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Carbohydrate Metabolism in *Spirochaeta stenostrepta*

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The pathways of carbohydrate metabolism in *Spirochaeta stenostrepta*, a free-living, strictly anaerobic spirochete, were studied. The organism fermented glucose to ethyl alcohol, acetate, lactate, CO_2, and H_2. Assays of enzymatic activities in cell extracts, and determinations of radioactivity distribution in products formed from ^{14}C-labeled glucose indicated that *S. stenostrepta* degraded glucose via the Embden-Meyerhof pathway. The spirochete utilized a clostridial-type clastic reaction to metabolize pyruvate to acetyl-coenzyme A, CO_2, and H_2, without production of formate. Acetyl-coenzyme A was converted to ethyl alcohol by nicotinamide adenine dinucleotide-dependent acetaldehyde and alcohol dehydrogenase activities. Phosphotransacetylase and acetate kinase catalyzed the formation of acetate from acetyl-coenzyme A. Hydrogenase and lactate dehydrogenase activities were detected in cell extracts. A rubredoxin was isolated from cell extracts of *S. stenostrepta*. Preparations of this rubredoxin stimulated acetyl phosphate formation from pyruvate by diethylaminoethyl cellulose-treated extracts of *S. stenostrepta*, an indication that rubredoxin may participate in pyruvate cleavage by this spirochete. Nutritional studies showed that *S. stenostrepta* fermented a variety of carbohydrates, but did not ferment amino acids or other organic acids. An unidentified growth factor present in yeast extract was required by the organism. Exogenous supplements of biotin, riboflavin, and vitamin B_{12} were either stimulatory or required for growth.

Few studies have dealt with the metabolism of anaerobic spirochetes, possibly because of the unavailability of strains which could be conveniently mass cultured. In such studies, the spirochetes were grown in the blood of infected hosts (19) or in media containing serum (3). The development of methods for the isolation of free-living, anaerobic spirochetes (12, 13, 52) has provided strains which grow abundantly in serum-free media. As a result, investigations of the metabolic pathways and energy-yielding mechanisms of anaerobic spirochetes are now greatly facilitated.

The present report describes the results of a study on the carbohydrate metabolism of *Spirochaeta stenostrepta* strain Z1, a strictly anaerobic spirochete isolated from sulfide-containing mud (12, 13). During this study, some aspects of the nutrition of *S. stenostrepta* Z1 were investigated for the twofold purpose of obtaining information on the overall physiology of the organism and of improving mass cultivation techniques.

MATERIALS AND METHODS

Media and growth conditions. *S. stenostrepta* strain Z1 was routinely grown at 30 C in Florence flasks (50 to 3,000 ml) filled with medium GYPT (12) consisting of (g/100 ml of distilled water): glucose, 0.5; yeast extract (Fisher) and peptone (Difco), 0.2 each; and sodium thioglycolate, 0.05. The *p*H of the medium was adjusted to 7.6 with 5% KOH (w/v) before sterilization. Generally, the inoculum consisted of 1 to 3 ml of broth culture (10^8 to 3×10^8 cells/ml) per 100 ml of medium GYPT.

The spirochete was mass-cultured in 20-liter carboys filled with a modified GYPT medium including 0.6 g of yeast extract per 100 ml. The *p*H of this medium was maintained above 6.0 during growth by addition of 0.1 g of $NaHCO_3$/100 ml five times, at intervals of 8 to 12 hr, beginning in the second half of the exponential phase of growth.

Vitamin requirements were studied in a vitamin-poor basal medium consisting of (g/100 ml of distilled water): glucose, 0.5; yeast extract, 0.05; vitamin-free casein hydrolysate, 0.5; and sodium thioglycolate, 0.05. This medium was supplemented with the following vitamins (mg of vitamin added/100 ml of medium): biotin, vitamin B_{12}, riboflavine, folic acid, 0.1 each; thiamine, calcium pantothenate, pyridoxal phosphate, *p*-amino-benzoic acid, and niacin, 1.0 each.

S. stenostrepta was harvested by centrifugation during the exponential-growth phase and the cells were used immediately.

Clostridium butyricum and *C. pasteurianum* were grown in the following medium (g/100 ml of distilled water): glucose, 1.0; casein hydrolysate, 0.5; yeast extract, 0.3; and K_2HPO_4, 0.2.

Preparation of cell suspensions. Cell pellets were suspended in three times their volume of cold (5 C)

0.03 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer solution (*p*H 7.0) containing 0.001 M dithiothreitol (DTT). Cells were not washed as washing caused cellular lysis and extensive loss of physiological activity.

Cell and protein concentrations. The turbidities of cultures and cell suspensions were determined by means of a Klett-Summerson photoelectric colorimeter equipped with a 660-nm filter. Colorimetric readings were converted to cell numbers or protein concentrations by using standard curves relating the reading to direct cell counts or to cell protein determinations, respectively. The protein content of whole cells was assayed by the method of Lowry et al. (31), and that of cell extracts by the method of Warburg and Christian (54).

Cell extracts. Cells of *S. stenostrepta* were disrupted at 5 C by means of either a French pressure cell (20- to 100-ml cell suspension; 2,500 psi) or a Bronwill 20-kc Biosonik probe (5-ml cell suspension; sonic treatment for three 5-sec intervals; power setting 45). Both methods resulted in complete cellular disruption, as determined microscopically. Cellular debris was removed by centrifugation (5 C) at 18,000 × *g* for 15 min after French pressure cell disruption, or at 39,000 × *g* for 20 to 30 min after sonic treatment. In manometric experiments, the cellular debris was not removed to minimize loss of enzymatic activity. Cell extracts were used immediately after preparation as enzymatic activity was lost during storage (at −25 C, in argon or other gases).

