Biochem. J. (1969) **111**, 679
Printed in Great Britain

A Nucleoside Triphosphate-Dependent Deoxyribonucleic Acid-Breakdown System in *Mycobacterium smegmatis*, and the Effect of Iron Limitation on the Activity of this System

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(*Received* 22 *August* 1968)

1. The presence of a nucleoside triphosphate-dependent DNA-breakdown system was demonstrated in extracts of *Mycobacterium smegmatis*. Its activity was increased substantially by iron limitation, apparently after the fall in DNA content that took place under these conditions. A maximal activity of about 0·2 μmole of deoxyribonucleotide/30min./mg. of protein was found in crude extracts. 2. After slight purification by streptomycin treatment, the enzyme showed maximal activity with undenatured DNA ($K_m \simeq 200\,\mu$g./ml.), ATP ($K_m \simeq 1{\cdot}2$mM) or UTP, CTP and GTP giving lower activity and pyrophosphate giving none, and Mg^{2+} ions (optimum concn. 12mM). The optimum pH was 8·5. 3. In the assay system there was proportionality between enzyme concentration and rate of reaction, but the rate fell off with time. 4. ATP was broken down in the reaction and monodeoxyribonucleotides were among the products, but the presence of some oligodeoxyribonucleotides was not excluded and the degree of phosphorylation of the primary products was uncertain.

It was reported previously that the concentration of DNA in *Mycobacterium smegmatis* is decreased by iron limitation to a value markedly below that observed during exponential growth, or when carbon, nitrogen or zinc is the limiting nutrient (Winder & O'Hara, 1962). One of the ways in which iron limitation could lead to this decrease in DNA would be through a requirement for this metal by the ribonucleotide reductase system of this organism, as is the case with ribonucleotide reductase systems from mammals and from *Escherichia coli* (Moore & Hurlbert, 1962; Moore & Reichard, 1964; Brown, Eliasson, Reichard & Thelander, 1968). Attempts to investigate this possibility in *Myco. smegmatis* led to the discovery of a highly active nucleoside triphosphate-dependent DNA-breakdown system. Evidence for the occurrence of this system and an account of some of its properties are presented in this paper. Some of these results have been presented in preliminary form (Winder & Coughlan, 1967*a*,*b*,*c*).

Enzymes rather similar to this have been reported from *Micrococcus lysodeikticus* (Tsuda & Strauss, 1964) and *Bacillus laterosporus* (Anai, 1967). These enzymes both probably have an absolute requirement for a nucleoside triphosphate. ATP, UTP, CTP and GTP are active, but there is variation between the enzymes in the relative effectiveness of these triphosphates and in their optimum concentrations. Deoxy-ATP is as active as ATP with the former enzyme, but deoxyribonucleotides were not tested with the latter. They have a similar optimum pH and optimum Mg^{2+} concentration, but the *B. laterosporus* enzyme differs from the other in being strongly stimulated by Mn^{2+}. They both show a preference for native rather than heat-denatured DNA, a preference which seens absolute with the highly purified *B. laterosporus* enzyme. With the *Micro. lysodeikticus* enzyme, most of the DNA broken down is converted into mononucleotides, and ATP yields orthophosphate, apparently 1 mole of ATP being used per mole of dexoyribonucleotide solubilized. With the *B. laterosporus* enzyme ATP yields ADP, but only about one-third of a mole of ADP is formed per mole of deoxyribonucleotide solubilized. This and other evidence was interpreted as indicating that oligonucleotides are the products in this case.

A phage-restriction enzyme from *E. coli* has also been identified as an ATP-dependent DNA-breakdown system (Meselson & Yuan, 1968). This enzyme requires *S*-adenosylmethionine in addition to ATP for activity, and is endonucleolytic in action.

With none of these systems has it been shown conclusively that the action on the DNA is purely hydrolytic, and with the *Myco. smegmatis* enzyme there is a possibility that the primary products from DNA are at a higher level of phosphorylation than monophosphates, as discussed below. Hence we avoid the term 'deoxyribonuclease' in referring to this enzyme.

MATERIALS AND METHODS

All nucleosides and nucleotides were obtained either from Sigma Chemical Co., St Louis, Mo., U.S.A., or from Calbiochem, Los Angeles, Calif., U.S.A. *E. coli* alkaline phosphatase, *Crotalus adamanteus* venom, phosphodiesterase (Sigma type II), deoxyribonuclease I (from bovine plasma), DNA (Sigma type I, from calf thymus), DNA (Sigma type VI, from fish roe), 2-deoxy-D-ribose and 2-mercaptoethanol were all obtained from Sigma Chemical Co. Aluminium oxide ('standardized for chromatographic adsorption analysis according to Brockmann, II–III'), used to adsorb metal ions from the growth medium, acetaldehyde, diphenylamine and glycerol were obtained from E. Merck A.-G., Darmstadt, Germany. Streptomycin sulphate (U.S.P. grade) was obtained from Calbiochem. Charcoal (Nuchar C-190, plus 30 mesh) was from Industrial Chemical Sales, Covington, Va., U.S.A. Acetic acid, thioglycollic acid and all the vitamins used in the micro-bioassay medium were from British Drug Houses Ltd., Poole, Dorset. L-Asparagine was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., Tween 80 (T.B. culture grade) from Honeywill and Stein Ltd., London W. 1, bovine plasma albumin from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, and Niacin Assay Medium from Difco Laboratories, Detroit, Mich., U.S.A.

