Inactivation of Glucose 6-Phosphate Dehydrogenase during Germination and Outgrowth of *Bacillus cereus* T Endospores

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The specific activity and total activity of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) under conditions of complete cell breakage fall 10–20-fold during a 3 h period of spore germination and outgrowth. The spores must germinate (lose refractility), but do not have to undergo outgrowth, for the loss of activity to occur. Glucose 6-phosphate dehydrogenase activity from cells at any stage of development is completely stable in extracts at 4°C or 30°C. All of the enzyme activity is found in a soluble (50 000g supernatant) fraction and remains completely soluble throughout development. Soluble protein and total cellular protein remain constant for about 2 h. Proteinases could not be detected or protein turnover demonstrated during the morphogenetic process. Phenylmethanesulphonyl fluoride and *o*-phenanthroline, inhibitors of proteolytic enzymes, do not prevent glucose 6-phosphate dehydrogenase inactivation when added to whole cells. Mixing experiments show no inhibitor of glucose 6-phosphate dehydrogenase to be present in late-stage cells. The enzyme is not excreted into the culture medium. Chloramphenicol and rifampicin immediately stop protein synthesis and development but not the inactivation of glucose 6-phosphate dehydrogenase. NaN₃, 2,4-dinitrophenol or anaerobiosis immediately stop development and prevent the loss of enzyme activity. A requirement for metabolic energy is therefore probable. Extracts of spores pre-labelled with L-[¹⁴C]leucine were made at various stages of morphogenesis and subjected to polyacrylamide-gel electrophoresis. Glucose 6-phosphate dehydrogenase, which was identified by a specific stain, did not lose ¹⁴C label, and therefore may not be degraded during the inactivation process.

The disappearance of specific enzyme activities is a common event during the morphogenesis of several micro-organisms (Bernlohr & Gray, 1969; Deutscher & Kornberg, 1968; O'Day, 1974; Orłowski *et al.*, 1972; Rosenberg *et al.*, 1973; Sussman & Lovgren, 1965). These microbial systems provide a convenient means to study the regulation of enzyme function by specific inactivation or degradation (Leitzmann & Bernlohr, 1968; Orłowski & White, 1974; Waindle & Switzer, 1973). By means of radiorespirometry, it was previously established that the hexose monophosphate shunt pathway operates maximally (25% of glucose catabolized) during germination and early outgrowth of *Bacillus cereus* T endospores (Goldman & Blumenthal, 1964). As outgrowth progresses, the functioning of the hexose monophosphate pathway diminishes until it is almost zero on completion of the first cell division (Goldman & Blumenthal, 1964). The specific activity of glucose 6-phosphate dehydrogenase (EC 1.1.1.49), the first enzyme of the hexose

monophosphate pathway, also goes from maximal to near minimal values during the same period (Orłowski & Goldman, 1974). The present paper describes experiments which examine the biochemical requirements and mechanism of the inactivation process *in vivo*.

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Experimental

Organism

Bacillus cereus strain T was used throughout these experiments.

Preparation of spores

Spores were prepared by growth on modified G-medium (Hashimoto *et al.*, 1960) as previously described (Goldman & Blumenthal, 1964). The spores were washed 20 times with water, freeze-dried and stored at –20°C.

Germination of spores

Freeze-dried spores (250 mg) in 100 ml of 0.05 M-sodium phosphate buffer (pH 7.3) were heat-treated

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at 70°C for 20 min. L-Alanine (100 μ mol), adenosine (100 μ mol) and dehydrated Tryptic Soy Broth (Difco, Detroit, Mich., U.S.A.) (2g) were added and the culture was incubated at 30°C on a rotary water-bath shaker (200 rev./min; 1.5 in-radius strokes).

Preparation of cell-free extracts

Cells at the appropriate stage of development were chilled on crushed ice (0°C), harvested by centrifugation (at 2°C), washed with and suspended in 0.05M-Tris-HCl buffer (pH 7.5). The cell suspension was subjected to homogenization with glass beads (0.11 mm diam.) for 1.0 min in a CO₂-cooled Bronwill MSK cell homogenizer (Bronwill Scientific, Rochester, N.Y., U.S.A.). Microscopic examination showed complete cell destruction. The preparation was centrifuged at 30000g for 10 min and the supernatant fluid used in enzyme assays.

