catalytic roles is a question to which future experiments will be addressed.

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Physical Studies on Ribonucleic Acid Polymerase from *Escherichia coli* B*

Darwin Berg and Michael Chamberlin†

ABSTRACT: The sedimentation properties of RNA polymerase from *Escherichia coli* B have been studied. At high ionic strengths core polymerase exists as a single sedimenting species ($s_{20,w}$ = 12.6 S) and has a molecular weight, determined by low-speed sedimentation equilibrium, of $3.8 \times 10^5 \pm 0.2 \times 10^5$. RNA polymerase holoenzyme, containing 1 equiv of σ component, also exists as a single sedimenting species ($s_{20,w}$ = 15.0 S) at high ionic strengths. Studies of the subunit composition of the protein indicate that these species are the protomeric forms of the RNA polymerase molecule.

In solutions which contain high concentrations of ammonium sulfate the apparent values of $s_{20,w}$ and molecular weight decrease for both forms of the enzyme. The effect is not due to dissociation of the polymerase, but appears to be due to multicomponent interactions in the concentrated salt solution. Both core polymerase and RNA polymerase holoenzyme aggregate at low ionic strengths. In the absence of added salt (μ = 0.04) core polymerase forms a mixture of aggregates having a mean sedimentation coefficient of 44–48 S. Thus core polymerase can form aggregates at least as large as a hexamer of the 12.6S species. RNA polymerase holoenzyme sediments as a single species having $s_{20,w}$ = 23 S under these conditions. Thus the presence of 1 equiv of σ component limits the aggregation of RNA polymerase to a dimer of the protomeric form.

Deoxyribonucleic acid directed RNA polymerase (EC 2.7.7.6.) plays a central role in gene expression. The *Escherichia coli* enzyme can be isolated in two different forms: a core polymerase, and a polymerase holoenzyme. The latter form contains an additional subunit which has been designated as σ component (Burgess *et al.*, 1969; Berg *et al.*, 1970). With an intact, bacteriophage DNA as template, RNA polymerase holoenzyme synthesizes asymmetric RNA which is thought to be gene specific (Summers and Siegel, 1969; Travers, 1969). In contrast, core polymerase shows a reduced and variable ability to initiate RNA synthesis with such templates and the RNA produced is not gene specific. σ component appears to confer specificity on holoenzyme prior to the initiation of RNA chains (Travers and Burgess, 1969; Berg *et al.*, 1969), and probably determines the site at which the enzyme binds to DNA (Hinkle and Chamberlin, 1970). After initiation has occurred, σ component is released from the enzyme and can participate in a second round of initiation. The remaining core polymerase continues elongation of the newly formed RNA chain (Travers and Burgess, 1969; Berg *et al.*, 1969). Thus both RNA polymerase holoenzyme and core polym-

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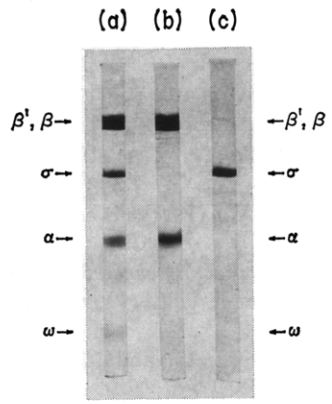

FIGURE 1: Analysis of RNA polymerase fractions by acrylamide gel electrophoresis in SDS buffer. Samples of protein were prepared and subjected to acrylamide gel electrophoresis in SDS buffer. The gels were stained with coomassie blue, and were destained electrophoretically as described in Methods. Electrophoresis is from top to bottom. (a) 20 μg of holoenzyme, (b) 20 μg of core polymerase, and (c) 5 μg of σ component.

erase appear to represent biologically important forms of the enzyme.

Because of the remarkable specificity of transcription exhibited by the isolated enzyme, the physical properties of RNA polymerase are of great interest. Early studies on the sedimentation behavior of the enzyme led to conflicting results. The enzyme appeared to aggregate at low salt concentrations but there was no agreement as to the number and nature of sedimenting species encountered under these conditions (Pettijohn and Kamiya, 1967; Fuchs *et al.*, 1964; Zillig *et al.*, 1966; Priess and Zillig, 1967; Colvill *et al.*, 1966; Lubin, 1969; Richardson, 1966; Stevens *et al.*, 1966; Maitra and Hurwitz, 1967; Chamberlin, 1963). A dissociated form of the enzyme was generally obtained at high salt concentrations but reports for the sedimentation coefficient of this species varied from 10 to 14 S (Richardson, 1966; Stevens *et al.*, 1966; Maitra and Hurwitz, 1967; Chamberlin, 1963). There was also considerable speculation as to which of the enzyme species, identified by sedimentation, might represent the active species.

The studies reported here were undertaken to identify the various species of the enzyme and to clarify the relationships among them. Initially studies were conducted with an enzyme preparation which comprised a mixture of holoenzyme and core polymerase. The sedimentation behavior of this preparation was examined in several salt solutions at various ionic strengths. The effects of preferential solvent interactions and of reversible equilibria were taken into account in identifying sedimenting species. Then the sedimentation properties and aggregation behavior of purified holoenzyme and of purified core polymerase were examined. The molecular weights of homogeneous species were determined by the method of low-speed equilibrium sedimentation. The subunit compositions of enzyme preparations were characterized by acrylamide gel electrophoresis in SDS[1] buffer. From these electrophoresis results molecular weights were estimated for holoenzyme and core polymerase, and these values were compared to those obtained from sedimentation equilibrium.

Materials

RNA polymerase holoenzyme was purified from *E. coli* B/1 as previously described (Berg *et al.*, 1970). It has maximal specific activities of 7300 units/mg with T2 DNA as template and 24,000 units/mg with d(A-T) as template. When the preparation was analyzed by acrylamide gel electrophoresis in SDS buffer (Shapiro *et al.*, 1967), it was found to contain five major components (Figure 1). Using the nomenclature of Burgess *et al.* (1969), we have designated these components as β', β, σ, α, and ω. Their molecular weights were estimated from their relative migrations during electrophoresis using the protein standards described below and are 150,000, 145,000, 86,000, 41,000, and 12,000 $\pm$ 10%, respectively (Berg, 1969). The relative amounts of each component were estimated by scanning the stained protein in the gels with a recording densitometer. These values, taken with the estimated molecular weights, give approximate molar ratios of the components present, if one assumes that each component stains equally on the basis of mass (De St. Groth *et al.*, 1963). By this criterion, holoenzyme has a composition of $\beta'/\beta/\sigma/2\alpha/2\omega$. Components β', β, and α are taken to be subunits of the enzyme since they migrate with the enzyme activity through all fractionation procedures and appear in constant ratio to each other (Berg, 1969). σ is also considered a subunit of the enzyme since it is bound by the enzyme and contributes a functional role (Burgess *et al.*, 1969; Berg *et al.*, 1970; Summers and Siegel, 1969; Travers, 1969). The relationship of ω to the enzyme is less clear. It is selectively lost when enzyme is subjected to zone sedimentation in 1 M urea. It is also partially removed when enzyme is analyzed by electrophoresis under nondenaturing conditions (Berg, 1969). Thus ω appears to be less tightly bound by the enzyme than are the other components and may not be a functional subunit of RNA polymerase.

