Effects of Biotin Deficiency on Growth, Morphology and Sporulation in *Bacillus megaterium* NCIB7581

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SUMMARY

Bacillus megaterium strain NCIB7581 grew at 30 °C in a simple chemically defined medium without biotin. At 37 °C the bacteria grew more slowly in the same medium and were deformed, especially during the earlier stages of growth, as filaments or with swollen ends. Addition of biotin restored the normal appearance and rate of growth at 37 °C. The organisms synthesized more biotin at 30 °C (11 ng l⁻¹ in a stationary phase culture) than at 37 °C (1.4 ng l⁻¹).

Addition of either aspartate, malate, fumarate or acetate to minimal medium also restored normal morphology and accelerated the growth rate in biotin-deficient medium at 37 °C. Neither acetate nor aspartate increased synthesis of biotin at 37 °C.

After growth in medium containing only 0.2% glucose almost every organism in the culture was able to sporulate at 30 °C whether or not biotin was added. About 50 times fewer spores were formed at 37 °C without biotin; the yield of spores increased about 20-fold at this temperature when biotin, aspartate or dipicolinate was added.

INTRODUCTION

White (1972) studied the nutritional requirements of 11 strains of *Bacillus megaterium*. Strain NCIB7581 required biotin to grow from small inocula at 37 °C in a chemically defined medium with glucose (or glycerol) and ammonium sulphate as the principal nutrients. However, the organisms could be propagated from large inocula at 37 °C without biotin. The effect of biotin deficiency (at 37 °C) ranged from production of some abnormally shaped organisms on solid medium to inability of a small inoculum to grow in liquid medium. In this study the role of inoculum size was examined further; conditions for the production of cultures containing only abnormally shaped organisms were devised; the synthesis of biotin was measured at both 37 °C and 30 °C; the abilities of the organisms to sporulate at 37 °C and 30 °C were compared with or without biotin; and the deformities caused by biotin deficiency at 37 °C were examined by electron microscopy.

A preliminary report of some of these results has already been published (Al-ssum & White, 1974).

METHODS

Organisms. *Bacillus megaterium* NCIB7581 was maintained at 37 °C on nutrient agar slopes that were incubated at 37 °C overnight, then stored at 2 °C and subcultured monthly. For day-to-day use, inocula were prepared from organisms grown overnight at 37 °C on slopes of medium A1. *Lactobacillus arabinosus* NCIB9085 was grown overnight in stabs of yeast extract (1%, w/v), Difco tryptone (1%, w/v), glucose (0.1%, w/v), KH₂PO₄ (0.2%, w/v)

CaCO₃ (0.3 %, w/v), and agar (1.5 %, w/v), at 30 °C and subcultured monthly. For day-to-day use, inocula were prepared from organisms grown overnight in yeast extract (1 %, w/v) plus glucose (1 %, w/v) at 30 °C.

Media. The chemically defined medium A1 was that of White (1972) with glycerol (usually 1 %, w/v) as carbon source. Medium A2 contained glucose (usually 1 %, w/v) instead of glycerol. Biotin assay medium (Difco no. 0419-15) was rehydrated by dissolving 7.5 g in 100 ml distilled water, with boiling.

Biotin extraction and assay. (i) Extraction [modified from Pai & Lichstein (1962)]. Each stationary phase culture was autoclaved at 121 °C for 20 min, centrifuged at 5000 g for 20 min and the supernatant liquid was kept. The killed organisms were washed twice with warm distilled water (50 to 60 °C) by centrifuging at 5000 g. All supernatant liquids were combined and dried at 50 °C in a rotary evaporator. The dried residue was extracted with 10 ml ethanol (100 %) three times, and the solid was discarded. Ethanol was evaporated and the residue was extracted with warm water (80 °C) five times. The water extracts were combined and concentrated to a small volume.

Presence of the vitamin in the extractable material was proved by its growth-promoting activity for *L. arabinosus*; this activity was lost after pre-incubation (of 0.1 ml extract containing 1 to 2 ng biotin) for 15 min with avidin (5 µg) at 30 °C.

