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Use of polylysine for adsorption of nucleic acids and enzymes to electron microscope specimen films

[RNA polymerase (RNA nucleotidyltransferase)/bacteriophage T7 DNA/poly(dA-dT)/binary complex/negative stain]

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ABSTRACT **Enzymes and nucleic acids, both free and as bound in binary complexes, adsorb to electron microscope specimen films in well-distributed fashion if a dilute solution of polylysine is previously applied to the films. Electron micrographs are exhibited that demonstrate the usefulness of the technique in visualizing double- and single-stranded DNA, *Escherichia coli* RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) in negative stain, and polymerase complexed to poly(dA-dT) and to an 1100 base-pair restriction fragment of bacteriophage T7 DNA containing the early promoters.**

The base-pair spacing of DNA prepared for electron microscopy by the polylysine method was found to be 0.326 nm. Four promoter sites on the T7 fragment were located at 215, 440, 560, and 670 base-pair distances from the left terminus. When poly(dA-dT) was incubated with a 20-to-1 weight ratio of polymerase the bound enzyme particles were found to be about two-thirds as closely packed as is sterically permissible.

Electron microscopy became routinely employed for the observation of nucleic acids only after 1962 when it was shown (1) that they could be incorporated into a monolayer film of denatured cytochrome *c* for subsequent transfer to a specimen support film. The effect of embedment of nucleic acids in the relatively rigid monofilm is to produce a well-spread display of strands, as distinct from the laterally aggregated mass generally found when a nucleic acid solution is simply allowed to dry upon carbon-coated specimen film. Although the protein monofilm technique has proven enormously useful and popular, it cannot be satisfactorily used for observation of the association of nucleic acids with protein molecules, such as enzymes, because of the obscuring effect of the bound cytochrome *c*. Electron microscopic studies of protein–nucleic acid association can be performed only with free nucleic acids or with ones that are incorporated into a monofilm of molecules much smaller than those of cytochrome *c*.

Griffith (2) and Highton and Whitfield (3) reported that DNA will be adsorbed without lateral clumping if the solution containing it is placed upon a carbon specimen film that has been pre-treated with a prolonged plasma discharge in a partial vacuum. Dubochet *et al.* (4) developed a method of adsorbing DNA to a carbon film by first exposing it to a plasma discharge in a partial atmosphere of pure amylamine. This treatment apparently creates upon the film a number of amylamine polymers with positively charged groups that firmly bind the nucleic acid strands and prevent their aggregation upon drying. Koller *et al.* (5) reported that DNA could be satisfactorily adsorbed to a freshly cleaved mica surface if a sufficient amount of ethidium bromide was added to the solution. Still another method of spreading nucleic acids for electron microscopy has been reported by Vollenweider *et al.* (6), who formed a monolayer from benzyldimethylalkylammonium chloride (BAC), a molecule whose weight is only about 350 daltons.

Preparations of purified *Escherichia coli* RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6), examined after negative staining, have disclosed particles with a variety of morphologies. Slayter and Hall (7) described them as "porous and stellate," an appearance similar to that found by Lubin (8). Fuchs *et al.* (9), Colvill *et al.* (10), and Zillig *et al.* (11) found ring-like particles (possibly with six protuberances) and ones appearing like hexagons with a central hole.

E. coli RNA polymerase molecules bound to bacteriophage T7 DNA have been shown in electron micrographs by Dubochet and collaborators (12), and by Portmann *et al.* (13). Similar findings were reported by Brack and Delain (14) for polymerase complexes with a mitochondrial DNA. Recently, Hirsh and Schleif (15) exhibited molecules of RNA polymerase bound to the *lac* and the *Pr* promoters of bacteriophage λ DNA, and Lescure *et al.* (16) showed RNA polymerase bound to polyoma virus DNA.

The present report deals with the elaboration of a method, mentioned by Laemmli (17) in visualizing condensed forms of DNA, for displaying naked nucleic acids and enzyme particles upon specimen support films pre-treated with polylysine. Results are shown of the application of the method to electron microscopy of T7 and bacteriophage fd DNA, of purified *E. coli* RNA polymerase negatively stained, and of binary complexes of the enzyme with T7 and with a synthetic DNA.