Enzyme assays

Glucose 6-phosphate dehydrogenase was assayed at room temperature (approx. 22°C) as previously described (Goldman & Blumenthal, 1964). One unit of activity was defined as that amount of enzyme which produces 1 nmol of NADPH/min. Proteolytic activity was assayed at pH 7.5 and 10.0 (0.05M-Na₂CO₃ buffer) by the method of Charney & Tomarelli (1947), by using azocasein as substrate.

Protein measurements

Soluble protein was determined by the method of Lowry *et al.* (1951). Total cellular protein was determined by the method of Spudich & Kornberg (1968). Crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) served as the standard.

Radioactive amino acid incorporation

L-[U-¹⁴C]Leucine (Volk Radiochemicals, Skokie, Ill., U.S.A.) was added at a final concentration of 1 μ Ci/ml (4.3 nmol/ml) to cultures at the same time as the germinants. Addition of the appropriate metabolic inhibitors followed immediately. Portions (1 ml) of culture fluid were collected immediately and at intervals, placed into 5 ml of 5% (w/v) trichloroacetic acid, treated at 90°C for 15 min, chilled at 0°C for 30 min, and filtered on membrane filters (Sartorius; Brinkman Instruments, Westbury, N.Y., U.S.A.; pore size 0.45 μ m). The filters were washed with 3 $\times$ 5 ml of cold 5% trichloroacetic acid, then with 5 ml of anhydrous diethyl ether, and dried thoroughly at 70°C. The dried filters were placed into a solution of ethanolamine (0.67 ml), 2-methoxyethanol (4.67 ml), toluene (6.67 ml) and diphenyloxazole (55 mg) and radioactive disintegrations counted in a Beckman liquid-scintillation spectrometer model LS-250.

Measurement of protein turnover

Vegetative cells were grown in Tryptic Soy Broth to the mid-exponential phase of growth. The cells (1 ml) were inoculated on to plates of AK 2 sporulation agar (BBL, Baltimore, Md., U.S.A.) containing L-[U-¹⁴C]leucine (1 μ Ci/ml; 4.3 nmol/ml) and incubated for 2 days at 30°C. Spores were scraped from the agar surface, washed 20 times with water, and stored frozen (-20°C) until use. Spores were germinated as described above with L-[¹²C]leucine (1000 μ g/ml) added to the medium. Samples were collected at intervals and treated as described above for the determination of L-[¹⁴C]leucine in protein. Culture supernatant solutions obtained after precipitation with cold trichloroacetic acid (1 ml) were extracted with 3 $\times$ 3 ml of anhydrous ether, evaporated to dryness and assayed for released ¹⁴C label.

Polyacrylamide-gel electrophoresis. Spores prelabelled with L-[U-¹⁴C]leucine were germinated in the presence of L-[¹²C]leucine, and samples were collected at intervals, broken, prepared and assayed for glucose 6-phosphate dehydrogenase activity, as described above. The cell-free extracts were supplemented with sucrose (20%, w/v, final concn.); 10 μ l of this was applied to the polyacrylamide gel. An EC 370 Minicell vertical gel-electrophoresis apparatus (EC Apparatus Corp., St. Petersburg, Fla., U.S.A.) was used with 7% polyacrylamide gel (slab dimensions 5 cm $\times$ 10 cm $\times$ 0.75 cm) and 0.18M-Tris-Na₂EDTA-boric acid buffer, pH 8.4 (in gel and reservoirs). Current was applied until migration of a Bromophenol Blue dye front approached the end of the gel (5 h; 7 cm). The apparatus was kept chilled in a cold-room (2°C). The gel slab was stained for glucose 6-phosphate dehydrogenase activity at pH 7.5 with the solution of Shaw & Barto (1965). Staining was dependent on the addition of both D-glucose 6-phosphate and NADP⁺. The narrow bands containing stain were cut out of the gel, heated to dryness at 70°C, pulverized, added to scintillation fluid, and assayed for radioactivity as described above. Other gel slabs thus stained for glucose 6-phosphate dehydrogenase activity were also stained for protein with Amido Black 10B. The enzyme bands appeared as discrete protein bands.

Anaerobiosis, antibiotics and inhibitors

Aeration was discontinued at appropriate times of development, and compressed methane was vigorously bubbled through the cultures for the duration of the experiment. The following compounds were used in all cases at the final concentrations indicated: chloramphenicol (250 μ g/ml); rifampicin (100 μ g/ml); *o*-phenanthroline (1 mM); phenylmethanesulphonyl fluoride (1 mM); NaN₃ (10 mM); and 2,4-dinitrophenol (0.2 mM). These compounds were added to cultures directly as a dry powder or as

an aqueous solution, except for phenylmethane-sulphonyl fluoride, which was dissolved in 5% (v/v) ethanol. All compounds were purchased from Sigma.

