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Induction of Stable Linkage between the Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase and $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ by Ultraviolet Light†

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ABSTRACT: *Escherichia coli* RNA polymerase bound to the synthetic polymer $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ can be induced with ultraviolet (uv) light to form stable binary complexes. The stability of the complex most likely is due to covalent linkage(s) between the enzyme and polymer as shown by resistance of the complex to high ionic strength solutions and stability to alkali or heat treatment. Formation of stable complexes was found to be a function of the incident uv dose, the input molar ratio of enzyme–polymer, and the components present in the irradiation solution. Ultraviolet light induced enzyme–polymer complexes were detected by retention on Millipore filters after washing with high ionic strength solutions (2 M) which dissociate nonlinked enzyme–polymer complexes. Analysis of uv-induced stable complexes on cesium chloride sedimentation equilibrium gradients resulted in formation of an enzyme-dependent peak which essentially floated on top of the gradient. For a particular dose of uv light, the per cent total polymer present in this floating peak compared favorably with the per cent total polymer retained on Millipore filters after a high salt wash for several molar ratios of the enzyme–polymer tested. From density distribution profiles of detergent-treated, uv-induced enzyme–polymer complexes in cesium chloride equilibrium gradients, a significant fraction of the denatured enzyme–polymer complex banded at a density of 1.58 g/cm^3. This fraction most likely contains only one linked polypeptide subunit of the multisubunit enzyme, and this is possibly the α or σ subunit.

The first step in ribonucleic acid (RNA) synthesis is association of the deoxyribonucleic acid dependent ribonucleic acid polymerase (RNA polymerase) with the deoxyribonucleic acid (DNA) template; this initial process is designated as the binding step. Upon mixing purified RNA polymerase with a nucleic acid polymer *in vitro*, a binary complex is formed in the absence of RNA synthesis (Fox *et al.*, 1965). The *Escherichia coli* RNA polymerase is a complex multisubunit enzyme; the holoenzyme is composed of the polypeptide subunits $\alpha_2\beta\beta'\sigma(\omega)$ (Zillig *et al.*, 1970; Burgess and Travers, 1971). Recent studies using partially dissociated fragments of the RNA polymerase have provided evidence as to the function(s) of the various subunits of the enzyme in the process of RNA synthesis (Zillig *et al.*, 1970; Ishihama, 1972). However, in order to understand better the molecular mechanisms of transcription, it is necessary to know the spatial relationship of the various subunits of the enzyme in the RNA polymerase–DNA complex. Several attempts have been made with the electron microscope to determine the fine structure of RNA polymerase and RNA polymerase bound to DNA (Fuchs *et al.*, 1964; Slayter and Hall, 1966; Colvill *et al.*,

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1966; Kitano and Kameyama, 1969; Lubin, 1969). The results of these studies have been too variable to warrant any detailed interpretation of the fine structure of the enzyme. Possible explanations for this included variability of purity of enzyme preparations and distortion of particles in the staining process used before examination. In addition, the monomer–dimer equilibrium of the enzyme and the question of which form of the enzyme is the physiologically active form have complicated interpretation of electron micrographs. It is thought generally from electron microscopic data that the dimeric form of the enzyme assumes a hexagonal structure surrounding an empty core; a somewhat similar structure was deduced from small-angle X-ray measurements (Pilz *et al.*, 1972), yet several lines of evidence suggest that it is the monomeric form of the enzyme which is the active one (Smith *et al.*, 1967; Krakow and von der Helm, 1970; Schäfer *et al.*, 1972) and that a considerable rearrangement of the subunits takes place upon dissociation of the dimer to the monomer form (Pilz *et al.*, 1972). To date, the geometric relationship of the RNA polymerase subunits to one another and to the polymer to which they are bound is unknown.

In this report we describe a procedure which may be useful in the determination of the morphology of RNA polymerase–DNA complexes. Basically the method involves the photoinduction of cross-links between the polymer and bound enzyme. Ultraviolet (uv) light can induce stable complex formation between protein and DNA (Smith, 1962; Alexander and Moroson, 1962). Smith (1970) provided a possible mechanism as to the nature of uv light-induced stable protein–nucleic acid complexes; the process evidently is mediated through formation of a stable covalent bond linkage. Markovitz (1972) recently reported that uv light could induce stable complexes between DNA polymerase and DNA isolated from *E. coli*. The experiments described below show that uv light can induce stable complex formation between RNA polymerase and a synthetic deoxyribonucleic acid polymer, $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$. Resistance of the complex to dissociate in high salt buffers or treatment with alkali or heat suggests that the complex is stabilized through a covalent link. Results from cesium chloride equilibrium gradients of uv-induced RNA polymerase–DNA complexes suggest that there may exist unique potential sites on the enzyme for photochemical induced linkage between the enzyme and polymer. Although there are limitations in this approach, as will be discussed, it would appear that this technique should prove to be valuable in elucidation of the structure of RNA polymerase–DNA complexes as well as other protein–nucleic acid complexes. In addition, this technique may be useful in providing information about the nature of sites on nucleic acid molecules to which proteins bind in a specific manner.

Experimental Section

Isolation of RNA Polymerase. The DNA-dependent RNA polymerase containing σ subunit was isolated from *E. coli*, strain B_{s-1}, according to a modified procedure of Burgess (1969a) as outlined previously (Strniste *et al.*, 1973a,b). The polymerizing activity of the isolated enzyme using calf thymus DNA template (Worthington Biochemical Corp.) was 600 units/mg of protein (one unit of enzyme incorporates 1 nmol of AMP in a 10-min incubation under conditions described elsewhere (Strniste *et al.*, 1973a)). Protein determinations were conducted using a microbiuret assay (Goa, 1953) or using the extinction coefficient for RNA polymerase, $\epsilon_{280\text{ nm}}^{1\%} = 6.5$ (Richardson, 1966). Values for protein concentration obtained from these two methods agreed within 10%. Electrophoresis of the purified RNA polymerase on 0.1% sodium dodecyl sulfate polyacrylamide gels as previously described (Shapiro *et al.*, 1967; Weber *et al.*, 1972) indicated homogeneity in the preparation.

Materials. All chemicals were of analytical grade unless otherwise specified. Optical grade cesium chloride was purchased from the Harshaw Chemical Company. Radioactive ribo- and deoxyribonucleoside triphosphates were purchased from Schwarz/Mann. Unlabeled ribonucleoside triphosphates and deoxyribonucleoside triphosphates were purchased from P-L Biochemicals, Inc. and Schwarz/Mann, respectively. Purity of nucleoside triphosphates was determined by paper chromatography as described by Vanderheiden (1968).

