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Effects of Iron Deficiency and of Zinc Deficiency on the Activities of some Enzymes in *Mycobacterium smegmatis*

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When the growth medium of *Mycobacterium smegmatis* is depleted of iron or zinc, limitation of growth of the culture is finally brought about by exhaustion of the supply of the depleted metal. When this occurs certain characteristic changes occur in the composition of the bacteria that give some indications of the synthetic pathways which are affected earliest by deficiencies of these metals (Winder & O'Hara, 1962; Ratledge & Winder, 1962). Further information on the effects of these deficiencies has been sought by following the activities of some enzymes in these depleted cultures and these results are reported in the present paper. A preliminary report on some of this work has been published (Winder, O'Hara & Ratledge, 1961).

EXPERIMENTAL

Organism. *Mycobacterium smegmatis* is a strain of unknown origin used for previous studies (Winder & O'Hara, 1962).

Growth and harvesting. The preparation of metal-depleted medium, treatment of glassware and growth of the bacteria were described by Winder & O'Hara (1962) and Ratledge & Winder (1962). Metals were added to the depleted medium to give the following concentrations (per ml.): in normal medium, 2.16 μg . of Fe^{3+} and 0.42 μg . of Zn^{2+} ions; in iron-deficient medium, 0.27 μg . of Fe^{3+} and 0.42 μg . of Zn^{2+} ions; in zinc-deficient medium, 2.16 μg . of Fe^{3+} and 0.035 μg . of Zn^{2+} ions. Surface cultures only were used. After 2, 3 and 4 days one or more flasks of each type of culture were taken, the cells were collected in a Buchner funnel on

Whatman no. 1 paper and washed with water under gentle suction.

Preparation of extracts. The cells were transferred to Mickle disintegrator tubes (about 50–100 mg. of cell protein/tube) and about 5 ml. of the buffer appropriate to the enzyme determination (usually 0.05 M-potassium phosphate, pH 7.5) was added together with a volume of Ballotini beads about equal to the volume of the cells. The tubes were placed in a refrigerator at -10° until just frozen and were then shaken in a Mickle disintegrator at maximum amplitude for three 10 min. periods, refreezing at -10° between periods. The extracts were centrifuged at 2000g for 15 min. at 4° and the supernatant was removed for assay of enzyme activity and for protein determination by the biuret method (Layne, 1957). Preliminary studies were carried out to determine for each enzyme the optimum disintegration time. The above procedure led to extraction of all the enzymes studied except aconitate hydratase, which was inactivated to an undesirable extent. Aconitate hydratase was extracted by grinding the cells with an equal volume of Ballotini beads in a chilled mortar for about 5 min. with gradual addition of chilled buffer. The extracts were centrifuged as before and the supernatants were used as enzyme. This method gave minimal inactivation of the enzyme.

The enzymes were assayed also in the residues after centrifuging but in all the enzymes the activity was much greater in the supernatant and it is this latter value which is given throughout this paper. All enzyme assays except that of the aminotransferase were done at $18-20^\circ$.

Assay procedures

Glycerol dehydrogenase (glycerol-NAD oxidoreductase, EC 1.1.1.6). This enzyme was determined according to the method of Burton (1955). The reaction mixture contained, in a final volume 3.0 ml.: 500 μmoles of glycerol, 0.5 μmole of NAD, 100 μmoles of sodium pyrophosphate buffer

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(pH 9.0) and usually 0.2 ml. of extract (i.e. about 2 mg. of protein). ΔE_{340} was determined during the initial 4 min. The control cell contained the above-mentioned constituents except glycerol. The number of units of enzyme present ($\mu\text{m-moles}$ of NADH formed/min.) was calculated from $\Delta\epsilon\ 6.3 \times 10^3$.

