STUDIES ON THE CARBOHYDRATE METABOLISM OF A GRAM-NEGATIVE ANAEROBE (*BACTEROIDES SYMBIOSUS*) USED IN THE CULTURE OF *ENTAMOEBA HISTOLYTICA*[1]

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ABSTRACT

BRAGG, P. D. (Louisiana State University, New Orleans) AND R. E. REEVES. Studies on the carbohydrate metabolism of a gram-negative anaerobe (*Bacteroides symbiosus*) used in the culture of *Entamoeba histolytica*. J. Bacteriol. **83**:76–84. 1962—Resting cells of *Bacteroides symbiosus* have been shown to utilize glucose and several other monosaccharides. The fermentation of the sugars is mediated by demonstrable kinases except in the case of mannitol. The main end products of metabolism of glucose are CO_2, H_2, ethanol, and acetic, butyric, succinic, and lactic acids. Changes in the thiol used in the growth media produce different enzyme complements in the cells. Thus, cells grown with cysteine as the thiol are unable to metabolize glucosamine, whereas those grown with thiomalate rapidly degrade the amino sugar. The results of the enzyme assay and the results from experiments with C^{14}-labelled glucose suggest that glucose is metabolized by resting cells mainly by the Embden-Myerhof pathway.

Luxuriant growth of the parasitic ameba, *Entamoeba histolytica*, has been obtained in several laboratories employing a fluid medium supplemented by a suspension of viable, penicillin-inhibited cells of an anaerobic bacterial species first isolated by Shaffer and Frye (1948). Recently, Reeves, Schweinfurth, and Frye (1960) successfully substituted radiation-inactivated for antibiotic-inhibited bacterial cells, and, with both types of nonmultiplying bacterial cells, the stimulation of amebic growth has appeared to be directly related to the ability of the bacterial cells to metabolize glucose. To explore the possible dependence of rapid amebic growth upon the metabolic capabilities of the associated bacterial cells, it was decided to initiate a comparative study of the carbohydrate metabolism of both the bacteria and the amebae. This report summarizes our current observations with respect to the bacterial organism.

MATERIALS AND METHODS

The organism is a gram-negative, obligatory anaerobe which has not as yet been adequately classified. Shaffer continues to employ it in the cultivation of amebae and refers to it as a streptobacillus, although he has noted that on the basis of its morphological and cultural characteristics it appears to belong to the genus *Bacteroides* (Shaffer, 1952). A culture obtained from Shaffer was included in a study carried out by Stevens (1956) on various species of the genus *Bacteroides*. Stevens first proposed the name *Bacteroides symbiosus*, which we use to refer to our cultures from the same source. A culture of this organism was supplied to Andre R. Prévot of the Pasteur Institute who stated (*personal communication*) that he regards it as belonging to the genus *Fusocillus* in his classification of anaerobic organisms. A culture has been deposited in the American Type Culture Collection (ATCC 12829).

The basal growth medium employed in all experiments had the following composition (Reeves, Meleney, and Frye, 1957): Trypticase (casein hydrolysate, Baltimore Biological Laboratory, Baltimore, Md.), 16 g; yeast extract (Difco Laboratories, Detroit, Mich.), 1.6 g; glucose, 8 g; NaCl, 2 g; K_2HPO_4, 1.2 g. To this mixture was added either 80 ml of 0.1 M sodium thiomalate or 1 g of cysteine hydrochloride. Distilled water was added, the solution adjusted to pH 7.0, and then

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diluted to 800 ml. The bacterial cells obtained were designated "thiomalate-grown" or "cysteine-grown" cells according to whether the thiol in the growth medium was sodium thiomalate or cysteine. The bacterial cells, after harvesting and twice washing with the storage fluid, were stored at 4 C in either 80 ml of 0.1 M sodium thiomalate (pH 7.0), in the case of the thiomalate-grown cells, or in 40 ml of phosphate buffer (pH 7.0) for cysteine-grown cells. The nitrogen content of the suspension was about 1 mg per ml for the former and 1.1 to 1.3 mg per ml for the latter.

Growth studies were carried out using 10 ml of the basal medium in screw-cap tubes. The tubes were incubated at 37 C and the turbidity measured in a Coleman 6 "Junior" spectrophotometer at 600 mμ.