Growth of Myco. smegmatis. The media used for the growth of *Myco. smegmatis* contained glycerol (20ml. in iron-limited medium and 2·5ml. in carbon-limited medium), L-asparagine (5g.), KH_2PO_4 (5g.), K_2SO_4 (0·5g.) and $MgSO_4,7H_2O$ (0·5g.) in 1l. of deionized water. The pH was adjusted to 6·8 with 10N-NaOH and the media were depleted of trace metals by autoclaving then with alumina as described by Winder & O'Hara (1962), except that 2% of Merck alumina (pre-washed to remove fines) was used. Zn^{2+} (0·4μg./ml.) and Fe^{2+} (2μg./ml. in the carbon-limited medium and 0·06μg./ml. in the iron-limited medium) as their sulphate salts were added to the media, which were then transferred to conical flasks that had been cleaned as described by Ratledge & Winder (1962).

Inocula were prepared from 3–4-day-old stationary cultures in Proskauer & Beck medium (American Trudeau Society, 1950). The pellicle was lightly ground in aq. 0·9% NaCl, and about 0·7μg. of bacterial N/ml. of medium was used. The cultures were then incubated at 37° on a rotary shaker.

Preparation of cell-free extracts and crude enzyme. Bacteria were washed with 0·9% NaCl and stored below −10°. About 2g. wet wt. of the cells was suspended in 5–10ml. of 0·05M-tris–HCl buffer, pH8·0, containing 1mM-mercaptoethanol, treated for four 30sec. periods near 0° with a 100w 20kcyc./sec. ultrasonic generator (Soniprobe type 1130A; Dawe Instruments Ltd., London, W. 3), and centrifuged at 25000*g* for 20min. near 0°. The supernatant provided the crude cell-free extract. Slight purification was achieved by adding streptomycin sulphate to this extract to a final concentration of 0·5%, stirring gently for 20min. and centrifuging at 25000*g* for 20min., all at 0°. The supernatant was dialysed for 18hr. against 2×200vol. of the original buffer and is referred to as the 'streptomycin-treated extract'.

Measurement of enzyme activity. The incubation mixtures usually contained 0·8–1·0ml. of streptomycin-treated extract (about 6mg. of protein, 40μmoles of tris–HCl buffer, pH8·0, and 0·8–1·0μmole of mercaptoethanol), 20μmoles of $MgCl_2$, 1mg. of the sodium salt of DNA and 4μmoles of ATP in a final volume of 1·0–1·4ml. After the appropriate period the reaction was stopped by the addition of 2ml. of N-$HClO_4$, the mixture was centrifuged and the supernatant was removed from the acid-insoluble residue. Modifications to this procedure are indicated in the appropriate places in the text. Usually the supernatant was assayed for acid-labile deoxyribose and the result doubled to allow for pyrimidine-bound deoxyribose. Occasionally total deoxyribose was determined directly. On other occasions deoxyribonucleotides were determined microbiologically.

Assay procedures. Protein was determined by the biuret method (Gornall, Bardawill & David, 1949) with bovine plasma albumin as standard. DNA in crude cell-free extracts was measured by treating a 1ml. sample with 5ml. of 5% (w/v) trichloroacetic acid, centrifuging, extracting the residue with 5ml. of 5% (w/v) trichloroacetic acid at 90° for 15min., determining acid-labile deoxyribose in the extract as described below and doubling the results to allow for pyrimidine-bound material.

Acid-labile deoxyribose was measured by a modification of the method of Giles & Myers (1965): to 1ml. of suitably diluted sample was added 1ml. of 50% (w/v) $HClO_4$, 2ml. of 4% (w/v) diphenylamine in acetic acid, and 1·6mg. of acetaldehyde; the mixture was incubated at 30° for 18hr., and the deoxyribose content was calculated from the difference between the extinction at 595mμ and that at 700mμ, by using a standard curve prepared with each assay. Total deoxyribose was measured by a slight modification of the method of Blakley (1966): to 0·5ml. of a sample in 0·5M-tris–HCl buffer, pH8·0, containing 1mM-mercaptoethanol, was added 0·2ml. of 1N-$HClO_4$ and 0·2ml. of saturated bromine water; the mixture was shaken and left for 10min., 0·5ml. of 5N-NaOH was added, and it was left for a further 10min., after which the deoxyribose content of a 1ml. sample was determined as described above. This procedure avoided the formation of a precipitate during the colour development, which occurred when the Giles & Myers (1965) method was used in conjunction with the original procedure of Blakley (1966). In these procedures diphenylamine from E. Merck A.-G. was used without recrystallization.

