tranase B) have been shown to hydrolyse *Leuconostoc mesenteroides* (Birmingham) dextran to complex mixtures of oligosaccharides.

2. Both dextranases produced glucose, isomaltose, isomaltotriose and 'branched' penta-, hexa-, hepta- and octa-saccharides. Dextranase B produced, in addition, a 'branched' tetrasaccharide. Unresolvable material of degree of polymerization greater than 8 was produced by both dextranases. The 'branched' oligosaccharides were shown to be members of an homologous series.

3. The tetrasaccharide produced by dextranase B was shown to be 3³- α -glucosylisomaltotriose.

4. The pentasaccharides produced by dextranases A and B were identical and shown to be a 3³-glucosylisomaltotetraose, alone or in admixture with 3⁴-glucosylisomaltotetraose. Likewise, the hexasaccharides were identical and shown to be 3³-glucosylisomaltopentaose, alone or in admixture with isomers in which the branching occurred further towards the non-reducing end of the isomaltopentaose unit.

5. The heptasaccharide and octasaccharide produced by dextranase A were shown to contain 3⁴-glucosylisomaltohexaose and 3⁴-glucosylisomaltoheptaose respectively. Both saccharides could have been in admixture with isomers in which the branching occurred further towards the non-reducing end of the isomalto-hexaose and -heptaose units respectively. The octasaccharide also contained a saccharide in which two glucose units are joined through (1 $\rightarrow$ 3)-linkages to isomaltohexaose.

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The Effects of Antimicrobial Agents on Deoxyribonucleic Acid Polymerase

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The synthesis of DNA is carried out by an enzyme, deoxyribonucleic acid polymerase (deoxyribonucleic acid nucleotidyltransferase; deoxynucleoside triphosphate-deoxyribonucleic acid deoxynucleotidyltransferase, EC 2.7.7.7), which re-

quires the presence of 4 mol.prop. of deoxyribonucleotide triphosphates and 1 mol.prop. of a DNA primer (Bessman, Lehman, Simms & Kornberg (1958). Kirk (1960) reported that the enzyme is inhibited by the antibacterial and antitumour agent, actinomycin D, at concentrations considerably in excess of those needed for marked effects on cell growth. This paper describes work in which

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this observation has been confirmed and extended and deals with the effect of other antimicrobial agents which have been tested on the system.

EXPERIMENTAL

Abbreviations. Deoxyadenosine 5'-monophosphate, deAMP; deoxycytidine 5'-monophosphate, deCMP; deoxyguanosine 5'-monophosphate, deGMP; thymidine 5'-monophosphate, TMP. The corresponding 5'-triphosphates are abbreviated as deATP, deCTP, deGTP and TTP respectively.

Materials. deAMP, deCMP, deGMP and TMP were obtained from Schwartz BioResearch Inc., Mount Vernon, N.Y., U.S.A. deATP, deCTP, deGTP and TTP were prepared by chemical phosphorylation of the mononucleotides by the method of Smith & Khorana (1958). The isolated tetralithium salts were analysed spectrophotometrically for nucleotide-base content and for total phosphorus by the method of Fiske & Subbarow (1925). The preparations gave the following base: total phosphorus ratios: deATP, 1:2.87; deCTP, 1:3.02; deGTP, 1:2.98; TTP, 1:3.08. The percentages of total phosphorus liberated by hydrolysis in *N*-HCl for 10 min. at 100° from deCTP and TTP were 63.2 and 59.6 respectively.

[³H]deCMP was obtained from Schwartz BioResearch Inc. and converted enzymically into [³H]deCTP by the method of Lehman, Bessman, Simms & Kornberg (1958), except that phosphoenolpyruvate and the appropriate kinase were used as the ATP-generating system. The final product was obtained in 29% yield and contained approx. 10⁶ counts/min./μmole. A sample of the preparation was chromatographed in isobutyric acid-ammonia solvent (Krebs & Hems, 1953) with unlabelled deCTP and deCMP as carriers, and the radioactivity of these spots and of an intermediate zone, presumed to be deCDP, was determined. Of the total radioactivity recovered in the preparation 91% was present as deCTP, 8% as deCDP and less than 1% as deCMP.

