The Inhibition of Ribonucleic Acid Polymerase by Acridines

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(Received 19 October 1965)

1. The aminoacridines, proflavine (3,6-diaminoacridine) and 9-aminoacridine, and a hydrogenated derivative, 9-amino-1,2,3,4-tetrahydroacridine, were shown to inhibit *in vitro* the DNA-primed RNA polymerase of *Escherichia coli*. The inhibition is strong with both proflavine and 9-aminoacridine, but weak with 9-amino-1,2,3,4-tetrahydroacridine. 2. The extent to which the three acridines bind to calf-thymus DNA in the enzyme medium was studied spectrophotometrically. The extent of binding decreases in the order: proflavine, 9-aminoacridine, 9-amino-1,2,3,4-tetrahydroacridine. Some evidence was also obtained for interaction between the nucleoside triphosphate substrates and proflavine or 9-aminoacridine; no such interaction was detectable with 9-amino-1,2,3,4-tetrahydroacridine. 3. Although the amount of acridine bound to DNA increases with increasing inhibition, a stage is reached where an increase in acridine concentration still causes an increase in inhibition, with practically no increase in the amount bound to DNA. 4. Plots of reciprocal rates against the reciprocal of DNA concentration were linear and had a common intercept when proflavine or 9-aminoacridine was present. Similar relations were obtained when the reciprocal concentration of nucleoside triphosphates was plotted. The observations are interpreted kinetically in terms of a competitive inhibition of the enzyme by proflavine or 9-aminoacridine and of a kinetic role for the DNA analogous to 'activation'. 5. This suggests that inhibitory acridine molecules can occupy the sites on the RNA polymerase that are specific for binding the nucleoside triphosphate substrate or the bases of the DNA, when these become accessible during the copying process.

Inhibition of the DNA-primed RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) has been observed both *in vitro* and *in vivo* with several compounds (Goldberg & Rabinowitz, 1962; Waring, 1964a; Hurwitz, Furth, Malamy & Alexander, 1962). Inhibition by actinomycin D has been shown to be the result of a specific combination with guanine residues in the DNA primer and the action appears to be the same *in vitro* and *in vivo* (Goldberg & Rabinowitz, 1962; Hamilton, Fuller & Reich, 1963). Other inhibitors, e.g. ethidium bromide (Waring, 1964b) and atebirin (Hochster & Chang, 1963), appear to be less specific, having no particular affinity for a base in the DNA. Their degree of inhibition, however, correlates well with their strong binding to DNA. The effect of proflavine (3,6-diaminoacridine) on the activity *in vitro* of RNA polymerase from *Escherichia coli* B has been reported briefly (Waring, 1964b; Goldberg & Rabinowitz, 1962; Goldberg, Rabinowitz & Reich, 1962). Hurwitz *et al.* (1962) found that the inhibitory activity of proflavine is not restricted to the RNA polymerase of *E. coli* and that, for a given

proflavine concentration, the inhibition is greater with the DNA-primed DNA polymerase (EC 2.7.7.7). From these results it has been inferred that inhibition of RNA synthesis is caused by the attachment of proflavine to the primer DNA, which would be consistent with the established strong affinity of the aminoacridines for nucleic acids (Peacocke & Skerrett, 1956). Nevertheless, other enzymes, not involving DNA, are also known to be inhibited by acridines, e.g. urease and hexokinase (Albert, 1951, p. 306).

The following describes a study of the action on *E. coli* RNA polymerase of the aminoacridines, proflavine and 9-aminoacridine, and of a hydrogenated derivative of the latter, 9-amino-1,2,3,4-tetrahydroacridine; the relation of the inhibition to the extent of binding was particularly sought. The results, though still allowing that inhibition may be partly due to binding of acridines by DNA, indicate that other mechanisms are involved.

MATERIALS AND METHODS

Materials. *E. coli* B (500g. wet wt.) was supplied by the Microbiological Research Establishment, Porton, Wilts. Cells were grown in continuous culture in a 20l. culture

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vessel with a dilution rate of 0.75 hr.^{-1} , giving a mean generation time of 0.93 hr. at 37° . The culture medium contained: yeast extract, 10 g. ; glycerol, 10 ml. ; K_2HPO_4 , 22 g. ; KH_2PO_4 , 17 g. ; tap water to 1 l. , giving approx. $\text{pH } 6.8$. Cells (2.7 g. dry wt./l.) were harvested by centrifugation and washed at 5° with buffer ($10 \text{ mM-tris-HCl-10 mM-magnesium acetate, pH } 7.4$). The suspension was recentrifuged, and the resulting slurry was frozen and stored at -20° .