Manometry

Germinated spores (1mg dry wt.), 30min old, were suspended in 0.05M-sodium phosphate buffer (pH7.3) containing an appropriate inhibitor and preincubated for 5min at 30°C. Tryptic Soy Broth (2% final concn.) was added and O₂ uptake determined with a Gilson differential respirometer (Gilson Medical Electronics, Racine, Wis., U.S.A.).

Chemicals

D-Glucose 6-phosphate and all nucleotides were purchased from Sigma. Cyanogum-41 acrylamide and the necessary polymerization catalysts were purchased from EC Apparatus Corp. All other chemicals were of reagent grade or better.

Results

Spore germination and outgrowth

The morphological characteristics of endospore germination and outgrowth in the genus *Bacillus* have often been described in the literature (Levinson & Hyatt, 1956; Levinson *et al.*, 1967). Under the conditions of these experiments, our spore preparations generally completed 'phase-darkening' (loss of optical refractility) within 5min, swelling within 60min, emergence within 90min, elongation within 150min and the first cell division within 180min. Thereafter vegetative growth proceeded.

Glucose 6-phosphate dehydrogenase activity during morphogenesis

Glucose 6-phosphate dehydrogenase activity first appeared and accumulated within *Bacillus cereus* T cells during endospore formation (M. Orlowski, unpublished work). The enzyme specific activity

Table 1. *Glucose 6-phosphate dehydrogenase activity in heat-treated and germinated spores of Bacillus cereus* T

Freeze-dried spores were suspended in 0.05M-sodium phosphate buffer (pH7.3) and heat-treated at 70°C for 20min. The culture was divided into two parts, with only one part receiving the germinants L-alanine and adenosine. Both parts were incubated at 30°C with shaking.

Time (min)	Specific activity (units/mg of protein)	
	Heat-treated	Germinated
0	67.5	89.0
90	70.0	50.0
180	65.0	34.0

was very high in dormant spores (Fig. 1). During germination and outgrowth of the spores, the enzyme specific activity decreased precipitously. By the time the outgrowing cells completed their first cell division, specific activity had fallen 10–20-fold (Fig. 1). If vegetative growth was allowed to continue, all activity disappeared (not shown). Total enzyme activity per volume of culture, measured after complete cell disintegration, displayed the same kinetics of disappearance as specific activity (Fig. 1). Spores which had been stimulated to germinate (lose refractility) with L-alanine and adenosine, but which were not allowed to undergo outgrowth (by omission of Tryptic Soy Broth from the medium), lost a significant amount of enzyme activity over a 3h period of incubation (Table 1). Spores which were merely heat-treated did not lose any enzyme activity over the same period of incubation (Table 1).

Glucose 6-phosphate dehydrogenase activity in cell-free extracts

Enzyme activity in cell-free extracts (30000g supernatant fractions) made at any stage of morphogenesis was completely stable. Extracts kept at 4°C retained 94% of their activity after 48h. Extracts kept at 30°C retained 100% of their activity for at least 12h. All of this activity remained in the supernatant fraction through successive centrifugation steps at 1000g, 30000g and 50000g (15min running time). The resultant pellets, which were washed with and resuspended in 0.05M-Tris-HCl buffer (pH7.5), contained no glucose 6-phosphate dehydrogenase activity at any stage of morphogenesis. Adding back unwashed 30000g pellets to the 30000g supernatant fractions did not alter the stability of the enzyme activity in extracts.

Protein content of spores during morphogenesis

Soluble protein content in the cell-free extracts (30000g supernatant fractions) used for enzyme

Fig. 1. Time-course of glucose 6-phosphate dehydrogenase specific activity and total activity/ml of culture during germination and outgrowth of *B. cereus* T endospores

Symbols: ○, specific activity; ●, total activity/ml of culture. The two curves represent separate experiments.

Fig. 2. Time-course of soluble protein and total cellular protein during germination and outgrowth of *B. cereus* T endospores

Symbols: ●, soluble protein (in 30000g supernatant fractions); ○, total cellular protein. The two curves represent separate experiments.

assays remained constant for approximately the first 2h of spore development. Thereafter an increase occurred (Fig. 2). The total amount of cellular protein per volume of culture also remained constant for approx. 2h and thereafter increased (Fig. 2).

