Control of the Production of Exo- β -N-acetylglucosaminidase by *Bacillus subtilis* B

DE-REPRESSION DURING GLUCONEOGENESIS AND INITIAL STAGES OF SPORULATION

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1. The control of exo- β -N-acetylglucosaminidase (EC 3.2.1.30) production by *Bacillus subtilis* B growing on a chemically defined medium was studied. 2. The enzyme was repressed during exponential growth by those carbon sources that enter the glycolytic pathway above the level of phosphoenolpyruvate. When exponential growth ceased as a result of low concentrations of the nitrogen, carbon or metal ion components of the medium, the enzyme was formed and its amount could be increased by the addition of cell-wall fragments as inducer. 3. The enzyme was de-repressed and could be induced during exponential growth on non-glycolytic compounds metabolized directly into pyruvate, acetyl-CoA or tricarboxylic acid cycle intermediates. 4. The major difference in the metabolism of the organism utilizing these two groups of compound was the existence of high activities of phosphoenolpyruvate carboxylase required for gluconeogenesis. 5. It is concluded that the de-repression of glucosaminidase occurs when the only principal change detected in the intermediary metabolism of the organism was the presence of high activities of phosphoenolpyruvate carboxylase. 6. When the organism was grown on media containing repressing compounds, the enzyme was only de-repressed on entry of the cells into the initial stages of sporulation, where phosphoenolpyruvate carboxylase activity, even in the presence of excess of glucose, increased in parallel with glucosaminidase, neutral proteinase and alkaline phosphatase activities. 7. These results suggest a strong link, at the level of the tricarboxylic acid cycle, between the control of phosphoenolpyruvate carboxylase and the control of the de-repression of glucosaminidase and sporulation.

Exo- β -N-acetylglucosaminidase (EC 3.2.1.30) is released by *Bacillus subtilis* B grown under certain conditions. Because of its potential ability to attack the cell-wall peptidoglycan and wide distribution amongst bacterial species (Berkeley *et al.*, 1969), studies were undertaken in an attempt to establish its physiological role, and in particular to investigate the possibility that it is involved with cell-wall metabolism.

The enzyme has been purified (Ortiz *et al.*, 1972) and characterized (Berkeley *et al.*, 1973). Initial work on the control of the enzyme suggested that it was both repressible by glucose and related compounds and inducible by cell-wall fragments (J. M. Ortiz, R. C. W. Berkeley & S. J. Brewer, unpublished work).

The glucosaminidase appears to be linked with the entry of the organism into sporulation [although not essential for it, as defined by Schaeffer (1969), since a mutant lacking the enzyme still sporulates (J. M. Ortiz, personal communication)]; therefore a knowledge of the control of glucosaminidase may also

yield useful information about the control of sporulation.

We report here a study of the changes in the metabolism of the organism which occur on de-repression of the glucosaminidase, and discuss the possible relationship of the control of this enzyme with the control of sporulation.

Materials and Methods

Azo-albumin, glyceric acid, ITP and GSH were obtained from Sigma (London) Chemical Co., London S.W.6, U.K.; *p*-nitrophenyl disodium orthophosphate was from British Drug Houses Ltd., Poole, Dorset, U.K.; *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucoside was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; malate dehydrogenase, lactate dehydrogenase, NADH, phosphoenolpyruvate cyclohexylammonium salt and ADP were from Boehringer Corp. (London) Ltd., London W.5, U.K.; and NaH¹⁴CO₃, [U-¹⁴C]glucose and [³H]acetate were from The Radiochemical

Centre, Amersham, Bucks., U.K. All other reagents where possible were purchased as analytical-reagent grade.

The minimal salts (MS medium) used as a basis for media contained K_2HPO_4 , 14.0 g/l; KH_2PO_4 , 6.0 g/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l; $(NH_4)_2SO_4$, 2.0 g/l; autoclaved at 69 kPa (10 lbf/in²) for 20 min; FEMMS medium was prepared by adding $FeSO_4 \cdot 7H_2O$ (0.02 g/l) and $MnCl_2$ (0.02 g/l) to the MS medium. Carbon sources were sterilized by filtration and added separately to the required concentration.

