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Growth and sporulation characteristics of *Bacillus megaterium* under different conditions of nutrient limitation

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The quantitative nutritional requirements to achieve specific cell densities have been studied for *B. megaterium*. Growth of batch cultures was separately limited by depletion of glucose, ammonium, sulphate, potassium, phosphate, manganese and magnesium. Maximum population density (E_{420}) for graded concentrations of each limiting nutrient was plotted against nutrient concentration and a linear plot was obtained below a critical concentration. Under conditions of magnesium depletion, two phases of growth occurred separated by a plateau. Proposals are made for the use of these cultures in drug resistance studies. Sporulation occurred in all cultures except those limited by potassium, manganese or magnesium. Spores were produced in magnesium-limited cultures provided that glucose was simultaneously depleted. Spores produced under different conditions of nutrient depletion varied in germination characteristics, heat resistance and spore volume.

The effects of cultural conditions and particularly medium composition on bacterial spore formation have been the subject of much work in recent years and have been regularly reviewed (Curran, 1957; Murrell, 1961, 1967; Halvorson, 1962; Vinter, 1969). Many workers have used chemically defined media. Nevertheless, the nature of the nutrient finally limiting vegetative growth has rarely been specified. Furthermore, we can find no reference in which any consideration has been given to the degree of excess of non-limiting nutrients.

The nutrient requirements for sporulation have been examined in several different ways. One approach is that of Grelet (1957), in which each of the components in a defined medium was in turn made the growth-limiting factor whilst other components were present in excess. He found sporulation to occur when the growth-limiting nutrient for *Bacillus megaterium* was glucose, nitrate, phosphate, iron or zinc, but not to occur when potassium, magnesium or manganese were the limiting factors. He also examined the germination requirements of spores formed through deficiency of glucose or nitrate. That sporulation does not occur when potassium, magnesium or manganese are deficient was substantiated by Lee & Weinberg (1971) and Rabinovitch (1971).

Since the work of Grelet, the effects of nutrient limitation have been studied in both batch and continuous culture and on both Gram-negative and spore-forming bacteria. Sporulation of *Bacillus subtilis* was studied in chemostat cultures under various limiting conditions by Dawes & Mandelstam (1970). Sporulation was observed when either glucose or ammonium were the limiting components but sporulation rates were low when either magnesium or phosphate were limiting. The relation between sporulation and availability of glucose has been reported for a species of *Clostridium* (Hsu & Ordal, 1969).

The phenomenon of endotrophic sporulation has also been used to study nutrient requirements for sporulation. The ability of vegetative cells of spore-formers to sporulate when transferred to distilled water was first reported by Buchner (1890) and developed largely by Foster & Perry (1954) and Foster (1956). The subject was reviewed by Black & Gerhardt (1963). The term "replacement technique" became used to describe experiments in which sporulation is not strictly endogenous and in which other substances were contained in the sporulation media. Replacement techniques have been used to study the roles of amino-acids in sporulation (Vinter, 1963; Buono, Testa & Lundgren, 1966), the requirements for metal cations and the effects of substitution of one cation for another (Black, Hashimoto & Gerhardt, 1960; Foerster & Foster, 1966). The minimal requirements for commitment to sporulation in *Bacillus megaterium* have recently been examined by a replacement technique (Greene & Slepecky, 1972).

In addition to information from growth limitation studies and replacement techniques, information on the nutrient and particularly metal cation requirements for sporulation has been obtained from batch culture experiments in which the material, not necessarily the growth-limiting factor, is incorporated in the medium before inoculation. Some specific ion requirements have been tabulated by Murrell (1961) and are discussed in subsequent reviews (Halvorson, 1962; Murrell, 1967).