The DNA-dependent RNA polymerase was prepared by the method of Chamberlin & Berg (1962). Their procedure was followed through the streptomycin and protamine precipitations, and the $(\text{NH}_4)_2\text{SO}_4$ fractionations. The chromatography on DEAE-cellulose was not found to be necessary and the enzyme pellet from the final $(\text{NH}_4)_2\text{SO}_4$ precipitation was dissolved in 2.8 ml. of storage medium [$0.1 \text{ M-(NH}_4)_2\text{SO}_4$ - $50 \text{ mM-tris-HCl (pH } 8.1)$ - $0.1 \text{ mM-EDTA-5 mM-glutathione-1 mM-MgCl}_2$] and stored in 5 ml. glass vials in 0.4 ml. portions at -70° . The activity of the RNA polymerase was followed during the purification by using the assay given below [with the addition of sufficient exogenous DNA ($50 \mu\text{g.}$) to saturate the priming requirement of the enzyme]. For all assays, other than during isolation, the enzyme was diluted by the addition of $10 \text{ mM-tris-HCl (pH } 7.9)$ - 10 mM-MgCl_2 - $10 \text{ mM-}\beta$ -mercaptoethanol- 0.1 mM EDTA containing bovine serum albumin (1 mg./ml.).

DNA was prepared from fresh frozen calf thymus by the procedure of Kay, Simmons & Dounce (1952) and extracted twice by the chloroform-gel method (Gulland, Jordan & Threlfall, 1947). Unless otherwise stated, concentrations of DNA are based on E_{260} values and expressed in terms of the molarity of DNA deoxyribonucleotides. A standard solution of DNA (1 mg. of DNA/ 1 ml. has $E_{1\text{cm}, 20}$) in 0.9% $\text{NaCl-10 mM-sodium citrate}$ was prepared at 0° . ATP, CTP and GTP were obtained from L. Light and Co. Ltd., Colnbrook, Bucks., UTP (grade Sigma), yeast RNA (commercial grade) was from the Sigma Chemical Co., St Louis, Mo., U.S.A., and $[8\text{-}^{14}\text{C}]\text{ATP}$ (disodium salt) was from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. Membrane filters (type DA, porosity 0.65μ) were obtained from the Millipore Corp., Bedford, Mass., U.S.A., and similar $0.5\text{--}1.0 \mu$ porosity filters from Oxoid Division, Oxo Ltd., London, E.C.4.

Proflavine hemisulphate (British Drug Houses Ltd., Poole, Dorset) was converted into the free base with aq. NH_3 . An 8 g. portion was redissolved in 3200 ml. of boiling distilled water and filtered. The crystals formed on cooling were reprecipitated from 400 ml. of 95% (v/v) ethanol and dried at 110° . The m.p. (sealed) was 282° . Approx. 6 g. of 9-aminoacridine hydrochloride from British Drug Houses Ltd. was dissolved in 275 ml. of boiling water, and brought to $\text{pH } 6$ with KOH ; a slight precipitate was removed by filtration. The free base was reprecipitated by addition of KOH to $\text{pH } 11$, filtered off and washed successively with 0.1 N-KOH , water and 15 ml. of 95% (v/v) ethanol. After being dried at 110° , it was dissolved in 200 ml. of acetone. The solution was evaporated to 40 ml. and left to crystallize. The yellow crystals were dried at 110° (m.p. 243°). 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride from the Aldrich Chemical Co., Milwaukee, Wis., U.S.A., had m.p. 285° and was used as supplied. Solutions of acridines were prepared by dissolving weighed quantities in water with the addition of HCl to maintain neutrality. Concentrations were checked either spectro-

photometrically or volumetrically by the method of Bolliger (1940). The solutions were prepared as soon as possible before use and kept in the dark.

Pancreatic ribonuclease was obtained from the Sigma Chemical Co. The T_1 ribonuclease was prepared from Taka-diestase powder by the method of Rushizky & Sober (1962). Specific activity was determined by incubating 0.8 ml. of a solution of 7.5 mg. of yeast RNA/ ml. in 125 mM-tris-HCl buffer, $\text{pH } 7.3$, with 0.2 ml. of enzyme solution for 1 hr. at 37° . Then 1 ml. of ice-cold 5% (w/v) trichloroacetic acid- 0.5% (w/v) $\text{La}_2(\text{SO}_4)_3$ was added to the chilled reaction mixture, and the extinction was measured of a sample of the supernatant made up to 5 ml. with 10 mM-HCl . The presence of Mg^{2+} or Mn^{2+} had no discernible effect on ribonuclease activity at the concentrations employed in the assays of RNA polymerase.