Synthesis of $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$. High specific activity [^{14}C]d-$(A\text{-}T)_n \cdot d(A\text{-}T)_n$ was prepared using *Micrococcus luteus* DNA polymerase which was isolated by the method of Harwood *et al.* (1970). The reaction mixture for synthesis of [^{14}C]d-$(A\text{-}T)_n \cdot d(A\text{-}T)_n$ contained per 2.00 ml: 100 μmol of Tris buffer[1] (pH 7.8), 2 μmol of HSEtOH; 10 μmol of $MgCl_2$; 1.0 μmol of dATP-*8*-^{14}C (2.10×10^7 cpm/μmol); 1.0 μmol of dTTP-*2*-^{14}C (2.1×10^7 cpm/μmol); 0.22 A_{260} unit of primer $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$; and 90 units of *M. luteus* DNA polymerase. The reaction was incubated at 37° for 90 min, at which time about 50% of the labeled deoxyribonucleoside triphosphates was acid insoluble. The reaction was then extracted with phenol as previously described (Smith *et al.*, 1970) and dialyzed exhaustively against 0.01 M NaCl. The labeled polymer was then eluted from a calibrated Bio-Gel A-50m column (Bio-Rad Laboratories) according to procedures outlined elsewhere (Hayes *et al.*, 1968; Hayes and Mitchell, 1969). The preparation of [^{14}C]$d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ had an average monomer unit length of 456. The specific activity of the preparation was 1.94×10^7 cpm/μmol when spotted on a Millipore filter and counted in Aquasol universal liquid scintillation cocktail (New England Nuclear Corp.) in a Beckman CPM-100 liquid scintillation system.

Binding Assay. The binding of RNA polymerase to the synthetic polymer $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ was similar to the procedures of Jones and Berg (1966) and Ishihama and Hurwitz (1969). Binding reaction mixtures contained per 0.100 ml (unless stated differently elsewhere): 5.0 μmol of Hepes buffer (pH 7.9); 0.4 μmol of $MnCl_2$; 0.4 μmol of HSEtOH; and varying amounts of RNA polymerase and ^{14}C-labeled polymer. Reactions were incubated at 37° for 5 min, immediately chilled to 0° with the addition of 1.5 ml of 0.01 M Tris buffer (pH 7.2 at 22°) and 0.05 M KCl (to be referred to as low salt washing buffer), and kept at 0° for an additional 10 min. The solution was then passed through a Millipore filter (type HA, 0.45 μ mean pore size), presoaked for over 10 min in washing buffer, with gentle suction (20 mm), and rinsed with 50 ml of low salt washing buffer (0°). In washing with buffers containing higher KCl concentrations, the filters were always rinsed with 10 ml of low salt washing buffer (0°) after passage of high salt washing buffer in order to remove residual salt from the filter. The filters were dried at 75°, placed in scintillation vials with 15 ml of Aquasol scintillation fluid, and counted.

Irradiation with Ultraviolet Light. Irradiation with uv light was performed using an unfiltered General Electric 30-W germicidal lamp (G30T8). The incident dose rate was deter-

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; HSEtOH, 2-mercaptoethanol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

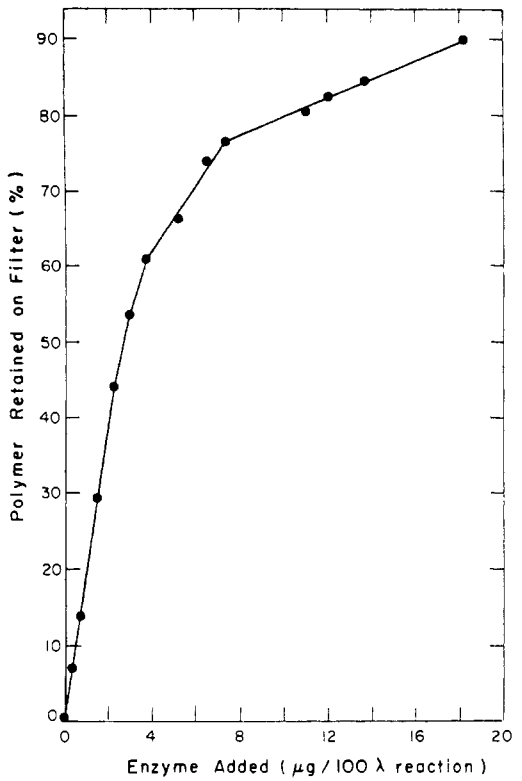

FIGURE 1: Binding of *E. coli* RNA polymerase to the synthetic polymer [^{14}C]d(A-T)$_n$·d(A-T)$_n$ as a function of amount of enzyme added to the binding reaction mixture. Each reaction (0.1 ml) contained 5 μmol of Hepes buffer (pH 7.9), 0.4 μmol of $MnCl_2$, 0.4 μmol of HSEtOH, 0.9 monomer nmol of ^{14}C-labeled polymer, and various amounts of RNA polymerase. Reactions were incubated for 5 min at 37°, diluted with 1.5 ml of chilled (0°) low salt washing buffer, applied to presoaked Millipore filters, rinsed with 50 ml of chilled low salt washing buffer, dried, and counted in 15 ml of Aquasol scintillation fluid.

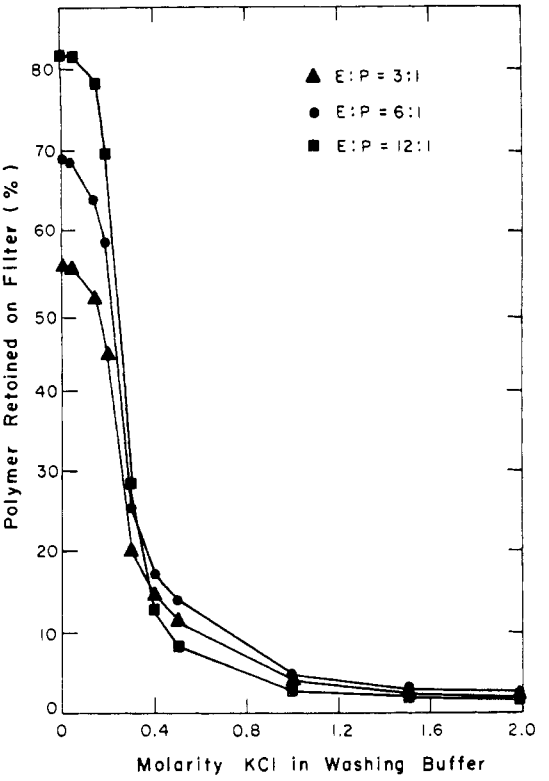

FIGURE 2: Dissociation of *E. coli* RNA polymerase-[^{14}C]d(A-T)$_n$·d(A-T)$_n$ complexes as a function of concentration of KCl in the washing buffer. Reactions (0.5 ml) contained 25 μmol of Hepes buffer (pH 7.9), 2.0 μmol of $MnCl_2$, 2.0 μmol of HSEtOH, 4.50 monomer nmol of ^{14}C-labeled polymer, and either 15, 30, or 60 μg of enzymes. Reactions were incubated at 37° for 5 min, and 0.05-ml aliquot were sampled into 1.5 ml of chilled (0°) washing buffer with various concentrations of KCl (0–2.0 M). Each sample was then applied to a presoaked Millipore filter, rinsed with 50 ml of chilled washing buffer of various concentrations of KCl (0–2.0 M), dried, and counted. Various symbols used in this plot represent input molar ratios of enzyme–polymer of 3:1, 6:1, or 12:1.