Although it has become apparent that resistance properties and the chemical composition of certain vegetative cells are substantially altered under different growth-limiting conditions (Brown & Melling, 1969; Meers & Tempest, 1970), there appears to have been virtually no attempt to establish whether spore properties are similarly affected. It therefore seemed desirable to produce spores from cultures, growth-limited by different medium components, and to examine the extent to which biological properties and chemical composition of the spores are dependent upon the nature of the limitation. Our main purpose was the development of a medium which may be manipulated such that any desired component is the growth-limiting factor and all others are present in controlled excess. Metabolic changes may precede an observed change in growth rate due to nutrient depletion (Brown & Melling, 1969). Consequently, where possible, each non-limiting nutrient was added in 5-fold excess to that necessary to achieve the final limited population.

Studies in this laboratory on mechanisms of antibacterial activity have shown that certain Gram-negative cells physiologically defined in terms of limiting nutrient exhibited a characteristic and reproducible resistance pattern depending upon the nature of the limitation. The development of a medium for *Bacillus megaterium* was desirable because it would also make available similarly defined Gram-positive cells of reproducible characteristics. Such cells may facilitate both antibiotic assay procedures and studies on mechanisms of antibacterial action as well as facilitate spore studies.

A preliminary report of part of this study has been published (Brown & Chesson, 1971). This preliminary work was with a strain derived from *B. megaterium* ATCC 8245. Technical difficulties due to clumping caused us to change to a laboratory strain of *B. megaterium*. All of the work reported here is with this latter strain.

MATERIALS AND METHODS

Organism. A laboratory strain of *Bacillus megaterium*, verified as such using the classification scheme of Wolf & Barker (1968), was used throughout.

Chemicals and glassware. All chemicals used in the preparation of media were of Analytical Reagent grade (Analar), with the exception of $FeCl_2 \cdot 4H_2O$ (Laboratory Reagent grade) and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid, assay >99%, BDH Chemicals Ltd., Poole, England). All water used in the preparation of media was deionized before glass distillation. All glassware was cleaned using a hot 5% *v/v* solution of Decon 90 concentrate (BDH Chemicals Ltd.), soaked in 1% *v/v* hydrochloric acid and finally rinsed repeatedly in distilled water.

Culture methods. Stock cultures were kept on agar slants stored at 0–4°. The organism was routinely subcultured in a medium of composition identical to that described for glucose limitation in Table 1 and containing 0·01M glucose. 25 ml volumes of media in 100 ml conical flasks were used throughout the work, both for daily subculturing and all growth limitation studies. Cultures were incubated at 35° on a reciprocating shaker operating at 100 × 3·8 cm throws per min.

Limitation studies. Growth curves were recorded for cultures containing graded concentrations of the limiting component. Growth was measured as the extinction at 420 nm (E_{420}) using a Unicam S.P.600 spectrophotometer.

The inoculum was always 0·25 ml of a dense log phase culture, passaged at least twice in defined medium, or a dilution thereof in prewarmed medium used to minimize any lag phase. The extinction of the inoculum was adjusted such that when 0·25 ml was added to 25 ml the E_{420} of the latter was just less than 0·01.

The inoculum culture medium was in each case similar to that used in the limitation experiment with the concentration of the limiting component adjusted so that carry-over was negligible. The composition of all media used is shown in Table 1. Where possible the concentrations of medium constituents were kept constant for each limitation. Thus the medium composition was not progressively modified as the data from each experiment became known, unless it was apparent that a particular component was deficient or present in gross excess.

