The Metabolism of Iron-, Zinc- and Manganese-Deficient *Nocardia opaca*

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SUMMARY

The metabolism of iron-, zinc- and manganese-deficient cells of *N. opaca* was studied. Zinc-deficient organism showed inability to utilize reserve carbohydrate as compared with organism grown under normal conditions. Manganese deficiency caused a much decreased DNA synthesis. Iron deficient organism lacked catalase and the oxygen uptake with certain substrates (pyruvate, phenylacetate, *n*-dodecane) was markedly affected.

INTRODUCTION

In a previous paper (Webley, 1960) it was shown that iron-, zinc- and manganese were required for optimum growth of *Nocardia opaca* and that deficiency of manganese caused marked morphological changes in the organism, irrespective of the substrate on which it was grown. In the present work a study of organisms deficient in these trace elements revealed several differences in their chemical composition and metabolism as compared with organisms grown on complete medium.

METHODS

Organism. *Nocardia opaca* strain T 16 described previously (Webley, 1954) was used.

Production of metal-deficient organisms. Details about the treatment of glassware, and the composition and preparation of media for individual deficiencies were given in a previous paper (Webley, 1960). After growth no special precautions in the harvesting of the organisms were necessary.

Manometry. Carried out by the conventional Warburg techniques at 25°.

Fractionation of the cell material. The Mickle disintegrator (Mickle, 1948) was used to break organisms, in suspensions containing 10-20 mg. dry wt. organism/ml., with ballotini beads (no. 12) for 15-20 min. The Vibrogen cell mill (E. Bühler, Tübingen, Germany) was used when large volumes (100 ml.) of suspension were to be broken. After removal of the glass beads the broken preparations were centrifuged at 35,000 *g* in a refrigerated centrifuge. The debris was washed twice with distilled water at 35,000 *g* and proved, on examination with the electron microscope, to be principally cell-wall material with some amorphous material. The cell-free extracts and debris were used directly or were freeze-dried, as required.

Carbohydrate determinations. Usually descending chromatograms were run in butan-1-ol+acetic acid+water (4+1+5, by vol.) on Whatman no. 1 paper with aniline phthalate or benzidine+trichloroacetic acid (Bacon & Edelman, 1951) as a spray for revealing sugars. The anthrone method of Fairbairn (1953) was used for

the estimation of total carbohydrate. The results were calculated in terms of glucose although paper chromatography showed that other sugars (particularly arabinose) were present in the hydrolysates of whole organisms. Therefore the results can only be regarded as comparative. Estimations on the paper chromatograms were done as described by Duff & Eastwood (1950).

Lipid determinations. The method of Reichert (1944) was used; the organisms were treated with boiling methanol and then extracted with diethyl ether (after removal of methanol). An apparatus was adapted for extracting smaller amounts of material (equiv. 50 mg. dry wt.) than required in the original method; two extractions were made.

Nucleic acid determinations. The freeze-dried cell contents were separated into fractions containing RNA and DNA as described by Elson, Gustafson & Chargaff (1954). The sediment containing the DNA fraction was hydrolysed with 12 N-perchloric acid (Marshak & Vogel, 1951). The solution containing nucleotides derived from RNA was evaporated to dryness *in vacuo* at 20°, taken up in N-hydrochloric acid and hydrolysed at 100° for 1 hr. (Markham & Smith, 1951). The purine and pyrimidine bases obtained from DNA were chromatographed in two dimensions on Whatman no. 1 paper: (a) upwards in isopropanol+hydrochloric acid+water (Wyatt, 1951); (b) downwards in *n*-butanol+ammonia+water (MacNutt, 1952). The mixture of purine bases and pyrimidine nucleotides from the RNA was chromatographed upwards in isopropanol+hydrochloric acid+water. Bases and nucleotides were detected under u.v. radiation, eluted with 0.1 N-HCl and estimated spectrophotometrically, using extinction coefficients quoted by Markham & Smith (1951) and Wyatt (1951).