mined as described elsewhere (Barnhart and Cox, 1970). Samples ($\leq$1.0 ml) to be irradiated were placed in shallow, chemically cleaned Pyrex watch glasses and covered with quartz windows. The watch glasses were set in beakers filled with ice and placed under the germicidal lamp 24 cm from the light source. The corrected incident dose rate was determined to be 36.2 ergs mm^{-2} sec^{-1}.

Cesium Chloride Equilibrium Sedimentation. Samples to be centrifuged were mixed with solutions of cesium chloride of known density. Density measurements either before or after centrifugation were made with a Bausch and Lomb precision refractometer; the density of the samples was determined from standard tables (Handbook of Biochemistry, 1968) relating refractive index and density. Centrifugation was performed in either a Beckman Type 40 general-purpose, fixed-angle rotor or a Beckman Type 50.1 six-tube, swinging-bucket rotor, using a Beckman Model L2-65 ultracentrifuge. To develop a shallow gradient ($\Delta\rho \simeq 100$ mg/cm^3), the Type 40 fixed-angle rotor was centrifuged for 66 hr at 33,000 rpm at 20°. Depending on the experimental conditions, rotor tubes [either polycarbonate (Beckman) or polypropylene (IEC)] were presoaked for 36 hr with 1% bovine serum albumin and 1.0 M NaCl, or 1% bovine serum ablumin, 0.01% denatured calf thymus DNA, and 1.0 M NaCl, respectively, emptied, and swabbed with cotton immediately before use. Treatment of the centrifuge tubes prior to centrifugation reduced considerably the retention of enzyme–polymer complexes in the tubes. To develop a steeper gradient ($\Delta\rho > 400$ mg/cm^3), the Type 50.1 swinging-bucket rotor was centrifuged for 66 hr at 44,000 rpm at 20°. Polyallomer tubes were used and were presoaked with 1% bovine serum albumin and 1.0 M NaCl, for 36 hr prior to use. Collection of gradients centrifuged in the Type 40 rotor entailed layering gently 1.0 ml of H_2O on top of the gradient after centrifugation and collecting 0.20-ml sample fractions from the bottom of the tube. Collection of gradients centrifuged in the Type 50.1 swinging-bucket rotor did not include the H_2O layer after centrifugation; however, fractions of 0.15 ml were collected from the bottom. Fractions were either assayed for index of refraction readings or combined with 1.0 ml of H_2O and 15 ml of Aquasol and counted.

Results

It has been shown that there exists a strong binding affinity between the RNA polymerase and the synthetic polymer d(A-T)$_n$·d(A-T)$_n$ as detected by retention of the complex on Millipore filters (Jones and Berg, 1966). Figure 1 shows the formation of [^{14}C]d(A-T)$_n$·d(A-T)$_n$–RNA polymerase complexes as detected by retention of the complex on Millipore filters as a function of amount of enzyme added to the reaction mixture. The ^{14}C-labeled polymer had an average monomer unit length of 456[2] (228 base pairs), and in all reactions there

[2] Quantities of synthetic polymer expressed in this paper have been determined in terms of monomer units. Therefore, dividing this quantity by the average monomer unit length of the polymer results in the molar quantity of the polymer of noted average size.

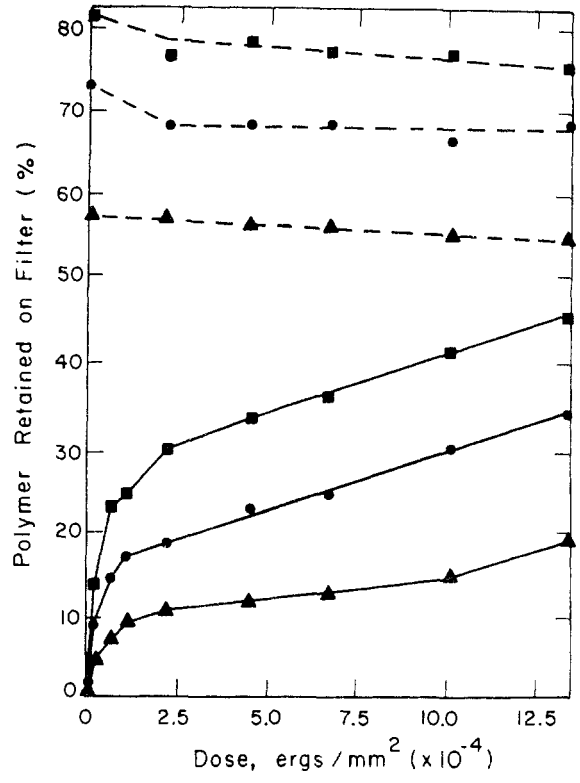

FIGURE 3: Induction of high salt stable complexes between *E. coli* RNA polymerase and [^{14}C]d(A-T)$_n$·d(A-T)$_n$ by uv light. Reactions (0.9 ml) contained 45 μmol of Hepes buffer (pH 7.9), 3.6 μmol of $MnCl_2$, 3.6 μmol of HSEtOH, 8.10 monomer nmol of ^{14}C-labeled polymer, and either 27, 54, or 108 μg of enzyme. Irradiation at 0° was conducted as outlined in the Experimental Section. At various times during irradiation, two 0.05-ml samples were removed and diluted with 1.5 ml of chilled low or high salt washing buffer. Samples were then applied to presoaked Millipore filters, washed with 50 ml of chilled low or high salt washing buffer, dried, and counted. Dashed curves represent stability of complexes to low salt washing buffer, while solid curves represent formation of high salt stable complexes. Symbols used represent input molar ratio of enzyme–polymer: (▲) 3:1, (●) 6:1, and (■) 12:1.

existed 0.9 monomer nmol/0.100 ml of sample or 2.0 pmol of polymer. Using a molecular weight for *E. coli* RNA polymerase plus σ subunit of 5×10^5, 1 μg of enzyme would equal 2 pmol of enzyme. Thus, linearity in the binding kinetics of RNA polymerase of d(A-T)$_n$·d(A-T)$_n$, as detected by the above described method, continues until the molar ratio of enzyme to polymer is equal to about 4, where approximately 60% of the polymer in solution is retained on the filter.

