Biochem. J. (1975) **147**, 401–410
Printed in Great Britain

The Mechanism of Inhibition of Ribonucleic Acid Synthesis by 8-Hydroxyquinoline and the Antibiotic Lomofungin

By RONALD S. S. FRASER and JAMES CREANOR
Department of Zoology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, U.K.

(*Received* 14 *October* 1974)

RNA synthesis in yeast is rapidly inhibited by 8-hydroxyquinoline and the phenazine antibiotic lomofungin (5-formyl-1-methoxycarbonyl-4,6,8-trihydroxyphenazine). It is shown that lomofungin, like 8-hydroxyquinoline, is a chelating agent for bivalent cations. The mechanism of inhibition of RNA synthesis by lomofungin and 8-hydroxyquinoline was investigated in experiments with isolated *Escherichia coli* RNA polymerase. The results show that both inhibitors are capable of inhibiting polymerase activity solely by chelating the dissociable cations Mn^{2+} and Mg^{2+}. Evidence is presented which shows that inhibition may occur in the absence of any direct contact between the RNA polymerase or DNA template and the inhibitor. The possibility that inhibition might also occur by chelation of the Zn^{2+}, which is tightly bound to the polymerase, is discussed: it is concluded that lomofungin or 8-hydroxyquinoline is likely to inhibit the enzyme by removal of Mn^{2+} and Mg^{2+} before chelating the Zn^{2+}. On the basis of inhibition by chelation of Mn^{2+} and Mg^{2+}, explanations are proposed for why lomofungin and 8-hydroxyquinoline inhibit synthesis of ribosomal and polydisperse RNA more than that of 5S RNA and tRNA, and for why protein synthesis is not immediately inhibited in the intact yeast cell.

Lomofungin (5-formyl-1-methoxycarbonyl-4,6,8-trihydroxyphenazine) is an antibiotic from *Streptomyces lomodensis* (Johnson & Dietz, 1969). It rapidly inhibits RNA synthesis in yeast, but has no immediate effect on cell growth or protein synthesis (Gottlieb & Nicolas, 1969; Lampen *et al.*, 1973; Fraser *et al.*, 1973; Cannon *et al.*, 1973). Lomofungin is proving useful in studies of yeast RNA metabolism and enzyme synthesis, as other antibiotics such as actinomycin D fail to inhibit yeast RNA synthesis or do only at extremely high concentrations (Hartwell & McLaughlin, 1968; Soskova *et al.*, 1970; Medoff *et al.*, 1972; Mitchison *et al.*, 1973; Tønnesen & Friesen, 1973). An interesting feature of lomofungin action on yeast is that it strongly inhibits the synthesis of rRNA and polydisperse RNA, but has comparatively less effect on synthesis of 5S RNA and tRNA (Fraser *et al.*, 1973; Cano *et al.*, 1973; Cannon & Jimenez, 1974).

In the present paper we report experiments on the mechanism of inhibition of RNA synthesis by lomofungin. Cano *et al.* (1973) found that lomofungin inhibited the synthesis of RNA *in vitro* in systems using RNA polymerases extracted from *Saccharomyces cerevisiae* and *Escherichia coli*, and suggested that the RNA polymerase was the site of action of the antibiotic.

We found that 8-hydroxyquinoline has effects on RNA synthesis in yeast similar to those of lomofungin (Fraser & Creanor, 1974). This, together with certain similarities in the molecular structures of lomofungin and 8-hydroxyquinoline, suggested a common mode of action of the inhibitors. 8-Hydroxyquinoline is a chelating agent for bivalent cations (Hollingshead, 1954). This suggested that lomofungin and 8-hydroxyquinoline might inhibit RNA synthesis by chelating bivalent cations required for RNA polymerase activity.

Two distinct types of bivalent cations are known to be involved in RNA polymerase activity: the dissociable cations Mn^{2+} and Mg^{2+} (Roeder & Rutter, 1969; Burgess, 1971; Ponta *et al.*, 1971), and Zn^{2+}, which is tightly bound to the enzyme (Scrutton *et al.*, 1971; Valenzuela *et al.*, 1973). Pavletich *et al.* (1974) have suggested that lomofungin acts by chelating the Zn^{2+}. In the present paper we show that lomofungin can chelate Mn^{2+} and Mg^{2+}. We report that lomofungin and 8-hydroxyquinoline are capable of inhibiting RNA polymerase solely by chelating the Mn^{2+} and Mg^{2+}, and that inhibition of enzyme activity can occur in the absence of direct contact between inhibitor and enzyme. The question of whether inhibition of RNA polymerase activity *in vivo* is a consequence of chelation of dissociable bivalent cations or Zn^{2+} is discussed. The knowledge that 8-hydroxyquinoline and lomofungin inhibit by chelation permits interpretation of some other aspects of their effects on yeast.

Materials and Methods

Lomofungin

Lomofungin was a gift from Dr. G. B. Whitfield of the Upjohn Co., Kalamazoo, Mich., U.S.A. It was purified from a contaminant, probably a bivalent or tervalent cation: the lomofungin was dissolved at 300μg/ml in 10mM-Tris adjusted to pH7.95 at 20°C with 1M-HCl. Insoluble material was removed by centrifugation for 1min at 12000g ($r_{av.}$ 4.5cm). 8-Hydroxyquinoline was added to 5mM, then (with any chelates formed) removed by chloroform extraction. The pH of the aqueous phase was lowered to 3.5 by addition of acetic acid. Most of the lomofungin precipitated was collected by centrifugation as above and dried under vacuum.

RNA polymerase assay

RNA polymerase (RNA nucleotidyltransferase, EC 2.7.7.6) from *E. coli* strain M.R.E. 600 was purchased from Boehringer Corp. (London), London W.5, U.K.