RESULTS

Chemical analysis of metal-deficient organisms

From Table 1 it can be seen that, whereas the total nitrogen contents of the complete and Mn deficient organisms were very similar, the Zn- and Fe-deficient cells had, respectively, lower and higher total nitrogen contents. The explanation of

Table 1. *Chemical analysis of Nocardia opaca grown in complete medium or in Fe-, Mn- or Zn-deficient medium*

N. opaca grown in 200 ml. complete medium or in Fe-, Mn- or Zn-deficient medium with 20 g. glucose/l. on a shaker at 25°. Organisms harvested after 7 days and washed 8 times with double-distilled water and made up to known volume. A sample of the washed suspension was used for determination of total nitrogen, carbohydrate and lipid content. The figures given as % of the dry wt. of the cell suspension.

	Total nitrogen	Carbohydrate (as glucose)	Lipid
	mg./100 mg. dry wt. organism		
Complete	7.97	13.4	18.9
—Fe	11.90	4.5	—
—Mn	7.65	11.5	18.1
—Zn	5.17	39.3	10.60

these differences is more clearly understood on examination of the carbohydrate figures. These show that the Zn-deficient organisms had a much higher carbohydrate content than the complete and Mn-deficient organisms while the Fe-

deficient organisms were much lower in carbohydrate. Similarly, when grown on other substrates (sucrose, glycerol, phenylacetate, acetate), the Zn-deficient organisms always had the highest carbohydrate content. Irrespective of growth conditions, when organisms were hydrolysed for 4 hr. at 100°, paper chromatography showed glucose and arabinose in all organisms. The glucose spot was much more intense in the hydrolysate of the Zn-deficient organisms than in the others. The lipid content of the Zn-deficient organisms was slightly less than that of the complete or Mn-deficient organisms. The equipment used did not permit the production of sufficient Fe-deficient organisms for their lipid content to be determined.

Table 2. *Carbohydrate content of Nocardia opaca during growth on Zn-deficient medium*

N. opaca grown in 200 ml. complete medium, or Zn-deficient medium with 20 g. glucose/l. on a shaker at 25°. Samples 5–10 ml. periodically removed aseptically and centrifuged. Organisms washed twice with distilled water and suspended in 2.0 ml. water. Carbohydrate analysis (anthrone reaction) on samples of suspension and supernatant fluid of original sample.

Days	Complete-medium organisms		Zn-deficient organisms	
	Residual glucose in medium (mg./ml.)	Carbohydrate in organism (mg./100 mg. dry wt.)	Residual glucose in medium (mg./ml.)	Carbohydrate in organism (mg./100 mg. dry wt.)
4	14.7	9.8	12.5	15.2
5	4.6	15.6	12.25	31.5
6	1.15	34.9	9.0	35.7
7	0.28	5.5	6.0	24.8
8	0.31	—	4.05	32.5
9	Nil	6.6	2.0	26.4
11	Nil	8.3	Nil	12.0

Effect of zinc deficiency on carbohydrate content of organisms

In view of the greater carbohydrate content of Zn-deficient organisms grown on all substrates tested, an experiment was set up to follow the carbohydrate content of the organisms during growth under these conditions. For this two 1 l. culture flasks (Jobling Cat. no. 1410) each containing 200 ml. of medium, one complete and the other Zn-deficient, were set up. At intervals 5–10 ml. samples were removed. The organisms were centrifuged, well washed and finally suspended in 2 ml. distilled water. A sample was removed from each suspension for determination of dry weight and for carbohydrate content (Table 2). In the complete-medium-grown organisms the carbohydrate content decreased rapidly after reaching its maximum, whereas in the Zn-deficient organisms it did not. The decrease in the carbohydrate content of the complete-medium organisms took place when the glucose had been almost exhausted. It appears that reserve carbohydrates are affected by zinc deficiency.

The polysaccharide material in the complete and Zn-deficient organisms was extracted by the procedure of Palmstierna (1956). The moisture content of a sample of wet organism was estimated and the necessary amounts of KOH and water added to give 300 g./l. solution of KOH. This was heated at 100° for 3 hr. After mixing with water, the solution was poured into ethanol and the precipitated crude glycogen dialysed in aqueous solution. The polysaccharide was recovered by pouring the

dialysed solution, after evaporation *in vacuo*, into ethanol. The crude polysaccharide was purified by reprecipitation from water with glacial acetic acid. The yield was 24 % of the dry weight of Zn-deficient organisms and 14 % of the dry weight of complete-medium grown organisms. Both specimens gave glucose, together with small amounts of mannose and arabinose, after hydrolysis and chromatography in the usual way. Paper chromatographic analyses of hydrolysates of the cell fractions were also carried out (Table 3). All the arabinose and most of the glucose was associated with the cell debris.