Although the RNA polymerase has a strong affinity for the synthetic polymer d(A-T)$_n$·d(A-T)$_n$, an increase in ionic strength of the washing buffer will cause dissociation of the enzyme–polymer complex. Figure 2 shows the ability of the complex to remain associated as a function of increasing concentrations of KCl in the washing buffer for three molar ratios of reacting enzyme–polymer. It is apparent from the results that concentrations of KCl in the washing buffer ≥1.0 M cause essentially complete dissociation of the enzyme–polymer complex. Background retention of the polymer alone on filters over the range of KCl concentrations noted was 1.0–2.5%. Dissociation of the enzyme–polymer complex by increasing the ionic strength in the washing buffer was essentially independent of the molar ratio of enzyme–polymer in the reaction mixture. That is, approximately 50% of the enzyme–polymer complexes retained on the Millipore filter with no KCl in the washing buffer remains on the filter at a KCl concentration of 0.28 M for molar ratios of enzyme–polymer equaling 3:1, 6:1, and 12:1. In experiments to be described below, low salt washing buffer consists of 0.01 M Tris buffer (pH 7.2) and 0.05 M KCl, whereas high salt washing buffer consists of 0.01 M Tris buffer (pH 7.2) and 2.0 M KCl.

When [^{14}C]d(A-T)$_n$·d(A-T)$_n$ was complexed with *E. coli* RNA polymerase at various molar ratios of enzyme–polymer and irradiated with uv light at 0°, formation of high salt (2.0 M) stable complexes was detected by retention on Millipore filters. The results of this experiment are shown in Figure 3. The formation of high salt stable complexes by uv light was found to be a function of uv dose and molar ratio of enzyme–polymer irradiated. In all three examples noted (molar ratios enzyme–polymer equaling 3:1, 6:1, and 12:1), there existed an initial rapid increase in formation of high salt stable complexes, after which formation proceeded more slowly with dose. Stability of the enzyme bound to d(A-T)$_n$·d(A-T)$_n$ at 0° is evident from the low salt wash curves (dashed lines); greater than 90% of

TABLE I: Stability of *E. coli* RNA polymerase–[^{14}C]d(A-T)$_n$·d(A-T)$_n$ Complexes Irradiated Separately with Uv Light and Then Combined to Either Low or High Salt Washing Buffers.[a]

	Total Cpm Retained on Millipore Filter (%)							
	0		3.25 × 10^4 ergs		6.5 × 10^4 ergs		13.0 × 10^4 ergs	
	Low salt[b]	High salt[b]	Low salt	High salt	Low salt	High salt	Low salt	High salt
Polymer	1.0	1.3	1.0	1.2	1.1	1.2	1.1	2.3
Enzyme–polymer = 3:1[c]	44.9	2.1	31.0	1.9	30.7	2.4	28.7	4.4
Enzyme–polymer = 6:1	69.4	2.1	59.0	2.1	57.0	2.4	55.7	4.5

[a] Reactions (0.250 ml) contained 12.5 μmol of Hepes buffer (pH 7.9), 1.0 μmol of $MnCl_2$, 1.0 μmol of HSEtOH, and either 0, 15, or 30 μg of RNA polymerase or 4.5 monomer nmol of labeled polymer (1.94×10^4 cpm/monomer nmol). Reactions were chilled to 0° and irradiated with uv light as described in the Experimental Section. During irradiation, 0.05-ml aliquots of the reaction mixtures containing either 0, 15, or 30 μg of RNA polymerase (total amount in 0.25 ml) were combined with 0.05-ml aliquots of the irradiated reaction mixture containing only the polymer. Combined irradiated samples were incubated at 37° for 5 min, and two 0.050-ml aliquots were removed from each reaction and diluted with either 1.5 ml of chilled low or high salt washing buffers. Samples were then applied to presoaked Millipore filters, washed, dried, and counted as outlined in Figures 1–3. Doses used are incident uv doses (ergs/mm^2). [b] Low and high salt columns represent the per cent retention of total polymer on the filter when washed with either low or high salt washing buffers, respectively. [c] Enzyme–polymer = 3:1 or 6:1 represents the final molar ratio obtained in the reaction mixture after combination of the separately irradiated enzyme and polymer mixtures.

the original complexes formed at the three molar ratios of enzyme–polymer are retained on filters over the duration of the experiment. At the highest dose of uv light used in this experiment (1.30×10^5 ergs/mm^2), 36, 49, and 60% of the enzyme–polymer complexed after low salt wash was stable to a high salt wash for enzyme–polymer complexes formed at molar ratios equaling 3:1, 6:1, and 12:1, respectively. Formation of high salt stable complexes between the RNA polymerase and d(A-T)$_n$·d(A-T)$_n$ by uv light is also a function of the irradiation solution components (see below).

It was noted by Smith (1964) that irradiation of DNA and protein separately with uv light resulted in a complex stable to high salt upon combining the two irradiated species. However, Markovitz (1972) conducted a similar experiment with purified *E. coli* DNA polymerase and DNA and found no high salt stable complex formation upon combination of the two separately irradiated species. We have conducted analogous experiments with highly purified *E. coli* RNA polymerase and d(A-T)$_n$·d(A-T)$_n$, and the results of this experiment are shown in Table I. Retention on the filters of irradiated polymer alone is essentially that obtained for the nonirradiated polymer. When RNA polymerase and d(A-T)$_n$·d(A-T)$_n$ were irradiated separately and then combined at molar ratios of 3:1 or 6:1, there exists a slight but detectable loss in binding of enzyme to polymer after a low salt wash (KCl = 0.05 M) when compared to control nonirradiated species. However, it is apparent that combination of separately irradiated enzyme and polymer does not result in the formation of high salt stable complexes over the uv dose range used.

Since uv light induces high salt stable complex formation between RNA polymerase and d(A-T)$_n$·d(A-T)$_n$, it was possible to analyze this complex in cesium chloride equilibrium gradients. In the following experiment, RNA polymerase was mixed and incubated with [^{14}C]d(A-T)$_n$·d(A-T)$_n$ at various molar ratios, irradiated with uv light, mixed with cesium chloride, and centrifuged to equilibrium. After centrifugation, the gradients were fractionated and analyzed for refractive index readings or radioactive counts per minute (cpm). The results of this experiment are shown in Figure 4. The profile of recovered cpm for the irradiated polymer is the same as that obtained for a nonirradiated polymer (data not shown); the average buoyant density for d(A-T)$_n$·d(A-T)$_n$ equaled 1.67 g/cm^3, in agreement with previously reported values for this polymer (Wells and Blair, 1967). However, when RNA polymerase–polymer complexes were irradiated with uv light at various molar ratios (enzyme–polymer) and examined on cesium chloride equilibrium gradients, there was an enzyme-dependent increase in amount of ^{14}C-labeled polymer which essentially floated on top of the gradient. Summation of the cpm in these peaks resulted in 12.9, 21.3, and 37.7% of the total cpm recovered from the gradient for molar ratios of enzyme–polymer equaling 3:1, 6:1, and 12:1, respectively. These results compare favorably with the high salt stable complexes retained on Millipore filters where, at the same uv dose of 65,000 ergs/mm^2, there existed 12.1, 23.5, and 35.2% polymer retained for molar ratios of enzyme–polymer of 3:1, 6:1, and 12:1, respectively.