Microbiological assay of deoxyribonucleotides was carried out after dephosphorylation as recommended by Larsson (1963). The sample (usually 3ml.) was adjusted to pH9·0, incubated at 37° with 0·5mg. of *C. adamanteus* venom for 1·5hr., and adjusted to pH7·0. The resulting deoxyribonucleosides were determined as described by Hoff-Jørgensen (1957), except that the assay medium was made as follows: 7·5g. of Difco Niacin Assay Medium, 1·0ml. of vitamin solution, 1·0ml. of thioglycollic acid solution, 1·0ml. of thymine solution (2mg./ml.), 1·0ml. of

Tween 80 (10%, w/v) and 10mg. of CMP were made up to 100ml. with water, which gave the required pH6·7. After inoculation and incubation for 24hr., the volume in each tube was brought to 3ml. and E_{650} was measured. Samples were assayed in triplicate and a standard curve with thymidine was prepared with each assay.

Investigation of reaction products. Incubations were carried out on five times the scale used for enzyme assay. The reaction was stopped after 0, 10 or 30min. After chilling and centrifugation, the supernatant was neutralized with KOH and centrifuged to remove perchlorate. A sample was removed, freeze-dried and dissolved in a small volume of water, and the solution was applied as a band to Whatman no. 1 paper, which was then developed in an ascending direction in isobutyric acid–aq. NH_3 soln. (sp.gr. 0·88)–water (66:1:33, by vol.) for 18hr. with appropriate markers. Spots were located by examination under light of wavelength 253·7mμ. To the major portion of the supernatant was added 0·5μmole each of CMP, GMP and UMP (to facilitate identification of peaks), and it was placed on a column (7mm. × 30mm.) of Dowex AG1 (X4, 200–400 mesh), from which fines had been removed by washing, and which had been converted into the formate form. Elution was carried out with a concave gradient of formic acid up to 4N (300ml.) followed by a similar gradient of ammonium formate up to 1M with formic acid maintained at 4N (400ml.) (Ingle, 1962). The flow rate was about 10ml./hr. and fractions (5ml.) were collected. All fractions were freeze-dried and formic acid and ammonium formate were removed at 40° under vacuum.

The fractions were dissolved and the extinctions of suitable dilutions were determined at 260mμ. The ribonucleotides present were readily identified by the positions of the peaks, by the ratios of extinctions at 250, 260 and 280mμ, and by paper chromatography with isobutyric acid–aq. NH_3 soln. (sp.gr. 0·88)–water (66:1:33, by vol.) as solvent. The deoxyribonucleotides were usually present in amounts too small to show distinct peaks in the plot of extinction at 260mμ, and hence total deoxyribose was determined in samples from all fractions by the diphenylamine method after bromination, and the values were plotted against tube number. These peaks were identified by their positions relative to ribonucleotide peaks, by using information derived from control mixtures of authentic ribonucleotides and deoxyribonucleotides run in the same column.

RESULTS

Evidence for nucleoside triphosphate-dependent DNA breakdown. During attempts to determine ribonucleotide reductase activity in cell-free extracts of *Myco. smegmatis*, it was observed that incubation of these extracts with ATP and CDP in the presence of Mg^{2+} ions resulted in marked production of acid-soluble deoxyribonucleotides, measured by chemical or microbiological means. Omission of CDP did not affect the activity provided that the ATP remained, no requirement for a reduced nicotinamide nucleotide could be

Table 1. *Effects of incubation with and without ATP on the amounts of acid-soluble and acid-insoluble deoxyribonucleotide and total deoxyribose in crude extracts of carbon-limited and of iron-limited cultures of* Myco. smegmatis

The basal incubation mixtures were as described in the text, except that DNA and ATP were omitted. The reaction was stopped by the addition of 2ml. of N-$HClO_4$. After centrifugation deoxyribonucleotides were determined in the supernatant by microbiological assay after dephosphorylation, and total deoxyribose was determined after bromination. The acid-insoluble residue was dissolved in 1ml. of 0·3N-KOH, the pH was adjusted to 7·0 with $HClO_4$, 0·5ml. of 0·5M-tris–HCl buffer, pH7·0, containing 0·1M-$MgCl_2$, and 0·5mg. of deoxyribonuclease were added, and the mixture was incubated for 2hr. at 37°. The mixture was then adjusted to pH9·0, incubated at 37° with 0·5mg. of snake (*C. adamanteus*) venom for 1·5hr. and adjusted to pH7·0, and samples were taken for the determination of deoxyribonucleoside and total deoxyribose.

Source of extract	Addition to basal incubation mixture	Period of incubation (min.)	Deoxyribonucleotide (mμmoles/mg. of protein)		Total deoxyribose (mμmoles/mg. of protein)	
			Acid-soluble fraction	Acid-insoluble fraction	Acid-soluble fraction	Acid-insoluble fraction
Carbon-limited cells	None	0	9·9	397	9·4	426
		30	15·3	374	20·6	407
		Change ...	+5·4	−23	+11·2	−19
	ATP	0	9·9	405	10·4	437
		30	62·1	325	83·8	358
		Change ...	+52·2	−80	+73·4	−79
Iron-limited cells	None	0	14·1	158	18·7	154
		30	21·1	144	25·8	148
		Change	+7·0	−14	+7·1	−6
	ATP	0	14·1	158	16·8	154
		30	107·1	42	105·5	43
		Change ...	+93·0	−116	+88·7	−111

shown even after dialysis of the extracts, and precipitation of endogenous nucleic acid in the extracts by streptomycin followed by dialysis decreased the activity almost to zero. These three findings strongly suggested that the soluble-deoxyribonucleotide formation took place by a mechanism other than ribonucleotide reduction and that the soluble deoxyribonucleotide formed arose from the acid-insoluble fraction.