The growth of the organism was followed by measuring the E_{675} . Provided that exponential growth was occurring, dry-weight estimates could be obtained from a calibration curve. During non-exponential growth, considerable lysis of the organism occurs and no correlation between dry weight and extinction was found. Therefore the E_{675} is quoted where exponential growth was not maintained throughout the duration of the experiment. Numbers of heat-resistant spores were determined by heating a 100-fold dilution of culture in an 80°C-water bath for 10 min, and after suitable further dilution counting the number of colonies growing on nutrient agar plates after incubation overnight at 37°C.

Glucosaminidase inducer was prepared from the non-diffusible material of a lysozyme digest of *Micrococcus lysodeikticus* cell walls. *M. lysodeikticus* cell walls were prepared by the method of Wadström & Hisatsune (1970). The cell-wall material was resuspended to a concentration of 5 mg/ml in 0.05 M-sodium phosphate buffer, pH 6.5 (30 ml). Lysozyme was then added to give a final concentration of 0.1 mg/ml and the digest was incubated with shaking at 37°C until no further decrease in absorbance occurred. Lower-molecular-weight material was then twice extracted by dialysis for 20 h against 200 ml of water. The non-diffusible material was sterilized by autoclaving at 69 kPa for 20 min, then centrifuged (MSE HS18, 8 × 50 ml rotor, 30 min at 4°C and 25 000 g), and the soluble material was flash-dried to yield a translucent flaky solid.

Glucosaminidase activity was determined as described by Ortiz *et al.* (1972). NADH oxidase activity was determined by polarographic measurement of the O_2 uptake of the particulate fraction of disrupted cells as detected by an O_2 electrode. Phosphoenolpyruvate carboxylase activity was determined by the method of Chang & Lane (1966) on the soluble fraction of disrupted cells, but by using the incorporation of $^{14}CO_2$ from 20 μ Ci of $NaH^{14}CO_3$ into malate and counting radioactivity of liquid samples (1 ml) in scintillant (10 ml), consisting of 4 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/l of toluene-Triton X-100 (2:1, v/v). Proteolytic activity at pH 7.2 was determined by adding culture (0.5 ml) to a solution of azo-albumin (10 mg/

ml) in MS medium (0.5 ml), and incubating at 37°C for 30 min. The reaction was stopped by the addition of 6% (w/v) trichloroacetic acid (4 ml), the precipitate centrifuged down and 5 M-NaOH (0.5 ml) added to the supernatant (2.5 ml) and E_{440} measured. Pyruvate kinase activity was determined by measuring the difference in rate of decrease in E_{340} of NADH on incubation of the soluble fraction of ultrasonicated cells (2.0 ml) with Tris, 0.59 ml (0.2 M-Tris adjusted to pH 7.5 with HCl); ADP, 0.2 ml (10 mg/ml); NADH, 0.05 ml (10 mg/ml); lactate dehydrogenase, 0.01 ml (2 mg/ml); in a 1 cm cuvette at 37°C in the presence and in the absence of phosphoenolpyruvate, 0.15 ml (5 mg/ml). Succinate dehydrogenase activity was determined in the particulate fraction of disrupted cells by the method of Bernath & Singer (1962). Alkaline phosphatase activity was determined by adding culture (1.5 ml) to 4 mM-*p*-nitrophenyl disodium orthophosphate (1.5 ml) in 1 M-Tris adjusted to pH 8.2 with 5 M-HCl, incubating at 37°C for 60 min, cooling on ice and determining E_{400} .

Units of enzyme activities are expressed as follows: glucosaminidase, μ mol/min; NADH oxidase, μ mol of O_2 /min; phosphoenolpyruvate carboxylase, c.p.m. incorporated into oxaloacetate/min; alkaline phosphatase, neutral proteinase, pyruvate kinase and succinate dehydrogenase, as the activity of enzyme causing a change in extinction of 0.001/min at 400, 440, 340 and 600 nm respectively. Specific activity is expressed as units/ E_{280} unit.