MATERIALS AND METHODS

Preparation of Support Films. Electron microscope specimen grids, 400-mesh Cohen-Pelco handle type (Ted Pelco, Inc.), are filmed with Formvar and coated with a thin layer of carbon. The films are then made hydrophilic by subjecting them for about 15 sec to a glow discharge at 70–80 millitorr (9–11 Pa) air pressure. The handles of the grids are prevented from becoming hydrophilic during the discharge by covering them with small pieces of metal. This precaution prevents any liquid drop subsequently applied to the surface of a grid from spreading to the tips of the forceps in which grids are held. A 5- to 8-μl drop of polylysine in water at 0.3–1.0 μg/ml is placed for about 30 sec upon each grid and drained as completely as possible. Poly(L-lysine) (Sigma) of molecular weight approximately 2000 is quite satisfactory, as are longer polymers. The drop of polylysine solution is drained by touching the edge of the grid with a vacuum-connected aspirator freshly made from a pasteur pipet flame-drawn to less than 0.5 mm bore diameter. The residual liquid on the grid can be inspected under a 10-power microscope as it dries; the surface should be so hydrophilic that drying takes several seconds, with the trailing edge of the liquid film showing one to two complete orders of interference colors. (It cannot be stressed too strongly that thorough cleanliness, as assessed by hydrophilicity of the specimen films, is essential to the consistent success of the method. Thus, no filters of any kind are used, either for water purification or for removal of liquid drops from the grid surface. Water at least

double-distilled is used.) After the residual polylysine is dried the grids are ready for use, and remain so for at least a few days if they are kept in a tightly covered container.

Adsorption of DNA to the Polylysine-Treated Films. Natural DNAs were the generous gift of J. C. Wang [the *Hae* III, K fragment of phage PM2; $(Hinf\ T7)_{1100}$, an 1100 base-pair fragment of T7 cleaved by the *Hinf* endonuclease and containing four RNA polymerase binding sites; phage fd]; poly-(dA-dT), $s_{20} \geq 7$, was purchased from Miles. Satisfactory concentrations for adsorption are in the range 0.1–2.0 μg/ml, when drops of about 6 μl are applied to the treated films and allowed to adsorb for upwards of 60 sec. After the drop is drained away the grid is rinsed, on its filmed surface only, with four or five drops of distilled water applied and drained *seriatim*. A 5-μl drop of 5% aqueous uranyl acetate is then applied for about 15 sec. After its removal the film is rinsed with two or three drops of water, drained, and allowed to dry. (Drops for rinsing are conveniently held on a freshly scraped Teflon block; an inverted grid touched to a drop will pick it up neatly.) It is important that, during the above operations, the film be prevented from drying until after the final rinse. The dried grids are rotary shadowed with tungsten (18). A 20-mil tungsten wire, of 3 cm free length, is initially heated with a 32.5-ampere current, after which the applied voltage is left constant until burn-out (about 90 sec). This schedule evaporates about 10 mg of tungsten. An oblique angle of 8°, with a source-to-sample distance of 9.5 cm, is satisfactory for contrast enhancement.

RNA polymerase was purified in M. Chamberlin's laboratory (19) and donated by him. It was stored in 50% (vol/vol) glycerol, at −20°, with aliquots appropriately diluted in buffer just before use. Its adsorption to the filmed grids was done in a manner the same as for DNA. If the preparation is to be negatively stained, the last step is the application of the uranyl acetate, added either as a drop (and later drained), or as spray droplets sprayed from a nebulizer during the last stage of rinse-water drying. A satisfactory concentration for the enzyme ranges from 1 μg/ml with 3 min adsorption time to 10 μg/ml for 20 sec. Binary complexes of DNA and RNA polymerase are adsorbed in a manner identical to that described for DNA alone.