RNA polymerase activity was measured by the incorporation of [^{3}H]CTP radioactivity into trichloroacetic acid-insoluble material. The standard incubation mixture contained, in a total volume of 0.5ml, 50mM-Tris–HCl buffer, pH7.95 at room temperature (20°C); 1.5mM-$MnCl_2$; 1.0mM-$MgCl_2$; 1.0mM-2-mercaptoethanol; 50mM-KCl; 0.1mM each of ATP, GTP and UTP; 0.025mM- [^{3}H]CTP (5μCi) (The Radiochemical Centre, Amersham, Bucks., U.K.); 42μg of denatured calf thymus DNA as template. The reaction was started by addition of 5μg of RNA polymerase. After various times of incubation at 37°C, 20μl samples were withdrawn and mixed with 40μg of yeast non-radioactive carrier RNA and 0.5ml of ice-cold 10% (w/v) trichloroacetic acid containing 40mM-sodium pyrophosphate. The precipitate was collected by filtration on Whatman GF/A glass-fibre paper, washed three times with ice-cold 10% trichloroacetic acid–40mM-sodium pyrophosphate and twice with 80% (v/v) ethanol containing 0.1M-NaCl. The filters were dried and incorporation of radioactivity into acid-insoluble polynucleotides was determined by counting in 5ml of 0.5% butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole]–toluene scintillator in a Packard liquid-scintillation counter. Counting efficiency was approx. 10%. At least 1000 counts above background were collected for each sample.

Single-point assays of enzyme activity were carried out by incubating 100μl reaction mixtures for 5min. The final concentrations of all the constituents were as described above. Carrier RNA (40μg) and 0.5ml of trichloroacetic acid were added to the entire reaction mixture to stop the reaction, and total incorporation was determined as above.

DNA-independent incorporation was measured in assays lacking DNA.

Purified lomofungin was dissolved directly in the polymerase reaction mixture before addition of polymerase, to give the required final lomofungin concentration.

8-Hydroxyquinoline was dissolved in ethanol at 100 times the required final concentration, and 0.01vol. was added to the reaction mixture before addition of polymerase. Control experiments showed that 1% ethanol had no effect on polymerase activity.

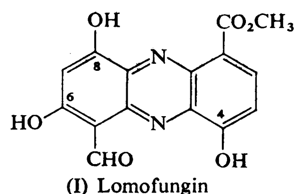
U.v.-absorption spectra

U.v-absorption spectra of lomofungin and 8-hydroxyquinoline were measured in a Unicam SP. 800 recording spectrophotometer, in 10mm path-length cells.

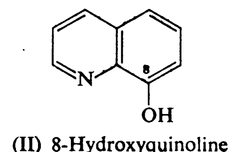
Results

Evidence that lomofungin is a chelating agent

The structural formulae of lomofungin (I) and 8-hydroxyquinoline (II) are shown. 8-Hydroxy-



(I) Lomofungin



(II) 8-Hydroxyquinoline

quinoline is able to chelate bivalent cations because of the location of the hydroxyl group relative to the ring nitrogen. The hydroxyl group acts as an acid; as the pH rises it dissociates to —O^-. Bivalent cations are bound by the dissociated acid group and the lone pair of electrons borne by the nitrogen atom (Hollingshead, 1954).

The structural formula of lomofungin shows two possible chelating sites, namely where the hydroxyl groups at positions 4 and 8 are located relative to ring nitrogen atoms (as in 8-hydroxyquinoline). Four lines of evidence confirm that lomofungin can chelate bivalent cations.

(1) 8-Hydroxyquinoline chelates of certain metal cations, such as Mn^{2+}, are insoluble within certain pH ranges (Hollingshead, 1954). Addition of Mn^{2+} (1.5mM) to lomofungin solution (1.25mM in 10mM-Tris–HCl buffer at pH7.95) caused formation of a heavy precipitate.

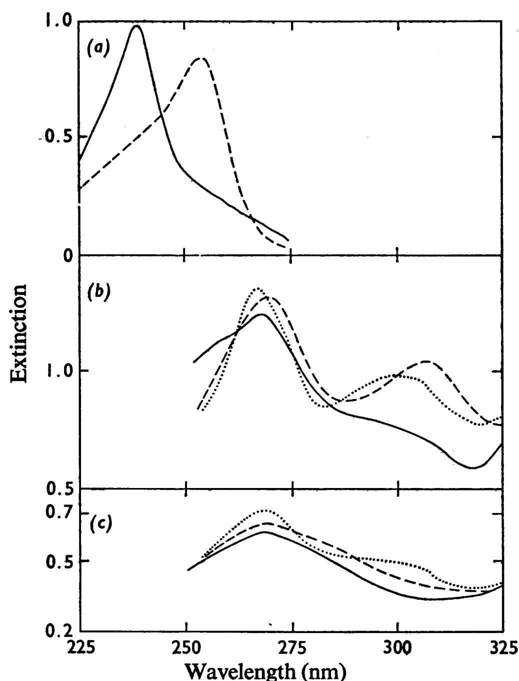


Fig. 1. *U.v.-absorption spectra of 8-hydroxyquinoline and lomofungin*

(*a*) 0.035mM-8-Hydroxyquinoline in 10mM-Tris–HCl buffer, pH7.95, alone (——) and with 5mM-$MgCl_2$ (----). (*b*) 0.025mM Purified lomofungin in 10mM-Tris–HCl buffer, pH7.95, alone (——) and plus 0.05mM-$MnCl_2$ (----) or 0.1mM-$MgCl_2$ (····). (*c*) 0.012mM Purified (——) and commercial (----) lomofungin in 20mM-sodium acetate adjusted to pH5.5 with 1M-acetic acid, and purified lomofungin plus 0.05mM-$MgCl_2$ (····).

(2) Chelation of bivalent cations by 8-hydroxyquinoline results in a change in the u.v.-absorption spectrum (Fig. 1*a*). Fig. 1(*b*) shows the u.v.-absorption spectrum of purified lomofungin. Addition of Mn^{2+} or Mg^{2+} caused changes in the absorption spectrum, with increased extinction in the 265–275 and 295–315nm regions. Univalent cations (Na^+, K^+) did not cause a change in the absorption spectrum. The absorption spectrum of the lomofungin-plus-bivalent-cation form could be restored to the free-lomofungin form by addition of EDTA to a concentration exceeding that of the bivalent cation.

(3) Fig. 2 shows the pH-dependence of the change in extinction of lomofungin at 308nm caused by extinction of lomofungin at 308 nm caused by addition of Mn^{2+}. Taking the change in extinction at 308nm as a measure of the amount of chelation, the results indicate that chelation commenced at about pH4.5, and that chelation capacity increased up to about pH7. These results are consistent with a dependence of chelation on dissociation of acidic hydroxyl groups, and suggest that half of the hydroxyl groups are dissociated and available for chelation at pH5.6. The pK_a for the dissociation of the hydroxyl group of 8-hydroxyquinoline is 5.5 (Hollingshead, 1954; O'Sullivan, 1969).

