Inhibition and Activation of Polynucleotide Phosphorylase by Acridine Orange*

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Preliminary studies by Beers et al. (1) have shown that the zinc salt of acridine orange can either inhibit or accelerate the rate of polymerization of polynucleotides by polynucleotide phosphorylase of Micrococcus lysodecticus, depending upon the experimental conditions. We have now confirmed these findings with the zinc-free dye. Some evidence for a reversal of inhibition with degraded ribonucleic acid by acridine orange has also been obtained (2). Acridine orange forms two classes of complexes with polynucleotides. The first complex (Complex I) occurs with the nucleotides along the chain of the polymer and presumably involves both the phosphate and base of each nucleotide unit (3, 4). The second complex (Complex II) apparently involves only the terminal phosphate group of the polymer (1, 5). The binding of the dye by the two kinds of sites of the polymer is influenced by such factors as Mg, ionic strength, and pH (5), all of which also influence the polymerization reaction of catalyzed polynucleotide phosphorylase.

In view of the specific nature of these reactions of acridine orange a more detailed study of the effects of the dye on polymerization of polynucleic acid has been undertaken. It was hoped that a correlation of the effects of Mg, KCl, and polymer on the action of the dye in the enzyme system and on the dye binding properties of polyadenylic acid would give some indication of the site or sites of action of the dye. To a limited extent this objective has been accomplished.

EXPERIMENTAL

Acridine orange, supplied by the National Aniline Division of the Allied Dye and Chemical Corporation, contains 1 mole of zinc per mole of dye, a fact not specified by the distributor of this dye.1 Crystallization of the free base in 1 M NH₄OH leaves the metal in solution as the ammonium complex, which can be washed free from the crystals of dye. The free base of the dye is relatively insoluble in water and can be converted to soluble hydrochloride form with HCl. Two crystallizations of the dye have been found to be sufficient to remove all the zinc, as evidenced by failure of the dye to produce a zinc complex with diphenylthiocarbazone in CCl₄ at pH 6.0 (aqueous phase). A stock solution of the dye was prepared as the hydrochloride, final pH 4.0, at a concentration of approximately 0.04 M. Specific concentrations of the dye were made by dilutions with distilled water. The concentration of the dye was estimated from its absorption at 495 nm, using the extinction coefficient calculated from the data of Zanker (6): 4.9 × 10⁴. The dye solution follows the Beer-Lambert law up to an optical density of 0.4, the region in which the concentrations were determined.

Methylene blue-HCl (National Aniline Division) was used in a few preliminary studies without purification. No metal was detected in this product. Concentrations of the dye were estimated from its absorption at 665 nm, with the extinction coefficient of 7 × 10⁴ estimated from the data of Wouterspoon and Oster (7).

Polynucleotide phosphorylase was prepared from M. lysodeiciticus as described elsewhere (8) with some modifications. After lysis of 10% suspensions of the cells (20- to 40-g lots) in 0.5% NaCl at pH 8.0, a broad (NH₄)₂SO₄ fractionation was made between 0.3 and 0.65 saturation. The precipitate was dissolved in 0.1 M Tris, pH 8.0, and dialyzed against distilled water for 8 to 12 hours in the cold. The solution was lyophilized and stored in the deep freeze. Solutions (1%) of the lyophilized material in the Tris buffer were fractionated between 0.43 and 0.57 saturation with (NH₄)₂SO₄. The precipitate was dissolved in the same buffer and fractionated stepwise with acetone at -5°. Considerable variation in the activity distribution pattern of the enzyme occurs in the acetone fractionation procedure, but the major activity of the enzyme is in the 40 to 50% acetone fraction. The acetone precipitate was dissolved in Tris buffer and refractionated with (NH₄)₂SO₄ between 0.43 and 0.57 saturation. The final precipitate was dissolved in a small volume of the Tris buffer and dialyzed against the same buffer for several hours. These preparations contained adenylate kinase activity, but no demonstrable phosphatase or nuclease activity toward the substrate or the “product.” Only trace quantities of nucleotide material were present: 280:260 nm ratios varied from 1.3 to 1.64.