The stability of uv-induced high salt stable complexes to either alkali or heat treatment was examined in the following experiment. Preincubated samples of polymer or enzyme–polymer at a molar ratio of 6:1 were irradiated with 65,000 ergs/mm^2 at 0°, and half of each reaction was treated with either KOH (final pH 12.6) for 1 hr or heat (93° for 15 min). After neutralization of the alkali sample or reincubation (37°) of the heated sample, all samples were combined with neutral

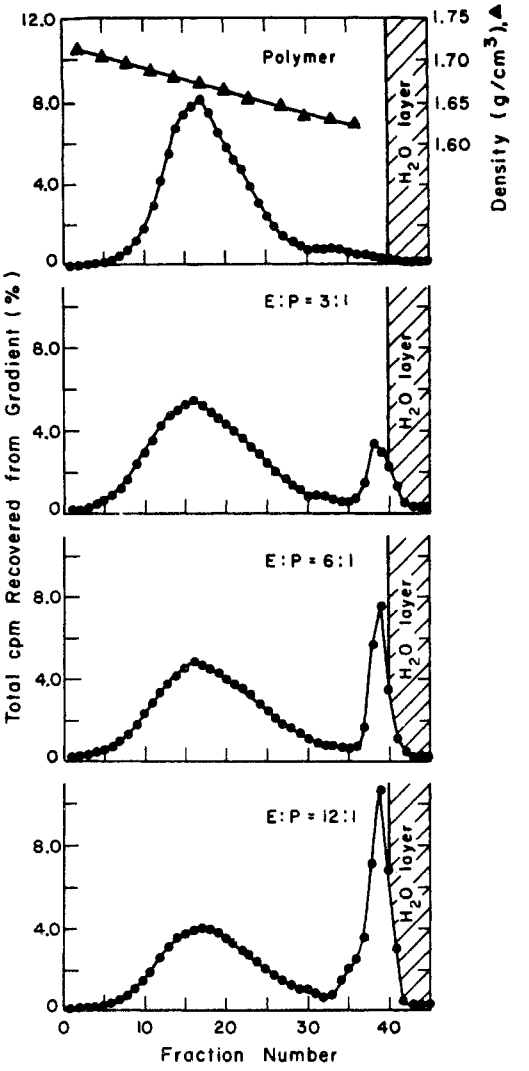

FIGURE 4: Cesium chloride equilibrium analysis of uv-induced stable complex formation between *E. coli* RNA polymerase and [^{14}C]d(A-T)$_n$·d(A-T)$_n$. Reactions (0.7 ml) contained 35 μmol of Hepes buffer (pH 7.9), 2.8 μmol of $MnCl_2$, 2.8 μmol of HSEtOH, 6.30 monomer nmol of ^{14}C-labeled polymer, and either 0, 21, 42, or 84 μg of RNA polymerase. Reactions were processed and irradiated with uv light (65,000 ergs/mm^2) as described in the Experimental Section. Samples of each irradiated reaction (0.4 ml) were added to cesium chloride solutions such that the final volume equaled 8.0 ml of average density of 1.655 g/cm^3. The mixtures were loaded into polycarbonate tubes which had been presoaked with 1% bovine serum albumin, 1.0 M NaCl, for 36 hr and were centrifuged in a Beckman Type 40 general-purpose, fixed-angle rotor for 66 hr at 33,000 rpm at 20°. After centrifugation, 1.0 ml of H_2O was gently layered on top of each gradient, and 0.2-ml samples were collected from the bottom directly into scintillation vials. Gradients were analyzed for radioactive cpm or refractive index readings. Recovery of input cpm from the gradients was 85–90%. Plots represent irradiated polymer alone or enzyme–polymer complexes of input molar ratios of 3:1, 6:1, or 12:1.

cesium chloride solution and centrifuged to equilibrium. In this experiment, presoaked polypropylene tubes were used; recovery of total input cpm was 75–80%. The results are shown in Figure 5. The alkali- or heat-treated, uv-irradiated polymer which had been neutralized or reincubated at 37° before centrifugation displayed a band profile and average buoyant density similar to a nontreated polymer sample (data not shown). However, when irradiated enzyme–polymer complex at a molar ratio of 6:1 was treated with either alkali or heat prior to centrifugation, approximately 20% of the