To confirm this interpretation, and to prove that DNA was the material in the acid-insoluble fraction from which the soluble deoxyribonucleotide arose, the experiment summarized in Table 1 was carried out. In this, DNA was measured in the acid-insoluble fraction both by chemical means and by enzymic hydrolysis followed by microbiological assay. Further confirmation came from the experiment illustrated in Table 2, which showed that the capacity of streptomycin-treated extracts to produce soluble deoxyribonucleotides could be restored by the addition of DNA to the incubation mixture. These experiments showed that the deoxyribose that was lost from the acid-insoluble material during incubation with ATP came from DNA.

Extracts of freshly harvested cells, particularly carbon-limited cells, had a considerable capacity to break down endogenous DNA in the absence of added ATP. As a result of storage of the cells at about −15°, extracts showed progressively decreased activity in the absence of added ATP, even though the activity in the presence of added ATP remained almost unchanged (Table 3).

Properties of the DNA-breakdown system. As shown below, iron-limited cultures about 72hr. old provided the best source of the enzyme system. Extracts of such cells were treated with streptomycin, as described in the Materials and Methods section, which removed endogenous DNA and increased the specific activity slightly. These streptomycin-treated extracts were used for a preliminary investigation of the properties of this enzyme system.

The system produced soluble deoxyribonucleotides from *Myco. smegmatis* DNA or from native, highly polymerized, DNA from calf thymus or from fish soft roe, and was about equally active with DNA that had been exposed to ultrasonic vibration. Heat denaturation of the DNA, however, markedly decreased its capacity to act as substrate (Table 2). The effect of DNA concentration on enzyme activity is shown in Fig. 1. Near-maximal activity was obtained with about 750 μg. of sodium deoxyribonucleate/ml. and half-maximal activity was obtained with about 200 μg./ml.

The results in Table 4 show that ATP can be replaced by UTP in the assay system without loss of activity, but that CTP and particularly GTP are poorer substrates. ADP gave much lower activity than ATP, suggesting that it probably had to be converted into ATP by phosphotransferase action

Table 2. *Formation of soluble deoxyribonucleotide by crude and streptomycin-treated extracts of iron-limited* Myco. smegmatis *with and without the addition of DNA*

The DNA was highly polymerized material from calf thymus. Ultrasonic treatment of the DNA was for four 30sec. periods near 0°, and heating was for 5min. at 100°. The basal incubation mixture was as described in the text, with the omission of ATP and DNA. Where appropriate, 4 μmoles of ATP and 1mg. of DNA (sodium salt) were added. Deoxyribose was determined by the diphenylamine method after bromination and alkaline hydrolysis. —, Not determined.

Additions to basal incubation mixture	Soluble deoxyribose formed (mμmoles/30min./mg. of protein)	
	Crude extract	Streptomycin-treated extract
None	4·3	0·0
ATP	107	0·0
ATP+native DNA	218	310
ATP+ultrasonically treated DNA	220	280
ATP+heated DNA	—	102

Table 3. *Effect of storage of carbon-limited* Myco. smegmatis *on the formation of soluble deoxyribonucleotide with and without addition of ATP*

Crude extracts were prepared from cells from carbon-limited cultures that had been stored at about −15° for the appropriate period. The basal incubation mixture was as described in the text, with the omission of ATP. Where appropriate, 4 μmoles of ATP were added. Deoxyribonucleotides were measured microbiologically.

Addition to basal incubation mixture	Soluble deoxyribonucleotide formed (mμmoles/30min./mg. of protein)		
Period of storage of cells (weeks)	1	2	4
None	31·2	18·6	5·4
ATP	63·6	65·6	52·2

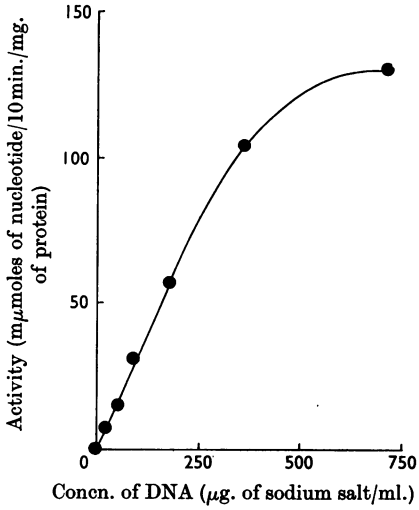

Fig. 1. Effect of the concentration of DNA on the rate of formation of soluble deoxyribonucleotide by a streptomycin-treated extract of iron-limited *Myco. smegmatis*. Incubation mixtures were as described in the text, except that the DNA concentration was varied. Soluble acid-labile deoxyribose was determined and correction was made for pyrimidine-bound material.

