Journal of General Microbiology (1985), **131**, 833–847. *Printed in Great Britain*

Properties of the Germination Inhibitor of *Streptomyces viridochromogenes* Spores

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(*Received 21 September 1984*)

Germinating spores of *Streptomyces viridochromogenes* excreted a substance into the surrounding medium which inhibited germination of another sample of the spores. The germination inhibitor (GI) was produced during submerged culture after exponential growth had ceased. The GI was purified 51-fold following extraction from growth liquor with chloroform. It was soluble in alcohol and water and had a molecular weight of less than 1000. The GI blocked growth and respiration of some Gram-positive bacteria and was an inhibitor of the membrane bound, but not solubilized, calcium-dependent ATPase of germinated spores and mycelia of the producing organism. Several sodium–potassium activated ATPases were also inhibited. All four activities (respiration, growth, germination inhibition, ATPase) co-purified during column and thin-layer chromatography. The GI activities released during germination and produced during growth were identical. A role for the GI antibiotic in regulation of dormancy of spores of the producing organism is discussed.

INTRODUCTION

Previous work has established that the conidiospores (referred to hereafter more simply as spores) of *Streptomyces viridochromogenes* are constitutively dormant (Hirsch & Ensign, 1978). Synchronous germination occurs following activation of the spores by heat shock (Hirsch & Ensign, 1976*b*) or detergent wash (Grund & Ensign, 1982). Initiation of germination of the activated spores requires only the addition of calcium ions (Eaton & Ensign, 1980). Early germination events involve a decrease in refractility of spores accompanied by excretion of approximately 20% of the total spore carbon, increased respiratory rate and a rapid rate of RNA synthesis (Hirsch & Ensign, 1975, 1978). Synthesis of protein begins after a short lag period and DNA synthesis begins at the time germ tubes emerge (Hirsch & Ensign, 1975).

In preliminary reports, germinating spores of *S. viridochromogenes* were shown to excrete into the surrounding medium some substance which inhibited germination of another suspension of the dormant spores (Ensign, 1976; Hirsch & Ensign, 1978). In this paper we report that this germination inhibitor (GI) is a low molecular weight substance with antibiotic activity against some bacteria. The GI was found to be an inhibitor of the calcium-dependent ATPase activity in membranes of mycelia and germinating spores of the producing organism and of ATP-hydrolysing enzymes from some other sources. These observations suggest that the GI plays a role in regulating spore dormancy and germination. The observation that the GI also has antibiotic activity suggests the idea that the GI is an antibiotic with a discrete function as a bioregulator of metabolic processes in the producing organism.

METHODS

Growth conditions, procurement of spores and germination conditions. Procedures for growth of *S. viridochromogenes* on solid media, procurement of spores, activation and germination of spores were described previously

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Abbreviations: GI, germination inhibitor; DCCD, *N,N'*-dicyclohexylcarbodiimide; DGM, defined germination medium (Hirsch & Ensign, 1976*b*).

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(Hirsch & Ensign, 1976*a*, *b*). For growth in submerged culture, 250 ml flasks containing 50 ml 0·5% (w/v) peptone, 0·1% (w/v) yeast extract, 2·0% (w/v) glucose, 10 mM-potassium phosphate buffer pH 7·0 and 20 mM-MOPS buffer pH 7·0 (PYGPM) were inoculated with several spore coated glass beads and incubated while shaking at 30 °C. For large volumes, incubation was done in 10–13 l batches of PYGPM in a New Brunswick Microferm fermenter at 30 °C while aerated at 3 l min^{-1} and agitated by impellers rotating at 500 r.p.m. The fermenters were inoculated with one-tenth volume exponential phase cultures.

Other organisms used were: *Acholeplasma laidlawii* B from Dr R. McElhaney, University of Alberta, USA, and *Streptococcus faecalis* 9796 from Dr F. Harold, University of Colorado, USA. Other bacteria and yeast cultures were obtained from the culture collection of the Department of Bacteriology, University of Wisconsin, USA. Beef heart mitochondria were a gift of Dr G. Blondin, University of Wisconsin, USA.

Assay of germination inhibitor. Assays for GI involved preincubation of spores in 1·0 ml 50 mM-Tris buffer pH 7·3, 0·001% (w/v) Triton X-100 with 10 µl of test sample for 15 min prior to addition of germination medium. The rate and amount of germination was measured by following the decrease in optical density at 600 nm and compared to a control reaction containing no inhibitor. A Beckman model 25 spectrophotometer was used. One unit of inhibitory activity is defined as the least amount of GI causing a complete inhibition (no OD decrease) of a 1·0 ml spore suspension.

Purification of germination inhibitor. Germination inhibitor was obtained either from the filtrate solutions following removal of germinated spores with 0·4 µm membrane filters or from growth liquor following removal of mycelia by filtration through a Teflon cloth filter. The filtrates were acidified to pH 1·0–2·0 with concentrated HCl. An equal volume of $CHCl_3$ was added and the two phases were mixed by stirring at 4 °C for 12–18 h. The aqueous layer was removed and once more extracted with $CHCl_3$. The combined $CHCl_3$ extracts were concentrated in a Buchler rotary flash evaporator and stored under nitrogen at 4°C.

To attempt extraction of GI from mycelia, the mycelial residue remaining after removal of growth liquor was washed twice with water and then extracted with $CHCl_3$ or other solvents. The extracts were freed from residual mycelia by filtration and concentrated 100-fold *in vacuo*.

Concentrated GI from growth liquor was passed through a 1·5 × 110 cm column of Sephadex LH-20 in methanol. The flow rate of methanol was 10 ml h^{-1}, 5 ml fractions were collected and assayed for GI activity and OD at 220 nm. The fractions showing activity were pooled and concentrated *in vacuo* to a volume of 10 ml. A 100 µl sample was retained for assays of ATPase and respiration inhibition activity and the remainder was applied to a 0·9 × 6 cm column of Dowex AG-1 in the chloride form. The column was washed with a 0·1 to 1·0 M gradient of HCl at a flow rate of 25 ml h^{-1} and 5 ml fractions were collected and assayed for GI activity and A_{220}. A 100 µl sample of each fraction showing GI activity was retained for assays of ATPase and respiration inhibitor activity and the remainder was pooled and dried *in vacuo*. The residue, a yellow oil, was dissolved in 25 ml of water.

Another purification procedure involved preparative scale thin-layer chromatography of the active material contained in the LH-20 column fractions. The concentrated material was applied as strips to plates with a layer of silica gel 500 µm thick (Analtech Co.). The plates were first precleaned by washing in methanol and developing in 1:1 (v/v) CH_3OH/H_2O and were activated by heating for 30 min at 100 °C just prior to use. The solvent system used was ethyl acetate/methanol, 4:1 (v/v). After development, the plates were observed with a short wavelength UV light to detect location of the fluorescent GI. The silica gel from 1 cm^2 sections was scraped from the plates and extracted with $CHCl_3$ for 2–3 h at ambient temperature while shaken. The silica gel was removed by filtration and the filtrate fractions were evaporated *in vacuo*. The residues were dissolved in approximately 5 ml methanol and tested for activity.

Spore carbon release. Spores labelled with ^{14}C were obtained by growing *S. viridochromogenes* for 10 d on sucrose/nitrate medium (Hirsch & Ensign, 1976*a*) containing 0·5 µCi ml^{-1} (18·5 kBq ml^{-1}) [^{14}C]sucrose as sole carbon source. Release of label from the spores was measured by filtering 1·0 ml samples of spores on 0·45 µm membrane filters (Millipore type HA) and counting the radioactivity of 0·1 ml samples of the filtrate in Aquasol (Packard Instruments) using a Packard tri-carb scintillation counter.