C. butyricum and *C. pasteurianum* were disrupted by means of a French pressure cell (10,000 psi, 5 C), and the cellular debris was removed by centrifugation at 27,000 × *g* for 30 min at 5 C.

Coenzyme A-depleted extracts. Cell extracts were depleted of coenzyme A (CoA) by stirring them with an equal volume of Dowex chloride resin (Dowex 1-X8, 50 to 100 mesh) for 15 min under argon (46) or by treatment with Sephadex resin. Approximately 5 to 10 ml of extract was rapidly passed through a Sephadex G-25 column (2.5 by 30 cm; Pharmacia Fine Chemicals, Piscataway, N. J.) previously equilibrated at 5 C with 0.03 M HEPES buffer solution (*p*H 7.0) containing 4.0×10^{-3} M DTT. The first 50% of the protein-containing effluent was collected and constituted the coenzyme A-depleted extract.

Ferredoxin- or rubredoxin-depleted extracts. Approximately 10 ml of cell extract was passed through a diethylaminoethyl cellulose (DEAE) column (1.8 by 20 cm) previously equilibrated with 500 ml of 0.05 M phosphate buffer solution (*p*H 6.6). The first 50 to 60% of the protein-containing effluent was collected, and this fraction constituted the ferredoxin- or rubredoxin-depleted extract.

Isolation of ferredoxin and rubredoxin. Ferredoxin was purified from cell extracts of *C. pasteurianum* and *C. butyricum* by methods similar to those described by Mortenson (33). Frozen-cell suspensions instead of dried cells were used as the starting material. The initial DEAE column was eluted extensively with 0.08 M tris(hydroxymethyl)aminomethane-hydrochloride (Tris) buffer solution (*p*H 8.0) before elution with 0.2 M Tris buffer.

Rubredoxin was isolated from *S. stenostrepta* cell extracts by the methods of Lovenberg and Sobel (30), with slight modifications in the ammonium sulfate steps. The rubredoxin-containing preparations were brought to 40% saturation (at 5 C) with granular ammonium sulfate and the resulting precipitate was removed by centrifugation. As the supernatant liquid was slowly taken to 90% saturation the rubredoxin-containing material rose to the surface of the ammonium sulfate solution. This material was carefully removed, suspended in a small amount of distilled water, and subjected to further purification (30).

Yields of rubredoxin purified from 500 g of wet cells ranged from 4.0 to 6.5 mg. Attempts to crystallize the purified rubredoxin were unsuccessful.

Manometry. Double-sidearm Warburg vessels were used in studies of gaseous and nongaseous products formed from glucose or pyruvate by whole cells or by cell extracts. All experiments were conducted at 30 C with an argon atmosphere in the vessel. Conventional Warburg manometric techniques were employed (50). Unless indicated otherwise, the following additions were made to each vessel (μmoles): substrate, 50 (sidearm 1); HEPES buffer (*p*H 7.0), 100; DTT, 10; H_2SO_4, 500 (sidearm 2); and either cells or cell extract in a final volume of 3.2 ml. Where appropriate, the center well contained 0.2 ml of 20% KOH.

In experiments measuring the fermentation of pyruvate by cell-free extracts, each vessel contained in addition to the above-mentioned components (μmoles): potassium phosphate buffer (*p*H 6.8), 30; $MgCl_2$, 10; thiamine pyrophosphate (TPP), 1; and coenzyme A, free of $[CoAS\text{-}]_2$, reduced form (CoASH).

Assay of fermentation products. Generally the combined contents of several Warburg vessels (containing identical reaction mixtures) were analyzed after incubation for fermentation products.

Carbon dioxide was determined gravimetrically as $BaCO_3$ (formed by addition of $BaCl_2$ to KOH solutions removed from the center wells of Warburg vessels by using carbon dioxide-free water washes).

Cells were separated from the vessel contents by centrifugation, and protein was precipitated from the supernatant liquid by adding $ZnSO_4$ (35). The glucose concentration in the resulting clarified solution was determined by the glucose oxidase method (Glucostat, Worthington Biochemical Corp., Freehold, N.J.). Lactate, pyruvate, acetoin, and diacetyl were assayed for by the methods of Barker and Summerson (5), Friedemann and Haugen (18), and Westerfeld (55), respectively.

Samples of the neutral, clarified fermentation solution were assayed by standard methods for fermentation products such as alcohols and volatile and nonvolatile organic acids (4, 8, 35, 36, 51). Ethyl alcohol was assayed enzymatically (22) and by dichromate oxidation (35, 36). Hydrogen sulfide was determined qualitatively by using filter paper strips impregnated with lead acetate.

Exchange experiments. Carbon dioxide-pyruvate and formate-pyruvate exchange activities with whole cells or cell extracts were determined by conventional

procedures (27, 58). The reaction mixture (3.2 ml) contained (μmoles): HEPES buffer, (*p*H 7.0 or 8.4), 100; $MnCl_2$, 10; DTT, 10; CoASH, 0.1; TPP, 10; potassium pyruvate, 100; $NaH^{14}CO_3$ or $Na^{14}COOH$, 100; and either whole cells or cell extract protein. Incubation was for 20 min at 30 C under argon.