(4) We attempted to measure the stoicheiometry of chelation of bivalent cations by lomofungin, by measuring the change in u.v. absorption when increasing amounts of Mn^{2+} or Mg^{2+} were added to lomofungin solutions. The measurements were made at pH7.95, where almost all the hydroxyl groups should be dissociated. Fig. 3 shows that with 0.025mM-lomofungin, a maximum increase in the extinction at 308nm was obtained with 0.05mM-Mn^{2+}. These data suggest that at saturation, two Mn^{2+} cations are bound per molecule of lomofungin, a result consistent with the two binding sites suggested by the structural formula. The slight decline in the extent of the change in extinction at 308nm at higher concentrations of Mn^{2+} was caused by some precipitation of the lomofungin–Mn^{2+} chelate.

A rather higher concentration of Mg^{2+} (0.1mM) was required to produce the maximum change of extinction of 0.025mM-lomofungin (Fig. 3). This result could mean that lomofungin can bind more than two Mg^{2+} cations per molecule, but is more likely to mean that the stability constant of the lomofungin–Mg^{2+} chelate is comparatively low, and that higher Mg^{2+} concentrations are required to ensure complete occupation of the available chelating sites. It is

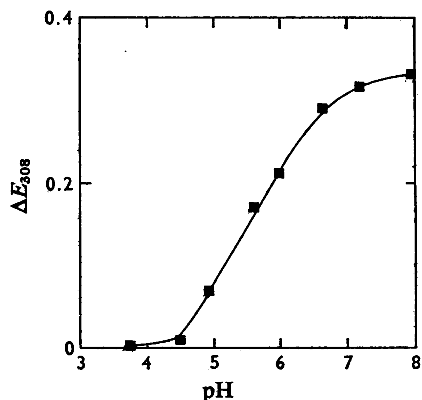


Fig. 2. *pH-dependence of chelation of Mn^{2+} by lomofungin*

Changes in the extinction at 308nm caused by adding 0.05mM-$MnCl_2$ to 0.025mM-purified lomofungin, at various pH values. Solutions were buffered by 20mM-sodium acetate adjusted with 1M-acetic acid to pH values between 3.7 and 6.0, by 20mM-2-(*N*-morpholino) ethanesulphonic acid adjusted with 1M-NaOH to pH6.6 or 7.2, or by 20mM-Tris–HCl buffer, pH7.95.

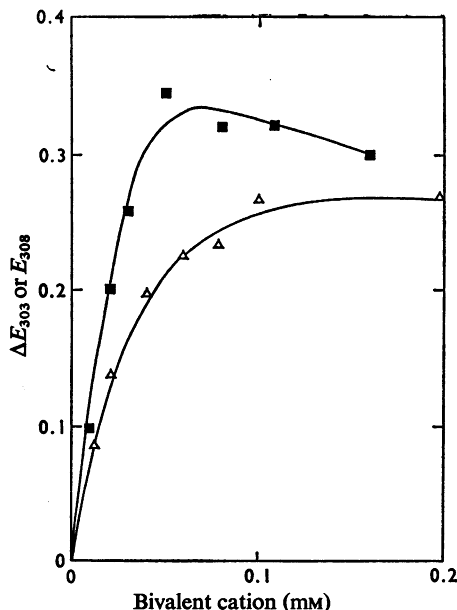


Fig. 3. *Stoicheiometry of chelation of Mn^{2+} (■) and Mg^{2+} (△) by lomofungin*

Various concentrations of $MnCl_2$ or $MgCl_2$ were added to 0.025mM-lomofungin in 10mM-Tris–HCl buffer, pH7.95. Chelation was measured by the change in extinction at 308nm (Mn^{2+}) or 303nm (Mg^{2+}).

consistent with this explanation that 8-hydroxyquinoline also binds Mn^{2+} more strongly than Mg^{2+} (O'Sullivan, 1969).

Purification of lomofungin

The experiments in this paper were carried out with lomofungin purified from the commercial sample. The absorption spectrum of commercial lomofungin (Fig. 1*c*) was similar to that obtained when bivalent cation was added to purified lomofungin. The absorption spectra shown in Fig. 1(*c*) were measured at pH5.5, as the contaminant–lomofungin complex is more soluble at pH5.5 than at pH7.95.

The absorption spectrum of commercial lomofungin could be shifted to the purified lomofungin form by addition of EDTA. These data therefore suggest that the commercial lomofungin contained a bivalent (or tervalent) cation as impurity. This impurity was found to inhibit RNA polymerase activity under certain circumstances, for example in the presence of very high concentrations of Mn^{2+} and Mg^{2+} (Fig. 7), which presumably liberated the impurity from lomofungin by competition for the chelating sites. At the normal bivalent cation concentration of the standard polymerase reaction mixture, the impurity remained bound to the lomofungin, and RNA polymerase activity was affected similarly by commercial and purified lomofungin, as shown in Fig. 6.

The purification scheme for lomofungin described in the Materials and Methods section attempted to remove the contaminant in three ways: by dissolving the lomofungin at high pH and high concentration, and removing insoluble chelates; by competing for cations with relatively high concentrations of 8-hydroxyquinoline, and by decreasing the pH to 3.5, where lomofungin does not chelate (Fig. 2), and where the antibiotic is insoluble, leaving the liberated cations in solution.

RNA polymerase assay

Fig. 4 shows a time-course of the incorporation of [^{3}H]CTP radioactivity into acid-insoluble polynucleotide by *E. coli* RNA polymerase. Depending on the batch of enzyme used, incorporation was

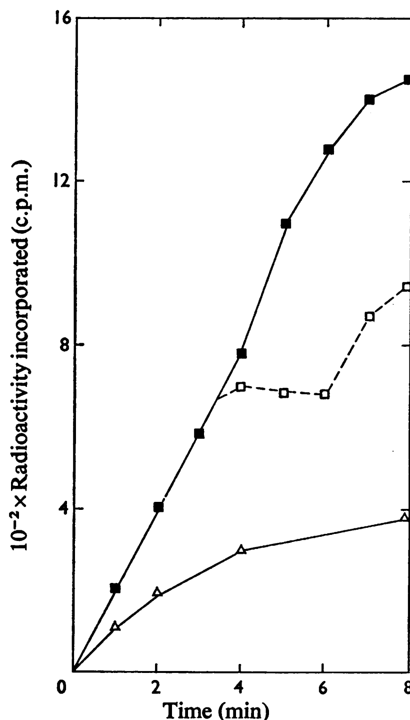


Fig. 4. *Kinetics of incorporation of [^{3}H]CTP by isolated E. coli RNA polymerase*

△, DNA-independent incorporation; ■, DNA-dependent incorporation, found by subtracting DNA-independent incorporation from total incorporation; □, DNA-dependent incorporation in an incubation to which 1.0mM-lomofungin was added after 3.5min and an additional 1.5mM-$MnCl_2$ and 1.0mM-$MgCl_2$ were added after 6min.