Cells were disrupted by ultrasonic treatment with a Dawe Soniprobe at 3A of a suspension of washed cells in MS medium (10 ml) for 1.5 min at 0°C. Soluble enzyme was prepared by centrifugation at 30 000 g in an MSE HS18, 8 × 50 ml rotor for 5 min at 4°C. Particulate (membrane and wall-bound) enzyme was prepared by differential centrifugation. Unbroken cells were sedimented by centrifugation in the same rotor for 3 min at 4°C and 4000 g followed by centrifugation of the supernatant for 5 min at 30 000 g to sediment the particulate fraction.

The uptake of [$U-^{14}C$]glucose by the organism was determined by incubating at 37°C with washed cells (2 ml) resuspended at 0.2 mg/ml in phosphate buffer (K_2HPO_4 , 14 g/l; KH_2PO_4 , 6.0 g/l) containing [^{14}C]glucose (100 nCi/mg of glucose per ml). Samples (0.5 ml) were removed at 0 and 15 min and washed on a membrane filter (Millipore type HA) with 2 M-glucose (3 × 5 ml) and water (3 × 5 ml). Membranes were dried in a scintillation vial (80°C, 2 h), immersed in a scintillant (10 ml) containing 4 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/l of toluene and ^{14}C radioactivity was counted in a Packard liquid-scintillation spectrometer. The uptake of [3H]acetate was determined by the same method except that cells (2 ml) were resuspended at 0.2 mg/ml in the phosphate buffer containing labelled acetate (100 nCi/mg of acetate per ml), washed with 2 M-sodium acetate

(3×5ml) and then water (3×5ml), and ³H radio-activity was determined in the scintillation counter.

Results

Development of growth medium

B. subtilis B grows on MS medium containing (NH₄)₂SO₄, potassium phosphate and MgSO₄ when provided with a wide variety of carbon sources. The addition of glucose (0.25%) allowed exponential growth of the cells up to a dry weight of 0.16mg/ml. Of the medium components only the addition of higher concentrations of phosphate increased the cell density to which exponential growth could be sustained (Table 1). At a cell density of 0.16mg/ml the phosphate was vastly in excess and hence it appeared unlikely that phosphate limitation prevented further growth. A search was therefore made for metal ions possibly present as contaminants in the potassium phosphate, addition of which would allow the organism to continue growing. Of the salts tested [Na₂B₄O₇, Co(NO₃)₂, CuSO₄, FeSO₄, MnCl₂, (NH₄)₆Mo₇O₂₄, NaCl, ZnSO₄], it was found that only MnCl₂ and FeSO₄ allowed higher cell densities to be obtained and that a mixture of Mn²⁺ and Fe²⁺ led to exponential growth to cell densities of greater than 1.5mg/ml (Table 2). Under these conditions phosphate was still well in excess and the medium, in which no component now appeared to be limiting, allowed reproducible growth up to cell densities of 1.6mg/ml. In subsequent studies the concentration of individual components of glucose-FEMMS medium were in turn decreased such that the final cell densities reached were well below this value and did not exceed 0.4mg/ml, thus ensuring an excess of all but one of the medium components.

Characteristics of enzyme production in glucose-FEMMS medium

A culture in glucose-MS medium produced glucosaminidase only when exponential growth

ceased before sporulation. It now appears that the enzyme was only released at this point owing to insufficient concentrations of Mn²⁺ and Fe²⁺. Growth on glucose-FEMMS medium can be stopped, by providing low concentrations of any one of the constituents of the medium. It was found that glucosaminidase was first detected at the point at which exponential growth ceased (Fig. 1), and enzyme production then continued for some 4-5h, after which no further increase in activity occurred. However, no enzyme was detected in weakly buffered, low-phosphate medium even 48h after the end of exponential growth. During exponential growth on glucose-FEMMS medium *B. subtilis* B grew in long filaments, but when exponential growth ceased in media containing low nitrogen, glucose, or metal ion concentrations, the organism broke down into shorter chains and pairs. However, this change in morphology on entering the stationary phase did not occur in the weakly buffered, glucose-FEMMS medium, where the filaments formed chains of organisms

Table 2. Additions of metal ions to glucose-MS medium and their effect on the cell densities at which exponential growth ceased

Growth of the organism was followed in MS medium containing 1% glucose with additions (0.02g/l) of MnCl₂ and/or FeSO₄.7H₂O and the cell densities at which the plot of the logarithm of cell density (mg dry wt./ml) against time was no longer linear were recorded.