Phosphoenolpyruvate was a crystalline cyclohexylamine salt kindly given by Dr V. M. Clark; the kinase was a crystalline preparation (Boehringer und Soehne G.m.b.H., Mannheim, Germany). Pancreatic deoxyribonuclease was a twice-crystallized product (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). Calf-thymus DNA was a 'highly polymerized' sodium salt (Sigma Chemical Co., St Louis, Mo., U.S.A.). Except where otherwise stated, the primer DNA used was an aqueous solution (0.5 mg./ml.) which had been heated at 100° for 10 min. and rapidly cooled in ice-water.

Actinomycin D was a gift from Professor A. W. Johnson. Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridium bromide) was a gift of Boots Pure Drug Co. Ltd., Nottingham. Other antibiotics were obtained from commercial sources. Usually these substances were dissolved in water at a concentration of 1.0 mg./ml.

Glycine buffer was a glycine-KOH mixture.

Enzyme preparation. This was an *Escherichia coli* preparation at the 67-fold purified stage (Lehman *et al.* 1958); it had been stored for several months at -10° and was kindly given by Mrs J. Kirk. The solution contained 4.0 mg. of protein/ml., as determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

Assay of deoxyribonucleic acid polymerase. The procedure used was, in principle, that of Lehman *et al.* (1958), involving incorporation of labelled nucleotide into an acid-insoluble form. The labelled nucleotide was [³H]deCTP and a modified method for measuring the incorporation was used. The standard incubation system, essentially that of Lehman *et al.* (1958), contained the following mixture: 0.02 ml. of 1.0 M-glycine buffer, pH 9.2; 0.03 ml. of 0.01 M-mercaptoethanol; 0.02 ml. of 0.1 M-MgCl₂; 0.02 ml. of 0.5 mM-deGTP; 0.01 ml. of 0.5 mM-deATP; 0.01 ml. of 0.5 mM-TTP; 0.05 ml. of heated DNA solution (0.5 mg./ml.); 0.01 ml. of 0.48 mM-[³H]deCTP (containing approx. 5.3 × 10⁶ counts/min.); 0.03 ml. of enzyme solution. The final volume was 0.3 ml. After incubation at 37°, normally for 30 min., 0.025 ml. of carrier DNA solution (0.5 mg./ml.) was added to each tube and the reactions were stopped with 0.5 ml. of cold *N*-HClO₄. After being kept at 0° for about 5 min. the contents of each tube were filtered through a 2.0 cm. diam. Oxid membrane filter. The tube was repeatedly washed with small amounts of cold 0.5 *N*-HClO₄, the washings being transferred to the filter. The filter was then rinsed with further amounts of 0.5 *N*-HClO₄, the total volume of the washings being 20 ml. The filter was finally washed with 5.0 ml. of cold 1% (v/v) acetic acid to remove HClO₄ and blotted on filter paper. After drying in air (until it began to curl slightly but was still not brittle) the filter was placed cylinderwise in small counting vials (1.0 cm. × 5 cm.) and dried at 70° for 2 hr. The filters were then covered with 2.5 ml. of scintillation mixture (in toluene) and the radioactivity was determined in a Packard liquid-scintillation counter.

RESULTS

Application of the modified procedure to the assay of deoxyribonucleic acid polymerase

As a check on the validity of the membrane-filtration procedure for determining deoxyribonucleic acid-polymerase activity and to establish that the experimental conditions were suitable for testing the effect of potential inhibitors, the general characteristics of the system were examined with this method. Table 1 shows that incorporation of [³H]deCMP from [³H]deCTP into DNA was almost linear with time up to 30 min. The replicate determinations show that the procedure gives values which usually agree to within ± 6%, although a single value at the shortest time-interval was 14% lower than the mean. In subsequent work duplicate determinations always gave values within the accuracy of ± 6%. The zero-time blank values were invariably small (about 25 counts/min.; approx. 0.5% of the total). Table 2 shows that with the modified assay procedure the system showed the same general characteristics reported by Bessman *et al.* (1958), although dependence on TTP was not complete, presumably owing to contamination of the enzyme with thymidine nucleotide(s). Fig. 1 shows that the assay method gives a satisfactory response to enzyme concentration. The falling-off at high concentrations could be due to the effect

of adding excessive amounts of deoxyribonuclease which is present as a contaminant in the enzyme preparation (Lehman *et al.* 1958), or to quenching effects during determinations of radioactivity. In all subsequent work 0.03 ml. of the enzyme was used in the assay system.