Lactate dehydrogenase. The method of Appleby & Morton (1959) was used. The reaction mixture contained, in a final volume 3.0 ml.: 1.34 μmoles of sodium lactate, 3 μmoles of $\text{K}_3\text{Fe}(\text{CN})_6$, 275 μmoles of sodium pyrophosphate buffer (pH 9.0) and usually 0.2 ml. of extract. ΔE_{420} was determined during the initial 4 min. The control cell lacked ferricyanide. A blank determination was done subsequently in which lactate was omitted from the reaction mixture. The number of units of enzyme present [half the number of $\mu\text{m-moles}$ of $\text{K}_3\text{Fe}(\text{CN})_6$ reduced/min.] was calculated from $\Delta\epsilon\ 0.88 \times 10^3$ and by making allowance for the blank rate.

Succinate dehydrogenase [succinate-(acceptor) oxidoreductase, EC 1.3.99.1]. The method of Bonner (1955) was used. The reaction mixture contained, in a final volume 3.0 ml.: 40 μmoles of sodium succinate (pH 7.2), 3 μmoles of $\text{K}_3\text{Fe}(\text{CN})_6$, 30 μmoles of KCN (pH 7.2), 320 μmoles of potassium phosphate buffer (pH 7.2) and usually 0.20 ml. of extract. ΔE_{400} was determined during the initial 4 min. The control cell lacked ferricyanide. A blank determination was also made, in which succinate was omitted from the reaction mixture. The number of units of enzyme present [half the number of $\mu\text{m-moles}$ of $\text{K}_3\text{Fe}(\text{CN})_6$ reduced/min.] was calculated from $\Delta\epsilon\ 0.88 \times 10^3$ and by making allowance for the blank rate.

Aconitate hydratase (citrate hydro-lyase, EC 4.2.1.3). The method of Anfinsen (1955) was used. The reaction mixture contained: 2.9 ml. of 0.03 M-citrate in 0.05 M-potassium phosphate buffer, pH 7.4, and 0.1 ml. of extract. ΔE_{240} was determined during the initial 4 min. Citrate was omitted from the control cell. The number of units of enzyme present ($\mu\text{m-moles}$ of *cis*-aconitate formed/min.) was calculated from $\Delta\epsilon\ 4.38 \times 10^3$.

NADH-cytochrome c reductase (NADH₂-cytochrome c oxidoreductase, EC 1.6.2.1). The method of Mahler (1955) was used. The reaction mixture contained, in a final volume 3 ml.: 0.6 μmole of NADH, 1.0 mg. of ferricytochrome c, 3 μmoles of KCN, 200 μmoles of potassium phosphate buffer (pH 7.5) and 0.2 ml. of extract. ΔE_{550} was followed for the first minute. Enzyme was omitted from the blank cell. The number of units of enzyme present ($\mu\text{m-moles}$ of cytochrome c reduced/min.) was calculated from $\Delta\epsilon\ 27.7 \times 10^3$.

Rate of oxidation of NADH. The reaction mixture contained, in a final volume 3 ml.: 0.2 μmole of NADH, 200 μmoles of potassium phosphate buffer (pH 7.4) and 0.1 ml. of extract. ΔE_{340} was followed for the first 4 min. The number of units of enzyme present ($\mu\text{m-moles}$ of NADH oxidized/min.) was calculated from $\Delta\epsilon\ 6.3 \times 10^3$.

Catalase (hydrogen peroxide-hydrogen peroxide oxidoreductase, EC 1.11.1.6). The reaction mixture contained, in a final volume 3 ml.: 20 μmoles of potassium phosphate buffer (pH 7.0), 1 ml. of extract suitably diluted and 100 μmoles of H_2O_2 . The reaction was stopped 2 min. after addition of the peroxide by rapidly running in 1.0 ml. of titanium sulphate reagent (conc. H_2SO_4 saturated near its boiling point with TiO_2 , cooled, diluted with an equal volume of water, cooled and the supernatant used), mixing

and centrifuging. The extinction of the supernatant was read at 400 m μ . A zero-time determination and a blank determination (without peroxide) were also made. The specific catalase activity of the preparation was calculated from the formula for a first-order reaction

$$\frac{2.3}{t \times c} \log \frac{E_0 - E_b}{E_t - E_b} \text{ l./g. of protein/min.}$$

where t is the 2 min. reaction time, c is the concentration of protein/l. of reaction mixture, and E_0 , E_t and E_b are the readings at zero time, after 2 min. and without peroxide respectively.