(ii) Assay. Test tubes were prepared containing 5 ml rehydrated biotin assay medium, increasing amounts of biotin (in the range 0 to 1 ng) or test material, plus water to give a total volume of 10 ml per tube. The tubes were autoclaved at 121 °C for 15 min. *Lactobacillus arabinosus* was grown overnight in yeast extract plus glucose (5 ml), washed in 0.05 M-Tris/HCl buffer, pH 7, then in distilled water, and suspended in 100 ml 0.9 % (w/v) NaCl. One drop of this suspension was used as the inoculum for each tube, and the tubes were incubated (upright, stationary) at 30 °C for 72 h. Extinctions were measured at 650 nm and the amount of biotin in test samples was calculated from the standard curve.

Growth of *B. megaterium* on solid and in liquid medium. Different sizes of inocula, washed first in 0.05 M-Tris/HCl buffer, pH 7.4, then in 0.9 % NaCl and finally in distilled water, were seeded on to the surface of 8 cm diam. plates of medium A1 (about 20 ml) with biotin (10 µg l⁻¹) or without biotin and incubated at 30 or 37 °C for 12 to 16 h. Liquid cultures were usually grown in 50 ml medium A1 with biotin (10 µg l⁻¹) or without biotin in 250 ml conical flasks. These were incubated with shaking (about 200 gyrations min⁻¹) at 30 or 37 °C. Turbidities of liquid cultures were measured with an EEL colorimeter as described by White (1972).

Sporulation. Spores of *B. megaterium* NCIB7581 were obtained by growing the organisms at 37 or 30 °C in liquid medium A2 containing glucose (0.2 %) and 1 mM-CaCl₂ (added to the medium after autoclaving), with or without supplements, for 35 to 48 h. The number of heat-resistant spores was assessed by incubating the suspension at 60 °C for 30 min, and then plating diluted samples on nutrient agar that was incubated at 30 °C for 24 h. Colonies were counted and the number of spores per ml culture was calculated.

Electron microscopy. The organisms were grown on plates of medium A1 with or without biotin at 37 °C for 10 to 16 h. They were then scraped off the plates, washed and fixed with 3 % (v/v) glutaraldehyde in 0.1 M-phosphate buffer pH 7.2 overnight. After washing in the same buffer containing sucrose (6.84 %, w/v), the organisms were treated for 90 min in 2 % (w/v) OsO₄ in 0.1 M-phosphate buffer pH 7.2. Dehydration was by treatments (each 30 min) with increasing concentrations of ethanol ranging between 20 % (v/v) and 100 %. The organisms were embedded in dry ethanol/epon (1:1, v/v) for 30 min; then ethanol/epon (1:3, v/v) for 30 min and full strength epon overnight. The samples were blocked out in

Fig. 1. Surface growth of *B. megaterium* NCIB7581 on medium A2 without biotin at 37 °C. About 2×10^6 organisms (from a nutrient agar slope; in 0.1 ml water) were spread over the surface of 30 ml solid medium in a Petri dish (8 cm diam.). The plate was incubated overnight at 37 °C. On longer incubation, growth in the zones of minute colonies (A and B) gradually became heavier until these zones were almost indistinguishable.

gelatine capsules using fresh epon resin and polymerized at 30 °C for 8 h. Sections, cut on a Reichert OmU3 ultramicrotome using a Dupont diamond knife, were collected on 200-mesh grids prepared with formvar support films and then stained with uranyl acetate followed by lead citrate (Echlin, 1964).

RESULTS

Bacillus megaterium NCIB7581 produced two types of growth on an agar surface (8 mm diam.) of medium A1 or A2 (30 ml without biotin) at 37 °C from an inoculum of about 10^6 organisms. These two types of growth occupied zones that were distinguished by their appearance: a zone (or zones) of heavy confluent growth and other zones of minute colonies (Fig. 1). An inoculum of 5×10^6 organisms per plate produced heavy and confluent growth with no sign of the zones of small colonies. On the other hand, smaller inocula of about 10^2 to 10^3 organisms required long incubation times (about 48 h) to grow at 37 °C to colonies of about 1 mm diam., and no smaller colonies were seen.

Organisms from the zones of heavy growth appeared normal under the phase contrast microscope, i.e. rod shaped and occurring singly or in short chains (Fig. 2*a*). Organisms from the small colonies were filamentous, twisted and often swollen at their ends (Fig. 2*b*).