Table 3. *Paper chromatographic analysis of sugars in cell debris and cell-free extract from complete and Zn-deficient Nocardia opaca*

N. opaca grown in 200 ml. complete medium or Zn-deficient medium with 20 g. glucose/l. on a shaker at 25°. Organisms harvested after 6 days, washed 3 times with double-distilled water. Suspensions (equiv. 10–20 mg. dry wt. ml.) disintegrated and centrifuged at 35,000 g. Cell debris and cell-free extract freeze-dried. Freeze-dried fractions hydrolysed with N-H₂SO₄ at 100° for 5 hr. in sealed tubes and analysed for sugars after chromatographic separation as described in Methods. The results are the average of two determinations.

	Organisms grown on			
	Complete-medium		Zn-deficient medium	
	Cell debris	Cell-free extract	Cell-debris	Cell-free extract
	Sugar (mg./100 mg. dry wt. preparation)			
Glucose	4.0 (8.2)*	7.6 (5.2)	21.7 (14.4)	5.7 (5.0)
Arabinose	12.6 (10.1)	Nil (Nil)	12.3 (10.5)	Nil (Nil)
Mannose	Trace (2.4)	3.4 (4.1)	Trace (2.6)	1.6 (4.1)
Ribose	Nil (Nil)	0.6 (2.0)	Nil (Nil)	2.7 (3.8)

* The results of a second experiment with a different batch of organisms are given in parentheses.

Nature of the carbohydrate in the cell-free extract. The crude polysaccharide from Zn-deficient organisms was fractionated by the Cetavlon procedure of German, Jones & Nadarajah (1961). From the major fractions at pH 7.5 and 9.5. Cetavlon was removed with 2 N acetic acid and the recovered glycogen reprecipitated from aqueous solution with glacial acetic acid. Both samples gave infrared absorption peaks at 930, 845 and 758 m μ , as obtained for glycogen by the above authors. Hydrolysis with N-H₂SO₄ at 100° for 5 hr. and subsequent paper chromatography showed that glucose was the main constituent, accompanied by a small amount of ribose (from nucleic acid), and mannose, in the pH 7.5 and 9.5 fractions, respectively.

The fractions were combined for optical rotation determination, the mixture had $[\alpha]_D + 165^\circ$ (conc. 5 g./l. of aqueous solution). The glycogen isolated gave a brown colour with iodine, having a maximum u.v. absorption at 360 m μ , E₃₆₀ 1.07 (an appropriate value due to light scattering). In these experiments we used a 8g./l. aqueous solution of glycogen (1.56 ml.) + 1.60 ml. saturated ammonium sulphate + 0.08 ml. potassium tri-iodide solution (1.25 g./l. I in 2.5 g./l. KL), the conditions are comparable to those used by Schlamowitz (1951).

Effect of trace element deficiency on RNA and DNA content of Nocardia opaca

Webley (1960) showed that Mn deficiency caused marked morphological effects during the growth of *Nocardia opaca*. The organisms did not fragment in the usual way but continued to grow in the mycelial phase. As the cultures became older, bulbous swellings frequently appeared on the filaments. Amino acid analysis (DeKock & Morrison, 1958) of cell debris from complete, Zn- and Mn-deficient organisms showed the following: aspartic acid, glutamic acid, serine, α -alanine, valine, leucine, glucosamine, diaminopimelic acid, muramic acid. Muramic acid has been associated only with bacterial cell walls (Strange, 1956; Work, 1957). The acid was present in the cell debris from complete, Zn- and Mn-deficient organisms in the ratio 1.4:1.3:1.0, respectively.