Assays of ATP concentration, ATPase activity, phosphatase activity and respiration. ATP was extracted from cells with 90% (v/v) dimethylsulphoxide (Leps & Ensign, 1979). The concentration of ATP in samples and standards was measured using luciferin–luciferase reagents in a DuPont 760 luminescence biometer.

Assays for ATPase activity involved measurement of inorganic phosphate following enzymic hydrolysis of ATP (Herbert *et al.*, 1971). For most assays of *S. viridochromogenes* ATPase activity, the reaction mixture contained 50 mM-Tris buffer, pH 8·0, 1·0 mM-$CaCl_2$, 5·0 mM-Ca^{2+}-ATP and enzyme sample in a final volume of 2·0 ml. Incubation was at 35 °C, usually for 60 min. Assays for ATPase activity in other organisms involved following published protocols. The procedures used for isolation and assay of ATPases were: for *Escherichia coli*, Fillingame (1975); for *Bacillus cereus*, Decker & Lang (1977); for *A. laidlawii* B., Jinks *et al.* (1978); for mitochondria; van de Stadt *et al.* (1973). Canine and porcine ATPases were assayed in 50 mM-Tris buffer, pH 7·0, 10 mM each of $MgCl_2$, NaCl and KCl, and 5 mM-Mg^{2+}-ATP.

Phosphatase activity was measured by the method of Garen & Levinthal (1980) using *p*-nitrophenylphosphate as substrate at pH 8·5 or 5·0.

Oxygen uptake was measured using a Yellow Springs Model 53 polarographic oxygen analyser. Spores or cells were suspended in 3·0 ml Tris buffer, pH 7·0, and stirred while incubating at 35 °C. All values were corrected for endogenous oxygen uptake.

Antibiotic assays. Test organisms were grown in a medium containing 0·5% (w/v) peptone, 0·1% (w/v) yeast extract to mid-late exponential growth. Samples of 0·1 ml were spread over the surface of agar medium of the same composition. Wells of 2·0 mm were cut from the agar and 50 µl samples of GI were added. After incubation for 24 and 48 h, zones of inhibition were measured.

Ionophore assays. The method of Harold & Baarda (1969) was used to test GI for monovalent ionophore activity. Calcium ionophore activity was tested by the method of Pressman (1972).

Disruption of spores and mycelia. Dormant and germinated spores were broken using a Bronson Model 350 sonifier. The large probe was used at 80% of maximum power output with pulsing 30 s bursts while immersed in ice. Spores were suspended in 50 mM-Tris, pH 8·5, containing 10 mM-dithiothreitol (DTT). Three volumes were mixed with one volume of 25 µm diameter glass beads. A total sonication time of 2 min disrupted virtually all the dormant or germinated spores. The beads were removed by centrifugation at 2000 *g* for 2 min. The supernatant fraction was then centrifuged at 190000 *g* at 2 °C for 60 min and the resulting particulate fraction was resuspended in 50 mM-Tris buffer, pH 8·5, containing 10 mM-DTT and centrifuged again at high speed. The supernatant and washed pellet fractions were used within 3–4 d for ATPase assays.

Mycelia of mid-exponential phase cells growing in PYGPM broth were lysed with lysozyme. Washed mycelia were suspended in 25% (w/v) sucrose, 50 mM-Tris buffer, pH 8·0, containing 10 mM-DTT and homogenized with a glass tissue grinder. Lysozyme was added to a concentration of 1·0 mg ml^{-1} and the mixture was incubated at 37 °C for 30 min. The mycelia were virtually completely lysed and protoplasts and membrane fragments were evident. The membranes were sedimented by centrifugation at 230000 *g* for 120 min at 2 °C, resuspended in 50 mM-Tris buffer pH 8·0 plus 10 mM-DTT, sedimented again and resuspended in the same buffer.

Source of chemicals. [^{14}C]Glucose (uniform label, 421 mCi mol^{-1}), ^{86}RbCl and ^{45}CaCl$_2$ (the last two used in ionophore assays) were purchased from New England Nuclear. Sephadex gels were from Pharmacia. Ionophore A-23187, *N,N'*-dicyclohexylcarbodiimide (DCCD) and oligomycin were from Calbiochem. Sigma was the source of Ca^{2+}- and Mg^{2+}-ATP, canine and porcine ATPase, and all inhibitors and enzymes not listed above. Myxobacter AL-1 enzyme was prepared as described previously (Ensign & Wolfe, 1966).

RESULTS

Demonstration of germination inhibitor activity

Previously published work demonstrated that germinating spores of *S. viridochromogenes* excrete one or more substances that inhibit germination of another sample of the spores (Ensign, 1976; Hirsch & Ensign, 1978). Heat-activated spores, or spores initiated for germination for as short a time as 5 min germinated normally in the presence of the inhibitor. The index for germination used in these experiments was the decrease in optical density of spore suspensions as the spores changed from phase bright to dark. An experiment was designed to test the effect of GI on the complete germination process culminating in formation of germ tubes. Suspensions (1 ml) of unactivated and heat-activated spores were incubated in 0·1% yeast extract medium in

Table 1. *Effect of GI on initiation of germination and outgrowth of S. viridochromogenes spores*

Spore treatment	OD decrease (percentage of maximum)*	Percentage of spores with germ tubes after incubation for (h): 0	1	2	3	4
Unactivated	68	0	0	0	10	100
Unactivated + GI	0	0	0	0	0	0
Heat activated	100	0	0	100	100	100
Heat activated + GI	82	0	0	18	100	100
Unactivated + GI washed†	76	0	0	0	25	100

* Based on maximum OD decrease for heat activated spores which was a decrease of 22% at 40 min. The maximum OD decrease in all experiments occurred between 40 and 60 min. Amount of GI was 1·0 unit ml^{-1}.

† Incubated with GI in 0·1% yeast extract medium for 1 h, then washed twice with 30 mM-phosphate buffer and reinoculated into 0·1% yeast extract. The observations reported are at times following washing and reinoculation.

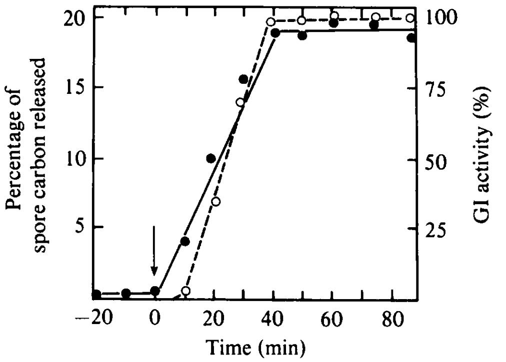

Fig. 1. Release of spore carbon and germination inhibitor by germinating spores of *S. viridochromogenes*. Activated spores that had been labelled with ^{14}C during growth were incubated in buffer for 20 min prior to addition of germinant (DGM: arrow). Spores (3 × 10^5 c.p.m. ml^{-1}) were removed from samples at various times by membrane filtration and the amount of radioactive label in 0·1 ml samples of the filtrate fluids was determined. Data are expressed as the percentage of the total amount of radioactivity of the spore suspension released. A 0·3 ml sample of the filtrate was tested for inhibition of germination of a 2·7 ml suspension of unactivated spores. Data are expressed as the percentage inhibition of germination after 60 min incubation (OD assay). ●, Spore carbon release; ○, germination inhibitor activity.