In liquid medium A1 at 37 °C an inoculum of 2×10^6 organisms per 50 ml produced two types of organisms: normally shaped rods that were similar to those seen from the heavy growth zones on solid medium, and long filaments that were twisted and swollen. An inoculum of 5×10^6 organisms produced only normally shaped rods. Decreased numbers of bacteria (10^2 or 10^3 organisms) gave no growth in liquid medium A1 at 37 °C.

Fig. 2. *Bacillus megaterium* NCIB7581 from surface growth on medium A2 without biotin at 37 °C. Unstained organisms were suspended in water containing 10 % (w/v) gelatine and photographed under phase contrast. (a) Normal organisms from a zone of confluent growth; (b) filamentous organisms from a zone of minute colonies. Bar markers represent 20 µm.

Biotin requirement

Abnormal morphology in the absence of biotin has not been observed below 33 °C, either in liquid or on solid medium A1. Above this temperature, deformity of the growing organisms, and a requirement for biotin to prevent this and to allow normal growth, (White, 1972) became noticeable if less than 1×10^6 organisms were used as the inoculum for 50 ml liquid medium. At 37 °C the phenomenon became more obvious and reproducible. However, the organisms could still grow at higher temperatures, e.g. 40 °C, without biotin, but a larger inoculum was required (4×10^7 organisms in 50 ml) and the growing organisms were always deformed.

The presence of two morphological types (normal and filamentous) in the same culture at 37 °C without biotin made it difficult to obtain the filamentous forms without the normal ones. On transfer to fresh medium without biotin, normal or deformed organisms continued to give a mixed population after incubation at 37 °C. Hence, conditions favourable for production of only the filamentous forms at 37 °C were sought. The inoculum was washed in sterile 0.05 M-Tris/HCl buffer, pH 7.4, then in 0.9% NaCl and finally in distilled water. The washed inoculum (10^6 organisms) was plated on medium A1 and incubated at 37 °C for 10 to 12 h. The organisms formed only minute colonies of filamentous forms. Occasionally there were a few colonies of normally shaped organisms, though never more than 10 colonies on one plate. Only when liquid medium A1 was brought to 37 °C before inoculation did washed organisms give cultures consisting solely of filamentous forms. However, in liquid cultures all the deformities in the absence of biotin at 37 °C were transient, and normally shaped organisms became predominant on longer incubation (24 h). Presumably, variants that can synthesize biotin at 37 °C arise frequently, and the biotin produced by these organisms then allows normal growth of the remaining bacteria that have limited ability to make the vitamin.

Swollen and twisted organisms similar to those formed in the absence of biotin were also produced when *B. megaterium* NCIB7581 was grown at 37 °C under certain conditions in

Table 1. *Effects of various additions to medium A1 on growth of B. megaterium* NCIB7581 at 37 °C

All the additions (except biotin) were sterilized separately and added to the molten medium at 45 °C to give the required concentration. Plates were spread with 0.1 ml bacterial suspension (about 2×10^6 organisms) and incubated at 37 °C for 12 to 24 h.

Addition	Concentration (mg l ⁻¹)	Growth after 12 to 24 h
Biotin	0.001	+++
L-Aspartate	100	+++
L-Glutamate	100	++M
D-Aspartate	100	—
D-Glutamate	100	—
L-Malate	100	+++
Na fumarate	100	+++
Na oleate	50	—
Na malonate	50	++M
Na oxoglutarate	100	++M
Na palmitate	50	—
Na succinate	100	+MM
Na ₃ citrate (2H ₂ O)	100	+MM
Pimelic acid	10	+MM
Na acetate (3H ₂ O)	500	+++
Na bicarbonate	50	—
None		+MM

+++ , Heavy confluent growth within 12 h incubation; ++M, heavy confluent growth within 12 h incubation but also a small zone (or zones) of minute colonies; +MM, zone or zones of minute colonies occupying most of the agar surface; —, no growth after 24 h incubation at 37 °C.

medium A1 containing biotin. For example, growth in the presence of 1 mM-dipicolinic acid gave very twisted organisms, as did growth with methicillin (about $0.2 \mu\text{g ml}^{-1}$) or the detergent Triton X-100 ($5 \mu\text{g ml}^{-1}$), and in stationary phase cultures on solid medium A1 many organisms became swollen at their ends, presumably as a consequence of autolysis without sporulation.