Table 1. *Composition of media used in growth limitation studies* †*

Constituent	Nature of limitation							
	Glucose	NH_4	SO_4	PO_4	K	Mn	Mg	Glucose and Mg‡
$Na_2HPO_4 12H_2O$	0·0539	0·0539	0·0539	**	2×10^{-4}	0·0539	0·0539	0·0539
KH_2PO_4	0·0128	0·0128	0·0128			0·0128	0·0128	0·0128
NH_4Cl	0·007	**	0·007	0·007	0·007	0·007	0·007	0·00635
KCl		0·007		0·0128	**			
Na_2SO_4	$2{\cdot}5 \times 10^{-5}$	$2{\cdot}5 \times 10^{-5}$	**	$2{\cdot}5 \times 10^{-5}$	$2{\cdot}5 \times 10^{-5}$	$2{\cdot}5 \times 10^{-5}$	$6{\cdot}35 \times 10^{-5}$	$8{\cdot}7 \times 10^{-5}$
$MgSO_4 7H_2O$	4×10^{-5}	4×10^{-5}		4×10^{-5}	4×10^{-5}	4×10^{-5}	**	5×10^{-6}
$FeSO_4 7H_2O$	2×10^{-6}	2×10^{-6}		2×10^{-6}	2×10^{-6}	2×10^{-6}	2×10^{-6}	2×10^{-6}
$MnSO_4 4H_2O$	4×10^{-7}	4×10^{-7}		4×10^{-7}	4×10^{-7}	**	4×10^{-7}	4×10^{-6}
Glucose	**	0·01	0·01	0·01	0·01	0·02	0·01	0·003
HEPES				0·025	0·025			
$MgCl_2 6H_2O$			4×10^{-5}					
$FeCl_2 4H_2O$			2×10^{-6}					
$MnCl_2 4H_2O$			4×10^{-7}					

* All concentrations expressed as molarities.
† All media adjusted to pH 7·4.
** Limiting constituent.
‡ Reformulated medium used to induce simultaneous glucose and magnesium depletion.

The basic media did not contain added calcium. However, the calcium content due to contamination was determined by atomic absorption spectrophotometry and was approximately 1·0 μg ml^{-1}.

Preparation of spore suspensions. Spores cultured on the surface of nutrient agar (Oxoid) plates at 35° for 72 h were harvested by the method of Long & Williams (1958). Liquid cultures (18–24 h) were harvested in the same way but with prior incubation with lysozyme 200 i.u. ml^{-1} followed 2–4 h later with trypsin 0·2 mg ml^{-1}.

Percentage sporulation. Cultures were prepared and grown in the same manner as those for limitation studies, except that the flask contents were aerated at the higher shaking rate of 120 throws per min to produce maximum sporulation. Percentage sporulation was measured after 24–30 h incubation by direct microscopical counting using a Helber bacterial chamber and a Wild model M20 phase contrast microscope.

Measurement of spore volume, germination rate and heat resistance. Spore volume was measured in a manner similar to that described by Hitchens, Greene & Slepecky (1972). The coefficient of variation for 5 replicate volume determinations was 5·5%.

The rate and extent of germination was measured by following the drop in E_{420} of 50 ml spore suspension in a 250 ml conical flask slowly agitated in a reciprocating shaker bath.

For determinations of heat resistance 0·25 ml of aqueous spore suspensions containing $2 \times 10^8 - 5 \times 10^8$ spores ml^{-1} were added to 49·75 ml of preheated phosphate buffer, pH 7·4, in a 250 ml stoppered conical flask with a side arm. The flask was maximally immersed in a polypropylene water bath, thermostatically controlled to $\pm 0{\cdot}05°$, such that the suspension in the flask was well below water level. The suspension was agitated slowly to avoid splashing, by use of a 3·0 cm Teflon coated magnetic stirring bar, and a magnetic stirrer directly below the conical flask and polypropylene bath. 1 ml samples were removed at time intervals, diluted in single strength nutrient broth (Oxoid), and 0·5 ml volumes spread on the surface of nutrient agar (Oxoid) plates.