Radioactivity determinations. Radioactivity in products accumulated by cell suspensions or cell extracts incubated with ^{14}C-labeled compounds was determined as follows. Ethyl alcohol was converted to acetate by dichromate oxidation (36), and the resulting acetate was recovered by steam distillation (35). Radioactivity in the individual acetate carbons was determined as described by Phares (39).

Radioactivity was measured by using a thin-window, gas-flow Geiger counter equipped with a scaler (Nuclear-Chicago Corp., Des Plaines, Ill.). Carbon dioxide was assayed for radioactivity as barium carbonate, pyruvate as the 2,4-dinitrophenylhydrazone, and glucose and sodium acetate were assayed directly. Corrections were made for self-absorption of radiation.

Determination of enzymatic activities. Various pyridine nucleotide-linked enzymatic activities of cell extracts were determined from rates of absorbancy changes measured at 340 nm, using a Gilford model 2000 spectrophotometer. The assay mixture (3.0 ml) contained 0.01 to 0.3 mg of cell extract protein, 10 μmoles of DTT, and the appropriate compounds (Table 1). The concentrations of compounds used and some of the assay conditions were similar to those described in the references listed in Table 1. Enzymatic activity was measured as the oxidation or reduction of pyridine nucleotide. Specific activity was defined as the nanomoles of pyridine nucleotide change per minute per milligram of cell extract protein.

Phosphotransacetylase (EC 2.3.1.8) activity was measured by determining the disappearance of acetyl phosphate in the presence of arsenate (46). The assay mixture (1.0 ml) contained (μmoles): HEPES buffer (*p*H 7.0), 20; potassium arsenate (*p*H 8.0), 20; DTT, 5; acetyl phosphate, 15; CoASH, 0.01; and 1.2 mg of cell extract protein or 0.9 mg of Sephadex-treated cell extract protein. Incubation was for 20 min at 30 C under argon.

Acetate kinase (EC 2.7.2.1) activity was determined in both the forward and reverse directions (40). Lactate dehydrogenase (EC 1.1.1.27) was determined as described by Kornberg (26) except that HEPES buffer (*p*H 8.4) was used instead of phosphate buffer.

The method of Peck and Gest (38) was used to measure hydrogen gas evolution by cell extracts from reduced dyes. Each Warburg vessel contained (μmoles): HEPES buffer (*p*H 7.0), 100; DTT, 10; methyl viologen, 20; and 21 mg of cell extract protein in a final volume of 3.2 ml. The dye (sidearm) was reduced by adding 40 μmoles of sodium hydrosulfite. Incubation was for 20 min at 30 C under argon. Uptake of hydrogen gas was measured by similar procedures except the sodium hydrosulfite was omitted and a hydrogen atmosphere in place of argon was employed.

Acetyl phosphate formation from pyruvate. Reaction mixtures used to determine formation of acetyl phosphate from pyruvate by ferredoxin- or rubredoxin-depleted extracts contained (μmoles): phosphate buffer (*p*H 6.6), 100; HEPES buffer (*p*H 7.0), 20; DTT, 5; TPP, 0.5; $MnCl_2$, 0.5; CoASH, 0.02; potassium pyruvate, 50; and 3.0 to 5.0 mg of cell extract protein in a total volume of 1 ml. Incubation was for 30 min at 30 C under argon. Similar reaction mixtures were used to measure acetyl phosphate formation from pyruvate by crude cell extracts. Acetyl phosphate was assayed by standard procedures (29).

Ethyl alcohol formation from acetyl phosphate. The assay mixture (3.0 ml) contained (μmoles): HEPES buffer (*p*H 8.4), 100; DTT, 20; acetyl phosphate, 34; CoASH, 0.5; reduced nicotinamide adenine dinucleotide (NADH), 37.5; and 11 mg of cell extract protein. After 2 hr of incubation at 30 C under argon, the mixture was chilled (3 C) and assayed for acetyl phosphate, ethyl alcohol, acetaldehyde, reduced pyridine nucleotide, and acetyl-CoA (29).

Chemicals. Piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (PIPES), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), *N*,*N*-bis(2-hydroxyethyl)glycine (BICINE), HEPES, and radioactive compounds were purchased from Calbiochem, Los Angeles, Calif.

RESULTS

Growth and nutrition. The doubling time of *S. stenostrepta* growing in medium GYPT was approximately 6 hr and the final yield ranged from 2 to 3 $\times$ 10^8 cells/ml. Growth ceased when the *p*H of the culture reached values between 5.5 and 5.3 (the initial *p*H was 7.5 to 7.6), even though fermentable carbohydrate was still available in the medium. Greater carbohydrate utilization, and cell yields from 0.5 to 1.0 $\times$ 10^9 cells/ml were obtained by buffering medium GYPT with 0.033 M HEPES, PIPES, TES or BICINE (initial pH of medium, 7.5), or with increased concentrations of yeast extract (i.e., 0.6 g/100 ml). Repeated additions of $NaHCO_3$ to *S. stenostrepta* cultures in medium GYPT resulted in final yields of approximately 5 $\times$ 10^9 cells/ml. When the buffer content of medium GYPT was augmented both by increasing the yeast extract concentration (to 0.6 g/100 ml) and by intermittent additions of $NaHCO_3$, the final growth yield approximated 10^{10} cells/ml. Addition of potassium (or sodium) phosphate or Tris buffers (*p*H 7, sterilized separately) to medium GYPT at levels as low as 0.02 M inhibited growth, the final yields being less than 10^7 cells/ml.