Core polymerase, prepared by chromatography on phosphocellulose (Berg *et al.*, 1970), has maximal specific activities of 300 units/mg with T2 DNA as template and 16,000 units/mg with poly d(A-T) as template. Enzyme prepared in this manner contains four major components (Figure 1), β', β, α, and ω; these are present in the same ratios as in holoenzyme. It contains 0.5% by weight of σ and therefore constitutes a preparation of core polymerase which is contaminated 2–3% with holoenzyme (Berg *et al.*, 1970).

σ component (Figure 1) was obtained from the initial eluate of the phosphocellulose column as previously described (Berg *et al.*, 1970).

Fraction VI′ enzyme is RNA polymerase purified from *E. coli* B/1 by an extension of the method of Chamberlin and Berg (1962). Fraction IV was sedimented through a glycerol gradient at low ionic strength (10–30% glycerol, 0.012 M ammonium sulfate, 0.01 M Tris (pH 8), 0.01 M magnesium chloride, and 0.01 M mercaptoethanol) where it has a sedimentation coefficient of about 24 S. Fractions with high enzyme specific activities were collected (fraction V′) and were resedimented through an identical gradient containing 0.20 M ammonium sulfate where the enzyme has a sedimentation coefficient of about 13 S. Fractions with high specific activities were pooled, precipitated with ammonium sulfate

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

and stored at −16° in liquid form as previously described (Berg *et al.*, 1970). Enzyme prepared in this manner is referred to as fraction VI′ enzyme. It contains the same subunit composition as holoenzyme with the exception that it has only 60% as much σ, as determined by acrylamide gel electrophoresis in SDS buffer. Thus fraction VI′ enzyme does not contain 1 equiv of σ component and appears to be a mixture of RNA polymerase holoenzyme and core polymerase. It has specific activities with d(A-T) and T2 DNA which are close to those expected for a mixture of core enzyme and holoenzyme.

Acrylamide reagents were purchased from Eastman Organic Chemicals. Sodium dodecyl sulfate was purchased from Matheson Coleman & Bell. Sephadex G-75 was obtained from Pharmacia.

Methods

Standard RNA polymerase assays using either poly d(A-T) or T2 DNA as templates were conducted as previously described (Berg *et al.*, 1970). One unit of RNA polymerase activity catalyzes the incorporation of 1 mμmole of AMP into acid-insoluble material in 1 hr at 37°. σ component was assayed by its ability to stimulate the activity of core polymerase when T2 DNA is template (Burgess *et al.*, 1969; Berg *et al.*, 1970). One unit of σ activity increases the rate of incorporation catalyzed by 15 d(A-T) units of core polymerase by 1 mμmole of AMP/hr in a standard assay.

Protein determinations were made according to the method of Lowry *et al.* (1951), using BSA as a standard.

Analytical sedimentation velocity studies were performed with a Spinco Model E ultracentrifuge equipped with a scanner attachment (Schachman *et al.*, 1962; Schachman, 1963). Sedimentation was followed with absorption optics at 280 mμ or with schlieren optics. Double-sector cells were used, containing sample and buffer in the two chambers. Samples were equilibrated with TMS buffer (0.01 M Tris (pH 8), 0.01 M magnesium chloride, and 0.002 M mercaptoethanol) and salt either by passage through a column of Sephadex G-75 or by dialysis in a "rapid dialysis" device (Englander and Crowe, 1965). Sedimentation coefficients, $s_{20,w}$, were calculated from the observed s values by correcting for the viscosity and density of the solution (Schachman, 1959).

In a multicomponent system preferential interactions between protein and solvent or protein and solutes such as salt can significantly affect both sedimentation and equilibrium measurements (Casassa and Eisenberg, 1964; Schachman, 1959). As we show below this is true for RNA polymerase in solutions of ammonium sulfate. We have treated this effect in the present case by measuring the sedimentation coefficient of the protein at several solvent densities and extrapolating to determine the solvent density at which the protein would be iso dense, using a plot of $s\eta$ *vs.* ρ (Schachman, 1959; Hill and Cox, 1965).[2] We define the reciprocal of this density as ϕ^*, the apparent specific volume of the protein (Schachman and Edelstein, 1966). The amount of solvent preferentially bound is then calculated from the expression (Williams *et al.*, 1958)

$$\Gamma' = \frac{(\bar{v} - \phi^*)}{(\phi^* - \bar{v}_s)} \quad (1)$$

where Γ' is in grams of water bound per gram of protein and $\bar{v}_s$ is the partial specific volume of the preferentially bound solvent, which in this case is water. The sedimentation coefficient of the preferentially solvated protein was then corrected for the viscosity and density of the solution and for the effects of preferential solvation by the formula

$$s_{20,w} = s(\eta/\eta_{20,w})(1 - \bar{v}\rho_{20,w})/[(1 - \bar{v}\rho) + \Gamma'(1 - \bar{v}_s\rho)] \quad (2)$$

This treatment assumes that the nature and extent of preferential interactions, in this case of preferential solvation, do not vary with ρ, and that ϕ^* is in fact equal to the partial specific volume of the protein in the salt solution. It has been found that this assumption is not justified in certain cases (Cohen and Eisenberg, 1968; Reisler and Eisenberg, 1969), and the effect of this assumption on our ultimate conclusions will be discussed below.

Sedimentation equilibrium experiments were performed by using absorption optics at 280 mμ and the method described by Schachman and Edelstein (1966). Experiments were performed at 2–4°. Overspeeding was used to achieve equilibrium within 18–20 hr. The distribution of protein was then determined at 4-hr intervals to ensure that equilibrium had been attained. A six-chamber Yphantis cell was used with water in the two middle chambers serving as a zero reference point for the scanner; the upper and lower pairs of chambers contained sample and buffer in each pair. Samples were prepared by passage over a Sephadex G-75 column. The protein concentration was evaluated in centimeters of pen deflection of the scanner. After the equilibrium distribution of protein had been determined, the base line of the sample was obtained in the following manner. The rotor was centrifuged at 40,000 rpm for 1 hr to sediment the protein to the bottom of the cell. Then the rotor was returned to the original speed used for the equilibrium sedimentation and the base line was measured. The middle 70% of the sample column was used to calculate the weight-average molecular weight of the protein.