RESULTS

Growth limitation studies. Growth curves for glucose and ammonium-limited cultures are shown in Figs 1A and B. Fig. 2A illustrates the relation between E_{420}

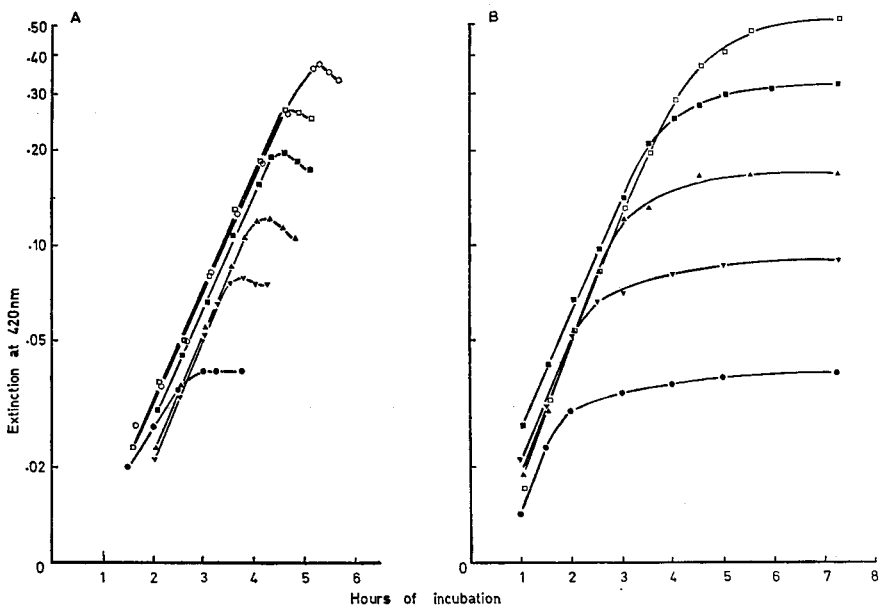

FIG. 1A. Growth curves of cultures limited by graded concentrations of glucose. ○ 0·0010 M; □ 0·0007 M; ■ 0·0005 M; ▲ 0·0003 M; ▼ 0·0002 M; ● 0·0001 M.

B. Growth curves of cultures limited by graded concentrations of ammonium (NH_4Cl). □ 0·0006 M; ■ 0·0004 M; ▲ 0·0002 M; ▼ 0·0001 M; ● 0·00004 M.

maxima and medium concentration of growth-limiting component for glucose and ammonium limitation. The corresponding plot for sulphate limitation is shown in Fig. 2B.

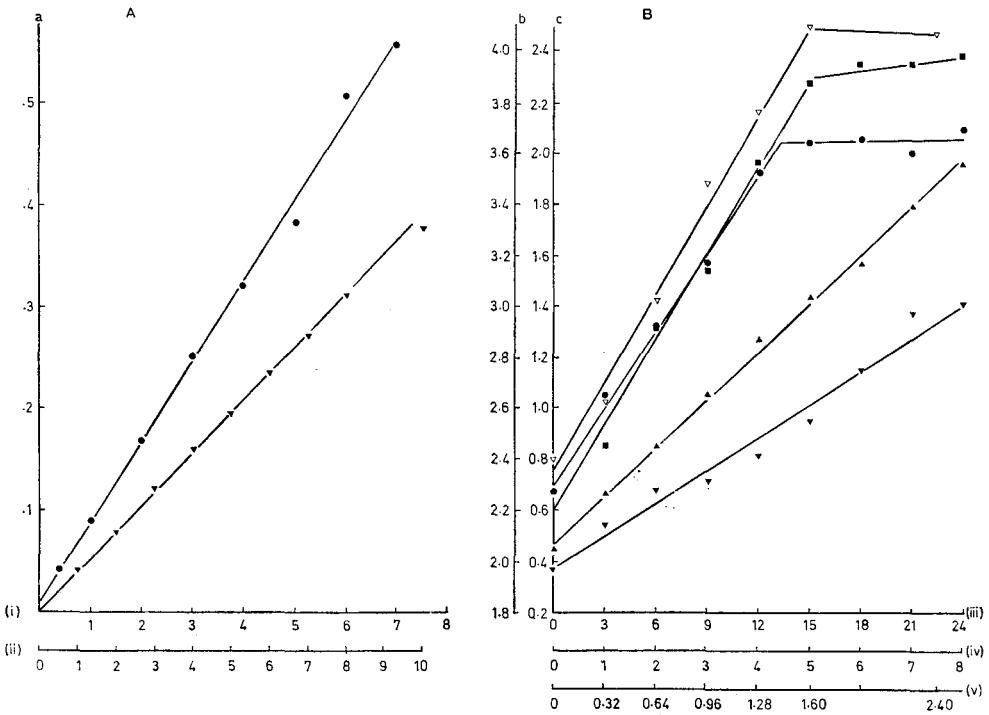

FIG. 2A. Glucose and ammonium limitation at different initial concentrations. ▼ Glucose; ● Ammonium.