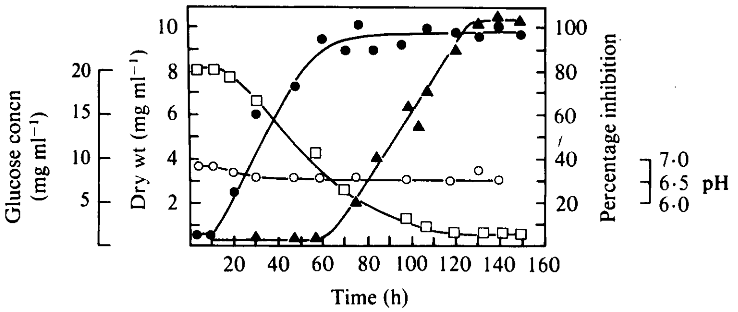

Fig. 2. Formation of germination inhibitor during growth of *S. viridochromogenes* in complex medium. Cells were inoculated into 12 l PYGPM medium and aerated vigorously while incubated at 30 °C. Samples were removed at various intervals, the cells sedimented by centrifugation and their dry weight measured. The pH, glucose content and GI activity of the supernatant fractions were measured. ●, Dry weight; △, GI activity; □, glucose concentration; ○, pH.

the absence and presence of 0·1 ml of the supernatant fluid obtained from centrifuging a suspension of spores (2·0 mg dry weight ml^{-1}) that previously had been germinated completely (incubated 60 min) in 0·1% yeast extract medium. The results (Table 1) show that some unactivated spores possessed germ tubes at 3 h and all did at 4 h. In the presence of GI there was no decrease in optical density of the spore suspension and no germ tubes were formed during the 4 h incubation period. All the heat-activated spores germinated in the absence of GI and had formed germ tubes at 2 h. The GI slightly diminished the optical density decrease of the spores and delayed by 1 h the time at which all spores contained germ tubes. The observation that all of the activated spores did form germ tubes in the presence of GI should be noted. The ability of GI to block germination was reversible. Unactivated spores incubated with GI for 1 h and then washed with buffer germinated normally when transferred to the germination conditions.

The decrease in refractility of germinating spores is accompanied by a concomitant excretion of carbon-containing materials (Hirsch & Ensign, 1978). The rates of excretion of carbonaceous materials and GI from heat activated spores before and during germination were determined. A negligible amount of ^{14}C label and no detectable amount of GI was released from the spores during 20 min incubation in buffer (Fig. 1). No further release of either carbon or GI occurred during incubation of spores for an additional 90 min period (data not shown). Release of spore carbon began immediately after addition of defined germination medium and ceased approximately 40 min later when 18% of the total spore carbon had been excreted. Examination with a phase contrast microscope verified that all the spores had become dark at that time. Activity of GI was first detected at 10 min. A sufficient amount of GI was present at 40 min in a 0·3 ml sample of filtrate from the germinating spore suspension to completely inhibit germination (no decrease in optical density of the spore suspension and no detectable loss of refractility) of a 2·7 ml sample of unactivated spores. The rates of release of GI and spore carbon were nearly identical. To determine if the amount of GI activity that had been released at 40 min and later time was the actual maximum amount, 0·01 and 0·05 ml samples of germination fluid obtained at 40, 50, 60 and 90 min were tested for GI activity. Each of the 0·05 ml samples caused a 90–100% inhibition of germination, while the 0·01 ml samples inhibited germination by 60–65% (data not shown). Thus, virtually all the GI activity had been released from germinating spores at 40 min, the time when spore carbon excretion had ceased.

Purification and properties of GI

Our initial attempts to isolate and purify GI were limited by the small amount of activity released from germinating spores. The difficulty inherent in obtaining sufficiently large batches of spores to make purification feasible was resolved by the discovery that GI activity was produced during growth in submerged cultures in a complex medium. The results of a determination of growth and formation of GI activity during cultivation of *S. viridochromogenes* in a 12 l fermentation vessel of PYGMP medium are shown in Fig. 2. Following a short lag period, growth was exponential until a maximum cell yield of approximately 10 mg dry weight ml^{-1} was reached at 60 h. Activity of GI was first detected at 60–70 h when the stationary growth phase had been reached. The GI activity increased progressively to a maximum amount at 120 h. At this time, a 0·1 ml sample of growth liquor completely inhibited germination of a 1·0 ml spore suspension. This was a maximum value for GI activity since 0·05 ml samples of growth liquor at times later than 120 h caused an approximate 50% inhibition of spore germination. The glucose content of the medium had decreased by approximately 70% at the time when GI activity appeared. Glucose continued to be utilized until about 0·1 mg ml^{-1} remained and then was not further utilized. This suggests that some parameter other than glucose limitation was limiting growth. The pH was not involved in growth limitation, as it decreased only slightly.

Prior to purification of GI, a study was made of some of its properties. The GI activity was extracted from either the supernatant fluid following germination of spores or from growth liquor into $CHCl_3$, ethyl acetate or methylene chloride. GI activity was not extracted into more non-polar solvents such as hexane, diethyl ether, benzene or toluene. Extraction into $CHCl_3$ was much more effective at pH 1·0–2·0 than at neutral or alkaline pH. The GI activity was removed from germination fluid or growth liquor by batch-wise treatment at pH 4·7 or 10 with DEAE-cellulose, silica gel and the anion exchange resin Dowex AG1-X8. Cation exchange resins did not adsorb GI. The following treatments did not inactivate GI from germinated spores or growth liquor: 5% (w/v) perchloric acid for 30 min at 0 °C; 0·5 M-NaOH for 30 min at 37 °C; 5% (w/v) trichloroacetic acid for 30 min at 80 °C; 1% (w/v) periodic acid for 30 min at 50 °C; or by heating at 100 °C for 30 min. Activity was destroyed by 30 min incubation in 1 M-HCl. GI activity from both sources was completely resistant to 2 h digestion at 37 °C by ribonuclease, deoxyribonuclease, pronase, *B. subtilis* protease, trypsin, lysozyme and lipase. There was a 25% loss of activity during storage of a methanol solution for 2 weeks at 4 °C or −20 °C. Virtually all activity was lost after 1 week at room temperature. Concentrated methanol solutions of GI were stable for at least 1 year when stored under nitrogen at −20 °C.

No GI activity was extracted by $CHCl_3$ or other solvents from mycelia either prior to or during the active period of GI production in the PYGMP medium.

GI was purified from PYGMP growth liquor (Table 2). Activity was completely extracted from the growth liquor by two 6 l $CHCl_3$ extractions at pH 2·0. The combined $CHCl_3$ extracts contained 12 × 10^4 units of activity. The $CHCl_3$ extract was evaporated to dryness and the

Table 2. *Purification of germination inhibitor activity*

Fraction	Volume (ml)	Total amount (mg)	10^{-4} × Total units*	Units per ml	Specific activity†	Recovery (%)	Purification‡ (fold)
Growth liquor	12000	—	12	10	—	—	—
Concentrated $CHCl_3$ extract	10	400	12	12×10^3	300	100	1·0
LH-20 column peak fractions	20	37	11·25	$5·6 \times 10^3$	3040	93	10·4
AG-l-Cl column peak fractions	25	5·5	8·49	$3·4 \times 10^3$	15436	75	51

* One unit of activity is the least amount of GI sample completely inhibiting germination of a 1·0 ml suspension of unactivated spores (OD assay).

† Units per mg sample.

‡ Relative to the $CHCl_3$ extract sample.

residue dissolved in 10 ml methanol. No loss of activity occurred and a 1·4-fold increase in specific activity was obtained. The methanol solution was applied to a Sephadex LH-20 column made up in methanol. The column was washed with methanol. A red pigment remained at the top of the column and was not removed by extensive washing of the column with methanol or chloroform. Most fractions contained material absorbing at 220 nm. The GI activity was eluted as a single peak in the four fractions comprising 60–80 ml of effluent (Fig. 3*a*).

A 10·4-fold purification; with a 71% recovery of activity was achieved. The pooled LH-20 fractions were concentrated *in vacuo* and applied to a Dowex-1 cation exchange column. The GI activity was eluted by HCl in a single peak (Fig. 3*b*), Most of the 220 nm absorbing material was associated with these fractions. An overall purification of 51-fold with 75% recovery of activity was achieved.