Cell-free extracts were prepared by suspending the washed cells obtained from 800-ml cultures in 20 ml of buffer and subjecting the suspension to sonic vibration in a 9 kc oscillator for 10 min at 0 C. The cell debris and unbroken cells were removed by centrifugation at 5,500 $\times$ *g* for 30 min at 4 C, and the supernatant fluid was used as the cell-free extract. The protein content of the bacterial extract was determined by the method of Lowry et al. (1951), using serum albumin as a standard. Cell-free extracts prepared in this way had about 6 mg protein per ml. For some experiments the extract was fractionated with $(NH_4)_2SO_4$. The precipitates produced at 50% saturation, and between 50 and 66% saturation, were dissolved in tris(hydroxymethyl)aminomethane (tris) buffer and dialyzed against more of the same buffer until the precipitates were free of salt. Unless specified, only the second of these two fractions was used.

The phosphate buffer employed in this study had the following composition: NaCl, 2 g; K_2HPO_4, 1.2 g; cysteine hydrochloride, 0.64 g. The pH was adjusted to the required value with NaOH and the solution diluted to 800 ml. The tris buffer contained: tris, 0.399 g; cysteine hydrochloride, 0.1 g. The pH was adjusted to the required value with HCl and the solution diluted to 100 ml. This buffer then contained 100 μmoles tris per 3 ml. It was frequently found to be more convenient to use double strength buffers and these are designated by the prefix 2$\times$. For some experiments the thiol was omitted and the buffer designated as "cysteine-free" buffer. For experiments using resting or penicillin-inhibited cell suspensions, where sugar utilization or gas production were to be measured, known amounts of substances were added to a medium consisting of the phosphate buffer containing Trypticase (2 g/100 ml) at pH 7.0.

Carbon dioxide and hydrogen evolution (Gest, Kamen, and Breghoff, 1950) were measured under a nitrogen atmosphere at 37 C using conventional manometric techniques (Umbreit, Burris, and Stauffer, 1957). Reactions involving the reduction of triphosphopyridine nucleotide (TPN) or diphosphopyridine nucleotide (DPN), or the oxidation of reduced DPN (DPNH) were carried out at room temperature (28 C) in an air atmosphere using 1-cm cuvettes, and changes in optical density (Δ OD) of the solutions were measured at 340 mμ in a spectrophotometer.

Total carbohydrate and also certain individual sugars were measured by the phenol-sulfuric acid method of Montgomery (1957). Glucose was used to give the standard curve for the measurement of total carbohydrates but in the other cases the respective sugars were used. Glucosamine was assayed by Rondle and Morgan's (1955) method, fructose by the method of Roe, Epstein, and Goldstein (1949), and total pentose by the orcinol method of Mejbaum (1939) with a boiling time of 40 min, and with the chromogen measured at both 620 and 670 mμ (Eltz and Van Demark, 1960) to detect the presence of ketoheptoses. Ketoheptoses were measured by the cysteine-sulfuric acid method, using mannoheptulose as the standard since this was found to give the same molar extinction coefficient as that determined for sedoheptulose by Newburgh and Cheldelin (1955). Triose phosphate was assayed by the method of Sibley and Lehninger (1949), pyruvic acid by the total hydrazone method of Friedemann and Haugen (1943), and ethanol determined by the procedure of Friedemann and Klaas (1936). Acetyl phosphate was determined by the hydroxamic acid method of Lipmann and Tuttle (1945), using succinic anhydride as a standard.

The acids produced by the breakdown of carbohydrate were separated by chromatography on Celite (Johns-Manville, New York, N. Y.), using either chloroform:*n*-butanol (9:1) saturated with 0.5 N sulfuric acid or diethyl ether saturated with 0.5 N sulfuric acid as solvents (Phares et al., 1952), and examined on paper chromatograms using *n*-propanol:ammonia (sp gr 0.88) (6:4) (Isherwood and Hanes, 1953).

The following crystalline enzymes used in this

work were prepared by C. F. Boehringer and Soehne, Mannheim, Germany, and purchased from the California Corporation for Biochemical Research (Los Angeles, Calif.): aldolase, lactic dehydrogenase, and G-6-P dehydrogenase.