B. Manganese, potassium, phosphate and sulphate limitation at different initial concentrations. ▽ Manganese-limited cultures after 7 h growth; ■ potassium-limited cultures after 18 h growth; ▲ phosphate-limited cultures after 8·5 h growth; ● phosphate-limited cultures after 20 h growth; ▼ sulphate-limited cultures after 10 h growth. Cultures containing no added KCl did not exhibit growth. Ordinates: (a) maximum extinction: (b) extinction of manganese-limited cultures; (c) extinction of sulphate-, phosphate- and potassium-limited cultures. Abscissae: (i) NH_4Cl (M × 10^4); (ii) glucose (M × 10^4); (iii) Na_2SO_4 (M × 10^6); (iv) $NaHPO_4 \cdot 12H_2O$ or KCl (M × 10^5); (v) $MnSO_4 \cdot 4H_2O$ (M × 10^7).

Growth curves for glucose-limited cultures, being characterized by an abrupt cessation of logarithmic growth followed by a fall in E_{420}, differed from those of ammonium- or sulphate-limited cultures. The latter were similar in shape to those for ammonium and are not illustrated. The growth rate of these cultures did not abruptly change at the onset of limitation but showed a progressive fall to zero over a 3–4 h period. The fact that media, to which no sulphate had been added nevertheless supported growth to an E_{420} in excess of 0·35, suggests that there was a relatively high level of sulphate contamination from other medium components.

For both potassium and phosphate limitation experiments, Sörensen's phosphate buffer was replaced by HEPES, 0·025 M. The growth rates and microscopical appearance of log phase cells growing in HEPES buffered media were found to be the same as those growing in phosphate buffered media. The growth curves for phosphate and potassium-limited cultures were similar. In each case the E_{420} did not exhibit a maximum value within an 8–10 h incubation period, as seen under other limitations.

The extinction value continued to increase slowly. Nevertheless the E_{420} at 8–10 h for phosphate-limited cultures was linearly related to phosphate concentration (Fig. 2B). E_{420} values after 18 and 20 h were also plotted for potassium- and phosphate-limited cultures respectively against concentration of limiting component. The non-linearity in the plots at E_{420} values $>2{\cdot}0$ is probably due to exhaustion of glucose.

In each case the intercept of the plots, corresponding to no added phosphate or potassium, revealed a high contamination level of these two ions. Cultures to which no potassium was added always failed to exhibit growth even after a 48 h incubation period, despite the intercept value suggesting that growth to an E_{420} of approximately 0·6 should occur.

The effects of manganese depletion were only observed at relatively high cell densities. To support growth to the required E_{420} values it was necessary to increase the concentration of glucose in the medium. At extinctions in excess of 1·0, the growth rates of the cultures were proportional to the manganese concentration in the medium. The E_{420} values after 7 h recorded growth are plotted against manganese concentration in Fig. 2B. It should be noted that no distinct E_{420} maxima were observed in the growth curves and the decision to plot the values at 7 h was arbitrary. Also, repetition of the experiment gave a plot similar in slope to that in Fig. 2B, but the intercept value varied with each batch of media. The results show that cell density was dependent upon manganese concentration, but that the effects of depletion may be observed only at relatively low concentrations of manganese.

