Effects of Hydroxyurea, Nalidixic Acid and Zinc Limitation on DNA Polymerase and ATP-dependent Deoxyribonuclease Activities of *Mycobacterium smegmatis*

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SUMMARY

Treatment of growing *Mycobacterium smegmatis* with hydroxyurea decreased the DNA/protein ratio and markedly increased the specific activities of DNA polymerase and ATP-dependent deoxyribonuclease. These changes were similar to those produced by iron limitation of growth. Treating *M. smegmatis* with nalidixic acid also decreased the DNA/protein ratio but had no effect on the activity of either enzyme. Growth of *M. smegmatis* under conditions of zinc limitation did not diminish the specific activity of DNA polymerase thus not favouring a role for zinc in the action of this enzyme. The activity of the ATP-dependent deoxyribonuclease was, however, slightly increased. In previous reports the specific activity of DNA polymerase in this organism has been seriously underestimated due to interfering deoxyribonuclease and nucleoside triphosphatase. This was corrected by sufficient dilution of the extract. Revised specific activities of DNA polymerase in carbon-limited, nitrogen-limited and iron-limited cultures are, respectively, 10, 14, 11 and 64 units/mg protein.

INTRODUCTION

Growth of *Mycobacterium smegmatis* under conditions of iron limitation leads to a diminution in the DNA/protein ratio and a marked increase in specific activities of an ATP-dependent deoxyribonuclease and a DNA polymerase (Winder & O'Hara, 1962; Winder & Coughlan, 1969; Winder & McNulty, 1970). As part of an investigation of the mechanism of these responses to iron limitation and of the regulation of the activities of these two enzymes, the effects of hydroxyurea and nalidixic acid, which are selective inhibitors of DNA synthesis (Gale, Kendall, McLain & DuBois, 1964; Goss, Deitz & Cook, 1964, 1965; Rosenkranz & Levy, 1965), were determined. The effects of zinc limitation on DNA polymerase were studied since Slater, Mildvan & Loeb (1971) reported that purified DNA polymerase I from *Escherichia coli* contains two atoms of zinc/molecule of enzyme and that DNA polymerase from sea-urchin embryos contains four atoms. They suggested that, as *o*-phenanthroline inhibits the polymerase *in vitro*, zinc plays an essential role in the enzyme's action. Huberman & Kornberg (1970) also have suggested a role for a divalent metal in binding at one of the sites in this enzyme. A possible test for this hypothesis is to determine whether zinc deficiency in a bacterium decreases the activity of DNA polymerase. It is difficult to demonstrate zinc deficiency in *E. coli* (Ratledge & Winder, 1964), but limitation of growth by zinc is readily observed in *Mycobacterium smegmatis* (Winder & O'Hara, 1962) and, consequently, provides an easy means of applying this test.

METHODS

Growth of organism. The strain of *Mycobacterium smegmatis* (Winder & O'Hara, 1962) was grown in a medium containing (g/l de-ionized water): glycerol, 20 ml; monosodium glutamate, 14; KH_2PO_4 , 5; K_2SO_4 , 0.5; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5. The pH was adjusted to 6.8 with 10 M-NaOH and the medium was autoclaved with alumina (Winder & Coughlan, 1969) to remove trace metals. Fe^{2+} (2 $\mu\text{g/ml}$) and Zn^{2+} (normally 0.4 $\mu\text{g/ml}$ but 0.012 $\mu\text{g/ml}$ in the zinc-limited medium) were added as their sulphate salts. Cultures were grown at 37 °C on a rotary shaker.

Nalidixic acid (Calbiochem Ltd, London), in 0.1 M-NaOH, and hydroxyurea, in water, were sterilized by membrane filtration and were added to a proportion of the cultures after the appropriate period of growth. Bacteria were harvested by filtration under suction on Whatman no. 1 paper, washed with 0.9% NaCl, removed from the paper and stored at about -15 °C. Crude bacteria-free extracts were prepared as described previously (McNulty & Winder, 1971).

Assays. In the assay of DNA, 2.5 ml M- HClO_4 was added to 1 ml of the bacterial extract and the mixture was heated at 95 °C for 15 min and centrifuged. Deoxyribose was measured in the supernatant as previously described (Winder & Coughlan, 1969), except that 0.3 mg of acetaldehyde, instead of 1.6 mg, was used.

DNA polymerase was assayed in the bacterial extracts as described by McNulty & Winder (1971), except that twofold serial dilutions of each extract were assayed and the specific activity was calculated from the results for the dilution which gave nearest to 0.35 unit of enzyme/assay. Units are defined by McNulty & Winder (1971).