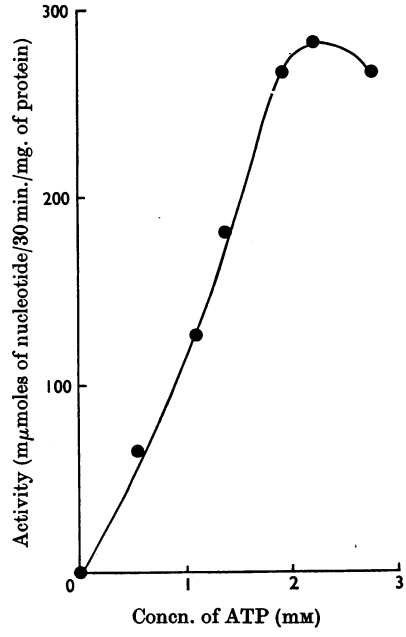

Fig. 2. Effect of concentration of ATP on the rate of breakdown of DNA by a streptomycin-treated extract of iron-limited *Myco. smegmatis*. The incubation mixtures were as described in the text, except that the ATP concentration was varied. Soluble acid-labile deoxyribose was determined and correction was made for pyrimidine-bound material.

Table 4. *Effectiveness of various nucleotides in stimulating the breakdown of DNA by streptomycin-treated extracts of iron-limited* Myco. smegmatis

Incubation mixtures were as described in the text, except that ATP was replaced by other nucleotides at the same concentration. Soluble acid-labile deoxyribose was determined and correction was made for the pyrimidine-bound material.

Nucleotide	Soluble deoxyribonucleotide formed (mμmoles/10 min./mg. of protein)	Activity (% of that given by ATP)
ATP	353	100
UTP	365	104
CTP	265	75
GTP	149	42
ADP	82·6	23·4
AMP	2·4	0·7
None	0·0	0·0

before it could serve as substrate. Deoxyribonucleoside triphosphates were not tested. A variety of substances, including AMP, UMP, CMP, GMP, adenine, adenosine, cytosine, cytidine, orthophosphate, NAD^+, $NADP^+$, NADH, NADPH and deoxyribose, were found to give no activity whatever. In view of the important possibility that ATP was serving merely to produce inorganic pyrophosphate and that this led to reversed action by a DNA polymerase (EC 2.7.7.7), pyrophosphate was added in place of ATP at various concentrations ranging from 3·0 to 30 mM without any evidence of activity being obtained. The effect of varying the concentration of ATP is shown in Fig. 2, which demonstrates that the optimum concentration of ATP was slightly over 2 mM and that half-maximal activity was obtained with about 1·2 mM. Two features about the curve are noteworthy: the facts that it is slightly sigmoid and that it passes through a maximum, features that were confirmed in experiments other than that illustrated. However, in view of the crude nature of the preparation and of the fact that initial rates were not determined, the significance of the first of these features is unknown.

A number of metal ions were tested for their capacity to replace Mg^{2+} and, of these, Mn^{2+} and Co^{2+} were about 20% as effective as Mg^{2+} at the concentration used, and the others were inactive (Table 5). All the ions tested in the presence of Mg^{2+} were partially or completely inhibitory (Table 5). The effect of varying the concentration

Table 5. *Effects of various metal ions on the ATP-dependent breakdown of DNA by streptomycin-treated extracts of* Myco. smegmatis *incubated with and without* Mg^{2+}

Incubation mixtures were as described in the text, except that in obtaining the left-hand column other metal ions at 16·6mM were used in place of Mg^{2+} and the activity was compared with that when Mg^{2+} was used at the same concentration. The ions were also used at 8·3mM (except where otherwise noted) together with 16·6mM-Mg^{2+} and the activity was compared with that in the presence of Mg^{2+} alone, the result being given as percentage inhibition in the right-hand column.

Activity (% of that given by Mg^{2+})	Metal ion	Inhibition compared with Mg^{2+} only (%)
100·0	Mg^{2+}	—
19·8	Mn^{2+}	27·2
18·3	Co^{2+}	35·2
—	Fe^{2+} (4·2mM)	45·0
—	Fe^{2+} (8·3mM)	60·0
—	Fe^{2+} (12·5mM)	72·0
0·0	Fe^{2+} (16·6mM)	82·0
2·6	Ca^{2+}	79·0
2·6	Hg^{2+}	98·6
0·7	Zn^{2+}	99·0
0·0	Sn^{2+}	100·0
0·0	Cu^{2+}	100·0
0·0	Fe^{3+}	100·0
5·1	None	0·0

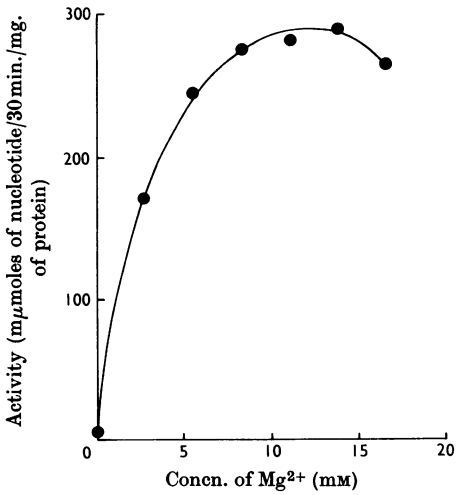

Fig. 3. Effect of the concentration of Mg^{2+} on the breakdown of DNA by streptomycin-treated extracts of iron-limited *Myco. smegmatis*. Incubation mixtures were as described in the text, except that the Mg^{2+} concentration was varied. Soluble acid-labile deoxyribose was determined and correction was made for pyrimidine-bound material.