The molecular weight of the preferentially solvated protein was calculated according to the formula

$$M^* = 2.303(2RT/\omega^2)(\mathrm{d}\log c/\mathrm{d}r^2)/(1 - \phi^*\rho) \quad (3)$$

where ϕ^* was determined from sedimentation velocity experiments as described above. The molecular weight of the protein, corrected for preferential solvation, was then calculated according to the formula

$$M = M^*/(1 + \Gamma') \quad (4)$$

where Γ' was determined from eq 1. Alternatively, ϕ^* and Γ' can be determined directly from sedimentation equilibrium studies (Schachman and Edelstein, 1966). With RNA polymerase, this latter method has limited usefulness since the enzyme appears to aggregate slightly at very high concentrations of salt, necessitating a hazardous extrapolation of the data to determine the solvent density at which the protein is iso dense. However the results of such a determination (Berg, 1969) give values of ϕ^* and Γ' quite close to those determined

[2] A more exact treatment (Casassa and Eisenberg, 1964) is possible if the true value of $\bar{v}$ is determined by equilibrium dialysis. This has not yet been possible with RNA polymerase due to the relatively small amounts of the homogeneous protein which are available.

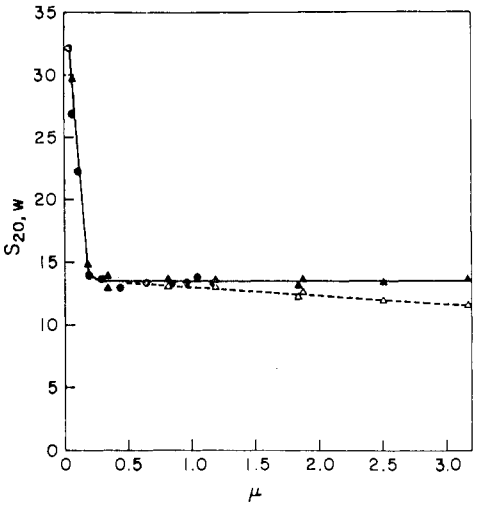

FIGURE 2: Sedimentation of fraction VI′ enzyme. Samples of fraction VI′ enzyme were prepared in TMS buffer plus the indicated concentrations of salt and were subjected to analytical centrifugation. The ionic strength, μ, includes the contributions of both the salt and the TMS buffer. Initial protein concentrations were 0.2 mg/ml. Sedimentation coefficients obtained in ammonium chloride solutions (●—●) were corrected for the viscosities and densities of the solutions, to values in water at 20°. Sedimentation coefficients obtained in ammonium sulfate solutions were either corrected similarly (△--△) or they were additionally corrected for the effects of preferential solvation, according to eq 2 (▲—▲).

by sedimentation analysis. A value of 0.738 was used for $\bar{v}$, the partial specific volume of RNA polymerase (Burgess, 1969).[3]

Densities of solutions were determined to ±0.1% by weighing aliquots in a 250-μl H. E. Pederson micropipet. The micropipet was calibrated by weighing it filled with water at the same temperature.

Viscosity measurements were made using an Ostwald viscometer in a large water bath controlled at 21°. The outflow time for water was 160 sec, and the volume of the viscometer was 1.0 ml.

Acrylamide gel electrophoresis of the dissociated protein was done in SDS buffer according to the procedure of Shapiro *et al.* (1967) using the fixing and staining procedure of Burgess *et al.* (1969). Samples were prepared in 0.10 ml containing 0.02 M sodium phosphate (pH 7.1), 0.1% SDS, 1% mercaptoethanol, 4% sucrose, and 0.002% tracking dye; samples were incubated at least 30 min at 37° prior to electrophoresis. The same electrophoresis pattern was obtained when the enzyme was denatured using several different procedures (Shapiro *et al.*, 1967; Weber and Osborn, 1969; Fine *et al.*, 1968). Electrophoresis was conducted at room temperature for 2 hr using 5% acrylamide gels which were 8 cm long. To calibrate the relationship between mobility and molecular weight for this system, a number of proteins of known molecular weights were individually subjected to electrophoresis under these conditions. The following proteins and molecular weight values were used: β-galactosidase, 130,000; isoleucyl-tRNA synthetase, 112,000; DNA polymerase, 109,000 (all from *E. coli*); BSA, 65,000; ovalbumin, 44,000; carboxypeptidase A, 34,000; myoglobin, 17,000; lysozyme, 14,400; pancreatic ribonuclease, 13,700. To quantitatively determine the amount of each component present, the stained gels were traced with a Densicord recording electrophoresis densitometer, equipped with integrater.

Results

Sedimentation Velocity Studies

Fraction VI′ Enzyme. Sedimentation studies were initially performed with fraction VI′ enzyme. It was subsequently found that fraction VI′ enzyme contains less than 1 equiv of σ component and, thus, appears to be a mixture of RNA polymerase holoenzyme and core polymerase. However, these studies of fraction VI′ were extremely useful in determining the general effects of salt on the sedimentation properties of RNA polymerase.

The sedimentation coefficient of fraction VI′ enzyme was measured in several concentrations of ammonium chloride and the results are shown in Figure 2, graphed as a fraction of the ionic strengths of the solutions. The data have been corrected for the viscosities and densities of the solutions to give values in water at 20°. In the absence of ammonium chloride (μ = 0.04) the enzyme sediments at 33 S.

Although at low protein concentrations a single sedimenting boundary is obtained under these ionic conditions (Figure 3a) the boundary is broader than that expected for diffusion of a single species. At elevated protein concentrations the boundary is resolved into several components. Approximately 70% of the material sediments at about 34 S, 25% at 38 S, and a trace at 14 S. These results suggest that fraction VI′ enzyme behaves as an interacting system under these ionic conditions. However the sedimentation coefficient of the boundary does not exhibit the strong positive dependence on protein concentration expected for a reversibly aggregating system. Thus as the protein concentration was increased from 0.08 to 7.6 mg per ml the mean sedimentation coefficient increased only from 32.5 to 35 S. This suggests that although fraction VI′ enzyme at μ = 0.04 is an interacting system, as the protein concentration is increased it reaches a limit of aggregation. If this is true then fraction VI′ must be heterogeneous and in particular must contain enzyme components with different limits of aggregation. This interpretation is supported by the studies on core polymerase and polymerase holoenzyme reported below.

