The RNA Polymerases from *Drosophila melanogaster*†

Robert H. Gross and Michael Beer*

ABSTRACT: The DNA-dependent RNA polymerases of *Drosophila melanogaster* have been extracted and partially purified. Extraction from 12- to 24-hr old embryos yields two enzyme species (Ia and II) which elute from a DEAE-Sephadex column at 0.04 and 0.25 *M* ammonium sulfate. When the extraction is carried out from third instar larvae, three enzyme peaks elute from the column (Ia′, Ib, and II) at 0.095, 0.145, and 0.25 *M* $(NH_4)_2SO_4$. Each of these enzymes may be distinguished by its characteristic behavior in the presence of varying concentrations of Mg, Mn, and $(NH_4)_2SO_4$, as well as its sensitivities to various inhibitors. Enzyme II from embryos is indistinguishable from the larval enzyme II in terms of $(NH_4)_2SO_4$, Mg, and inhibitor sensitivities. It is concluded that these two enzymes are the same and correspond to the RNA polymerase II found in other eukaryotes. Embryonic enzyme Ia and larval enzyme Ia′ have the same Mg, $(NH_4)_2SO_4$, and inhibitor sensitivities, but differ in elution point. Since it is possible (by incubation at 25° or storage at −70°) to alter the elution point of embryonic enzyme Ia to approach that of larval enzyme Ia′, it is concluded that these two enzymes are the same and correspond to the RNA polymerase I found in other eukaryotes. It is implied that enzyme Ia is modified during development to become Ia′. RNA polymerase Ib is unique in several respects. Unlike other eukaryotic RNA polymerases, *Drosophila* RNA polymerase Ib is very sensitive to rifampicin, but is not bacterial and probably not mitochondrial. Furthermore, RNA polymerase Ib represents the appearance of a new enzyme during development, which has not previously been observed in any eukaryotic organism.

In the past few years, RNA polymerases have been successfully isolated from several eukaryotes (Jacob, 1973). In contrast to prokaryotes which have only one RNA polymerase (Burgess, 1971), eukaryotic organisms have usually been found to possess from two to four RNA polymerases (Jacob, 1973), but as many as eight have been reported (Wasserman et al., 1972).

The different eukaryotic RNA polymerases can be distinguished from one another by their different response characteristics to varying concentrations of Mg, Mn, $(NH_4)_2SO_4$, and transcription inhibiting drugs (Meilhac et al., 1972). Furthermore, RNA polymerase I has been shown to be localized within the nucleolus and RNA polymerases II and III to be extranucleolar (Beerman, 1971; Siev et al., 1969; Jacob et al., 1970; Lindell et al., 1970; Roeder and Rutter, 1970b; Schwartz and Roeder, 1974). Enzyme III has been shown to be responsible for tRNA and 5S RNA synthesis (Weinmann and Roeder, 1974) and enzymes I and II are thought to be responsible for rRNA and HnRNA[1] synthesis, respectively (Beermann, 1971; Siev et al., 1969; Jacob et al., 1970; Lindell et al, 1970; Roeder and Rutter, 1970b).

The compartmentalization of the RNA polymerase species and their supposed functional differences indicates a possible role for RNA polymerase in genetic control, albeit on a crude level. Quantitative (but not qualitative) changes in RNA polymerase composition have been observed during development in sea urchin (Roeder and Rutter, 1970a) and *Xenopus* (Roeder et al., 1970).

There have been three reports of the isolation of RNA polymerase from *Drosophila melanogaster*. Natori et al. (1973) find only a single enzyme species in either tissue culture cells or imaginal discs that seems to correspond to RNA polymerase II in other eukaryotes. Adoutte et al. (1974) found that by varying the extraction procedure they could obtain either one or two RNA polymerases from larvae. While the present work was in progress, Phillips and Forrest (1973) reported the extraction of two RNA polymerase activities (I and II) from 12- to 24-hr embryos.

In this paper, we report the extraction and partial purification of the RNA polymerases from *Drosophila melanogaster* embryos (12–24 hr), and larvae (third instar). Two species of enzyme are found in embryos (in agreement with Phillips and Forrest (1973)) but three are found in larvae. One of the larval enzymes represents the appearance of a new RNA polymerase during development and is therefore unique. All the enzymes were completely characterized with respect to $(NH_4)_2SO_4$, Mg, and Mn requirements, and were examined for their sensitivities to various drugs.

Materials and Methods

Buffers. SSC: 0.15 *M* sodium chloride and 0.015 *M* sodium citrate (pH 7.0). Buffer E (extraction buffer): 50 m*M* Tris-HCl (Sigma, Trizma HCl) (pH 7.9 at 2°); 0.1 m*M* EDTA (Baker); 5 m*M* $MgCl_2$ (Baker); 1 m*M* dithiothreitol (Sigma); 25% (v/v) glycerol (Baker). The dithiothreitol was added immediately before use from a stock solution of 0.1 *M* dithiothreitol in 0.01 *M* sodium acetate (pH 5.2). Buffer C (column buffer): buffer E with 0.1% (v/v) 2-mercaptoethanol. Buffers EP and CP: buffers E and C supplemented with 2 m*M* phenylmethyanesulfonyl fluoride ($PhCH_2SO_2F$, Sigma) added from a 7-mg/ml stock in ethanol immediately before use.

