Utilization of Glucose in Heterotrophic Media by *Thiobacillus intermedius*

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The growth yield of *Thiobacillus intermedius* is greater in glucose-yeast extract or glucose-casein hydrolysate broth than in comparable media without glucose. The quantity of glucose utilized in the glucose-supplemented media is much greater than the increase in cell yield observed relative to the unsupplemented media. Addition of glucose to cell-free extracts of glucose-yeast extract or glucose-casein hydrolysate grown cells results in the reduction of endogenous cytochrome *c*. Thus, in these media, glucose serves as a source of energy. This is in contrast to thiosulfate-glucose broth in which glucose provides only cell carbon. The presence of thiosulfate in glucose-casein hydrolysate broth results in a marked decrease in glucose consumption. Cytochrome *c* in extracts of cells grown in this medium is not reduced by glucose addition. The data suggest that thiosulfate prevents the utilization of glucose for energy generation. The final growth yield in glucose-casein hydrolysate broth is directly proportional to the initial glucose concentration, although not all the glucose was utilized even at the lowest concentration tested. This effect may be due to an inefficient glucose transport in this organism.

Thiobacillus intermedius, a mixotrophic chemolithotroph (8), grows in autotrophic or complex heterotrophic medium, but optimal growth occurs in a mixotrophic medium, i.e., in the presence of thiosulfate and organic substrates (2). Among other compounds, the presence of glucose in thiosulfate mineral salts medium or in yeast extract broth markedly stimulates the growth rate and yield of this organism (2). Ribulose diphosphate carboxylase is repressed during growth in thiosulfate-glucose broth, and glucose provides over 90% of cell carbon under these conditions (3). Since the amount of glucose utilized approximated the amount of cell material formed, it was concluded (3) that glucose serves as a carbon source but not as an energy source in thiosulfate-glucose broth.

The basis for the glucose-stimulated growth in glucose-yeast extract broth has not been investigated, but it was suggested (2) that in this medium glucose might serve as an energy source for assimilation of carbon furnished by compounds in yeast extract. An analogous situation has been described for *Desulfovibrio desulfuricans* growing in an isobutanol-yeast extract medium (6). If this were the case, a paradox exists in that glucose would provide *T. intermedius* carbon in one nutritional environment, energy in another, and yet would not serve as the sole carbon and

energy source (2). Since the organism grows in autotrophic medium or in synthetic media with thiosulfate plus single organic compounds, a need for specific growth factors cannot explain the paradox. The following study was undertaken to delineate the role of glucose in the growth of *T. intermedius* in complex heterotrophic media.

MATERIALS AND METHODS

Growth media. The mineral salts (MS) base used in the preparation of various media had the following composition in gram per cent (w/v): NH_4Cl, 0.1; $MgCl_2 \cdot 6H_2O$, 0.05; $MgSO_4$, 0.03; KH_2PO_4, 0.04; $K_2HPO_4 \cdot 3H_2O$, 0.06; $FeCl_3 \cdot 6H_2O$, 0.002. This base was supplemented with $Na_2S_2O_3 \cdot 5H_2O$ or organic substrates, or both, at concentrations specified in the text. Media to which complex organic substrates were not added also contained 3% (v/v) Pfennig's (7) trace salts. Bromothymol blue (0.003%) was used as an internal pH indicator. Distilled, deionized water was used throughout. Phosphates, $FeCl_3$, bromothymol blue, $Na_2S_2O_3 \cdot 5H_2O$, and the organic supplements were sterilized separately and added aseptically to the medium. Parallel stock cultures were maintained on autotrophic, mixotrophic, and heterotrophic media. Those on heterotrophic media were periodically checked for their ability to grow autotrophically.

Growth procedures. Growth experiments were done in 250-ml culture flasks with side arms (Nephalo flasks, Bellco Glass Inc., Vineland, N.J.) containing 30 to 35 ml of medium. The inocula used were grown

in homologous media. Growth was followed by measuring increase in turbidity of cultures by using a Klett-Summerson colorimeter with a 540-nm filter. A standard curve was prepared relating Klett units to dry weight of cells. Cultures were shaken on a rotary shaker at 30 C. Incubation was continued until the stationary period was reached and growth yields were calculated from maximal turbidities. Acid produced in media containing thiosulfate was periodically neutralized with sterile Na_2CO_3 solution. The pH of heterotrophic media was maintained at about 6.8 by periodic addition of dilute H_2SO_4.

Fernbach flasks containing 700 to 800 ml of medium were employed for obtaining large cell crops. Starter cultures on thiosulfate and thiosulfate-glucose agar media were grown in Roux bottles. Growth harvested from six to eight bottles was used to inoculate a single flask. Otherwise, 100 ml of culture grown in the homologous medium was used per flask.

