References

Bellamy, A. R., Shapiro, L., August, J. T., and Joklik, W. K. (1967), *J. Mol. Biol. 29*, 1.
Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S. (1967), *J. Mol. Biol. 26*, 373.
Bram, S., and Beeman, W. W. (1971), *J. Mol. Biol. 55*, 311.
Chrambach, A., and Rodbard, D. (1971), *Science 172*, 440.
Crawford, L. V., and Waring, M. J. (1967), *J. Mol. Biol. 25*, 23.
Dahlberg, A. E., Dingman, C. W., and Peacock, A. C. (1969), *J. Mol. Biol. 41*, 139.
Dingman, C. W., Kakefuda, T., and Aronow, A. (1970), *Biochim. Biophys. Acta 224*, 114.
Dingman, C. W., and Peacock, A. C. (1968), *Biochemistry 7*, 659.
Dingman, C. W., and Sporn, M. B. (1967), *Cancer Res. 27*, 938.
Eigner, J., and Doty, P. (1965), *J. Mol. Biol. 12*, 549.
Ferguson, K. A. (1964), *Metabolism 13*, 985.
Fisher, M. P., and Dingman, C. W. (1971), *Biochemistry 10*, 1895.
Green, M., Piña, M., Kimes, R., Wensink, P. C., MacHattie, L. A., and Thomas, C. A., Jr. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1302.
Haselkorn, R. (1964), *Science 143*, 682.
Josse, J., and Eigner, J. (1966), *Annu. Rev. Biochem. 35*, 789.
Kurland, C. G. (1960), *J. Mol. Biol. 2*, 83.
Loening, U. E. (1969), *Biochem. J. 113*, 131.
Loening, U. E., and Ingle, J. (1967), *Nature (London) 215*, 363.
MacHattie, L. A., and Thomas, C. A., Jr. (1964), *Science 144*, 1142.
Neville, D. M., Jr. (1971), *J. Biol. Chem. 246*, 6328.
Peacock, A. C., and Dingman, C. W. (1967), *Biochemistry 6*, 1818.
Peacock, A. C., and Dingman, C. W. (1968), *Biochemistry 7*, 668.
Richards, E. G., and Lecanidou, R. (1971), *Anal. Biochem. 40*, 43.
Rodbard, D., and Chrambach, A. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 970.
Rodbard, D., and Chrambach, A. (1971), *Anal. Biochem. 40*, 95.
Rosset, R., Monier, R., and Julien, J. (1964), *Bull. Soc. Chim. Biol. 46*, 87.
Sinsheimer, R. L. (1959), *J. Mol. Biol. 1*, 43.
Sporn, M. B., and Dingman, C. W. (1963), *Biochim. Biophys. Acta 68*, 389.
Sporn, M. B., Dingman, C. W., Phelps, H. L., and Wogan, G. N. (1966), *Science 151*, 1539.
Strauss, J. H., Jr., and Sinsheimer, R. L. (1963), *J. Mol. Biol. 7*, 43.
Takahashi, M., Ogino, T., and Baba, K. (1969), *Biochim. Biophys. Acta 174*, 183.
Thorne, H. V. (1967), *J. Mol. Biol. 24*, 203.
Trilling, D. M., and Axelrod, D. (1970), *Science 168*, 268.
Vinograd, J., Lebowitz, J., Radloff, R., Watson, R., and Laipis, P. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 1104.
Waring, M. J. (1966), *Biochim. Biophys. Acta 114*, 234.

# Subunits of Ribonucleic Acid Polymerase in Function and Structure. I. Reversible Dissociations of *Escherichia coli* Ribonucleic Acid Polymerase†

Akira Ishihama

ABSTRACT: Two systems for reversibly dissociating DNA-dependent RNA polymerase from *Escherichia coli* have been developed by use of *p*-chloromercuribenzoate and urea. A subunit complex which retains the binding activities of ribonucleoside triphosphates and an antibiotic rifampicin was isolated by treatment of the enzyme with *p*-chloromercuribenzoate, and found to be composed of $\beta$ and $\beta'$ subunits. Thus, the site on the enzyme participating in the substrate-binding reaction appeared to reside in $\beta$ and/or $\beta'$ subunit. On the other hand, low concentrations of urea dissociated the enzyme into $\alpha$–$\beta$ and $\alpha$–$\beta'$ complexes. The observation that both fragments were capable of binding template DNA suggest that not only $\beta'$ but also $\beta$ subunit may possess the DNA-binding site. Although these are all reversible reactions, the retention of the activity to carry out part of the polymerase reactions was essential for reassociations to form active enzyme.

The complex structure of DNA-dependent RNA polymerase of *Escherichia coli* has received considerable attention. The studies of Burgess and coworkers (Burgess *et al.*, 1969; Burgess, 1969) have demonstrated that the enzyme is composed of at least four different polypeptide chains, $\alpha$, $\beta$, $\beta'$, and $\sigma$. There is also the possibility that another component, called $\omega$, is a part of the holoenzyme structure. Based on the known molecular weight and the relative content of these components, it appears that the holoenzyme has the structure $\alpha_2\beta\beta'\sigma(\omega)$. The subunit $\sigma$ can be dissociated from the enzyme and the resulting core enzyme with the structure $\alpha_2\beta\beta'(\omega)$ has been shown to possess all of the enzymatic activities associated with the polymerase. However, it has been shown that the core enzyme can hardly initiate RNA synthesis from intact

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duplex DNA (Vogt, 1969; Ishihama *et al.*, 1971). Thus, the $\sigma$ subunit is essential for physiologic transcription.

We have concerned ourselves with an attempt to define the sites on the enzyme responsible for the different catalytic activities characteristic of the polymerase system. In previous communication (Ishihama and Hurwitz, 1969a) chemical alteration of the enzyme was shown to result in preparations capable of catalyzing only some of the discrete reactions of the polymerase system. The present study is concerned with the isolation of fragments of RNA polymerase which can catalyze one or more of these discrete reactions. It was found that the holoenzyme treated with *p*CMB,[1] resulting in complete inactivation in terms of the overall reaction, yields a $\beta\beta'$ complex which retains the binding activities of ribonucleoside triphosphates and antibiotic rifampicin.

When the holoenzyme was treated with low concentrations of urea the enzyme was dissociated into 9S and 4.5S fragments. The 9S component retains the ability to bind template DNA. It has been found that the 9S material is a mixture of two different complexes of the structure $\alpha\beta$ and $\alpha\beta'$. On the basis of the subunit composition of the two partially active fragments, the distribution of some of the active sites among the different polypeptide chains has been proposed. In addition, reconstruction of fully active enzyme has been achieved from the isolated fragments.

The forthcoming paper will describe studies on the reconstruction of active enzymes from isolated subunits. Some of this work has been reported previously (Ishihama and Hurwitz, 1969a,b; Ishihama, 1969).

