Transcriptional and Sedimentation Properties of Ribonucleic Acid Polymerase from *Micrococcus lysodeikticus**

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ABSTRACT: Ribonucleic acid polymerase has been prepared from *Micrococcus lysodeikticus* and *Escherichia coli*, and several properties of these two similar enzymes have been examined. Whereas the *E. coli* enzyme exhibits both fast and slowly sedimenting forms, depending upon salt concentration, the *M. lysodeikticus* enzyme shows only a slowly sedimenting form in solutions of both low and high ionic strength. Purified *M. lysodeikticus* ribonucleic acid polymerase may transcribe intact deoxyribonucleic acids asymmetrically under appropriate reaction conditions. The degree of symmetry of the deoxyribonucleic acid directed ribonucleic acid synthesis is a function of several factors including the choice of template deoxyribonucleic acids. With ϕX174 RF deoxyribonucleic acids as template the concentration of KCl, $MgCl_2$, and spermidine in the reaction system markedly influences strand selection by the *M. lysodeikticus* enzyme. With T4 deoxyribonucleic acid as template, however, these reagents appear to have little effect on the type of transcription.

It is generally accepted that cellular synthesis of RNA occurs by the DNA-directed reaction of RNA polymerase (nucleotidyltransferase, EC 2.7.7.6). The wide distribution of this activity in nature and its mechanism of action have been extensively reviewed in a number of articles (Grunberg-Manago, 1962; Hurwitz and August, 1963; Elson, 1965; Singer and Leder, 1966; Georgiev, 1967; Hayes, 1967; Geiduschek, 1969). The most thoroughly studied RNA polymerases are those that have been isolated and purified from microbial sources. The polymerases from different microorganisms show remarkable similarity in their cofactor requirements, reaction kinetics, and ability to utilize various nucleic acids as templates. On the other hand, differences between various bacterial enzyme preparations have been observed; *in vitro*, RNA polymerase from *Micrococcus lysodeikticus* transcribes *both strands* of native DNA templates, whereas the *Escherichia coli* enzyme transcribes only one of the DNA strands. An additional difference appears to be that the *E. coli* enzyme will assume a rapidly sedimenting form in buffers of low ionic strength, while the *M. lysodeikticus* enzyme appears to maintain a slowly sedimenting form in buffers of either high or low ionic strength. This paper examines in detail some of the reported differences between the RNA polymerases of *E. coli* and *M. lysodeikticus*.

Materials and Methods

Assay of RNA Polymerase. The assay is based on the incorporation of radioactive ribonucleoside triphosphate into an acid-insoluble product. The standard assay mixture (0.5 ml) contains 50 μmoles of Tris·HCl (pH 7.5), 1 μmole of spermidine, 1.25 μmoles of $MnCl_2$, 0.4 μmole each of ATP, CTP, GTP, and UTP (one of these being radioactive), 100 μg of calf thymus DNA, and 5–10 units of RNA polymerase. The assay mixture is incubated for 10 min at 30° and the reaction is terminated by the addition of 0.1 ml of 50% trichloroacetic acid. After standing for 5 min at 0°, 2 ml of 5% trichloroacetic acid is added, the acid-insoluble material is collected by filtration through a membrane filter (Millipore, type HA, 0.45-μ pore size), and the filter is washed with five successive 5-ml portions of cold 5% trichloroacetic acid. The filters are dried, and the radioactivity is determined by counting in a windowless gas-flow counter.

A unit of RNA polymerase activity is defined as the incorporation of 1 mμmole of CMP or GMP into the acid-insoluble fraction during 10-min incubation at 30° with calf thymus DNA as template.

Preparation of RNA Polymerase. M. lysodeikticus enzyme was purified by a modification of the procedure of Nakamoto *et al.* (1964), and is reported in detail elsewhere (Weiss, 1968). One important modification which helped in removing nucleases was treatment of the enzyme preparation with CM-cellulose. The final preparation had a specific activity of 500–700 units/mg of protein and a 280/260 mμ absorption ratio of 1.6. The stock enzyme solution had a concentration of 7–15 mg/ml in a solution containing 150 mM ammonium sulfate, 10 mM Tris·HCl (pH 7.5), and 50% glycerol and was stored at −20°. All dilutions of the stock enzyme were made in 150 mM ammonium sulfate and 10 mM Tris·HCl (pH 7.5) unless otherwise stated.

E. coli RNA polymerase was prepared either by the method of Chamberlin and Berg (1962) or by modifications of that procedure (Richardson, 1966a,b). The final enzyme preparation had a concentration of 2–3 mg of protein in a solution containing 10 mM Tris·HCl (pH 7.9), 1 mM $MgCl_2$, 5 mM glutathione, and 100 mM ammonium sulfate; the stock enzyme was stored at −85° in 0.2-ml aliquots. When the stock en-

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zyme was thawed for use, an equal volume of glycerol was added and the solution was subsequently stored at −20°. All dilutions of the stock enzyme were made in 10 mM Tris·HCl (pH 7.9), 5 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM mercaptoethanol, and 50 mM KCl. The *E. coli* preparation had a specific activity ranging from 700 to 900 units per mg.

DNA Preparations. Bacteriophage φX174 SS[1] DNA was prepared by phenol extraction of φX174 phage as described by Sinsheimer (1966). After dialysis against a solution containing 10 mM Tris·HCl (pH 7.5), 10 mM NaCl, and 0.5 mM EDTA, the volume of the DNA solution was reduced by rotary evaporation and centrifuged through a 5–20% linear gradient of sucrose containing the same buffer solution as above. The fractions in the symmetrical peak were collected and then dialyzed against three changes of the same Tris–NaCl–EDTA buffer. DNA concentration was determined by the relationship A_{260} of 1 = 36 μg of DNA in 200 mM NaCl, pH 7.0 (Sinsheimer, 1966).

φX174 RF DNA was prepared essentially as described by Jansz *et al.* (1966). The final RF DNA preparation from 2 l. of infected *E. coli* C was concentrated to 3 ml and centrifuged for 18.5 hr through a linear 5–20% sucrose gradient containing 10 mM Tris (pH 7.5), 10 mM NaCl, and 0.5 mM EDTA at 25,000 rpm in a Spinco No. SW25.1 rotor. Two peaks of 260-mμ-absorbing material were observed (21 and 16 S). The fractions in each of these peaks were combined separately, dialyzed, concentrated, and recentrifuged through an identical sucrose gradient. The fractions containing the 21S and 16S forms of the RF DNA were combined separately, dialyzed against Tris–NaCl–EDTA buffer, and stored at 4°. Electron microscopic examination showed that the 21S RF DNA contained predominantly supercoiled structures while the 16S RF DNA fraction consisted primarily of closed circles; some open circles and DNA fragments (<10%) were observed in both RF DNA species. Only 21S RF DNA was used in the studies described here.

T4 DNA was prepared by phenol extraction of T4 phage as described for λ phage by Sinsheimer (1965). MS2 RNA was isolated from MS2 phage by phenol extraction in sterile glassware according to the procedure of Gierer and Schramm (1956).

Preparation of cRNA for Nearest-Neighbor Frequency Analysis. The product of a DNA-directed RNA polymerase reaction which has a base composition and nearest-neighbor frequencies similar to the DNA template and which will hybridize with the template DNA under appropriate conditions is defined as cRNA (Geiduschek *et al.*, 1961). The cRNA was isolated from 0.5- to 1.0-ml polymerase reaction mixtures, utilizing ^{32}P-labeled nucleoside triphosphates, by the addition of carrier *E. coli* sRNA (1 mg), and precipitation with 4 ml of 10% trichloroacetic acid. After standing in ice for 5 min, the precipitate was collected by centrifugation, redissolved in 1.5 ml of 0.02 N NaOH, and precipitated again with trichloroacetic acid. This procedure was repeated twice. The final trichloroacetic acid precipitate was washed successively with 4 ml of 95% ethanol, 4 ml of ethanol–ether (1:1), and 4 ml of ether, and then dried *in vacuo*. Nearest-neighbor frequency analysis was done on this precipitate as previously reported (Weiss and Nakamoto, 1961). The base composition of the cRNA formed was determined from the nearest-neighbor data.