ATP-dependent deoxyribonuclease was assayed in the extracts (Winder & Lavin, 1971) except that 4 μmoles of ATP were used/incubation mixture. Units are defined as previously.

The assay of glycerol dehydrogenase and the units employed were as described by Winder & O'Hara (1964).

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as the standard.

RESULTS

Re-examination of the specific activity of DNA polymerase in Mycobacterium smegmatis. While investigating the reliability of assays of DNA polymerase in crude extracts of *M. smegmatis* considerable interference was observed which caused a serious underestimation of the activity. This was demonstrated to be due, at least partially, to nucleoside triphosphatase and deoxyribonuclease activities in these extracts: pre-incubation of either the deoxyribonucleoside triphosphates or the DNA with the crude extracts, before adding the remainder of the assay components, decreased the apparent activity still further.

When the extracts were serially diluted, the observed specific activity of the enzyme increased to a plateau value, which was maintained over about an eightfold range of dilution. When dilutions which gave specific activities in this plateau region were employed, interference by deoxyribonuclease and nucleoside triphosphatase activities was almost eliminated, as shown by pre-incubating the diluted extracts with the DNA or with the deoxyribonucleoside triphosphates which led to very little diminution in polymerase activity.

This plateau region, in which the observed total incorporation of [*methyl*- ^3H]thymidine 5'-phosphate responded in linear fashion to the amount of enzyme used, varied somewhat depending on the growth conditions of the bacteria. However, an observed activity of

Table 1. *Specific activity of DNA polymerase in extracts prepared from Mycobacterium smegmatis grown with different nutrient limitations*

Bacteria were grown, extracts were prepared and assays were carried out as described under Methods.

Type of culture	Age of culture (h)	Specific activity of DNA polymerase (units/mg of protein)
'Fully sufficient'	48	8.6
	72	10.5
	96	9.0
Carbon-limited	48	10.4
	72	14.0
	96	12.1
Nitrogen-limited	48	7.9
	72	10.1
	96	11.2
Iron-limited	72	64.1

0.35 unit of enzyme/assay mixture always fell within this region. For this reason, each extract was assayed in serial dilution and the specific activity was calculated from the results for the dilution which gave nearest to 0.35 unit of enzyme/assay mixture.

Because these findings indicated that earlier determinations (Winder & McNulty, 1970; McNulty & Winder, 1971) had given seriously low values for the specific activity of DNA polymerase in crude extracts of *Mycobacterium smegmatis*, a re-investigation under various growth conditions was undertaken. A comparison of the DNA polymerase activity of cultures of *M. smegmatis* at various stages of growth shows that the activity did not vary much during growth of carbon-limited or nitrogen-limited cultures, or cultures grown in a medium in which excess nutrients were supplied (Table 1). This accords with previous results (Winder & McNulty, 1970; McNulty & Winder, 1971), though the actual values were about 20 times higher. Extracts of iron-limited bacteria had much higher specific activities than did extracts of the other cultures, in keeping with previous observations, though in this case the activities were about 40 times higher.

Effects of nalidixic acid treatment. Much higher concentrations of nalidixic acid were required to exert effects on *Mycobacterium smegmatis* than on *Escherichia coli*; over 100 µg/ml was required to partially inhibit an inoculum of *M. smegmatis* in a tube dilution test. When it was added at 200 µg/ml to young, growing cultures of *M. smegmatis*, and these harvested 19 h later, substantial inhibition of growth was observed (Table 2). However, as the DNA/protein ratio was decreased, either DNA synthesis had been strongly inhibited or some degradation of DNA had taken place. The decline in this ratio though was less marked than has been reported for *E. coli* (Goss *et al.* 1964), and no obvious elongation of the bacteria occurred. No appreciable change in DNA polymerase or ATP-dependent deoxyribonuclease was observed.

Effects of hydroxyurea treatment. In a tube-dilution test, 200 µg hydroxyurea/ml allowed virtually no growth of an inoculum of *Mycobacterium smegmatis*, while 50 µg/ml was partially inhibitory. When it was added at 50 and 200 µg/ml to young, growing cultures of this organism and these were harvested 16 h later, the DNA/protein ratio of the bacteria was markedly decreased with the specific activities of DNA polymerase and ATP-dependent deoxyribonuclease in the extracts being increased (Table 3). These effects were more marked at the higher drug concentration.

Table 2. *Effects of nalidixic acid on growing cultures of Mycobacterium smegmatis*

Mycobacterium smegmatis was grown for 28 h in 'fully sufficient' medium. Nalidixic acid (200 µg/ml) was added after 28 h and after a further 19 h the control and treated cultures were harvested. Extracts were prepared and assays carried out as described under Methods.