Table 4. *RNA compositions of freeze-dried cell-free extracts from Nocardia opaca grown in complete medium or Zn- or Mn-deficient medium*

N. opaca grown in 200 ml. complete medium or Zn- or Mn-deficient medium with 20 g. glucose/l. on a shaker at 25°. Organisms harvested after 6 days and washed 3 times with double-distilled water. Cell suspensions (10–20 mg./dry wt. ml.) disintegrated and centrifuged at 35,000 g. Cell-free extract freeze-dried. Purine bases and pyrimidine nucleotides obtained from the RNA fraction and estimated as described in Methods.

	Organisms from media		
	Complete	Zn-deficient	Mn-deficient
	Base proportions (moles %)		
Guanine (G)	33.0*	32.1	30.1
Adenine (A)	23.0	23.8	22.2
Cytosine (C)	23.8	24.6	25.4
Uracil (U)	20.2	19.6	22.2
Purines/pyrimidines	1.27	1.26	1.10
G + U/A + C	1.14	1.07	1.10

* Values are the means of at least two chromatographic analyses of hydrolysis products.

Table 5. *DNA composition of freeze-dried cell-free extract from Nocardia opaca grown in complete medium or in Zn-deficient medium*

N. opaca grown in 200 ml. complete medium or in Zn- or Mn-deficient medium with 20 g. glucose/l. on a shaker at 25°. Organisms harvested after 6 days and washed 3 times with double-distilled water. Suspensions (equiv. 10–20 mg./ml. dry wt.) disintegrated and centrifuged at 35,000 g. Cell-free extract freeze-dried. Purine and pyrimidine bases obtained from DNA fraction as described in Methods.

	Organisms from media		
	Complete	Zn-deficient	Mn-deficient
	Base proportions (moles %)		
Guanine (G)	33.5*	36.4	35.0
Adenine (A)	19.7	19.5	20.6
Cytosine (C)	30.4	26.7	35.0
Thymine (T)	16.4	17.5	9.8
Purines/pyrimidines	1.14	1.26	1.26
A + T/G + C	0.56	0.59	0.43

* Values the means of two chromatographic analyses of hydrolysis products.

Hydrolysis of the cell debris with perchloric acid gave only traces of purine and pyrimidine bases on paper chromatograms (see Table 3), no ribose was contained in this fraction.

The relative proportions of the bases in the RNA and DNA fractions from freeze-dried cell-free extract are shown in Tables 4 and 5. The composition of the RNA was similar for the differently grown organisms. The DNA was similar in the complete and Zn-deficient organisms but the recovery of pyrimidines appears to be slightly low in both cases. The amounts of bases in the Mn-deficient organisms were so low that only approximate values could be obtained. To confirm the deficiency of thymine relative to adenine in the DNA, large-scale fractionation and isolation would be necessary. The nucleic acid contents of the cell-free extracts are given in Table 6. The absolute amounts of DNA and the ratio of DNA to RNA were lower in the Mn-deficient organisms.

Table 6. *Nucleic acid content of cell-free extracts from Nocardia opaca grown in complete medium or Zn- or Mn-deficient medium*

Values calculated from analysis of the amounts of bases and nucleotides obtained by hydrolysis of DNA and RNA fractions.

	Organisms grown in media		
	Complete	Zn-deficient	Mn-deficient
	mg./100 mg. freeze-dried preparation		
DNA	3.41 (2.97)	1.96	1.43 (1.09)
RNA	3.46 (1.98)	1.97	2.45 (4.75)
DNA/RNA	0.99 (1.50)	0.99	0.58 (0.23)

The results for a second batch of organisms given in parentheses. The second Mn-deficient batch was harvested after 10 days, the others after 6 days.

Warburg experiments with trace element deficient organisms of Nocardia opaca

Catalase activity. Table 7 shows that Fe-deficient organisms lacked catalase activity. A similar result was reported for *Aerobacter aerogenes* by Waring & Werkman (1944).