As the concentration of ammonium chloride is increased sedimentation analysis shows that the enzyme dissociates to give a single boundary with an $s_{20,w}$ = 13.5 S (Figure 3b). Further increases in the ammonium chloride concentration do not lead to a further reduction in the sedimentation coefficient of the preparation. In 0.4 M ammonium chloride solutions variation of the protein concentration from 0.05 to 0.5 mg per ml did not detectably vary the value of $s_{20,w}$ obtained.

Enzyme preparations which have been stored for long

[3] The amino acid composition of *E. coli* B RNA polymerase holoenzyme and core polymerase has been determined in this laboratory. The values differ very little from those obtained by Burgess (1969) for the *E. coli* K12 core polymerase and give values of $\bar{v}$ = 0.737 and 0.739, respectively (Schachman, 1959). Thus the value of $\bar{v}$ = 0.738 obtained by Burgess is probably valid for both preparations. Our original calculations (Berg, 1969) were carried out using a value of $\bar{v}$ = 0.722 which was obtained by an erroneous calculation. The assumption that $\bar{v}$ calculated from the molar volumes of the amino acids is the true value of $\bar{v}$ has not been tested with this protein.

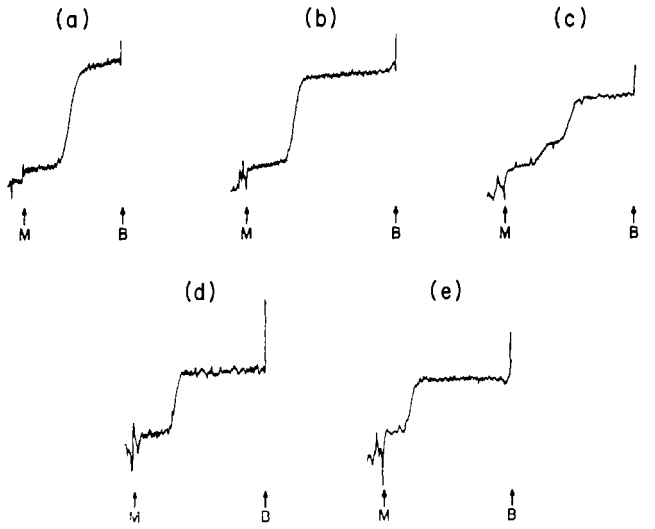

FIGURE 3: Sedimentation velocity patterns of fraction VI′ enzyme. Samples of fraction VI′ enzyme were prepared in TMS buffer containing the indicated concentrations of salt and were subjected to analytical centrifugation. Absorption optics were used to scan at 280 mμ. Sedimentation is from left to right. The meniscus and base of the sample are marked with "M" and "B," respectively. (a) 12 min at 44,000 rpm at 6° in the absence of added salt (TMS buffer alone) with an initial protein concentration of 0.5 mg/ml; (b) 16 min at 60,000 rpm in 0.4 M ammonium chloride at 4° with an initial protein concentration of 0.5 mg/ml. (c) This is duplicate sample as in part b after storage 2 days at 4°. Sedimentation is for 28 min at 60,000 rpm in 0.40 M ammonium chloride at 4° with an initial protein concentration of 0.5 mg/ml; (d) 20 min at 60,000 rpm at 4° in 0.10 M ammonium sulfate with an initial protein concentration of 0.2 mg/ml; and (e) 20 min at 60,000 rpm at 7° in 1.04 M ammonium sulfate with an initial protein concentration of 0.2 mg/ml.

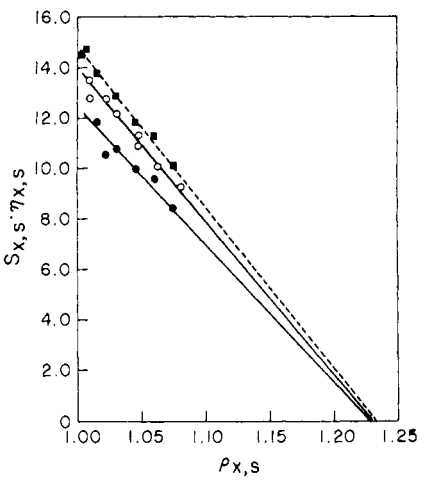

FIGURE 4: Dependence of sedimentation coefficient on solvent density. Samples of enzyme were sedimented at 60,000 rpm in TMS buffer containing 0.10–1.04 M ammonium sulfate. Absorption optics were used to scan at 280 mμ. Initial protein concentrations were approximately 0.2 mg/ml. $s_{x,s}$ is the uncorrected sedimentation coefficient for enzyme in solution s at temperature x; $\eta_{x,s}$ and $\rho_{x,s}$ are the viscosity and density, respectively, of solution s at temperature x. A least-squares fit of the data was extrapolated to determine the intercept at which $\phi^* = 1/\rho_0$. Fraction VI′ enzyme (O—O), core polymerase (●—●), and holoenzyme (■- -■).

times at 4° accumulate a protein component which sediments at 8 S (Figure 3c). This 8S material appears to be a breakdown product of the native enzyme molecule. The component does not reassociate to form the native enzyme molecule under a variety of salt concentrations, and, when isolated by zone sedimentation, it is found to be inactive in both the RNA polymerase and σ assays.

The sedimentation properties of fraction VI′ enzyme were studied further in ammonium sulfate solutions. Ammonium sulfate was chosen to vary the ionic strength since the enzyme is routinely prepared and stored in this salt and appears to be more stable under these conditions. The sedimentation coefficients obtained in ammonium sulfate solutions were corrected for the viscosities and densities of the solutions to give values for water at 20° and were plotted as a function of the ionic strengths of the solutions (Figure 2). When expressed in this way the results obtained are similar to those obtained in ammonium chloride solutions. Thus the effect of salt on aggregation of the enzyme is due to variation of the ionic strength. At low ionic strength the enzyme aggregates extensively while at high ionic strength it dissociates, sedimenting at 13.5 S.