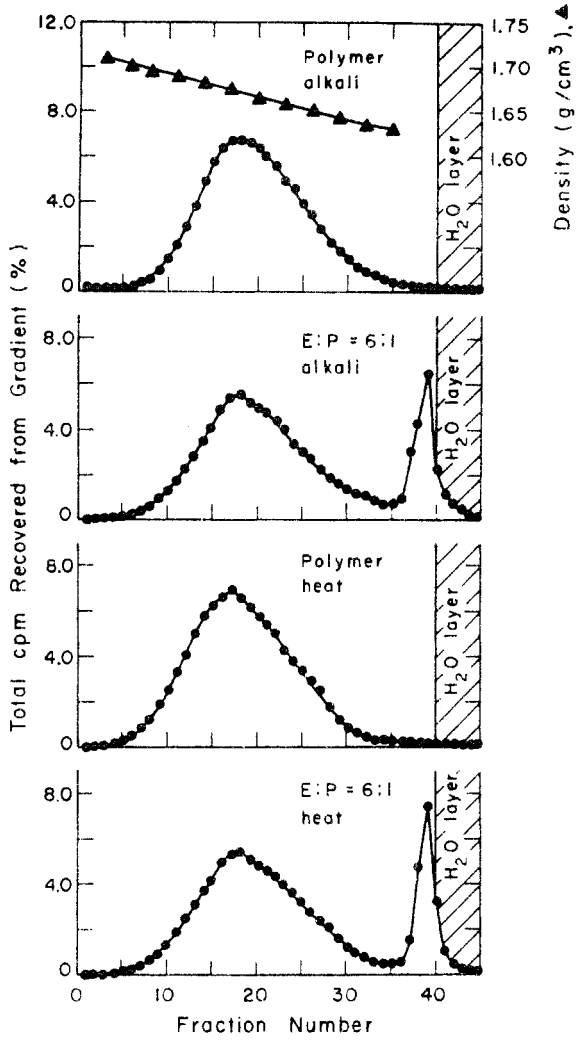

FIGURE 5: Cesium chloride equilibrium analysis of alkali- or heat-treated, uv-induced stable complexes between *E. coli* RNA polymerase and [^{14}C]d(A-T)$_n$·d(A-T)$_n$. Reactions (0.9 ml) contained 45 μmol of Hepes buffer (pH 7.9) and 8.0 monomer nmol of ^{14}C-labeled polymer plus 0 or 54 μg of RNA polymerase and were processed as in Figure 4. From each irradiated sample, two 0.4-ml samples were removed, and one of each was heated to 93° for 15 min, reincubated at 37° for 10 min, and chilled to 0°. To the other 0.4-ml samples, 0.1 ml of 1 N KOH was added (final pH 12.6), incubated at 37° for 1 hr, and neutralized to pH 7.6 with Tris buffer. The samples were then combined with neutral cesium chloride solutions, and the final volume of each was 8.0 ml with an average density of 1.655 g/cm^3. All samples were loaded into pretreated polypropylene tubes and centrifuged in a Beckman Type 40 general-purpose, fixed-angle rotor for 66 hr at 33,000 rpm at 20°. After centrifugation, gradients were fractionated and analyzed for either radioactive cpm or refractive index readings. Recovery of input cpm from the gradients was 75–80%. Plots represent irradiated polymer or enzyme–polymer complexes at an input molar ratio of 6:1 which had been treated with alkali or heat.

recovered cpm floated on top of the cesium chloride gradient. Since the irradiation medium in this experiment contained no $MnCl_2$ or HSEtOH, the amount of non-alkali- or non-heat-treated high salt stable complexes formed at a molar ratio enzyme–polymer of 6:1 after 65,000 ergs/mm^2 as detected by retention on Millipore filters is 28% (see Table II). This variance in amount of high salt stable complex formation, a function of the components in the irradiation medium, will be discussed below.

It should be mentioned that we were unable to use the Millipore filter assay for detection of alkali- or heat-treated, uv-induced complexes even after neutralization or incubation at 37° due to high background cpm retained for the polymer control. The reason(s) for this is not understood but would possibly suggest that renaturation of the polymer is not complete, since single-stranded DNAs bind tenaciously to cellulose filters (Nygaard and Hall, 1963). However, it would appear that at least 70% of the complexes formed by uv light are stable to alkali or heat treatment as detected by cesium chloride gradient analysis. At this point, it could be argued that 70% of the uv-induced linkages formed between the enzyme and polymer are stable to alkali or heat, the remaining 30% being sensitive to either form of treatment. However, as has been mentioned, recovery of input cpm from these gradients averaged about 75–80%. Upon sectioning these tubes into several slices, a significant amount of cpm was always found bound to the tube in the vicinity of the floating complex peak in the gradient. If one combines these bound cpm with the recovered cpm in the floating peak and compares this total to the total input cpm, the results agree favorably with the per cent retention as detected by the Millipore filter technique for non-alkali- or non-heat-treated, uv-induced, high salt stable complexes. Thus, we believe that uv-induced stable complex formation between RNA polymerase and d(A-T)$_n$·d(A-T)$_n$ is not only resistant to high ionic strength but also to alkali and heat treatment.

The RNA polymerase isolated from *E. coli* is a complex enzyme consisting of several polypeptide subunits. The objective of the following experiment was to determine if a particular subunit(s) of the enzyme participated in the uv-induced cross-linking between the enzyme and polymer. Probably only those subunit(s) with a certain chemical structure and spatial proximity can interact in the photochemical cross-linking phenomenon between the enzyme and polymer. Either preincubated polymer or enzyme–polymer complexes at molar ratios of 3:1 or 6:1 were irradiated with uv light (65,000 ergs/mm^2) at 0° and then treated with sodium laurylsarcosine and HSEtOH to denature the protein and reduce available sulfhydryl groups. All samples were then mixed with cesium chloride solutions to obtain a final volume of 4.5 ml and average density of 1.480 g/cm^3. Centrifugation was performed in a Beckman SW50.1 swinging-bucket rotor; collection entailed fractionation of the gradient into 0.15-ml aliquots. Gradients were assayed for radioactive cpm or index of refraction readings; the results are plotted in Figure 6.

In comparing the distribution of the uv-irradiated, detergent-treated enzyme–polymer complexes (molar ratio 3:1 or 6:1) with the distribution of the irradiated and detergent-treated polymer, it is evident that there exists a population of material whose buoyant density ranges from about 1.35 to 1.58 g/cm^3. If a particular subunit(s) is involved in the uv-induced enzyme–polymer complex, then it is possible to calculate the buoyant density of this protein–DNA complex. Using a simple relationship reported elsewhere (Brutlag *et al.*, 1969) and ignoring hydration differences, then $(1 + X)\bar{\nu}_c = \bar{\nu}_{DNA} + X\bar{\nu}_s$, where $\bar{\nu}_c$, $\bar{\nu}_{DNA}$, and $\bar{\nu}_s$ are the partial specific volumes of the DNA–subunit complex, DNA, and subunit, respectively, and X is the weight ratio of protein to DNA in the complex. Assuming $\bar{\nu} \simeq 1/\rho$, where ρ is the buoyant density, and using the molecular weight and $\bar{\nu}$ for the specific subunits of the RNA polymerase listed elsewhere (Burgess, 1969a,b; King and Nicholson, 1971) and the molecular weight for the cesium salt of d(A-T)$_n$·d(A-T)$_n$ of 456 monomer units equaling 2.0×10^5 daltons, a range of buoyant densities is obtained for a complex of polymer and a particular RNA polymerase subunit of 1.51–1.66 g/cm^3, depending on the subunit used in the calcula-

TABLE II: Formation of High Salt Stable Complexes between RNA Polymerase and [^{14}C]d(A-T)$_n$·d(A-T)$_n$ with Uv Light as a Function of Components in the Irradiation Solution.[a]

	Total Cpm Retained on Millipore Filter (%)							
	Binding mixture complete[b]		Binding mixture – HSEtOH		Binding mixture – $MnCl_2$		Binding mixture – HSEtOH – $MnCl_2$	
	Low salt[c]	High salt[c]	Low salt	High salt	Low salt	High salt	Low salt	High salt
Polymer	1.1(±0.3)	1.2(±0.3)	0.6	2.6	0.6	0.8	1.4(±0.2)	1.8(±0.2)
Enzyme–polymer = 3:1[d]	56.3(±2.4)	12.1(±0.5)	59.3(±5.2)	25.9(±1.5)	62.5(±5.6)	12.1(±0.5)	67.8(±4.6)	15.6(±0.8)
Enzyme–polymer = 6:1	70.5(±2.8)	23.5(±0.8)	76.6(±2.6)	37.7(±2.1)	79.5	25.2(±0.1)	79.3(±1.1)	28.3(±0.4)
Enzyme–polymer = 12:1	76.6(±1.0)	35.2(±0.9)					83.2	40.3