Bovine serum albumin (fraction V; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) was used for enzyme dilutions and as a standard for protein estimations, which were made by the method of Lowry, Rosebrough, Farr & Randall (1951).

Procedure for the assay of RNA-polymerase activity. RNA-polymerase activity was determined by measuring the amount of radioactive precursor rendered acid-insoluble. The incubation mixture contained, in 0.25 ml. : tris-HCl buffer, $\text{pH } 8.0$, $10 \mu\text{moles}$; MgCl_2 , $1 \mu\text{mole}$; MnCl_2 , $0.25 \mu\text{mole}$; CTP, UTP and GTP, each $0.1 \mu\text{mole}$; $[8\text{-}^{14}\text{C}]\text{ATP}$, $0.1 \mu\text{mole}$ and 50000 counts/min.; β -mercaptoethanol, $3.0 \mu\text{moles}$; DNA, $0\text{--}50 \mu\text{g.}$; enzyme, 11 units (1 unit is the amount of enzyme that catalyses the incorporation of $1 \mu\text{mole}$ of AMP/ 10 min. in the presence of $30 \mu\text{g.}$ of DNA under the conditions of assay). When required, the acridine was added to the combined salt and nucleoside triphosphate solutions before the DNA and enzyme. The acridine solutions were prepared at various concentrations which, when added as a 0.05 ml. portion, gave the desired final concentration in 0.25 ml. In control experiments, 0.05 ml. of distilled water replaced the acridine solution. The reaction mixtures were incubated for 10 min. in 10 ml. conical centrifuge tubes held in a water bath at 37° .

After incubation the tubes were transferred to a boiling-water bath; the inclusion of this denaturation step, recommended by Waring (1964b), led to a more finely divided precipitate and better reproducibility of results. After 3 min. the tubes were placed in an ice-water bath for a further 5 min. Then 0.1 ml. of yeast RNA (2 mg./ml.) was added as a co-precipitant followed by 0.35 ml. of ice-cold 10% (w/v) trichloroacetic acid. After 15 min. at 0° the suspension was applied to 13 mm. -diam. membrane filters and filtered under vacuum. The filters were washed with five successive 3 ml. portions of 5% (w/v) trichloroacetic acid and finally with 2 ml. of 95% (v/v) ethanol. The filters were dried under an infrared lamp and mounted on 15 mm. planchets. Counting was effected in a windowless gas-flow counter and was continued until the error was within $\pm 3\%$. Incorporation in $\text{m}\mu\text{moles}$ was measured by dividing the counts so obtained by the specific activity of the precursor. All samples were assumed to be infinitely thin.

Acridine binding by DNA. Investigation of the binding of acridines to DNA by the spectrophotometric method was possible only over the concentration range where Beer's law was obeyed. Conditions were chosen that simulated the conditions in the enzyme assay as nearly as possible. Bovine

serum albumin of the same concentration (w/v) replaced enzyme in the incubation mixture. A solution of acridine in 2.5 ml. of this modified incubation medium was equilibrated at 37°. Small volumes of DNA solution were added and the decreases in extinction measured in a Unicam SP.500 spectrophotometer with a Gilford attachment. Measurements were taken at appropriate wavelengths where the maximum change in absorption occurred on the addition of DNA to the acridine solution. These wavelengths were: for proflavine, 440 m μ ; for 9-aminoacridine, 400 m μ ; for 9-amino-1,2,3,4-tetrahydroacridine, 320 m μ . From the decrease in extinction at these wavelengths when DNA was added, it was possible to calculate the no. (r) of acridine molecules bound/nucleotide (Peacocke & Skerrett, 1956).

Acridine binding by nucleoside triphosphates. The possibility of complex-formation between acridines and nucleoside triphosphates was investigated by using the spectrophotometric method. The spectra of the acridine and of the nucleoside triphosphate were compared with that of the mixture of acridine and nucleoside triphosphate over the wavelength range 220–750 m μ in a Perkin-Elmer continuous-recording spectrophotometer (model 137 UV).

The spectrum of proflavine (36 μ M) in 40 mM-tris-HCl buffer, pH 8.0, was determined; 0.05 ml. of 10 mM-ATP was added to 5 ml. of the same proflavine solution, and the spectrum determined again. A 0.05 ml. sample of 10 mM-ATP was also diluted with 5 ml. of buffer and the spectrum was recorded on the same chart. The same procedure was followed with excess of the other three nucleoside triphosphates, at a final concentration of 0.1 mM, and with the other two acridine derivatives, at final concentrations of 36 μ M for 9-amino-1,2,3,4-tetrahydroacridine and 69 μ M for 9-aminoacridine. All spectra were determined at room temperature.