The $C^{14}O_2$ samples were precipitated as $BaC^{14}O_3$ and the precipitates were collected as layers of finite thickness on filter paper discs (Henriques et al., 1946). Samples thus obtained were assayed for radioactivity with an end-window Geiger-Müller counter.

RESULTS

Glucose in the basal medium was replaced by various sugars and the extent of growth, as measured by the turbidities of the solutions obtained after 24 hr, was compared with that obtained in the presence of glucose (100). The following results were obtained: L-arabinose (65), D-sorbitol (15), D-mannitol (88), sodium D-gluconate (50), D-fructose (86), D-glucosamine (80). After two transfers growth could also be obtained on D-mannose, probably due to the adaptive formation of a specific mannokinase. L-Rhamnose, L-sorbose, D-arabinose, D-xylose, methyl α- and β-D-glucosides, lactose, maltose, sucrose, and glycerol were unable to replace glucose. Therefore, the glucose-grown cells probably do not produce extracellular glycosidases.

Similar results on sugar utilization were obtained if the disappearance of the individual sugars was measured after the addition of a thiomalate-grown cell suspension and penicillin to the basal medium. L-Arabinose and D-galactose were metabolized at 44 and 55% of the rate for glucose, respectively, and mannose was utilized only after a lag-period of 3 hr.

In the presence of 10^{-2} M fluoride the rate of sugar utilization was reduced: D-glucose (75% of normal rate), D-fructose (66%), D-galactose (64%), and L-arabinose (70%). In the case of cells grown on the cysteine-containing medium, this concentration of fluoride caused complete inhibition of glucose utilization.

End products of sugar breakdown. Paper and column chromatography showed that the same acids were formed from glucose during growth as were formed by resting cells. However, the ratios of the acids produced were different (Table 1). In the presence of added iron the rate of cell growth and of glucose breakdown was increased, and the acids were produced in a different ratio to those produced without addition of iron (Table 1).

The ratio of acetic to butyric acids was unaffected by the addition of $FeSO_4$ but there was a threefold increase in the production of these acids relative to that of lactic acid. If the free iron content of the growth medium was decreased by the addition of cyanide to a concentration of 10^{-2} M, no growth occurred.

The presence of a thiol was necessary for growth, but in the case of cysteine there was an optimum concentration (0.1 to 0.4 mg/ml medium) above and below which growth rapidly decreased. The decrease in growth at the higher cysteine concentrations may possibly be explained as being due to the removal of iron by complexing with cysteine.

Resting cysteine-grown cells rapidly degraded D-glucose, D-galactose, and D-fructose with the production of CO_2 and H_2 (Table 2). D-Mannitol and pyruvic acid also gave CO_2 and H_2 but quantitative estimations were not made in these cases to relate gas production to substrate utilization. D-Glucosamine was not metabolized by these cells, although thiomalate-grown cells rapidly utilized glucosamine with the liberation of quantitative amounts of ammonia (Table 3).

Experiments were made to relate acid production to the amount of glucose metabolized by cysteine-adapted cells during 1 hr. Ethanol and CO_2 were determined in separate experiments. The results (Table 4) show that most of the glu-

TABLE 1. *Ratio of acids (moles) formed from glucose under different conditions*

Conditions	Acetic acid	Butyric acid	Lactic acid	Succinic acid	Unidentified acids	
					A	B
Resting cells (22 hr)	1	0.52	0.67	0.014	0	0.007
Growing cells	1	0.21	0.96	0.10	0.11	0.10
Growing cells in the presence of 3.6×10^{-3} M $FeSO_4$	1	0.21	0.32	0.011	0.065	0

TABLE 2. *Production of CO_2 and H_2 from sugar by whole cells of cysteine-grown Bacteroides symbiosus*

Substrate	Moles gas produced per mole of substrate utilized*	
	CO_2	H_2O
D-Glucose	2.7; 2.61	1.05; 0.86
D-Galactose	2.48	0.78
D-Fructose	2.07	0.80

* These results are not corrected for endogenous CO_2, H_2 production, or carbohydrate utilization, which are included in the measurements.