In high concentrations of ammonium sulfate, however, the enzyme exhibits a puzzling behavior. Over the range of 0.35–3.5 in ionic strength, $s_{20,w}$ for the protein decreases in a linear manner from 13.4 to 11.5 S. This decrease is less apparent, if present at all, in ammonium chloride solutions where ionic strengths up to 1.2 were used. Three possible explanations for the decrease in sedimentation coefficient in ammonium sulfate were considered: (1) the enzyme is irreversibly dissociated by this treatment; (2) the enzyme is reversibly dissociated by this treatment; and (3) the variation of the sedimentation coefficient is due to preferential solvation. It should be noted that the heterogeneity of fraction VI′ enzyme does not appear to be involved here since both core polymerase and holoenzyme give similar results at high concentrations of ammonium sulfate as is discussed below.

The possibility of irreversible dissociation can be ruled out since the enzyme can be dissolved in high concentrations of ammonium sulfate and, when subsequently transferred to a solution having an ionic strength of 0.35, for example, sediments at 13.4 S once again. Furthermore, a single, sharply sedimenting boundary is obtained both at 0.35 and at 3.5 ionic strength in ammonium sulfate solutions (Figure 3d,e), suggesting that only a single species is present instead of a disaggregating mixture. If reversible dissociation occurred, one might expect to obtain a positive dependence of sedimentation coefficient on protein concentration. In an ammonium sulfate solution with an ionic strength of 1.8, however, $s_{20,w}$ values of 12.2 and 11.8 S were obtained at initial protein concentrations of 0.1 and 4 mg per ml, respectively. Thus over a 40-fold range in protein concentration the enzyme exhibited the slight negative dependence of $s_{20,w}$ on protein concentration expected for a single sedimenting species. This argues against the possibility that reversible dissociation is occurring in high concentrations of ammonium sulfate. The possibility remains that the decrease in the observed sedimentation coefficient at elevated ammonium sulfate concentrations is due to preferential interactions in this three-component system.

Where preferential interactions are present, one expects to obtain an apparent partial specific volume for the sedimenting

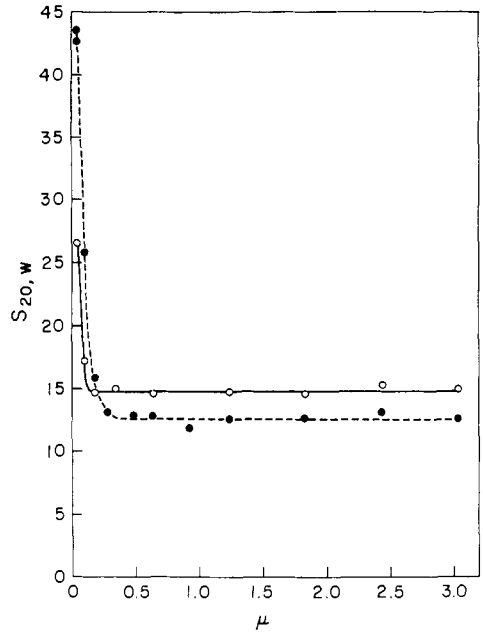

FIGURE 5: Sedimentation of core polymerase and holoenzyme. Samples of core polymerase and RNA polymerase holoenzyme were prepared in TMS buffer containing the indicated concentrations of ammonium sulfate and were subjected to analytical centrifugation. The ionic strength, μ, includes the contributions of both the ammonium sulfate and the TMS buffer. Initial protein concentrations were 0.2 mg/ml. The sedimentation coefficients were corrected for the viscosities and densities of the solutions to obtain values for water at 20° and were also corrected for the effects of preferential hydration according to eq 2. Core polymerase (●--●), and RNA polymerase holoenzyme (○—○).

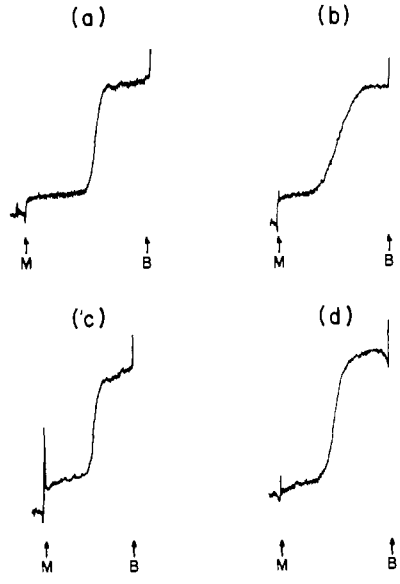

FIGURE 6: Sedimentation velocity patterns of core polymerase and RNA polymerase holoenzyme. Samples of enzyme were prepared in TMS buffer containing the indicated concentrations of salt and were subjected to analytical centrifugation as described in Methods. Absorption optics at 280 mμ were used. Sedimentation is from left to right. The meniscus and base of the sample column are marked with "M" and "B," respectively. (a) Core polymerase in 0.20 M ammonium sulfate, 40 min at 60,000 rpm with an initial protein concentration of 0.5 mg/ml; (b) core polymerase in the absence of added salt (TMS buffer alone), 16 min at 40,000 rpm with an initial protein concentration of 0.5 mg/ml; (c) holoenzyme in 0.20 M ammonium sulfate, 24 min at 60,000 rpm with an initial protein concentration of 0.7 mg/ml; and (d) holoenzyme in the absence of ammonium sulfate, 28 min at 44,000 rpm with an initial protein concentration of 0.5 mg/ml.

species which is different from the partial specific volume for the protein calculated from its amino acid composition. When the sedimentation data are graphed as $s\eta$ *vs.* solvent density as shown in Figure 4, an apparent specific volume (ϕ^*) of 0.81 is obtained for fraction VI′ enzyme in ammonium sulfate solution. This is markedly higher than the value of 0.738 obtained from amino acid analysis. If one assumes this difference is due to preferential solvation, and that the extent of this solvation is constant over the density range studied, one can calculate from eq 1 that $\Gamma' = 0.39$ g of water preferentially bound per g of protein. Similar results have been obtained by other workers using both complex proteins consisting of several subunits (Aune and Timasheff, 1970) and proteins which contain only a single polypeptide chain (Hill and Cox, 1965; Ifft and Vinograd, 1966). Similar results are obtained when the apparent molecular weight of core polymerase is determined at a variety of ionic strengths using sedimentation equilibrium (Berg, 1969). Thus there is a decrease in the apparent molecular weight as the ionic strength is increased. When the data are plotted according to Schachman and Edelstein (1966) a value of $\phi^* = 0.8$ is also obtained. Although this procedure is less accurate than that employing measurement of s and η the result rules out the possibility that the variation of s with ionic strength is due to a change in shape of the protein (Berg, 1969).