Although a variety of enzyme preparations have been used in these studies, only the results obtained with E133 and E134 are described in this paper. In an effort to reduce still further the possible contamination of the enzyme preparations by the polynucleotides, several procedures involving specific adsorption of the nucleotide material have been examined, the details of which will be presented in a later paper. The polynucleotide phosphorylase preparation, E134, has been treated with Norit A; final 280:260 nm ratio, 1.66.

A crude preparation of polynucleotide phosphorylase was also prepared from lysed cells of Bacillus subtilis. After lysis in 0.5% NaCl with lysozyme at pH 8.0 the preparation was centrifuged at 15,000 r.p.m. for 30 minutes. The supernatant was fractionated between 0.3 and 0.5 saturation with (NH₄)₂SO₄,

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The precipitate was dissolved in 0.1 M Tris, pH 8.0. The 280/290 m\textsuperscript{u} ratio of the preparation was 0.5.

Polyadenylic acid was synthesized with \textit{M. lysodeikticus} poly-nucleotide phosphorylase and isolated as described elsewhere (9). The polymer was dissolved in distilled water and the concentration determined from the organic phosphate content. Enzyme assays based on the rate of inorganic orthophosphate production were performed as described elsewhere (8). Protein and nucleic acid content of the enzyme preparations were estimated from the optical densities at 280 and 260 m\textsuperscript{u}, with the data reported by Warburg and Christian (10) for mixtures of enolase and RNA.

**RESULTS**

\textbf{Preliminary Studies}—Although much of the early work with acridine orange was performed with the zinc salt of the dye, the influence of the zinc at the concentration of dye used was negligible because of the high pH used in these studies (pH 9 to 9.5). However, at pH 7 to 8 the metal competes effectively with the dye for the Complex II sites. Moreover, it can replace Mg as the activating metal in the low pH region. The activating effect of the dye in the polymerization of polyuridylic acid at low pH, reported previously to occur in the absence of Mg (11), resulted from the activating effect of the metal associated with the dye. Studies with the free dye under similar experimental conditions failed to show any activation.

\textbf{Acridine Orange-activity Curve}—The shape of the activity curve of polynucleotide phosphorylase with increasing concentrations of acridine orange depends upon several factors which will be considered below. Fig. 1, Curve a, gives the results of an experiment with an uncomplicated system.

An apparent "threshold" concentration of the dye is required before any significant inhibition of the enzyme occurs. At intermediate concentrations of the dye, the system is markedly sensitive to the action of the dye. When corrected for the threshold quantity of dye, the concentration of dye giving 50% inhibition, I\textsubscript{50}, is approximately 8 \textmu{}M. However, at higher concentrations of the dye the inhibition reaches a limiting value of only 80%. The "residual activity" as shown below is an artifact.

RNA or polyadenylic acid can reverse the inhibition by the dye (1). Therefore, it is possible that the contaminating polynucleotides in the enzyme preparation protect the enzyme against low dye concentrations. We should expect that varying the enzyme concentration would vary the threshold concentration of the dye. However, the concentration of the polynucleotide material in the assay mixture is too low to account for this effect (approximately 0.6 \textmu{}M compared with 10 \textmu{}M dye). Moreover, a 3-fold increase in the concentration of the enzyme does not significantly change the threshold concentration of the dye, provided the extent of the reaction in the absence of the dye is the same with the different enzyme concentrations, i.e. the reaction time is decreased to one third with the higher enzyme concentration.

The second possibility is a trimolecular reaction involving 2 dye molecules. Indeed, some of the experimental curves obtained coincided very closely with a theoretical curve derived from the equation for a trimolecular reaction. However, these findings were fortuitous and could not always be duplicated.

Preincubation of the enzyme does not change the general characteristics of the acridine orange-activity curve, although the flat plateau region at the low dye concentration is often replaced by a sloping curve. Comparison of the shape of the dye-activity curves in Fig. 1 with those to be discussed below points out marked variation in the steepness of the slope of the threshold region and of the inhibitory region of the dye in different experiments.