TABLE 3. *Formation of ammonia during the metabolism of D-glucosamine by thiomalate-grown Bacteroides symbiosus*

Time	Glucosamine degraded	Ammonia formed
hr.	*μmoles*	*μmoles*
0.5	0.43	0.53
1	1.26	1.13
1.5	2.26	2.26
3	4.78	4.77
5	5.35	5.43

cose carbon may be accounted for in CO_2, ethanol, butyric, succinic, lactic, and acetic acids.

The formation and interconversion of 6-carbon phosphates. The kinase activities of the cell-free extracts of *B. symbiosus* grown on glucose in the presence of cysteine were assayed by the technique of Colowick and Kalckar (1943) using 10 μmoles of adenosine triphosphate (ATP) to 2 mg substrate, except for glucose and gluconic acid when 5 μmoles of ATP were used. The extracts showed strong D-gluco- and D-galactokinase activities with lesser D-glucono-, D-fructo-, and D-phosphofructokinase activities. The initial kinase activities corrected for endogenous activity (substrates replaced by water) were, respectively: 2.0, 3.2, 0.44, 0.57, and 0.76 μmoles substrate phosphorylated per hr per mg protein of the extract.

D-Glucokinase and D-fructokinase were also assayed by coupling the kinase reaction to the reduction of TPN in the presence of added glucose-6-phosphate (G-6-P) dehydrogenase. It was not possible to obtain quantitative activities from these experiments, nor, in fact, in any experiments using reduction of the phosphopyridine nucleotides as the assay method, since the extracts had high DPNH oxidase activity.

TABLE 4. *End products of glucose breakdown after 1 hr by cysteine-adapted cells of Bacteroides symbiosus*

End products	Amount	Total carbon
	moles	%
CO_2	2.65	44.2
Butyric acid	0.026	1.7
Succinic acid	0.059	4.0
Lactic acid	0.21	10.3
Acetic acid	0.51	16.9
Ethanol	0.55	18.4

Phosphoglucomutase and phosphohexoisomerase were demonstrated in the cysteine-grown cells by coupling these reactions to the reduction of TPN in the presence of added G-6-P dehydrogenase (Fig. 1). A measurement of the activity of phosphohexoisomerase was obtained by measuring the formation of fructose-6-phosphate (F-6-P) from G-6-P by a colorimetric method (Roe et al., 1949). The enzyme showed high activity (>32 μmoles fructose formed per hr per mg protein at 37 C) and the equilibrium mixture consisted of 67.6% G-6-P and 32.4% F-6-P.

Although phosphoglucomutase was present in the cysteine-grown cells it could not be detected in extracts of the cells grown in the presence of thiomalate.

A further difference between the cells grown in the presence of thiomalate and those grown in the presence of cysteine was that only in extracts of the latter could G-6-P and 6-phosphoglucuronic acid (6-P-G) dehydrogenase be detected (Fig. 1 and 2). However, this may not mean that these enzymes are absent from thiomalate-grown cells since, if the activities of these enzymes were less than that of reduced phosphopyridine nucleotide oxidases, the method employed would not detect them. Various procedures were used in an attempt to inactivate DPNH oxidase. These included high speed centrifugation (105,000 × *g* for 2 hr), ammonium sulfate fractionation, and carrying out the reactions in the presence of 10^{-2} M KCN (Sutton and Starr, 1960). All these methods were unsuccessful in inactivating DPNH oxidase.

The 6-P-G dehydrogenase of the cysteine-grown cells was activated by iron (Fig. 2). If an excess of cysteine was present in the solution to be assayed, this enzyme could not be detected, probably because the iron was complexed by the

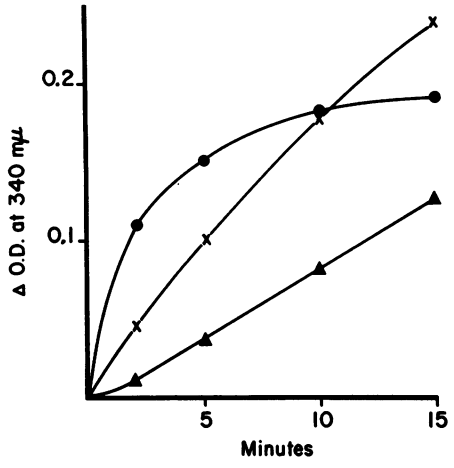

FIG. 1. *TPN reduction by extracts of cysteine-grown Bacteroides symbiosus as a demonstration of the formation of G-6-P. Each cuvette contained G-6-P (X), 5 μmoles, or F-6-P (●), 10 μmoles, or glucose-1-phosphate (▲), 10 μmoles; TPN, 0.3 μmoles; nicotinamide, 30 μmoles; 2 × phosphate buffer, pH 7.0, 1.5 ml; extract, 1.5 mg protein; water to total volume of 3.4 ml. Crystalline G-6-P dehydrogenase was present in ● and ▲ cuvettes. The OD was compared to solutions in which the substrate had been replaced by water.*