Measurements of proteolytic activity and protein turnover in spores during morphogenesis

Cell-free extracts from concentrated spore suspensions were assayed at pH7.5 and 10.0 for proteolytic activity with azocasein as substrate. Only negligible activity ($\Delta E_{440}/30\text{min} = 0.010/\text{ml}$ of extract) was detected at either pH value at any stage of morphogenesis. The proteinase inhibitors, phenylmethanesulphonyl fluoride and *o*-phenanthroline, did not affect this small activity. Protein turnover during morphogenesis was determined by measuring the loss of hot-trichloroacetic acid-insoluble radioactivity and the accumulation of cold-trichloroacetic acid-soluble radioactivity from spores pre-labelled with L-[^{14}C]leucine. Protein turnover was not detected at any stage of development by using these methods (Table 2).

Effect of proteinase inhibitors on glucose 6-phosphate dehydrogenase inactivation

In spite of the previous data, which suggested the absence of proteolytic activity during germination and outgrowth, experiments were done to test the effects of proteinase inhibitors on the loss of glucose 6-phosphate dehydrogenase activity. Phenylmethanesulphonyl fluoride, an inhibitor of serine proteinases, and *o*-phenanthroline, an inhibitor of metal proteinases, were added to cultures at various stages of morphogenesis. Both of the compounds permeated the cells and inhibited protein synthesis and respiration (Figs. 3 and 4). The cells attained 'phase-darkening' in the presence of the substances.

Table 2. Protein turnover during germination and outgrowth of *B. cereus* T spores

Spores, which had been pre-labelled with L-[^{14}C]leucine, were suspended in 0.05M-sodium phosphate buffer (pH 7.3), heat-treated (70°C for 20min) and stimulated to germinate with L-alanine, adenosine and Tryptic Soy Broth. The medium contained a chase of L-[^{12}C]leucine (1000 $\mu\text{g}/\text{ml}$). Samples were withdrawn from the culture at intervals, heated in trichloroacetic acid (90°C for 15min), chilled (0°C for 30min) and filtered. The precipitate was assayed for radioactivity. Duplicate samples were treated with cold trichloroacetic acid and filtered. The filtrate was extracted with anhydrous ether, evaporated to dryness and assayed for radioactivity. The experiment was also carried out in the presence of 1mM-phenylmethanesulphonyl fluoride or 1mM-*o*-phenanthroline, inhibitors of proteolytic enzymes, with identical results.

Time after addition of germinants (min)	Hot-trichloroacetic acid-precipitated (c.p.m./ml of culture)*	Cold-trichloroacetic acid-soluble (c.p.m./ml of culture)†
0	7.9×10^4	24
30	7.9×10^4	30
60	7.8×10^4	51
120	7.9×10^4	37
180	7.9×10^4	20

* Data include a standard error of <1%.

† Data include a standard error of >20%.

Fig. 3. Effect of metabolic inhibitors on L-[^{14}C]leucine incorporation into hot-trichloroacetic acid-insoluble material during germination and outgrowth of *B. cereus* T endospores

A suspension of spores was divided into several cultures which were stimulated to germinate as described in the text. L-[^{14}C]Leucine was added to the cultures at the same time as the germinants. Chloramphenicol (250 $\mu\text{g}/\text{ml}$), rifampicin (100 $\mu\text{g}/\text{ml}$), *o*-phenanthroline (1mM) and phenylmethanesulphonyl fluoride (1mM) were added to separate cultures at 0min and 30min. Symbols: ○, control culture; ●, effect of all inhibitors added at 0min; □, effect of all inhibitors added at 30min.

However, further morphological conversion was blocked by the inhibitors. The inhibitors did not prevent the loss of glucose 6-phosphate dehydrogenase activity at any time of development (Fig. 5).

Fig. 4. Effect of metabolic inhibitors on oxygen uptake of germinated ('phase-dark') spores of *B. cereus* T

Germinated spore suspensions were pre-incubated for 5 min in the presence of a particular inhibitor before the addition of substrate. Symbols: ○, control; ●, 0.2 mM-2,4-dinitrophenol; □, 10 mM- NaNO_3 ; ■, 1 mM-*o*-phenanthroline; △, 1 mM-phenylmethanesulphonyl fluoride.