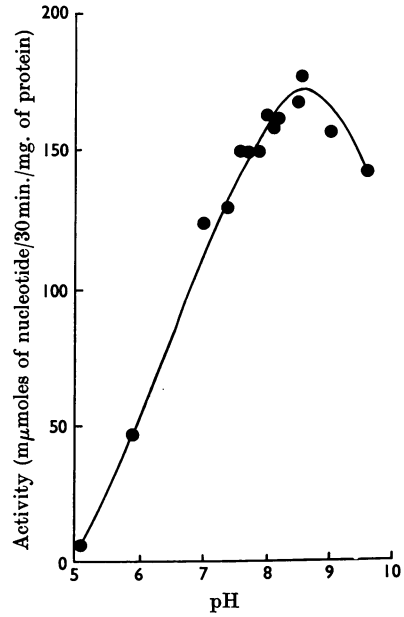

Fig. 4. Effect of pH on the breakdown of DNA by streptomycin-treated extracts of iron-limited *Myco. smegmatis*. The incubation mixture contained 0·5ml. of extract (containing 2·5μmoles of tris–HCl buffer, pH 8·0, and 1μmole of mercaptoethanol), 50μmoles of tris containing various amounts of HCl, 20μmoles of $MgCl_2$, 4μmoles of ATP and 1mg. of DNA (sodium salt) in a final volume of 1·4ml. The incubation period was 30min. Soluble acid-labile deoxyribose was determined by the diphenylamine method and allowance was made for pyrimidine-bound material. Duplicate incubation mixtures were set up for the determination of the pH of each mixture.

of Mg^{2+} is shown in Fig. 3. The effect of varying the pH of the incubation mixture is shown in Fig. 4.

The effect of varying the amount of streptomycin-treated extract in the incubation mixture on the rate of breakdown of DNA is illustrated in Fig. 5. The results indicated that there was a reasonable proportionality between the amount of enzyme and the rate of the reaction under the assay conditions used. The reaction rate fell off more markedly with time at lower than at higher enzyme concentrations. This appeared to be due to inactivation of the enzyme. The enzyme was inactivated by heating: 100° for 5min. destroyed all activity.

Nature of the products of DNA breakdown. The equivalence between the formation of soluble deoxyribose and of soluble deoxyribonucleoside derivatives as determined by the highly specific microbiological assay after enzymic dephosphorylation is shown in Table 1. That the deoxyribonucleosides were released in phosphorylated form was shown by their adsorption on Dowex 1 and by

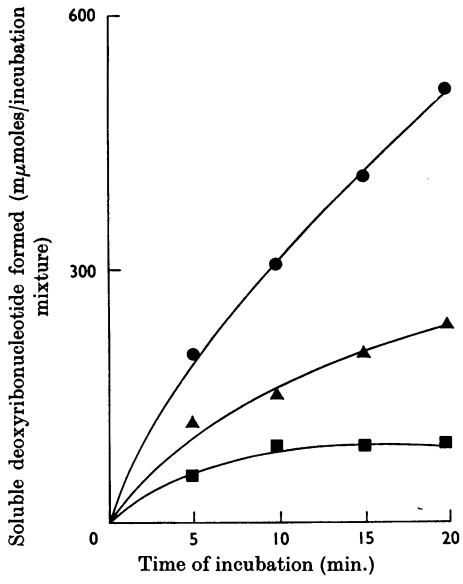

Fig. 5. Amount of breakdown of DNA after various periods of incubation with various concentrations of streptomycin-treated extracts of iron-limited *Myco. smegmatis*. The incubation mixtures were as described in the text except that one contained 2mg. of protein (●), another 1mg. of protein (▲), and the other 0·5mg. of protein (■). Soluble acid-labile deoxyribose was determined by the diphenylamine method and corrected for pyrimidine-bound material.