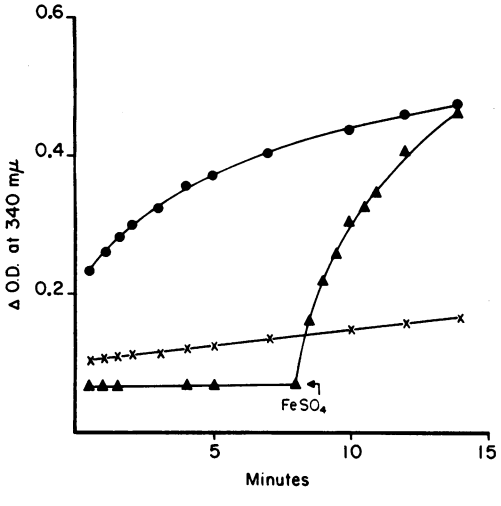

FIG. 2. *TPN reduction by fractionated extracts of cysteine-grown Bacteroides symbiosus with 6-P-G as substrate. Each cuvette contained 6-P-G (except in X) 5 μmoles; $MgCl_2$, 10 μmoles; $FeSO_4$, 0.7 μmoles (except in ▲); TPN, 0.3 μmoles; cysteine-free tris buffer, pH 7.0, 2 ml; extract, 0.7 mg protein; water to total volume of 3.1 ml. After 8 min $FeSO_4$, 0.7 μmoles, was added to ▲.*

thiol. Since the cells are grown in the presence of cysteine, 6-P-G dehydrogenase may not be functional in their metabolism. This would mean that the pentose phosphate cycle would be of little importance to the growing cells.

The Embden-Myerhof pathway. Aldolase and phosphofructokinase are the two enzymes unique to the Embden-Myerhof pathway. Both were detected in extracts prepared from both cysteine- and thiomalate-grown cells of *B. symbiosus*. Aldolase, as determined by the method of Sibley and Lehninger (1949), had an activity of 0.21 μmoles of fructose-1,6-diphosphate (HDP) split per hr per mg protein of an extract of the cysteine-grown cells. A modification of this technique was used to assay phosphofructokinase. HDP was replaced by F-6-P (10 μmoles), ATP (20 μmoles), and $MgCl_2$ (10 μmoles), and an excess of crystalline aldolase was added. For the cysteine-grown cells 1.6 μmoles of substrate was phosphorylated per hr per mg protein of the extract. Phosphofructokinase was also assayed manometrically.

The oxidation of 3-phosphoglyceraldehyde in the presence of cysteine and arsenate, with DPN as the electron acceptor, was demonstrated with extracts from both types of cells. The triose phosphate was formed by the action of crystalline aldolase on HDP.

In the presence of DPNH, pyruvate was rapidly reduced (Fig. 3). This result, together with the fact that lactic acid was a major end product of glucose metabolism, suggested that a lactic dehydrogenase was present in the cells. The pathway to pyruvate is probably from 3-phosphoglyceraldehyde by way of the remaining reactions of the Embden-Myerhof pathway. The presence of these enzymes was suggested by the oxidation of DPNH in the presence of 3-phosphoglycerate (Martinez and Rittenberg, 1959). There was an appreciable endogenous oxidation of DPNH by the extracts, paralleled by the assay system in the absence of adenosine diphosphate (ADP). Addition of ADP, however, resulted in a marked increase in the rate of oxidation of DPNH (Fig. 4). The enzymes (phosphoglyceromutase, enolase, phosphopyruvic transphosphorylase, and lactic dehydrogenase) involved in these reactions are present in extracts from both thiomalate- and cysteine-grown cells.