At each purification step, samples with GI activity were also analysed for ATPase and respiration inhibition activities. All three activities were present in each sample of purified GI (Table 3). More significant is the observation that the relative proportion of ATPase and respiration inhibition activity to GI activity was essentially constant at each step of purification. This is a strong indication that the same substance is responsible for all three activities. Moreover, GI activity was obtained as a single band with R_F 0·8 after silica gel thin layer chromatography (data not shown). This purified GI also contained ATPase and respiration inhibitory activity.

The GI activity purified from growth liquor was compared with that present in the supernatant fluid of germinated spores. Both activities migrated identically during analytical thin-layer chromatography on silica gel plates developed with 1:1 ethyl acetate/methanol (R_F 0·86), 1:1:1 ethyl acetate/hexane/chloroform (R_F 0·53) and 2:1 hexane/ethyl acetate (R_F 0·21). A mixture of $CHCl_3$ extracts of growth liquor and germinated spore liquor each with 100 units of GI activity were applied to an LH-20 column. The combined fractions showing GI activity that were eluted with methanol contained 170 units of activity. GI activity from both sources passed through a Diaflo membrane YM2 (Amicon) and was retained by a Diaflo UMO5 membrane. The molecular weight of GI is thus between 500 and 1000. The most important evidence that both GI activities involve the same active substance was their identical mode of action (see below).

Mode of action of GI

Tests were made of the effects of GI purified from growth liquor on two early germination events: release of spore carbon and increased respiratory activity. A sample of GI containing 0·5 units of activity retarded both processes by approximately 50% in unactivated spores and had virtually no effect on heat activated spores (Table 4). Twice the concentration of GI (1·0 unit) completely blocked release of spore carbon and respiratory activity of unactivated spores and caused a 42% decrease in release of spore carbon and a 23% decrease in respiration of activated

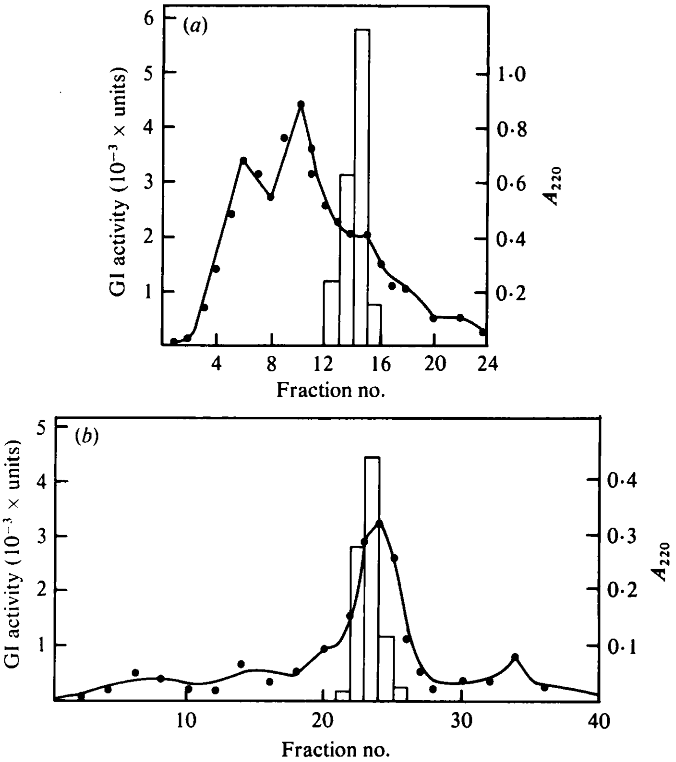

Fig. 3. Purification of germination inhibitor by column chromatography using Sephadex LH-20 and Dowex-1. (*a*) Concentrated GI from growth liquor was applied to a 1·5 × 110 cm column of LH-20 and eluted with methanol. Fractions of 5·0 ml were collected and assayed for A_{220} and GI activity. (*b*) Fractions with GI activity from the LH-20 column were applied to 0·9 × 6 cm column of Dowex AG-1 and eluted with HCl. Fractions of 5·0 ml were collected and assayed for A_{220} and GI activity.

Table 3. *Inhibition of respiration and ATPase by fractions obtained during purification of GI*

	Specific Activity			Ratio of inhibitor activities	
Fraction	GI*	ATPase† inhibitor	Respiration‡ inhibitor	GI:ATPase	GI:Respiration
$CHCl_3$ extract	300	14000	73	0·021	4·11
LH-20 column	3040	135000	675	0·022	4·50
AG-l-Cl column	15346	650000	3000	0·024	5·11

* As defined in Table 2.

† Units per mg sample. One unit is the smallest amount of sample producing a 90–100% inhibition of porcine ATPase activity.

‡ Units per mg sample. One unit is the smallest amount of sample producing a 80–100% decrease in respiratory rate of *A. crystallopoietes*.

spores. Not shown in the table are the observations that similar results were obtained using GI concentrated from germination fluid and spores activated by detergent rather than heat.

Methanol solutions of the $CHCl_3$ extract of supernatant fluid of germinated spores and of the 51-fold purified GI from growth liquor were tested for antibiotic activity. Growth of some but not all of the Gram-positive bacteria tested was inhibited by both sources of GI (Table 5). There was no inhibition of growth of the Gram-negative bacteria or the one yeast strain. Respiration of those organisms whose growth was inhibited by GI was completely inhibited by the GI purified from growth liquor. Respiration of some of the Gram-positive bacteria whose growth was not

Table 4. *Effects of GI on early germination events in unactivated and activated spores*

Spores were incubated in buffer for 10 min with or without 0·5 or 1·0 units purified GI ml^{-1} prior to initiation of germination. The amount of radioactivity in the supernatant fluid following removal of ^{14}C-labelled spores was determined 60 min later. Values reported are the percent of initial label of spores released. Respiration was monitored for 60 min using a polarographic oxygen monitor.

Experimental conditions	Percentage of spore carbon released	Respiration Q_{O_2}*
Unactivated spores, no GI	8·8	2·7
Unactivated spores, 0·5 units GI ml^{-1}	4·9	1·2
Unactivated spores, 1·0 unit GI ml^{-1}	0·6	0
Activated spores, no GI	15·9	8·3
Activated spores, 0·5 units GI ml^{-1}	14·6	8·3
Activated spores, 1·0 units GI ml^{-1}	9·2	6·4

* μM O_2 uptake (mg dry wt spores)$^{-1}$ h^{-1}.

Table 5. *Antibiotic activity and effect on respiration of GI*

Organism	Germination fluid*	Growth liquor†	Percentage inhibition of respiration‡
Bacillus cereus	+	+	100
B. subtilis	+	+	100
Arthrobacter crystallopoietes	+	+	100
Streptomyces parvulus	+	+	100
S. viridochromogenes	+	+	100
S. griseus	+	+	100
Nocardia corallina	−	−	0
Mycobacterium smegmatis	+	+	100
Staphylococcus aureus	−	−	20
Micrococcus luteus	−	−	33
Azotobacter vinelandii	−	−	NT
Pseudomonas aeruginosa	−	−	0
Klebsiella pneumoniae	−	−	NT
Escherichia coli	−	−	0
Saccharomyces cerevisiae	−	−	NT

NT, Not tested.

+, Zone of inhibition caused by a 0·05 ml of sample methanol solution of GI applied to paper discs. Discs were dried and placed on lawn of test organisms; −, no zone of inhibition.

* Acidified germination fluid (10 ml) was extracted with $CHCl_3$, the $CHCl_3$ extract evaporated and the residue dissolved in 0·5 ml methanol.