It has not proven possible to adsorb DNA unless Mg^{2+} is present at a concentration ≥3 mM, but when sufficient Mg^{2+} is present normal adsorption will occur in the presence of several auxiliary substances, so long as they are readily soluble in water. For example, glycerol (≤20% vol/vol), formamide (≤85% vol/vol), and glyoxal (about 0.5 M) may be added to the standard buffer used: 50 mM KCl/10 mM $MgCl_2$/10 mM Tris·HCl/0.1 mM EDTA/0.2 mM dithiothreitol, pH 7.9. Bovine serum albumin may be included in solutions if its concentration when applied to the grid does not exceed 3 μg/ml. Adsorption is unaffected if reactions between DNA and enzyme are carried out at high concentrations of the reactants followed by appropriate dilution immediately before adsorption.

Micrographs were obtained on a JEOL 100-B electron microscope, with primary magnification between ×20,000 and ×50,000, and photographic exposures on Electron Image Plates (Eastman). Enlargements for half-tone reproduction were made directly on Kodabromide RC(F-EH) paper.

RESULTS

DNA

Double-Stranded. Fig. 1 shows the left-terminus fragment of T7 DNA, designated as $(Hinf\ T7)_{1100}$, adsorbed at a concentration low enough to minimize strand entanglement. The width of the strands including the shadow cap (estimated to be 1.0 nm thick on each side) is about 4.0 nm, a value similar to that reported by Vollenweider *et al.* (6) for double-stranded DNA in a benzyldimethylalkylammonium chloride film. Longer DNA polymers (not shown here), such as the entire T7 genome or the relaxed form of PM2 DNA, exhibit several crossover points, as would be expected in the absence of any hydrodynamic shear forces. An estimate has been made of the "yield" of adsorption of (*Hinf* T7), i.e., the number of strands per unit area found attached to the film compared with the number expected if all the strands in the applied drop were adsorbed. This fraction is a function of adsorption time, of course, but at the end of a reasonable time (10 min) it is estimated to be 10%.

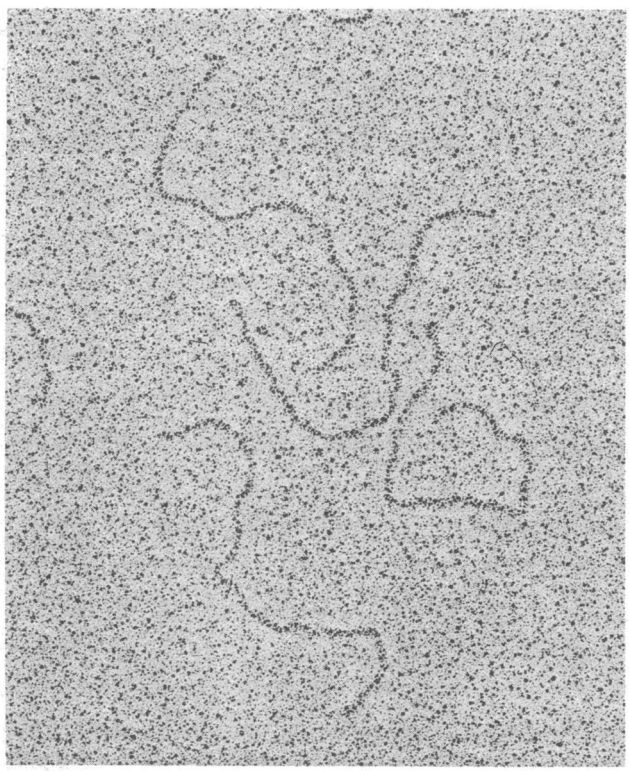

FIG. 1. $(Hinf\ T7)_{1100}$ DNA adsorbed at 0.4 μg/ml upon a polylysine-treated carbon film and rotary shadowed with tungsten. Procedure is as described in *Materials and Methods*. DNA strands are about 0.360 μm long and 4.0 nm across. (×200,000.)