Cell suspensions exhibited an analogous response to buffers (*p*H 7.0; final concentration, 0.06 M). The volume of gas evolved by cells suspended in glucose solutions markedly increased when the mixture included HEPES, TES, PIPES, or Tris-maleate. Slight or no increase occurred

TABLE 1. *Determination of enzymatic activities in cell extracts*

Enzymatic activity	Assay mixture[a]	Reference
Hexokinase (EC 2.7.1.1)	Tris (*p*H 8.5), 200; $MnCl_2$, 2; NAD, 2; ATP, 15; glucose, 15; KH_2AsO_4, 50	15
Glucose 6-phosphate dehydrogenase (EC 1.1.1.49)	Tris (*p*H 7.8), 150, or HEPES (*p*H 8.4), 200; NADP, 8.4; $MnCl_2$, 10; G-6-P, 10	43
Phosphogluconate dehydrogenase (EC 1.1.1.43)	HEPES (*p*H 8.4), 200; $MgCl_2$, 20; NADP, 10; 6-P-G, 10	44
Glucosephosphate isomerase (EC 5.3.1.9)	HEPES (*p*H 8.5), 200; $MnCl_2$, 2; NAD, 2; G-6-P, 10; ATP, 5; KH_2AsO_4, 50	42
Phosphofructokinase (EC 2.7.1.11)	HEPES (*p*H 8.5), 200; $MnCl_2$, 2; NAD, 2; F-6-P, 5; ATP, 5; KH_2AsO_4, 50	28
Fructosediphosphate aldolase (EC 4.1.2.13)	HEPES (*p*H 8.5), 200; F-1,6-diP, 10; NAD, 1; KH_2AsO_4, 50	49
Triosephosphate isomerase (EC 5.3.1.1)	Triethanolamine (*p*H 8.5), 200; NAD, 2; DHAP, 2; KH_2AsO_4, 50	6
Phosphoglyceromutase (EC 2.7.5.3), phosphopyruvate hydratase (EC 4.2.1.11)	Triethanolamine (*p*H 8.5), 100; PGA, 5; NADH, 1; $MgCl_2$, 2; ADP, 2	9, 54
Pyruvate kinase (EC 2.7.1.40)	Triethanolamine (*p*H 8.5), 100; PEP, 5; NADH, 1; $MgCl_2$, 2; ADP, 2	10
Glycerolphosphate dehydrogenase (EC 1.1.99.5)	Tris or HEPES (*p*H 8.0), 150; NADH, 1; DHAP, 1	7
Glycerol dehydrogenase (EC 1.1.1.6)	Tris, HEPES, or triethanolamine (*p*H 8.0), 200; glycerol or DHAP, 5; NADH or NAD, 1	11
Glycerol kinase (EC 2.7.1.30)	Tris (*p*H 8.5), 200; glycerol, 10; ATP, 5; NAD, 1; $MnCl_2$, 1; KH_2AsO_4, 50	23
Glyceraldehydephosphate dehydrogenase (EC 1.2.1.12)	HEPES (*p*H 8.4), 150; NAD, 1; GAP, 5; KH_2AsO_4, 50	53
Gluconokinase (EC 2.7.1.12)	Tris or HEPES (*p*H 8.0), 200; $MgCl_2$, 20; ATP, 10; potassium gluconate, 6 or 20; NADP or NAD, 10	14

[a] Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; F-6-P, fructose 6-phosphate; F-1,6-diP, fructose 1,6-diphosphate; GAP, glyceraldehyde 3-phosphate; G-6-P, glucose 6-phosphate; NAD and NADH, oxidized and reduced nicotinamide adenine dinucleotide; NADP and NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; 6-P-G, 6-phosphogluconate, Tris, tris(hydroxymethyl)aminomethane. Numbers refer to micromoles of compound in the 3.0-ml assay mixture.

in the presence of BICINE, triethanolamine or Tris, whereas phosphate was inhibitory. Microscopic examination of the cell suspensions showed extensive round-body formation (12) and cellular lysis in mixtures containing phosphate, Tris, or triethanolamine.

Attempts to cultivate *S. stenostrepta* in a chemically defined medium were not successful. Although medium GYPT supported growth when the yeast extract concentration was decreased to 0.05 g/100 ml, there was no growth when the yeast extract was omitted. Replacement

of the yeast extract with mixtures of vitamins, purines, pyrimidines, and amino acids did not permit growth. Apparently a growth factor present in yeast extract was required by the organism. A similar requirement has been reported for *S. zuelzerae* (13, 52).

Other nutritional studies were conducted using medium GYPT from which glucose was omitted (medium YPT). This medium supported a low yield (approximately 2×10^7 cells/ml) as the result of fermentation of sugars present in the yeast extract. Substrates under test were added to medium YPT (in various concentrations up to 0.4 g/100 ml) and substantial increases in the final cell yield (to 10^8 to 3×10^8 cells/ml) were taken as an indication that the substrate was being utilized. These experiments indicated that D-fructose, D-galactose, D-mannose, cellobiose, lactose, maltose, sucrose, L-arabinose, D-ribose, and D-xylose were fermented. Similar results were obtained by measuring gas evolution by cell suspensions. Addition to medium YPT of amino acids, singly or in combinations, or of casein hydrolysate, or of various organic acids did not increase the final yield. Correspondingly, cell suspensions evolved little or no gas in the presence of amino acids or other organic acids.