RESULTS

Inhibition studies. The effect of different concentrations of DNA primer on the activity of the enzyme is shown in Fig. 1. From these results, the optimum concentration was taken to be 30 μ g. of DNA/0.25 ml. of assay mixture. At the enzyme concentration normally employed (11 units), incorporation up to 6000 counts/min. proceeded linearly during the 10 min. incubation, in the absence of acridines. Hence the incorporation after 10 min. was proportional, within the experimental limits, to the initial rate of incorporation and so to the initial velocity of RNA formation. The initial rate of incorporation, with 30 μ g. of DNA, was determined in this way with different enzyme concentrations between 0.1 and 16 units and was shown to be proportional to the enzyme concentration. To obtain the main features of the kinetics, the highly probable assumption was made that the decreased incorporation in the presence of acridine was likewise linear for the first 10 min. under the same experimental conditions.

In the absence of DNA primer, incorporation was less than 120 counts/min. above the background of 18 counts/min., which shows that the presence of DNA increases the rate 50–100-fold. Table 1

Fig. 1. Incorporation of AMP into acid-precipitable material by 11 units of *E. coli* RNA polymerase in the presence of different amounts of calf-thymus DNA. The conditions of incubation are given under 'Procedure for the assay of RNA-polymerase activity' in the Materials and Methods section.

Table 1. Incorporation of [8-¹⁴C]AMP into acid-precipitable material by the action of *E. coli* RNA polymerase

The conditions of incubation were as described under 'Procedure for the assay of RNA-polymerase activity' in the Materials and Methods section, with the further variations indicated (incubation time was 10 min.). In general, 11 enzyme units were primed by 30 μ g. of calf-thymus DNA.

Incubation temp.	Variations to conditions	Incorporation (counts/min.)
37°	None	5400
37	100° for 5 min., 30 min. at 37° with pancreatic ribonuclease	660
37	100° for 5 min., 30 min. at 37° with T ₁ ribonuclease	1320
37	100° for 5 min., 30 min. at 37° with both pancreatic and T ₁ ribonucleases	420
0	With proflavine (250 μ M)	240
37	No DNA primer	120

presents some of the results of experiments undertaken to test whether the system had the expected characteristics. In the experiments with ribonuclease, the amount of enzyme added was that calculated, on the basis of specific activity, to be sufficient to hydrolyse the RNA formed by the polymerase. It had previously been shown, by

Fig. 2. Extent of the inhibition by proflavine (O), 9-aminoacridine (□) and 9-amino-1,2,3,4-tetrahydroacridine (●) of the activity of 11 units of *E. coli* RNA polymerase primed by 30 μ g. of DNA. The conditions of incubation are given under 'Procedure for the assay of RNA-polymerase activity' in the Materials and Methods section.

reference experiments with polyadenylic acid as substrate for the ribonuclease, that, under such conditions, if all the [8- 14 C]AMP were incorporated as polyadenylic acid, the amount split by the ribonuclease would be less than 0.1% (i.e. less than 6 counts/min.).

The effect of the addition of various amounts of acridines to enzyme mixtures, containing a constant amount of DNA, is shown in Fig. 2. On a molar basis, proflavine inhibited more than did 9-aminoacridine, whereas 9-amino-1,2,3,4-tetrahydroacridine inhibited very little. The 25% decrease in incorporation observed at a concentration of 1.0 mM-9-amino-1,2,3,4-tetrahydroacridine was observed with 9-aminoacridine at 35 μ M.

Binding studies. The extent of binding of the acridines with DNA in the assay medium is in agreement with previous work (Fig. 3): the binding to DNA was shown to decrease with increase in salt concentrations (Peacocke & Skerrett, 1956; Drummond, Simpson-Gildemeister & Peacocke, 1965), and in the plot of r against the concentration (c) of free unbound acridine there was a plateau region at r 0.14, corresponding to the completion of strong binding on calf-thymus DNA in 0.1 M-sodium chloride (pH 6.9, 25°). In 60 mM-sodium chloride the plateau occurred at r 0.16. The type of ion present also had an effect on the binding: the addition of Mg^{2+} (5 mM-magnesium chloride) decreased binding by approx. 20% more than would be expected from considerations of ionic strength alone (Peacocke & Skerrett, 1956). Thus the depression of the binding (Fig. 3) to r 0.135 for proflavine in the