Preparation of cRNA for Annealing Analysis. The cRNA product was prepared essentially as described by Cohen *et al.* (1967). After RNA polymerase reactions (containing radioactive nucleoside triphosphates) were stopped by chilling in a salt–water ice bath, an aliquot was removed for acid precipitation. The remainder was then heated at 65° for 5 min to destroy RNA polymerase activity, its $MgCl_2$ content was adjusted to 10 mM, DNase (free of RNase) was added to 10 μg/ml, and the mixture was incubated at 37° for 30 min. It was then heated for 3 min at 100°, an equal volume of 2× SSC (pH 7.0) containing 0.05% SDS was added, and it was extracted twice with an equal volume of phenol in the cold. The phenol was preequilibrated with the SSC–SDS buffer. The aqueous phase was dialyzed twice against 1 l. of 2× SSC (pH 7.0), 0.05% SDS for 2 hr at room temperature, and twice for 8 hr in the cold against 2 l. of 0.01× SSC (pH 7.0). The total recovery of acid-precipitable radioactivity was 75–85%.

RNA–RNA Hybridization Assays. The cRNA prepared for the annealing assay was heated for 5 min in a boiling-water bath, quick cooled, and then placed in a 85° water bath which cooled to 65° in 2 hr, and held at 65° for 3–4 hr more, as described by Robinson *et al.* (1964). The annealing mixture, 0.3-ml total volume, contained 0.3 μg/ml of cRNA in 2× SSC (pH 7.0). After annealing, the mixtures were diluted to 2 ml with 2× SSC (pH 7.0), adjusted to contain 10 μg/ml of pancreatic RNase and 1 μg/ml of T1 RNase, and incubated at 37° for 30 min. The mixtures were then chilled in ice, 70–100 μg of sRNA was added as carrier, and the preparation was precipitated with trichloroacetic acid, collected on nitrocellulose membranes, and washed with trichloroacetic acid as described under assay of RNA polymerase. Controls, in which annealed samples were diluted with 2 ml of 10 mM Tris (pH 7.5), heated at 100° for 5 min, quick cooled, adjusted to contain 300 mM NaCl, and treated with RNase as above, showed that more than 98% of the input RNA was rendered acid soluble. Samples were counted in a windowless gas-flow counter if ^{32}P was used, and in a scintillation counter if ^{3}H was used.

DNA–RNA Hybridization Assays. Hybridization of RNA to DNA was performed as described by Gillespie and Spiegelman (1965). Denaturation of the various DNAs (φX174 RF DNA and T4 DNA) was performed by a modification of the method of Studier (1965) in which 0.1 volume of 1 N NaOH was added to the DNA solution (200 μg/ml or less). After standing for 10 min at room temperature, 25 volumes of 6× SSC (pH 7.0) and an equivalent amount of 0.1 N HCl to neutralize the NaOH were added. The 21S RF DNA was converted into its 16S allomorph by the DNase procedure of Roth and Hayashi (1966) before it was denatured. The denatured DNA preparations were used to load the nitrocellulose membranes (type B-6, Carl Schleicher & Schuell Co.) as described by Gillespie and Spiegelman (1965). The amount of DNA on the filters was determined by measuring A_{260} before and after filtration in the case of T4 DNA and φX174 SS DNA, and by using ^{3}H-labeled RF DNA. The nitrocellulose membrane bound 86, 92, and 95% of the RF DNA, SS DNA, and T4 DNA, respectively. Hybridizations were carried out in scintillation counting vials containing 1 ml of 2× SSC (pH 7.0) for

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: SS, single stranded; cRNA, complementary RNA; EC, *Escherichia coli*; ML, *Micrococcus lysodeikticus*; RF, replicate form; SDS, sodium dodecyl sulfate; SSC, 150 mM NaCl–15 mM sodium citrate (pH 7.0).

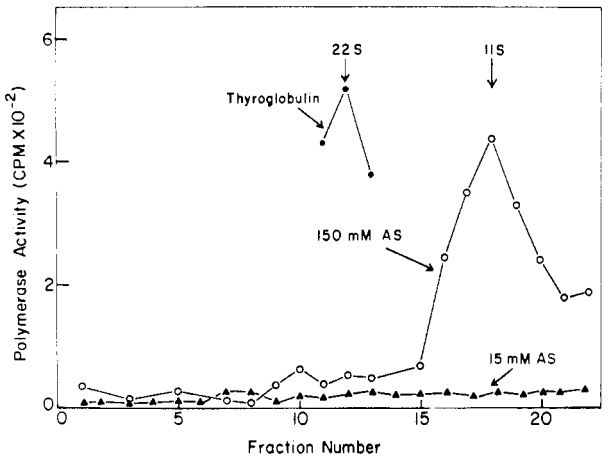

FIGURE 1: Zone sedimentation of purified ML RNA polymerase in a sucrose gradient. A 0.1-ml sample containing 27 μg of ML RNA polymerase was layered on a 4.6-ml 20–5% sucrose gradient containing 10 mM Tris (pH 7.5) and either 150 or 15 mM AS ($(NH_4)_2SO_4$). A 0.1-ml sample containing 0.5 A_{280} unit of porcine thyroglobulin was layered on an identical gradient containing 150 mM AS. After centrifugation for 11 hr at 25,000 rpm at 4° in a SW39 rotor, fractions were collected and 0.05-ml aliquots were assayed for RNA polymerase activity. The thyroglobulin marker was located by its absorbance at 280 mμ.

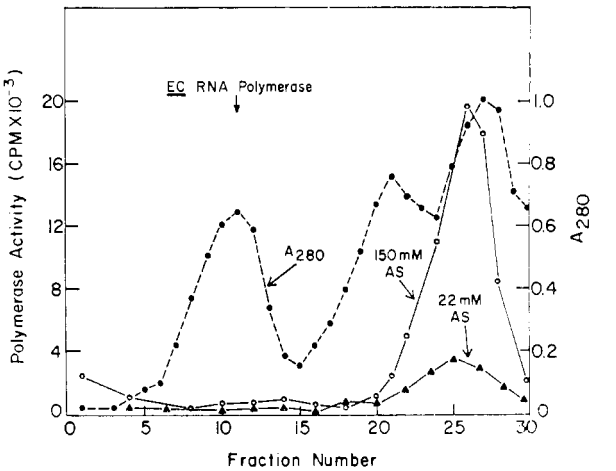

FIGURE 2: Zone sedimentation of ML RNA polymerase. A 1.0-ml sample of ML RNA polymerase containing 15 A_{280} units prepared by the procedure of Fuchs *et al.* (1964) was layered on a 30-ml 35 to 10% glycerol gradient containing 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 150 or 22 mM AS, and 1 mM MSH. After centrifugation for 12 hr at 25,000 rpm at 4° in a SW25.1 rotor, fractions were collected and assayed for RNA polymerase activity and absorption at 280 mμ. The labeled substrate for the assay of RNA polymerase was [^{32}P]GTP (3.5 × 10^6 cpm/μmole).

18–22 hr at 66–67° as described by Cohen *et al.* (1967).