The method gives a convenient and rapid procedure for assaying deoxyribonucleic acid-polymerase activity which is particularly useful since tritiated compounds are used as substrate. Difficulties could, however, be encountered if the system employed were very crude and contained sufficient protein or other material to block the membrane filter or to cause excessive quenching during counting. Bollum (1959) has described an alternative filter-paper-disk method for measuring the incorporation of [^3H]thymidine into DNA. This, however, was not convenient for the usual deoxyribonucleic acid-polymerase assay because of the relatively large volume of solution that has to be applied to the filter paper.

Table 1. *Time-course of incorporation of [^3H]-deoxycytidine 5'-monophosphate from [^3H]deoxycytidine 5'-triphosphate into deoxyribonucleic acid*

Experimental details are given in the text. The values for the radioactivity incorporated into DNA at 10, 20 and 30 min. have been corrected for the zero-time blank of 19 counts/min.

Time of incubation (min.)	Radioactivity in DNA (counts/min.)	Error (% of mean)
0	20 } 19	—
10	150 } 142	{ +5.6 +4.2 -14
20	298 } 283	{ +5.0 -2.4 -2.4
30	398 } 374	{ +3.7 -1.0 -5.4

Table 2. *Characteristics of deoxyribonucleic acid polymerase determined by the modified assay procedure*

Experimental details are given in the text. All values for the radioactivity incorporated into DNA have been corrected for a zero-time blank of 25 counts/min.

Expt.	Radioactivity in DNA (counts/min.)
1. Complete system	420
2. No deGTP	38
3. No TTP	161
4. No deATP	4
5. No DNA	0
6. No Mg^{2+} ions	27

Inhibition of deoxyribonucleic acid polymerase by actinomycin D

Fig. 2 shows the effect of actinomycin D on DNA synthesis. In agreement with the findings of Kirk (1960), the drug has very little effect at the concentrations needed (less than 1.0 $\mu\text{g./ml.}$) for growth inhibition of *E. coli* or for marked inhibition of

Fig. 1. Effect of enzyme concentration on the assay of deoxyribonucleic acid polymerase. Experimental details are given in the text.

Fig. 2. Inhibition of deoxyribonucleic acid-polymerase activity by actinomycin D. ●, Unheated DNA used as primer; ○, heated DNA used as primer. Experimental details are given in the text.

RNA synthesis *in vivo* (Kirk, 1960). At higher concentrations, however, a pronounced inhibition of deoxyribonucleic acid polymerase is obtained. Fig. 2 also shows that the degree of inhibition is independent of whether heated or unheated DNA is used as primer.

Inhibition by ethidium bromide

Ethidium bromide is also a powerful inhibitor of deoxyribonucleic acid polymerase (Fig. 3). The amount required for 80% inhibition (approx. 15 $\mu\text{g./ml.}$) is of the same order as that used by Newton (1957) to obtain inhibition of DNA synthesis by cultures of the protozoan *Strigomonas oncopelti*. When ethidium bromide is added to DNA a marked change of colour occurs; the shift of the absorption spectrum is shown in Fig. 4. Actinomycin also shows a spectral shift when mixed with DNA (Kawamata & Imanishi, 1961).

Test to see whether actinomycin D and ethidium bromide stimulate breakdown of deoxyribonucleic acid

The possibility existed that the apparent inhibition of deoxyribonucleic acid-polymerase activity by actinomycin D or ethidium bromide is due to an indirect effect such as the destruction of DNA primer by causing a stimulation of deoxyribonuclease activity in the enzyme preparation, or to a destruction of the newly synthesized labelled DNA for the same reason. Mitomycin C has been reported to cause a release of acid-soluble fragments from DNA in *E. coli* (Reich, Shatkin & Tatum, 1960) as well as inhibition of DNA synthesis in susceptible organisms (Shiba, Terawaki, Taguchi & Kawamata, 1959). To check the first of these possibilities, namely that the inhibitors cause a very rapid destruction of DNA primer, a number of assay systems were set up, complete except for the omission of [^3H]deCTP, and incubated for 15 min. both with and without inhibitors. The primer DNA was re-isolated by acid precipitation, washed free from inhibitors and retested as primer in standard assay systems. Table 3 shows that fully active primer can be recovered from incubation systems containing either actinomycin D or ethidium bromide. For convenience, the preincubations were carried out with 75 $\mu\text{g.}$ of primer DNA present; under these conditions (Expts. 5 and 6, Table 3) the amounts of the two inhibitors present in the preincubation systems were sufficient to cause marked inhibition of deoxyribonucleic acid-polymerase activity. As a further check against the possibility that the drugs might be stimulating breakdown of the newly synthesized labelled DNA, standard assay systems were 'stopped' at 15 min. with excess of