Peroxidative activity towards pyrogallol. A portion (1 ml.) of 0.1 M-potassium phosphate buffer, pH 7.4, and 1 ml. of extract were placed in each of two 1 cm. cuvettes; 1.0 ml. of 1% (w/v) pyrogallol was added to the blank cuvette and the same addition was made 10 sec. later to the analytical cuvette, both cuvettes being stirred immediately after the addition. The spectrophotometer was rapidly balanced at 430 m μ with the blank cuvette in position and a reading taken 10 sec. later with the analytical cuvette in position. A portion (40 $\mu\text{l.}$) of 1% (w/v) H_2O_2 was added to the analytical cuvette, a stop-clock started, and the contents of the cuvette were stirred rapidly. After 4 min. 50 sec. the spectrophotometer was balanced with the blank cuvette in position and the reading on the analytical cuvette taken 10 sec. later. The slit width was kept as near to constant as possible. The number of μmoles of purpurogallin formed was calculated from a standard curve obtained from solutions of purpurogallin in 33 mM-potassium phosphate buffer, pH 7.4.

Alanine aminotransferase (L-alanine-2-oxoglutarate aminotransferase, EC 2.6.1.2). This enzyme was assayed at 37° on 0.2 ml. of extract according to the method of Reitman & Frankel (1957) as described in Sigma Technical Bulletin no. 505 and by using Sigma Kit no. 505-P (Sigma Chemical Co., St Louis, Mo., U.S.A.). Activity was calculated in terms of $\mu\text{m-moles}$ of pyruvic acid formed/min.

Materials. NAD⁺ (98–100%), NADH (98–100%), cytochrome c (horse-heart, type II) and purpurogallin were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Calcium L(+) lactate (grade B) was obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Other chemicals were of analytical reagent grade except for pyrogallol, which was B.P. grade.

Instruments. A Beckman DU2 spectrophotometer was used for all photometric work and Radiometer 21 and 22 pH-meters (Radiometer, Copenhagen) were used for pH determinations.

Statistical treatment of results. Each mean value given was based on several separate experiments (the number is given with each figure), not on replication within an experiment. The significance of differences between means was determined by the t test.

RESULTS AND DISCUSSION

The growth curves in these experiments were close to those reported by Winder & O'Hara (1962). Cultures in all three types of medium grew at about the same rate for the first 48 hr. Shortly after this the growth rate in these metal-deficient cultures

dropped below that in the normal cultures and after 72 hr. their growth had practically ceased. In the normal cultures the corresponding fall in growth rate (in this case due to exhaustion of the carbon and nitrogen sources) took place about 12 hr. later than in the deficient cultures. The results for the normal cultures (Table 1) showed that exhaustion of the carbon and nitrogen sources had comparatively little effect on the activity of any of the enzymes studied. Some significant changes were observed, but in no case did the mean value at the time of highest activity differ from that at the time of lowest activity by a factor as high as two.