Replacement of biotin

Medium A1 was supplemented with several compounds structurally unrelated to biotin (Table 1). Compounds that allowed normal growth at 37 °C without biotin were L-aspartate, L-malate, fumarate, glutamate and acetate. Pimelic acid, which is a precursor of part of the biotin molecule (see review by Eisenberg, 1973) as a CoA derivative, was ineffective. The rates of growth of the organisms at 37 °C in liquid medium A1 with various additions were compared with the rate in unsupplemented medium A1. L-Aspartate (100 mg l^{-1}) or L-malate (100 mg l^{-1}) allowed growth that was about as rapid and heavy as with biotin ($10 \mu\text{g l}^{-1}$). When the glycerol in medium A1 was entirely replaced by equimolar sodium acetate, growth in liquid culture was poor. The medium became alkaline (above pH 9), as would be expected from the complete oxidation of sodium acetate to water, CO₂ and NaOH. Better growth with sodium acetate (trihydrate; 0.5 g l^{-1}) occurred when some glycerol (0.25 %) was also present. With higher concentrations of glycerol (1 %), addition of acetate still restored the normal morphology (and rate of growth) in the absence of biotin.

Biotin content of stationary phase cultures grown at 30 °C or 37 °C

Growth of the organisms at 30 °C in medium A1 was normal (only short rods) regardless of the inoculum size, and there was no requirement for biotin. These observations suggested

Table 2. *Sporulation of biotin-sufficient and biotin-deficient organisms*

Bacillus megaterium was grown in medium A2 (containing only 0.2 % glucose, instead of 1 %) with 1 mM-CaCl₂, and supplemented as indicated. All cultures grew (before sporulation) to about the same turbidity (colorimeter reading 1.8 to 2.0) within 20 h. After 36 h samples were taken to determine the number of heat-resistant spores that survived heating at 60 °C for 30 min and were able to grow at 30 °C when plated on nutrient agar. This count did not increase on longer incubation. Results show the mean number of spores $\pm$ the standard deviation, with the number of measurements shown in parentheses.

Medium	10 ⁻⁶ $\times$ no. of spores (ml culture) ⁻¹	
	Growth temperature 30 °C	Growth temperature 37 °C
A2	41 $\pm$ 8 (3)	1.3 $\pm$ 0.5 (4)
A2 + biotin (1 μ g l ⁻¹)	45 $\pm$ 4 (3)	21 $\pm$ 7 (4)
A2 + L-aspartate (100 mg ml ⁻¹)	39 $\pm$ 2 (3)	15 $\pm$ 0.6 (4)
A2 + dipicolinic acid (1 mM)	ND	8* $\pm$ 2.8 (8)

ND, Not done.

* Spores from this culture showed an increased lag period (2 days instead of 1 day) before forming visible colonies on nutrient agar.

either that more biotin was needed at 37 °C than could be met by endogenous synthesis, or that biotin synthesis at 37 °C fell below the level needed for normal growth. Hence, the biotin contents of stationary phase cultures were measured after growth at 30 or 37 °C.

The culture grown in medium A1 at 37 °C contained only about 0.1 as much biotin (1.4 ng l⁻¹) as did that grown at 30 °C (11 ng l⁻¹). This measured biotin represents the biotin content of the medium, plus biotin that could be released from the organisms by autoclaving. Covalently-bound biotin was released by acid hydrolysis of the killed organisms and was 0.01 ng l⁻¹ from organisms grown at 30 °C, and much less from organisms grown at 37 °C (only traces).

Surprisingly, compounds that allowed normal growth in the absence of biotin at 37 °C decreased biotin synthesis. In medium A1 plus L-aspartate (100 mg l⁻¹), biotin synthesis was 1.5 ng l⁻¹ at 30 °C and 0.25 ng l⁻¹ at 37 °C. In medium A1 (only 0.25 % glycerol) plus sodium acetate trihydrate (500 mg l⁻¹), biotin synthesis was 6.6 ng l⁻¹ at 30 °C and 0.3 ng l⁻¹ at 37 °C. Thus, the effects of these supplements were probably made by supplying products of biotin-dependent enzyme systems.