\textbf{Effect of Acridine Orange on Time Course of Polymerization Reaction}—In Fig. 2 is shown the time course of the polymerization reaction with different concentrations of acridine orange. With the exception of the highest dye concentration, 40.8 \textmu{}M (Curve e), the rate of polymerization shows an initial lag (Curves a and b) or a marked autocatalytic character (Curves c and d). The inhibition of the dye decreases with increasing concentration of polymer during the course of the reaction. Curve a actually shows a short period of activation by the dye after the initial inhibitory phase is overcome, a finding observed on several occasions. Significantly, however, unless the concentration of the polymer exceeds the concentration of the dye during the 30 minutes of the reaction time, the inhibition of the dye is not reversed. In Curve e, for example, the final concentration of the polymer at 30 minutes is 33\% m, whereas that of the dye is 40.8 \textmu{}M.

Also shown in Fig. 2 is the reaction in presence of 40.8 \textmu{}M acridine orange with 1.25 mm ADP to which polyadenylic acid has been added after 10 minutes of reaction time, final concentration 0.95 mm. The inhibition is promptly and completely reversed by the polymer. (The data have been normalized to permit the use of the same control curve for both sets of experiments).

These studies show that the observed inhibition by the dye during a given period of time depends upon the rate of polymerization reaction and the time allowed for assay. The inhibition is not constant but decreases during the reaction. Thus, any variable which decreases or increases the rate of the reaction will increase or decrease, respectively, the observed degree of inhibition of the dye. It is apparent, therefore, that
effectively reverses the inhibition by the dye during the polymerization reaction, it is necessary to measure the relative extent of inhibition by the dye under conditions in which the amount of polymer produced in the controls during the assay time is the same for each change in rate resulting from a change in one or more variables (other than dye). This condition is inherent in a single dye-activity curve in which all the variables but the dye are constant. However, to compare different dye-activity curves it is necessary to vary either the time or the concentration of the enzyme to assure a constant amount of polymer produced in each of the controls without dye. The reaction with the dye is allowed to proceed for the same length of time as is the corresponding control.

In Fig. 1 is shown the effect of varying ADP concentration on the acridine orange-activity curve. The time of the reactions was varied in the controls. Polynucleotide phosphorylase preparation E134 was used. Both the level of activity during and the extent of the “threshold” region increases with increasing ADP concentration. These results indicate that the magnitude of the “threshold” region reflects both the continual reversal of the inhibition by the polymer being synthesized and some other factor which increases with ADP concentration. The possibility that this additional factor represents an increase in the concentration of polymer molecules is discussed below.

Effect of ADP on Acridine Orange-activity Curve—Earlier, it was shown that, with the zinc dye, increasing ADP concentration partially reversed the inhibition by the dye (time of assay constant, polymer production increasing) (1). These studies
were repeated with zinc-free dye and gave the same results. In Fig. 4 is shown the amount of inorganic orthophosphate released in 20 minutes at varying ADP concentrations with different levels of dye concentration. At low dye concentration there is a loss of polymer independent of the ADP concentration. The "rates" of the reaction are corrected for this loss of polymer, the Michaelis and maximum velocity constants are independent of the dye concentration. At a higher concentration this relationship is replaced by a continuous curve which represents the "residual activity" of the enzyme, a value that increases with ADP concentration.

**Effect of Polyadenylic Acid on Inhibition of Acridine Orange**—Polyadenylic acid activates the polymerization reaction if the salt concentration is sufficiently high to produce inhibition (5). The activating effect is more marked at low than at high Mg concentration which suggests a reciprocal relationship between ionic strength and Mg that involves a Mg-polymer complex. The shape of the polyadenylic acid-activity curve is similar to that observed with KCl or Mg (Fig. 5A, dashed curves). It is not known whether this activation by polyadenylic acid represents a reversal of inhibition by residual RNA contaminates (2), a primer effect (12), or a nonspecific effect associated with the polyelectrolyte character of the polymer. Similar activation can be demonstrated with yeast RNA at high salt concentration, provided the period of incubation of this enzyme with the RNA is brief.