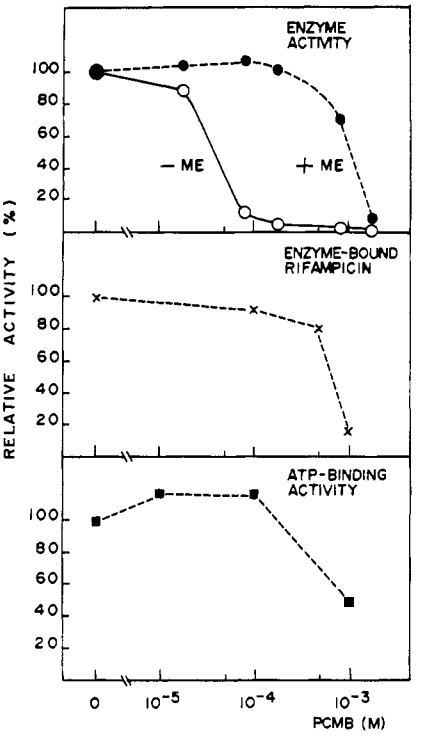


FIGURE 1: Inactivation of RNA polymerase by *p*CMB. (A, top) RNA polymerase previously dialyzed for 15 hr *vs.* 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, and 0.1 mM EDTA was treated with the indicated concentrations of *p*CMB for 10 min at 0° and aliquots were used to measure the incorporation of [$^{14}$C]ATP with and without 10 mM 2-mercaptoethanol. (B, middle) Dialyzed [$^{14}$C]rifampicin–enzyme complex isolated as in Figure 5 was treated with *p*CMB at the indicated concentrations and then subjected to electrophoresis in standard gels at pH 8.7 as shown in Figure 6. (C, bottom) The polymerase was treated as in part A and subjected to the assay of interaction with [$^{14}$C]ATP in the absence of 2-mercaptoethanol as described in Methods and Table I. The ATP-binding activities are represented as the relative values compared to untreated enzyme.

## Materials

Unlabeled and labeled ribonucleoside triphosphates were obtained from Schwarz BioResearch and Sigma, while $^{32}$P-labeled-$PP_i$ was prepared as described by Berg (1959). Calf thymus and *Escherichia coli* DNA preparations were isolated as described by Ishihama and Kameyama (1967). T7 phage DNA labeled with $^{14}$C or $^{3}$H was prepared by phenol extraction from T7 phage stock grown in the presence of [$^{14}$C]- or [$^{3}$H]thymidine (Yudelvich and Gold, 1969).

Protamine sulfate was obtained from Eli Lilly and Co., Ind., and Yuki Gosei Kogyo, Japan. Sephadex G-200 (fine grade), Sephadex G-25 (fine grade), Sepharose 6B, and DEAE-Sephadex A50 were purchased from Pharmacia. Phosphocellulose was a product of Whatmann Co. (P 11, 7.4 mequiv/g).

Acrylamide and *N,N'*-methylenebisacrylamide were purchased from Eastman. Solutions of recrystallized urea were freshly prepared before use and deionized by passing through columns of mixed resin of Amberlite IRC-50 and IRA-400, and Bio-Rad AG 501-X8, 20–50 mesh.

$^{14}$C-Labeled rifampicin was a product of Daiichi Pure Chemicals, Japan.

RNA polymerase of *E. coli* W was purified by a new procedure which includes stepwise elution of the enzyme from protamine precipitate by ammonium sulfate (Kameyama *et al.*, 1969) followed by DEAE-Sephadex A-50 column chromatography and gel filtration through Sepharose 6B column. The specific activity of the holoenzyme was 1000–2000, or 3000–6000 units per mg of protein using *E. coli* or T7 DNA as templates. In some experiments reported below, the enzyme was obtained as described by Maitra and Hurwitz (1967). The two enzyme preparations showed the same subunit pattern in polyacrylamide gel electrophoresis in the presence of urea, or SDS.

## Methods

*Assay Conditions.* To detect the RNA polymerase–DNA complex, modification of the nitrocellulose membrane method developed by Jones and Berg (1966) was employed. The standard reaction mixture contained, in a final volume of 0.25 ml, 12.5 $\mu$moles of Tris-HCl (pH 8.0 at 4°), 1 $\mu$mole of $MnCl_2$, 1 $\mu$mole of 2-mercaptoethanol, $^{14}$C-labeled T7-DNA, and RNA polymerase or its subunits. The reaction was carried out as described previously (Ishihama and Hurwitz, 1969a).

The reversible interactions of RNA polymerase or subunit complexes with nucleotide substrates were studied by the gel filtration technique (Hummel and Dreyer, 1962). A Sephadex G-50 column (1 × 40 cm) was equilibrated with 4 mM $MgCl_2$, 4 mM 2-mercaptoethanol, and 50 mM Tris-HCl buffer (pH 7.8 at 4°), containing [$^{14}$C]ATP, and the enzyme or subunit complexes were then applied to the column and eluted with the same buffer according to Ishihama and Hurwitz (1969a).

The procedure of Ishihama and Kameyama (1967) was used for the assay of RNA synthesis. The standard reaction

[1] Abbreviations used are: *p*CMB, *p*-chloromercuribenzoate; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; ATP, adenosine 5′-triphosphate; XTP, nucleoside 5′-triphosphate; RNA polymerase, nucleoside 5′-triphosphate·RNA·nucleotidyl transferase (DNA dependent) (EC 2.7.7.6).