Mixing experiment

Extracts of cells made at later stages of morphogenesis, when glucose 6-phosphate dehydrogenase activity was low, were added to extracts made at early stages of development, when activity was high. Table 3 shows that the resulting activities were strictly additive. Thus there was no indication of a soluble enzyme inhibitor present in the late-stage cells.

Glucose 6-phosphate dehydrogenase activity in the culture medium

The cell-free culture fluid was assayed during morphogenesis to determine whether the loss of

Table 3. Mixing experiment

Extract from cells with high glucose 6-phosphate dehydrogenase activity (heat-treated spores) was mixed (1/1, v/v) with extract from cells with low activity (germinated spores having completed the first cell division). The extract mixture was kept at 4°C for 30 min before assay.

Source of extract	Specific activity (units/mg of protein)
0-min cells (heat-treated spores)	58.96
180-min cells (completed first cell division)	4.32
0-min+180-min cells	31.86
Expected specific activity if no inhibition on mixing (average of 0-min and 180-min)	31.64

Fig. 5. Effect of proteinase inhibitors on the inactivation of glucose 6-phosphate dehydrogenase during germination and outgrowth of *B. cereus* T spores

A suspension of spores was divided into several cultures which were stimulated to germinate as described in the text. Protease inhibitors were added to cultures at 0 min, 1 h or 2 h. (a) Cultures receiving 1 mM-phenylmethanesulphonyl fluoride. (b) Cultures receiving 1 mM-*o*-phenanthroline. Symbols: ○, control; ●, addition of inhibitor at 0 min; □, addition of inhibitor at 2 h.

glucose 6-phosphate dehydrogenase activity is due to excretion of the enzyme from the cells. No activity was detected in the culture medium at any time of development (Table 4). Because of the possibility that any excreted glucose 6-phosphate dehydrogenase may have been inactivated in the culture medium, the 'reconstruction' experiment presented in Table 4

Table 4. Assay for glucose 6-phosphate activity in culture fluid and 'reconstruction' experiment

Cells were centrifuged from the culture medium at various times during germination and outgrowth of *B. cereus* T spores. The cell-free supernatant fluid was assayed for glucose 6-phosphate dehydrogenase activity. For the 'reconstruction' experiment, spores were broken in 0.05M-sodium phosphate buffer (pH 7.3) and centrifuged (30000g for 10min). The supernatant fluid, containing glucose 6-phosphate dehydrogenase activity, was added to a culture after heat-treatment (70°C for 20min) and addition of germinants. Cell-free culture fluid was assayed as described above.

Time after addition of germinants (min)	Enzyme activity (units/ml)	
	Culture fluid	Culture fluid + added extract
0	0	31.8
60	0	31.8
120	0	31.8
180	0	30.9

was performed. The results indicate that the enzyme was not inactivated.

Effect of inhibitors of protein and RNA synthesis on inactivation of glucose 6-phosphate dehydrogenase

Chloramphenicol and rifampicin immediately and quantitatively inhibited the incorporation of L-[¹⁴C]-leucine into hot-trichloroacetic acid-insoluble material during germination and outgrowth (Fig. 3). The morphological development of the cells did not proceed beyond 'phase-darkening' in the presence of these compounds. Neither of the compounds had an effect on the loss of glucose 6-phosphate dehydrogenase activity during development (Fig. 6). The synthesis of protein or RNA is apparently not a requirement for inactivation of the enzyme.

Effect of inhibitors of energy metabolism on inactivation of glucose 6-phosphate dehydrogenase

Previous investigations have shown that differentiating cultures of *Bacillus subtilis* (Waindle & Switzer, 1973), *Bacillus licheniformis* (Bernlohr & Gray, 1969) and *Myxococcus xanthus* (Orlowski & White, 1974), when deprived of oxygen by sparging with nitrogen or argon, failed to display the characteristic loss of specific enzyme activities. Developing cultures of *B. cereus* T spores made anaerobic by sparging with methane immediately stopped morphogenesis and did not show the usual loss of glucose 6-phosphate dehydrogenase

Fig. 6. Effect of chloramphenicol and rifampicin on the inactivation of glucose 6-phosphate dehydrogenase during germination and outgrowth of *B. cereus* T spores

A suspension of spores was divided into several cultures which were stimulated to germinate as described in the text. Chloramphenicol or rifampicin were added to cultures at 0min, 1h or 2h. (a) Cultures receiving chloramphenicol (250 µg/ml). (b) Cultures receiving rifampicin (100 µg/ml). Symbols: ○, control; ●, addition of inhibitor at 0min; □, addition of inhibitor at 1h; ■, addition of inhibitor at 2h.