Results

Several laboratories have observed that RNA polymerase isolated from *E. coli* sediments with a value of from 21 to 25 S in buffers with low ionic strength, and from 11 to 13 S in buffers with high ionic strength (Fuchs *et al.*, 1964; Richardson, 1966a,b; Pettijohn and Kamiya, 1967). When similar sedimentation studies were conducted with purified ML RNA polymerase, a single peak of 11–12S was observed with sucrose gradients containing high ionic strength buffers, whereas no activity was recovered in low ionic strength gradients (Figure 1). Solutions of low ionic strength inactivate ML RNA polymerase preparations, and multiple peaks both slower and faster than 12 S have been observed by analytical ultracentrifugation (unpublished results). We have not succeeded in reactivating ML polymerase after exposure to low salt.

Fuchs *et al.* (1964) have described a procedure for the partial purification of EC RNA polymerase without the use of streptomycin or protamine. After cell lysis, the procedure involves differential centrifugation, DEAE chromatography, and centrifugation in a linear sucrose gradient. Since 24S EC polymerase is obtained by this relatively simple method of purification, it was adapted to cell lysates of *M. lysodeikticus*. The ML enzyme prepared by this procedure showed a single sedimenting peak of enzyme activity in a glycerol gradient containing 150 mM ammonium sulfate which moved significantly more slowly than reported for EC polymerase (Figure 2). In a similar gradient containing 22 mM ammonium sulfate, the activity, although greatly reduced, appeared in the same position as with the higher salt concentration. ML enzyme prepared in this way had a specific activity of 5–50 units/mg of protein, while EC polymerase so prepared has a much higher activity (Fuchs *et al.*, 1964). The reason for the lower specific activity of the ML enzyme is that it does not separate from the bulk of the contaminating protein, most of which remains at the top of the gradient.

Nearest-Neighbor Studies. The DNA of the bacteriophage ϕX174 was selected for these studies because it can be isolated intact without single-strand interruptions and also because its circular structure obviates any difficulties that might arise from the effects of free ends (Berg *et al.*, 1965). The studies of Swartz *et al.* (1962) have provided the nearest-neighbor frequencies for ϕX174 SS DNA (positive strand) and its complementary strand (negative strand). This information permits a test of whether one or both of the strands of ϕX174 RF DNA serve as template for cRNA synthesis.

ϕX174 RF DNA prepared by the procedure of Jansz *et al.* (1966) showed little or no contamination with host DNA by several different criteria (nearest-neighbor frequencies, hybridization of cRNA product, and electron microscopic analysis). When ϕX174 SS DNA is used as a template, the cRNA products formed with either EC or ML RNA polymerase have very similar nearest-neighbor frequencies (Table I). The values are those expected from the data of Swartz *et al.* (1962), assuming that the RNA product is complementary and antiparallel to the template strand.

When ϕX174 RF DNA is used as a template, the cRNAs synthesized with both enzymes have distinctly different nearest neighbor frequencies (Table II). The cRNA formed by EC RNA polymerase has nearest-neighbor frequencies similar to those of the positive strand, as might be expected for asymmetric transcription and as was found by Hayashi *et al.* (1964). Conversely, the cRNA synthesized by ML RNA polymerase has the nearest-neighbor frequencies that would be predicted if symmetrical transcription had occurred. Similar results are found with ML polymerase when denatured ϕX174 RF DNA is employed as a template. Nearest-neighbor frequency analy-

TABLE I: Nearest-Neighbor Frequencies of cRNA Prepared with ϕX174 SS DNA as Template.[a]

Dinucleotide	Expected[b]	Found Using ML RNA Polymerase[c]	Found Using EC RNA Polymerase
ApA, UpU	0.101, 0.069	0.101, 0.067	0.107, 0.076
CpA, UpG	0.096, 0.048	0.092, 0.044	0.088, 0.046
GpA, UpC	0.054, 0.064	0.062, 0.060	0.057, 0.062
CpU, ApG	0.052, 0.069	0.048, 0.082	0.048, 0.072
GpU, ApC	0.047, 0.068	0.048, 0.060	0.049, 0.060
GpG, CpC	0.040, 0.053	0.048, 0.050	0.039, 0.054
UpA	0.061	0.056	0.058
ApU	0.072	0.068	0.072
CpG	0.045	0.050	0.050
GpC	0.061	0.065	0.061

[a] The reaction system (0.5 ml) for cRNA synthesis contained 100 mM each of three unlabeled nucleoside triphosphates and 60 mM of one ^{32}P-labeled nucleoside triphosphate (4.4–10.7 $\times$ 10^6 cpm/μmole), 5 mM $MgCl_2$, 5 mM KCl, 0.5 mM 2-mercaptoethanol, 3.6 μg of ϕX174 SS DNA, and either 20 units or 33 units of ML and EC RNA polymerase, respectively. Four reactions, each using a different ^{32}P-labeled triphosphate, were carried out for each determination. The reactions were incubated for 20 min at 37°. The cRNA was isolated for nearest-neighbor frequency analysis as described under Methods. [b] Data for the limited replication of ϕX174 DNA by Swartz *et al.* (1962). [c] Average of two determinations.

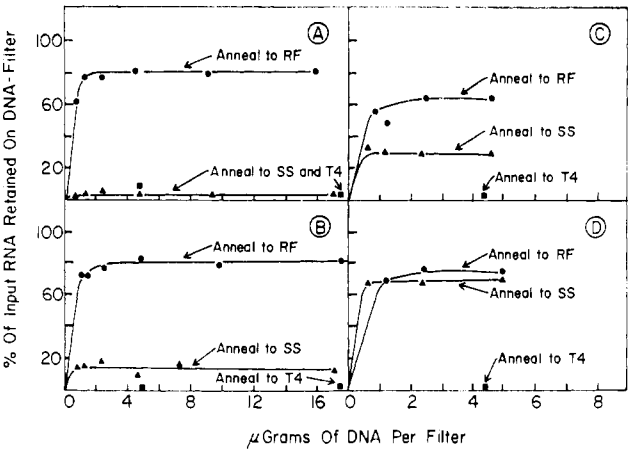

FIGURE 3: DNA–RNA hybridization analysis of EC and ML product cRNA. The reaction mixtures (1 ml) contained 30 mM Tris (pH 7.9), 400 nmoles each of ATP, GTP, and UTP, 325 nmoles of ^{32}P-labeled CTP (56 $\times$ 10^6 cpm/μmole), 5 mM $MgCl_2$ in all except B which was 10 mM, 5 mM KCl, 0.5 mM MSH, and template DNA and enzyme as indicated below. (A) EC RNA polymerase, 14.8 units; ϕX174 RF DNA, 3.27 μg. (B) ML RNA polymerase, 16.7 units; ϕX174 RF DNA, 3.27 μg. (C) ML RNA polymerase, 16.7 units; heat-denatured ϕX174 RF DNA, 3.39 μg. (D) ML RNA polymerase, 16.7 units; ϕX174 SS DNA, 3.44 μg. The mixtures were incubated at 37° for 15 min. The amounts of cRNA formed were: 17.1 μg in A, 3.4 μg in B, 1.32 μg in C, and 1.05 μg in D. The isolation and hybridization of cRNA was as described in Methods. The cRNA was present at a concentration of 0.05 μg/ml in A–D in a total annealing volume of 1.0 ml.

sis does not lend itself to determining the degree of symmetrical transcription because the method is not sufficiently sensitive and considerable variations ($\pm$10%) are encountered in such determinations. For these reasons analysis of the cRNA product by the hybridization assay technique was employed in the following studies.