Single-Stranded. Many attempts have been made to display single-stranded DNA (such as fd) in a form similar to that seen when it is encased in a cytochrome *c* film. Despite the use of extreme denaturing conditions (high temperature, low ionic strength, glyoxal, and high concentrations of formamide) the single strands, while appearing generally circular, appear knotted or kinked (Fig. 2). This is probably to be expected of a two-dimensional projection (upon the grid surface) of the three-dimensional form taken by a single-stranded polynucleotide free in solution. The appearance of the fd molecules is consistent with the fact that their measured contour length is generally less than one-half of that found when they are in a cytochrome *c* film.

Base-Pair Repeat Distance. Measurements were made of the base-pair axial repeat distance in a restriction fragment of DNA from PM2, in the buffer specified in *Materials and Methods*, after adsorption by the polylysine method. The K fragment produced by the *Hae* III restriction enzyme, known to contain 263 ± 5 base pairs (20), was adsorbed and, after very light rotary shadowing, was photographed at primary magni-

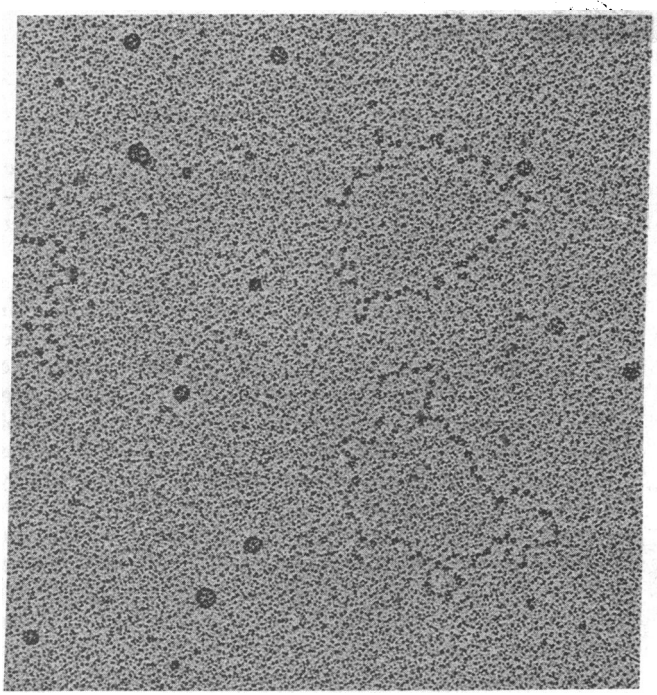

FIG. 2. Single-stranded fd DNA. The sample was adsorbed at 1.5 μg/ml to specimen film in an 80% (vol/vol) formamide solution containing 3 mM $MgCl_2$, 5 mM KCl, and 3 mM Tris·HCl at pH about 8.0. (×120,000.)

fication of ×46,100. The magnification was determined with the aid of fragments of a crossed diffraction-grating replica of 2160 lines/mm (E. Fullam, Inc.) deposited upon the back surface of the specimen film. The lengths of the DNA strands were measured with a Numonics Graphics Calculator at a projected magnification of ×461,000. The mean length and standard deviation of the 360 measured strands was 85.6 ± 4.5 nm, or 0.326 ± 0.018 nm per base pair.