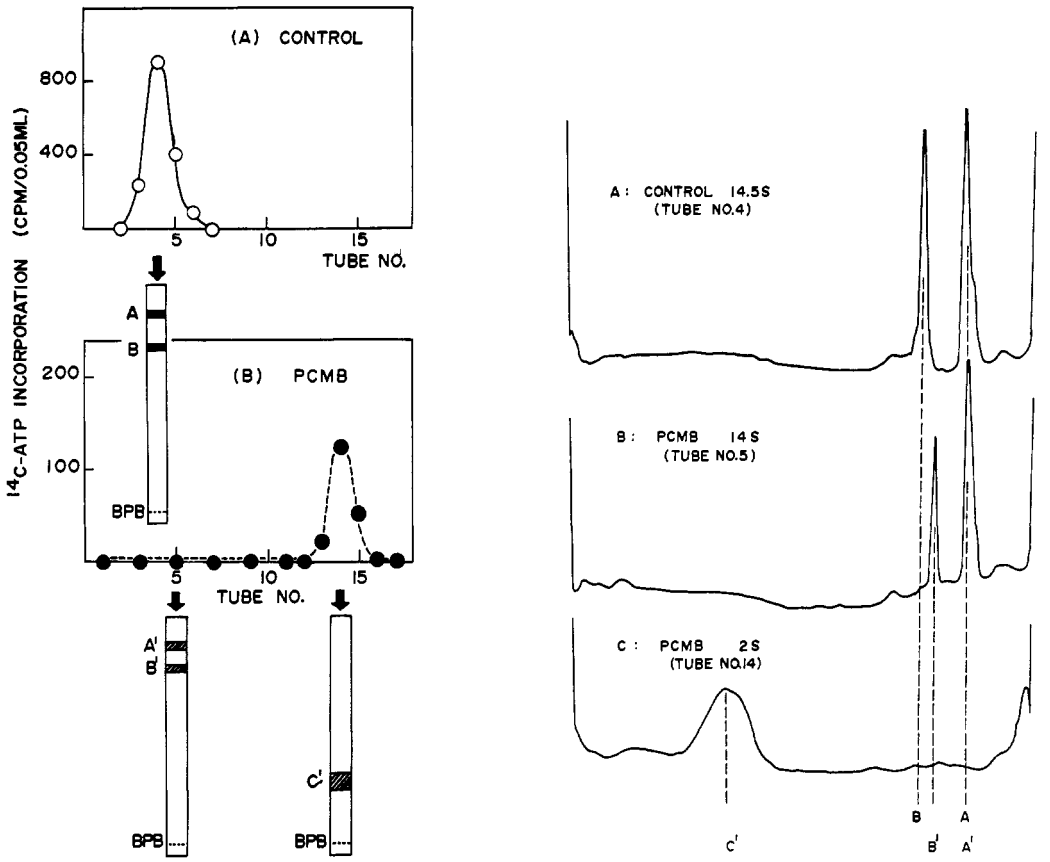


FIGURE 2: Separation and reconstruction of the units obtained by *p*CMB treatment. RNA polymerase (0.12 mg of protein) previously dialyzed against 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 0.1 mM EDTA was treated with $10^{-4}$ M *p*CMB and subjected to centrifugation at 60,000 rpm for 7 hr at 4° in 5 ml of 10–35% glycerol gradient containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 M KCl, and $10^{-4}$ M *p*CMB. After centrifugation, 0.01 ml of each fraction was subjected to electrophoresis in standard gels at pH 8.7. The 14S component thus obtained (tubes 3–6) gave two bands (A′ and B′) in electrophoresis as shown in part B (right) and in the illustrations of part A (left). An aliquot (0.05 ml) of the pooled 14S component (A′ and B′) was mixed with 0.05 ml from each of the fractions and was applied to the assay of RNA polymerase reaction after preincubation for 5 min at 30° in the presence of 10 mM of 2-mercaptoethanol (parts A and B). The 14.5S native polymerase was centrifuged in a separate gradient as a reference marker and gave 2 bands (A and B) on disc gel electrophoresis (part A).

mixture (0.25 ml) contained: Tris-HCl buffer (pH 7.8 at 37°, 30 μmoles), magnesium acetate (1.25 μmoles), manganese sulfate (0.5 μmole), 2-mercaptoethanol (1.25 μmoles), 40 nmoles each of GTP, CTP, and UTP; [$^{14}$C]- or [$^{3}$H]ATP (specific activity (3–5) × $10^6$ cpm per μmole, 40 nmoles), *E. coli* DNA (10 μg/tube), and RNA polymerase. The reaction mixture was incubated at 37° for 10 min, and one unit of enzyme activity is defined as that amount of protein which catalyzes the incorporation of 1 nmole of labeled ATP into RNA in 60 min at 37° under the above conditions.

*Polyacrylamide Gel Electrophoresis.* Polyacrylamide gels at pH 8.7 were prepared by the methods of Ornstein (1964) and Davis (1964). For the analysis of the subunit structure, deionized 8 M urea was added to all solutions and samples as described by Jovin *et al.* (1964). Gels containing 0.1% SDS were prepared and run according to the method of Shapiro *et al.* (1967). Gels were stained with Coomassie Brilliant Blue or Naphthol Blue Black, and scanned with Joyce-Loebl microdensitometer MK III. For the assay of RNA polymerase in gels, poly(AU) synthesis and its detection with ethidium bromide were carried out as described by Krakow *et al.* (1968). The detection of labeled rifampicin in gels was measured by liquid scintillation counting after extraction with a solution containing 0.1% sodium dodecyl sulfate–0.05 M NaOH–0.25 mM EDTA.

*Ultracentrifugal Analysis.* A Spinco Model E analytical ultracentrifuge with schlieren optical system was employed. Velocity sedimentation was carried out at 15° with the use of either a single- or double-sector 12-mm cell filled with Epon centerpiece in an An-D rotor. The schlieren patterns were photographed on Kodak metallographic plates and the plates measured on Nikon microcomparator. The sedimentation coefficients were converted to *s* values with corrections for KCl concentration from International Critical Tables (1962) and for urea solutions from the data of Kawahara and Tanford (1966).

## Results

*Reversible Dissociation of RNA Polymerase by p-Chloromercuribenzoate.* Treatment of RNA polymerase (holoenzyme) with reagents which react with sulfhydryl groups yields enzyme preparations that are unable to catalyze ribonucleotide incorporation, initiation of RNA synthesis, and binding reaction to template DNA (Ishihama and Hurwitz 1969a). However, such treated enzyme is still capable of binding nucleoside triphosphate substrates, as well as an antibiotic, rifampicin. The *p*CMB treatment has also been shown to cause the dissociation of a small subunit with a sedimentation velocity of 2 S from the holoenzyme molecule. As shown in Figure 1A, the

TABLE I: Interaction between Subunit Complexes and ATP.[a]

| Treatment of Enzyme | Subunit Component (Glycerol Fraction) | Rel Amt[b] of $\alpha$ Subunit (%) | Added Protein (mg) | Bound ATP (nmoles) |
|---|---|---|---|---|
| None | 15S native enzyme | 100 | 1.50 | 5.50 |
| $10^{-4}$ M *p*CMB | 14S fraction | 15 | 1.05 | 4.81 |
| | 2S fraction | | 0.15 | <0.01 |
| $10^{-4}$ M *p*CMB | 14S fraction | 10 | 0.85 | 3.89 |
| | 2S fraction | | 0.11 | <0.01 |

[a] Subunits of RNA polymerase treated with the indicated concentrations of *p*CMB were fractionated through glycerol gradient centrifugation as in Figure 2. The pooled fractions of each peak were concentrated with a Sartorius collodion bag and applied onto Sephadex G-50 columns to measure the binding activities of ATP as described in Methods. [b] Amounts of $\alpha$ subunit in 14S fraction were measured by SDS gel electrophoresis and compared to untreated RNA polymerase.

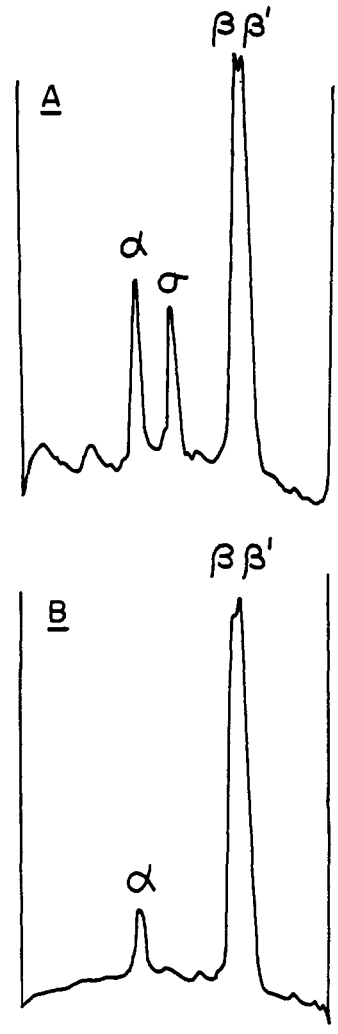


FIGURE 3: SDS–gel electrophoresis of the 14S unit obtained by *p*CMB treatment. (A) RNA polymerase (35 $\mu$g) was subjected to subunit analysis on a SDS gel. Protein migration is toward the anode, shown at the left. (B) The 14S unit (28 $\mu$g) obtained as described in Figure 2 was analyzed on a SDS gel following addition of 10 mM 2-mercaptoethanol. This preparation contained about 15% of the $\alpha$ band compared to the untreated polymerase though a 10% value was obtained in an another preparation.

effects of *p*CMB at concentrations less than $5 \times 10^{-4}$ M are completely reversible. Thus, if the 2S material is essential for activity, it must have been reassociated with the larger molecular weight component upon reversal of inhibition by 2-mercaptoethanol.