Fig. 3. Dependence of the no. (r) of acridine molecules bound/DNA nucleotide on the free acridine concentration (c): O, proflavine; □, 9-aminoacridine; ●, 9-amino-1,2,3,4-tetrahydroacridine. Values were determined at 37° in the modified incubation medium by the spectrophotometric method described under 'Acridine binding by DNA' in the Materials and Methods section.

complex assay medium (10.05, 4 mM-magnesium chloride) was in accord with observations with simpler solvents. This resemblance at the lower r values suggested that the binding curve for this enzyme medium obtained by the spectrophotometric method might be extended to higher r values (greater than 0.2) by using previous results obtained by equilibrium-dialysis measurements in this range of r (Fig. 9 in Peacocke & Skerrett, 1956). These results consist of a complete binding curve for herring-sperm DNA (10.5 mg. atoms of phosphorus/l., 0.1 M-sodium chloride, pH 6.9, 20°), which had a plateau region at r 0.22. The binding curve determined here for the enzyme assay medium was extended by multiplying these r values found by equilibrium dialysis by the ratio of r at the respective plateau regions (0.135/0.22).

Since the binding curve (r against c) does not vary significantly with changes in DNA concentration (T_A , in g. atoms of nucleotide phosphorus/l.) (Peacocke & Skerrett, 1956), it was possible to find the total acridine concentration (T_L) corresponding to a particular r value at a given DNA concentration by using the relation:

$$T_L = T_A \cdot r + c$$

By substituting corresponding pairs of r and c values from Fig. 3, and with T_A equal to that in the enzyme assay, a series of T_L values was obtained and plotted against r . Values of r could then be read from this graph for the particular total proflavine concentrations (T_L) used in the inhibition

Fig. 4. *E. coli* RNA-polymerase activity (control 100%) of 11 units of enzyme primed by 30 μ g. of DNA in the presence of proflavine, plotted against: $\square$, fraction bound (r); $\circ$, total concentration (T_L); $\bullet$, free concentration (c) of proflavine. The conditions of incubation are given under 'Procedure for the assay of RNA-polymerase activity' in the Materials and Methods section. Points below the horizontal double lines were obtained by calculation from previous data (see the text).

Fig. 5. *E. coli* RNA-polymerase activity (control 100%) of 11 units of enzyme primed by 30 μ g. of DNA in the presence of 9-aminoacridine, plotted against: $\square$, fraction bound (r); $\circ$, total concentration (T_L); $\bullet$, free concentration (c) of 9-aminoacridine. The conditions of incubation are given under 'Procedure for the assay of RNA-polymerase activity' in the Materials and Methods section.

experiments. The corresponding free proflavine concentration (c) could also be calculated by substitution in the above equation.

Relation of inhibition to the binding of the acridines on DNA. Fig. 4 shows the decrease in activity, expressed as a percentage of the control (no acridine), at different total proflavine concentrations (T_L) when 30 μ g. of DNA is used as primer in 0.25 ml., i.e. T_A is 369 μ M. After the activity has dropped to 36% of the control, no further inhibition was observed, even at the highest proflavine concentrations employed.

Fig. 4 also shows the variation of activity with r and with the concentration (c) of free proflavine. The curves above the horizontal double lines in Fig. 4 are based on the experimental observations and r values determined for the enzyme assay medium; the curves below the double lines are derived from r values on the binding curve extended by the procedure described above. The results obtained in similar experiments with only 5 μ g. of DNA as primer gave similar curves to those of Fig. 4, with inhibition reaching 80%. The corresponding results with 9-aminoacridine and 30 μ g. of DNA as primer are shown in Fig. 5. No equilibrium-dialysis measurements were available with 9-aminoacridine, so that no r or c values are known accurately above the plateau region, where r is 0.087 (Fig. 3). This limit is also indicated by the horizontal double lines in Fig. 5.

The binding curve of 9-amino-1,2,3,4-tetrahydroacridine was limited by the range available to the spectrophotometric method, i.e. up to the plateau

region where r is 0.033, T_A is 369 μ M and T_L is 37.2 μ M. The enzyme reaction mixture, which contained the same concentration (T_A) of DNA, showed no significant inhibition until T_L was 150 μ M (Fig. 2) and, at T_L as high as 1.0 mM, 74% of the control activity still remained.