Vitamin requirements were studied by growing cells in a vitamin-poor basal medium to which a vitamin supplement was added. Omission of the supplement of biotin, riboflavine, or vitamin B_{12} from the culture medium drastically reduced the final cell yield (from 10^8 cells/ml in the fully supplemented medium to approximately 9×10^6 cells/ml), indicating that exogenous supplies of these vitamins are either required or stimulatory for the growth of the organism. Omission of other vitamins did not appreciably affect the final growth yield.

In nature, *S. stenostrepta* occurs in sulfide-rich mud. This suggested that the organism might carry out anaerobic respiration, using sulfate as the electron acceptor. However, H_2S was not evolved by *S. stenostrepta* growing in various media to which were added ammonium sulfate (0.3 g/100 ml) and possible electron donors, such as pyruvate, lactate, malate, or glucose (0.5 g/100 ml). A relatively small amount of H_2S was evolved when the organism was cultured in media containing sodium thioglycolate (0.05 g/100 ml), which probably originated from the sulfhydryl group of this compound.

Fermentation of glucose. The main products of glucose fermentation by *S. stenostrepta* Z1 are ethyl alcohol, acetate, lactate, CO_2, and H_2 (12, 13). The metabolic pathways active in this organism were studied by determining the distribution of radioactivity in products accumulated by cell suspensions fermenting glucose-*1*-^{14}C or glucose-*6*-^{14}C (Tables 2 and 3). Radioactivity was detected almost exclusively in carbon 2 of ethyl alcohol and acetate, whereas no significant label was present in CO_2. This labeling pattern excluded the presence of active hexose monophosphate or Entner-Doudoroff pathways, and was consistent with patterns observed in organisms utilizing the Embden-Meyerhof pathway for glucose oxidation.

Determinations of enzyme activities in freshly prepared cell extracts of *S. stenostrepta* Z1 indi-

TABLE 2. *Fermentation of glucose-1-^{14}C by cell suspensions of Spirochaeta stenostrepta*[a]

Products	Amt of products[b]	Distribution of label[c]
CO_2	187.5	36.0
H_2	27.2	
Ethyl alcohol	146.2	788.0
Carbon 1		3.4
Carbon 2		760.0
Acetate	20.4	468.0
Carbon 1		4.8
Carbon 2		310.0
Lactate	8.2	

[a] Specific activity of glucose-*1*-^{14}C: 1,313 counts per min per μmole; 5.4×10^{10} cells were added per Warburg vessel and incubated for 2 hr.

[b] Expressed as micromoles of product per 100 μmoles of glucose fermented; carbon recovery: 90.8%.

[c] Expressed as counts per minute per micromole of product.

TABLE 3. *Fermentation of glucose-6-^{14}C by cell suspensions of Spirochaeta stenostrepta*[a]

Products	Amt of products[b]	Distribution of label[c]
CO_2	124.0	18.3
H_2	86.9	
Ethyl alcohol	74.3	664.0
Carbon 1		1.4
Carbon 2		582.0
Acetate	73.8	675.0
Carbon 1		2.7
Carbon 2		551.0
Lactate	20.8	

[a] Specific activity of glucose-*6*-^{14}C: 1,620 counts per min per μmole; 4×10^{10} cells were added per Warburg vessel and incubated for 4.5 hr.

[b] Expressed as micromoles of product per 100 μmoles of glucose fermented; carbon recovery: 80.4%.

[c] Expressed as counts per minute per micromole of product.

TABLE 4. *Enzyme activities in cell extracts of Spirochaeta stenostrepta*

Enzyme[a]	Specific activity[b]
Hexokinase	6
Glucosephosphate isomerase	18
Phosphofructokinase	35
Fructosediphosphate aldolase	54
Glyceraldehydephosphate dehydrogenase	171
Triosephosphate isomerase	28
Phosphoglyceromutase, phosphopyruvate hydratase	186
Pyruvate kinase	105
Phosphogluconate dehydrogenase	12

[a] Not detected: glucose-6-phosphate dehydrogenase, gluconokinase, glycerol kinase, glycerolphosphate dehydrogenase, and glycerol dehydrogenase.

[b] Expressed as nanomoles of pyridine nucleotide change per minute per milligram of protein.

cated that enzyme activities associated with the Embden-Meyerhof pathway were present (Table 4). The glyceraldehydephosphate dehydrogenase activity was specific for nicotinamide adenine dinucleotide (NAD), was strongly inhibited by sodium iodoacetate (10^{-3} M), and was stimulated by potassium arsenate (10^{-3} to 10×10^{-3} M). In the absence of added substrates, NADH or reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidizing activities were not present.

Glucose-6-phosphate dehydrogenase and other enzyme activities associated with gluconate metabolism were not detected in extracts prepared from glucose-grown cells or from cells incubated in the presence of gluconate after initial growth on glucose (potassium gluconate, substituted for glucose in medium GYPT, did not support growth). On the other hand, NADP-specific phosphogluconate dehydrogenase activity was present (Table 4). This activity may be associated with the synthetic processes of the organism.

Enzymes of glycerol metabolism were not detected in cell extracts (Table 4). This finding is consistent with the observation that *S. stenostrepta* does not ferment glycerol.

Results of experiments in which cell suspensions were incubated with metabolic inhibitors are in agreement with the conclusion that *S. stenostrepta* ferments glucose via the Embden-Meyerhof pathway. The addition of sodium iodoacetate (10^{-3} M) or sodium fluoride (10^{-2} M) to cells fermenting glucose caused immediate cessation of gas evolution. Sodium azide, at similar concentrations, had no effect.