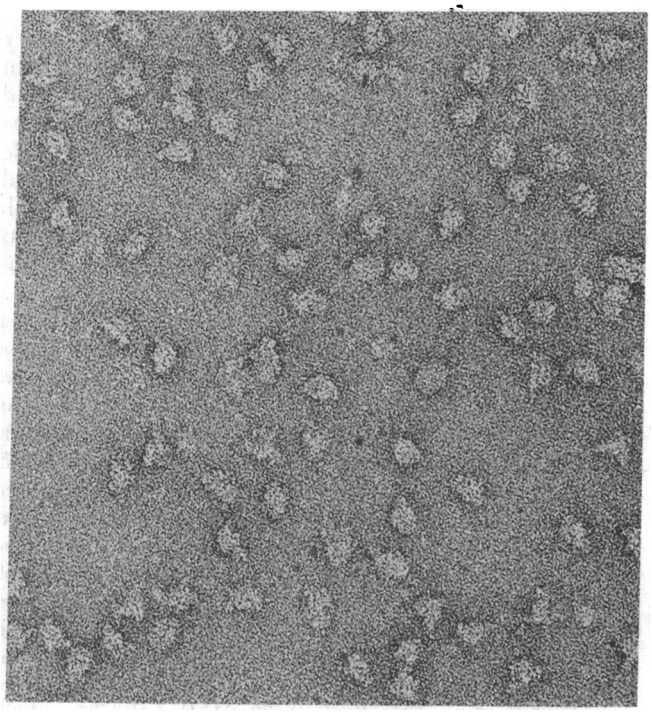

FIG. 3. *E. coli* RNA polymerase, adsorbed at 5 μg/ml to specimen film and negatively stained as described in *Materials and Methods*. (×275,000.)

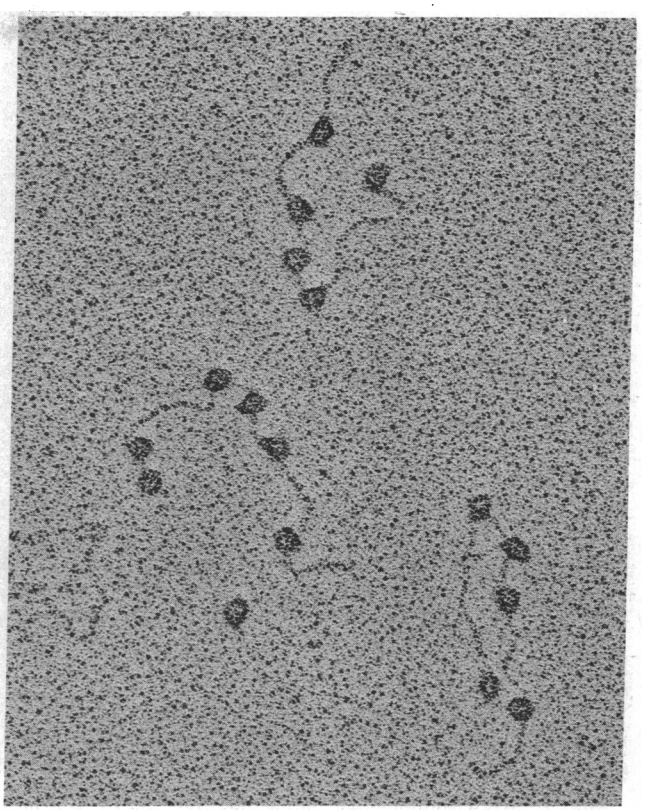

FIG. 4. RNA polymerase holoenzyme seen at four early promoter sites of T7 DNA. (*Hinf* T7)$_{1100}$ DNA at 0.4 μg/ml and enzyme at 1.7 μg/ml were incubated 2 min at 37° in binding buffer and allowed to adsorb to polylysine-treated specimen film for 2 min (see *Materials and Methods*). The three uniformly separated particles are at the A_1, A_2, and A_3 binding sites; the fourth is at the D site. Enzyme molecules also attach indiscriminately at either end of the DNA strands. Micrograph has been chosen to show only one free enzyme, and one attached to DNA at a position other than the promoter sites and the ends. (×200,000.)

RNA polymerase

Fig. 3 is a micrograph of the holoenzyme of *E. coli* RNA polymerase. As might be expected of a macromolecule of about 480,000 daltons and containing five subunits of four different sizes, no sign of symmetry is apparent. This micrograph, and others taken of the enzyme at greater concentration, indicates that dimers and higher aggregates do not form in the solvent employed. This behavior is distinct from that of the core enzyme (not shown here) where, at about 50 mM KCl, dimer and larger oligomeric forms are commonly seen. The only notable feature of the particle images shown in Fig. 3, aside from their reasonably uniform size, is a tendency toward three-sidedness.