† 51-fold purified GI from growth liquor.

‡ Inhibition of endogenous respiration tested by adding 0·1 μl of 51-fold purified GI from growth liquor to 2·9 ml cell suspension. Respiration measured for 60 min using a polarographic oxygen monitor.

inhibited was repressed to a lesser extent. Respiration of the Gram-negative test organisms was not inhibited. Vegetative cells of the *Bacillus* and *Streptomyces* species were tested in these experiments. Germination of a 1·0 ml spore suspension of each of the organisms in 1% yeast extract medium was inhibited by 0·1 μl of the purified GI from growth liquor and 0·1 ml of crude exudate from germinated spores (data not shown).

The effect of GI on the rate of oxidation of glucose and pyruvate by *A. crystallopoietes*, *B. cereus* and *E. coli* was tested. Oxidation of the substrates by the first two organisms was completely blocked by 10 units of purified GI ml^{-1} and 0·1 ml of germination exudate ml^{-1} while there was no inhibition of respiration by *E. coli* (data not shown).

At this point it seemed likely that GI inhibits spore germination by interfering with respiratory processes. Consequently, a variety of inhibitors of energy production were tested for their effects on germination of *S. viridochromogenes* spores as measured by OD decrease. The

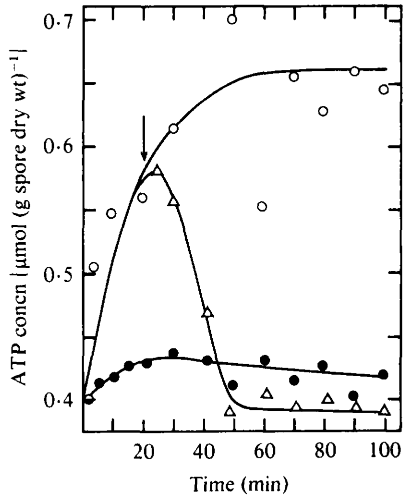

Fig. 4. Effect of germination inhibitor on formation of ATP by germinating spores. Spores were incubated in DGM only, or DGM to which purified GI (1·0 unit ml^{-1}) was added at time zero or 20 min. Samples were removed at various times, the ATP extracted from cells with 90% dimethylsulphoxide and quantified. ○, no GI added; ●, GI added at zero time; △, GI added at 20 min (arrow).

respiratory chain inhibitors $HgCl_2$ and KCN at concentrations of 5 mM completely inhibited germination, while NaN_3 at 5 and 10 mM inhibited germination by approximately 50%. The uncoupling agents carbonylcyanide *m*-dichlorophenylhydrazone, pentachlorophenol, 3,3′,4′,5′-tetrachlorosalicylanilide and dicumerol at concentrations of 0·1–0·2 mM completely inhibited germination. Dinitrophenol at 1·0 mM was only slightly inhibitory. The ionophore molecules gramicidin S, valinomycin and nonactin did not inhibit germination when tested at 5–10 µM in both the presence and absence of 50 mM-potassium ions. The ATPase inhibitors oubain, oligomycin and digitoxin at concentrations of 1·0 mM were not inhibitory while DCCD completely inhibited germination at a concentration 0·1 mM. These results reinforce the idea that energy production may be involved in initiation of spore germination and suggest that establishment of a protonmotive force and ATPase activity are key reactions. This idea and the previous demonstration that calcium ions initiate germination of *S. viridochromogenes* spores (Eaton & Ensign, 1980) led to testing GI for monovalent and divalent cation ionophore activity. The GI activity from both growth liquor and germinated spores did not demonstrate either K^+ or Ca^{2+} ion activity (data not shown).

The effect of GI on ATP production by germinating spores was determined by measuring the ATP content in the presence and absence of 2·5 units GI ml^{-1}. The results (Fig. 4) show that the ATP concentration in germinating spores nearly doubled during the first 60 min of germination and then remained constant. The ATP content of spores incubated in the presence of GI increased by only approximately 10%. Addition of GI after germination had begun, at 20 min, resulted in a rapid decrease in the amount of ATP to a level below that maintained in spores to which GI had been added at time zero.

A test was made of the possibility that GI is an ATPase inhibitor. A variety of ATPases were incubated for 10 min with 2·5 units partially purified (through the LH-20 column step) GI ml^{-1} obtained from either growth liquor or germination fluid. ATP was then added and the amount of ATPase activity was compared to control reactions with no GI added (Table 6). The canine and porcine Na^+-K^+-activated ATPases were completely inhibited by GI. The Na^+-activated ATPase of *A. laidlawii* was nearly completely inhibited. The Ca^{2+}–Mg^{2+}-activated ATPases of *E. coli* and *B. cereus* membrane preparations were resistant to GI while the mitochondrial enzyme was only partially inhibited. The *E. coli*, *B. cereus* and *A. laidlawii* ATPases were completely inhibited by 0·2 mM-DCCD (data not shown). The mitochondrial ATPase was inhibited by only 37% by the DCCD. This is an important point because DCCD-sensitive

Table 6. *Effect of GI on various ATPases*

ATPases were incubated for 10 min (prior to addition of ATP) with 2·5 units purified GI ml^{-1} obtained from growth liquor, or fluid after removal of germinated spores. The activity of ATPase was determined 30 min later and compared to the values obtained by reactions without GI.

Source of ATPase	Metal activators	Percent inhibution by GI from: Growth liquor	Germination fluid
Canine	Na^+, K^+	100	88
Porcine	Na^+, K^+	100	100
A. laidlawii	Na^+	94	78
E. coli	Ca^{2+}, Mg^{2+}	0	0
B. cereus	Ca^{2+}, Mg^{2+}	0	0
Beef heart mitochondria	Ca^{2+}, Mg^{2+}	22	20

ATPases become insensitive to this inhibitor when disassociated from their membrane bound complexes (Downie *et al.*, 1979). This signifies that the *E. coli* and *B. cereus* ATPases were membrane bound and DCCD sensitive. The mitochondrial ATPase may have contained both soluble and bound membrane activities. It will be shown later that GI inhibits a Ca^{2+}-activated ATPase activity of *S. viridochromogenes* only when it is membrane associated. The partial inhibition of mitochondrial ATPase activity by GI could thus represent the membrane associated enzyme. The GI insensitive activity would be due to soluble enzyme.

The GI target: ATPase of S. viridochromogenes membranes

A study was made of the ATPase activity of dormant and germinated spores and of vegetative mycelia of *S. viridochromogenes*. The goal was to form a basis for understanding the action of GI. The first task was to develop a procedure for disrupting spores. Attempts to gently lyse dormant spores to obtain membrane-bound ATPase activity were not successful. The dormant spores were not disrupted by lysozyme treatment even in the presence of reagents such as 8 M-urea, 10% (w/v) 2-mercaptoethanol, 10% (w/v) sodium dodecylsulphate and 0·1 M-dithiothreitol. These treatments render spores of some *Bacillus* species sensitive to lysozyme (Sargent, 1980). Various chelating agents and detergents failed to either lyse or render dormant spores susceptible to lysozyme. Spores germinated for 40–60 min did become susceptible to lysozyme digestion. Various mechanical means for disrupting spores were tested. This involved subjecting dormant and germinated spores to a French pressure cell at various pressures, grinding with alumina, sonic oscillation and rapid shaking in a dental amalgamator (Caulk Vari-Mix II-M). The only treatments effective in disrupting both dormant and germinated spores were sonic oscillation in the presence of 25 μm glass beads and shaking with 10 μm beads; greater than 99% disruption of spores followed 5 min and 2 min treatments, respectively. Vegetative-stage mycelia were lysed readily by lysozyme.