For magnesium-depleted cultures the duration of the lag phase was inversely related to the magnesium concentration. This effect was also observed in sulphate-limiting media but to a lesser degree. There was a progressive decrease in the growth rate to zero over a 2–3 h period, following the exponential growth phase. E_{420} maxima occurred for the growth curves of cultures containing 1·5 or $3{\cdot}0 \times 10^{-6}$ M

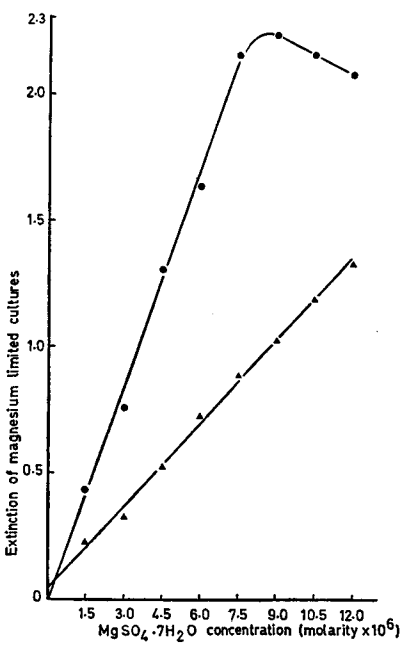

FIG. 3. Magnesium limitation at different initial concentrations. ▲ E_{420} after first growth phase (7 h); ● E_{420} after second growth phase (18 h).

$MgSO_4 \cdot 7H_2O$. At higher magnesium concentrations the curves exhibited a plateau at which the growth rates approximated to zero. Once more, linear relations occurred if these maxima or plateaux were plotted against magnesium concentration (Fig. 3, lower line). After further incubation, however, growth was resumed. When the E_{420} values were recorded after 18 h and plotted similarly, the upper line in Fig. 3 resulted. Thus two very distinct phases of growth were observed in magnesium-depleted cultures. It is possible that a change in light reflecting characteristics of the cell surface may contribute to this phenomenon. It is nevertheless unlikely to account entirely for a 2–3-fold increase in E_{420}.

The shape of the growth curve was examined over an extended incubation period to record the second phase of E_{420} increase. Fig. 4 shows the growth of three replicate cultures each containing $3{\cdot}0 \times 10^{-6}$ M $MgSO_4 \cdot 7H_2O$. The growth curves are displaced by 15 min on the time scale to improve display. The relation between E_{420} values after 18 h and magnesium concentration in the medium was linear up to an E_{420} value of approximately 2·2, corresponding to 8×10^{-6} M $MgSO_4 \cdot 7H_2O$. The subsequent fall at higher concentrations may be due to lysis occurring in the cultures as glucose in the medium became exhausted. The growth rates of cultures attaining high E_{420} values of about 1·5 were much reduced, due in part to insufficient aeration. Relatively more of the available glucose is utilized supporting the respiration of a slowly growing population. Consequently the cell density attained was lower than that achieved when the culture had grown at the faster rate until glucose exhaustion.

Microscopical appearance of cells under different limiting conditions. No appreciable differences were observed in the appearance of log phase cells from the different media.

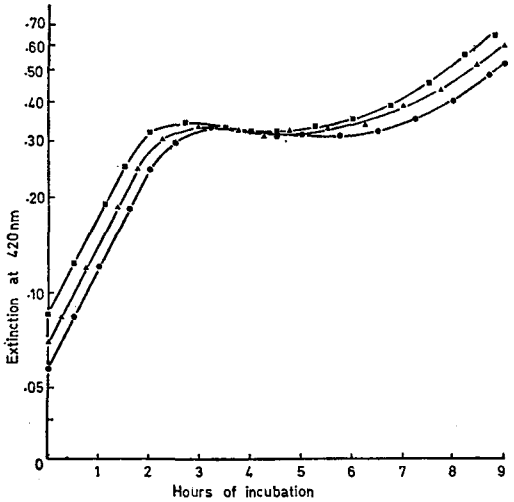

FIG. 4. Replicate magnesium-limited cultures showing two phases of growth. The concentration of $MgSO_4 \cdot 7H_2O$ is 3×10^{-6}M in each case. Displaced by 15 min on time scale for display.