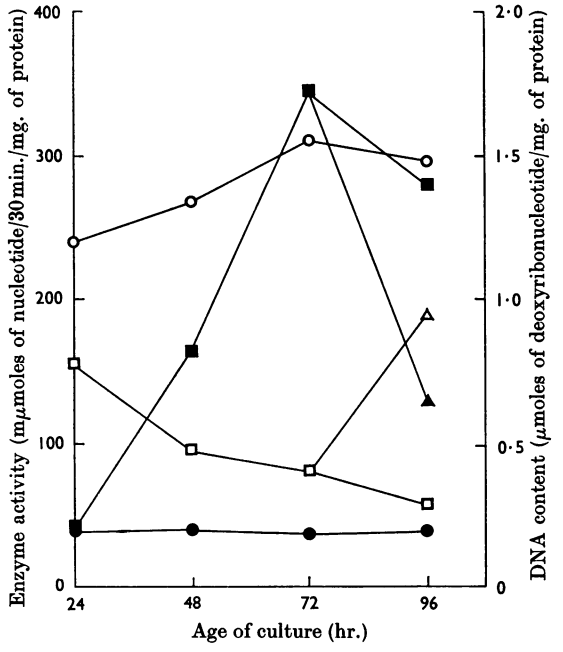

Fig. 6. Comparison between the DNA content (○) and the ATP-dependent DNA-breakdown activity (●) of carbon-limited cultures of *Myco. smegmatis*, the DNA content (□) and DNA-breakdown activity (■) of iron-limited cultures, and the DNA content (△) and DNA-breakdown activity (▲) of iron-limited cultures supplemented with 1·94μg. of Fe^{2+}/ml. after 72hr. The DNA content/mg. of protein was determined by using crude cell-free extracts, and the DNA-breakdown activity/mg. of protein was determined by using streptomycin-treated extracts.

their precipitation by Ba^{2+} at pH 9 in the presence of ethanol.

The amount of total deoxyribose solubilized in the presence of ATP was exactly twice the amount of acid-labile deoxyribose solubilized, demonstrating that equal amounts of purine and pyrimidine deoxyribonucleotides were produced from DNA. When CTP was used, the amount of pyrimidine-bound deoxyribose was slightly higher than that of purine-bound, suggesting that a limited base exchange might also have taken place.

When the products of the reaction were examined by paper chromatography it appeared, from comparison of the u.v. densities of the ribonucleotide bands from mixtures before and after incubation with DNA, that ATP was broken down mainly to AMP during the incubation. Incubation in the absence of DNA resulted in substantially less conversion of ATP into AMP. Since deoxyribonucleotides were present in substantially smaller amount than ribonucleotides, their formation could not be assessed by this method and attempts to measure them by elution and deoxyribose measurement gave very poor recoveries.

When the products of the reaction were separated by chromatography on columns of Dowex 1 as described in the Materials and Methods section, it was found that about 85% of the ATP was converted into a mixture of ADP and AMP in the ratio about 1·3:1. Deoxyribosides of all four bases were recovered in mono-, di- and tri-phosphorylated forms, with somewhat greater quantities of the more phosphorylated forms. However, the total recovery of deoxyribonucleotides was less than 20% of the amount of ATP broken down. The cause of this apparent discrepancy was not discovered.

There was no evidence of a substantial proportion of oligonucleotides in the eluates from the columns, although the possibility that there are oligonucleotides among the reaction products was not eliminated.

Hence ATP was broken down in the course of the reaction. It appears probable that one or two of its phosphate residues were incorporated into the primary products of the DNA breakdown, and that subsequent phosphatase and phosphotransferase

action in the crude preparation led to the multiplicity of products.

Effects of iron limitation on the activity of the DNA-breakdown system. Fig. 6 shows that the ATP-dependent DNA-breakdown activity/mg. of protein in streptomycin-treated extracts of carbon-limited cells was little affected by the age of the culture. In the case of iron-limited cultures, the activity of this enzyme in the streptomycin-treated extracts increased markedly after 24hr. Addition of Fe^{2+} to these cultures rapidly decreased the activity towards the value characteristic of the carbon-limited cultures. Fig. 6 also shows that the response of the enzyme activity to the depletion and addition of Fe^{2+} was clearly the inverse of the response of the DNA concentration. However, the DNA concentration fell before the enzyme activity rose, suggesting that this fall was the cause rather than the effect.

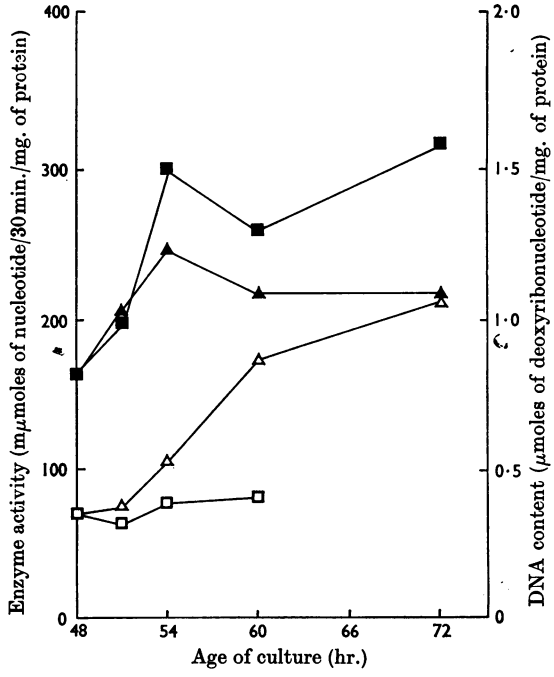

Fig. 7. Comparison between the DNA content (□) and the ATP-dependent DNA-breakdown activity (■) of iron-limited cultures of *Myco. smegmatis* and the DNA content (△) and DNA-breakdown activity (▲) of iron-limited cultures supplemented with 1·94μg. of Fe^{2+}/ml. after 48hr. The DNA content/mg. of protein was determined by using crude cell-free extracts, and the DNA-breakdown activity/mg. of protein was determined by using streptomycin-treated extracts.