The results of analyses of the effects of metal ions, GI and DCCD on ATPase activity in the particulate and soluble fractions obtained from sonic disruption of dormant and germinated spores and of lysozyme treated mycelia are shown in Table 7. The supernatant fraction of germinated spores had no ATPase activity in the absence of Ca^{2+} and high activity in the presence of Ca^{2+}. The soluble Ca^{2+}-dependent ATPase activity was insensitive to DCCD and GI but was inhibited significantly in the presence of 10 mM of either Mg^{2+} or Mn^{2+}. The particulate fraction of germinated spores contained Ca^{2+}-dependent ATPase activity which was completely inhibited by GI, DCCD, Mg^{2+} or Mn^{2+}. No ATPase activity was detected in either the particulate or soluble fractions obtained from dormant spores. The particulate fraction obtained from mycelia contained Ca^{2+}-dependent ATPase activity which was partially inhibited by Mg^{2+}, GI and DCCD. The soluble fraction contained high Ca^{2+}-activated ATPase activity which was DCCD and GI insensitive but was partially inhibited by Mg^{2+} or Mn^{2+}. In none of the spore or mycelial fractions was any Na^+- or K^+-activated ATPase activity detected (data not shown). All fractions contained low levels of a Mn^{2+}-dependent phosphatase activity that was potently inhibited by Ca^{2+} (data not shown).

Table 7. *Effect of metal ions and inhibitors on ATPase activity of S. viridochromogenes*

Fraction tested	Additions† ...	Activity*					
		None	Ca^{2+}	$Ca^{2+} + Mg^{2+}$	$Ca^{2+} + Mn^{2+}$	Ca^{2+} + GI	Ca^{2+} + DCCD
Germinated spore supernatant		0	1300	450	118	1300	1300
Germinated spore particulate		0	118	0	0	0	0
Dormant spore supernatant		0	0	—	—	0	0
Dormant spore particulate		0	0	—	—	0	0
Mycelial supernatant		0	3200	1800	1400	3150	3200
Mycelial particulate		0	1240	525	—	514	828

—, Not tested.

* Units of ATPase activity: µg P_i released from ATP (mg protein)$^{-1}$ h^{-1}. Metal ions, 10 mM; GI, 10 units purified from growth liquor; DCCD, 0·2 mM.

These data establish that germinated spores and mycelia contain a Ca^{2+}-dependent ATPase which is sensitive to GI and DCCD when membrane bound but insensitive when soluble. This is a specific ATPase and not a phosphatase. Failure to detect ATPase activity in extracts of dormant spores may be due to inhibition of the enzyme by the endogenous GI. Experiments were designed to activate this presumed latent ATPase activity. The wall-membrane residue and supernatant fractions following sonic and amalgamator disruption of the dormant spores were tested for ATPase activity with 10 mM-Ca^{2+} added at pH 7·5. Neither fraction had ATPase activity (data not shown). Heat shock treatments of the fractions, varying the time of heating from 5–30 min at temperature between 45 and 60 °C, were not effective in releasing ATPase activity. Dialysis at 4 °C for 24 h released no activity. No ATPase activity occurred after treatment of the fractions with the following enzymes: trypsin, pronase, myxobacter AL-1 protease, lipase, phospholipases A, C and D, neuraminidase, lysozyme, and a mixture of lysozyme and pronase. Incubation with various amounts of an extract of 48 h mycelia or an extract of spores that had been germinated for 1 h also released no activity from the dormant spore fractions. We have no explanation at this time for this absolute failure of ability to activate the putative latent ATPase activity of the dormant spore extract fractions.

Characterization of the GI target

The soluble ATPase activity obtained after sonic disruption of germinated spores was further studied. A study was made of the effect of various metal ions at 10 mM concentration. A significant amount of activity was obtained only with Ca^{2+}, Sr^{2+} and Cd^{2+}: the latter two ions gave 32% and 24%, respectively, of the Ca^{2+}-dependent activity. The following ions did not stimulate activity significantly: Mg^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}, Fe^{2+}, Cu^{2+}, Na^+ or K^+. The Ca^{2+}-stimulated activity was drastically inhibited by Mg^{2+} and Mn^{2+} but not by Na^+ or K^+. The Mg^{2+} inhibition was found to have noncompetitive kinetics (data not shown).

The optimum Ca^{2+} : ATP ratio was determined by incubating the enzyme with 5·0 mM-ATP and various concentrations of Ca^{2+}, and incubating 0·25 mM-Ca^{2+} with varied concentrations of ATP. The optimum ratio was 1 : 1. The activity was essentially halved at Ca^{2+} : ATP ratios of 1 : 5 and 4 : 1 but was only 10% less at 1 : 2 and 2 : 1. A Lineweaver–Burke plot of enzyme velocity versus substrate (ATP) concentration demonstrated Michaelis-Menten kinetics with a K_m of approximately 2 mM and V_{max} of 12 µmol ATP hydrolysed (mg protein)$^{-1}$ h^{-1}. GTP and UTP were hydrolysed at 30 and 22%, respectively, of the ATP hydrolysis rate. The optimum ATPase activity occurred at pH 8·0. Activity declined rapidly at more acidic pH values but more slowly at more alkaline pH values.

DISCUSSION

Self-inhibitors of germination are common in fungal spores (Macko *et al.*, 1976). The mechanisms of action of these germination inhibitors are, with a few exceptions, unknown. The inhibitor in wheat stem rust spores blocks digestion of spore cell wall material in such a way as to prevent germ tube emergence (Hess *et al.*, 1975). A germination inhibitor in *Blastocladiella* *emersonii* zoospores is associated with ribosomes and blocks translation processes prior to initiation of germination (Adelman & Lovett, 1974). The germination inhibitor of *Dictyostelium discoideum* was identified as 3-(3-amino-3-carboxypropyl)-6-(3-methyl-2-butenylamino) purine (Abe *et al.*, 1976).

Self-inhibitors of germination are more rare in dormant prokaryotic cells. The GI of *S. viridochromogenes* spores is the only known example of inhibition of germination due to a self-contained germination inhibitor. In some spores of bacilli, alanine racemase converts exogenously added L-alanine to the D isomer which in turn is an inhibitor of germination (Fey *et al.*, 1964). This reaction is only involved in germination when L-alanine is the germinant. Gramicidin S, an antibiotic produced by *B. brevis*, has been reported to inhibit biosynthetic processes associated with outgrowth of germinating spores of the producing organism (Piret & Demain, 1982). However, initiation of germination (loss of refractility) does occur in the presence of the antibiotic.

The observation that the GI of *S. viridochromogenes* spores is a specific ATPase inhibitor suggests a mechanism for dormancy and initiation of germination of spores of this organism. Our hypothesis is that dormancy of the spores is maintained due to binding of the inhibitor to a membrane-associated ATPase complex. The result would be an inactive or latent system for ATP production. The primary event in initiation of germination, which requires only calcium ions (Eaton & Ensign, 1980), involves disassociation of GI from the target site, thus allowing coupling of transport and ATP synthesis. The hypothesis is consistent with the observations that the respiratory activity and ATP levels remain low during incubation of spores and increase rapidly following addition of germinants (Hirsch & Ensign, 1978). Why adding GI to dormant spore suspensions blocks their subsequent germination and why heat or detergent activation makes the spores immune to the added GI are questions for which we currently have no answer. It is possible that inhibition of germination of neighbouring spores by the first spore to respond to a germination stimulus has potential positive selective value. Should the first spore to germinate find conditions favourable and complete the life cycle, the newly formed spores would add to the gene pool of the organism. Should germination have been a lethal mistake, perhaps due to harsh conditions, the other spores whose germination was delayed by GI could try their luck at a later time. Inhibition of germination by GI is reversible since washed spores regain germinability.