To increase cell yields, additional thiosulfate was added to thiosulfate-containing media during culture development until 2 to 3 g/100 ml had been consumed, at which point the cells were harvested. No additions were made to the heterotrophic media during growth. Cells in these media were harvested toward the end of exponential phase. All cultures were maintained near pH 6.8 as already described.

Every culture was tested for purity at the time of harvesting by microscopic examination and by streaking on thiosulfate-yeast extract plates. The speed of colony development, colonial morphology, and acid production on thiosulfate-yeast extract plates readily distinguished *T. intermedius* from any heterotrophic contaminants.

Preparation of cell-free extracts. Cells from various media were harvested by centrifugation at 4 C and washed twice in cold 0.006 M potassium phosphate buffer (pH 6.8). The washed cells (about 0.7 g, dry-weight basis) were suspended in about 10 ml of the same buffer. Depending on the medium used, growth from 2 to 12 Fernbach flasks was required to make such suspensions. The cell suspensions were either used immediately or stored at −40 C.

Cells were broken by passing the suspension twice through a chilled French pressure cell at about 20,000 psi. One passage was sufficient for cells grown in most media, but autotrophically grown cells were not effectively broken by a single treatment. To keep the procedure constant, all suspensions were processed twice. After treatment, the mixture was centrifuged at 11,000 × g for 50 min at 4 C to sediment unbroken cells and debris. Such extracts (crude cell extract) were used to study cytochrome-linked reactions.

Reduction of endogenous cytochrome c in cell extracts was shown by difference spectra between systems containing and lacking substrate. With glucose as the substrate, the reaction mixture contained, in a total volume of 0.25 ml, tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (pH 7.0), 2.5 μmoles; glutathione, 1.25 μmoles; $MgCl_2$, 0.65 μmole; $MnSO_4$, 0.2 μmole; nicotinamide adenine dinucleotide (NAD), 0.10 μmole; nicotinamide adenine dinucleotide phosphate (NADP), 0.10

μmole; adenosine triphosphate (ATP), 0.25 μmole; glucose, 5 μmoles, and crude cell extract protein, 2.0 to 2.5 mg. With thiosulfate as the substrate, the reaction mixture consisted of Tris-maleate buffer, 2.5 μmoles; $Na_2S_2O_3 \cdot 5H_2O$, 5 μmoles, and crude cell extract protein, 2.0 to 2.5 mg. Spectrophotometric measurements were made with a Cary 15 recording spectrophotometer.

Protein was determined by the Lowry et al. (4) method; glucose with the glucostat reagent (Worthington Biochemical, Freehold, N.J.), or, in media containing thiosulfate, with the anthrone reagent; and thiosulfate by iodometric titration.

RESULTS

The effect of glucose on the growth of *T. intermedius* in complex heterotrophic media was reinvestigated. Two complex media, yeast extract and casein hydrolysate broths, were used. Addition of glucose to either yeast extract or casein hydrolysate broth enhances both the growth rate and yield of this organism (Table 1).

The amount of glucose consumed at the termination of growth in glucose-yeast extract or glucose-casein hydrolysate broth ranged between 25 to 45% of that added (Table 1). Since considerable glucose remained and the pH was maintained near optimum, it was not clear why growth stopped in these media. Three possible reasons were considered to explain this cessation of growth: (i) lack of effective aeration due to high cell density of the cultures; (ii) accumulation of toxic end product(s) in the medium; and (iii) exhaustion of some component(s) of yeast extract or casein hydrolysate which is essential for growth.

To decide among these alternatives, two cultures of *T. intermedius*, one in glucose-yeast extract broth, and the other in yeast extract broth, were allowed to reach stationary phase of growth while an optimal pH level was maintained. After maximal turbidity was reached, 10 ml of each culture were transferred to Nephalo flasks containing 20 ml of sterile MS solution lacking carbon and energy sources. The diluted cultures were shaken for 3 days during which time there was no detectable change in their turbidity. Then, 0.1% yeast extract was added to the diluted glucose-yeast extract culture and 0.5% glucose was added to the diluted yeast extract culture. There was rapid growth in the former but none in the latter (Fig. 1). The latter culture was viable since growth commenced on addition of yeast extract. It can be concluded that some component(s) of yeast extract is essential for the utilization of glucose by *T. intermedius* in glucose-yeast extract broth; when this is exhausted, growth ceases. This conclusion also applies to the cessa-

TABLE 1. *Thiobacillus intermedius: growth yield and glucose consumption in various media*