Additions to MS medium (g/l)		Cell densities at which exponential growth ceased (mg/ml)
MnCl ₂	FeSO ₄ .7H ₂ O	
0.0	0.0	0.16
0.02	0.0	0.32
0.0	0.02	0.41
0.02	0.02	1.62

Table 1. Variation in the concentration of the components of MS medium and its effect on the cell density at which exponential growth ceased

Growth was followed with time and the cell densities at which the plot of the logarithm of cell density against time ceased to be linear were recorded.

Medium components (g/l)					Cell density when exponential growth ceased (mg/ml)	Final pH
K ₂ HPO ₄	KH ₂ PO ₄	MgSO ₄ .7H ₂ O	(NH ₄) ₂ SO ₄	Glucose		
14.0	6.0	0.5	2.0	2.5	0.16	7.15
42.0	18.0	0.5	2.0	2.5	0.41	7.18
14.0	6.0	2.5	2.0	2.5	0.15	7.15
14.0	6.0	0.5	10.0	2.5	0.17	7.15
14.0	6.0	0.5	2.0	8.0	0.16	7.15

Fig. 1. Growth and glucosaminidase production on low concentrations of individual components of glucose-FEMMS medium

Growth plotted as $\log(10^3 \times E_{675})$ (—) and glucosaminidase activity as units/ml of culture (----). Complete medium, 1.0% glucose-FEMMS medium (Δ); weakly buffered, K_2HPO_4 , 0.7 g/l, KH_2PO_4 , 0.3 g/l, in glucose-FEMMS medium ($\circ$); low magnesium, $MgSO_4 \cdot 7H_2O$, 0.015 g/l in glucose-FEMMS medium ($\square$); low nitrogen, $(NH_4)_2SO_4$, 0.2 g/l, in glucose-FEMMS medium ($\bullet$); low glucose, 1.0 g/l of FEMMS medium ($\blacksquare$).

which, after further incubation, became distorted in appearance.

Characteristics of enzyme induction in a glucose-FEMMS culture

The addition of crude material from the non-diffusible fraction of a lysozyme digest of *M. lyso-deikticus* cell walls caused a tenfold increase in the rate of glucosaminidase production at the end of exponential growth on glucose-MS medium. Similar results were obtained with the glucose-FEMMS medium and inducing with 0.2 mg of inducer/ml under the same conditions described for Fig. 1. On the weakly buffered glucose-FEMMS medium, how-

ever, no enzyme was produced. During exponential growth enzyme could not be induced on any of these media.

Characteristics of enzyme induction on various carbon sources

It was found that the addition of inducer to a culture growing exponentially in FEMMS medium, utilizing succinate as its sole carbon source, led to the rapid induction of the enzyme within 0.5 h of its addition (Fig. 2), the production lasting for some 1.2 h. In contrast, no induction of the glucosaminidase occurred if the inducer was added to a culture growing exponentially on glucose-FEMMS medium.

Because of this difference it was decided to study the inducibility of the enzyme in FEMMS medium with other carbon sources. The compounds fell into two groups, depending on whether or not induction occurred in cells growing on them exponentially (Table 3). The addition of glucose (0.1%) to an induced culture caused an immediate increase in growth rate and decreased the final amount of enzyme (Fig. 2).