In order to identify which component possessed the substrate binding activity, the *p*CMB-treated preparation was subjected to glycerol gradient centrifugation in $10^{-4}$ M *p*CMB, and the two components (14S and 2S) was isolated as can be seen in Figure 2A. The 14S component was separated completely from the 2S component and neither fraction alone possessed enzymatic activity even after addition of 2-mercaptoethanol. When these two discrete fractions were combined, in the presense of 2-mercaptoethanol all the enzymatic activity was regained.

Since RNA polymerase contains the subunit structure $\alpha_2\beta\beta'\sigma(\omega)$, where the molecular weights of the subunits and chains are 39,900, 155,000, 165,000, 90,000 and 9000, respectively (Burgess, 1969), it was of particular interest to determine which of these subunits were present in the separated protein fractions. Figure 2B shows the gel patterns obtained when the 14S and 2S components were subjected to electrophoresis in standard gels at pH 8.7. The 14S components gave two bands, A′ and B′ in positions similar to A and B bands of the native enzyme, though B′ moved slightly slower than B. On the other hand, the 2S material gave a broad peak C′. The 14S and 2S components were then subjected to SDS gel electrophoresis after removal of *p*CMB with 2-mercaptoethanol (Figure 3). The 14S material contained $\beta$, $\beta'$, and a small amount of $\alpha$ subunit, while the latter yielded $\alpha$, $\omega$, and $\sigma$ subunits. Since about 15% of $\alpha$ subunit remained in 14S component, it appears to be either a mixture of a $\beta$–$\beta'$ complex and $\alpha$–$\beta$–$\beta'$ complex at a ratio of 2:1 or a mixture of a $\beta$–$\beta'$ complex and $\alpha_2$–$\beta$–$\beta'$ enzyme at a ratio of 5:1. The isolated 14S component was as active in binding of nucleoside triphosphate as the native enzyme as can be seen in Table I, but was almost totally inactive in proceeding RNA synthesis. Thus, it is suggested that the 14S material did not contain $\alpha_2$–$\beta$–$\beta'$ enzyme. Another preparation consisting of $\beta$–$\beta'$ and $\alpha$–$\beta$–$\beta'$ complexes at a ratio of 4:1 also showed full activity in binding substrate (Table I). Thus, the $\beta$–$\beta'$ complex must be fully active in the substrate-binding reaction. The results suggest that the $\beta$ and/or $\beta'$ subunits are the possible candidates for the unit which possess substrate-binding sites.

The antibiotic, rifampicin, is known to be a potent inhibitor of the initiation reaction of RNA synthesis by binding to the RNA polymerase (Umezawa *et al.*, 1968; Lancini *et al.*, 1969). It has been proposed that the drug binds to the initiation site on the enzyme and, in fact, this effect has been shown to be prevented by preincubation of the enzyme with template DNA prior to rifampicin addition (Lill *et al.*, 1970). These results suggest that the substrate-binding sites and rifampicin binding sites on the enzyme may be related in some way. This relationship was then investigated by comparing the influence of *p*CMB on both activities.

A [$^{14}$C]rifampicin–RNA polymerase complex was isolated by Sephadex chromatography after incubation of the enzyme

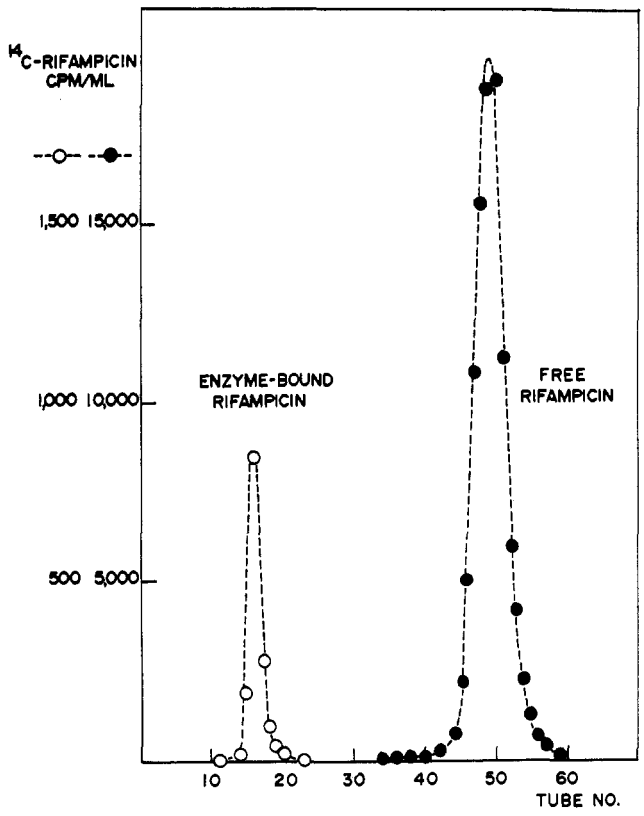


FIGURE 4: Isolation of [$^{14}$C]rifampicin–RNA polymerase complex. A mixture of [$^{14}$C]rifampicin (94 nmoles; specific activity 1.49 × $10^4$ cpm/nmole) and RNA polymerase (0.338 mg) was incubated for 10 min at 30°, and passed through 0.8 × 12 cm of Sephadex G-25 (fine) column. The enzyme and the rifampicin–enzyme complex were eluted with 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.2 M KCl. Aliquots (0.02 ml) from each 1.0-ml fraction were used to measure the radioactivity.

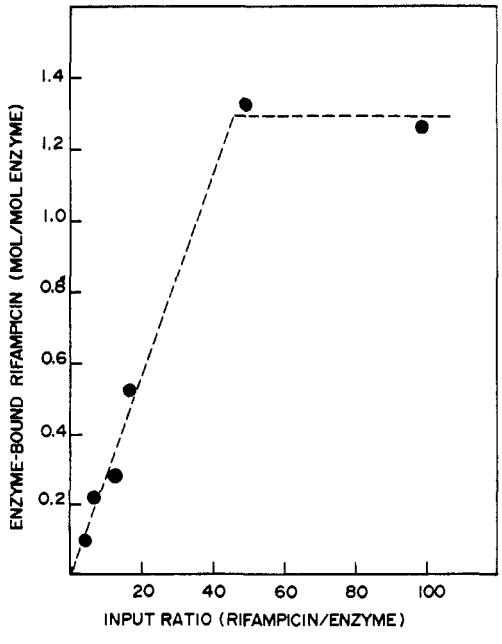


FIGURE 5: Binding of [$^{14}$C]rifampicin to RNA polymerase. RNA polymerase was mixed with [$^{14}$C]rifampicin at the indicated input ratios and the complex was isolated as described in Figure 3. The molar concentration of the enzyme was calculated assuming the molecular weight of 5 × $10^5$ for the enzyme and that the enzyme preparation was pure and 100% active.