[a] Reactions (0.1 ml) contained 5 μmol of Hepes buffer (pH 7.9), ±0.4 μmol of $MnCl_2$ ± 0.4 μmol of HSEtOH, 0.9 monomer nmol of ^{14}C-labeled polymer, and 0, 3, 6, or 12 μg of RNA polymerase. All reactions were incubated for 5 min at 37°, chilled to 0°, and irradiated with uv light for 65,000 ergs/mm^2 (0°). From each irradiated reaction, two 0.05-ml aliquots were mixed into either 1.5 ml of low salt wash or high salt wash buffers. Diluted samples were filtered through Millipore filters with either low or high salt washing buffers (0°), and the filters were assayed for radioactive cpm retained. [b] Binding mixture complete contains Hepes buffer, $MnCl_2$, and HSEtOH. Other mixtures contain Hepes buffer minus $MnCl_2$ or HSEtOH or both. [c] Low salt and high salt washing buffers contain 0.01 M Tris buffer (pH 7.2)–0.05 M KCl or 0.01 M Tris buffer (pH 7.2)–2.0 M KCl, respectively. [d] Enzyme–polymer = 3:1, 6:1, or 12:1 represent original molar ratios of enzyme–polymer in the binding reaction. Values containing ± include the standard deviation calculated from data from six or more independent experiments.

tion. However, since the input molar ratio of enzyme–polymer is greater than 1.0, it is likely that some polymer molecules have more than one subunit attached, in which case, the buoyant densities of the various combinations of a particular subunit complexed to the polymer become more heterogeneous (1.43–1.66 g/cm^3) if one considers three or less of a specific subunit attached. Furthermore, it is possible that more than one type of subunit is involved in the cross-linking phenomenon; a variety of combinations of various subunits complexed to the polymer would also account for heterogeneity in the buoyant density of uv-induced complex. We are currently examining sodium dodecyl sulfate acrylamide gel band distributions of isolated uv-induced RNA polymerase–d(A-T)$_n$·d(A-T)$_n$ complexes in order to determine which subunits are bound to the polymer.

As was mentioned previously, formation of stable complexes between RNA polymerase and d(A-T)$_n$·d(A-T)$_n$ was a function of the components present in the irradiation medium. In the following experiment, polymer or enzyme–polymer solutions which had been preincubated were irradiated with uv light (65,000 ergs/mm^2) in Hepes buffer alone or with the addition of $MnCl_2$ or HSEtOH or both. Samples were assayed for retention of uv-induced stable complexes on Millipore filters using both low and high salt washing buffers, and the results are shown in Table II. Filtration of uv-irradiated polymer alone is independent of the medium in which it was irradiated; retention of the polymer is within the range of per cent retention of nonirradiated polymer for both low and high salt washes. However, formation of uv-induced high salt stable RNA polymerase–polymer complexes was 60–110% greater when irradiated in binding mixtures minus HSEtOH compared to irradiation in complete binding mixture (Hepes buffer–$MnCl_2$–HSEtOH). This increase was also dependent on the molar ratio of enzyme–polymer irradiated. The reduction in formation of uv-induced polymerase–polymer stable complexes in the presence of HSEtOH can possibly be explained due to the fact that HSEtOH does absorb light at 254 nm, the principal emission line from a germicidal lamp, therefore reducing the relative uv dose to the sample. Furthermore, it is possible that HSEtOH may compete with active sites on the polymerase in the cross-linking process, since Smith (1967) noted that the amino acid cysteine could complete with bovine serum albumin in the uv-induced

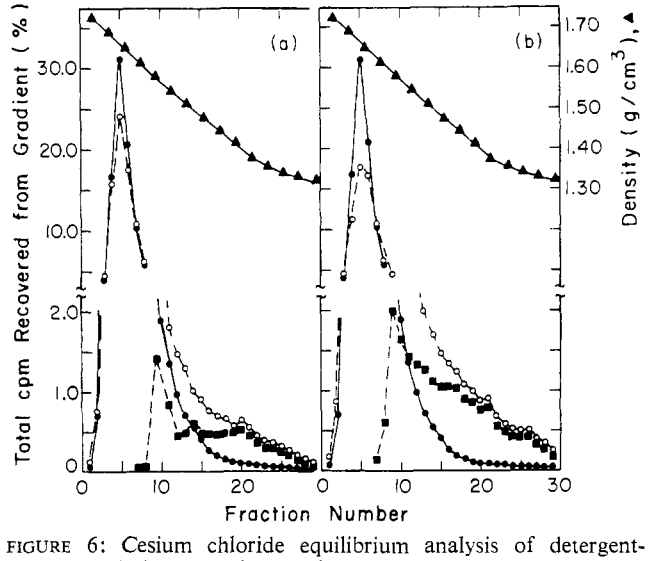

FIGURE 6: Cesium chloride equilibrium analysis of detergent-treated, uv-induced stable complexes of *E. coli* RNA polymerase and [^{14}C]d(A-T)$_n$·d(A-T)$_n$. Reactions (0.450 ml) contained 22.5 μmol of Hepes buffer (pH 7.9), 1.8 μmol of $MnCl_2$, 1.8 μmol of HSEtOH, 4.5 monomer nmol of ^{14}C-labeled polymer, and either 0, 13.5, or 27 μg of RNA polymerase. Reactions were incubated at 37° for 5 min, chilled to 0°, and irradiated with uv light at 0° for 65,000 ergs/mm^2. To 0.4-ml of each reaction, 0.4 ml of 2% sodium laurylsarcosine and 2% HSEtOH were added, and the mixtures were incubated at 37° for 10 min. Cesium chloride was added to each reaction; the final volume equaled 4.5 ml of average density of 1.480 g/cm^3. Each sample was placed in a pretreated polyallomer tube and centrifuged in a Beckman SW50.1 swinging-bucket rotor at 44,000 rmp for 66 hr at 20°. After centrifugation, 0.15-ml fractions were analyzed for radioactive cpm or refractive index readings as outlined in the Experimental Section. Recovery of input cpm was greater than 95%. (a) Distribution of per cent total cpm for detergent-treated, uv-induced enzyme–polymer complexes at a molar ratio of 3:1 (○); (●) the detergent-treated, uv-irradiated polymer control; (■) the difference in cpm distribution of the enzyme–polymer complex and polymer alone. (b) Distribution of per cent total cpm for detergent-treated, uv-induced enzyme–polymer complexes at a molar ratio of 6:1. Symbols used are the same as described in (a).

cross-linking of this protein to DNA. Either of these possibilities can probably account for the 15–30% increase in high salt stable complexes formed when enzyme–polymer complexes are irradiated in Hepes buffer (minus $MnCl_2$ and HSEtOH). Omitting only $MnCl_2$ from the irradiation solution results in no change in the formation of uv-induced high salt stable complexes when compared to irradiation in complete binding mixture. The reason(s) for this variance in uv-induced enzyme–polymer complexes on the irradiation solution composition is not understood at this time but is the subject of continuing investigation.