Medium[a]	Generation time	Glucose[b]			Dry weight cells[b]	
		Initial	Residual	Used	Final yield	Increase[c]
	hr					
0.5% YE	12				26.0	
0.5% YE + 0.5% G	9	250	187	63 (25)[d]	37.0	11.0
0.5% T + 0.5% G[e]	8	250	228	22 (8.8)	14.0	
0.5% C	27				14.5	
0.5% C + 0.5% G	22	250	172	88 (35)	26.0	11.5
0.5% C + 0.5% G + 1.0% T	13	250	227	23 (9.2)	33.2	
0.5% C + 0.1% G		50	29	21 (42)	18.6	4.1
0.5% C + 0.3% G		150	97	53 (36)	21.2	6.7
0.5% C + 0.5% G	22	250	172	88 (35)	26.0	11.5
0.5% C + 0.6% G		300	197	103 (35)	29.2	14.7
0.5% C + 0.8% G		400			32.8	18.3
0.5% C + 1.0% G		500	277	223 (45)	37.6	23.1

[a] Additions to mineral salts base: C, casein hydrolysate; G, glucose; T, $Na_2S_2O_3 \cdot 5H_2O$; and YE, yeast extract.

[b] Milligrams per 50 ml of culture.

[c] Caused by glucose addition to complex media.

[d] Numbers in parentheses represent glucose consumed expressed as per cent of that added.

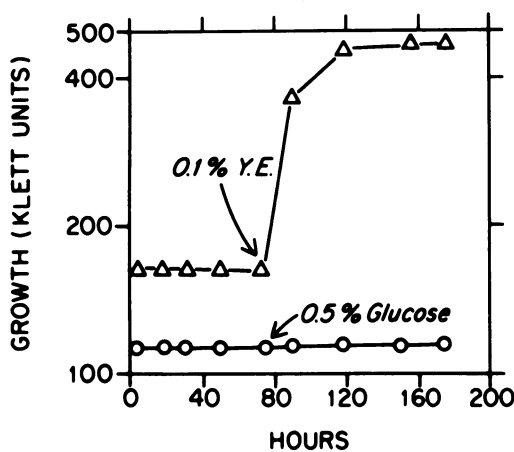[e] Data of London and Rittenberg (3) reproduced for reference.

FIG. 1. *Effect of addition of yeast extract (YE) and glucose to diluted stationary glucose-yeast extract and yeast extract cultures, respectively, of Thiobacillus intermedius. Symbols:* ○, *cells pregrown in yeast extract (0.5%) broth;* △, *cells pregrown in glucose (0.5%)-yeast extract (0.5%) broth. Glucose and yeast extract were added as indicated. One Klett unit is equivalent to approximately 0.11 mg (dry weight) of cells per 50 ml of culture.*

tion of growth in glucose-casein hydrolysate broth, since similar results were obtained when yeast extract was replaced by casein hydrolysate in the above experiment.

Glucose is consumed to a greater extent in the complex heterotrophic media than in thiosulfate-glucose broth (Table 1). In the latter medium, in which most of the cell carbon is derived from the hexose (3), the amount of glucose consumed is roughly equal to the cell material synthesized. In the former media, in which the complex substrates presumably continue to serve as carbon sources, however, glucose consumed is substantially in excess of the increase in the quantity of cell material synthesized when glucose is added (Table 1).

The ability of extracts of cells grown in glucose-supplemented complex heterotrophic media and in thiosulfate-glucose broth to couple glucose oxidation with the reduction of endogenous cytochrome c was compared. Addition of glucose to cell-free extracts of glucose-yeast extract or glucose-casein hydrolysate grown cells resulted in the rapid appearance of a reduced cytochrome c spectrum; cytochrome c in these extracts was also reduced by yeast extract, casein hydrolysate, or thiosulfate. In contrast, no reduction of endogenous cytochrome c could be detected within 40 min after the addition of glucose to extracts of thiosulfate-glucose-grown cells, even in the presence of 5 μmoles of KCN in the reaction mixture. The addition of thiosulfate to these extracts with and without KCN rapidly caused such a reduction. It was shown that glucose is phosphorylated in the latter extracts under the conditions of the cytochrome reduction experiment. Thus an enhanced adenosine triphosphatase activity cannot account for lack of cytochrome c reduction.