Production of proteinase, alkaline phosphatase and spores during growth on various carbon sources

The end of exponential growth in *B. subtilis* is usually indicative of the onset of the initial stages of

sporulation. Two marker enzymes which always increase in specific activity at this point in the development of the organism are alkaline phosphatase (Warren, 1968) and neutral proteinase (Mandelstam & Waites, 1968). At the point where exponential growth ceased in a low-nitrogen glucose-FEMMS medium, assays of the whole culture showed an increased activity of both these enzymes, concurrent with glucosaminidase release (Fig. 3). This type of situation was found with glucose, maltose, pyruvate and succinate as carbon sources on FEMMS medium low in nitrogen, metal ion and carbon. In weakly buffered FEMMS medium, pyruvate and succinate were the only carbon sources that allowed the production of these enzymes. When differences in the

Fig. 2. Induction of glucosaminidase during exponential growth on succinate-FEMMS medium and the effect of addition of glucose

Growth on FEMMS medium containing 0.4% sodium succinate plotted as $\log(10^3 \times E_{675})$ (—) and glucosaminidase activity as units/ml of culture (----). *M. lysodeikticus* inducer was added to a final concentration of 0.5 mg/ml. Addition of inducer: none (Δ); at 0.75 h ($\blacksquare$); at 0.75 h + 0.1% glucose at 0.75 h ($\triangle$); at 0.75 h + glucose at 1.4 h ($\square$), as shown by the arrows.

Table 3. *Differences in glucosaminidase induction during exponential growth on various carbon sources*

Glucosaminidase induction, as measured by the maximum activity of glucosaminidase reached during exponential growth, was determined on various carbon sources (2.5 g/l of FEMMS medium) following the addition of inducer (0.4 mg/ml). Inducer was added at a cell density of 0.15 mg/ml.

Carbon source	Mean generation time (h)	Glucosaminidase induction (munit/ml)
Maltose	1.2	0.00
Glucose	0.8	0.00
Glycerol	1.0	0.00
Glycerate	1.9	0.00
Pyruvate	1.2	0.29
Lactate	2.2	0.42
Acetate	1.8	0.83
Citrate	2.0	1.00
Glutamate	1.9	1.52
Succinate	2.2	1.47

activities of these enzymes and the number of heat-resistant spores were studied it was found that weakly buffered glucose-FEMMS medium led to only 1% of the amount of spores found with low-nitrogen glucose-FEMMS medium, and no alkaline phosphatase, neutral proteinase or glucosaminidase activities were detected. All three of these enzyme activities were found on low-nitrogen FEMMS medium on both carbon sources, and in weakly buffered FEMMS medium on pyruvate along with higher numbers of heat-resistant spores (Table 4). Further investigation of the effect of low phosphate concentrations on inhibition of sporulation indicated that this was a pH effect caused by the lowered buffering power of this medium. In growth on maltose, glucose and glycerol the pH fell to approx. 4.3, and remained at this value, when no glucosaminidase could be detected. Growth on the repressing compound glycerate, and the non-repressors pyruvate and succinate, did not lead to this pH fall, and in these cases sporulation occurred and glucosaminidase was released when growth ceased.

Fig. 3. *Growth and glucosaminidase, alkaline phosphatase and neutral proteinase production on low-nitrogen glucose-FEMMS medium*

Growth on 0.25% glucose-FEMMS medium [containing 0.2 g of (NH₄)₂SO₄/l] plotted as log(10³ × E₆₇₅) (●) and glucosaminidase (Δ), phosphatase (○), and proteinase (□) activities as units/ml of culture.

Table 4. Activities of two sporulation marker enzymes, glucosaminidase and spore numbers in low-nitrogen or weakly buffered FEMMS medium

Cultures were inoculated at 0.02mg/ml and grown for 22h on glucose and pyruvate (5.0g/l) in FEMMS medium with the same low-nitrogen or weakly buffered conditions described in Fig. 1. Activities of alkaline phosphatase, neutral proteinase, glucosaminidase and concentration of heat-resistant spores were determined as described in the Materials and Methods section, along with the corresponding cell densities (mg dry wt./ml) at this point.