Culture	Yield (g wet wt/l)	DNA content (µmol of nucleotide/mg of protein)	DNA polymerase activity (units/mg of protein)	ATP-dependent deoxyribonuclease activity (units/mg of protein)
Control	2.09	0.257	12.4	92.6
Nalidixic acid-treated	0.94	0.188	12.8	87.2

Table 3. *Effects of hydroxyurea on growing cultures of Mycobacterium smegmatis*

Mycobacterium smegmatis was grown in 'fully sufficient' medium. Hydroxyurea (50 µg/ml or 200 µg/ml) was added after 35 h. Treated and untreated cultures were harvested after a further 16 h of incubation. Extracts were prepared and assays carried out as described under Methods.

Culture	Yield (g wet wt/l)	DNA content (µmol of nucleotide/mg of protein)	DNA polymerase activity (units/mg of protein)	ATP-dependent deoxyribonuclease activity (units/mg of protein)
Control (35 h)	1.03	0.249	16.4	55.7
Control (51 h)	—	0.250	13.9	58.0
Hydroxyurea (50 µg/ml) (5 h)	3.60	0.141	23.4	90.0
Hydroxyurea (200 µg/ml) (51 h)	2.74	0.095	36.4	136

Microscopic examination showed that the vast majority of the bacteria grown in 50 µg hydroxyurea/ml were longer than the control bacteria, though the elongation was less than when the bacteria were grown under iron limitation. When 200 µg hydroxyurea/ml was used, elongation was less marked, presumably because of more complete inhibition of growth.

Effects of zinc limitation. When *Mycobacterium smegmatis* was grown in zinc-limited medium the growth rate began to diverge from that of the unlimited cultures after about 48 h (Fig. 1), indicating that zinc limitation began to affect growth at about that time. Zinc limitation had, however, begun to affect other processes earlier than this; by 48 h the specific activity of glycerol dehydrogenase, which serves as a sensitive indicator of zinc deficiency in this organism (Winder & O'Hara, 1964), was below that in the control bacteria. The DNA/protein ratios were not substantially different from those in the controls. The specific activity of the ATP-dependent deoxyribonuclease rose markedly above that in the control bacteria after about 48 h. In this, and other similar experiments, the specific activity of DNA polymerase never fell below that in the control bacteria, even when zinc deficiency had been established for a considerable period. In fact, DNA polymerase activity in the zinc-deficient bacteria invariably tended to rise slightly above that in the control bacteria.

Our main interest in the effects of zinc limitation was concerned with a possible role of zinc in the action of DNA polymerase. The case for such a role is based partly on the demonstration that DNA polymerases from several sources are inhibited by *o*-phenanthroline (Slater *et al.* 1971). Hence, if the results with the *Mycobacterium smegmatis* were to be relevant in this context, its DNA polymerase must also be inhibited by *o*-phenan-

Fig. 1. Comparison of 'fully sufficient' and zinc-limited cultures of *Mycobacterium smegmatis*. Fully sufficient cultures are indicated by open symbols and zinc-limited by closed symbols. Extracts were prepared and assays carried out as described under Methods. (a) Yield of bacteria (○, ●) and specific activity of glycerol dehydrogenase in extracts (□, ■). (b) DNA content of bacteria (○, ●); specific activity of DNA polymerase in extracts (△, ▲); specific activity of ATP-dependent deoxyribonuclease in extracts (□, ■).

Table 4. Inhibition of partially purified DNA polymerase of *Mycobacterium smegmatis* by *o*-phenanthroline

The DNA polymerase was purified from iron-limited *Mycobacterium smegmatis* as described by McNulty & Winder (1971). Assays were carried out as described under Methods, in the presence of the specified concentrations of *o*-phenanthroline.

Concentration of <i>o</i> -phenanthroline (μM)	Activity of DNA polymerase (units)	Inhibition (%)
0	0.854	0
21	0.795	7
41	0.536	37
83	0.169	80
165	0.107	88
370	0.102	88
660	0.093	89

tholine; with a partially purified DNA polymerase from *M. smegmatis* 50% inhibition was caused by 50 μM *o*-phenanthroline (Table 4). This sensitivity to *o*-phenanthroline is similar to that of the enzyme from *Escherichia coli* and greater than that of the other DNA polymerases reported (Slater *et al.* 1971).