The fate of glucose in the presence of both

thiosulfate and complex organic matter was also examined. In thiosulfate-glucose-casein hydrolysate broth, both the growth rate and growth yield were greater than in glucose-casein hydrolysate broth, but there was a marked reduction in the amount of glucose consumed (Table 1). Attempts to couple glucose oxidation with the reduction of endogenous cytochrome *c* in the cell-free extracts of thiosulfate-glucose-casein hydrolysate grown cells were unsuccessful. These extracts rapidly linked thiosulfate or casein hydrolysate oxidation with endogenous cytochrome *c* reduction.

Although most of the glucose remains unused in thiosulfate-glucose broth, London and Rittenberg (3) showed that growth yields in this medium are directly proportional to glucose concentration in the range of 0.1 to 0.7%. The same relation was found for glucose-casein hydrolysate broth: the growth yield increased linearly with increasing glucose concentration over the entire range tested, i.e., 0.1 to 1.0% (Fig. 2). The amount of glucose consumed at the termination of growth also increased with increasing glucose concentration in the medium (Table 1).

DISCUSSION

The evidence presented here indicates that in complex heterotrophic media *T. intermedius* uses glucose as a source of energy, presumably for the assimilation of carbon compounds in the complex substrates. This conclusion is supported by the radiorespirometric experiments, presented in the following paper (5), which demonstrate release of $^{14}CO_2$ from ^{14}C-glucose added to these media. The data on glucose consumption and cytochrome *c* reduction show that glucose in thiosulfate-glucose or thiosulfate-glucose-casein hydrolysate broth provides cell carbon and no, or little, energy for the growth of *T. intermedius*. Presumably this is because of the repression of several enzymes of glucose metabolism (5). Thus, although *T. intermedius* is unable to derive energy from glucose in one nutritional environment, it does so in another.

The regulatory mechanisms which enable this organism to use glucose differently in various nutritional environments will be considered in detail in the following paper (5). Evidence presented here, however, demonstrates that the presence of thiosulfate in a complex medium interferes with the utilization of glucose. Cells consume much less glucose in a thiosulfate-supplemented glucose-casein hydrolysate broth and extracts of cells harvested from this medium do not exhibit reduced cytochrome *c* spectra upon the addition of glucose. In both these respects,
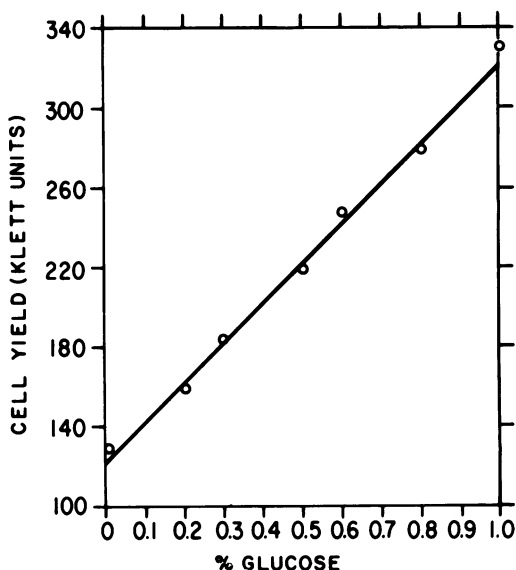

FIG. 2. *Growth yields of Thiobacillus intermedius in glucose-casein hydrolysate (0.5%) broth as a function of glucose concentration. One Klett unit is equivalent to approximately 0.11 mg (dry weight) of cells per 50 ml of culture.*

thiosulfate-glucose-casein hydrolysate grown cells are identical to those grown in thiosulfate-glucose broth.

If thiosulfate interferes with the metabolism of glucose by *T. intermedius*, the converse apparently is also the case, i.e., glucose, or some product of its metabolism, interferes with thiosulfate oxidation. London and Rittenberg (3) have reported that cell suspensions harvested from thiosulfate-glucose broth oxidize thiosulfate at a much lower rate than suspensions of cells grown in thiosulfate-mineral salts medium. It was also shown that with increased glucose concentration in the medium there is a decrease in thiosulfate-oxidizing activity. This mutual antagonism between glucose metabolism and thiosulfate oxidation, the chemolithotrophic energy-generating mechanism in *T. intermedius*, is reminiscent of a similar antagonism between fructose metabolism and the hydrogen-oxidizing system which has been reported in *Hydrogenomonas* H-16 (1).

The effect of glucose concentration on the growth yields of *T. intermedius* in thiosulfate-glucose and glucose-casein hydrolysate broths is peculiar. In both cases, even though most of the added glucose remains unused during growth, cell yields increase in direct proportion to the glucose concentration of the medium. This effect suggests an inefficient, concentration-dependent glucose transport in *T. intermedius*. A study of

glucose transport in this organism might provide an explanation for its inability to grow in an unsupplemented glucose-MS medium.

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