Cleavage of pyruvate. Cells and extracts of *S. stenostrepta* degraded pyruvate to the same major products formed by cells fermenting glucose (Table 5). In addition to these products, intact cells and especially extracts produced acetoin from pyruvate. A similar situation has been reported for *Streptococcus faecalis* (17) and *Bacillus macerans* (20); cells of these organisms do not produce acetoin anaerobically from glucose, whereas their extracts form acetoin from pyruvate.

Cell suspensions and cell extracts evolved CO_2 and H_2 from pyruvate, but not from formate. These results suggested that *S. stenostrepta* metabolized pyruvate by means of a clastic system similar to that present in clostridia (25, 57), *Micrococcus lactilyticus* (56) and *Sarcina maxima* (27). This system cleaves pyruvate to acetyl-CoA, CO_2, and H_2 without production of formate. Cells or cell extracts possessing this system catalyze an exchange reaction between CO_2 and pyruvate for which CoA and TPP are required (34, 56, 58).

CO_2-pyruvate exchange activity was found both in whole cells and cell extracts of *S. stenostrepta* (Table 6). No formate-pyruvate exchange was detected at *p*H 7.0, or at *p*H 8.4, which is near the optimum *p*H for the formate-pyruvate exchange in other bacteria (32, 47). Coenzyme A stimulated CO_2-pyruvate exchange activity in the crude cell extracts of *S. stenostrepta* (Table 7). Addition of TPP resulted in low levels of stimulation (Table 7), probably because of the presence of endogenous TPP in the extracts. Reducing

TABLE 5. *Fermentation of pyruvate by cell suspensions and by cell-free extracts of Spirochaeta stenostrepta*[a]

Products	Amt of products[b]	
	Whole cells	Cell extracts
CO_2	110.5	73.0
H_2	19.5	27.9
Ethyl alcohol	35.5	31.3
Acetate	57.5	19.9
Lactate	3.7	4.2
Acetoin	0.2	12.5
Acetaldehyde, formate, butyrate, and succinate	0.0	Trace

[a] Each Warburg vessel contained 7.6×10^{10} cells or 28 mg of cell extract protein; 50 μmoles of potassium pyruvate was added to mixtures containing whole cells, 100 μmoles to mixtures containing cell extracts; incubation for mixtures including whole cells was 2.5 hr, for mixtures including extracts, 3.5 hr.

[b] Expressed as micromoles of product per 100 μmoles of pyruvate fermented.

agents and divalent cations such as Mg^{2+}, which are required for CO_2-pyruvate exchange in *M. lactilyticus* (56), were stimulatory (Table 7). Electron-accepting compounds inhibited the exchange reaction of *S. stenostrepta* (Table 7) at concentrations similar to those at which they inhibit CO_2-pyruvate exchange in *M. lactilyticus* (56).

Exchange activity in *S. stenostrepta* cell extracts treated with Dowex 1-X8 (to remove endogenous CoA) was completely dependent on the addition of CoA (Fig. 1). Optimum exchange occurred at CoA concentrations of 10^{-5}: to 10^{-6} M. Higher concentrations of Co A (i.e., 3×10^{-4} M) inhibited the CO_2-pyruvate exchange (Fig. 1), as was observed in other organisms (34, 48, 56).

Cell extracts of *S. stenostrepta* catalyzed formation of acetyl phosphate from pyruvate and exhibited phosphotransacetylase and acetate kinase activities. The phosphotransacetylase activity in CoA-depleted extracts was stimulated by addition of CoA (Table 8). The amount of acetyl phosphate formed from pyruvate was increased two-fold or more by the addition to the reaction mixture of $MnCl_2$ (10^{-4} M), TPP (2×10^{-4} M), or CoA (2×10^{-6} M) and was dependent on the presence of phosphate.

It was concluded that *S. stenostrepta* degrades pyruvate to acetyl-CoA, CO_2, and H_2 via a clostridial-type clastic reaction. Phosphotransacetylase and acetate kinase catalyze the formation of acetate from acetyl-CoA.

Formation of ethyl alcohol and lactate. Ethyl alcohol production and a rapid oxidation of NADH were catalyzed by *S. stenostrepta* cell extracts incubated with acetyl-CoA or acetyl phosphate (Table 9). A similarly rapid oxidation

TABLE 6. *$^{14}CO_2$-pyruvate and $H^{14}COOH$-pyruvate exchange by whole cells and extracts of Spirochaeta stenostrepta*[a]

Exchange	pH	Specific activity of residual pyruvate[b]	
		Whole cells	Cell extracts
$^{14}CO_2$-pyruvate	7.0	451.0	204.5
$^{14}CO_2$-pyruvate	8.4	444.0	111.5
$H^{14}COOH$-pyruvate	7.0	0.1	0.7
$H^{14}COOH$-pyruvate	8.4	0.3	0.4

[a] Specific activity (counts per minute per micromole): $NaH^{14}CO_3$, 1,045; $Na^{14}COOH$, 585; 6×10^{10} cells or 6 mg of cell extract protein were added per Warburg vessel.

[b] Counts per minute per micromole of residual pyruvate.