The formation of acetohydroxamic acid from acetate in the presence of hydroxylamine, ATP, and extracts of cysteine-grown cells was taken as

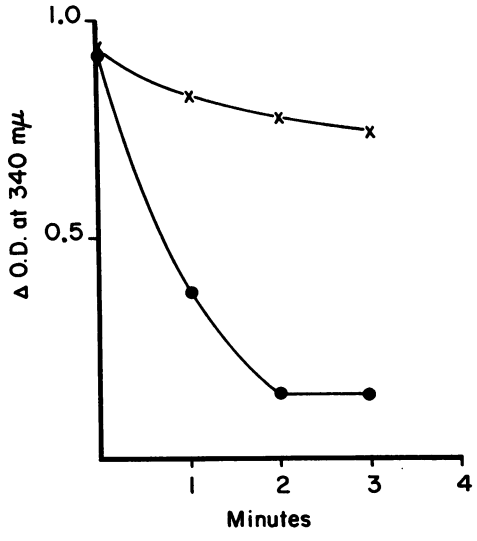

FIG. 3. *DPNH oxidation by fractionated extract of cysteine-grown cells of Bacteroides symbiosus with pyruvate as substrate. Each cuvette contained sodium pyruvate, 5 μmoles; DPNH, ca 0.5 μmoles; tris buffer, pH 7.0, 2.5 ml; extract, 2.1 mg protein; water to total volume of 3.5 ml. In the control (X) the substrate was replaced by water.*

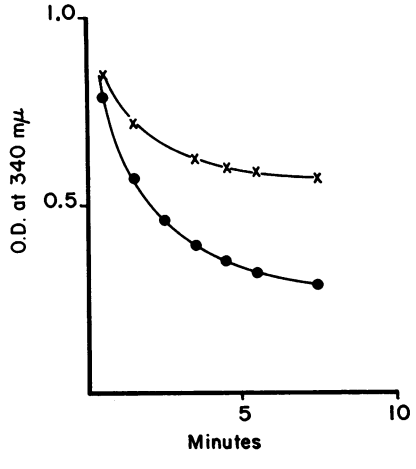

FIG. 4. *DPNH oxidation by fractionated extracts (50% $NH_4(SO_4)_2$) of Bacteroides symbiosus with 3-phosphoglycerate as substrate, and the effect of ADP. Each cuvette contained 3-phosphoglycerate, 5 μmoles; DPNH, ca 0.5 μmoles; $MgCl_2$, 10 μmoles; tris buffer, pH 7.0, 2.5 ml; extract, 2.3 mg protein; water to total volume of 3.5 ml. X = basic mixture. ● = basic mixture and 1 μmole ADP. The rate of endogenous oxidation of DPNH is similar to curve X.*

evidence of an acetokinase (Lipmann and Tuttle, 1945). The standard curve was constructed using succinohydroxamic acid. Acetohydroxamic acid was formed at the rate of 1.0 μmole/hr/mg protein.

Pentose phosphate pathway. Cell extracts prepared from cysteine-grown *B. symbiosus* did not utilize ribose-5-phosphate (R-5-P). Repeated experiments with the original extract and the extract after dialysis, with the addition in various combinations of cysteine hydrochloride, phosphate, iron, potassium, sodium and magnesium ions, and thiamine pyrophosphate, have failed to show that any degradation of R-5-P had occurred. In contrast to this, extracts from thiomalate-grown cells readily transformed R-5-P to ketohexose, ketoheptose, and triose (Fig. 5), and degraded 6-P-G to pentose and ketohexose (Fig. 6). When samples from the incubation mixture with 6-P-G as substrate were submitted to the cysteine-carbazole test (Dische and Borenfreund, 1951) for ketopentoses and ketohexoses, the absorption spectra showed a gradual change from the region characteristic of ketopentoses (545 mμ) to that characteristic of ketohexoses (565 mμ). This is more clearly shown by comparison of the ratios of the optical density at 565 mμ to that at 545 mμ: 30 min, 0.43; 60 min, 0.92; 120 min, 1.03 (Agosin and Aravena, 1960). Since these compounds are all components of the pentose phosphate cycle, the results suggest that this pathway may be present in thiomalate-grown cells.