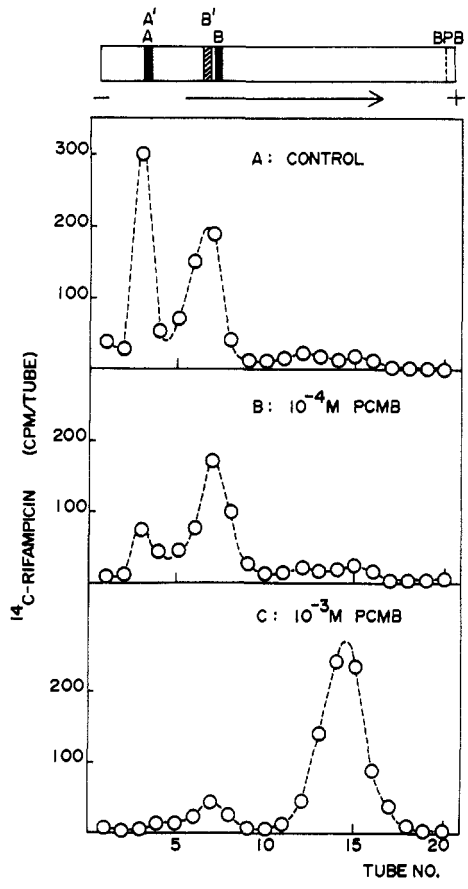


FIGURE 6: Disc gel electrophoresis of [$^{14}$C]rifampicin–RNA polymerase complex. The [$^{14}$C]rifampicin–enzyme complex (0.94 mole of rifampicin/mole of enzyme as calculated in Figure 4) isolated as shown in Figure 3 was subjected to electrophoresis in standard gels (6 cm length). In the figure, the following are presented: (A) control untreated complex, (B) the complex treated with $10^{-4}$ M *p*CMB, and (C) the complex treated with $10^{-3}$ M *p*CMB. After electrophoresis, the gels were divided into 3-mm fractions and the radioactivity of [$^{14}$C]rifampicin was measured after overnight elution with 0.5 ml of a solution containing 0.1% sodium dodecyl sulfate–0.05 M NaOH–0.25 mM EDTA.

with an excess of [$^{14}$C]rifampicin (Figure 4). Employing this technique, it was found that the amount of [$^{14}$C]rifampicin bound to RNA polymerase plateaued at a molar input ratio of approximately 50 (Figure 5). At this plateau value it was found that 1 mole of enzyme protein contained 1.3 moles of rifampicin suggesting that the active enzyme may bind 2 moles of rifampicin. The complex isolated after Sephadex chromatography was subjected to electrophoresis on acrylamide gels. The $^{14}$C-labeled rifampicin of the complex migrated at the same rate as free RNA polymerase. These results indicate little dissociation of rifampicin from the complex in keeping with a high affinity of the antibiotic for the enzyme (Figure 6). However, rifampicin could be released from the complex by high concentrations of *p*CMB ($10^{-3}$ M), but not by low concentrations ($10^{-4}$ M) as shown in Figure 6B,C. It should be noted that the concentrations of *p*CMB required for the release of rifampicin coincides well with those required to inactivate the enzyme with respect to its ability to bind substrates (Figure 1B,C). These results suggest that both the rifampicin- and the substrate-binding activities of the 14S component may reside in the subunit(s), $\beta$ and/or $\beta'$.

*Reversible Dissociation of RNA Polymerase by Urea.* The RNA polymerase has been shown to dissociate into several

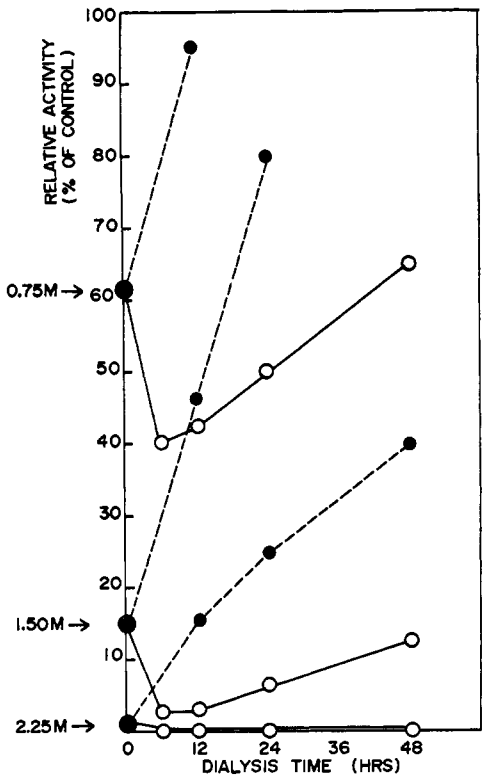


FIGURE 7: Reactivation of urea-treated RNA polymerase. RNA polymerase (0.045 mg of protein) was incubated in the presence of 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.1 mM EDTA, 50 mM 2-mercaptoethanol, and the indicated concentrations of deionized urea in a total volume of 0.2 ml for 10 min at 30°. After incubation, an aliquot of the mixture was used to determine the residual activity in a standard assay system. The low concentration of urea present in the assay did not interfere with this measurement. The denatured enzyme was dialyzed at 4° against the same buffer without urea and aliquots were removed for activity measurements at the time intervals indicated on the abscissa. Control enzyme was also treated as the denatured enzymes except urea was omitted during incubation. The enzymic activities are represented as the relative values compared with the control which showed 5 units immediately after incubation and 4 units after dialysis for 48 hr. The curves represented by ●—● included 75 mmoles of calf thymus DNA during dialysis; the curves represented by O–O indicate dialysis without DNA.

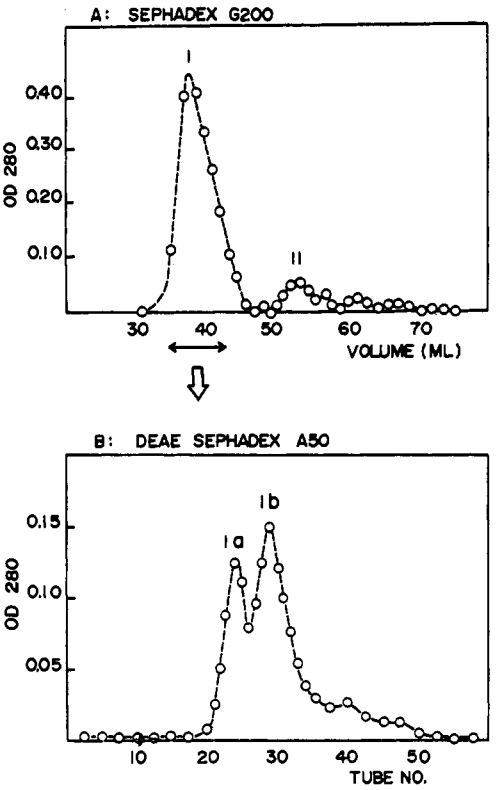


FIGURE 8: Separation of the subunits of RNA polymerase treated with 2 M urea. (A) RNA polymerase (4.35 mg of protein) was brought to 2 M urea by addition of deionized 9 M urea, incubated for 30 min at 30° and subjected to gel filtration chromatography on Sephadex G-200 (2 × 80 cm) equibrated with 2 M urea containing 10 mM Tris-HCl (pH 7.5), 0.05 M NaCl, 0.1 mM EDTA, and 0.05 M 2-mercaptoethanol. (B) The pooled fractions of peak I obtained after chromatography on Sephadex G-200 were applied to a DEAE-Sephadex A-50 column (2 × 30 cm) equibrated with the same 2 M urea buffer and eluted with a linear gradient (300 ml) from 0.05 to 0.5 M KCl. Gel analysis of the fractions allowed identification of the polypeptide chains present in each peak as follows: Ia, $\alpha\beta'$; Ib, $\alpha\beta$; II, $\omega$ and $\sigma$.

