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NOTES

Phosphotransferase Activity in *Clostridium acetobutylicum* from Acidogenic and Solventogenic Phases of Growth

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***Clostridium acetobutylicum* cells, when energized with fructose, transported and phosphorylated the glucose analog 2-deoxyglucose by a phosphoenolpyruvate-dependent phosphotransferase (PT) system. Butanol up to 2% did not inhibit PT activity, although its chaotropic effect on the cell membrane caused cellular phosphoenolpyruvate and the 2-deoxyglucose-6-phosphate to leak out. Cells harvested from the solventogenic phase of batch growth had a significantly lower PT activity than did cells from the acidogenic phase.**

The factors that limit the production of butanol, acetone, and ethanol by *Clostridium acetobutylicum* are the subject of much current interest because of the industrial importance of these metabolic end products (19). One focus of study is the possible direct effect of the end products of fermentation on sugar catabolism (9, 13); decreased rates of glycolysis are thought to result in inhibition of growth. Indeed, it has been shown that butanol addition inhibits growth and glucose uptake of *C. acetobutylicum* cells (3, 15, 16). For another clostridium, *Clostridium thermocellum*, it has been proposed that membrane-associated glycolytic enzymes may be the sites of inhibition by the end product ethanol (9).

Clostridia transport many sugars, including glucose, by phosphoenolpyruvate (PEP)-dependent phosphotransferase (PT) systems (reviewed in references 4, 6, and 18). PT activity for several sugars has been demonstrated in cell-free preparations and toluene-permeabilized cells (2, 5, 10, 14, 17). Thus, in *C. acetobutylicum* these transport systems may be inhibited by butanol present in the medium during the solventogenic phase of growth; this would be responsible for the lower glycolytic rates observed in the later growth phase.

To define a possible butanol-sensitive step of glucose catabolism in *C. acetobutylicum*, we investigated whether the first step of glucose utilization, uptake, is inhibited by the alcohol. Butanol may inhibit this membrane-linked function directly, or indirectly at a later step of glycolysis, and thus decrease the supply of PEP. In this communication we report that *C. acetobutylicum* cells harvested during the solventogenic phase have a decreased glucose PT activity compared with acidogenic-phase cells, but that butanol is not responsible for the decrease. In addition, we conclude that the assay for PT activity cannot be used to determine whether butanol inhibits glucose catabolism.

Cells of *C. acetobutylicum* ATCC 4259 were grown anaerobically (1) in a medium consisting of 0.5% yeast extract, 0.5% tryptone (both from Difco Laboratories, Detroit, Mich.), 0.5% sodium acetate, 0.0001% resazurin, and 6% glucose (autoclaved separately). The medium was inoculated with 0.02 volume of a stationary-phase culture containing spores, heated for 3 min in a boiling water bath, and incubated at 34°C. For sugar uptake experiments the cells were harvested by centrifugation from the acidogenic or solventogenic phase of batch growth (as determined by gas chromatography), washed twice with 20 mM potassium phosphate buffer (pH 6.7), containing 10 mM $MgCl_2$, and resuspended in the same buffer. The uptake assays were carried out at 28°C in mixtures consisting of the potassium phosphate buffer, cells at a final density of 1.0 to 1.4 mg (dry weight)/ml, radiolabeled substrate, either D-[U-^{14}C]glucose ([^{14}C]glucose) or 2-[G-^{3}H]deoxy-D-glucose (^{3}H-DG) (both from Dupont-New England Nuclear Corp., Boston, Mass.) at the indicated concentrations, and, where indicated, 50 mM D-fructose as the energy source. Samples (1 ml) were removed at various time intervals, and the cells were separated from the incubation mixtures by centrifugation through silicone oil, as described previously (1, 11). All manipulations were carried out within an anaerobic glove box, as described previously (1). The cell pellets and aliquots of the supernatants were counted for radioactivity.

To assay PT activity, we first sought a nonmetabolizable glucose analog. The effect of three potential competitors was tested. 2-Deoxyglucose (DG) was found to inhibit the uptake of 0.5 mM [^{14}C]glucose by 43% at 5.0 mM DG, and by 85% at 25 mM DG (Table 1). In contrast, addition of α-methyl glucoside (1-*O*-methyl-α-D-glucopyranoside) or 3-*O*-methyl glucose (3-*O*-methyl-D-glucopyranose) had little effect, even when added at 50 times the glucose concentration. These results indicate that the *C. acetobutylicum* glucose uptake system had a greater affinity for DG than for the other two analogs tested.