DNA–RNA and RNA–RNA Hybridization Analysis. When ϕX714 RF DNA is used as a template for purified EC RNA polymerase, approximately 80% of the product cRNA an-

TABLE II: Nearest-Neighbor Frequencies of cRNA Prepared with ϕX174 RF DNA as Template.[a]

Dinucleotide	Expected if Symmetric Transcription[b]	Found Using ML with RF DNA	Found Using ML with Denatured RF DNA	Expected if Asymmetric Transcription[c]	Found Using EC with RF DNA
ApA, UpU	0.085, 0.099	0.084, 0.088	0.086, 0.083	0.069, 0.101	0.075, 0.108
CpA, UpG	0.070, 0.070	0.066, 0.071	0.072, 0.062	0.048, 0.096	0.049, 0.086
GpA, UpC	0.058, 0.065	0.058, 0.063	0.060, 0.060	0.064, 0.054	0.060, 0.060
CpU, ApG	0.064, 0.058	0.064, 0.056	0.060, 0.063	0.069, 0.052	0.075, 0.048
GpU, ApC	0.053, 0.053	0.056, 0.054	0.050, 0.056	0.068, 0.047	0.058, 0.049
GpG, CpC	0.041, 0.041	0.044, 0.048	0.050, 0.055	0.053, 0.040	0.046, 0.043
UpA	0.059	0.059	0.052	0.061	0.058
ApU	0.075	0.074	0.066	0.072	0.069
CpG	0.045	0.050	0.056	0.045	0.051
GpC	0.061	0.063	0.071	0.061	0.063

[a] The cRNA synthesized using EC and ML RNA polymerase was as described in Table I, except that 3.6 μg of ϕX174 RF RNA served as template. Denatured ϕX174 RF DNA was prepared by mild DNase treatment (Roth and Hayashi, 1966), heating for 5 min in a boiling water bath and rapid cooling in ice–water. The cRNAs were isolated and analyzed for their nearest-neighbor frequencies as described under Methods. [b] Data for extensive replication of ϕX174 DNA found by Swartz *et al.* (1962). [c] Data for limited replication of ϕX174 DNA found by Swartz *et al.* (1962).

neals to denatured RF DNA while only about 2% anneals to φX174 SS DNA (Figure 3A). Control hybridizations (annealing of the cRNA product to T4 DNA) also show 2% RNase resistance. These results indicate that transcription of φX RF DNA is asymmetric and derives almost exclusively from the "negative" strand. Transcription of RF DNA with purified ML RNA polymerase also results in a cRNA product in which 80% hybridizes to denatured RF DNA, but 14–18% of the cRNA also anneals to the positive strand, suggesting that some of the cRNA formed is transcribed from the DNA strand that does *not* serve as a template for EC RNA polymerase (Figure 3B). Control reactions show that when denatured RF DNA is used as template (Figure 3C), about half as much of the cRNA product anneals to SS DNA as to RF DNA (30 and 64%, respectively), indicating that denaturation allows both strands to be copied equally well. Moreover, when SS DNA functions as template, 65–70% of the cRNA product anneals to either RF or SS DNA, as would be expected (Figure 3D). The somewhat lower hybridization values obtained in the control reactions may be due to the smaller size in cRNA product when denatured RF or SS DNA serves as template; the efficiencies of hybridization of all the species of cRNA may not be the same under the annealing conditions used. In addition, the saturation curves of Figure 3 show that a DNA/RNA weight ratio of 40 is sufficient to ensure maximum hybridization under these conditions.

If a preparation of RNA contains molecules with nucleotide sequences that are complementary to one another, RNase-resistant RNA–RNA hybrids can be formed under appropriate self-annealing conditions (Geiduschek *et al.*, 1962). This technique should also allow us to determine the degree of symmetrical transcription by an RNA polymerase preparation (Geiduschek *et al.*, 1962; Colvill *et al.*, 1965). When cRNA isolated from a reaction mixture in which RF DNA is used as a template for EC RNA polymerase is tested for self-complementarity, 6% is RNase resistant. On the other hand, cRNA formed by ML RNA polymerase with RF DNA as template is 36% RNase resistant, indicating a higher degree of symmetrical copying than for the EC enzyme.

In Table III we have compiled the results of several experiments, and the two hybridization assay techniques are compared for EC and ML enzymes with different φX DNA templates. Altogether the data indicate that although there is some variability, the DNA–RNA and RNA–RNA hybridization assays give qualitatively similar results that are more specific and sensitive than those obtained by nearest-neighbor frequency determinations. The hybridization assays show that when SS DNA or denatured RF DNA are used as templates for the two different RNA polymerases, the amounts of DNA–RNA and RNA–RNA hybrids formed by the cRNAs are similar. However, ML and EC RNA polymerases transcribe native RF DNA somewhat differently; the EC enzyme copies one of the RF DNA strands almost exclusively while the ML enzyme transcribes more symmetrically, although the degree of symmetry is less than complete and less than previously reported with other native DNA templates (Geiduschek *et al.*, 1961, 1962; Colvill *et al.*, 1965).

The observed difference in the *in vitro* transcription of native RF DNA by the two enzymes might be due to a contaminant of the ML preparation which alters the integrity of the DNA template, *e.g.*, a DNase activity that could open the ring structure or cause partial denaturation of the DNA. This

TABLE III: Hybridization of cRNA to φX174 SS DNA, to φX174 RF DNA, and to Itself.[a]

Reaction		Hybridization				
		% RNA–RNA Hybrid			% DNA–RNA Hybrid	
Template	RNA Polymerase	Individual Values[b]	Av Values	DNA on Filter	Individual Values[b]	Av Values
φX174 RF DNA	ML	62, 61, 51, 57	58	RF	61, 80, 75, 50, 48	63
				SS	16, 25, 18, 12, 17	18
	EC	6, 6, 14, 5	8	RF	69, 79, 66	70
				SS	1, 2, 2	2
Denatured φX174 RF DNA	ML	50		RF	43, 62	52
				SS	19, 30	24
	EC	70		RF	46	
				SS	24	
φX174 SS DNA	ML	2		RF	61, 73	67
				SS	37, 65	51
	EC	16, 8		RF	64	
				SS	71	

[a] The reaction system (1.0 ml) and conditions for cRNA synthesis were similar to those described for Table I. ^{32}P-labeled CTP or UTP (15–52 × 10^6 cpm/μmole) served as the radioactive substrates. The cRNA products were isolated, hybridized, and assayed as described under Methods. DNA–RNA hybridizations were carried out in DNA excess (DNA/RNA greater than 40). Self-annealing of cRNAs were at concentrations greater than 0.3 μg/ml. The results are expressed as the per cent of the acid-precipitable cRNA which becomes RNase resistant (RNA–RNA) or which becomes specifically bound to φX174 DNA-impregnated filters (DNA–RNA) after annealing under conditions described in Methods. [b] Each value was derived from a separately synthesized cRNA sample.

