The Effect of Deficiency of Iron, Zinc and Manganese on the Growth and Morphology of Nocardia opaca

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Summary

A study of the trace element requirements of a strain of Nocardia opaca was made. Iron, zinc and manganese were required for optimum growth of the organism on glucose, sucrose, glycerol, gluconate and phenylacetate. Deficiency of manganese caused marked morphological changes in the organism during growth on all the substrates.

Introduction

The trace element requirements of the actinomycetes, as compared with the fungi (see Foster, 1949; Cochrane, 1958) have received little attention in the past. Studies of this nature which have been made have been directed principally towards the elucidation of the trace element requirements of specific Streptomyces strains used for the production of specific antibiotics. Literature dealing with this subject was reviewed by Villemin, Lechevalier & Waksman (1958). Heim & Lechevalier (1956) studied the effect of iron, zinc, manganese and calcium on the growth of eight different strains of Streptomyces. As far as can be ascertained no such study has been made with organisms of the genus Nocardia. In the present work it has been found that iron, zinc and manganese are required for optimum growth of a strain of N. opaca and also that deficiency of the latter two elements (particularly manganese) causes marked morphological changes in the organism.

Methods

Organism. Nocardia opaca, strain T18, described previously (Webley, 1954), was used throughout.

Growth experiments. These were carried out in 100 ml Erlenmeyer or 1 l. culture flasks containing 25 and 200 ml. medium, respectively. All glassware used was of Pyrex brand and was soaked for at least 18 hr. (overnight) in 5 N-HCl before being rinsed many times with double-distilled water obtained from a glass still. The flasks were subsequently drained dry. The basal mineral salts were those of Donald, Passey & Swaby (1952) (g./l.: 5, KNO₃; 2·5, K₂HPO₄; 0·5, MgSO₄·7H₂O) with 0·024% (w/v) L-glutamic acid (sodium salt). The substrates used for carbon and energy source were glucose, sucrose, glycerol, sodium gluconate, all at 2% (w/v), and sodium phenylacetate (1%, w/v). The method of treating the basal medium + substrate, to remove as much of the contaminating trace metals as possible, was that of Donald et al. (1952) using 2% (w/v) alumina. The basal mineral salts + substrate (except glucose) were adjusted to pH 7·5 and the sodium glutamate solution
(to be added separately) to pH 7·8–8·0 (see Heim & Lechavalier, 1956) after addition of the alumina—both solutions being treated separately. When glucose was used, it was also treated separately, and the pH adjusted to 6·8 to avoid excessive breakdown. The strengths of the solutions were made so that when they were finally mixed, and the trace-elements added, the desired final concentrations were obtained. The pH value of the final mixture was adjusted to 7·2 with trace element-free NH₄OH or HCl solutions. After purification, complete medium was obtained by adding to the basal mineral salts + substrate solution (per litre): 200µg. Fe (as FeSO₄·7H₂O; 180µg. Zn (as ZnSO₄·7H₂O)); 40µg. Cu (as CuSO₄·5H₂O); 20µg. Mn (as MnSO₄·5H₂O); 10µg. Mo (as (NH₄)₂MoO₄·4H₂O); 10µg. B (as H₃BO₃), unless otherwise stated. The salts used to supply trace elements were spectrographically standardized substances ('Specpure', Johnson, Matthey and Co. Ltd., 78/83 Hatton Garden, London). A specific trace element deficiency was obtained by omitting the salt containing the element in question. The small and large flasks were inoculated with one drop and 0·1 ml., respectively, of a suspension of the organism obtained from a 6-day surface colony on nutrient agar. This suspension was washed 3–5 times with double-distilled water and made finally to contain 2·5 x 10⁶ viable units/ml. The flasks were incubated at 25° statically or on a reciprocal shaking machine. The organisms were harvested after incubation for 7–12 days and washed 2–3 times with double-distilled water before being resuspended in a known volume. A sample was removed and dried to constant weight at 100° for determination of the total dry weight of cell material.

Morphology and viable counts. Morphological changes were followed by phase-contrast microscopy and by examination of heat-fixed preparations stained with crystal violet. For cell walls the tannic acid-crystal violet technique described by Hale & Bisset (1956) was used. Electron microscopic examination was also occasionally carried out. Viable counts were performed by the method of Miles & Misra (1938).