The enzyme activities fell into three groups in their response to iron deficiency: those elevated or not significantly affected (lactate dehydrogenase, alanine aminotransferase and NADH-oxidizing activity); those unaffected on the second day but significantly below normal thereafter (glycerol dehydrogenase, catalase and peroxidative activity towards pyrogallol); and those below normal even on the second day (succinate dehydrogenase, aconitate hydratase and NADH-cytochrome *c* reductase). Those in the first group may reasonably be assumed to have no requirement for iron for their synthesis or action. This tends to confirm that there is no lactate dehydrogenase of the cytochrome b_2 type (EC 1.1.2.3) in this organism. Two of the activities in the second group depend on haem compounds. The occurrence of glycerol dehydrogenase in this group suggests that it also might require iron for its synthesis or activity in this organism, but this has not been shown for this enzyme from other organisms (Burton, 1955; Edson, 1961). It is possible that the effect of iron deficiency is indirect, such as through repression brought about by the accumulation of an intermediate: this enzyme is inducible in some bacteria (Lin & Magasanik, 1960; Jacobs & VanDemark, 1960).

Each of the enzymes in the third group, which respond very rapidly to iron deficiency, has been shown, in some organisms, to contain non-haem iron (Slater, 1961; Dickman, 1961; Massey, 1961; Neilands, 1961), though this does not appear to be the case with the NADH-cytochrome *c* reductase of bacteria (Brodie, 1955; Neilands, 1961).

Aconitate hydratase, alanine aminotransferase and NADH-oxidizing activity were not decreased by zinc deficiency during the period of the experiments. Succinate-dehydrogenase, catalase and peroxidative activity towards pyrogallol showed a small fall in activity on the fourth day whereas NADH-cytochrome *c* reductase showed it somewhat earlier. This late fall may be due to slight proteolysis, which seems to occur at a fairly late stage of zinc deficiency (Winder & O'Hara, 1962).

The earlier and larger fall in glycerol-dehydrogenase activity suggests a special requirement for zinc for the synthesis or function of this enzyme. This is the case with a number of other NAD⁺-dependent dehydrogenases (Vallee, 1960), although it has not been demonstrated for glycerol dehydrogenase (Burton, 1955; Edson, 1961) and in fact the form extracted from *Aerobacter aerogenes* is inhibited by zinc (Lin & Magasanik, 1960). Again the effect may be due to repression. Lactate-dehydrogenase activity fell still earlier but we, like most other workers (Edson & Cousins, 1953; Cousins, 1956), have found no evidence for the presence of a NAD⁺-dependent lactate dehydrogenase (EC 1.1.1.27), which might account for the effect of zinc (Vallee, 1960). The activity assayed was probably due entirely to the decarboxylating lactate oxidase of mycobacteria (EC 1.1.3.2) (Yamamura, Kusunose & Kusunose, 1952; Cousins, 1956; Sutton, 1954, 1955, 1957). These workers failed to show the presence of any metal in this enzyme but it is possible that zinc is involved in a cofactor coupling the lactate oxidase with ferri-cyanide. Such a cofactor is necessary to couple the purified oxidase with acceptors having a redox potential less than +0.19v at pH 7.0 (Sutton, 1955), though the redox potential of ferri-cyanide is higher than this.

The activity of NADH-cytochrome *c* reductase could account for part only of the high rate of NADH oxidation, and there was no correlation between the responses of these two activities to different cultural conditions. There are several possible explanations for this, including the possibility that some of the NADH disappearance took place by a mechanism other than oxidation. A deficiency of either iron or zinc stimulated the cytochrome *c*-independent route of NADH disappearance.

Peroxidative activity towards pyrogallol correlated closely with catalase activity: the regression coefficient of peroxidase activity on catalase activity was highly significant ($P < 0.001$) when tested by the methods of variance analysis. Further, variance analysis (with the *F* test and the *t* test) on the ratios of the catalase activity to the peroxidative activity for each extract showed that there was no significant difference between the response of catalase activity and that of peroxidative activity to the progressive deficiencies of iron, zinc, or carbon and nitrogen induced during these experiments. These results are in keeping with the suggestion, which has been made on other grounds, that both activities in mycobacteria are functions of one enzyme (Winder, 1960). Studies on isoniazid-resistant strains of mycobacteria have shown that the peroxidative activity is not due to the other major haem enzymes (Winder, 1963).