FEMMS media	Carbon source	Alkaline phosphatase activity (units/ml)	Neutral proteinase activity (units/ml)	Glucosaminidase activity (munits/ml)	$10^{-5} \times$ Spore concn. (spores/ml)	Cell density (mg/ml)
Low nitrogen	Glucose	1.15	3.46	1.24	368	0.17
Weakly buffered	Glucose	0.20	0.00	0.00	3.1	0.20
Low nitrogen	Pyruvate	4.07	3.50	3.05	78	0.16
Weakly buffered	Pyruvate	13.10	2.11	5.28	82	0.18

Table 5. Activities of intracellular enzymes involved in catabolism and anabolism in cultures grown with glucose, maltose, pyruvate and succinate as sole carbon source

All carbon sources were at 5.0g/l in FEMMS medium. Glucosaminidase induction was determined by addition of inducer (0.4mg/ml) during exponential growth and measuring enzyme activity as described in Table 3. Other enzyme activities were determined on ultrasonically disrupted cells harvested at cell densities of 0.2mg/ml during exponential growth. NADH oxidase and succinate dehydrogenase were determined on the particulate fraction prepared from 80 and 40ml of culture respectively, and pyruvate kinase and phosphoenolpyruvate carboxylase activities were determined on the soluble fraction obtained from 80ml of culture.

Carbon source	Mean generation time (h)	Glucosaminidase induction (munits/ml)	NADH oxidase activity (munits/ E_{280})	Succinate dehydrogenase activity (units/ E_{280})	Pyruvate kinase activity (units/ E_{280})	Phosphoenolpyruvate carboxylase activity (units/ E_{280})
Glucose	0.9	0.00	1.76	260	0.16	1.2
Maltose	1.1	0.00	2.98	240	0.74	2.7
Pyruvate	1.2	0.29	0.89	900	2.42	241
Succinate	2.2	1.47	1.39	410	0.00	163

NADH oxidase, succinate dehydrogenase, pyruvate kinase and phosphoenolpyruvate carboxylase activities on different carbon sources

The activities of these enzymes, involved in anabolic and catabolic pathways, were compared during exponential growth on two examples of each of the compounds which repress and de-repress the glucosaminidase. In this way it was hoped to find indications of gross differences in the activities of these enzymes which correlated with the de-repression of glucosaminidase. The compounds maltose and glucose, and pyruvate and succinate, were chosen for this study. On maltose and pyruvate mean generation times were 1.1 and 1.2h respectively, and it was hoped that any differences owing to growth rates would be eliminated in these two cases. Although variations of enzyme activities were found with the different carbon sources, the only gross differences correlating with the ability

of the compounds to repress glucosaminidase were in the activities of phosphoenolpyruvate carboxylase (Table 5). An 80-fold increase in activity of this enzyme occurred in cultures where glucosaminidase was de-repressed (pyruvate and succinate) over cultures in which it was repressed (maltose and glucose).

Phosphoenolpyruvate carboxylase activities during growth on low-glucose, -nitrogen, -manganese or weakly buffered glucose-FEMMS medium

On entering the stationary phase in low-glucose, -nitrogen or -manganese glucose-FEMMS medium, there was an increase in the activity of phosphoenolpyruvate carboxylase at the same time as the appearance of glucosaminidase (also of phosphatase and proteinase, see Fig. 3). The specific activity of

phosphoenolpyruvate carboxylase was not as high on low-nitrogen and -manganese as that occurring on low-glucose glucose-FEMMS medium (Fig. 4). No increase in phosphoenolpyruvate carboxylase activity occurred when growth ceased on weakly buffered glucose-FEMMS medium.

Uptake of [^{14}C]glucose and [^3H]acetate on low-glucose glucose-FEMMS medium

During growth of *Bacillus cereus* on glucose, accumulation of pyruvate and acetate in the medium cause a fall in pH. These acids are utilized when glucose is exhausted and growth ceases, and the pH again rises (Nakata & Halvorson, 1960). A similar pattern of

pH change can be detected during growth of *B. subtilis* B on glucose-FEMMS medium, and therefore investigations were made to discover if the uptake of [^3H]acetate was always correlated with the increase in phosphoenolpyruvate carboxylase activity. The results indicated that the cell always had the ability to take up [^{14}C]glucose, but [^3H]acetate was only taken up when growth ceased on low-glucose glucose-FEMMS medium (Fig. 5). On low-nitrogen glucose-FEMMS medium, glucose could still be accumulated, but in this case no uptake of acetate occurred when growth ceased. Therefore, although the increase in phosphoenolpyruvate carboxylase could be explained by the cell requiring gluconeogenesis from acetate in the absence of carbohydrates,