Discussion

The results of this study show that RNA polymerase bound to the synthetic polymer $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ can be induced with uv light to form stable binary complexes. The stability of the complex is most likely due to covalent linkage(s) between the enzyme and polymer as shown by the resistance of the complex to high ionic strength solutions and stability to alkali or heat treatment. Formation of stable RNA polymerase–polymer complexes was found to be a function of uv dose and the molar ratio of enzyme–polymer. Stable complex formation as a function of dose was rapid initially and then proceeded much more slowly. This biphasic response in stable complex formation is evidently a function of the synthetic polymer used, since preliminary studies using RNA polymerase and the synthetic polymer $d(A)_n \cdot d(T)_n$ show high salt stable complex formation proceeding linearly with dose over a range from 0 to 130,000 ergs/mm^2 (G. F. Strniste, V. E. Mitchell, A. M. Martinez, and D. A. Smith, unpublished results).

From density distribution profiles of uv-induced enzyme–polymer complexes in cesium chloride gradients, an attempt was made to determine which subunit(s) of the RNA polymerase was involved in the cross-linking phenomenon. After extensive detergent treatment to denature and dissociate all nonlinked protein, the protein–polymer stable complexes displayed a heterogeneous buoyant density distribution in cesium chloride (1.35–1.58 g/cm^3). This distribution was similar for enzyme–polymer complexes irradiated at two molar ratios: 3:1 and 6:1. However, in each case, a significant fraction of the enzyme–polymer complex banded at a density of 1.58 g/ml. The density of $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ is 1.672 g/cm^3 in neutral cesium chloride (Wells and Blair, 1967, see Figure 6). Using the formula $(1 + X)\bar{\Gamma}_c = \bar{\Gamma}_{DNA} + X\bar{\Gamma}_s$ (Brutlag *et al.*, 1969), as described in the Results, and a molecular weight of 2.0×10^5 for the cesium salt of $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$, where $n = 114$, the buoyant density of the polymer linked to one β' subunit would be 1.511 g/ml. Buoyant densities calculated for the polymer linked to either one β subunit, one σ subunit, or one α subunit are 1.515, 1.553, or 1.610 g/ml, respectively. If more protein were bound to the polymer, the buoyant density of the complex would be lower. Therefore, it seems likely that the peak seen at a buoyant density of 1.58 g/cm^3 contains one linked subunit and that this is most likely α or σ. Experiments designed to investigate more directly which subunit(s) is linked to the polymer are in progress.

There exist certain limitations in this approach (uv induction of protein–nucleic acid complexes) in determining the morphology of the RNA polymerase–nucleic acid complex. Smith (1969) has reported that, of the 22 common amino acids present in protein, only 11 photochemically react significantly with uracil. Furthermore, the photochemical addition of the active amino acid cysteine to various polynucleotides and DNA was found to be dependent on the pnysical structure of the polymer (Smith, 1968; Smith and Meun, 1968). Thus, it is conceivable that a subunit of the RNA polymerase may be properly oriented adjacent to the nucleic acid polymer yet lack the necessary potentially reactive amino acid residue at this site necessary for photochemical induced cross-linking. On the other hand, the secondary structure of the polymer at the site where the RNA polymerase is bound may influence significantly the probability of cross-linking to potentially reactive amino acid residues of adjacent subunits of the bound enzyme.

It was found also that the photochemical cross-linking of RNA polymerase to $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ was a function of the components present in the irradiation buffer. The presence of $MnCl_2$ yet the absence of the sulfhydryl reducing agent HSEtOH substantially increased the formation of uv-induced enzyme–polymer stable complexes. The reason(s) for this is not understood at the present time; however, it is known that $MnCl_2$ dramatically influences the secondary structure of DNA (Luck and Zimmer, 1972). Therefore, destabilizing the structure of the polymer possibly could create new sites for potential cross-link formation between the enzyme and polymer.

Recent advances in the understanding of the fine structure of complex protein–nucleic acid structures using a variety of chemical cross-linking agents have been reported (Kenner, 1973; Lutter and Kurland, 1973; Tikchonenko *et al.*, 1973). Utilization of other modifying agents in conjunction with uv light should provide a more complete understanding of the molecular morphology of RNA polymerase–DNA complexes. This approach is being used in experiments in progess at this Laboratory.

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Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerases from Two T4 Phage-Infected Systems†

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ABSTRACT: The properties of DNA-dependent RNA polymerases from two T4 phage-infected systems have been studied and compared with those of RNA polymerase from uninfected *Escherichia coli* (host polymerase). For the T4 phage-infection systems, T4 double amber mutants were used, one being a gene 42⁻Xgene 47⁻ mutant and the other a gene 42⁻Xgene 55⁻ mutant. The contents of the σ subunit and of four new T4-specific polymerase-binding proteins, and the activities of the enzymes, were surveyed at different stages of the purification procedure. In subunit content, the two enzymes from the T4-infected systems differ; the enzyme from the T4 gene 55⁻ system lacks a new binding protein of mol wt 22,000. Considerable loss of both the σ subunit and the small binding protein of mol wt 10,000 (as detected by gel electrophoretic analysis) occurs during the purification of the T4 enzymes. Little loss of the small binding proteins of mol wt 12,000, 15,000, and 22,000 is found during routine purification steps, but chromatography of the enzymes on phosphocellulose results in loss of the mol wt 12,000 protein from the core enzyme fractions and some loss (the amount being dependent on the purification stage at which chromatography is carried out) of the mol wt 22,000 protein. The enzymes from both T4-infected systems have a reduced activity with T4 DNA as compared to host polymerase, and the same activity is sharply reduced by high salt concentration. Results are presented which show that the T4 enzymes contain a material, found in the unadsorbed fraction on phosphocellulose chromatography, which inhibits host σ stimulation of core enzyme activity.

Complex changes occur in the transcription process after T4 phage infection of *Escherichia coli*, and many of the studies of these changes have recently been reviewed (Losick, 1972; Bautz, 1972). The changes accommodate the synthesis of at least four different classes of T4 mRNA and the cessation of *E. coli* RNA synthesis. The studies suggest that changes in the host's transcription enzyme, the DNA-dependent RNA polymerase of *E. coli*, are very important to the new transcription program, although the synthesis of a new transcription enzyme which takes place after T7 and T3 phage in-

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