The possibility that 6-P-G might be metabolized via the Entner-Doudoroff pathway was examined for both cysteine- and thiomalate-grown cells. The reaction mixture had the same composition as that of Fig. 6. Hydrazine was present to trap 3-phosphoglyceraldehyde, and arsenite to inhibit utilization of pyruvate. Both triose and pyruvate were estimated for the extracts of cysteine-grown cells but only pyruvate was measured with thiomalate-grown cells. In both cases no evidence was obtained of cleavage of 6-P-G to 3-phosphoglyceraldehyde and pyruvic acid. In support of these experiments it was found that extracts of cysteine-grown cells were unable to produce CO_2 from 6-P-G.

Experiments with glucose-C^{14}. Both cysteine- and thiomalate-grown cells were permitted to metabolize glucose-C^{14}-labeled uniformly or at carbon atom 1 or 2 or 6, and the CO_2 which was

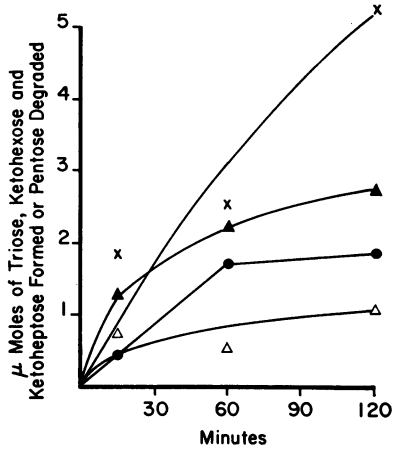

FIG. 5. *Ribose-5-phosphate utilization by extracts of thiomalate-grown Bacteroides symbiosus. The reaction mixture, incubated at 37 C, contained R-5-P, 50 μmoles; tris, 330 μmoles; thiamine pyrophosphate, 1 μmole; $MgCl_2$, 50 μmoles; $FeSO_4$, 3.5 μmoles; extract, 12.5 mg protein; taken to pH 7.4 and water added to give total volume of 10 ml. Pentose (X), ketohexose (▲), triose (△), and ketoheptose (●) were assayed as described in Methods.*

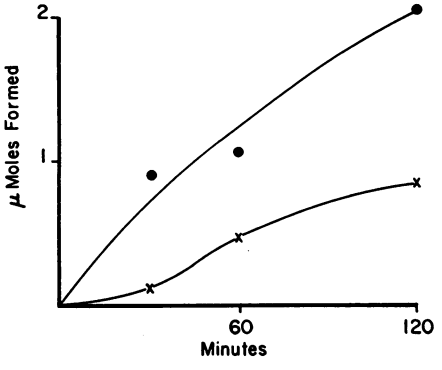

FIG. 6. *Formation of pentose and ketohexose (X) from 6-P-G by extracts of thiomalate-grown Bacteroides symbiosus. The reaction mixture incubated at 37 C contained 6-P-G, 50 μmoles; tris, 330 μmoles; hydrazine sulfate, 560 μmoles; arsenite, 20 μmoles; thiamine pyrophosphate, 1 μmole; $MgCl_2$, 50 μmoles; $FeSO_4$, 3.5 μmoles; extract, 14.8 mg protein; taken to pH 7.4; water to total volume of 10 ml.*

produced was collected in 2 N sodium hydroxide in the center well of a Warburg flask. From the activity of the CO_2 formed from the uniformly labeled glucose, the extent of the breakdown of the labeled glucose was determined. By using this value, the activities of the CO_2 released from the

TABLE 5. *Liberation of $C^{14}O_2$ from labelled glucose by resting cells of Bacteroides symbiosus*

Position of label on glucose molecule	Theoretical activity recovered as $C^{14}O_2$	
	Thiomalate cells	Cysteine cells
	%	%
C-1	3.62	6.27
C-2	1.86	1.42
C-6	2.76	4.68
Activity $\frac{C\text{-}1}{C\text{-}6}$	1.31	1.34

variously labeled glucose substrates were expressed as percentages of the theoretical amount which should have appeared during the experiment if the particular carbon atom had been completely converted to CO_2 by the bacteria (Table 5). Carbon dioxide was not released preferentially from C-1 over C-6 to any great degree, thus showing that the pentose phosphate cycle was of low activity in both cells and suggesting that either the Embden-Myerhof or Entner-Doudoroff pathway was present. The slightly greater liberation of CO_2 from C-1 and C-6 over C-2 supported the presence of the Embden-Myerhof pathway, since it suggested that there had been a symmetrical cleavage of the hexose molecule into two triose moieties which had then undergone a limited cleavage of the primary carbon atom.