subunits by treatment with urea and sodium dodecyl sulfate. Upon exposure to these reagents, the enzyme is dissociated primarily into 9S and 4.5S components, and further exposure can dissociate these subunits into 3.5S and 2S subunits (Ishihama, 1969). Since each of 3.5S and 2S subunits could not be further dissociated with urea (Burgess, 1969), they were considered to be monomeric polypeptide chains.

Figure 7 summarizes the recovery of enzymatic activity of dissociated RNA polymerase preparations after treatment with varying concentrations of urea followed by dialysis against 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 50 mM KCl, and 0.1 mM EDTA buffer containing 0.05 M 2-mercaptoethanol. It can be seen that the enzyme activity is slowly recovered when it was treated with urea at concentrations of approximately 2 M and lower. The recovery rate was stimulated by the addition of DNA to denatured enzyme preparations. This finding is consistent with the observation that enzyme exposed to low concentrations of urea still retains its DNA-binding activity (Ishihama and Hurwitz, 1969). The reactivation of dissociated polymerase and its stimulation by DNA was also observed by Lill and Hartman when RNA polymerase was treated with 6.5 M urea (1970). In contrast to the observations noted above, the reactivation of enzyme preparations treated with urea at concentrations higher than 2 M has been achieved if glycerol was added throughout the procedure (A. Ishihama, unpublished data).

In order to characterize the subunit structure of the enzyme dissociated by treatment with different concentrations of urea and to reconstruct the enzyme from isolated subunits, attempts were made to isolate individual subunits. Polymerase preparations treated with different concentrations of urea were applied to columns of Sephadex G-200 or Sepharose 6B in the presence of urea followed by ion-exchange chromatography on DEAE-Sephadex A-50 in urea. The subunit composition of each peak was determined by gel analysis in the presence of 8 M urea or 0.1% SDS. Figure 8 shows two succesive column profiles of RNA polymerase treated with 2 M urea indicating the degree of separation of each component. The 9S component was found to be a mixture of two fragments (Ia and Ib in Figure 8B) whose subunit composition was analyzed to be $\alpha\beta'$ (Ia) and $\alpha\beta$ (Ib), respectively, as can be seen in Figure 9. The DNA-binding activity of the enzyme treated with 2 M urea resided in both 9S fragments (Figure 10). Thus, it is clear that $\alpha$–$\beta$ and $\alpha$–$\beta'$ complexes are the units participating in the binding of RNA polymerase to DNA.

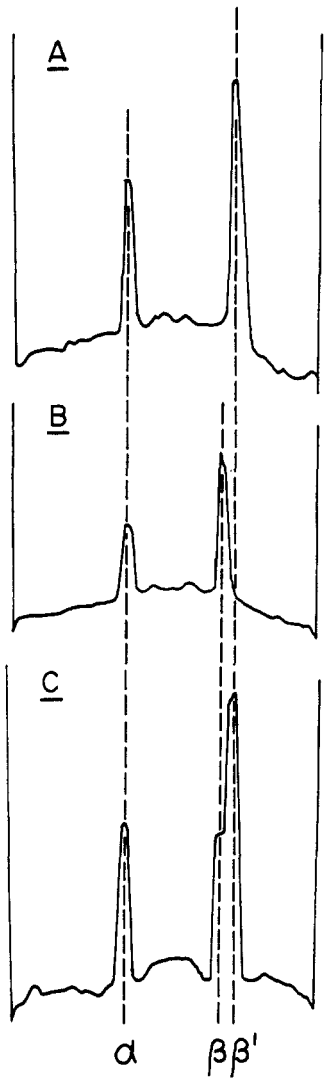


FIGURE 9: SDS–gel electrophoresis of subunit complexes obtained by urea treatment. (A) Peak Ia (21 μg of protein) from Sephadex G-200 column shown in Figure 8 was subjected to SDS–gel electrophoresis. Migration is from right to left. (B) Peak Ib (8 μg, of protein) was analyzed on a SDS gel. (C) The mixture of peak Ia (21 μg of protein) and peak Ib (8 μg of protein) was applied to a SDS–gel electrophoresis.

Complete separation of the enzyme into single peptide chains was carried out by treatment with 8 M urea followed by the same procedure as described above (Figure 11). Column chromatography on DEAE-Sephadex A-50 separated peak I of the eluates from Sephadex G-200 into two fragments that were found to be $\beta'$ and $\beta$ subunits without the $\alpha$ subunit. None of the subunits thus obtained had the biological activity.

The isolated 9S and 4.5S units obtained after Sephadex chromatography only retained DNA-binding activity in the 9S component. However, when these two fragments were mixed together, 35% of the nucleotide incorporating activity was regained (Table II). In contrast to this, when higher concentrations of urea were used and the 9S component was further dissociated, virtually all activities were irreversibly lost, and any combination of the isolated subunit regained no activity even after dialysis of urea. However, as will be described in the forthcoming paper, the enzymic activities could be regained if the subunits were isolated in the presence of glycerol.

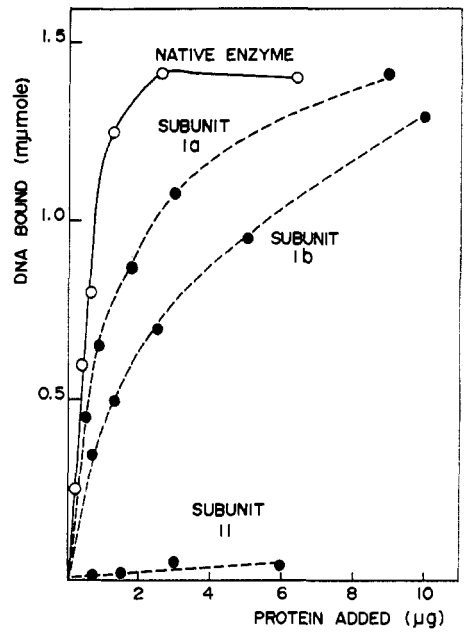


FIGURE 10: DNA-binding activity of isolated subunits RNA polymerase was treated with 2 M urea and the subunits were isolated as described in Figure 7. The indicated amounts of the subunits were added to the reaction mixture containing 2.43 nmoles (specific activity 1400 cpm/nmole) of [$^{14}$C]T7 DNA. Bound DNA was measured as previously described (Ishihama and Hurwitz, 1969a).