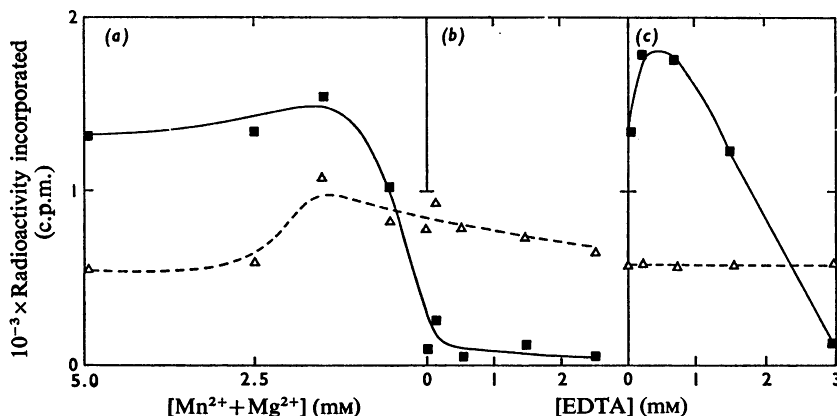


Fig. 5. *Bivalent-cation requirements of E. coli RNA polymerase*

■, DNA-dependent incorporation; △, DNA-independent incorporation in a 5min incubation. (*a*) Incubations containing no EDTA and various concentrations of bivalent cation in the molar ratio 1.5 $MnCl_2$: 1.0 $MgCl_2$. (*b*) Incubations containing no bivalent cation and various concentrations of EDTA. (*c*) Incubations containing 1.5mM-$MnCl_2$, 1.0mM-$MgCl_2$ and various concentrations of EDTA.

linear with time for 7 to 40min. Also depending on the batch of enzyme, between 10 and 30% of total incorporation was found to be independent of a DNA template. Negligible amounts of radioactivity were rendered acid-insoluble when no enzyme was present.

Fig. 5 shows the bivalent-cation requirements for enzyme activity. DNA-independent incorporation was relatively independent of bivalent cation concentration (Fig. 5*a*) and also continued in the complete absence of bivalent cations (Fig. 5*b*). The cause of this DNA-independent incorporation is not fully understood.

To test whether lomofungin was capable of inhibiting RNA polymerase activity solely by removing bivalent cations, it was necessary to correct for this amount of bivalent cation-independent DNA-independent incorporation. This was done by running each assay in parallel, with and without DNA. The amount of DNA-dependent incorporation was found by subtracting DNA-independent incorporation from total incorporation.

DNA-dependent incorporation did not occur in the absence of bivalent cations (Fig. 5*b*); it rose to a maximum at a total Mn^{2+}+Mg^{2+} concentration of 1.5mM (Fig. 5*a*), then remained fairly steady with increasing bivalent cation concentration over the range tested. In the presence of 1.5mM-Mn^{2+} and 1.0mM-Mg^{2+}, DNA-dependent RNA polymerase activity could be decreased by addition of EDTA. At low concentrations of added EDTA, up to 0.5mM, enzyme activity was increased with some batches of enzyme (Fig. 5*c*). This was presumably by removal of traces of toxic metal-cation contaminants in the assay mixture.

Effects of lomofungin and 8-*hydroxyquinoline on RNA polymerase activity*

As agents chelating Mn^{2+} and Mg^{2+}, lomofungin and 8-hydroxyquinoline would be expected to inhibit DNA-dependent RNA polymerase by decreasing the concentrations of these cations to values insufficient for enzyme activity (Fig. 5*a*). However, this does not exclude the possibility that lomofungin and 8-hydroxyquinoline might inhibit the enzyme activity in another way, possibly by binding to the DNA, by direct interaction with the polymerase as suggested by Cano *et al.* (1973), or by chelating the Zn^{2+} tightly bound to the polymerase (Pavletich *et al.*, 1974). If lomofungin and 8-hydroxyquinoline are capable of inhibiting RNA polymerase by chelation of the dissociable bivalent cations, three predictions should be fulfilled. (1) The concentration of inhibitor giving complete inhibition of polymerase activity should be that required to lower the Mn^{2+}+Mg^{2+} concentration to a value insufficient for enzyme activity. (2) The inhibition of polymerization should be reversed by addition of more Mn^{2+} and Mg^{2+}. (3) Inhibition should occur in the absence of direct contact between inhibitor and DNA or polymerase. These predictions have been tested experimentally.

(1) The effects of various concentrations of lomofungin or 8-hydroxyquinoline on DNA-dependent DNA polymerase activity are shown in Fig. 6. The two dose–response curves share certain features. At low concentrations of lomofungin or 8-hydroxyquinoline, there was no inhibition of enzyme activity; indeed, with some samples of enzyme, a small stimulation was found. Inhibition commenced at concentrations of 0.2mM-lomofungin and 1.0mM-

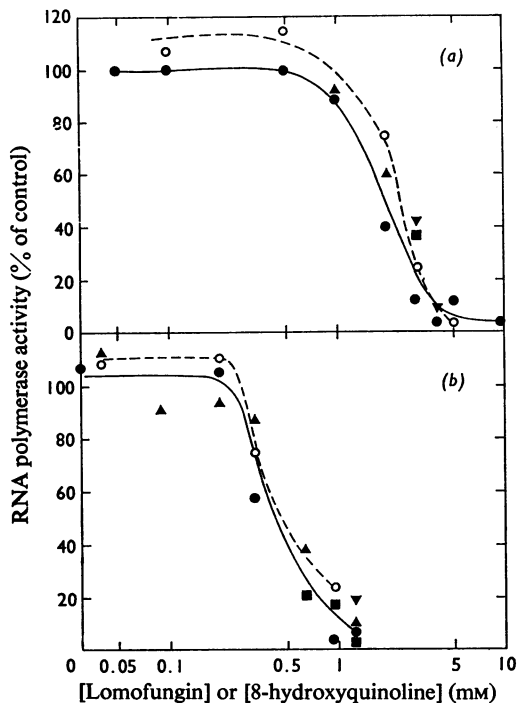


Fig. 6. *Effects of various concentrations of* (*a*) *8-hydroxyquinoline and* (*b*) *lomofungin on DNA-dependent RNA polymerase activity*