DISCUSSION

The specific activity of DNA polymerase in crude extracts of *Mycobacterium smegmatis* is much higher than previously reported (Winder & McNulty, 1970; McNulty & Winder, 1971). The revised figures are 10 units of DNA polymerase/mg protein in extracts of normal bacteria and over 60 units/mg protein in iron-limited bacteria. Thus, allowing differences in units employed, the specific activities are of the same order as those found with crude extracts of *Escherichia coli* (Richardson, Schildkraut, Aposhian & Kornberg, 1964), *Micro-*

coccus lysodeikticus (Zimmerman, 1966; Litman, 1968) and *Bacillus subtilis* (Okazaki & Kornberg, 1964), though differences in assay conditions make exact comparisons impossible. Iron-limited *M. smegmatis* appears to be an unusually rich source of the enzyme.

The effects produced in *Mycobacterium smegmatis* by hydroxyurea treatment are remarkably similar to those produced by iron limitation (Winder & O'Hara, 1962; Winder & Coughlan, 1969; Winder & McNulty, 1970). In both cases there is bacterial elongation, a fall in the DNA/protein ratio (indicative of a selective inhibition of DNA synthesis) and a marked increase in the specific activities of DNA polymerase and of ATP-dependent deoxyribonuclease. The increases in enzymatic activities on hydroxyurea treatment were not as great as in iron limitation, though further adjustment of drug concentration and exposure time might increase the effects in the former case. Thus, two conditions inhibitory to DNA synthesis increase the activities of these two enzymes in this organism. However, the two mechanisms of inhibition of DNA synthesis are possibly closely related, since iron limitation (Winder & Coughlan, 1971) and hydroxyurea (Neuhard, 1967; Krakoff, Brown & Reichard, 1968) both may inhibit DNA synthesis by inhibiting ribonucleotide reductase. In fact, hydroxyurea could act by chelating iron in ribonucleotide reductase (Moore, 1969).

The increases in activity of the two enzymes in iron limitation and on hydroxyurea treatment do not appear to be due to a fall in the DNA/protein ratio or inhibition of DNA synthesis *per se*, since nalidixic acid can lead to a similar, though smaller, fall in the DNA/protein ratio without increasing the specific activities of these enzymes. If both of these treatments do act by inhibiting ribonucleotide reductase, then the increases in activity may be a direct consequence of the altered deoxyribonucleotide pool. Perhaps the most plausible explanation, however, is that such an alteration in the deoxyribonucleotide pool may lead to the formation of abnormal DNA and this may provoke increased production of these enzymes: thymine starvation leads, not only to altered deoxyribonucleotide pools, but also to the formation of abnormal DNA (Gold & Hurwitz, 1963; Luzzati, 1966; Freifelder, 1969; Walker, 1970).

The DNA polymerase of *Mycobacterium smegmatis* is similar to DNA polymerase I of *Escherichia coli* (McNulty & Winder, 1971) and, hence, is likely to be involved in excision repair (Kelly, Atkinson, Huberman & Kornberg, 1969). Similarly, the ATP-dependent deoxyribonuclease of *M. smegmatis* is similar to ATP-dependent deoxyribonucleases in *E. coli* and *Diplococcus pneumoniae* (Winder & Lavin, 1971) and, hence, is likely to be involved in recombination repair (Rupp, Wilde, Reno & Howard-Flanders, 1971) and perhaps also excision repair (Cooper & Hanawalt, 1972). Hence, a reasonable hypothesis is that iron limitation and hydroxyurea treatment leads to the formation of abnormal DNA in *M. smegmatis* and that this induces increased production of repair enzymes. A marked increase in the specific activity of DNA polymerase in *Tetrahymena pyriformis* follows u.v. irradiation, thymine starvation or ethidium bromide treatment (Crerar & Pearlman, 1971; Keiding & Westergaard, 1971), and has been interpreted as a response to an increased need for repair, while in *Ustilago maydis* irradiation is thought to induce a recombination repair mechanism (Holliday, 1971).

Neither iron limitation nor hydroxyurea treatment increases the DNA polymerase in *Escherichia coli*. Although iron limitation in *E. coli* produces bacterial elongation (Ratledge & Winder, 1964) reminiscent of that in *Mycobacterium smegmatis*, no appreciable alteration either in DNA/protein ratio or in specific activity of DNA polymerase has been observed (F. G. Winder & D. S. Barber, unpublished observations). Hydroxyurea selectively inhibits DNA synthesis in *E. coli*, but this is not followed by an appreciable alteration in the DNA polymerase level (F. G. Winder & P. B. Davis, unpublished observations).

Investigations with zinc-limited *Mycobacterium smegmatis* provide no evidence in favour of a role for zinc in the action of DNA polymerase, since the activity of this enzyme did not decline even in severe zinc deficiency. Although this evidence does not disprove such a role, since the polymerase may have much higher affinity for zinc than most other zinc-requiring components of the bacteria, it does make the role less likely.

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