Table 1. *Activities of enzymes in normal, iron-deficient and zinc-deficient Mycobacterium smegmatis*

M. smegmatis was harvested at the ages shown, washed, disintegrated and the activities of the following enzymes were determined on the extracts. Values given are means $\pm$ s.e.m., the number of experiments being shown in parentheses. The significance of the difference between each value and that for the 2-day-old cultures in normal medium was determined by means of the *t* test: s., indicating $P < 0.05$, and h.s., indicating $P < 0.01$.

Activity	Units $\mu\text{m-moles of NADH formed/}$ $\text{min./mg. of protein}$	Culture medium	Culture after 2 days	Culture after 3 days	Culture after 4 days
Glycerol dehydrogenase	$\mu\text{m-moles of Fe(CN)}_6^{3-}$ reduced/min./mg. of protein	Normal	8.78 $\pm$ 1.06 (6)	6.52 $\pm$ 0.73 (9)	9.16 $\pm$ 0.96 (9)
		Iron-deficient	9.17 $\pm$ 0.70 (6)	2.90 $\pm$ 0.39 (9) h.s.	3.30 $\pm$ 0.64 (9) h.s.
		Zinc-deficient	7.99 $\pm$ 0.73 (6)	1.93 $\pm$ 0.39 (9) h.s.	1.62 $\pm$ 0.31 (9) h.s.
Lactate dehydrogenase	$0.5 \times \mu\text{m-moles of Fe(CN)}_6^{3-}$ reduced/min./mg. of protein	Normal	28.07 $\pm$ 2.03 (9)	23.60 $\pm$ 2.76 (9)	21.29 $\pm$ 1.68 (9) s.
		Iron-deficient	24.75 $\pm$ 2.51 (9)	24.66 $\pm$ 2.88 (9)	23.56 $\pm$ 2.76 (9)
		Zinc-deficient	14.57 $\pm$ 1.21 (9) h.s.	7.60 $\pm$ 1.20 (9) h.s.	3.65 $\pm$ 0.50 (9) h.s.
Succinate dehydrogenase	$0.5 \times \mu\text{m-moles of Fe(CN)}_6^{3-}$ reduced/min./mg. of protein	Normal	14.40 $\pm$ 1.76 (4)	12.45 $\pm$ 0.88 (4)	13.40 $\pm$ 1.10 (4)
		Iron-deficient	5.12 $\pm$ 0.14 (4) h.s.	2.86 $\pm$ 0.99 (4) h.s.	3.40 $\pm$ 0.41 (4) h.s.
		Zinc-deficient	12.80 $\pm$ 0.46 (4)	10.40 $\pm$ 1.06 (4)	8.58 $\pm$ 1.07 (4) s.
Aconitate hydratase	$\mu\text{m-moles of cis-aconitate}$ formed/min./mg. of protein	Normal	26.39 $\pm$ 5.77 (4)	32.29 $\pm$ 4.40 (5)	40.75 $\pm$ 6.15 (5)
		Iron-deficient	10.46 $\pm$ 2.56 (4) s.	9.51 $\pm$ 1.80 (5) s.	15.85 $\pm$ 3.85 (5)
		Zinc-deficient	40.84 $\pm$ 9.39 (4)	23.66 $\pm$ 4.74 (5)	26.90 $\pm$ 2.48 (5)
NADH-oxidizing activity	$\mu\text{m-moles of NADH dis-}$ appearing/min./mg. of protein	Normal	7.76 $\pm$ 0.66 (3)	14.00 $\pm$ 1.38 (3) h.s.	10.97 $\pm$ 0.40 (3) h.s.
		Iron-deficient	8.03 $\pm$ 0.40 (3)	13.16 $\pm$ 1.02 (3) h.s.	16.13 $\pm$ 3.00 (3) s.
		Zinc-deficient	9.90 $\pm$ 2.25 (3)	12.98 $\pm$ 3.54 (3)	18.82 $\pm$ 2.99 (3) h.s.
NADH-cytochrome <i>c</i> reductase	$\mu\text{m-moles of cytochrome } c$ reduced/min./mg. of protein	Normal	7.87 $\pm$ 0.06 (3)	6.36 $\pm$ 0.11 (3) h.s.	8.51 $\pm$ 0.96 (3)
		Iron-deficient	5.55 $\pm$ 0.88 (3) s.	4.80 $\pm$ 0.48 (3) h.s.	4.58 $\pm$ 0.49 (3) h.s.
		Zinc-deficient	7.77 $\pm$ 0.57 (3)	5.70 $\pm$ 0.60 (3) s.	3.39 $\pm$ 0.15 (3) h.s.
Catalase	First-order reaction constant ($\text{l.g.}^{-1} \text{ min.}$)	Normal	3.49 $\pm$ 0.66 (6)	3.16 $\pm$ 0.20 (6)	4.96 $\pm$ 0.24 (6)
		Iron-deficient	3.89 $\pm$ 0.58 (6)	0.89 $\pm$ 0.14 (6) h.s.	0.66 $\pm$ 0.12 (6) h.s.
		Zinc-deficient	7.62 $\pm$ 1.88 (6)	2.80 $\pm$ 0.48 (6)	1.60 $\pm$ 0.21 (6) s.
Peroxiidative activity towards pyrogallol	$\mu\text{moles of purpuragallin formed/}$ $\text{min./mg. of protein}$	Normal	2.14 $\pm$ 0.59 (5)	2.34 $\pm$ 0.54 (5)	2.96 $\pm$ 0.64 (5)
		Iron-deficient	2.08 $\pm$ 0.41 (5)	0.54 $\pm$ 0.17 (5) s.	0.42 $\pm$ 0.53 (5) s.
		Zinc-deficient	2.93 $\pm$ 0.50 (5)	2.03 $\pm$ 0.61 (5)	0.98 $\pm$ 0.21 (5) s.
Alanine aminotransferase	$\mu\text{m-moles of pyruvate formed/}$ $\text{min./mg. of protein}$	Normal	3.43 $\pm$ 0.39 (5)	3.39 $\pm$ 0.28 (5)	3.06 $\pm$ 0.21 (5)
		Iron-deficient	4.33 $\pm$ 0.28 (5)	4.57 $\pm$ 0.49 (5)	4.41 $\pm$ 0.45 (5)
		Zinc-deficient	3.90 $\pm$ 0.17 (5)	3.22 $\pm$ 0.46 (5)	3.07 $\pm$ 0.31 (5)