Continuous lines and solid symbols show results obtained when 8-hydroxyquinoline or lomofungin was present during the enzyme assay. The different solid symbols represent results from different batches of enzyme. For lomofungin, results shown by (▲) were obtained with commercial lomofungin; those shown by (●, ■, ▼) were obtained with purified lomofungin. The broken lines and open circles show results obtained when 8-hydroxyquinoline or lomofungin was removed from the incubation before addition of DNA and RNA polymerase, as explained in the Results section. All data are expressed as percentages of activity in control cultures containing no inhibitor. The incubation time was 5min.

8-hydroxyquinoline, and was complete at about 1.25mM-lomofungin and between 3 and 5mM-8-hydroxyquinoline.

The total bivalent cation concentration of the reaction mixture was 2.5mM (1.5mM-Mn^{2+}+1.0mM-Mg^{2+}). The concentration of 8-hydroxyquinoline required for complete chelation of the Mn^{2+} and Mg^{2+} is 5mM, since each bivalent cation can bind two molecules of 8-hydroxyquinoline in the chelate (Hollingshead, 1954). The data of Fig. 3 suggest that 1.25mM-lomofungin should chelate most of the 2.5mM bivalent cation, as each lomofungin molecule binds two Mn^{2+} ions or rather less than two Mg^{2+} ions at pH7.95.

As both lomofungin (Fig. 3) and 8-hydroxyquinoline (O'Sullivan, 1969) bind Mn^{2+} more strongly than Mg^{2+}, it is likely that Mn^{2+} would be removed more effectively than Mg^{2+} when the total chelator concentration is less than required for complete chelation of Mn^{2+} and Mg^{2+}. Mg^{2+} alone is much less effective as an activator of RNA polymerase than Mn^{2+}+Mg^{2+}. Pavletich *et al.* (1974) reported maximum RNA polymerase activity with 15–20mM-Mg^{2+}, whereas maximum activity was obtainable with 1.5–2.5mM of the combined cations (Fig. 5*a*). The concentrations of lomofungin and 8-hydroxyquinoline required to give near-complete inhibition of RNA polymerase activity (Fig. 6) were thus slightly less than those necessary for complete removal of all Mn^{2+} and Mg^{2+}. However, these inhibitor concentrations should have removed practically all of the Mn^{2+} and much of the Mg^{2+}, leaving the polymerase with too little Mg^{2+} for significant activity.

(2) Inhibition of RNA polymerase activity by 8-hydroxyquinoline could be prevented by adding extra Mn^{2+}+Mg^{2+} to the incubations (Fig. 7*a*). At the highest inhibitor concentration, it was not possible to get complete reversal of inhibition.

It proved less easy to demonstrate reversibility of inhibition by lomofungin. With the unpurified lomofungin, addition of extra Mg^{2+} and Mn^{2+} typically increased the inhibition of RNA polymerase activity (Fig. 7*b*), probably by releasing the presumed toxic contaminant from lomofungin (Fig. 1*c*). Very high concentrations of extra Mn^{2+} and Mg^{2+} gave some reversal of inhibition (Fig. 7*b*). The inhibition of RNA polymerase by purified lomofungin was readily reversible by addition of extra Mn^{2+} and Mg^{2+}.

The experiments described in Figs. 6 and 7 were carried out by adding polymerase to incubation mixtures already containing lomofungin or 8-hydroxyquinoline and bivalent cations. Fig. 4 shows that when purified lomofungin was added to an incubation in which the polymerase was already in action, inhibition occurred immediately. Polymerase activity recommenced immediately on addition of extra Mn^{2+}+Mg^{2+} to the incubation.

(3) As a check on the ability of 8-hydroxyquinoline and lomofungin to inhibit the polymerase solely by chelation of Mn^{2+} and Mg^{2+}, polymerase activity was assayed in reaction mixtures from which lomofungin or 8-hydroxyquinoline had been removed before addition of DNA and enzyme.

Incomplete polymerase-assay mixtures were prepared, containing the normal final amounts of Tris buffer, KCl, mercaptoethanol and bivalent cations, but lacking DNA, nucleoside triphosphates and RNA polymerase. 8-Hydroxyquinoline or lomofungin

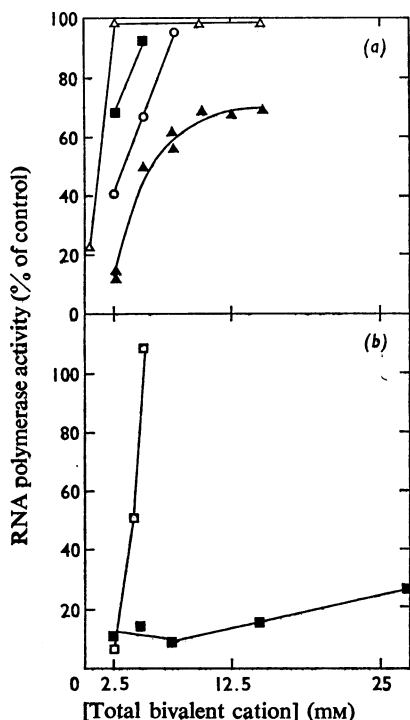


Fig. 7. *Reversal of inhibition of RNA polymerase by 8-hydroxyquinoline or lomofungin*

Bivalent cations in the molar ratio $1.5Mn^{2+}$: $1.0Mg^{2+}$ were added to various final total concentrations before addition of RNA polymerase to reaction mixtures containing lomofungin or 8-hydroxyquinoline. (*a*) △, DNA-dependent RNA polymerase activity in control incubations containing no inhibitor, and polymerase activity in the presence of (■) 2mM-, (○) 3mM- and (▲) 4mM-8-hydroxyquinoline. (*b*) RNA polymerase activity in incubations containing 1.0mM purified (□) or commercial (■) lomofungin. All data are expressed as percentages of incorporation in a control assay with 2.5mM total bivalent cation. The incubation time was 5min.