DISCUSSION

Glucose, fructose, galactose, gluconic acid, and mannitol are readily utilized by cells of *B. symbiosus*. In all cases, except that of mannitol, metabolism is preceded by phosphorylation. A mannitol kinase was not detected, which infers that mannitol may be initially dehydrogenated to give fructose, since mannitol is readily converted to CO_2 by resting cells. Opposed to this hypothesis is the fact that a mannitol dehydrogenase, linked to either DPN or TPN, was not found. The reactions whereby mannitol enters into the known pathways of carbohydrate breakdown are therefore still obscure.

The possibility that glucose might be utilized without prior phosphorylation was examined, but no evidence for DPN- or TPN-linked dehydrogenases was found. Gluconic acid itself is phosphorylated at an appreciable rate by an extract of cysteine-grown cells, and the enzymes necessary for its conversion to G-6-P are present in the cell-free extracts.

Carbon dioxide, ethanol, lactic, butyric, succinic, and acetic acids are the main end products of glucose breakdown by cysteine-grown cells. Hydrogen is also produced. The ratio and amount of the acids produced is dependent upon the iron concentration of the medium. A similar result has been observed with *Clostridium welchii* (*C. perfringens*) (Pappenheimer and Shaskan, 1944). When pyruvate was the substrate CO_2 and hydrogen were still produced, suggesting that pyruvate was the more immediate source of hydrogen production. Although the details of the enzymes involved in these reactions are unknown (Gest, 1954), the constant ratio of acetic to butyric acid, and the varying amount of lactic acid produced during growth under the different states of iron nutrition, suggest that the iron-sensitive reactions occur at a lower level in the pathway than pyruvate but preceding the reactions of acetyl phosphate or acetyl-CoA. The amount of lactic acid formed would then be dependent upon the activity of the pyruvate-decarboxylating system or upon the activity of lactic dehydrogenase. The ratio of butyric to acetic acids would remain unchanged, although the total amount of these acids could vary. The pathway from glucose to pyruvate, therefore, needs elucidation.

In extracts of both cysteine- and thiomalate-grown cells, all enzymes of the Embden-Myerhof pathway were present; glucose might be converted to pyruvate by this means. This pathway appears to be the major route of carbohydrate breakdown in cysteine-grown cells, since fluoride completely abolishes glucose utilization. With thiomalate-grown cells, however, the rate of sugar utilization is merely lowered to about 60 to 75% of the normal rates by equivalent concentrations of fluoride. In support of these results with fluoride, R-5-P was metabolized only by extracts of thiomalate-grown cells to yield products characteristic of the pentose phosphate cycle. Repeated attempts to find DPN- or TPN-linked G-6-P and 6-P-G dehydrogenases were not successful, although 6-P-G was metabolized to pentose and ketohexose.

G-6-P and 6-P-G dehydrogenases were found in extracts of cysteine-grown cells, although the remainder of the pentose phosphate pathway appeared to be absent. These enzymes do, however, provide the pathway by which gluconate, which is readily phosphorylated by these extracts, can enter the Embden-Myerhof pathway. An additional point at which iron appears to regulate the carbohydrate metabolism is shown by 6-P-G dehydrogenase, which is strongly activated by iron but only to a negligible extent by magnesium ions.

The Entner-Doudoroff pathway, as measured by the production of equimolecular quantities of 3-phosphoglyceraldehyde and pyruvic acid in the presence of hydrazine and arsenite, was not detected in either of the extracts.

Experiments with glucose, labeled at various positions with carbon-14, showed that, although certain enzymes of the pentose phosphate cycle were present in sonic extracts of thiomalate- and cysteine-grown cells, this pathway was of minor importance during the metabolism of glucose by resting cells. These results, taken together with the enzyme assay experiments, suggested that glucose was predominantly utilized by resting cells via the Embden-Myerhof pathway.

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