RESULTS

Effect of deficiency of trace elements on yield of organism and viable counts of Nocardia opaca

Table 1 shows the effect of deficiency of iron, zinc and manganese on the growth of the organism on four different substrates. Iron deficiency produced the greatest effect and the organisms en masse were frequently greyish white in colour in contrast to the orange-salmon pink of those grown in complete medium. A similar effect of iron deficiency on pigmentation of Aerobacter aerogenes was described by Waring & Werkman (1944). Deficiency of Mn or Zn did not produce this change in pigmentation. The pH value of the iron-deficient medium at the end of the growth period, particularly with glucose as substrate, was always acid (pH 4·0–5·0), in contrast to complete medium and Mn and Zn-deficient media which did not show this effect. The pH values of these cultures rose from the initial pH 7·2 to 8·0–8·5 at the end of the experiments. The omission of Cu, Mo or B did not affect the yields of organism. The addition of 40–400µg. Ca (as CaCl₂; prepared from ‘Specpure’ CaCO₃) per l. complete medium did not markedly affect the growth of the organism. Zn was not replaced by cadmium (as ‘Specpure’; cadmium sulphate). In fact, cadmium above 36µg./l. was toxic to the organism on Zn-deficient medium. Figure 1 shows
Trace element deficiencies and N. opaca

Table 1. Effect of deficiency of iron, manganese and zinc on growth of Nocardia opaca on different substrates

N. opaca grown in 200 ml. complete, or Fe-, or Mn-, or Zn-deficient medium with 2% (w/v) glucose, sucrose or glycerol or 1% (w/v) phenylacetate on shaking machine. Organisms harvested after 7 days with glucose or glycerol, and after 10 days with sucrose or phenylacetate, washed 8 times with double-distilled water, made up to known volume and dry weights determined on suitable samples.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glucose</th>
<th>Glycerol</th>
<th>Sucrose</th>
<th>Phenylacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>256.0</td>
<td>187.5</td>
<td>146.0</td>
<td>188.0</td>
</tr>
<tr>
<td>-Fe</td>
<td>22.8</td>
<td>6.0</td>
<td>11.4</td>
<td>7.2</td>
</tr>
<tr>
<td>-Mn</td>
<td>67.5</td>
<td>18.0</td>
<td>64.0</td>
<td>62.5</td>
</tr>
<tr>
<td>-Zn</td>
<td>140.0</td>
<td>27.8</td>
<td>51.0</td>
<td>48.0</td>
</tr>
</tbody>
</table>

Table 2 shows the viable counts obtained on the deficient media. Although these figures are not altogether reliable, because of the morphology of the organism, they closely reflect the differences in total yields of organism (see Table 1).

Determinations of the Zn and Mn contents of organisms from the corresponding deficient media were carried out by the Spectrochemistry Department of the

the growth response of Nocardia opaca to different amounts of Mn, Zn and Fe. It is clear that the requirement for Mn was very much smaller than that for Fe and Zn. Table 2 shows the viable counts obtained on the deficient media. Although these figures are not altogether reliable, because of the morphology of the organism, they closely reflect the differences in total yields of organism (see Table 1).

Determinations of the Zn and Mn contents of organisms from the corresponding deficient media were carried out by the Spectrochemistry Department of the
Institute. It was found that the Zn and Mn deficient organisms contained approximately $\frac{1}{3}$ and $\frac{1}{4}$, respectively, of the amount of these metals present in an equivalent weight of organisms grown on complete medium.

Table 2. Effect of deficiency of iron, manganese and zinc on viable counts of Nocardia opaca

<table>
<thead>
<tr>
<th>Medium</th>
<th>5 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>2638</td>
<td>1438</td>
</tr>
<tr>
<td>Fe</td>
<td>19</td>
<td>0.9</td>
</tr>
<tr>
<td>Zn</td>
<td>710</td>
<td>882</td>
</tr>
<tr>
<td>Mn</td>
<td>82</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Effect of deficiency of trace elements on the morphology of Nocardia opaca