Binary complex of DNA and RNA polymerase

T7 DNA. The left-most fragment, (*Hinf* T7)$_{1100}$ DNA, contains the D (21, 22), A_1, A_2, and A_3 binding sites in the early region of the genome. Fig. 4 shows this fragment of T7 DNA after incubation with *E. coli* RNA polymerase, with the enzyme particle concentration so adjusted as to provide an average of six per DNA strand. Some attachment of the enzyme at the DNA ends is seen, as well as at four interior positions (presumably the promoter binding sites). At the concentrations used about 20% of the strands show the pattern seen in Fig. 4. The location of the presumptive promoter sites has been determined on about 100 strands. The results, expressed in base pairs from the left terminus, are: 215 ± 25 (D), and 445, 560, and 670 for

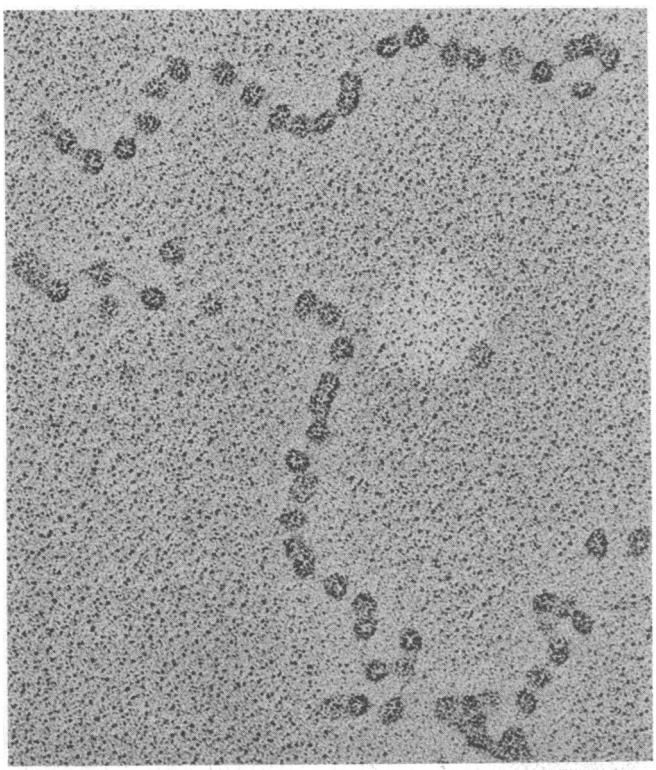

FIG. 5. RNA polymerase molecules bound to synthetic poly(dA-dT) at 0.1 μg/ml; enzyme at 2.0 μg/ml. Binding and adsorption are as for specimen shown in Fig. 4. The average spacing of the polymerase particles seen on the two longer strands is 18.0 nm. (×200,000.)

A_1, A_2, and A_3, respectively, with standard deviations of about ±35 base pairs.

When RNA polymerase is added to $(Hinf\ T7)_{1100}$ DNA at four times the concentration shown in Fig. 4, the average number of enzyme particles bound to the strands is increased less than 2-fold (data not shown).

Poly(dA-dT). RNA polymerase incubated with synthetic poly(dA-dT) in a weight ratio of 20 to 1 exhibited an abundance of enzyme binding (Fig. 5). The two long strands shown contain attached enzyme particles at an average spacing of 18.0 nm, or 55 base pairs. Similar measurements have been made on a total length of 4.3 μm of what appeared to be unkinked molecules, about 40-fold the length shown here, and disclose an average spacing of 64 base pairs.

DISCUSSION

Polylysine-treated carbon films can be used for producing well-distributed DNA and large protein molecules reproducibly, so long as contamination of solutions and surfaces with surface-active substances is avoided. The merit of the method, compared with previously employed techniques (4–6), lies primarily in the speed and simplicity with which an adsorbing surface can be prepared. An additional advantage is that the polylysine-treated surface is hydrophilic, allowing specimen objects to be negatively stained under optimal conditions.