Fig. 4. Growth and phosphoenolpyruvate carboxylase production on low concentrations of individual components of a glucose-FEMMS medium

Growth plotted as $\log(10^3 \times E_{675})$ (—) and phosphoenolpyruvate carboxylase activity in units/ E_{280} (----). Complete medium containing 1.0% glucose-FEMMS medium was modified as in Fig. 1 to give: weakly buffered glucose-FEMMS medium ($\circ$); low-nitrogen glucose-FEMMS medium ($\bullet$); low-glucose glucose-FEMMS medium ($\blacksquare$); and also low-manganese (MnCl_2 , 0.0 g/l) glucose-FEMMS medium ($\square$).

Fig. 5. Growth and uptake of [³H]acetate and [U-¹⁴C]glucose on low-glucose or -nitrogen glucose-FEMMS medium

Growth plotted as $\log(10^3 \times E_{675})$ (—) and uptake of [³H]acetate (---) and [U-¹⁴C]glucose (----) in c.p.m./15 min per 200 μ g dry wt. of cells, determined as described in the Materials and Methods section. Low-nitrogen glucose-FEMMS medium (●), and low-glucose glucose-FEMMS medium (■) concentrations as in Fig. 1.

this same effect cannot explain the rise in phosphoenolpyruvate carboxylase when growth ceases on low-nitrogen FEMMS medium in the presence of excess of glucose.

Discussion

Studies on the inducibility of the exo- β -N-acetylglucosaminidase during growth indicate the existence of two categories of catabolites, those that repress the formation of enzyme and those that de-repress the enzyme during exponential growth (Magasanik, 1961). If we assume the presence of the Embden-Meyerhoff pathway and the tricarboxylic acid cycle in this organism, then it appears that the actual ability of a catabolite to repress the formation of enzyme is a function of the catabolite's position in these major anabolic pathways. All repressing compounds are precursors of glucose 6-phosphate, 3-phosphoglycerate and phosphoenolpyruvate which are mutually interconvertible. Phosphoenolpyruvate is converted by an irreversible reaction into pyruvate, a compound that does not repress the glucosaminidase. All com-

pounds tested that do not cause repression enter the pathways after this irreversible reaction. This suggests that differences in the metabolism of these two groups of compounds must determine whether or not repression of the enzyme will occur.

A study of the differences in metabolism of repressing and non-repressing compounds should yield useful information about the control of glucosaminidase by catabolite repression. One potential major difference will be the extent to which these two groups of compounds will be utilized via glycolysis and/or the tricarboxylic acid cycle. The enzyme pyruvate kinase catalyses the only ATP-yielding step common to the metabolism of all glucosaminidase-repressing compounds. This theoretically is an unnecessary enzyme for the metabolism of non-repressing compounds, and indeed succinate-grown organisms have no detectable amounts of pyruvate kinase. However, organisms grown on pyruvate as sole carbon source do in fact possess high activities of this enzyme (Table 5). Further, the presence of NADH oxidase and succinate dehydrogenase, together with the necessity when glutamate is not supplied to the cell of using enzymes of the tricarboxylic acid cycle

to produce amino acids (Hanson & MacKechnie, 1969), suggests that similar oxidative-phosphorylation pathways and activities of tricarboxylic acid-cycle enzymes are present during growth on both classes of compound. Therefore it seems unlikely that the effect of these two classes of compound on catabolic repression of this enzyme can be explained in terms of any large differences in the means of energy production or the degree of tricarboxylic acid-cycle utilization.

All the repressing compounds are potentially interconvertible with glucose via the glycolytic pathway. However, to produce carbohydrates when growing on compounds found not to repress glucosaminidase formation, a means of reversing the essentially irreversible pyruvate kinase step is required. The indirect reversal of this step occurs in *Escherichia coli* by two routes utilizing the enzyme phosphoenolpyruvate synthase (Cooper & Kornberg, 1967) and phosphoenolpyruvate carboxylase (Sanwal, 1970). The latter enzyme is always found in *B. subtilis* B growing on non-repressing carbon sources, but is virtually absent when growing on those causing repression (Table 5).