The absence of Fe from the culture medium produced the least effect on morphology as seen with the optical microscope, the only noticeable change being that the organisms tended to fragment somewhat sooner than organisms from complete medium. The electron micrographs of Fe-deficient organisms, however, showed that they were generally not so opaque to the electron beam and the presence of electron-dense granules could be clearly seen, often in the polar positions (Pl. 1, fig. 2). These granules have already been reported in *Nocardia opaca* (Webley, 1954). The most noticeable effect on the morphology of the organism was obtained in Mn-deficient cultures. Manganese deficiency usually resulted in a more pronounced filamentous growth of the organism, with subsequent failure to fragment into short elements (Pl. 1, figs. 3–5). On further incubation bulbous swellings frequently appeared at the end, and sometimes also along the length of filaments (Pl. 1, figs. 4–6). As the culture became older the bulbous structures tended to rupture. With Zn deficiency the filamentous growth and swellings, although sometimes present (Pl. 1, fig. 7) were usually not so marked. The bulbous swellings in Mn deficiency were opaque to the electron beam (Pl. 1, fig. 6), and stained deeply with crystal violet (Pl. 1, fig. 4). They also showed evidence of cell walls when stained with tannic acid-crystal violet (Pl. 1, fig. 5). This abnormal morphology was prevented by the addition of as little as 4–10 μg Mn/l. (i.e. 0.004–0.01 p.p.m.) to Mn-deficient medium, but it was necessary to add about ten times this amount of Zn (0.04–0.1 p.p.m.) to correct a zinc-deficient medium. The morphological effects due to Mn deficiency were observed with all the substrates tested (glucose, sucrose, glycerol, gluconate, phenylacetate). The effects were usually seen after incubation for 5 days. With shaken cultures a similar picture was obtained, the bulbous swellings also developing under these conditions. When the organisms were harvested, washed and resuspended in double-distilled water the abnormal structures did not rupture.
DISCUSSION

The most interesting feature of the present work is the striking morphological effects obtained with *Nocardia opaca* when grown in Zn- and Mn-deficient media (particularly the latter). There appears to be no previous record of similar morphological changes in the actinomycetes. Winder & Denneny (1959) reported that the cells of *Mycobacterium smegmatis*, when grown on a modified Proskauer & Beck medium partially freed from trace elements, were elongated and even filament like. The addition of 2μg./ml. iron + 0·4μg./ml. zinc gave the normal morphology. These workers did not test these metals singly and did not detect a response to manganese. Heim & Lechevalier (1956) found that iron was the only element that permitted a substantial increase in mycelial weight, when added singly, in the growth of eight different strains of Streptomycetes; a mixture of Fe+Zn, however, gave a striking increase in mycelial weight. These workers were uncertain whether manganese was needed and did not examine the morphology of the organism. These differences in related organisms might be due either to the degree of deficiency obtained in the media or to differences in the requirements of the different organisms. The medium used here is simpler in composition than that used by Heim & Lechevalier and therefore might be somewhat more easily depleted of trace metals.

I am indebted to Mr W. Hodgkiss of the Torry Research Station, Aberdeen, for taking the electronmicrographs, to Mrs I. M. Johnston of this Institute for the spectrochemical analyses and to Miss I. Taylor and Mr M. Davidson for technical assistance.

REFERENCES


EXPLANATION OF PLATE

Fig. 1. *Nocardia opaca*, grown for 7 days on complete medium containing glucose. Static culture. Electron micrograph. ×14,500.

Fig. 2. *N. opaca*, grown for 7 days on Fe-deficient medium containing glucose. Static culture. Electron micrograph. ×14,500.

Fig. 3. *N. opaca*, grown for 8 days on Mn-deficient medium containing sucrose. Static culture. Crystal violet. ×2640.

Fig. 4. *N. opaca*, grown for 5 days on Mn-deficient medium containing sucrose. Static culture. Crystal violet. ×2640.

Fig. 5. *N. opaca*, grown for 7 days on Mn-deficient medium containing sucrose. Static culture. Cell wall stain. ×2640.

Fig. 6. *N. opaca*, grown for 10 days on Mn-deficient medium containing glucose. Shaken culture. Electron micrograph. ×14,500.

Fig. 7. *N. opaca*, grown for 7 days on Zn-deficient medium containing gluconate. Static culture. Crystal violet. ×2640.