was added to various concentrations. 8-Hydroxyquinoline and its chelates were removed by shaking the incomplete reaction mixtures twice with equal volumes of chloroform. The residual chloroform was removed under vacuum. Examination of the u.v.-absorption spectrum of the incomplete reaction mixture after 8-hydroxyquinoline treatment and chloroform extraction confirmed that removal of 8-hydroxyquinoline had been complete. Lomofungin was more difficult to extract, as it does not readily partition into organic solvents at high pH. Insoluble chelates formed in incomplete reaction mixtures with high lomofungin concentrations at pH7.95 were removed by centrifugation. The pH was then lowered to 5.5 by addition of acetic acid; most of the remaining lomofungin precipitated and was removed by centrifugation. The reaction mixture was then shaken with chloroform, which removed the last of the lomofungin, and the pH was restored to 7.95 with Tris. Incomplete reaction mixtures, without added chelating agent, were extracted by both above schemes as controls.

RNA, nucleoside triphosphates and RNA polymerase were added to the chelating-agent-treated or control partial incubation mixtures, and polymerase activity was measured as usual. The results were dose–response curves similar to those obtained when lomofungin or 8-hydroxyquinoline was present during the polymerase assay (Fig. 6). These results therefore prove that lomofungin and 8-hydroxyquinoline are capable of inhibiting RNA polymerase solely by chelation of Mn^{2+} and Mg^{2+}, and show that inhibition can occur in the absence of direct contact between the chelating agent and the DNA or RNA polymerase.

Discussion

We have shown that lomofungin can chelate Mg^{2+} and Mn^{2+} (Fig. 3). 8-Hydroxyquinoline will also chelate these cations (O'Sullivan, 1969). The *E. coli* RNA polymerase used in our experiments was inactive in the absence of $Mn^{2+}+Mg^{2+}$ (Fig. 5*a*). The data for polymerase activity with different concentrations of lomofungin or 8-hydroxyquinoline (Fig. 6) show that complete inhibition was achieved at the inhibitor concentrations required for chelation of most of the Mn^{2+} and Mg^{2+} present. Fig. 6 also shows that the same dose–response curves were obtained when there was no direct contact between enzyme and inhibitor. Finally, enzyme activity could be restored by addition of extra Mn^{2+} and Mg^{2+} to reactions inhibited by lomofungin or 8-hydroxyquinoline (Figs. 4 and 7). These results show unequivocally that lomofungin and 8-hydroxyquinoline are capable of inhibiting isolated *E. coli* RNA polymerase solely by chelating essential Mn^{2+} and Mg^{2+}. Our data do not exclude the possibility that other mechanisms of inhibition by lomofungin and 8-hydroxyquinoline might exist *in vivo*.

In contrast with our conclusions, Pavletich *et al.* (1974) claim to have shown that lomofungin does not act by chelating Mg^{2+} or Mn^{2+}. They suggested that lomofungin inhibits RNA synthesis by preventing the initiation of transcription, by chelating the Zn^{2+} which is tightly bound to the RNA polymerase (Scrutton *et al.*, 1971). We now discuss experimental evidence against these conclusions.

The suggestion by Pavletich *et al.* (1974) that lomofungin does not act by chelating Mn^{2+} or Mg^{2+} stems from their finding that yeast cells could be protected from inhibition of growth by lomofungin, by preincubation for 2h in culture medium con-

taining extra Cu^{2+}, Zn^{2+} or Fe^{2+}, but no protection was obtained when the medium contained extra Mn^{2+} or Mg^{2+}. In contrast, we have found that the inhibition of RNA synthesis in yeast by lomofungin does bear an inverse relationship to the Mg^{2+} concentration in the medium, but growth periods of longer than 2h under different Mg^{2+} concentrations are required to show the maximum effect (R. S. S. Fraser & J. Creanor, unpublished work). The protection of yeast from inhibition afforded by Cu^{2+}, Zn^{2+} and Fe^{2+} occurs because lomofungin chelates these cations (Pavletich *et al.*, 1974). In the cell this must result in a decrease in the concentration of free lomofungin available to inhibit RNA synthesis.

Pavletich *et al.* (1974) investigated the effects of Mg^{2+} concentration on the inhibition of isolated *E. coli* RNA polymerase by lomofungin, and found a significant increase in the activity of lomofungin-inhibited enzyme with higher Mg^{2+} concentrations. We attribute their failure to obtain complete recovery of polymerase activity to the use of unpurified lomofungin, from which high Mg^{2+} concentrations probably cause the release of a metal cation toxic to polymerases (Fig. 1*c*). Fig. 7 shows that with purified lomofungin, addition of extra Mn^{2+} and Mg^{2+} gave much higher recovery of polymerase activity than when unpurified lomofungin was used.

Pavletich *et al.* (1974) quoted no direct experimental evidence that lomofungin does not chelate Mn^{2+} and Mg^{2+}; we have shown that lomofungin does chelate these cations (Figs. 1 and 3). We therefore consider that these results of Pavletich *et al.* (1974) do not exclude the chelation of Mn^{2+} and Mg^{2+} by lomofungin as a means of inhibition of RNA polymerase.

The activity of RNA polymerase depends on the dissociable cations Mn^{2+} and Mg^{2+}, and on Zn^{2+}, which is tightly bound to the enzyme (Scrutton *et al.*, 1971; Valenzuela *et al.*, 1973). The ability of lomofungin and 8-hydroxyquinoline to chelate all three bivalent cations poses the question of whether the inhibition of RNA polymerase by these agents is a result of chelation of Zn^{2+}, the dissociable cations, or all three. Our data show that inhibition by chelation of Mn^{2+} and Mg^{2+} alone is possible, but do not rule out the chelation of Zn^{2+} as a further contribution to inhibition. Pavletich *et al.* (1974) concluded that inhibition was by chelation of Zn^{2+}, from experiments showing that Zn^{2+} and Cu^{2+} prevented inhibition of RNA synthesis in yeast by lomofungin, and from a demonstration that RNA polymerase pretreated with lomofungin failed to form the DNA–RNA polymerase initiation complex. Three types of experimental evidence suggest that chelation of Zn^{2+} is unlikely to contribute to the primary mechanism of inhibition of RNA polymerase by lomofungin or 8-hydroxyquinoline.