## Discussion

In previous studies chemical manipulation of RNA polymerase yielded preparations capable of catalyzing part of the discrete steps of RNA synthesis (Ishihama and Hurwitz, 1969). A number of reagents which reacted with sulfhydryl groups rendered the enzyme inactive in binding to DNA, initiation of RNA synthesis, and elongation of RNA chains, but were without effect on the binding of nucleoside triphosphates to the enzyme. Since initiation and elongation of RNA chains are dependent on the binding of the enzyme to DNA, the sulfhydryl groups in the enzyme appear to be essential for this binding reaction.

An enzyme preparation capable of carrying out only the binding reaction was obtained by treatment of the enzyme with reagents which modify amino groups. The results suggest that amino groups play an essential role in the substrate-binding reaction. Modifications of histidine residues of the enzyme resulted in the formation of an enzyme preparation able to bind DNA and nucleoside triphosphates and catalyze the initiation reaction but was blocked specifically in the elongation reaction. These experiments suggested that multiple active sites corresponding to at least three different regions on the enzyme are involved in the enzymatic synthesis of RNA.

The monomeric form of RNA polymerase has the subunit structure $\alpha_2\beta\beta'\sigma(\omega)$ whereas the core enzyme isolated after chromatography on phosphocellulose yielded an enzyme preparation devoid of the subunit $\sigma$ (Burgess *et al.*, 1969; Burgess, 1969).

At present the precise role of each of the subunits of the polymerase is only beginning to be understood. The $\sigma$ subunit has been considered to be the unit involved in initiation. Since the core polymerase ($\alpha_2\beta\beta'(\omega)$) is capable of catalyzing all the reactions characteristic of RNA polymerase, it is evident that $\sigma$ *per se* is not essential for binding DNA or triphosphates, initiation, elongation, and termination. The case for

TABLE II: Reconstruction of RNA Polymerase from Isolated Subunits.[a]

| Treatment of RNA Polymerase | Subunit Component (Sephadex Eluate) | Input Ratio | Recov of Enzyme Act. (%) |
|---|---|---|---|
| 2 M Urea | Peak I + peak II | 80:20 | 35 |
| 4 M Urea | Peak I + peak II + peak III | 75:25:1 | 2.5 |
| 6 M Urea | Peak I + peak II + peak III | 60:35:5 | 1.0 |
| 8 M Urea | Peak I + peak II + peak III | 60:30:10 | 1 |

[a] Subunits of RNA polymerase treated with the indicated concentrations of urea were fractionated in Sephadex G-200 columns as in Figures 7 and 9. The pooled fractions of each peak were concentrated with a Sartorius collodion bag and mixed at the stoichiometric ratios calculated from their distribution after chromatography on Sephadex columns, Aliquots (0.05–1.0 mg of protein), after mixing with 100 nmoles of calf thymus DNA, were dialyzed for 2 days against 10 mM Tris-HCl (pH 7.6 at 4°), 10 mM $MgCl_2$, 0.1 mM EDTA, and 50 mM 2-mercaptoethanol, and were used to measure the enzymatic activity.

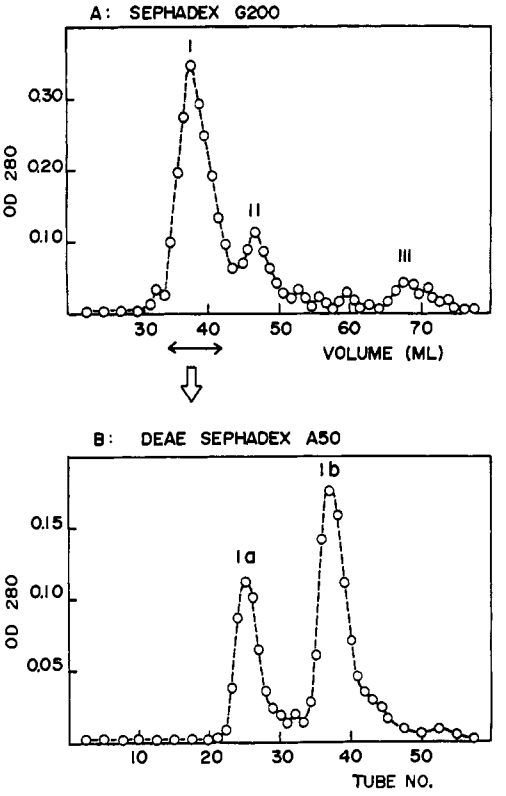


FIGURE 11: Separation of the subunits of RNA polymerase treated with 8 M urea. Subunits of the polymerase (4.15 mg) treated with 8 M urea were fractionated as in Figure 7. The elution buffer contained 6 M urea throughout the experiments. Peak Ia was determined to be $\beta'$ and peak Ib contained $\beta$ while peak II was a mixture of $\alpha$ and $\sigma$. Subunit $\omega$ was eluted in peak III.

the latter two reactions is clearly indicated by the findings of Krakow *et al.* (1969) that the $\sigma$ factor is not associated with enzyme actively engaged in RNA synthesis. Holoenzyme (containing $\sigma$) incubated with native DNA at 37° is bound irreversibly in contrast to the core enzyme (Hinkle and Chamberlin, 1971) suggesting that $\sigma$ facilitates binding at particular sites on the DNA (presumably constitutive promoter sites). It is a well established fact that polymerase binds more avidly to single-strand DNA; this binding reaction to single-stranded DNA results in the dissociation of $\sigma$ factor from RNA polymerase (Krakow *et al.*, 1969). Thus $\sigma$ must facilitate the interaction of the core components with DNA and is more likely to be an agent which aids specifically in binding of the enzyme to DNA most likely by facilitating the melting of duplex DNA structures (Ishihama *et al.*, 1971). However, this is still conjecture and more information is required.

In this paper two systems for reversibly dissociating the polymerase into subunits were reported. Protein subunits complexes consisting of $\beta$–$\beta'$ and $\alpha$–$\beta$–$\beta'$ obtained by treatment with $10^{-4}$ M *p*CMB bind nucleoside triphosphates almost as efficiently as the native enzyme. Furthermore, the antibiotic, rifampicin, which is known to react with the polymerase and inhibit initiation of RNA synthesis was found to be retained on the $\beta$–$\beta'$ or $\alpha$–$\beta$–$\beta'$ complexes. This is consistent with the observations that one of the *E. coli* mutant resistant to rifampicin contained a modified $\beta$ subunit different from wild-type $\beta$ in electrophoretic mobility (Rabussy and Zillig, 1969) and, furthermore, the reconstituate containing $\beta$ subunit from resistant enzyme was resistant to the drug (Heil and Zillig, 1970). Since more than one rifampicin are retained on these complexes, it is rather likely that there are two binding sites present on the enzyme for this drug though it is uncertain whether both sites reside on $\beta$ subunit or not. Under the conditions where *p*CMB-treated enzyme retains rifampicin and the capacity to bind nucleoside triphosphates, SH reagents reactivated the biological activity of the enzyme, indicating that $\beta$–$\beta'$ and $\alpha$–$\beta$–$\beta'$ complexes easily reassociate with other components.