Kinetics of inhibition. In a further series of experiments the inhibition by the same concentration of dye with various amounts of DNA primer was investigated. The chosen concentrations of acridine caused approx. 50% inhibition with 30 μ g. of DNA primer (150 μ M-9-aminoacridine and 48 μ M-proflavine). 9-Amino-1,2,3,4-tetrahydroacridine was not tested because of its lack of inhibitory activity at low concentrations and because of its limited solubility. The results from these experiments were expressed in the form of a reciprocal plot, (incorporation of $[8\text{-}^{14}\text{C}]\text{ATP}$) $^{-1}$ versus (DNA concentration) $^{-1}$ (Fig. 6). These resemble the usual Lineweaver & Burk plots (1934), although DNA in this system is not a true substrate.

The results of similar experiments in which the concentration of nucleoside triphosphates was alone varied are shown in Fig. 7; constant volumes of different dilutions of a combined stock solution of all four nucleoside triphosphates were added to each assay. At very low concentrations of the nucleoside triphosphates, above 0.5 on the abscissa, there was a tendency for the line to curve upwards, which may have been due to the large change in the ratio of Mg^{2+} to nucleoside triphosphates.

Fig. 6. Reciprocal plot of the activity (counts/100sec.) of 11 units of *E. coli* RNA polymerase and of the concentration of DNA primer in the presence of acridines: ●, control; ○ 48.1 μM -proflavine; □, 150 μM -9-aminoacridine. The conditions of incubation are given under 'Procedure for the assay of RNA-polymerase activity' in the Materials and Methods section. Each point represents the mean of three values.

Fig. 7. Reciprocal plot of the activity (counts/100sec.) of 3.5 units of *E. coli* RNA polymerase primed by 5 $\mu\text{g.}$ of DNA and of the concentration of the nucleoside triphosphates in the presence of acridines: ●, control; ○ 50 μM -proflavine; □, 50 μM -9-aminoacridine. The conditions of incubation are given under 'Procedure for the assay of RNA-polymerase activity' in the Materials and Methods section. Each point represents the mean of three values.

DISCUSSION

Several enzymes are known to be inhibited by acridines. For example, the antimalarial drug atebirin [6-chloro-9-(4-diethylamino-1-methylbutyl-amino)-2-methoxyacridine] inhibits cholinesterase

to the extent of 50% at 10 μM , and lactate dehydrogenase is strongly inhibited at a concentration of 1.0 mM (Albert, 1951, p. 305). Many of the enzymes inhibited possess coenzymes that in excess prevented inhibition or even reversed it, if added after atebirin (e.g. D-amino reductase with FAD, cytochrome reductase with riboflavin, glucose 6-phosphate dehydrogenase with NADP). Atebrin inhibits RNA polymerase (Hochster & Chang, 1963) and Acridine Orange inhibits phosphorolysis of polyadenylic acid by polynucleotide phosphorylase (Beers, Hendley & Steiner, 1958). RNA polymerase in preparations from HeLa cells was inhibited by actinomycin (2 $\mu\text{g./ml.}$), proflavine (200 $\mu\text{g./ml.}$) and acriflavine (200 $\mu\text{g./ml.}$) to the extents of 51, 35 and 69% respectively, i.e. incorporation of nucleotides into RNA was decreased by this fraction (Goldberg & Rabinowitz, 1962). This inhibition could be completely reversed by adding denatured DNA and partly reversed by adding native DNA. It was also shown that low concentrations of proflavine (1.5 μM) depressed to half its original value the synthesis of RNA in this system, when primed by a copolymer of the deoxyribonucleotides of adenine and thymine (Goldberg *et al.* 1962). Hurwitz *et al.* (1962) compared the action of proflavine on RNA polymerase and DNA polymerase and showed that proflavine (30 μM) decreased the activity of RNA polymerase by 30% and that of DNA polymerase by 85%.

The inhibitory activities towards *E. coli* RNA polymerase of the three acridines used in the present study were in the order: proflavine $\approx$ 9-aminoacridine $\gg$ 9-amino-1,2,3,4-tetrahydroacridine, which is parallel with their antibacterial activity (Albert, 1951, pp. 251-263) and with their decreasing ability to bind to DNA (Fig. 3). This seems to implicate the binding of the acridine to the DNA as the cause of the inhibition, as appears to be the case with ethidium bromide (Waring, 1964a). However, when the fraction of the original enzyme activity is plotted (Fig. 4) against the no. (r) of proflavine molecules bound/DNA nucleotide in the enzyme system, the inhibition is seen to increase not only with r up to a value corresponding to the plateau region of the DNA-binding curve (Fig. 3) but also beyond this point. That is, inhibition increases with proflavine concentrations when r hardly changes at all. It is therefore possible to decrease the level of incorporation of nucleotides into RNA in this system by increasing the proflavine concentration beyond 80 μM , even though, over this range, very little of the extra proflavine is binding to the DNA. The same situation is observed with proflavine inhibition of the same amount of enzyme at lower concentrations (5 $\mu\text{g.}$) of DNA primer and with 9-aminoacridine (Fig. 5). It is clear that inhibition at the