unlabelled deCTP, the inhibitors added, and the incubations continued for a further 15 min. Although a considerable destruction of labelled DNA took place after the addition of unlabelled deCTP (Table 4), neither actinomycin D nor ethidium bromide stimulated this; in fact, the

Fig. 3. Inhibition of deoxyribonucleic acid-polymerase activity by ethidium bromide. Experimental details are given in the text.

Fig. 4. Effect of deoxyribonucleic acid on the absorption spectrum of ethidium bromide. Upper curve, ethidium bromide (50 $\mu\text{g./ml.}$) in 0.66 M-glycine buffer. Lower curve, as above, plus heated DNA (125 $\mu\text{g./ml.}$).

drugs appeared to prevent the loss of radioactivity from DNA. No attempt has been made to investigate this latter point further.

Inhibition by antrycide

Antrycide, 4-amino-6-(2-amino-6-methyl-4-pyrimidinylamino)quinoline 1,1'-dimethosulphate, a trypanocidal drug which Newton (1962) has shown

to interfere with the normal ribosome pattern of *S. oncopelti*, inhibited deoxyribonucleic acid polymerase. This, however, occurred only at concentrations which caused an obvious precipitation of the DNA primer. The inhibition by this drug in this system is presumably therefore due to simple physical removal of primer.

Tests with other antimicrobial agents

The following compounds had either no or insignificant effects on deoxyribonucleic acid-polymerase activity when tested at a concentration of 20 µg./0.3 ml. of incubation mixture: chloramphenicol, erythromycin, aureomycin, neomycin, Terramycin, puromycin, actidione and streptomycin. Streptogramin had no effect at a concentration of 2 µg./0.3 ml.; higher concentrations were not tried because of the insolubility of this antibiotic in water.

DISCUSSION

The concentration of actinomycin D required to inhibit deoxyribonucleic acid polymerase makes it unlikely that this is the primary site of attack by the drug inside living cells. This conclusion is in agreement with the evidence available *in vivo*. Thus Kirk (1960) showed that, in *E. coli*, RNA synthesis is stopped immediately the cells are treated with the drug, whereas inhibition of DNA synthesis occurs only at a much later stage and to a lesser extent. Reich, Franklin, Shatkin & Tatum (1961) have likewise shown that the antibiotic prevents RNA synthesis in cultured mammalian cells, and Harbers & Müller (1962) have arrived at the same conclusion from studies with ascites-tumour cells. These latter authors also provided evidence that actinomycin combines with nuclear DNA within the living cell and concluded that the drug probably inhibits the DNA-dependent synthesis of RNA. At higher concentrations of actinomycin a significant inhibition of DNA synthesis was also observed in these experiments.

With ethidium bromide, it seems possible that the inhibition *in vitro* of deoxyribonucleic acid polymerase reported here is related to the effect of the drug *in vivo*. Newton (1957) showed that, in *S. oncopelti*, DNA synthesis is stopped immediately on the addition of ethidium bromide, whereas RNA and protein synthesis continue for a considerable time. The concentration of drug used by Newton (approx. 20 µg./ml.) is comparable with the concentration required for the inhibition of deoxyribonucleic acid polymerase. However, some caution must be exercised in this interpretation, since other cellular components may take up the drug and, indeed, Newton (1962) has found that ethidium bromide combines with ribosomes. Moreover, in the present work, *E. coli* deoxyribo-

Table 3. *Experiments to test whether actinomycin D and ethidium bromide stimulate destruction of deoxyribonucleic acid primer by deoxyribonuclease in the enzyme preparation*