When the sedimentation data are corrected for this amount of preferential hydration, according to eq 2, a constant value of 13.5 S is obtained for the sedimentation coefficient over the range of 0.35–3.5 in ionic strength (Figure 2). Thus we conclude that high concentrations of ammonium sulfate do not cause the enzyme to dissociate beyond the 13.5S species obtained in ammonium chloride solutions. The decrease in the sedimentation coefficient of fraction VI′ enzyme under these conditions can, instead, be ascribed to preferential hydration.

Core Polymerase. The sedimentation properties of core polymerase were studied in ammonium sulfate solutions at a number of ionic strengths. The results obtained previously with fraction VI′ enzyme suggested that preferential hydration would occur under these conditions. Accordingly, the data obtained with core polymerase at high ionic strengths were graphed as $s\eta$ *vs.* solvent density (Figure 4) and a preferential hydration of 0.40 g of water bound per g of protein was calculated for core polymerase. This is similar to the results obtained with fraction VI′ enzyme. The data were corrected for this preferential hydration according to eq 2 and the corrected sedimentation coefficients were graphed as a function of ionic strengths of the solutions (Figure 5).

The corrected results show that at ionic strengths above 0.26, core polymerase behaves as a single sedimenting species ($s_{20,w} = 12.6$ S). The dependence of $s_{20,w}$ on protein concentration supports the notion that core polymerase does not aggregate under these conditions. Thus at 0.64 ionic strength, sedimentation coefficients of 12.7 and 12.2 S are obtained with initial protein concentrations of 0.2 and 5 mg per ml,

respectively. At ionic strengths below 0.26, core polymerase aggregates extensively. In the absence of ammonium sulfate (μ = 0.04) the enzyme exhibits a very broad sedimenting boundary which appears to contain a number of different species (Figure 6b). Such a boundary is characteristic of a protein undergoing reversible equilibrium among different states of aggregation. The average sedimentation coefficient under these conditions is 44–48 S, which taken with the molecular weight of the core polymerase protomer determined below allows one to estimate that there must be at least six of these protomers in the average aggregate molecules.

The dependence of $s_{20,w}$ on protein concentration at μ = 0.04 is complex. A strong positive dependence of s on protein concentrate is expected; however, values $s_{20,w}$ of 47.8 and 44.7 S were obtained at protein concentrations of 0.2 and 5 mg per ml, respectively. It should be noted, however, that the variation of $s_{20,w}$ with μ is very steep in this range of ionic strength and at a concentration of 5 mg per ml the contribution of the protein itself to the ionic strength would be substantial. An estimate of the magnitude of this correction from the amino acid composition of *E. coli* B RNA polymerase (Berg, 1969) suggests that the contribution of the protein itself to the ionic strength of the solution could lower $s_{20,w}$ at the higher protein concentration by as much as 6 S. The possibility that core polymerase may only aggregate to a limited size—for example, to give an aggregate containing six core polymerase protomers—must also be considered in interpreting this low positive dependence of $s_{20,w}$ on protein concentration.

RNA Polymerase Holoenzyme. The sedimentation of holoenzyme was also examined in ammonium sulfate solutions. The data obtained at high ionic strengths were treated as described above to determine the amount of preferential hydration (Figure 4). Again a value of 0.39 g of water preferentially bound per g of protein was found. The sedimentation data, corrected for the preferential hydration, are graphed in Figure 5 *vs.* the ionic strength of the solutions.

At ionic strengths greater than 0.12, holoenzyme exists as a single sedimenting species with an $s_{20,w}$ of 15.0 S (Figure 6c). If one assumes that core polymerase and holoenzyme have the same frictional coefficient, this value is essentially that expected for the addition of 1 equiv of a protein subunit of mol wt 90,000 to a mole of core polymerase having $s_{20,w}$ = 12.6 S. As in the case of core polymerase, at low ionic strengths the RNA polymerase holoenzyme aggregates (Figure 6d). In the absence of ammonium sulfate (μ = 0.04) and in the presence of excess σ component, holoenzyme sediments at $s_{20,w}$ = 23 S. A dimer of a gloubular protein having a sedimentation coefficient of 15.0 S would have a predicted sedimentation coefficient of 23–24 S (Schachman, 1959). This indicates that RNA polymerase, in the presence of saturating amounts of σ component, aggregates maximally to give a dimer in the absence of added salt. Some preparations of holoenzyme (Berg *et al.* (1970) give slightly higher sedimentation coefficients (23–25 S) when excess σ component has not been added. This probably reflects the presence of small amounts of contaminating core polymerase in the holoenzyme preparation.

In summary, both RNA polymerase holoenzyme and core polymerase exist as single sedimenting species at high ionic strengths, having sedimentation coefficients of 15.0 and 12.6 S, respectively. At low ionic strengths both forms of the enzyme undergo aggregation. Holoenzyme, however, appears to be limited in aggregation to a dimer of the 15.0S species, whereas core polymerase can form large aggregates of the 12.6S species under these conditions. Fraction VI′ enzyme, containing less than 1 equiv of σ component, exhibits sedimentation behavior which is intermediate between that obtained for core polymerase and holoenzyme, as would be expected. All three preparations of RNA polymerase appear to preferentially interact with water in ammonium sulfate solutions, requiring a correction of the sedimentation data obtained in high ionic strengths under these conditions.

TABLE I: Sedimentation Equilibrium Results with Core Polymerase.[a]

Prepn	Rotor Speed (rpm)	Protein Concn (mg/ml)	Mol Wt
A	4800	0.5	377,000
		1.0	355,000
	6000	0.2	363,000
		0.4	376,000
	8000	0.5	365,000
		1.0	365,000
B	4800	0.2	407,000
		0.4	352,000
	6000	0.2	366,000
		0.4	355,000
		Av	= 368,000
Correction for dissociation during equilibration			× 1.04
Corrected average			= 383,000

[a] Sedimentation equilibrium studies were conducted with core polymerase in TMS buffer containing 0.20 M ammonium sulfate as described in Methods. The values listed have been corrected for a preferential solvation using Γ' = 0.39. The amount of irreversible dissociation of the protein to 8S material was determined by preparing a duplicate sample in each case and analyzing it for 8S material after storage on ice for the period of the sedimentation equilibrium experiment. Preparation A was purified as previously described (Berg *et al.*, 1970). Preparation B was further purified by subjecting it to differential zone sedimentation at high and low ionic strength as described for fraction VI enzyme (see Materials).