higher acridine concentrations cannot be caused by binding to the DNA, although this undoubtedly occurs. Since the extension and partial unwinding of the DNA molecule caused by the binding of acridines also reaches a maximum at the plateau value of r (Drummond, Pritchard, Simpson-Gildemeister & Peacocke, 1966), this extension cannot be the cause of the inhibition of the RNA polymerase by the acridines, since it is constant in the range of acridine concentrations over which inhibition is still increasing. This behaviour is manifest even if the decrease in activity is plotted as a function of total (T_L) or of free (c) acridine concentrations (middle and lower curves in Figs. 4 and 5).

When the reciprocal of the rate of incorporation is plotted against the reciprocal of the total DNA concentration, at constant nucleotide concentration, a linear plot is obtained that has the same intercept, with an enhanced slope, if proflavine or 9-aminoacridine is present (Fig. 6). This is the type of relation characteristic of competitive-inhibition kinetics. When the reciprocal of the amount of 'uncomplexed DNA' was plotted as the abscissa against the reciprocal of the rate (by 'uncomplexed DNA' is meant total DNA minus DNA inactivated by acridine, on the basis of one nucleotide inactivated/acridine molecule bound) then straight lines were again obtained. These had almost the same intercept on the ordinate but a decreased slope. Presumably, therefore, within the limits of the experimental error of the kinetic experiments, the amount of 'uncomplexed DNA' is approximately proportional to the total DNA present, and so this latter quantity has been used in the kinetic analysis.

The corresponding plot of reciprocal rate against reciprocal nucleotide concentration, at constant DNA concentration, was also linear and again exhibited the form characteristic of competitive inhibition when proflavine or 9-aminoacridine was present (Fig. 7). These curves can be explained on the following basis. In the absence of inhibitor, both DNA and the nucleotide triphosphates are essential for the reaction, but only the latter is the strict substrate and reacts to give the product, RNA. The role of DNA is therefore kinetically analogous to that of an activator and the equilibria may be formulated as follows, where E is enzyme (RNA polymerase), S is substrate (nucleoside triphosphates) and D is DNA primer:

Square brackets denote molar concentrations, [D] meaning the DNA concentrations in g.atoms of phosphorus/l. and [E] the concentration of free enzyme.

The assumptions are made that: (i) the equilibria (1)–(3) are established and not disturbed by the formation of product (i.e. Michaelis kinetics); (ii) $K_t' = K_t$, i.e. the binding of substrate on enzyme is not affected by the binding of DNA; (iii) only the complex EDS can yield the product (RNA); (iv) the DNA is not affected by this last stage, i.e. the DNA is an 'activator' and at the end remains unchanged. (The further equilibrium,

is redundant.) It is readily shown (Dixon & Webb, 1964) that:

$$v = k[EDS] = \frac{k[E]_0}{\left(1 + \frac{K_s}{[S]}\right)\left(1 + \frac{K_d}{[D]}\right)} \quad (5)$$

where $[E]_0$ is total concentration of enzyme. Eqn. (5) predicts the observed linearity, in the absence of acridine, of the Lineweaver-Burk plots of Figs. 6 and 7 for constant [S] and [D] respectively.

As mentioned above, the kinetics of inhibition by proflavine and 9-aminoacridine are of the competitive form, both for variable [D] at constant [S] and for variable [S] at constant [D]. It is therefore proposed that the inhibition occurs as a result of the acridine (I) combining with the enzyme and that the enzyme-acridine complex (EI) cannot yield the active form, EDS. Thus:

and, more fully:

In this case:

$$[E]_0 = [E] \left(1 + \frac{[S]}{K_s} + \frac{[D]}{K_d} + \frac{[S][D]}{K_s K_d} + \frac{[I]}{K_i} \right)$$

whence:

$$v = k[EDS] = \frac{k[S][D][E]}{K_s K_d}$$

and:

$$\frac{1}{v} = \frac{1}{k[E]_0} \left(1 + \frac{K_s}{[S]} \right) \left(1 + \frac{K_d}{[D]} \right) + \frac{K_s K_d}{K_i} \cdot \frac{[I]}{[S][D]} \quad (8)$$

If [D] is varied at constant [S] and constant [I], the plot of v^{-1} versus $[S]^{-1}$ is linear. As [I] increases

from zero, the intercept is unaltered but the slope should increase, since eqn. (8) may be written:

$$\frac{1}{v} = \left\{ \frac{K_d \left(1 + \frac{K_i}{[S]} \right)}{k[E]_0} + \frac{K_i K_d [I]}{K_i [S]} \right\} \frac{1}{[D]} + \frac{\left(1 + \frac{K_i}{[S]} \right)}{k[E]_0} \quad (9)$$

This is the behaviour observed (Fig. 6).