Sporulation occurred in all cultures except those growth-limited by potassium manganese or magnesium. All cultures in which sporulation occurred were similar in appearance, when examined both before and following sporulation. Manganese-limited cells resembled those in other limited cultures before the appearance of spores.

Potassium-limited cells differed greatly from those under other limiting conditions.

They were characteristically long convoluted filaments, equivalent in length to 20–50 individual log phase cells. The filaments were almost featureless. No mature spores or any structures resembling developing spores were observed. Accumulations of what was tentatively assumed to be poly β-hydroxybutyric acid (P.H.B.) were observed within the cells of other limited cultures, but they were absent from those limited by potassium. Magnesium-depleted cells which had completed the first growth phase but not the second were slightly shorter but otherwise little different in appearance from log phase cells. Those having completed both phases of growth differed in that they contained large quantities of P.H.B. and were present as filaments equivalent in length to 5–10 cells from the log phase. Again, no mature or developing spores were observed.

Sporulation in limited cultures. On the basis of the results from the limitation studies, the medium composition was adjusted such that the concentrations of NH_4^+, Mg^{2+} and SO_4^{2-} were sufficient to support growth to an E_{420} of 5·0. The concentrations of phosphate or potassium could not be adjusted without altering the buffer capacity of the medium. To enhance sporulation, 4×10^{-6} M $MnSO_4 \cdot 4H_2O$ was used. Higher concentrations did not result in a greater percentage of spores.

When glucose was at a growth-limiting concentration in the reformulated medium, percentage sporulation was 20–30%. This was not increased by the addition of calcium or glutamic acid to the medium. This figure is relatively low. Identical media supported extensive sporulation (in excess of 90%) in two other laboratory strains of *B. megaterium*. These, however, were unsuitable for further study because of a tendency to clump in defined media.

The effects of variation in magnesium concentration in the medium over the range 1×10^{-6} to 5×10^{-3} M were examined on glucose-limited cultures. An increase in percentage sporulation was observed within the concentration range 1×10^{-6} to 1×10^{-5} M. This was investigated further (Fig. 5). The peak corresponds to a magnesium concentration sufficient to give an E_{420} of approximately 0·6 after the first growth phase (see Fig. 3). The glucose content of the medium would support exponential

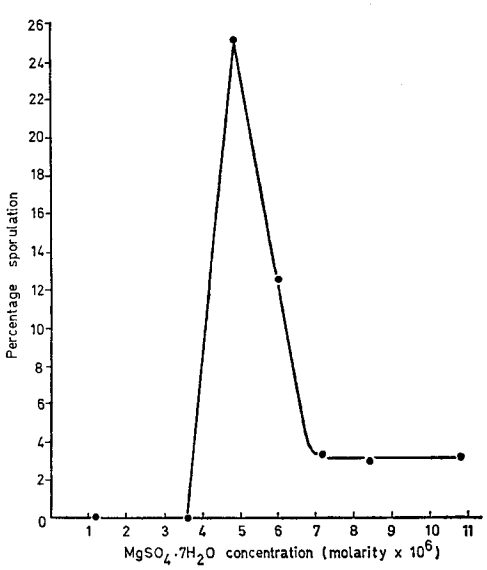

FIG. 5. Effect of magnesium concentration on percentage sporulation in glucose-limited cultures.

growth to an E_{420} value of 1·0. If growth ceases to be exponential, glucose becomes exhausted before an E_{420} of 1·0 is reached. Thus the culture must also become glucose-limited before the second growth phase is complete. Sporulation therefore occurred in conditions of simultaneous magnesium and glucose limitation. At concentrations of magnesium $>5 \times 10^{-6}$ M the percentage of cells producing spores was reduced. The reason for this is unknown.