Sedimentation Equilibrium Studies

Core Polymerase. Since core polymerase behaves as a single sedimenting species at high ionic strengths, it is possible to determine the molecular weight of this species by low-speed sedimentation equilibrium. Accordingly, core polymerase was subjected to equilibrium sedimentation in TMS buffer containing 0.20 M ammonium sulfate. Ten determinations were carried out at two speeds and a variety of protein concentrations (Table I). The concentration of ammonium sulfate used was chosen to minimize the effects of preferential hydration

while maintaining the enzyme in the 12.6S species. A correction of only 3% is required for preferential hydration under these conditions. Graphs of log c *vs.* r^2 obtained with core polymerase in these experiments were linear over the entire portion of the cell used for calculations. The average value obtained for the molecular weight of core polymerase is 368,000. During the course of sedimentation, however, approximately 12% of the total material breaks down irreversibly to an 8S species as described above. Assuming that the 8S material is half the size of the 12.5S material and assuming that it redistributes independently, this material would be expected to lower the observed molecular weight by approximately 3–4% under the conditions employed (Berg, 1969). Thus, when the results are corrected for the presence of the 8S material, a value of 3.8×10^5 is obtained for the molecular weight of core polymerase.

From the relative amounts of each component present during acrylamide gel electrophoresis in SDS buffer (see Materials), core polymerase appears to have a subunit composition of $\beta'\beta\alpha_2\omega_2$. A species with this composition would have a molecular weight of $4.0 \times 10^5 \pm 0.4 \times 10^5$, as calculated from the subunit molecular weight estimates obtained from electrophoresis in SDS buffer. This value is comparable to that obtained from sedimentation equilibrium determinations, indicating that the 12.6S species of core polymerase can be considered to be the protomeric unit (Monod *et al.*, 1965). However, the exact stoichiometry of α and of ω[4] in the molecule must still be considered somewhat uncertain. The accuracy of procedure used here to determine the subunit composition is uncertain since De St. Groth *et al.* (1963) found a variation of up to 20% in the efficiency with which proteins are stained with coomasie blue. Furthermore, neither method of molecular weight determination is sufficiently accurate to determine the exact stoichiometry of these smaller components. Burgess (1969) has also concluded that the composition of core polymerase is $\beta'\beta\alpha_2$ from his studies of the amino acid composition of the protein and of the separated subunits.

RNA Polymerase Holoenzyme. RNA polymerase holoenzyme was subjected to low-speed equilibrium sedimentation at 0.2 M ammonium sulfate under conditions where the 15.0S species of the enzyme was known to exist. The results were corrected for preferential hydration and contaminating low molecular weight material as described above for core polymerase. The values obtained for the molecular weight were in the range of $4.0–4.2 \times 10^5$. These results are considerably lower than the expected molecular weight of 4.7×10^5 which is predicted from the equilibrium sedimentation molecular weight for core polymerase to which 1 mole of σ component (mol wt 90,000) has been added. We attribute the low values obtained by sedimentation equilibrium for the molecular weight of holoenzyme to a partial dissociation of the holoenzyme to give core polymerase and σ component during sedimentation. This interpretation is supported by two observations. First, when holoenzyme is subjected to prolonged zone sedimentation in glycerol gradients as described for preparation of fraction V′ or VI′, a partial dissociation of σ from the enzyme can be observed. Thus, assays for RNA polymerase and for σ activity show that the latter activity trails slightly behind the polymerase activity in the gradient, indicating that dissociation is significant during long periods of sedimentation. Secondly, we recall our original finding that fraction VI′ enzyme is not saturated with σ component. We attribute this primarily to loss of σ during enzyme purification due to dissociation; again the conclusion is that although binding between σ component and core polymerase is tight, it is reversible and the loss of σ due to dissociation is significant both in sedimentation equilibrium and in very long sedimentation velocity experiments.

RNA Polymerase Aggregates. It would be of considerable interest to obtain reliable estimates for the molecular weights of the several RNA polymerase aggregates obtained at low ionic strength. Unfortunately, sedimentation equilibrium studies have limited usefulness here, since all enzyme preparations examined so far exhibit significant heterogeneity in the aggregated state. Several preparations of fraction VI′ enzyme were subjected to sedimentation equilibrium in TMS buffer where the enzyme preparation sediments at 33 S. Values in the range of $1.9–2.1 \times 10^6$ were obtained for the weight-average molecular weight under these conditions with definite signs of heterogeneity. Thus fraction VI′ enzyme appears to form aggregates containing at least four or five protomer units. As discussed above, the aggregates of core polymerase, which sediment at 44–48 S, must be even larger, containing on the order of six or more protomer units.

Discussion

There have been a number of conflicting reports in the literature describing the sedimentation behavior of RNA polymerase (Pettijohn and Kamiya, 1967; Fuchs *et al*, 1964; Zillig *et al.*, 1966; Priess and Zillig, 1967; Colvill *et al.*, 1966; Lubin, 1969; Richardson, 1966; Stevens *et al.*, 1966; Maitra and Hurwitz, 1967; Chamberlin, 1963). The present work evaluates four major factors which affect this behavior and which serve to reconcile previous reports. These factors are (1) ionic strength, (2) subunit composition, (3) preferential hydration, and (4) irreversible dissociation of the enzyme.

At high ionic strengths both RNA polymerase holoenzyme and core polymerase exist in the protomer form. As the ionic strength is lowered this molecule reversibly associates to produce aggregates of various sizes. At intermediate ionic strengths sedimentation analysis gives boundaries which appear to reflect sedimentation of reversible equilibrium mixtures of components rather than of single sedimenting species. Thus the ionic strength must be carefully controlled in order to obtain meaningful sedimentation coefficients.

The subunit composition of the enzyme can vary in its relative content of either σ component or ω component. We have not yet studied the effect that variation of the amount of ω[4] in the enzyme has on the sedimentation properties of the molecule. However, the amount of σ component in the enzyme affects the sedimentation behavior of the enzyme both at high ionic strength, where it contributes its mass to the sedimenting species, and at low ionic strength, where it has the additional effect of limiting aggregation of the enzyme to a dimer. Preparations of enzyme containing less than 1 equiv of σ exhibit sedimentation behavior intermediate between that obtained for core polymerase and holoenzyme. Thus the subunit composition of an RNA polymerase preparation is

[4] As discussed in Materials, designation of ω as a subunit of RNA polymerase is still open to question. It appears to be less tightly bound to the molecule than are the other components and it is not currently known to have a functional role in RNA synthesis.

important in the exact interpretation of sedimentation data.