If $[S]$ is varied at constant $[D]$, the plot of v^{-1} versus $[S]^{-1}$ is also linear and, if $[I]$ increases from zero, the intercept is again constant (but now equal to $(1 + K_d/[D])/k[E]_0$), and the slope should increase. This is as observed (Fig. 7).

Eqns. (8) and (9) therefore fit the observed phenomena and support the kinetic scheme shown in eqn. (7). The values of the various constants deduced from this analysis are: K_d 2.2×10^{-4} moles/l.; K_d 4.2×10^{-5} moles/l.; K_i for proflavine 5.4×10^{-9} moles/l.; K_i for 9-aminoacridine 2.4×10^{-9} moles/l. The DNA bases are more strongly bound (K_d/K_d is approx. 5) than the free nucleoside triphosphate bases and the dissociation constant of proflavine from the enzyme sites is twice that of 9-aminoacridine.

The form of the kinetic relationship indicates that molecules of the inhibitory acridine can occupy sites on the enzyme to which either the DNA or the nucleoside triphosphates normally attach themselves. The recognition process required to produce RNA related to the DNA in nucleotide sequence implies that the DNA is in some way opened out so that its bases are close to the enzyme. The site on the enzyme that binds the DNA therefore needs only to be specific for the base moiety of the deoxynucleotide, and so it is conceivable that the similarly sized flat ring of the acridine could compete for both this site and that normally occupied by the nucleoside triphosphate or its base. This also makes it clear why the inhibitory activity of the various acridines should be parallel to their ability to bind to DNA, since the same stereochemical factors would influence the ability to interact with the base rings of DNA and with the enzyme sites capable of holding such rings. This parallelism therefore need not be interpreted to mean that the binding to DNA is itself necessarily the cause of the inhibitory action on RNA synthesis. Although such binding may still make some contribution to the inhibition, it cannot explain the main features of the inhibition, in particular the competitive form of the Lineweaver-Burk plots. The existence of a weak interaction between the acridines and the nucleoside triphosphates is also a possibility since the extinction of a proflavine solution decreased when a solution of nucleoside triphosphates was added to it, although there was no shift in the wavelength of maximum absorption.

The inability of the buckled molecule of 9-amino-1,2,3,4-tetrahydroacridine to inhibit RNA synthesis in the DNA-primed system must result from its inability to attach to the enzyme sites whose configuration normally sequesters the flat base rings of either DNA or the nucleoside triphosphates. This lack of inhibitory power in the enzyme system has the same structural basis as its low affinity for DNA (Peacocke & Skerrett, 1956) and, presumably, its low bacteriostatic power (Albert, 1951, pp. 259–263).

In a similar study of the inhibition of RNA polymerase by ethidium bromide, Waring (1964a) found that complete inhibition could be achieved and that a plot of percentage of control activity against r gave the same straight line for different experimental conditions. This was consistent with the view that the binding of ethidium bromide to the DNA was the cause of inhibition, rather than interference with the binding of DNA (or nucleoside triphosphates) to the enzyme. However, even this correlation does not entirely eliminate the latter possibility, which appears from the foregoing to be the likelier explanation of the characteristics of inhibition by the acridines. This difference between ethidium bromide and the acridines is unexpected in view of the close similarity of their binding characteristics to DNA (Waring, 1965). The cause of the difference is probably therefore to be sought in the greater ease with which the acridines occupy the active sites on the enzyme, and this could reasonably be attributed to the ethidium cation being larger in area than one heterocyclic base ring of either DNA or nucleoside triphosphate.

The authors acknowledge the help afforded by a Research Fellowship of the Department of Scientific and Industrial Research (to B. H. N.), and the facilities provided by Mr J. R. P. O'Brien, Director of the Nuffield Department of Clinical Biochemistry, University of Oxford. They were particularly helped by the co-operation of the Director and staff of the Microbiological Research Establishment, Porton, Wilts., in providing adequate amounts of *E. coli* cells for the enzyme preparation.

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