There would appear to be a very strong correlation between the presence of phosphoenolpyruvate carboxylase, indicating the occurrence of gluconeogenesis, and the de-repression of the enzyme. In all cases so far studied, glucosaminidase is only de-repressed during exponential growth when the carbon source is such that gluconeogenesis is required to produce carbohydrates.

When growth on a repressing compound ceases owing to the lowering of the concentration of a medium component, which results in the de-repression of glucosaminidase, there is again an increase in the specific activity of phosphoenolpyruvate carboxylase. If we look at the individual cases, then it is possible tentatively to propose metabolic states resulting in this de-repression.

In low-glucose medium labelled acetate was taken up by *B. subtilis* B when growth ceased. This is a non-repressing compound, and phosphoenolpyruvate carboxylase activity increases in response to the requirement for carbohydrate and the glucosaminidase is de-repressed. In low-nitrogen or -metal-ion media there is still plenty of repressing compound present, and glucose is still taken into the cell. In spite of this, phosphoenolpyruvate carboxylase increases in specific activity. When the growth of a culture ceases or its rate is decreased on low-nitrogen or -metal-ion media, α -oxoglutarate for amino acid biosynthesis will presumably, because of the decrease in demand, not be removed from the tricarboxylic acid cycle at such a high rate. A build-up of tricarboxylic acid-cycle intermediates similar to the concentrations involved in growth on non-repressing compounds may occur, resulting in an increase in

phosphoenolpyruvate carboxylase and allowing de-repression of enzyme. Since *E. coli* phosphoenolpyruvate carboxylase is inhibited by NADH (Wright & Sanwal, 1969) it is possible that gluconeogenesis in *B. subtilis* B does not occur in the presence of excess of glucose.

It therefore appears that any condition leading to an increase in activity of phosphoenolpyruvate carboxylase also causes de-repression of glucosaminidase. Further, it is suggested that these conditions are those that result in the occurrence of high intracellular concentrations of tricarboxylic acid-cycle intermediates, either during growth on non-repressing compounds, or at the end of growth on a repressing compound. This results in the observed increase in phosphoenolpyruvate carboxylase, even when not required for gluconeogenesis, and the decrease in the concentration, below its critical value, of a glucosaminidase repressor, allowing induction of the enzyme.

The initiation of sporulation is considered to be catabolically repressed (Schaeffer *et al.*, 1965) and its control closely linked to the tricarboxylic acid cycle (Fortnagel, 1970; Elmerich & Aubert, 1972). One of the first consequences of the de-repression of sporulation is an increase in activity of alkaline phosphatase and neutral proteinase (Warren, 1968). After exponential growth of *B. subtilis* B on a repressing carbon source ceases, the production of glucosaminidase parallels that of these two enzymes. If, however, no sporulation occurs, as after growth on weakly buffered media none of these three enzymes are produced. These points suggest that during growth on repressing compounds, the enzyme may only be de-repressed during the initial stages of sporulation. The ability to induce the glucosaminidase during growth on non-repressing compounds leads to a consideration of the state of the organism under these conditions. Mandelstam *et al.* (1971) have reported a thymine-negative mutant of *B. subtilis* which when transferred from a rich medium to a sporulation medium lacking thymine was trapped, unable to enter the initial stages of sporulation. However, sporulation was initiated on transfer of cultures grown on a poor medium, where chromosome morphology was that of stage I of sporulation. It is possible that in *B. subtilis* B a similar situation exists during growth on the relatively poor media containing non-repressing compounds, where the organism would be growing with the initial stages of sporulation de-repressed, permitting the induction of the glucosaminidase. If the above reasoning is correct then there might also be a strong correlation between the presence of phosphoenolpyruvate carboxylase and the development of the initial stages of sporulation in *B. subtilis* B. If work on phosphoenolpyruvate carboxylase-negative mutants and on the control of this enzyme confirm that it is intimately linked with sporulation, then this should elucidate

the relationship of the tricarboxylic acid cycle with the control of this important example of morphogenesis.

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