Reaction mixtures (full details of which are given in the text), lacking [³H]deCTP and containing 75 µg. of primer DNA together with inhibitors as shown, were incubated for 15 min. at 37°. The DNA of each tube was then precipitated by the addition of 0.5 ml. of cold 1N-HClO₄ and, after 5 min. at 0°, centrifuged. The precipitates were washed with 0.5 ml. of cold 0.1N-HClO₄ and recentrifuged. The supernatants were poured off, the tubes thoroughly drained, and the precipitates dissolved in 0.1 ml. of water by the addition of 0.01 ml. of M-glycine buffer, pH 9.2. Samples (0.05 ml.) of these solutions were added as primer DNA for standard assay systems. No correction has been made for a zero-time blank.

Expt.	Radioactivity in DNA (counts/min.)
1. DNA preincubated without inhibitor	457
2. DNA preincubated with 10 µg. of ethidium bromide	464
3. DNA preincubated with 20 µg. of actinomycin D	445
4. No preincubation; 75 µg. of DNA primer added	440
5. No preincubation; 75 µg. of DNA primer + 10 µg. of ethidium bromide added	130
6. No preincubation; 75 µg. of DNA primer + 20 µg. actinomycin D added	161
7. No DNA primer added	11

Table 4. *Experiment to test whether actinomycin D or ethidium bromide stimulates breakdown of labelled deoxyribonucleic acid*

Standard assay systems (full details of which are given in the text) were preincubated for 15 min. and the reactions 'stopped' by the addition of a 50-fold excess of unlabelled deCTP; actinomycin D or ethidium bromide was then added as shown and the incubations were continued for a further 15 min. Then HClO₄ was added and the radioactivity determined in the normal way.

Additions after unlabelled deCTP	Radioactivity in DNA (counts/min.)
1. None	84
2. 10 µg. of ethidium bromide	120
3. 20 µg. of actinomycin D	108
4. Control: HClO ₄ added at 15 min.	149
5. Control: 10 µg. of ethidium bromide added at zero time, HClO ₄ added at 15 min.	6

nucleic acid polymerase was used and much less is known about the effects of ethidium bromide on bacteria; Richmond (1959) found that the synthesis of a lytic enzyme by *Bacillus subtilis* was inhibited 50% by this drug at a concentration of 350 µg./ml. B. A. Newton (unpublished work) has observed that the growth of *E. coli* is inhibited by the drug at a concentration of 35–75 µg./ml.

The shift in the absorption spectrum of ethidium bromide when mixed with DNA observed in the present work shows that this compound is capable of forming a complex with DNA, but there is no evidence that the action of the drug is related to this capability. However, the biological action of actinomycin is believed to be related to its ability to form a complex with DNA (Rauen, Kersten & Kersten, 1960; Kawamata & Imanishi, 1961).

SUMMARY

1. The action of a number of antibiotics and other drugs on deoxyribonucleic acid polymerase of *Escherichia coli* has been studied by a modified assay procedure.

2. Actinomycin D inhibits at relatively high concentration, whereas the phenanthridine drug, ethidium bromide, is a powerful inhibitor at growth-limiting concentrations. The absorption spectrum of ethidium bromide shows a marked shift on mixing with DNA, showing complex-formation between the two, although it is not known whether the inhibitory effect of the drug is related to this capability. Neither ethidium bromide nor actinomycin D causes irreversible destruction of DNA primer.

3. The trypanocidal drug, antrycide, inhibits DNA synthesis but this is probably due to simple physical removal of DNA primer by precipitation.

A range of other antibiotics tested had no significant effect on the enzyme system.

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Relation of Structure to the Inhibitory Activity of Purines against Urate Oxidase

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In a previous paper (Bergmann, Ungar-Waron & Kwietny-Govrin, 1963) on the interaction of thiouric acids with mammalian urate oxidase it was reported that the position of the sulphur atom has a decisive influence. The sequence of decreasing affinity is as follows: 6,8-dithiouric acid, 8-thiouric

acid, 6-thiouric acid, 2-dithiouric acid. These results made it desirable to determine separately the binding power of the thiocarbonyl group in each of the three available positions in the purine molecule in order to evaluate their contribution to the formation of the enzyme-inhibitor complex. Such