To investigate the relationship more closely, a number of samples were taken at various periods after addition of Fe^{2+} to iron-limited cultures. In these experiments also, the DNA concentration appeared to respond to the addition of Fe^{2+} before the enzyme activity did (Fig. 7).

Since streptomycin-treated extracts were used for the determination of enzyme activity throughout this series of experiments, it was possible that the lower enzyme activity in the extracts of carbon-limited cells was due to a larger proportion of the enzyme being lost during streptomycin treatment in this case. Table 6 shows the results of an experiment to test this. With both extracts, streptomycin treatment activated the enzyme, since the combined activities of the supernatant and precipitate after this treatment exceeded that of the crude extract. This activation was greater in the iron-limited cultures (1·7-fold) than in the carbon-limited cultures (1·2-fold). However, even with the crude extracts, iron-limited cells gave markedly more activity than carbon-limited ones.

Table 6. *Effect of streptomycin treatment on ATP-dependent DNA-breakdown activity of cell-free extracts of carbon-limited and iron-limited* Myco. smegmatis

Carbon-limited cultures were 96hr. and iron-limited cultures were 87hr. old. Crude extracts prepared in the normal fashion were dialysed for 18hr. at 2° against 2×200vol. of 0·05M-tris–HCl buffer, pH8·0, containing 1mM-mercaptoethanol. After precipitation with 5% (w/v) streptomycin, the residues were suspended in the same buffer and both these suspended residues and the supernatants were dialysed as described above. The incubation mixtures were as described in the text. Soluble acid-labile deoxyribose was determined by the diphenylamine method and corrected for pyrimidine-bound material.

Culture	Preparation	Enzyme activity (mμmoles of soluble deoxyribonucleotide formed/30min./mg. of protein in original extract)
Carbon-limited	Dialysed crude extract	69·4
	Dialysed supernatant after streptomycin treatment	45·8
	Dialysed precipitate after streptomycin treatment	37·2
Iron-limited	Dialysed crude extract	155·4
	Dialysed supernatant after streptomycin treatment	177·8
	Dialysed precipitate after streptomycin treatment	70·2

DISCUSSION

The nucleoside triphosphate-dependent DNA-breakdown system from *Myco. smegmatis* described above proves to be similar to the enzymes from *Micro. lysodeikticus* and *B. laterosporus* referred to in the introduction. On present evidence it appears to be closer to the former enzyme in that it is not strongly stimulated by Mn^{2+} and in that at least part of the deoxyribonucleotide solubilized is recovered as mononucleotide. ATP is converted into a mixture of ADP and AMP, and deoxyribonucleoside mono-, di- and tri-phosphates are formed. This multiplicity of products is probably due to the crudeness of the preparation. However, it does raise the possibility that the products of the reaction are not monophosphates, which is our reason for avoiding the term 'deoxyribonuclease'. Recently it has been found that deoxyribonucleoside triphosphates are as effective as ribonucleoside triphosphates in supporting DNA breakdown with this system (F. G. Winder & M. Lavin, unpublished work).

The discovery of these three systems and of the somewhat similar phage-restriction enzyme from *E. coli*, also referred to in the introduction, indicates that systems capable of breaking down DNA in the presence of nucleoside triphosphates are probably widely distributed in bacteria. Their function is not obvious. First, the breakdown of extracellular DNA, perhaps for nutritional purposes, is a possibility, and would be in keeping with the increased activity when the cellular DNA concentration falls in iron deficiency. However, the use of a nucleoside triphosphate for extracellular action on DNA seems to present problems. Secondly, digestion of endogenous DNA for error correction, repair purposes, or phage control is a possibility, though the purpose of nucleoside triphosphate requirement is not obvious.

Thirdly, the possibility must be considered that the activity is due to an abnormal action of one of the known DNA-metabolizing enzymes under the assay conditions employed: DNA nucleotidyltransferase, either replicating (DNA polymerase, EC 2.7.7.7) or terminal (Gottesman & Canellakis, 1966; Kato, Gonçales, Houts & Bollum, 1967), DNA ligase (Weiss & Richardson, 1967; Olivera & Lehman, 1967; Zimmerman, Little, Oshinsky & Gellert, 1967), or polynucleotide kinase (Novogrodsky & Hurwitz, 1966). For example, we have mentioned the possibility that we are measuring the reversed action of a DNA nucleotidyltransferase that can use a nucleoside triphosphate in place of pyrophosphate, with a consequent shift of equilibrium towards depolymerization (Winder & Coughlan, 1967*c*). Recently, however, DNA polymerase from *Myco. smegmatis* has been obtained essentially free from the DNA-breakdown activity (F. G. Winder & S. A. McNulty, unpublished work).

In view of the uncertainty about the function of this enzyme, little comment can be made about the significance of its increased activity in iron-limited cells. This increase appears to be a consequence rather than a cause of the fall in DNA concentration in these cells. It has been found that DNA polymerase activity undergoes a similar increase under these conditions (F. G. Winder & S. A. McNulty, unpublished work).

We thank Miss Anne Murphy for technical assistance. The work was supported by grants from the Medical Research Council of Ireland and Shell International Petroleum Co. Ltd. M.P.C. received a maintenance grant from the Department of Education, Irish Republic.

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