Sporulation of biotin-sufficient and biotin-deficient organisms

In medium A1 plus 1 mM-CaCl₂ with glycerol (1 % or 0.2 %) as carbon source, *B. megaterium* NCIB7581 did not sporulate at 30 or 37 °C whether or not biotin was present. The organisms were made to sporulate by limiting glucose in medium A2 to 0.2 % and adding 1 mM-CaCl₂. The number of spores per ml in a culture grown at 37 °C without biotin was only about 0.05 of that in a culture grown at 37 °C with biotin (Table 2). Furthermore, if L-aspartate was added instead of biotin, the same number of spores was formed per ml at 37 °C. Addition of dipicolinate also markedly increased the yield of spores at 37 °C in the absence of biotin. Cultures grown at 30 °C formed approximately the same number of spores per ml culture whether or not biotin was present, and this yield was always higher than at 37 °C.

Electron microscopy

Organisms grown at 37 °C in the presence of biotin had the appearance of normal *B. megaterium* by electron microscopy. Organisms grown at 37 °C without biotin were

Fig. 3. Deformed *B. megaterium* NCIB7581 grown on medium A1 at 37 °C without biotin. (a) Terminal swelling; (b) terminal swelling and thinning of wall (at arrow); (c) gross swelling and thinning of wall (at arrows). Bar markers represent 1 µm.

frequently swollen at the ends (Fig. 3). The wall was thinner round the swelling though of normal thickness in other parts of the organism. Satisfactory photographs of filamentous organisms were not obtained, probably because only a relatively short length of a twisted filament would lie in the plane of a section.

DISCUSSION

Synthesis of biotin by *B. megaterium* NCIB7581 in a chemically defined medium at 37 °C is limited, so that there is insufficient biotin to supply all the needs of the organisms for normal growth. The most obvious consequences are altered colonial and microscopic appearances, and a limited ability to sporulate. It is important to emphasize that biotin deficiency is not the only cause of morphological change in *B. megaterium*. Nevertheless, several other workers (e.g. Chan, 1964; Ahmad & Rose, 1962) have found altered morphology in biotin-deficient organisms.

The deformities caused at 37 °C by biotin deficiency may be the result of altered wall synthesis, or of a decreased rate of synthesis that cannot keep pace with autolysis. Electron microscopy showed apparently weak, thin regions of wall where swelling occurred. Biotin, however, is not known to play a direct role in the biosynthesis of peptidoglycan or other wall polymers of Gram-positive bacteria (see review by Osborn, 1969). Possibly the supply (from aspartate) of amino acids that are needed for peptidoglycan synthesis (glutamate and diaminopimelate in *B. megaterium*) is inadequate if carboxylation of pyruvate (to form oxaloacetate, and hence aspartate) is restricted by biotin deficiency (Cazzulo, Sundaram & Kornberg, 1969). That this supply may be inadequate seems probable from the finding that aspartate (or other C₄ compounds) can allow normal growth at 37 °C in the absence of biotin. Growth with normal morphology on acetate might result from the production of glyoxylate cycle enzymes, which allow C₄ compounds to be made without participation of pyruvate carboxylase.

Biotin is concerned in the synthesis of higher fatty acids, as a cofactor of acetyl-CoA carboxylase (see review by Vagelos, 1964). Deficiency of biotin might, therefore, lead to disturbances in lipid synthesis and hence to a defective cytoplasmic membrane. Damage to the membrane at 37 °C cannot be very drastic, otherwise the organism would not grow without biotin at this temperature. The membrane is the site of several of the enzymes of wall synthesis (Salton, 1974), and a defective membrane might synthesize an abnormal wall. Nevertheless, provision of aspartate in the medium ought not to help directly in lipid synthesis, yet aspartate does restore normal morphology. This suggests either that aspartate spares the limited amount of biotin which is made at 37 °C, so that more biotin is available as a cofactor for acetyl-CoA carboxylase, or that the main result of biotin deficiency is impaired synthesis of C₄ compounds, and the lipids are relatively unaffected.

It is not entirely clear why biotin deficiency suppresses sporulation on glucose limitation. At 37 °C in the absence of biotin, only about one organism in 20 forms a spore, and in most organisms no structure resembling a forespore can be seen. This suggests that formation of a sporulation septum and/or engulfment may be disturbed, rather than a later stage, such as cortex formation or synthesis of dipicolinate. On the other hand, aspartate allowed sporulation in the absence of biotin, as did dipicolinic acid to a lesser extent. Consequently, it seems possible that aspartate stimulates sporulation partly by acting as a precursor of dipicolinic acid.

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