Uptake of ^{3}H-DG by *C. acetobutylicum* is shown in Fig. 1. Only cells supplied with an exogenous energy source, i.e., fructose, took up ^{3}H-DG. This suggests that fructose and glucose do not share the same uptake system in this organism, as has been found in *Clostridium pasteurianum* (2). Moreover, it seems that the cells were easily depleted of energy stores. The failure of *C. thermocellum* cells incubated in buffer to take up α-methyl glucoside may also be due to energy depletion (7). Moreover, uptake of radioactive glucose by *C. acetobutylicum* has been found to be less sensitive to butanol than is the incorporation of radiolabel (16). This effect may be due to the lower concentration of glucose (which is an energy source as well as a substrate) used in the uptake experiments (25 μM) than in the incor-

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TABLE 1. Effect of sugars on the rate of glucose uptake by *C. acetobutylicum*[a]

Sugar	mM	% of control rate
α-Methyl glucoside	5	97
	25	72
3-*O*-Methyl glucose	5	109
	25	87
DG	5	57
	25	15

[a] [^{14}C]glucose concentration was 0.5 mM. The control uptake rate was 6.46 nmol/min per mg (dry weight) of cells.

poration experiments (28 mM) (16). Typically, in *C. acetobutylicum* intracellular ^{3}H-DG levels were found to be 20- to 30-fold greater than those in the medium and were reached within 10 min (Fig. 1). These uptake rates, about 6.5 nmol of glucose taken up per min per mg (dry weight) of cells, are similar to those reported for this organism (3, 16) and for other clostridia (2, 5, 7). The rates of ^{3}H-DG uptake by *C. acetobutylicum* were first order for at least 2 min. Initial rates obtained after 0.7 to 0.8 min of incubation were used to calculate the K_m and V_{max} (data not shown). The DG uptake system had an apparent K_m of 0.17 ± 0.01 mM DG and a V_{max} of 8.81 ± 1.03 nmol/min per mg (dry weight) of cells. The K_m for DG uptake is at least 10-fold higher than the K_m of 12.5 nM for glucose uptake reported by Ounine et al. (16).

Identification of the radioactive product accumulated after DG uptake was performed by anion exchange and paper chromatography. Acidogenic-phase cells were incubated for 10 min in buffer, as for uptake assays, with 50 mM fructose and 0.5 mM ^{3}H-DG, 2.2 Ci/mol. Cells centrifuged through silicone oil were permeabilized with 5% (vol/vol) butanol for 20 min at room temperature, and then the mixture was centrifuged. Aliquots of the supernatant were applied to columns (0.5 by 8 cm) of Dowex 1-X8 resin, formate form, and 1.0-ml fractions were eluted with 4 ml of water and then with 4 ml of 0.5 M ammonium formate. Aliquots of the eluates were counted for radioactivity. Over 70% of the extracted material was eluted by ammonium formate from the anion-exchange resin, indicating that DG had accumulated as an anionic derivative. After treatment with calf intestine alkaline phosphatase (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.), 99% of the extract was eluted by water and only 1% by ammonium formate, consistent with a phosphorylated derivative of DG. The ammonium formate-eluted fractions containing radioactivity were concentrated and chromatographed on paper (no. 1; Whatman, Inc., Clifton, N.J.) in the descending direction, using three solvent systems: methanol-acetic acid-water (80:15:5), methanol-formic acid-water (80:15:5), and butanol-acetic acid-water (50:30:20). Sugar-containing spots were detected with a molybdate spray reagent (12), compared with known standards, and counted for radioactivity. The radioactive derivative cochromatographed with 2-deoxyglucose-6-phosphate (DGP); treatment with alkaline phosphatase of the ammonium formate-eluted material resulted in release of DG. These results indicate that in *C. acetobutylicum* DG is accumulated as DGP.

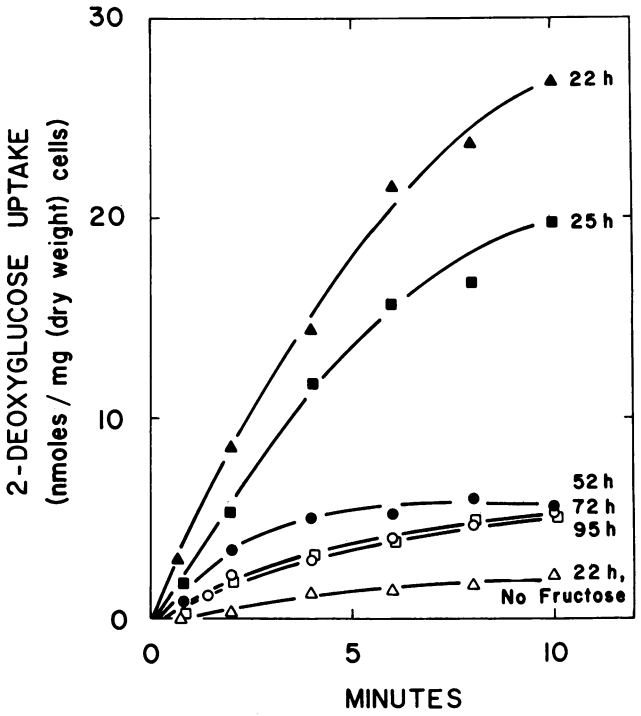

FIG. 1. Effect of growth stage on DG uptake by *C. acetobutylicum*. Cells were harvested after the indicated time of growth in batch cultures. The uptake of 1.0 mM ^{3}H-DG was assayed as described in the text.