Oxidation of added substrates. Table 8 shows that the oxidation of glucose by Fe-deficient organisms was not nearly so much affected as was that of other substrates. A similar result was obtained by Waring & Werkman (1944) with iron-deficient *Aerobacter aerogenes*. The marked effect on pyruvic oxidation suggested that this acid might accumulate in iron-deficient cultures. Iron-deficient growth experiments were set up with glucose, gluconate, sucrose or glycerol as substrates. After growth for 7 days a strong reaction for pyruvic acid was obtained with the Rothera test when applied to the supernatant fluid after removal of organisms by centrifugation. Similar tests on the supernatant fluids from complete, Zn- and Mn-deficient cultures gave negative results. Further confirmation of the presence of pyruvic acid in Fe-deficient cultures was obtained as follows. 2:4-Dinitrophenylhydrazine in 2 N-HCl was added to a suitable sample of the supernatant fluid from an Fe-deficient culture in which glucose was the substrate. The precipitated hydrazone, when examined by infrared analysis, was identical with the hydrazone prepared from pure pyruvic acid. No similar pyruvic acid derivative was obtained

from corresponding supernatant fluids of organisms grown on complete medium, Zn- or Mn-deficient media.

The oxygen uptakes of Zn- or Mn-deficient organisms in the presence of the substrates used by complete and Fe-deficient organisms (Table 8) were generally inferior to those of complete organisms. But the results were too variable to draw conclusion about effects on specific substrates. Addition of the respective trace elements to the Warburg vessels in a form and concentration similar to those used for the complete medium in the growth experiments did not affect the oxygen uptake.

Table 7. *Catalase activity of trace element-deficient Nocardia opaca*

Organisms grown in complete medium or in Fe-, Mn- or Zn-deficient medium containing 20 g. glucose/l. Harvested washed, and suspended in double-distilled water (equiv. 10 mg. dry wt./ml.). In main compartment of Warburg vessel 0.1 ml. suspension + 0.5 ml. M/15 buffer (pH 7.2) and distilled water to give 2.5 ml. In side arm 0.2 ml. H₂O₂ (3 g./l.). H₂O₂ tipped after 15 min. equilibrium time.

Medium	O ₂ output in 3 min. (μ l.)
Complete	57.0
Fe-deficient	2.1
Mn-deficient	59.0
Zn-deficient	54.0

Table 8. *Oxidation of various substrates by Fe-deficient Nocardia opaca*

N. opaca grown in complete medium or in Fe-deficient medium containing 20 g. glucose/l. After 7 days cells harvested and washed twice and suspended in double-distilled water to contain equiv. 10–12 mg. dry wt. organism/ml. In Warburg vessels 1.0 ml. suspension + 0.5 ml. M/15 buffer (pH 7.2) + 0.5 ml. M/50 substrate and distilled water to give 2.5 ml.

	Organisms grown on	
	Complete medium	Fe-deficient medium
	Q _{O₂} -Endogenous*	
Glucose	26.9	18.1
Gluconate	14.3	6.1
Butyrate	34.4	4.5
Phenylacetate	10.9	3.2
Pyruvate	11.5	1.1
n-Dodecane	26.3	Nil

* Endogenous Q_{O₂} was 9.12 and 5.3 for complete medium grown organisms and Fe-deficient medium grown organisms, respectively.

DISCUSSION

The results recorded in Table 2 suggest that Zn-deficient *Nocardia opaca* is unable to utilize its reserve polysaccharide as rapidly as organisms grown in the complete medium. Stein & Fischer (1960) showed that crystalline α -amylase from *Bacillus subtilis* is a zinc protein complex. Perhaps the polysaccharide splitting enzyme in *N. opaca* is also dependent on zinc.

The effect of manganese deficiency on DNA synthesis by *Nocardia opaca* is

interesting in the light of previous work (Webley, 1960) on the morphology of this organism when grown in Mn-deficient medium. It is well established that in normal growth the DNA content increases before cell division (Belozersky & Spirin, 1960). Jeener & Jeener (1952) showed that the deoxyribonucleoside-dependent *Thermobacterium acidophilus* R 26, when cultured in the absence of DNA, produced elongated cells which did not divide. In the light of these observations it is reasonable to suggest that when *N. opaca* is cultured under conditions of Mn deficiency the production of filamentous forms is caused by failure of normal DNA synthesis.

The presence of pyruvic acid in Fe-deficient cultures suggests that the pyruvic oxidase of the organism may be iron dependent. O'Kane (1954) showed that the pyruvate oxidase of *Clostridium butyricum* required ferrous ion.

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