Spores of *S. viridochromogenes* contain approximately 10 times the calcium concentration of vegetative cells (Eaton & Ensign, 1980). Calcium is expelled from the spores during the initiation process (unpublished results). This calcium excretion could be mediated by a Ca^{2+}-dependent ATPase pump mechanism which supplies an initial burst of ATP synthesis needed for germination. Calcium pumps associated with ATPase activity have been demonstrated in erythrocyte membranes (Schatzmann & Vincenzi, 1969) and appear to be involved in the maintenance of low Ca^{2+} levels in cells of bacteria (Kobayashi *et al.*, 1978). The observation that calcium effects the release of endogenous peptide inhibitor from the F_1-ATPase of muscle mitochondria (Yamada *et al.*, 1980) is perhaps also relevant. A critical test of our hypothesis would be the demonstration of the putative ATP–GI complex in dormant spores and definition of the mechanism of dissociation upon Ca^{2+} ion initiation of germination. The failure so far to detect ATPase activity in disrupted spores preparations has thwarted our attempts to test the hypothesis.

The GI of *S. viridochromogenes* can be considered an antibiotic because it is produced late in growth and inhibits growth of some bacteria. Sporulation does not occur during submerged culture in the medium used for GI production. Synthesis of GI might still be a sporulation-related process associated with sporulation events preceding the unknown block occurring in these conditions. During microcycle germination–sporulation of *S. viridochromogenes* in nitrogen-limited submerged culture, formation of spores and GI occur simultaneously (Koepsel & Ensign, 1984). This GI antibiotic can thus be considered a candidate for defining the role of an antibiotic for the producing organism, a subject discussed thoroughly by Demain (1974).

The mechanism of action of the antibiotic activity, inhibition of respiration of susceptible organisms, is consistent with its being an ATPase inhibitor. The well-characterized ATPase inhibitors DCCD and oligomycin block respiration in mitochondria (Heytler, 1979). Also, DCCD inhibits respiration during germination of *B. megaterium* spores (Rossignol & Vary, 1979). A Na^+- and K^+-activated ATPase inhibitor was recently isolated from mycelia of a *Streptomyces* species (Hensens *et al.*, 1983). The inhibitor is a 16-membered macrocyclic lactone. Werner *et al.* (1984) reported that an antibiotic purified from growth liquor of an *S. griseus* strain is identical in structure to this ATPase inhibitor. The antibiotic inhibits growth of many Gram-positive bacteria and fungi, but not Gram-negative bacteria. We have not yet been able to obtain a sample of the antibiotic or cultures of the organisms to determine if the antibiotic is the same as the GI–ATPase inhibitor of *S. viridochromogenes* or if the antibiotic has germination inhibitor activity in producing organisms.

Despite considerable effort in past years, the biochemical mechanisms of dormancy and triggering of germination of bacterial spores are not known. Recent work with spores of *B. megaterium* showed that D-glucose or L-proline triggered germination without being metabolized or permeating the spores (Racine *et al.*, 1979; Rossignol & Vary, 1979). The trigger compounds caused anisotropy changes in the spore membranes which may involve changes in conformation of membrane-bound proteins. Initiation of germination of *B. megaterium* spores does not involve respiration-linked processes involved in energy generation (Dills & Vary, 1978). The observation that initiation of germination of *S. viridochromogenes* spores is accompanied by ATP synthesis and that initiation is blocked by energy production inhibitors and protonmotive force uncouplers (Hirsch & Ensign, 1975) indicates a different basis for triggering germination than for *B. megaterium*.

Latent ATPase activity caused by peptide inhibitors has been demonstrated in mitochondria and chloroplasts (see reviews Cross, 1981; Pedersen *et al.*, 1981). Trypsin activated ATPase activity has been demonstrated in membranes of *Micrococcus lysodeikticus* (Carreira *et al.*, 1976), *Mycobacterium phlei* (Higashi et *al.*, 1975), *Escherichia coli* (Nieuwenhuis *et al.*, 1974) and *Azotobacter vinelandii* (Bhattacharyya & Barnes, 1976). The inhibitor was isolated from the F_1 subunit of *E. coli* ATPase and shown to have a molecular weight of 16000. Dreyfus & Satre (1984) demonstrated that the inhibitor in *E. coli* is the ε subunit of the ATPase. The possible function of these natural peptide regulators of ATPase activity in controlling or modulating ATP synthesis and hydrolytic processes was discussed in the review of Pedersen *et al.* (1981). In a recent study, Sanchez-Bastamante *et al.* (1982) reported that 70% of the ATPase molecules in mitochondrial membranes are inhibited by the endogenous inhibitor peptide during steady state oxidative phosphorylation. They suggest that the inhibitor may exert an active role in oxidative phosphorylation but the mechanism of such a role remains unexplained.

Of potential relevance to the apparent role of ATPase during Ca^{2+}-induced initiation of germination of *S. viridochromogenes* spores are the reports of trypsin activated ATPase activity associated with calcium transport in *A. vinelandii* (Bhattacharyya & Barnes, 1976) and *M. phlei* (Deves & Brodie, 1981). Release of spore components during the initiation stage of Ca^{2+}-induced germination of *S. viridochromogenes* ceases immediately upon addition of EDTA and resumes following removal of the chelator and addition of Ca^{2+} ions (Eaton & Ensign, 1980). This absolute requirement for Ca^{2+} ions during the entire initiation process could involve a dependence on calcium transport, possibly to supply ATP. The key to understanding the role of GI and the Ca^{2+}-dependent ATPase in initiation of germination awaits detection of the ATPase and GI in the dormant spores or extracts thereof. We are continuing this quest.

This investigation was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and by Public Health Service grant 5 RO1 AI 13948-06. A. D. G. was supported by Cellular and Molecular Biology training grant 5 T32 GM07215.

REFERENCES

ABE, H., UCHIYAMA, M., TANAKA, Y. & SAITO, H. (1976). Structure of discadenine, a spore germination inhibitor from the cellular slime mold *Dictyostelium discoideum*. *Tetrahedron Letters* **42**, 3807–2810

ADELMAN, T. G. & LOVETT, J. S. (1974). Evidence for a ribosome-associated translation inhibitor during differentiation of *Blastocladiella emersonii*. *Biochimica et biophysica acta* **335**, 236–245.

Bhattacharyya, P. & Barnes, E. M., Jr (1976). ATP-dependent calcium transport in isolated membrane vesicles from *Azotobacter vinelandii*. *Journal of Biological Chemistry* **251**, 5614–5619.

Carreira, J., Andreu, J., Nieto, M. & Muñoz, E. (1976). Membrane adenosine triphosphatase of *Micrococcus lysodeikticus*. Isolation of two forms of the enzyme complex and correlation between enzymatic stability, latency and activity. *Molecular and Cellular Biochemistry* **10**, 67–76.

Cross, R. L. (1981). The mechanism and regulation of ATP synthesis by F_1-ATPases. *Annual Review of Biochemistry* **50**, 681–714.

Decker, S. V. & Lang, D. R. (1977). *Bacillus megaterium* mutant deficient in membrane-bound adenosine triphosphatase activity. *Journal of Bacteriology* **131**, 98–104.

Demain,. A. L. (1974). How do antibiotic-producing microorganisms avoid suicide? *Analytical Biochemistry* **235**, 601–612.

Deves, R. & Brodie, A. F. (1981). Active transport of Ca^{++} in bacteria: bioenergetics and function. *Molecular and Cellular Biochemistry* **36**, 65–84.

Dills, S. S. & Vary, J. C. (1978). An evaluation of respiration chain-associated functions during initiation of germination of *Bacillus megaterium* spores. *Biochimica et biophysica acta* **541**, 301–311.

Downie, J. A., Gilson, F. & Cox, G. B. (1979). Membrane adenosine triphosphatases of prokaryotic cells. *Annual Review of Biochemistry* **48**, 103–131.