Fig. 7. Effect of anaerobiosis and inhibitors of energy metabolism on the inactivation of glucose 6-phosphate dehydrogenase during germination and outgrowth of *B. cereus* T spores

A suspension of spores was divided into several cultures which were stimulated to germinate as described in the text. Anaerobiosis was initiated by sparging cultures with methane at 0 min or 1 h. Energy poisons were added to cultures at 0 min, 1 h or 2 h. (a) Cultures made anaerobic. Symbols: ○, control; ●, anaerobiosis beginning at 0 min; □, anaerobiosis beginning at 1 h. (b) Cultures receiving 10mM- NaN_3 . (c) Cultures receiving 0.2mM-2,4-dinitrophenol. Symbols: ○, control; ●, addition of inhibitor at 0 min; □, addition of inhibitor at 1 h; ■, addition of inhibitor at 2 h.

activity (Fig. 7a). This response could indicate a requirement for either the continuous presence of molecular oxygen or a source of energy (ATP) dependent on aerobic metabolism in order for inactivation of glucose 6-phosphate dehydrogenase to occur. The following experiments were designed to distinguish between these possibilities.

NaN_3 , an inhibitor of terminal oxidation, and 2,4-dinitrophenol, an uncoupling agent of oxidative phosphorylation, were tested for their effect on the pattern of glucose 6-phosphate dehydrogenase activity during germination and outgrowth. Both compounds prevented morphogenesis from proceeding beyond 'phase-darkening'. NaN_3 completely stabilized glucose 6-phosphate dehydrogenase activity whenever added to a developing culture (Fig. 7b). This could reflect a need for continuous ATP synthesis or electron transport (possibly to eliminate accumulated reducing power from the cell) in order for inactivation of glucose 6-phosphate dehydrogenase to proceed. Addition of 2,4-dinitrophenol to the cultures at any time completely stabilized the glucose 6-phosphate dehydrogenase activity (Fig. 7c). This suggests the dependence of enzyme inactivation on continuous ATP synthesis rather than electron transport itself.

Respirometry experiments showed that NaN_3 inhibited, and 2,4-dinitrophenol stimulated, oxygen uptake under the conditions of these experiments (Fig. 4). Attempts to re-establish the inactivation of glucose 6-phosphate dehydrogenase by addition

of ATP (Gajdos *et al.*, 1968), GTP cyclic, AMP, dibutyl cyclic AMP, dibutyl cyclic GMP or NADP^+ (all at 1mM concn. and in the presence of 10mM- MgCl_2) to cultures containing NaN_3 were unsuccessful. None of the above nucleotides had any effect on the course of normal development or glucose 6-phosphate dehydrogenase inactivation.

In view of the apparent energy requirement indicated by the above experiments *in vivo*, an attempt was made to stimulate inactivation of glucose 6-phosphate dehydrogenase *in vitro* through the addition of several likely compounds to cell-free extracts. The extracts were incubated at 30°C with and without a 30000g unwashed particulate fraction. ATP, GTP, cyclic AMP, dibutyl cyclic AMP, dibutyl cyclic GMP and NADP^+ (all at 1mM concn. and in the presence of 8mM- MgCl_2) were added to the extracts without effect. ATP was inhibitory to enzyme activity at higher concentrations (Table 5), but the effect was immediate and did not increase with time. The ability of the germinants L-alanine and adenosine (1mM concns.) to inhibit glucose 6-phosphate dehydrogenase activity directly was tested, also without effect.

Fate of ^{14}C -labelled glucose 6-phosphate dehydrogenase protein during inactivation *in vivo*

Cell-free extracts subjected to polyacrylamide-gel electrophoresis and stained for glucose 6-phosphate dehydrogenase activity showed a single discrete blue-purple band whose location on the gel did not

Table 5. *Effect of ATP on glucose 6-phosphate dehydrogenase activity in cell-free extract*

A cell-free extract of dormant spores was prepared in 0.05M-Tris-HCl buffer (pH 7.5) containing 8mM-MgCl₂ by the method described in the text. ATP was added to the extract at the indicated concentrations before assay of glucose 6-phosphate dehydrogenase.