TABLE 7. *Effect of compounds on the CO_2-pyruvate exchange activity in cell extracts of Spirochaeta stenostrepta*[a]

Additions	Final concentration	CO_2-pyruvate exchange[b]
	M	
None		86.0
Coenzyme A	10^{-5}	235.0
Thiamine pyrophosphate	4×10^{-4}	95.0
$MgCl_2$	3×10^{-4}	154.0
Dithiothreitol	3×10^{-3}	194.0
β-Mercaptoethanol	3×10^{-3}	134.0
Methyl viologen	2×10^{-3}	8.3
Benzyl viologen	2×10^{-3}	38.0

[a] Specific activity of $NaH^{14}CO_3$ was 1,044 counts per min per μmole; 5 mg of cell extract protein was used per reaction mixture.

[b] Counts per minute per micromole of residual pyruvate.

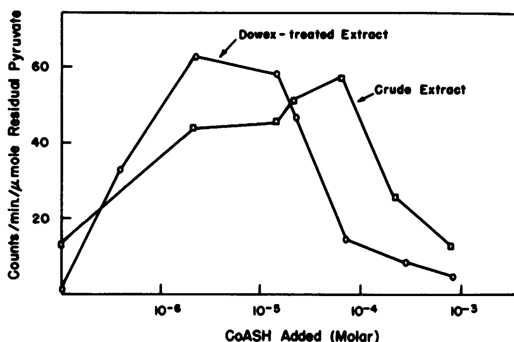

FIG. 1. *Effects of coenzyme A on CO_2-pyruvate exchange activity in cell extracts of S. stenostrepta. Crude cell extract protein (1.5 mg) and Dowex-treated cell extract protein (1.4 mg) were used. Specific activity of $NaH^{14}CO_3$ was 524 counts per min per μmole.*

TABLE 8. *Phosphotransacetylase activity in crude and in Sephadex-treated extracts of Spirochaeta stenostrepta*

Extract	Assay conditions	Acetyl phosphate utilized
		μmoles
Crude	Complete	1.17
	Acetyl phosphate omitted	0.00
	Arsenate omitted	0.99
Sephadex-treated	Acetyl phosphate omitted	0.00
	CoASH omitted	0.90
	10^{-5} M CoASH added	2.19
	3×10^{-5} M CoASH added	6.60
	5×10^{-5} M CoASH added	11.46

TABLE 9. *Ethyl alcohol formation from acetyl phosphate and from acetyl-coenzyme A by cell extracts of Spirochaeta stenostrepta*

Assay conditions	Acyl compound degraded (μmoles)		Pyridine nucleotide oxidized (μmoles)		Products formed (μmoles)	
	Ac-PO$_4$	Ac-CoA	NADH	NADPH	Acetal-dehyde	Ethyl alcohol
Complete	16.22		27.7		1.8	13.8
Acetyl phosphate omitted			5.9		1.4	2.1
CoASH omitted	9.18		14.2		2.1	7.4
NADH omitted, 30 μmoles of NADPH added	13.0			22.7	1.5	4.2
Ac-PO$_4$ omitted, 15 μmoles of acetyl-CoA added		14.7	12.9		2.6	7.5

of NADH occurred in the presence of acetaldehyde or pyruvate. According to the data (Table 9), one molecule of ethyl alcohol was formed from acetyl phosphate per two molecules of NADH oxidized. Apparently the overall reaction was NAD-specific, as ethyl alcohol formation from acetyl phosphate drastically decreased when NADPH was substituted for NADH (Table 9). These observations indicated that *S. stenostrepta* forms ethyl alcohol through enzymatic mechanisms similar to those present in *Escherichia coli* (16, 41) and involving the conversion of acetyl-CoA to ethyl alcohol by NAD-dependent acetaldehyde and alcohol dehydrogenase activities (EC 1.2.1.10 and EC 1.1.1.1, respectively).

Lactate dehydrogenase could not be demonstrated in freshly prepared *S. stenostrepta* extracts, which invariably catalyzed the decarboxylation of pyruvate to two-carbon compounds, such as acetyl phosphate. However, during storage the extracts lost the ability to degrade pyruvate to acetyl phosphate (usually 50% of the activity was lost in 4 hr at 5 C under argon). In these "aged" extracts, NAD-specific lactate dehydrogenase activity was detected.

Hydrogen evolution. Hydrogenase activity was present in cell extracts of *S. stenostrepta* (Table 10). Hydrogen gas was evolved from reduced methyl viologen, but not from reduced benzyl viologen or tetrazolium chloride. The hydrogenase activity was reversible inasmuch as cell extracts catalyzed gas uptake and reduction of methyl or benzyl viologen, methylene blue or triphenyltetrazolium chloride, when incubated in a H_2 atmosphere in the presence of one of these dyes.

Rubredoxin. Experiments were conducted to determine whether nonheme iron proteins, which frequently occur in hydrogen-producing clostridia and other anaerobes, were present in *S. stenostrepta*. A rubredoxin, which exhibited an absorption spectrum similar to that of rubredoxin of

TABLE 10. *H_2 evolution from reduced methyl viologen by cell extracts of Spirochaeta stenostrepta*

Assay conditions	H_2 evolved
	μmoles
Complete	18.80
Hydrosulfite omitted	1.70
Methyl viologen omitted	3.32
Extract omitted	5.10
Complete (boiled extract)	1.47
Methyl viologen omitted, benzyl viologen added (20 μmoles)	2.32
Methyl viologen omitted, triphenyltetrazolium chloride added (20 μmoles)	0.07

other bacteria (1, 2, 30, 37) was isolated from cell extracts of *S. stenostrepta* (Fig. 2). The absorption spectrum of the oxidized *S. stenostrepta* rubredoxin had maxima in the 275, 370 to 375, and 490 to 500 nm regions. The latter two absorption maxima were abolished when the rubredoxin was reduced by sodium hydrosulfite.