Dreyfus, G. & Satre, M. (1984). The ε subunit as an ATPase inhibitor of the F_1-ATPase in *Escherichia coli*. *Archives of Biochemistry and Biophysics* **229**, 212–219.

Eaton, D. & Ensign, J. C. (1980). *Streptomyces viridochromogenes* spore germination initiated by calcium ions. *Journal of Bacteriology* **143**, 377–382.

Ensign, J. C. (1976). Properties and germination of *Streptomyces* spores and a suggestion for a function of antibiotics. In *Microbiology 1976*, pp. 531–533. Washington, DC: American Society for Microbiology.

Ensign, J. C. & Wolfe, R. S. (1966). Characterization of a small proteolytic enzyme which lyses bacterial cell walls. *Journal of Bacteriology* **91**, 524–534.

Fey, G., Gould, G. W. & Hitchens, A. D. (1964). Identification of D-alanine as the autoinhibitor of germination of *Bacillus* spores. *Journal of General Microbiology* **36**, 229–236.

Fillingame, R. M. (1975). Identification of the DCCD-reactive protein component of adenosine triphosphate energy-transducing system of *Escherichia coli*. *Journal of Bacteriology* **124**, 870–883.

Garen, A. & Levinthal, C. (1980). A fine structure genetic and chemical study of the enzyme alkaline phosphatase of *Escherichia coli*: I. Purification and characterization of alkaline phosphatase. *Biochimica et biophysica acta* **38**, 470–483.

Grund, A. D. & Ensign, J. C. (1982). Activation of *Streptomyces virodochromogenes* spores by detergents. *Current Microbiology* **7**, 223–228.

Harold, F. M. & Baarda, J. R. (1969). Inhibition of membrane-bound ATPase and of cation transport in *Streptococcus faecalis* by *N,N'*-dicyclohexylcarbodiimide. *Journal of Biological Chemistry* **244**, 2261–2268.

Hensens, O. D., Monaghan, R. L., Huang, L. & Albers-Schönberg, G. (1983). Structure of the sodium and potassium ion activated adenosine triphosphatase inhibitor L-681,110. *Journal of American Chemical Society* **105**, 3672–3679.

Herbert, D., Phipps, P. J. & Strange, R. E. (1971). Chemical analysis of microbial cells. *Methods in Microbiology* **5B**, 210–344.

Hess, S. L., Allen, P. J., Nelson, D. & Lestor, H. (1975). Mode of action of methylcisferulate, the self-inhibitor of stem rust uredospore germination. *Physiological Plant Pathology* **5**, 107–112.

Heytler, P. G. (1979). Uncouplers of oxidative phosphorylation. *Methods in Enzymology* **55**, 462–472.

Higashi, T., Kalra, V. K., Lee, S. H., Bogin, E. & Brodie, A. F. (1975). Energy-transducing membrane-bound coupling factor ATPase from *Mycobacterium phlei*. *Journal of Biological Chemistry* **250**, 6541–6548.

Hirsch, C. F. & Ensign, J. C. (1975). Germination of *Streptomyces viridochromogenes* spores. In *Spores VI*, pp. 28–35. Edited by P. Gerhardt, R. N. Costilow & H. L. Sadoff. Washington, DC: American Society for Microbiology.

Hirsch, C. F. & Ensign, J. C. (1976*a*). Nutritionally defined conditions for germination of *Streptomyces viridochromogenes* spores. *Journal of Bacteriology* **126**, 13–23.

Hirsch, C. F. & Ensign, J. C. (1976*b*). Heat activation of *Streptomyces virodochromogenes* spores. *Journal of Bacteriology* **126**, 24–30.

Hirsch, C. F. & Ensign, J. C. (1978). Some properties of *Streptomyces viridochromogenes* spores. *Journal of Bacteriology* **134**, 1056–1063.

Jinks, D. C., Silvius, J. R. & McElhaney, R. N. (1978). Physiological role and membrane lipid modulation of the membrane-bound (Mg, Na)-adenosine triphosphatase activity in *Acholeplasma laidlawii*. *Journal of Bacteriology* **136**, 1027–1036.

Kobayashi, H., Van Brunt, J. & Harold, F. M. (1978). ATP-linked calcium transport in cells and membrane vesicles of *Streptococcus faecalis*. *Journal of Biological Chemistry* **253**, 2085–2092.

Koepsel, R. & Ensign, J. C. (1984). Microcycle sporulation in *Streptomyces viridochromogenes*. *Archives of Microbiology* **140**, 9–14.

Leps, W. T. & Ensign, J. C. (1979). ATP pool levels and endogenous metabolism in *Arthrobacter crystallopoietes* during growth and starvation. *Archives of Microbiology* **122**, 61–67.

Macko, V., Staples, R. C., Yaniv, Z. & Granados, R. R. (1976). Self-inhibitors of fungal spore germination, pp. 73–100. Edited by D. J. Weber & W. M. Hess. In *The Fungal Spore*, New York: John Wiley.

Nieuwenhuis, F. J. R. M., Drift, J. A. M. vd, Voet, A. B. & Van Dam, K. (1974). Evidence for a naturally occurring ATPase inhibitor in *Escherichia coli*. *Biochimica et biophysica acta* **368**, 461–463.

Pedersen, P. L., Schwerzmann, K. & Cintrón, N. (1981). Regulation of the synthesis and hydrolysis of ATP in biological systems: role of peptide inhibitors of H^+-ATPases. *Current Topics in Bioengineering* **11**, 149–199.

Piret, J. M. & Demain, A. L. (1982). Germination initiation and outgrowth of spores of *Bacillus brevis* strain Nagano and its gramicidin S-negative mutant. *Archives of Microbiology* **133**, 38–43.

PRESSMAN, B. C. (1972). Properties of ionophores with broad range cation selectivity. *Federation Proceedings. Federation of American Societies for Experimental Biology* **32**, 1698–1703.

RACINE, F. M., DILLS, S. S. & VARY, J. C. (1979). Glucose-triggered germination of *Bacillus megaterium* spores. *Journal of Bacteriology* **138**, 442–445.

ROSSIGNOL, D. P. & VARY, J. C. (1979). Biochemistry of L-proline-triggered germination of *Bacillus megaterium* spores. *Journal of Bacteriology* **138**, 431–441.

SANCHEZ-BASTAMANTE, V. J., DARSZON, A. & GOMEZ-PAYOU, A. (1982). On the function of the natural ATPase inhibitor protein in intact mitochondria. *European Journal of Biochemistry* **126**, 611–616.

SARGENT, M. G. (1980). A procedure for isolating high quality DNA from spores of *Bacillus subtilis* 168. *Journal of General Microbiology* **116**, 511–514.

SCHATZMANN, H. J. & VINCENZI, F. F. (1969). Calcium movements across the membrane of human red cells. *Journal of Physiology* **201**, 369–395.

VAN DE STADT, R. J., DEBOER, B. L. & VAN DAM, K. (1973). The interaction between the mitochondrial ATPase (F_1) and the ATPase inhibitor. *Biochimica et biophysica acta* **292**, 338–349.

WERNER, G., HAGENMAIER, H., DRAUTZ, H., BAUMGARTNER, A. & ZÄHNER, H. (1984). Metabolic products of microorganisms. 224. Bafilomycins, a new group of macrolide antibiotics. Production, isolation, chemical structure and biological activity. *Journal of Antibiotics* **37**, 110–117.

YAMADA, E. W., SHIFFMAN, F. H. & HUZEL, N. J. (1980). Ca^{++}-regulated release of an ATPase inhibitor protein from submitochondrial particles derived from skeletal muscle of the rat. *Journal of Biological Chemistry* **255**, 267–273.