SUMMARY

1. *Mycobacterium smegmatis* was grown on the surface of normal, iron-deficient and zinc-deficient media. The concentrations in the deficient media permitted a normal growth rate for 2 days. A number of enzymes were determined in cell-free extracts prepared from these cultures at the end of 2, 3 and 4 days' growth.

2. In the iron-deficient medium, lactate-dehydrogenase, alanine-aminotransferase and NADH-oxidizing activity were elevated or were not significantly affected; glycerol-dehydrogenase, catalase and peroxidative activity towards pyrogallol were unaffected on the second day but were significantly below normal thereafter; succinate dehydrogenase, aconitate hydratase and NADH-cytochrome *c* reductase were below normal throughout.

3. In the zinc-deficient medium, aconitate-hydratase, alanine-aminotransferase and NADH-oxidizing activity were normal or elevated; succinate dehydrogenase, NADH-cytochrome *c* reductase, catalase and peroxidative activity towards pyrogallol fell slightly about the fourth day, probably owing to autolysis; an earlier and larger fall occurred in glycerol-dehydrogenase and lactate-dehydrogenase activity.

4. NADH-cytochrome *c* reductase can account for only part of the NADH-oxidizing activity of the extracts; the two activities varied independently.

5. Peroxidative activity towards pyrogallol followed catalase activity closely, in keeping with the concept that both activities are functions of one enzyme.

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