Ferredoxin was not detected in cell extracts of *S. stenostrepta*. Ammonium sulfate fractionation of cell protein eluted from DEAE columns did not yield precipitates or supernatant liquids exhibiting the absorption spectrum characteristic of ferredoxin.

Passage of *S. stenostrepta* extracts through DEAE columns removed rubredoxin, which was retained as a reddish band at the top of the column. The eluted extracts possessed little or no ability to catalyze acetyl phosphate formation from pyruvate. Activity resulting in the production of acetyl phosphate from pyruvate was restored to the DEAE-treated extracts by the addition of *S. stenostrepta* rubredoxin preparations (Table 11). Addition to these extracts of ferredoxin isolated either from *C. butyricum* or *C. pasteurianum* stimulated acetyl phosphate formation from pyruvate, but the degree of stimulation was smaller than in the presence of

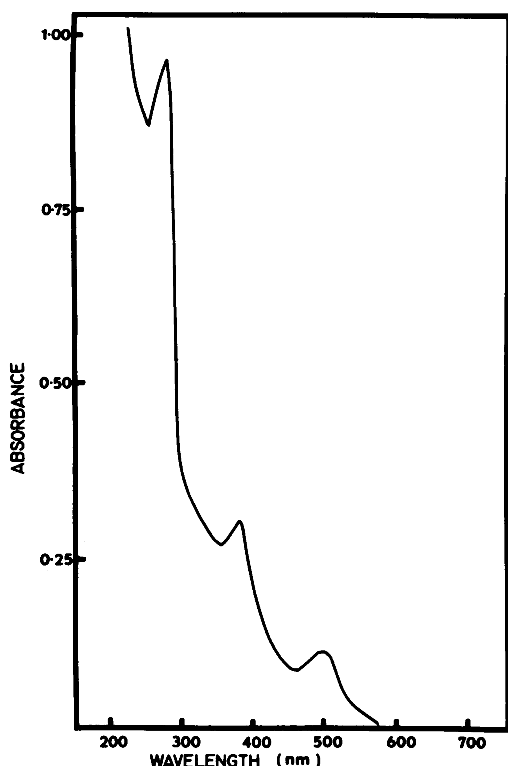

FIG. 2. *Absorption spectrum of oxidized rubredoxin isolated from S. stenostrepta.*

rubredoxin preparations (Table 11). These results suggest that rubredoxin participates in pyruvate cleavage in *S. stenostrepta*. Addition of *S. stenostrepta* rubredoxin preparations to DEAE-treated *C. pasteurianum* extracts similarly restored acetyl phosphate formation from pyruvate (Table 11).

DISCUSSION

Fulton and Smith (19, 45) reported the only extensive study previously conducted on the pathways of carbohydrate metabolism of an anaerobic spirochete. These investigators found that *Borrelia duttonii* ferments glucose primarily to lactic acid via the Embden-Meyerhof pathway and that the organism has no aerobic respiration. Similarly, as our results indicate, *S. stenostrepta* utilizes the Embden-Meyerhof pathway for carbohydrate degradation and has a strictly fermentative metabolism. However, in *S. stenostrepta*, pyruvate does not act as the major terminal electron acceptor, but is mostly decarboxylated yielding two-carbon compounds. Charge balance is maintained through the generation of hydrogen gas and the reductive steps leading to the formation of ethyl alcohol and lactate. Furthermore, *S. stenostrepta* gains one mole of adenosine triphosphate (ATP) per mole of acetate formed from acetyl phosphate, in addition to the two moles of ATP gained per mole of glucose degraded to pyruvate through the Embden-Meyerhof pathway.

Although our experiments suggest that a rubredoxin participates in the pyruvate clastic reaction of *S. stenostrepta*, the specific role (or roles) of this protein in the metabolism of the organism remains to be determined. The apparent absence of ferredoxin in *S. stenostrepta* was unexpected, inasmuch as this electron carrier has generally been found in anaerobes possessing rubredoxin.

At present, two species of strictly anaerobic, free-living spirochetes, *S. stenostrepta* and *S. zuelzerae*, have been cultured and studied (12, 13, 52). Both organisms were isolated from natural environments rich in hydrogen sulfide, which was probably of biological origin. Neither organism, however, is capable of carrying out anaerobic respiration resulting in the reduction of sulfate to sulfide. The metabolism of both species is eminently saccharolytic and, presumably, their main chemical role in nature is limited to the turnover of carbohydrates. Sulfide-rich environments may be favorable to the development of these spirochetes both because of the scarcity of molecular oxygen and because these organisms may not be sensitive to sulfide concentrations toxic to many bacteria.

TABLE 11. *Stimulation of acetyl phosphate formation from pyruvate by rubredoxin or ferredoxin*

Iron protein	Iron protein added	Acetyl phosphate formed	
		Spirochaeta stenostrepta extract[a]	*Clostridium pasteurianum* extract[b]
	μg	*μmoles*	*μmoles*
Rubredoxin from *S. stenostrepta*	0	0.15	0.40
	5	0.75	2.15
	15	1.50	5.60
	25	1.73	7.22
	50	2.10	9.35
Ferredoxin from *C. butyricum*	0	0.12	0.35
	5	0.24	3.65
	15	0.43	5.71
	25	0.55	7.00
	50	0.84	7.62

[a] DEAE-treated extract, 4 mg.
[b] DEAE-treated extract, 6 mg.

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