The strain of bacteria employed may affect the behavior of RNA polymerase in this regard. In our hands preparations of *E. coli* B RNA polymerase isolated by two different procedures (Berg *et al.*, 1970; Chamberlin and Berg, 1962) invariably contain less than 1 equiv of σ after purification. This is probably due to partial dissociation of the holoenzyme during the preparation. In contrast similar preparations of RNA polymerase from *E. coli* K contain 1 equiv of σ component (Travers and Burgess, 1969) and physical studies of *E. coli* K RNA polymerase show that the enzyme aggregates essentially to a dimer form (Fuchs *et al.*, 1964; Zillig *et al.*, 1966; Priess and Zillig, 1967; Richardson, 1966). RNA polymerase from *Azobacter vinelandii* also appears to aggregate maximally to give a dimer (Huang and Warner, 1969) and appears to contain essentially 1 full equiv of σ component (J. Krakow, personal communication). These differences may well be due to differences in the intrinsic affinity of the different polymerases for their σ factors.

The third effect, preferential hydration, plays a significant role only in solutions containing high concentrations of divalent salts. However, since elevated ionic strengths are required to minimize aggregation of the enzyme protomer the effect is a significant one and probably accounts for reports of sedimentation coefficients as low as 10–11 S for the RNA polymerase holoenzyme (Maitra and Hurwitz, 1967; Chamberlin, 1963). The effect has been studied in some detail in this work since the observation that the sedimentation coefficient of RNA polymerase decreases at elevated ionic strengths was initially interpreted both in the laboratory and by others as showing that the protein dissociated to a lower molecular weight form under these conditions. The danger of such a misinterpretation when dealing with complex proteins in three-component systems has been set forth recently by others (Aune and Timasheff, 1970).

The conclusion that preferential interactions are present in the multicomponent system RNA polymerase–water–ammonium sulfate can be made with some certainty from our studies of the variation of sedimentation coefficient with solvent viscosity and solvent density. In this instance we obtain an approximate value of the partial specific volume for RNA polymerase of 0.81 from which we conclude that preferential solvation must be present. This preferential solvation could result from steric exclusion of salt ions from the domain of the protein (Hill and Cox, 1965) or from direct preferential binding of water to groups on the protein. The former model predicts that the size and charge of the salt ion would be of importance in determining the amount of preferential solvation and it seems significant that with RNA polymerase preferential hydration is greatly reduced if present at all when ammonium chloride replaces ammonium sulfate. While preferential solvation can result from Donnan effects which appear to contribute appreciably to preferential solvation with DNA (Cohen and Eisenberg, 1968) it probably does not make a major contribution here since RNA polymerase carries a net negative charge at the pH employed in these studies and the extent of preferential solvation depends on the anion present.

It should be stressed that a rigorous interpretation of the preferential interactions in this system must await direct studies of binding by equilibrium dialysis (Casassa and Eisenberg, 1964). In particular we have assumed for the purpose of calculation that the extent of preferential solvation does not vary with solvent density and thus that ϕ^*, the apparent partial specific volume of the protein, is a valid measure of the partial specific volume of RNA polymerase in ammonium sulfate solutions. Neither assumption can be theoretically justified, and in fact where careful studies of preferential interactions have been made in some other three-component systems, it has been found that these assumptions are not justified (Cohen and Eisenberg, 1968; Reisler and Eisenberg, 1969). While these considerations must affect the exact interpretation of the cause and the extent of the preferential interactions in the RNA polymerase–ammonium sulfate system they do not change our qualitative picture of the effect of variations of ionic strength on the structure of the protein, nor do they affect in any way our calculation of $s_{20,w}$ or of the molecular weight for the two forms of polymerase since the correction due to preferential interactions is negligible at low concentrations of ammonium sulfate and in ammonium chloride, that is, as we approach a simple two-component system.

Finally, in determinations of the molecular weight of the enzyme protomer by sedimentation equilibrium, irreversible breakdown of the enzyme needs to be considered. The enzyme dissociates irreversibly to an 8S product at a slow but constant rate under all of the conditions we have examined. The amount of this component can be estimated and a correction can be made for its presence when determining the molecular weight of the enzyme protomer by the method of low-speed sedimentation equilibrium. Methods which emphasize the contribution of low molecular weight material, however, such as the meniscus depletion method of sedimentation equilibrium (Yphantis, 1964), may lead to considerable errors when applied to RNA polymerase because of this breakdown of the molecule during the course of sedimentation.

The aggregation behavior of RNA polymerase presents an interesting problem in protein interactions. First, the fact that holoenzyme does not aggregate beyond a dimer indicates that the protomer interactions for holoenzyme must be isologous (Monod *et al.*, 1965). Core polymerase utilizes either heterologous interactions or isologous interactions of a different nature (or both) since it aggregates beyond a tetramer stage and gives indications of forming polydisperse polymers. Two rather simple models for the aggregation behavior are attractive at this time. One possibility is that RNA polymerase aggregates in general by first forming dimers through isologous protomer interactions. These dimers then aggregate through a different set of interactions to produce long chains. σ component could be envisaged as blocking aggregation beyond the dimer stage by occupying a site on the dimer necessary for dimer–dimer interactions. By this model one would expect the aggregates formed by core polymerase to contain even numbers of protomers, *e.g.*, tetramers, hexamers, etc. Alternatively, one can imagine that the dimers are unique to holoenzyme and that core polymerase aggregates by adding individual protomers to the growing chain, utilizing quite different protomer interactions from those found with holoenzyme. We cannot at this time discriminate between these two possibilities.

Whether the RNA polymerase aggregates have significant biological functions is not yet clear. From the subunit composition data and from the molarity of σ component it appears

very likely that the protomer form of the enzyme is the active species. It is perhaps significant, however, that core polymerase produces aggregates at ionic strengths below 0.26 whereas holoenzyme forms aggregates only at ionic strengths below 0.12. Since the ionic strength of the cell appears to be in this range (Schultz and Solomon, 1961), it is possible that *in vivo* core polymerase exists in an aggregated form while enzyme in the holoenzyme form remains as protomer. It is known that the σ component is released by holoenzyme at some step after the initiation of an RNA chain *in vitro* (Travers and Burgess, 1969; Berg *et al.*, 1969). The remaining core polymerase continues elongation of the RNA chain and is presumably released from the template as core polymerase protomer after termination of the RNA chain. The released molecule of core polymerase could then either bind a molecule of σ component to reconstitute the holoenzyme protomer or it could associate with other core polymerase molecules to produce an aggregate. It has been observed that if σ component is added to the core polymerase aggregates *in vitro* at low ionic strength the enzyme rapidly dissociates to the holoenzyme protomer (D. Berg, unpublished results). One might suppose then that control over the distribution of RNA polymerase in its different forms in the cell might depend on both the ionic strength and on the amount of σ component present.

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