TABLE IV: Effect of Preincubation of ϕX174 RF Template with ML RNA Polymerase on Transcription by EC RNA Polymerase.[a]

% RNA–RNA Hybrid[b]	DNA on Filter	% DNA–RNA Hybrid[b]
	ϕX174 SS	1.2
6.1	ϕX174 RF	84.4
	T4	1.2

[a] In a volume of 1.0 ml, 6.56 μg of ϕX174 RF DNA was preincubated with 30.2 μg of ML RNA polymerase (550 units/mg), 60 mM Tris (pH 7.9), 200 nmoles of GTP, 200 nmoles of UTP, 10 mM $MgCl_2$, 20 mM KCl, and 10 mM MSH for 15 min at 37°. The mixture was heated at 60° for 5 min and cooled; 500 nmoles of GTP, 600 nmoles of UTP, 800 nmoles of ATP, and 97.4 nmoles of ^{32}P-labeled CTP (52.3×10^6 cpm/μmole) were added to a final volume of 1.91 ml. A 0.2-ml aliquot of this mixture was removed, 39.1 μg of EC RNA polymerase (717 units/mg) was added to the original mixture, and a 0.2-ml aliquot of this final mixture was removed and 5 μmoles of EDTA was added. The three reaction mixtures were incubated for 15 min at 37°, aliquots were removed for the determination of the extent of synthesis, and the cRNA from the main mixture was isolated and assayed for RNA–RNA and DNA–RNA hybridization as described under Methods. No cRNA was synthesized in the mixture containing EDTA; 25.6 μg of dRNA was synthesized in the main mixture containing EC RNA polymerase; and the mixture containing no added EC RNA polymerase showed that only 1.33 μg or 5.2% of the cRNA made in the main mixture could be due to residual ML RNA polymerase activity. [b] Values are averages of two hybridizations.

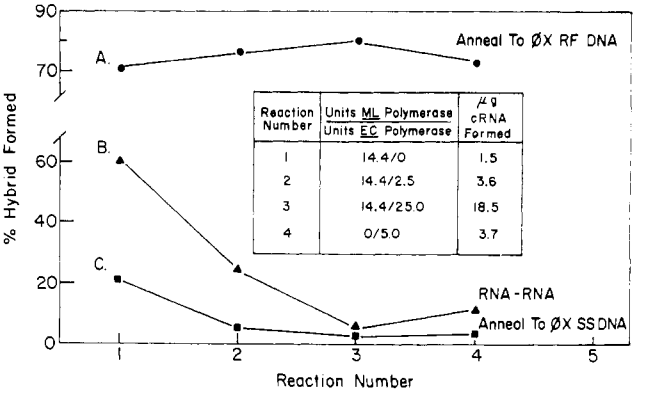

FIGURE 4: Symmetry of transcription of cRNAs synthesized by mixtures of EC and ML RNA polymerases. The cRNA was synthesized in 1.0 ml as described in Figure 3 except that 3.2 μg of ϕX174 RF DNA was the template, and varying units of ML and EC RNA polymerase were added as indicated above in the insert. The reaction mixtures were incubated at 37° for 10 min. The cRNAs were prepared and assayed for RNA–RNA and DNA–RNA hybrid formation as described in Methods after aliquots were removed from the reaction mixtures for the determination of the extent of synthesis. RNA–RNA hybridizations were carried out at 0.25–0.31 μg/ml to ensure maximum hybridization. RNA–DNA hybridizations were done with excess DNA, *i.e.*, DNA/RNA was greater than 50.

possibility was tested by preincubating RF DNA with ML RNA polymerase for 15 min at 37°, inactivating the ML enzyme by heating at 60°, and then transcribing the treated-RF DNA with EC RNA polymerase. Hybridization analysis of the cRNA formed after the above treatment shows that the RF DNA is still transcribed asymmetrically by EC RNA polymerase (Table IV). This indicates that the ML enzyme preparation does not render the DNA incapable of being asymmetrically transcribed.

When a reaction catalyzed by ML RNA polymerase is carried out in the presence of increasing amounts of EC RNA polymerase, the degree of asymmetric transcription approaches that seen when EC RNA polymerase is used alone (Figure 4). Regardless of the amounts of EC and ML enzyme in the reaction mixture, the product hybridizes to denatured RF DNA to about the same extent (Figure 4A). However, as the amount of EC enzyme increases the degree of symmetric transcription decreases, as determined by RNA–RNA and SS DNA–RNA hybridization (Figure 4B and C). These results suggest that neither ML RNA polymerase nor any contaminant in the ML preparation is interacting with the template to prevent asymmetric transcription by a competent RNA polymerase molecule. Since it can be calculated that there are about 80 ML RNA polymerase molecules per molecule of DNA in the reaction mixture, it is unlikely that the results are due to the selective transcription of free DNA molecules of EC RNA polymerase. In addition, since the amount of cRNA formed is roughly proportional to the combined units of enzyme activity added (Figure 4, insert), the EC enzyme does not seem to be inhibiting the ML enzyme. These data also reveal that EC RNA polymerase utilizes RF DNA as a template more efficiently than does ML RNA polymerase. The units given in Figure 4 are standard units of enzyme activity with calf thymus DNA as a template. When RF DNA is used as a template, seven to eight times as many units of ML RNA polymerase as EC RNA polymerase are required to synthesize the same amount of cRNA in the same time.

Fox *et al.* (1964) have demonstrated that both synthetic and natural RNA molecules can function as templates for ML RNA polymerase. If the ML enzyme utilized its cRNA product as a template it could possibly account for the symmetrical transcription observed. When 33 units of ML RNA polymerase are used in a reaction system similar to that described in Table I, approximately 3.4 μg of cRNA is formed. When half that amount of MS2 RNA was incubated under the conditions depicted in Table I, but lacking DNA, for 20 min at 37°, only 0.07 μg of RNA was made. This suggests that at most about 2% of the amount of cRNA made in the usual DNA-primed reaction could have been copied from itself, which would account for only 4% RNase resistance in the RNA–RNA annealing assay. Moreover, RNA should be a poor template for ML RNA polymerase when DNA is present, because DNA inhibits RNA priming and, in addition, the reaction conditions optimal for DNA priming are not optimal for RNA priming (Fox *et al.*, 1964). The substitution of MS2 RNA for ϕX174 cRNA is not likely to have introduced a serious error since it was previously shown that MS2 RNA

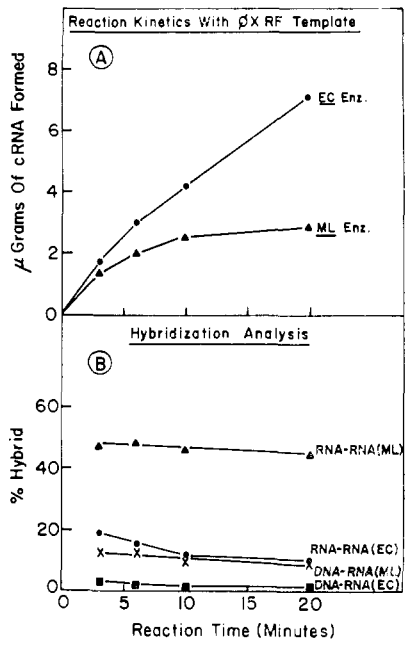

FIGURE 5: The effect of extent of synthesis on symmetry of transcription. Both reactions were incubated in 2.0-ml mixtures at 37°. The ML RNA polymerase-catalyzed reaction mixture was the standard enzyme assay system described under Methods except that 14.4 units of enzyme was present and 3.28 μg of φX174 RF DNA served as template. The EC polymerase system was the same as described for Figure 3 except that [^{32}P]UTP, 3.28 μg of φX174 RF DNA, and 5.1 units of EC enzyme were employed. Aliquots of the reaction mixtures were removed at the indicated times, a sample was acid precipitated to determine the extent of synthesis, and the remainder prepared and assayed as described in Figure 4. The cRNA was hybridized to φX174 SS DNA.