Since glucose uptake has been reported to be greater in acidogenic-phase cells than in solventogenic-phase cells (16), we examined DG uptake in cells harvested at various times during batch culture. It should be noted that it was not possible to measure DG uptake by cells during growth because of the high glucose concentration (6% or 333 mM) present in the medium. Uptake of DG was found to be greater in acidogenic-phase cells than in solventogenic-phase

TABLE 2. Effect of butanol on the formation of DGP by *C. acetobutylicum*

Phase	% Butanol	nmol of DGP formed/mg (dry wt) of cells in 5 min			
		Without PEP		With PEP	
		Cell-associated	Total	Cell-associated	Total
Acidogenic	0	56	64	50	67
	0.5	39	51	55	77
	1.0	44	62	41	70
	1.5	32	54	20	79
	2.0	2	29	5	101
Solventogenic	0	7	9	8	10
	1.5	3	6	4	14

cells (Fig. 1). After 22 h of growth in batch culture (when no butanol had been produced) cells took up 27 nmol/mg (dry weight) of cells in 10 min, and after 24 h (<0.03% butanol), 20 nmol/mg (dry weight) of cells. In contrast, solventogenic-phase cells harvested after 52 h (0.5% butanol produced), 72 h (0.7% butanol), or 95 h (0.95% butanol) took up 6 nmol of DG or less per mg (dry weight) of cells in 10 min. Thus, the glucose transport system operates optimally during the early stages of growth.

It has been shown that glucose uptake and incorporation by *C. acetobutylicum* (16), as well as uptake of 3-*O*-methyl glucose (15), were inhibited by added butanol. Moreira et al. (15) and Ounine et al. (16) suggested that the levels of butanol reached during batch fermentation were sufficiently high to inhibit glucose uptake and incorporation. We therefore tested the effect of butanol on DG PT activity (Table 2) and found that butanol decreased the amount of DG taken up by *C. acetobutylicum* cells. However, we noted that DGP was present extracellularly when butanol was added, so that when the total DGP formed was considered, DGP formation was not decreased by the alcohol. In contrast, the published studies have only reported the radioactivity associated with cells separated from the incubation medium by filtration and washing. The butanol effect is consistent with permeabilized cells releasing intracellular charged molecules to which untreated cell membranes normally are impermeable. It was therefore likely that other intracellular compounds were released by solvent-treated cells. This was tested by adding PEP (10 mM) to energized cells treated with 0.5 to 2.0% butanol (Table 2). Butanol-treated cells, supplemented with PEP, formed up to three times more DGP than did cells without added PEP, suggesting that the latter cells had released intracellular PEP. In the butanol-treated cells most of the DGP was extracellular. Thus, the ability of cells to phosphorylate DG was essentially the same or greater in PEP-supplemented, butanol-treated cells as in intact cells. We conclude that, even if butanol has other, specific effects on sugar uptake and utilization, the alcohol acts primarily as a chaotropic agent which disrupts the cell membrane, allowing release of intracellular metabolites, including PEP.

Finally, the decreased PT activity for DG seen in solventogenic-phase cells cannot be attributed to the presence of inhibitory concentrations of butanol in the later phase of batch culture. Solventogenic-phase cells were harvested after 48 h of growth, when the culture contained about 0.5% butanol, washed, and assayed in the same manner as the acidogenic-phase cells. These cells formed DGP at about one-seventh the rate as acidogenic-phase cells (Table 2), but their responses to butanol and PEP were the same. In the absence of butanol and PEP, 9 nmol of DGP were produced in 5 min/mg (dry weight) of cells, of which less than 2 nmol was extracellular. The addition of PEP had no significant effect on DGP formation. When treated with 1.5% butanol, the cells formed about 6 nmol of DGP, of which 3 nmol was extracellular; when 10 mM PEP was included, 14 nmol of DGP was formed, of which 10 nmol was extracellular. Thus, solventogenic-phase cells had a lower PT activity than did acidogenic-phase cells, but not because of inhibitory effects of the butanol generated by them.

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