The activating effect of polyadenylic acid is even more marked in the presence of the dye. Fig. 5A shows the rate of polymerization in the presence and absence of 0.27 mM dye. Fig. 5B shows the percentage of activity of the reaction in presence of the dye as compared with the reaction in the absence of dye.

In one set of experiments (X——X the concentration of Mg was very high, 3.3 mM with 0.4 mM KCl. In the other set of experiments (●——●) the Mg concentration was limiting, 0.157 mM, with no KCl.

The inhibition by the dye is reversed when the dye polymer ratio is approximately one. A slightly higher concentration of polymer is required at the low Mg and KCl concentration. At polymer dye ratios greater than one, the rate of polymerization is increased by as much as 90%. When the polymer-dye ratio exceeds 2 the activating effect of the dye is considerably reduced. The similarity in the position of the optimum polymer concentration in the absence of the dye and optimum dye-polymer ratio in the activity curve is coincidental, inasmuch as, at the lower dye concentrations the optimum concentration of polymer activation is lower.

**Acridine Orange-activity Curve with B. subtilis Polynucleotide Phosphorylase**—The activating effect of the dye in the presence of slight excess of polyadenylic acid can also be demonstrated with a B. subtilis polynucleotide phosphorylase preparation.
containing a substantial quantity of RNA (280:260 nm ratio 0.5). This emphasizes the fact that the quantity of dye required to inhibit a particular enzyme preparation will depend upon the concentration of polynucleotide material present.

*Effect of Mg on Acridine Orange-activity Curve*—Fig. 6 shows the effect of two concentrations of Mg on the acridine orange-activity curve. The prevention of dye inhibition by Mg is clearly evident.

**Effect of Acridine Orange on Mg-activity Curve**—The shape of the Mg-activity curve in the presence and absence of acridine orange is shown in Fig. 7. In this experiment the change of activity of the enzyme with varying Mg concentration was not corrected for the variation in the amount of polymer formed at the end of the assay period, a constant interval. Consequently, some of the reversal of inhibition observed at the suboptimal Mg concentration is the result of the autocatalytic reversal mechanism. However, the experiment shows the reversal of Mg inhibition at higher dye concentrations. This is a variable finding with different enzyme preparations that may be related to the RNA content of the enzyme preparation. One enzyme preparation, E129, showed a 2-fold increase in the rate with the addition of acridine orange at the high Mg concentration. A second variable is the degree of inhibition by Mg at high concentrations.

In Table I are recorded the extent of polymer synthesis at three Mg concentrations with and without acridine orange. The experiment was performed with E134. The concentration of the enzyme was varied to give approximately the same rates for each Mg concentration in the controls, except for the lowest concentration where the range of polymer production straddled the values obtained with higher Mg concentration. The ability of Mg to reverse the inhibition by the dye independently of the catalytic effect of the metal is clearly shown.

**Effect of KCl on Acridine Orange-activity Curve**—The major effect of increasing or decreasing the KCl concentration from 0.2 M appears to be to decrease or increase, respectively, the threshold concentration of the dye, i.e. in the ability of a given amount of polymer to reactivate the enzyme.

**Effect of Acridine Orange on KCl-activity Curve**—Fig. 8 shows the salt activation curve of E134 without acridine orange and the percentage of inhibition by the dye as a function of KCl concentration. The presence of the dye sharpens the KCl

<table>
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<th>MgCl₂ × 10⁻⁴ M</th>
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<th>4</th>
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<td>Rate of control × 10⁻⁴ M per min</td>
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<td>1.08</td>
<td>1.21</td>
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<tr>
<td>Rate of inhibited enzyme × 10⁻⁴ M per min</td>
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<td>0.59</td>
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<td>Per cent inhibition</td>
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<td>45</td>
<td>28</td>
<td>10</td>
</tr>
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</table>

* Reaction mixture contained 0.2 M KCl, 17 mM Tris, pH 9.0, 0.101 mM ADP, 0.0175 mM acridine orange or water, temperature, 37°.