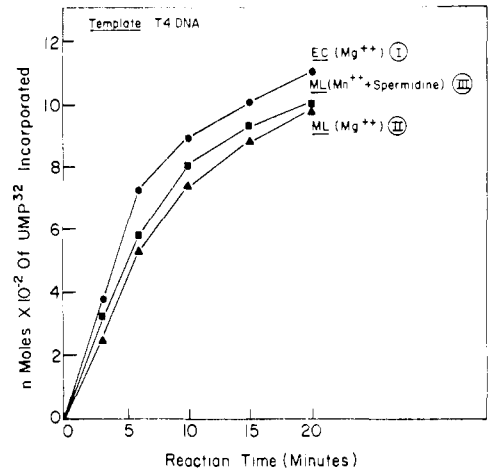

FIGURE 6: The kinetics of formation of cRNA by EC and ML RNA polymerases with T4 DNA. Reactions were carried out in 1.0 ml at 37°. At the indicated times 0.1-ml aliquots were withdrawn and acid precipitated for counting. Reactions I and II were as described for the EC RNA polymerase reaction in Figure 6 except that 2.58 μg of T4 DNA was used as template. Reaction III was as described for the ML RNA polymerase reaction in Figure 5 except that 2.58 μg of T4 DNA served as template. (●—●) I, EC RNA polymerase with 5 mM $MgCl_2$; (▲—▲) II, ML RNA polymerase with 5 mM $MgCl_2$; (■—■) III, ML RNA polymerase with 2.5 mM $MnCl_2$ + 2.0 mM spermidine.

and T7 cRNA function with nearly the same template efficiency (Fox *et al.*, 1964).

The difference in the symmetry of transcription of the two enzymes might be related to the extent of product synthesis. The kinetics of cRNA formation using RF DNA as template for the ML and EC RNA polymerases is illustrated in Figure 5A. The amount of cRNA produced by ML RNA polymerase reaches a maximum earlier than that produced in the EC polymerase-catalyzed reaction. In addition, the amount of cRNA formed is less than that produced by the EC enzyme even though 2.8 times more standard units of enzyme activity were used, which indicates, once more, that the ML enzyme does not utilize template RF DNA as efficiently as does the EC enzyme.

If one assumes that only one of the RF DNA strands is primarily copied, then the amount of cRNA synthesized by the ML enzyme represents on the average from once to twice the amount of DNA template. Thus, cRNA synthesis probably did not stop simply because the ML enzyme was unable to complete more than one cycle of transcription. When the cRNA produced at the different times by both enzymes is subjected to hybridization analysis, it appears that the degree of symmetrical transcription is somewhat dependent upon the extent of cRNA synthesis (Figure 5B). However, the decrease in symmetrical transcription by both enzymes with increasing amounts of cRNA made is not sufficient to account for the differences between the two enzymes when different amounts of cRNA are formed. Since RNA polymerase shows a greater affinity for denatured than for native DNA (Chamberlin and Berg, 1962; Hurwitz *et al.*, 1962; Fox and Weiss, 1964), the slight decrease in the degree of symmetrical transcription with increasing synthesis of cRNA may represent the initial preferential copying of denatured, and/or partially denatured, molecules contaminating the DNA preparation before the remaining intact DNA molecules are copied.

A comparison of the kinetics of cRNA formation with T4 DNA as template under the conditions described in Figure 5 shows relatively little difference between the two enzymes (Figure 6, I and III). This difference is increased slightly when the ML RNA polymerase reaction mixture contains magnesium ion, but no manganese or spermidine, as in the EC enzyme reaction mixture (Figure 6, II). These results indicate that EC and ML polymerases show similar kinetics of cRNA formation when the large linear T4 DNA is used as a template; with the small circular RF DNA the kinetics are different. However, as with RF DNA, the ML enzyme is not as efficient as the EC enzyme in synthesizing cRNA with T4 DNA as template.

Since the symmetrical transcription properties of ML polymerase could not be explained by impurities in the enzyme preparation, by product cRNA serving as template, or by the limited extent of product synthesis, the effects of the reaction conditions were examined. It was found that the nature or concentration of divalent metal ion and the presence of spermidine could modify the degree of symmetrical transcription by ML RNA polymerase with RF DNA as template (Table V). In the presence of 5 mM $MgCl_2$, the cRNA product catalyzed by ML RNA polymerase showed the expected high degree of symmetry, 62% RNA–RNA hybrid. Under the usual ML enzyme assay conditions ($MnCl_2$ + spermidine) a decrease to

TABLE V: Effect of Reaction Conditions on the Symmetry of Transcription.[a]

Reaction Conditions	cRNA Synthesized (μg)	RNA–RNA Hybrid (%)	DNA on Filter	DNA–RNA Hybrid (%)
I. $MgCl_2$	3.55	62	RF	50
			SS	12
II. $MnCl_2$ + spermidine	4.10	35	RF	48
			SS	7
III. High $MgCl_2$ + spermidine[b]	4.82	18	RF	67
			SS	3
IV. EC RNA polymerase, $MnCl_2$, + spermidine	14.10	14	RF	66
			SS	2

[a] All reactions were incubated in 1.0-ml systems for 20 min at 30°. Part I contained 3.3 μg of φX174 RF DNA, 0.8 mM ATP, CTP, and GTP, 226 nmoles of [^{32}P]UTP (13.3 × 10^6 cpm/μmole), 5 mM $MgCl_2$, 5 mM KCl, 0.5 mM MSH, and 30.2 μg of ML RNA polymerase (550 units/mg). Part II was the standard enzyme assay system with the exception that 3.3 μg of RF DNA was the template. Part III was identical with part II except that 10 mM $MgCl_2$ was substituted for 2.5 mM $MnCl_2$. Part IV was identical with part II except that 36 μg of EC RNA polymerase (717 units/mg) was substituted for the ML enzyme. The cRNAs were prepared and assayed as described in Table III. [b] Values are the average of two determinations.

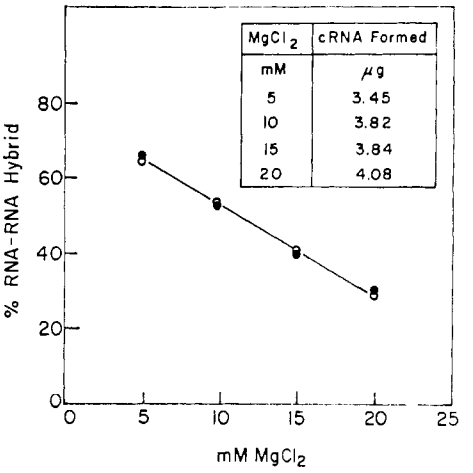

FIGURE 7: The effect of $MgCl_2$ concentration on the symmetry of transcription. The reaction mixtures were incubated for 20 min at 37°. The reaction system was as described in Figure 3 except that the concentration of $MgCl_2$ was varied as shown above. The cRNA was prepared, hybridized, and assayed as described in Methods. All annealing assays were carried out in duplicate. In addition, samples prepared and hybridized as described were treated with the usual amount of RNase plus 20 μg ml of pancreatic DNase in 10 mM $MgCl_2$ for 30 min at 37° before being acid precipitated and counted in the usual way. A control experiment in which a DNA–RNA hybrid was subjected to the DNase plus RNase treatment indicated that 55% of the RNase resistant hybrid could be destroyed by the RNase plus DNase treatment. (○—○) Regular RNA–RNA hybrid RNase treatment; (●—●) RNase plus DNase treatment of RNA–RNA hybrid.