(1) Inhibition of RNA polymerase by specific chelation of Zn^{2+} is very slow, with reported delays before full inhibition of 5–10min for *E. coli* RNA polymerase (Scrutton *et al.*, 1971) and 2h for eukaryote RNA polymerases (Valenzuela *et al.*, 1973). Inhibition by chelation of Zn^{2+} may be slow both because Zn^{2+} is involved in chain initiation rather than elongation, and because the Zn^{2+} is very tightly bound to the enzyme (Scrutton *et al.*, 1971) In contrast, lomofungin inhibited isolated *E. coli* RNA polymerase immediately (Fig. 4). Cano *et al.* (1973) also reported an immediate inhibition of isolated yeast RNA polymerase by lomofungin, and suggested that lomofungin inhibited chain elongation.

(2) The chelating agent of choice in studies of polymerase-bound Zn^{2+} is *o*-phenanthroline, which chelates Zn^{2+} but not Mg^{2+} (O'Sullivan, 1969). Hence *o*-phenanthroline does not interfere with the dissociable bivalent-cation requirement of the polymerase. 8-Hydroxyquinoline and EDTA chelate Zn^{2+} more strongly than *o*-phenanthroline does, but 8-hydroxyquinoline and EDTA also chelate Mg^{2+}. Scrutton *et al.* (1971) and Valenzuela *et al.* (1973) found that RNA polymerase was strongly inhibited by 0.5–1.0mM-*o*-phenanthroline, in the presence of 5–10mM-Mg^{2+}. Scrutton *et al.* (1971) found that 1mM-EDTA or -8-hydroxyquinoline did not inhibit polymerase activity. Valenzuela *et al.* (1973) reported that up to 10mM-EDTA or 8-hydroxyquinoline was required for strong inhibition. The clear implication of these results is that Mg^{2+} saturates the chelating sites of EDTA or 8-hydroxyquinoline, and prevents chelation of the Zn^{2+}. The concentrations of EDTA or 8-hydroxyquinoline reported by Valenzuela *et al.* (1973) to give strong inhibition of RNA polymerase activity were those required for chelation of the Mg^{2+}.

(3) Pavletich *et al.* (1974) found that incubation of RNA polymerase with lomofungin in the absence of Mg^{2+} prevented formation of the initiation complex between DNA and the polymerase. This result shows only that lomofungin is capable of chelating the Zn^{2+} of the polymerase in the absence of competing bivalent cations. It does not show that lomofungin will prevent formation of the initiation complex *in vivo* or in a complete system containing Mg^{2+} *in vitro*. Indeed, Pavletich *et al.* (1974) found that when lomofungin was preincubated with DNA, with subsequent addition of Mg^{2+} and polymerase, there was no inhibition of formation of the initiation complex by lomofungin. This result strongly suggests that Mg^{2+} prevented the chelation of polymerase-bound Zn^{2+} by lomofungin.

From these considerations, and from the experimental evidence reported in this paper, we suggest that the primary mechanism of inhibition of RNA polymerase by lomofungin and 8-hydroxyquinoline is by the chelation of the dissociable bivalent cations Mn^{2+} and Mg^{2+}, and not by chelation of the Zn^{2+} tightly bound to the enzyme. Our data do not exclude

the possibility that concentrations of chelating agent higher than required to chelate the available Mn^{2+} and Mg^{2+} might chelate the Zn^{2+}. However, by this stage the enzyme is already inactivated by lack of dissociable bivalent cation. Chelation of Zn^{2+} is a possible explanation of the failure totally to restore polymerase activity by addition of Mn^{2+} and Mg^{2+} to enzyme inhibited by the highest concentration of 8-hydroxyquinoline (Fig. 7).

Demonstration of the mode of action of an inhibitor in a simplified system *in vitro* provides a strong indication, though not complete proof, of the way it acts in the intact cell. We have shown that lomofungin and 8-hydroxyquinoline inhibit *E. coli* RNA polymerase *in vitro* by chelating Mn^{2+} and Mg^{2+}. Yeast RNA polymerases also require Mn^{2+} and Mg^{2+} (Ponta *et al.*, 1971; Brogt & Planta, 1972; Adman *et al.*, 1972) and lomofungin inhibits the activity of yeast RNA polymerases *in vitro* (Cano *et al.*, 1973). These data suggest that lomofungin and 8-hydroxyquinoline might inhibit RNA synthesis in intact yeast cells by chelation of the dissociable bivalent cations required for polymerase activity. There is experimental evidence which is consistent with chelation as the mechanism of action of the inhibitors *in vivo*. The ability to chelate falls with decreasing pH. When the internal pH of yeast cells was decreased by growing cells in culture medium at low pH, the inhibition of RNA synthesis by 8-hydroxyquinoline was also lessened (Fraser & Creanor, 1974).

Our suggestion that chelation is the mode of action of lomofungin and 8-hydroxyquinoline permits explanation of their effects on some other aspects of metabolism in yeast.

The synthesis of 5S RNA and tRNA is inhibited less than synthesis of rRNA and polydisperse RNA by 8-hydroxyquinoline (Fraser & Creanor, 1974) and lomofungin (Cano *et al.*, 1973; Fraser *et al.*, 1973; Cannon & Jimenez, 1974). This may be related to the different bivalent-cation requirements of different RNA polymerases. Yeast polymerase III has a lower total requirement for dissociable bivalent cations than polymerases I and II, and is fully active with Mg^{2+} alone, whereas polymerases I and II require Mn^{2+} as well as Mg^{2+} for full activity (Ponta *et al.*, 1972). The decrease in bivalent cation concentration, and particularly in Mn^{2+} concentration, likely with lomofungin or 8-hydroxyquinoline, should inhibit polymerase III less than polymerases I and II. This suggests that yeast polymerase III may be responsible for 5S RNA and tRNA synthesis. Polymerase III in animal cells, which also has a lower requirement for dissociable bivalent cations than polymerases I and II (Roeder & Rutter, 1969; Price & Penman, 1972), has been shown to synthesize 5S RNA and tRNA (Weinmann & Roeder, 1974).

We are unable to offer any explanation of the comparative resistance of synthesis of yeast 5S RNA and tRNA to inhibition by lomofungin based on primary inhibition of yeast RNA polymerases by chelation of the tightly bound Zn^{2+} (Pavletich *et al.*, 1974). Valenzuela *et al.* (1973) found that sea-urchin RNA polymerases I, II and III were inhibited to similar extents by the Zn^{2+} chelator *o*-phenanthroline.