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**Fig. 6.** Effect of Mg on the acridine orange-activity curve. Enzyme E133; 3 ml of reaction mixture contained 0.012 M Tris at pH 9.0, 0.2 mM ADP, 0.2 mM KCl; the temperature was 37°; 0.33 mM MgCl₂, 0.101 mM polyadenylic acid synthesized during 10-minute assay of control; 1.7 mM MgCl₂, 0.109 mM polyadenylic acid synthesized during assay of control.

**Fig. 7.** Effect of acridine orange on the Mg-activity curve. Enzyme E132; 3 ml of reaction mixture contained 0.017 mM Tris at pH 9.0, 0.2 mM KCl, 1 mM ADP; the temperature was 37°. ○—○, Control; ●—●, 68 μg acridine orange; X—X, % activity of dye treated system.
curve and shows a minimum inhibition at a KCl concentration corresponding approximately to the optimum value. The increase in inhibition at high KCl concentration may be related to the activating effect of the dye at critical polymer concentrations.

Effect of pH on Inhibition of Acridine Orange—Three acridine orange-activity curves at pH 7, pH 8, and pH 9 were determined. Both the time and concentration of the enzyme were varied to produce identical rates of polymerization at each pH. The inhibition by the dye is greatest at low pH.

Methylene Blue Inhibition. Methylene blue forms the same two classes of complexes with polynucleotides (5). Therefore, if the mechanism of inhibition by acridine orange is through specific reactions with polynucleic acid, similar results should be expected with methylene blue. A few preliminary experiments have been performed to confirm this. Methylene blue shows the same kind of inhibitor-activity curve with the threshold and residual activity regions. With E133 0.66 mM MgCl₂ and 0.2 mM KCl at pH 9.0, 48 µM inhibited the reaction 84.6%. The increase of the Mg concentration to 6.6 mM decreased the inhibition by methylene blue concentration to only 24%. The addition of polyadenylic acid reverses the inhibition completely.

DISCUSSION

These results show that when the concentration of polyadenylic acid nucleotide units exceeds the concentration of the dye by a few per cent, no inhibition by acridine orange occurs. Therefore, the binding of the dye by the majority of sites of the polymer does not result in any inhibition. Indeed, as shown in Fig. 5D the dye appears to increase the reactivity of the polymer. The activating effect of the dye disappears when the ratio of nucleotide bases to dye exceeds 2. The mechanism of activation by the dye cannot be clearly understood until the mechanism of activation by the polymer is known. It is possible in this instance that the added polymer acts as a buffer to maintain the proper ratio of dye to polymer during a significant portion of the assay period. This would result in a prolonged activating effect by the dye which could exist only briefly with lower concentrations of polymer (cf. Fig. 2, Curve a). A possible mechanism of activation by the dye may be the neutralization of the negative charges of the polymer by the cation dye. A similar method of activation by the shielding effect of KCl has been proposed (8). The activating effect of the dye on B. subtilis polynucleotide phosphorylase resembles the findings with mixtures of polyadenylic acid and M. lysodeikticus polynucleotide phosphorylase.

Prompt and nearly complete inhibition occurs when the dye to polymer ratio exceeds one, but this ratio increases with Mg concentration. Small quantities of dye produce little or no inhibition. These results mitigate against the possibility that inhibition occurs via the formation of Complex II as proposed previously from preliminary studies (1). The increase in inhibition at low pH does not conform to the pattern of dye binding observed in the formation of Complex II. Finally, and most important, it is difficult to see how the formation of Complex I could reverse the inhibition caused by Complex II, since the former does not exist until the available Complex II sites are filled (5). There remains the possibility, however, that under the experimental conditions used, the major fraction of Complex II is not formed until the Complex I sites have been saturated. The shape of the inhibition curve with acridine orange and varying KCl and Mg would suggest the Complex II is the mechanism of inhibition.