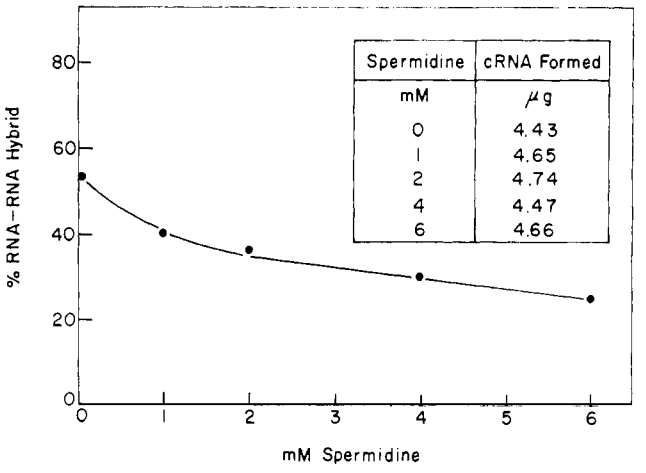

FIGURE 8: The effect of spermidine concentration on the symmetry of transcription. The 1.0-ml reaction mixtures with ML RNA polymerase were incubated for 20 min at 37°. The reaction system was the same as described in Figure 3 except that [^{3}H]UTP (21.4 × 10^6 cpm/μmole) was used as the labeled substrate and spermidine was varied as indicated above. The cRNA was prepared, hybridized, and assayed as described in Methods. All annealing assays were carried out in duplicate.

35% RNA–RNA hybrid formation occurred. When the reaction mixture was adjusted to contain 10 mM $MgCl_2$ and 2 mM spermidine, a still lower degree of symmetrical transcription was observed (18% RNA–RNA hybrid), which was nearly the same as when EC RNA polymerase was used (14% RNase resistant of the self-annealed product). The RNA–RNA hybrid results are also reflected in the DNA–RNA hybrid analysis.

Effect of $MgCl_2$ Concentration. An examination of the effect of $MgCl_2$ concentration on the degree of product symmetry showed that with increasing magnesium ion, the cRNA product demonstrated a significantly lower tendency for self-annealing, suggesting an increase in asymmetrical transcription of RF DNA by ML polymerase (Figure 7). These results cannot be attributed to differences in the amount of cRNA formed since similar amounts were made in each reaction (Figure 7, insert). The addition of DNase after annealing does not alter the degree of RNase resistance of the self-annealed products indicating that little or no DNA–RNA hybrid is present as a result of possible incomplete removal of template DNA during cRNA isolation.

Effect of Spermidine Concentration. Similar effects on transcription were observed with spermidine (Figure 8). When the spermidine concentration was increased in reaction mixtures containing RF DNA and ML enzyme, there was a significant decrease in the amount of cRNA product capable of being self-annealed, suggesting a decrease in the degree of symmetrical copying. Equivalent amounts of cRNA were synthesized in each reaction (Figure 8, insert).

Effect of KCl Concentration. Increasing the KCl concentration in a RF DNA primed ML RNA polymerase reaction from 5 to 50 mM caused a decrease in the self-annealing of

TABLE VI: Effect of Increasing KCl Concentration on the Symmetry of Transcription.[a]

Reaction Conditions	cRNA Synthesized	% RNA–RNA Hybrid[b]
I. 5 mM KCl	4.88	47
II. 50 mM KCl	4.77	33
III. 200 mM KCl	2.55	33
IV. EC RNA polymerase, 5 mM KCl	13.50	7

[a] The reaction system (0.5 ml) was incubated for 20 min at 37°, and was the same as described in Figure 4 except that all tubes contained 5 mM $MgCl_2$, 15.1 μg of ML polymerase or 5.4 μg of EC polymerase, and KCl as indicated above. The cRNA was prepared, hybridized, and assayed as described under Methods. [b] All annealing assays were carried out in duplicate.

product cRNA from 47 to 33% (Table VI). Still higher concentrations of KCl appeared to cause no further decreases, but inhibition of product cRNA formation became significant. EC RNA polymerase transcribed the same RF DNA asymmetrically at the lower salt concentration, indicating that the symmetrical transcription observed with the ML enzyme was not due to the loss of DNA integrity.

Kinetics of cRNA Formation under Conditions of High and Low Symmetrical Transcription. Figure 9, curves II and III, indicates that a slightly higher rate of cRNA formation is observed in a ML RNA polymerase reaction with RF DNA as template when conditions are adjusted (as previously described in Table V) so that symmetrical transcription is minimal. Even under these conditions, however, the increased rate of cRNA formation with the ML enzyme is still lower than the reaction rate of the EC enzyme (Figure 9, curve I). These data suggest therefore that there may be a slight, but not strong, correlation between the kinetics of cRNA formation and the degree of symmetrical transcription by ML RNA polymerase.

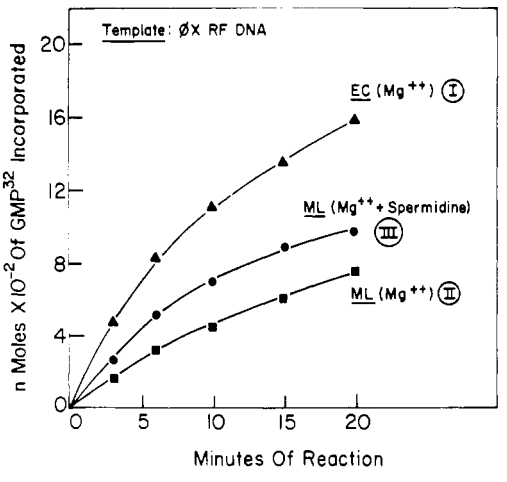

FIGURE 9: The kinetics of formation of cRNA by ML RNA polymerase under conditions of greatest and least symmetry of transcription. All reactions were incubated in 0.5 ml at 37°. At the indicated times 0.05-ml aliquots were removed and acid precipitated for the determination of the amount of cRNA formed. Reactions I and II contained 30 mM Tris (pH 7 9), 1.64 μg of RF DNA, 800 nmoles of ATP, CTP, UTP, and 134 nmoles of [^{32}P]GTP (178.9 × 10^6 cpm/μmole), 5 mM $MgCl_2$, 5 mM KCl, 0.5 mM 2-mercaptoethanol, and 4.3 μg of EC RNA polymerase in I and 15.1 μg of ML RNA polymerase in II. Reaction III was the same as reaction II except 10 mM $MgCl_2$ was present and 2 mM spermidine was added.

TABLE VII: Effect of Reaction Conditions on the Symmetry of Transcription of T4 DNA by ML RNA Polymerase.[a]

Reaction Conditions	Enzyme	cRNA Synthesized (μg)	% RNA–RNA Hybrid[b]
I. $MgCl_2$	ML	3.7	8.1
II. High $MgCl_2$ + spermidine	ML	3.4	8.0
III. $MnCl_2$ + spermidine	ML	5.1	7.1
IV. $MgCl_2$	EC	3.1	5.2

[a] All reactions were incubated at 37° for 20 min in 1.0-ml volumes. Reactions I and IV contained 30 mM Tris (pH 7.9), 400 nmoles of ATP, CTP, GTP, 173 nmoles of [^{3}H]UTP (21.4 × 10^6 cpm/μmole), 3.12 μg of T4 DNA, 5 mM $MgCl_2$, 5 mM KCl, and 0.5 mM MSH. Reaction II was the same as I except 10 mM $MgCl_2$ and 2 mM spermidine were present and reaction III contained 2.5 mM $MnCl_2$ and 2 mM spermidine. Reactions I, II, and III contained 30.2 μg of ML RNA polymerase. Reaction IV contained 10 μg of EC RNA polymerase. cRNA was isolated, purified, and assayed as described under Methods. cRNA concentrations were from 0.5 to 0.7 μg per ml in the annealing assays. [b] Values are the average of duplicate annealing assays of two separately synthesized cRNA samples for each reaction.