Properties of spores grown under different limiting conditions. Preliminary studies on spores from glucose-limited cultures (G-), and those from which magnesium was also depleted (G- Mg-), showed that they differed in several properties, and both differed from spores produced on nutrient agar.

There was a significant difference in size between G- spores (0·88 μm^3) and G-Mg- spores (1·39 μm^3). The former germinated more rapidly and more extensively when suspended in a solution of phosphate buffered L-alanine 100 mM, and glucose 10 mM, pH 7·4. After 15 min at 35° the E_{420} of G- spores had been reduced to 34% of its original value, whilst that of a suspension of G-Mg- spores was reduced to 77% of its original value.

Preliminary results show that heat resistance was also affected. G-spores heated at 95° exhibited a log-linear survivor curve. G-Mg- spores under the same conditions gave a survivor curve with a marked shoulder.

Detailed studies are proceeding on the dormancy, resistance and chemistry of these spores.

DISCUSSION

Manipulation of a defined glucose-salts medium permitted growth limitation of *Bacillus megaterium* by glucose, ammonium, sulphate, magnesium, phosphate, potassium and manganese ions. In each case a linear relation existed within limits, between population density and the concentration of limiting component in the medium.

Under conditions of magnesium depletion two phases of growth were observed and a plateau occurred in the growth curve, during which the growth rate approximated to zero. It seems possible that the plateau represents a period of adaptation and redistribution of magnesium within the cell. It has been well documented that for certain functions, and particularly as an enzyme activator, magnesium may be replaced by other metal cations, notably manganese (Dixon & Webb, 1964). Similar considerations apply to the possible redistribution of wall magnesium. Magnesium is also an integral component of ribosomes (Tissieres & Watson, 1958) a function for which it cannot be replaced. Further growth may possibly be permitted by redistribution and concentration of magnesium in the ribosome.

The observations that sporulation did not occur in cultures limited by potassium, magnesium or manganese are in agreement with those of Grelet (1957). However, spores were produced in magnesium-depleted cultures, provided that glucose also became simultaneously depleted.

Use of the data obtained has enabled the formulation of media with a controlled excess of all medium components except that which is growth-limiting at a predictable population density. Reproducible batches of spores may be produced which are defined, both in terms of the medium and of the nutrient limitation. Preliminary studies indicate that relatively minor changes in medium composition, which may, for

example, result in the simultaneous depletion of two medium components rather than one, also result in significant changes in spore properties.

Spore volume is one of the biological properties markedly affected by the nature of the limitation. Spores produced under conditions of glucose and magnesium depletion were larger than spores produced from media in which glucose alone was growth limiting. Hitchins & others (1972) have reported that the volume of *Bacillus megaterium* spores is dependent upon the nature of the carbon source, in a defined simple salts medium. They noted that by varying the carbon source, variations in spore size and chemical composition may be produced, apparently without altering the genetic make-up of the spore. Similar variations apparently occur under different conditions of nutrient limitation.

Anderson & Friesen (1972) have investigated the growth and sporulation characteristics of *Bacillus stearothermophilus* in a chemically defined medium. They describe a medium for the production of a standardized spore suspension suitable for heat sterilization control purposes. Percentage sporulation was reduced in media supporting the highest cell densities and the optimum sporulation medium finally recommended was one in which glucose would appear to be at a limiting concentration.

The current edition of the British Pharmacopoeia (1973) specifies that suspension of spores of *Bacillus* species used as inocula in antibiotic assay procedures should be prepared using complex media solidified with agar. Similarly complex media have been used for the preparation of spore suspensions of *Bacillus subtilis var niger* and *Bacillus pumilus* used in studies on the control of ethylene oxide and radiation sterilization processes respectively (Beeby & Whitehouse, 1965; Darmady, Hughes & others, 1961).

We suggest that greater reproducibility of resistance characteristics may be obtained in spores of *Bacillus* species used for antibiotic assay and sterilization control processes, by their production in chemically defined media, enabling their physiological definition in terms of nutrient limitation.

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