The appearance of single-stranded DNA, as shown in Fig. 2, differs from the linear forms found by Hart (23) and by Highton and Beer (24) for single strands of RNA from tobacco mosaic virus (TMV), and by Nanninga *et al.* (25) for ribosomal RNA. In those earlier experiments, however, the molecules seem to have been fully extended during specimen preparation by the action of hydrodynamic forces (streaking).

The appearance of the RNA polymerase molecules in negative stain (Fig. 3) seems not to throw much light on the arrangement of their subunits. No signs of symmetry are anticipated, of course, because of the subunits' uneven number, particularly because only two of them (αs) have identical molecular weights. The holoenzyme particles seen in Fig. 3 are probably in random orientation; the only hint of regularity (aside from a reasonably uniform size) is a three-sided aspect to, perhaps, one half of the particles.

The binary complex between DNA and *E. coli* RNA polymerase is readily visualized (Fig. 4) after rotary shadowing with tungsten, without special photographic enhancement. The DNA strands themselves have increased visibility if they are exposed to uranyl acetate for several seconds during the rinsing cycle. For a given ratio of enzyme and DNA the degree of binding seems unaffected by time of incubation so long as it is more than about 20 sec. Localization of strong binding sites by electron microscopy is imprecise, at best, because of the large size of the enzyme particle. All that can be measured is the position of some definable point such as its "center of mass" in the electron image; in general this may not lie over the center of the approximately 40 nucleotides in the binding site after the specimen has dried. As a consequence, histograms leading to determinations of the position of sites are rather broad.

The positions of the three strongest binding sites, A_1, A_2, and A_3, reported in this paper (445, 560, and 670 base pairs from the left terminus of T7 DNA) are in as close accord with those found by others, as would be expected from the above remarks. Values of 440, 540, and 630 have been reported by Bordier and Dubochet (12); 440, 570, and 700 by Portmann *et al.* (13); and 400, 530, and 630 by Darlix and Dausse (26) by a method in which electron microscopy was used to localize the initiation sites for transcription. Recently, Hsieh and Wang (27) have localized the sites by electrophoretic gel analysis of transcripts and have found them to be at 460, 580, and 720 base pairs. The minor promoter, D, has been localized at 220 base pairs by Portmann *et al.* (13) and by Hsieh and Wang (27); 215 is reported in this paper.

Particles of the RNA polymerase holoenzyme, after rotary shadowing, seem to show some of the variability in outline seen in negatively stained preparations. An occasional three-sided shape is seen in Fig. 4; in fact, only a rare particle appears round. In the studies reported here, there is no discernible difference in appearance between free enzyme particles and those bound to DNA. Treatment of the incubated material with 0.1% glutaraldehyde has been tried from time to time, but its effect (if any) on the appearance of the enzyme seems negligible.

The high affinity of poly(dA-dT) for non-specific binding with RNA polymerase is strikingly illustrated in Fig. 5. At the weight ratio here used, enzyme at 2.0 μg/ml and polymer at 0.1 μg/ml, the average separation of enzyme particles was found to be 20 ± 2 nm. The length of DNA protected from nucleolytic attack by an endodeoxyribonuclease is about 45 residues (see, for example, ref. 28) and about 60 residues after attack by an exonuclease (29). If the value of 0.326 nm per base pair is taken, the length of the protected region would lie between 15 and 20 nm. Thus, even at the low concentration of enzyme used, the observed spacing of the polymerase molecules on the poly(dA-dT) strands is about as small as the size of the nuclease-protected regions would seem to allow. The extensive polymerase binding exhibited by the d(AT) copolymer is hardly surprising in view of the finding (30, 31) that the T5 and T7 DNA regions recognized as promoters by the holoenzyme are rich in A+T. As mentioned earlier, the $(Hinf)_{1100}$ fragment of

T7 DNA shows distinctly less extensive general binding than does poly(dA-dT). Electron microscopic findings consistent with these results are those of Brack and Delain (14), who found in a preparation of mitochondrial DNA that the regions rich in A+T, as determined by alkaline denaturation, correlated well with those observed to bind RNA polymerase.

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