Transcription of T4 DNA by ML RNA Polymerase. No significant differences in the amounts of RNA–RNA hybrid formation could be detected when T4 DNA was used as template under reaction conditions that had previously yielded high and low degrees of symmetrical transcription with φX174 RF DNA (Table VII). Whether the reaction mixture contained high or low magnesium ion, manganese ion, or spermidine, the transcription of T4 DNA by both ML and EC RNA polymerase was essentially asymmetric. The results suggest that the ML enzyme behaves differently with different DNA templates with respect to its transcription properties.

Discussion

Sedimentation studies of ML RNA polymerase indicate the absence of a rapidly sedimenting form (21–25 S) of the enzyme. With EC RNA polymerase, under conditions of low ionic strength which favors the "fast" form, substantial loss of

enzyme activity was observed (Stevens *et al.*, 1966). It is possible, therefore, that the rapidly sedimenting forms of the ML enzyme might be quickly inactivated in low ionic strength buffers and, hence, difficult to detect.

The experiments presented here indicate that the degree of symmetry of transcription by ML RNA polymerase is probably a property of the enzyme itself and not due to a contaminant of the enzyme preparation which renders the template susceptible to symmetrical copying. They also show that a number of factors can influence the extent of symmetrical copying of the ML enzyme with RF DNA as template. Since the ionic strength of the maximum amount of $MgCl_2$ used was comparable with that of the minimum amount of KCl used, it is unlikely that the increase in asymmetrical transcription observed with increasing concentrations of $MgCl_2$, KCl, or spermidine was a simple ionic strength phenomenon. Other factors that might have influenced strand selection, *e.g.*, temperature, pH, triphosphate concentrations, the order of reagent additions, were not investigated.

Conditions that lead to substantial symmetry of transcription with RF DNA allow essentially asymmetric copying with T4 DNA, suggesting that the template plays a role in determining the type of transcription exhibited by the ML enzyme. With RF DNA as template, the different behavior of the two enzymes is reflected in the kinetics of cRNA formation and the efficiency of template utilization. These differences prevail with the ML enzyme even when the reaction conditions are altered to allow transcription to be almost as asymmetric as with the EC enzyme. The behavior of the ML polymerase might be related to the heterology of the system, *i.e.*, EC is the host for ϕX174 phage. Colvill *et al.* (1965), however, have shown that homology between template and RNA polymerase is not necessary for strand selection in a number of systems, and, as shown here, the ML enzyme does copy the RF DNA asymmetrically under certain conditions. These results indicate that the ML enzyme responds to various substances by altering its strand-selective properties with a low molecular weight DNA template containing a superhelical tertiary structure. Superhelical DNA molecules have unusual strand unwinding (denaturation) properties (Vinograd and Lebowitz, 1966) and the ML and EC enzymes appear to respond differently to such templates.

Previous studies of strand selection by the ML enzyme indicated that both strands were transcribed with large linear DNA templates (Geiduschek *et al.*, 1961, 1962; Weiss and Nakamoto, 1961; Robinson *et al.*, 1964; Colvill *et al.*, 1965). Under similar reaction conditions, the results reported here indicate that the transcription of T4 DNA is essentially asymmetric. One explanation for these contradictory results is that the enzyme used in this report was purified by a slightly different procedure which employed two carboxymethylcellulose treatments to remove basic contaminants including nucleases. If the preparations used in the earlier studies were contaminated with a nuclease activity similar to the EC exonuclease III which degrades helical linear DNA molecules from the 3′-hydroxyl ends to produce partially single-strand regions, symmetrical transcription would have been most probable. Another difference relates to enzyme storage. The enzyme used in this study was stored in a solution containing 150 mM $(NH_4)_2SO_4$, 10 mM Tris (pH 7.5), and 50% glycerol, whereas previous preparations were stored in a solution containing 5 mM Tris (pH 7.5) and 50% glycerol. The high ionic strength of this storage solution may have altered the strand-selective properties of the enzyme.

Under the proper *in vitro* conditions, ML RNA polymerase will mimic *in vivo* single-strand selection. This suggests that the purification procedure has not irreversibily altered the enzyme's ability to copy one strand of the template. The ability to alter the strand selectivity of the enzyme by altering the reaction conditions might be a useful tool in the study of the initiation of transcription and the factors that affect it.

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Equilibrium Mechanochemistry of Collagen Fibers*

Merry M. Rubin,† Karl A. Piez, and Aharon Katchalsky‡

ABSTRACT: The isothermal shrinkage of a fiber under constant tensile force by varying the concentration of a component of the surrounding medium has been demonstrated previously. The thermodynamic corollary of this process states that the extent of chemical interaction between the fiber and such a component should be altered by applying force to the fiber. The interaction between collagen fibers and the denaturant LiBr, its variation with tensile force or relative length of the fiber, and the analysis of the mechanochemical properties of this system in terms of a two-state model are the subjects of this report. The experimental procedure included (1) preliminary exposure of the fibers to extremes of denaturant concentration and force to enhance reversibility of measured effects, (2) mercurimetric titration of LiBr, (3) use of a mechanochemically inert tracer [^{14}C]glycerol in evaluating excess LiBr in the fiber phase relative to the external solution (enrichment), and (4) correlation of length and enrichment measurements for individual fibers. The variations of fiber length with tensile force and denaturant activity were semiquantitatively consistent with computations according to the two-state model of Hill. In solutions of constant denaturant activity, the measured enrichment decreased linearly with fiber length; in 5 M LiBr the molar ratio of excess LiBr:amino acid residue varied from 1:4 in relaxed fibers to 1:7 in fully stretched fibers. The relationship between the sigmoidal force dependence of the enrichment and the sigmoidal dependence of fiber length upon denaturant activity fulfilled the thermodynamic predictions.

Conformational changes of fibrous macromolecules, accompanying the reversible interaction with specific reagents, provide a general mechanism for the isothermal conversion of chemical energy into mechanical work (Katchalsky *et al.*, 1960). A well-documented example for such conversion is the contractility of collagen fibers, which shrink appreciably by reversible combination with several neutral salts, and which develop concomitantly large mechanical forces (Flory and Spurr, 1961; Yonath *et al.*, 1965; Puett *et al.*, 1965; von Hippel and Schleich, 1969). It was found that LiBr in particular causes a rapid chemical melting of collagen molecules, and that the forces developing during the process could be utilized for the operation of a mechanochemical engine (Steinberg *et al.*, 1966). The interaction with LiBr will serve as the basic example of the equilibrium studies of this paper.

On *a priori* grounds the isothermal contractile behavior of collagen fibers may be characterized by four parameters: by the length, l, and the force, f, by the chemical potential, μ, of the reagent, and by the extent of reagent combination with the protein molecules. A full set of quantitative relations among these four parameters, whether empirical or theoretical, would constitute an "equation of state" for the collagen–salt system. Although for different fibrous proteins and salts, different numerical parameters would enter the equation of state, it is hoped that the basic form of the equation might hold for many systems; and hence the interest in evaluating the behavior of a reproducible, and well-defined example.

While the mechanical measurements of the dependence of force upon length are easily carried out, and may be readily interpreted by existing theories of rubberlike behavior, it has proven difficult to determine the binding of salt to collagen and to evaluate its dependence upon mechanical force. Ciferri *et al.* (1967) studied the selective interaction of [^{14}C]KCNS with collagen at different temperatures but did not correlate the binding with conformational changes under variable mechanical stresses. Oplatka and Yonath (1968) measured total KCNS and water uptake during the first denaturation cycle of strips of natural collagen. Since our aim was to obtain reversible data suitable for thermodynamic analysis, we used reconstituted collagen fibers which had undergone several contraction–expansion cycles, and developed a procedure to measure LiBr combination with the fiber under variable stresses. The method will be described in more detail in the experimental part.

A quantitative correlation between force and length in media of different salt concentrations was carried out by Flory and

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