ATP concn. (mM)	Enzyme activity	
	(units/ml)	(% of control)
0	131.2	100
1	131.2	100
2	131.2	100
3	106.1	81
5	40.5	31

Fig. 8. *Localization of glucose 6-phosphate dehydrogenase activity on polyacrylamide gels*

Cell-free extracts were made at various times during germination and outgrowth of *B. cereus* T spores. Electrophoresis was carried out as described in the text. The solution of Shaw & Barto (1965) was used to stain glucose 6-phosphate dehydrogenase. The gels were scanned at 540nm with a Gilford model 2400 recording spectrophotometer equipped with a model 2410 linear transport scanner (Gilford Instruments, Oberlin, Ohio, U.S.A.). The spectrophotometer traces were identical for all samples measured (therefore only one is shown). Key: (1) origin; (2) enzyme band; (3) location of dye front. The distance from (1) to (3) represents 7.3cm on the gel.

change during morphogenesis (Fig. 8). Data in Table 6 indicate that the amount of radioactively pre-labelled protein necessary to yield one unit of enzyme activity increased during morphogenesis. That is to say, enzyme activity was lost but enzyme protein was not. Apparently, inactivation of glucose 6-phosphate dehydrogenase is not dependent on gross degradation or modification of the enzyme protein.

Table 6. *Fate of ¹⁴C-labelled 6-phosphate dehydrogenase protein during inactivation in vivo*

Spores, which had been pre-labelled with L-[¹⁴C]leucine, were suspended in 0.05M-sodium phosphate buffer (pH 7.3), heat-treated (70°C for 20min) and stimulated to germinate with L-alanine, adenosine and Tryptic Soy Broth. The medium contained a chase of L-[¹²C]leucine (1000μg/ml). Extracts made from cells collected at various stages of germination and outgrowth were assayed for enzyme activity and subjected to electrophoresis on 7% polyacrylamide gel. Glucose 6-phosphate dehydrogenase was located with a specific stain, cut from the gel, and assayed for radioactivity.

	Time after addition of germinants (min)		
	0	60	180
Specific activity (units/mg of protein)	89.6	67.8	11.8
% change in sp. activity	—	-24	-87
Radioactivity (c.p.m.)* / unit activity	4.2 × 10 ⁵	6.9 × 10 ⁵	22.2 × 10 ⁵
% change in c.p.m. / unit activity	—	+64	+440
% of total c.p.m. applied in enzyme protein	7	8	8

* Includes a standard error of 1%.

Discussion

The hexose monophosphate shunt is a metabolic pathway known to cease functioning over the course of germination and outgrowth of bacterial endospores (Goldman & Blumenthal, 1964). One of the component parts of the pathway, glucose 6-phosphate dehydrogenase, offered the opportunity to examine the inactivation of a specific enzyme during a sequence of morphogenetic events. The observed fall in enzyme specific activity (Fig. 1) was proportional to the reported decrease in activity of hexose monophosphate pathway function (Goldman & Blumenthal, 1964) during morphogenesis. The correlation of these data indicates the possibility that functioning of the pathway is determined by the activity of the enzyme.

Several experiments indicate that the decrease in specific activity represents a genuine loss of enzyme activity rather than mere dilution of the enzyme by accumulation of other protein. Data in Fig. 1 show that total enzyme activity per volume of culture decreases greatly and in parallel with specific activity. Data in Fig. 2 show that the amount of protein in the culture does not change significantly over the first 2h of development, a period of rapid enzyme-activity loss. The final twofold increase in cellular protein could not account for the 10-20-fold decrease in enzyme specific activity. Further, if dilution due to

added protein were responsible for the loss of specific activity, chloramphenicol would be expected to prevent the loss. It did not (Fig. 6).

The fact that the enzyme was completely stable in extracts is important. It indicates that the inactivation during morphogenesis is a process actively carried out inside living cells. One of the more obvious possible mechanisms of inactivation would be proteolytic cleavage or degradation of the enzyme. Proteolytic enzymes were not detected in cell-free extracts, protein turnover was not demonstrated in whole cells (Table 2), and known inhibitors of proteolytic enzymes did not prevent the inactivation of glucose 6-phosphate dehydrogenase (Fig. 5). The tentative conclusion based on these observations would be that glucose 6-phosphate dehydrogenase inactivation is not caused by proteolytic enzymes. However, there are limitations to the above experiments which should be mentioned. Azocasein may not be a suitable substrate for intracellular proteinases of *B. cereus* T spores. The intracellular pool of L-leucine may not be washable during germination and outgrowth of the organism. Hypothetical intracellular proteinases may be present in very small amounts and may not be affected by the usual inhibitors of such enzymes. Previous reports have described large amounts of proteinase activity in spores of *Bacillus subtilis* measurable with azocasein (Bishop & Doi, 1966), but no proteinase activity could be found in washed, activated spores of *B. cereus* (Torriani *et al.*, 1969). Further, spores of *B. cereus* pre-labelled with a mixture of ^{14}C -labelled amino acids, germinated in the presence of excess of unlabelled amino acids (chase), and fractionated by polyacrylamide-gel electrophoresis did not show a loss or change in distribution of ^{14}C label over a 2 h period of incubation. The authors concluded that no significant degradation of proteins takes place (Torriani *et al.*, 1969).