The hypothesis for a Complex II site as distinct from Complex I sites has been discussed elsewhere (5). The fact that it is inert in the inhibition and activation studies makes the arguments for or against (13) this hypothesis of less importance in these studies. However, in the interpretation of these studies, the inert feature of the 5'-phosphate group in both polymerization and phosphorylysis of polyadenylic acid has been shown by the studies of Singer et al. (12). In this respect the present studies are consistent with the hypothesis for a specific Complex II site, i.e. the 5'-phosphate group at the end of the polymer chain.
The dye could inhibit by reacting with some active site other than on the polymer. As pointed out elsewhere (5), little free dye exists in the solution until all of the Complex I and most of the Complex II sites are filled. This small quantity of “free dye” increases with ionic strength. Thus, increasing the salt concentration should cause only an increase in inhibition. The effect of salt on dye inhibition is not consistent with this. Furthermore, the inhibition is not complete until after the initiation of the polymerization reaction. Since the major new species appearing in the reaction mixture is the polymer (orthophosphate has no effect on the dye properties with or without Mg aside from that attributable to the ionic contribution of the phosphate), it is most probable that the dye inhibits either by reacting with the polymer, which then inhibits the enzyme, or the dye reacts with the polymer-enzyme complex.

The third possible mechanism of inhibition is the formation of Complex I with the terminal nucleotide bases. We have no data at present to evaluate this possibility because we know nothing about the reactivity of the terminal Complex I sites. Inhibition may occur when the dye binds the last two or three sites at the 3'-OH end of the chain, the point of attachment of new nucleotide units (12).

The ability of the enzyme ADP system to overcome the inhibition by the dye is a function of the rate and extent of production of polymer. For a given dye concentration and over a wide range of ADP concentration, there is a net constant loss of polyadenylic acid synthesis compared with that of the control. This is true provided the inhibition of the polymerization in the presence of the dye has been overcome during the time of assay. The reason for this phenomenon is inherent in the mechanism of reversal of inhibition. Irrespective of the rate at which polymerization occurs or the manner in which the rate is varied, i.e. enzyme or substrate concentration, the inhibition is effectively reversed when the polymer concentration equals the dye concentration. Moreover, the extent of inhibition during this recovery phase is a function of the polymer concentration at any given time. Therefore, a constant lag in the production of polymer will take place. At higher dye concentrations where the inhibition is not successfully overcome during the period of assay, either because of the excess dye or because the rate of reactivation by the polymer is too slow, this constant relationship between net loss of polymer and dye concentration is lost.

An additional factor in the recovery process also exists. This is illustrated in Fig. 1. The recovery resulting from the synthesis of polymer has been compensated for in this experiment. Nevertheless, an increase in the substrate concentration reverses the inhibition slightly. One possible explanation for this phenomenon is the increase in the concentration of polymer molecules with increasing ADP concentration (5). If, as has been postulated elsewhere (10), the major limiting component in this system is the concentration of polymer molecules, then increasing this concentration with increasing ADP concentration could result in some reversal of dye inhibition. Studies now in progress show that at high salt concentration the activating effect of polyadenylic acid is associated with decrease in the Michaelis constant for ADP and no significant change in the maximum velocity constant.

**SUMMARY**

The inhibition and activation of polyadenylic acid phosphorylase by acridine orange has been studied as a function of several variables known to influence the enzymatic reaction and the binding of the dye by polynucleotides. The rate of polymerization reaction is autocatalytic in the presence of intermediate concentrations of the dye, but the inhibition is not complete until after the synthesis of some polymer has occurred. When the concentration of polymer bases exceeds that of the dye, the inhibition is reversed; at polymer dye ratios of approximately unity the dye strongly activates the enzyme. Inhibition is reversed by increasing or decreasing the salt concentration to approximately 0.1 to 0.2 m KCl. It is concluded that inhibition occurs via the formation of a polymer-dye complex that probably involves the phosphate groups at the 3'-OH end of the chain; activation occurs via the formation of dye-polymer complexes along the remainder of the chain and may be the result of a neutralization of the negative charges.

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