On the other hand, low concentrations of urea dissociate the enzyme into $\alpha$–$\beta$ and $\alpha$–$\beta'$ complexes, both of which possess the ability to bind DNA. Under the conditions employed, the retention of this activity appears to be essential for reassociation of these complexes to form the active enzyme, since reassociation was markedly stimulated by adding DNA as reported previously (Ishihama and Hurwitz, 1969b; Ishihama,

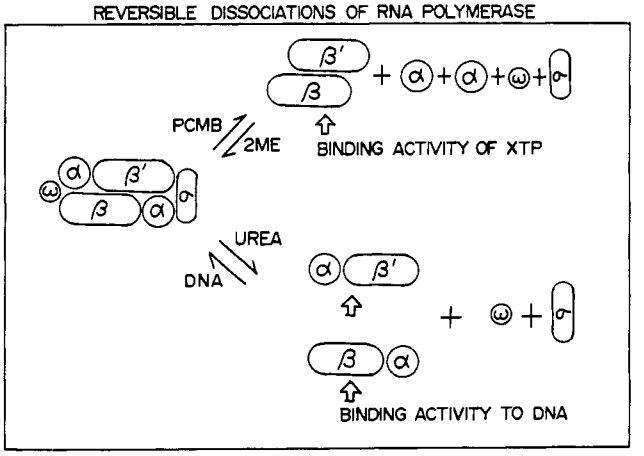


FIGURE 12: Reversible dissociation of RNA polymerase. This model summarizing the results is described in the text.

1969; Lill and Hartman, 1970). Lill and Hartman (1970) reported considerable reactivation of RNA polymerase from enzyme which had been treated with 6.5 M urea. The discrepancy between their observations and those reported here might be due to the degree of alteration of the intramolecular conformation of the individual subunits. In fact, the enzymic activities could be regained if the subunits were prepared in the presence of glycerol (Heil and Zillig, 1970; A. Ishihama, unpublished data).

The ability of $\alpha$–$\beta$ and $\alpha$–$\beta'$ units to bind DNA only reflects one parameter of the polymerase activity. The observed binding with these complexes does not imply that the sites on DNA to which they are attached are the same as those which bind holoenzyme. As the isolated single polypeptides ($\alpha$, $\beta$, and $\beta'$) or even the $\beta$–$\beta'$ complex obtained by treatment with low concentrations of *p*CMB lacked the ability to complex with DNA, it is suggested that $\alpha$ plays an important role in the DNA-binding reaction. Recently a subunit complex having the activity to bind DNA was observed upon sedimentation of the enzyme treated with LiCl (Sethi *et al.*, 1970). However, such a complex was suggested to be a mixture of $\alpha$–$\beta$–$\beta'$ and $\beta$–$\beta'$ complexes and the DNA-binding activity was shown to reside even on the isolated $\beta$–$\beta'$ complex. Thus, the potential sites for DNA binding are supposed to reside on the $\beta$ and $\beta'$ subunit and require $\alpha$ subunit or a combination of $\beta$ and $\beta'$ subunit to activate them.

The results obtained here may be summarized as shown in Figure 12. Treatment of the enzyme with *p*CMB leads to the isolation of the $\beta$–$\beta'$ (and $\alpha$–$\beta$–$\beta'$) complex and the formation of the other individual subunits of the native enzyme. Thus, it appears that $\beta$ and/or $\beta'$ subunits possess the ability to bind nucleoside triphosphates. In contrast, low concentrations of urea cause the enzyme to form the $\alpha$–$\beta$ and $\alpha$–$\beta'$ complexes that are still capable of binding to DNA. As depicted in the figure, these are all reversible reactions. Further work is required to establish the function and structure of these different subunits. The precise configuration of the subunits as summarized in Figure 12 is unknown. The isolation of $\beta$–$\beta'$ and $\alpha$–$\beta$ and $\alpha$–$\beta'$ complexes suggests that these units are linked together. Since we have not detected $\alpha$–$\alpha$ component, the configuration as suggested in the figure has taken this fact into consideration. The positioning of $\sigma$ and $\omega$ is unknown.

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## References

Berg, P. (1959), *J. Biol. Chem. 222*, 1025.

Burgess, R. R. (1969), *J. Biol. Chem. 244*, 6168.

Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. K. F. (1969), *Nature (London) 221*, 43.

Davis, B. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Heil, A. and Zillig, W. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 11*, 165.

Hinkle, D. C., and Chamberlin, M. (1971), *Cold Spring Harbor Symp. Quant. Biol. 35*, 65.

Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63*, 530.

International Critical Tables (1962), Vol. 10, New York, N. Y., McGraw-Hill.

Ishihama, A. (1969), *J. Cell. Phys. 74, Suppl. 1*, 223.

Ishihama, A., and Hurwitz, J. (1969a), *J. Biol. Chem. 244*, 6680.

Ishihama, A., and Hurwitz, J. (1969b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 659.

Ishihama, A., and Kameyama, T. (1967), *Biochim. Biophys. Acta 138*, 480.

Ishihama, A., Murakami, S., Fukuda, R., Matsukage, A., and Kameyama, T. (1971), *Mol. Gen. Genet. 111*, 66.

Jones, O. W., and Berg, P. (1966), *J. Mol. Biol. 22*, 199.

Jovin, T., Chrambeach, A., and Naughton, M. (1964), *Anal. Biochem. 9*, 351.

Kameyama, T., Kitano, Y., Kawakami, H., Iida, Y., Murakami, S., Tanaka, Y., and Ishihama, A. (1969), *in* Nucleic Acid Metabolism, Cell Differentiation and Cancer Growth, Cowdrey, E. V., and Seno, S., Ed., New York, N. Y., Pergamonn Press, p 29.

Kawahara, K., and Tanford, C. (1966), *J. Biol. Chem. 241*, 3228.

Krakow, J. S., Daley, K. and Fronk, E. (1968), *Biochem. Biophys. Res. Commun. 32*, 98.

Krakow, J. S., Daley, K., and Karstadt, M. (1969), *Proc. Nat. Acad. Sci. U. S. 67*, 432.

Lancini, G., Pallanza, R., and Silvestri, L. (1969), *J. Bacteriol. 97*, 761.

Lill, U. I., and Hartmann, G. R. (1970), *Biochem. Biophys. Res. Commun. 39*, 930.

Lill, H., Lill, U., Sippel, A., and Hartmann, G. (1970), *Lepetit Collect. Biol. Med. 1*, 55.

Maitra, U., and Hurwitz, J. (1967), *J. Biol. Chem. 242*, 4897.

Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.

Rabussy, D. and Zillig, W. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 5*, 104.

Sethi, V. S., Zillig, W., and Bauer, H. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 6*, 339.

Shapiro, A. L., Viñuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.

Umezawa, H., Mizuno, S., Yamazaki, H., and Nitta, K. (1968), *J. Antibiotics 21*, 234.

Vogt, V. (1969), *Nature (London) 223*, 854.

Yudelvich, A., and Gold, M. (1969), *J. Mol. Biol. 40*, 77.