Several other possible mechanisms of inactivation were investigated. Excretion of active enzyme from the cell is known to be involved in the regulation of intracellular enzyme activities during development in *Dictyostelium discoideum* (Sussman & Lovegren, 1965). *Polysphondylium pallidum* (O'Day, 1973) and *Myxococcus xanthus* (Sudo *et al.*, 1972). The experiment represented in Table 4 tested the possibility that glucose 6-phosphate dehydrogenase activity is lost by excretion from the cell. Enzyme activity was never detected in the culture medium. The 'reconstruction' experiment established that active glucose 6-phosphate dehydrogenase would be detectable if excreted into the medium. It was possible that a soluble enzyme inhibitor accumulated in the cells during morphogenesis. Such a hypothetical inhibitor could be a small molecule or a protein. It could be synthesized *de novo* or unmasked. The mixing experiment described in Table 3 eliminates

the possibility of such an inhibitor unless it is very tight-binding and present in stoichiometric amounts. Another possibility was that the enzyme was converted from a soluble into a particulate form during morphogenesis, that is, it became bound up in membranes, vesicles or aggregates which were removed by centrifugation. The fact is that all of the measurable activity remained in a 50000g supernatant fraction throughout development. The enzyme was never present in a particulate fraction, unless it was there in an inactive form.

Some of the metabolic prerequisites for the inactivation process were studied with use of specific inhibitors. The addition of chloramphenicol or rifampicin to the cultures immediately and completely stopped protein synthesis and further morphogenesis, but did not prevent glucose 6-phosphate dehydrogenase inactivation. Apparently the inactivation is not dependent on the on-going synthesis of proteins or RNA. Whatever macromolecules may be involved in the process must be pre-formed and unmasked at the time of germination. The stabilization of glucose 6-phosphate dehydrogenase activity in anaerobic cultures and in cultures containing NaN_3 or 2,4-dinitrophenol demonstrates the need for a continuous supply of metabolic energy (ATP) in order for inactivation of the enzyme to occur. The requirement for a continuous supply of energy for enzyme inactivation has been described in other systems (Bernlohr & Gray, 1969; Orlowski & White, 1974; Waindle & Switzer, 1973) and may have a basis in several possible mechanisms. We may be justified in rejecting the hypothesis that the energy poisons stabilize the enzyme by preventing the continuation of morphological development. The proteinase inhibitors and the inhibitors of biosynthesis stopped morphogenesis but not inactivation of the enzyme. Further, spores which had germinated (lost refractility) did not need to continue further morphological development for inactivation to occur (Table 1). The requirement for metabolic energy in the present case cannot simply reflect the demands of an enzyme transport system (Table 4) or the synthesis *de novo* of an 'inactivating protein' (Fig. 6). Inactivation of the enzyme could be due to direct covalent modification such as an ATP-dependent adenylation or phosphorylation (Holtzer & Duntze, 1971). Adenylation has been reported in the mechanism of inactivation of glutamine synthetase in *Escherichia coli* (Shapiro & Stadtman, 1970). Alternatively, adenylate nucleotide species or other metabolites in flux with the intracellular energy charge (Atkinson, 1969) may act as effectors or modifiers of the enzyme protein itself or other molecules involved in the inactivation mechanism.

The electrophoresis experiment (Table 6) showed that ^{14}C -labelled protein was not lost from the bands containing glucose 6-phosphate dehydrogenase

activity during morphogenesis and accompanying inactivation of the enzyme. If it is assumed that the protein bands containing glucose 6-phosphate dehydrogenase activity were not contaminated with extraneous protein of varying amount during the time-course, the experiment indicates that wholesale degradation of the enzyme protein is probably not the direct cause of enzyme inactivation.

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