Lomofungin and 8-hydroxyquinoline might be expected to inhibit any cellular process requiring bivalent cations. This is certainly true for DNA synthesis. 8-Hydroxyquinoline inhibits DNA synthesis in fission yeast (Fraser & Creanor, 1974). Cannon & Jimenez (1974) reported inhibition by lomofungin of DNA synthesis in budding yeast, and suggested that this might be caused by the especial sensitivity to lomofungin of RNA synthesis particularly involved in DNA synthesis. This suggestion is made unlikely by the observation by Pavletich *et al.* (1974) that DNA polymerase is directly inhibited *in vitro* by lomofungin.

Protein synthesis, which requires Mg^{2+}, is not immediately inhibited in yeast by lomofungin (Cano *et al.*, 1973; Cannon *et al.*, 1973; Fraser *et al.*, 1973) or by 8-hydroxyquinoline (Fraser & Creanor, 1974). However, in vertebrate cells both 8-hydroxyquinoline (Fraser & Creanor, 1974) and lomofungin (R. S. S. Fraser & J. Creanor, unpublished work) inhibit protein synthesis as rapidly as they inhibit RNA synthesis. These findings suggest that there is nothing intrinsic to lomofungin or 8-hydroxyquinoline which makes them selective inhibitors of nucleic acid synthesis in yeast. In an earlier paper (Fraser & Creanor, 1974), we suggested that the pH of yeast cytoplasm might be too low for significant chelation by 8-hydroxyquinoline. Under these circumstances protein synthesis would not be inhibited. There is evidence that the pH of the yeast nucleus may be higher than that of the cytoplasm (Conway & Downey, 1950) which would permit chelation in the nucleus and thus selective inhibition of RNA synthesis. In vertebrate cells the average internal pH is much higher than that of yeast (Caldwell, 1956). 8-Hydroxyquinoline and lomofungin would be expected to be capable of chelating in both nucleus and cytoplasm, thus inhibiting both RNA and protein synthesis.

The suggestion by Pavletich *et al.* (1974) that lomofungin inhibits RNA synthesis solely by chelating the Zn^{2+} tightly bound to the RNA polymerase might appear an attractive explanation of why lomofungin does not inhibit yeast protein synthesis. However, their model does not explain the rapid inhibition of protein synthesis in vertebrate cells (Fraser & Creanor, 1974) and does not allow for the ability of lomofungin to chelate Mg^{2+} as well as Zn^{2+} (Figs. 1 and 3).

This work was supported by the Medical Research Council and Science Research Council. We thank Professor J. M. Mitchison and Dr. M. E. Bindloss for useful discussions.

References

Adman, R., Schultz, L. D. & Hall, B. D. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 1702–1706

Brogt, T. M. & Planta, R. J. (1972) *FEBS Lett.* **20**, 47–52

Burgess, R. R. (1971) *Annu. Rev. Biochem.* **40**, 711–740

Caldwell, P. C. (1956) *Int. Rev. Cytol.* **5**, 229–277

Cannon, M. & Jimenez, A. (1974) *Biochem. J.* **142**, 457–463

Cannon, M., Davies, J. E. & Jimenez, A. (1973) *FEBS Lett.* **32**, 277–280

Cano, F., Kuo, S.-C. & Lampen, J. O. (1973) *Antimicrob. Ag. Chemother.* **4**, 23–34

Conway, E. J. & Downey, M. (1950) *Biochem. J.* **47**, 355–359

Fraser, R. S. S. & Creanor, J. (1974) *Eur. J. Biochem.* **46**, 67–73

Fraser, R. S. S., Creanor, J. & Mitchison, J. M. (1973) *Nature (London)* **244**, 222–224

Gottlieb, D. & Nicholas, G. (1969) *Appl. Microbiol.* **18**, 35–40

Hartwell, L. H. & McLaughlin, C. S. (1968) *J. Bacteriol.* **96**, 1664–1671

Hollingshead, R. G. W. (1954) *Oxine and its Derivatives*, vol. 1, Butterworth, London

Johnson, L. E. & Dietz, A. (1969) *Appl. Microbiol.* **17**, 755–761

Lampen, J. O., Kuo, S.-C. & Cano, F. (1973) in *Yeast, Mould and Plant Protoplasts* (Villanueva, J. R., Garcia-Acha, I., Gascon, S. & Uruburu, F., eds.), pp. 143–156, Academic Press, London and New York

Medoff, G., Kobayashi, G. S., Kwan, C. N., Schlessinger, D. & Venkov, P. V. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 196–199

Mitchison, J. M., Creanor, J. & Sartirana, M.-L. (1973) in *Yeast, Mould and Plant Protoplasts* (Villanueva, J. R., Garcia-Acha, I., Gascon, S. & Uruburu, F., eds.), pp. 229–245, Academic Press, London and New York

O'Sullivan, W. J. (1969) in *Data for Biochemical Research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M., eds.), 2nd edn., pp. 423–434, Oxford University Press, Oxford

Pavletich, K., Cano, F. & Lampen, J. O. (1974) *Biochem. Biophys. Res. Commun.* **60**, 942–950

Ponta, H., Ponta, U. & Wintersberger, E. (1971) *FEBS Lett.* **18**, 204–208

Ponta, H., Ponta, U. & Wintersberger, E. (1972) *Eur. J. Biochem.* **29**, 110–118

Price, R. & Penman, S. (1972) *J. Mol. Biol.* **70**, 435–450

Roeder, R. G. & Rutter, W. J. (1969) *Nature (London)* **224**, 234–237

Scrutton, M. C., Wu, C. W. & Goldthwait, D. A. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 2497–2501

Soskova, L., Soska, J. & Svoboda, A. (1970) *Folia Microbiol. (Prague)* **15**, 442–447

Tønnesen, T. & Friesen, J. D. (1973) *J. Bacteriol.* **115**, 889–896

Valenzuela, P., Morris, R. M., Faras, A., Levinson, W. & Rutter, W. J. (1973) *Biochem. Biophys. Res. Commun.* **53**, 1036–1041

Weinmann, R. & Roeder, R. G. (1974) *Proc. Nat. Acad. Sci. U.S.* **71**, 1790–1794