Extraction and Fractionation of the RNA Polymerases. RNA polymerases were extracted essentially by the method of Roeder and Rutter (1969). All procedures were done in plastic containers at 0–2° unless stated otherwise. Larvae were mixed with cold buffer E and homogenized in a glass–Teflon homogenizer at high speed (5000 rpm) until homo-

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[1] Abbreviations used are: mRNA, messenger RNA; HnRNA, heterogeneous nuclear RNA; $PhCH_2SO_2F$, phenylmethylsulfonyl fluoride.

geneous (fraction H, FH). Fraction H was sonicated for 36 sec (12-sec bursts) at setting 8 of a Branson sonifier (Model LS75), $(NH_4)_2SO_4$ (Schwarz/Mann, Enzyme Grade) was added to 0.3 *M* and sonication was continued for an additional 60 sec (fraction 1, F1). If embryos were being used instead of larvae, they were first dechorinated in 3% sodium hypochlorite for 5 min (the embryos are completely viable after this treatment) and the sonication bursts were shortened to 6 sec. Immediately following sonication, F1 was diluted 1:2 with buffer E and centrifuged at 60,000 rpm for 60 min at 2° in a Type 65 rotor (Beckman). The RNA polymerases were precipitated from the supernatant (fraction 2, F2) with $(NH_4)_2SO_4$ (0.45 g of $(NH_4)_2SO_4$/ml of F2) and collected by centrifugation for 90 min at 60,000 rpm in the Type 65 rotor at 2°. The pellet containing the RNA polymerase activity was resuspended in buffer E (fraction 3), broken into 1-ml aliquots, and frozen at −70°. Fraction 3 can be stored several months without significant loss of activity.

The RNA polymerases were fractionated by column chromatography on DEAE-Sephadex A-25 that had been equilibrated with buffer C (ten column volumes). Fraction 3 (containing 1–1.5 × 10^6 cpm by the UTP limited assay—see below) was diluted to approximately 0.03 *M* $(NH_4)_2SO_4$ with buffer C and applied directly to a 0.9 × 15 cm plastic column at a rate of 1.6 ml/min. After a wash of 0.03 *M* $(NH_4)_2SO_4$ in buffer C (1 column volume), the column was developed with a 50-ml gradient (5 column volumes) from 0.03 to 0.4 *M* $(NH_4)_2SO_4$ in buffer C (flow rate of 1 ml/min). 1-ml fractions were collected into tubes containing 0.5 ml of glycerol and 1 mg of bovine serum albumin (as 0.1 ml of a 10 mg/ml bovine serum albumin (Sigma type V) stock in Buffer C), mixed immediately, and kept at −20°.

For extractions done in the presence of the protease inhibitor $PhCH_2SO_2F$, buffers EP and CP were used instead of buffers E and C in the extraction procedure.

Special conditions were required for the growth of sterile larvae. Eggs were sterilized and then allowed to develop in a sterile environment. Aliquots of FH were analyzed for bacterial content by plating out a dilution series on nutrient agar petri dishes and incubating 18–24 hr at 25°.

Sucrose-Glycerol Gradient. Sucrose-glycerol gradients were run as 0–15% (w/w) sucrose in 20% (v/v) glycerol, 0.1 *M* Tris-HCl (pH 7.9), 1 m*M* EDTA, 10 m*M* $MgCl_2$, 50 m*M* NaCl, 5 m*M* dithiothreitol, 0.1% (v/v) 2-mercaptoethanol, and 0.2 mg/ml of lysozyme (Sigma). Centrifugation was for 13 hr at 40,000 rpm in a SW50L rotor (Beckman) at 2°.

Assay for RNA Polymerase Activity. The standard assay mix contained, in 115 μl: 20 μg of calf thymus DNA (Sigma type I), 2.5 μg of pyruvate kinase (Sigma, type II), 7 μmol of Tris-HCl (Sigma, Trizma HCl) (pH 7.9), 0.2 μmol of $MnCl_2$, 1 μmol of KCl, 0.75 μmol of NaF, 0.5 μmol of phosphoenol pyruvate (Sigma, type II), 0.2 μmol of 2-mercaptoethanol, 0.05 μmol of UTP (Sigma, type III), 0.05 μmol of ATP (P-L Biochemicals), 0.05 μmol of CTP (Sigma, type III), 0.05 μmol of GTP (Sigma, type I shipped in Dry Ice), 200 pmol of [^{3}H]-UTP (New England Nuclear, 27 Ci/mmol), and 50 μl of sample in buffer E or C at 0.1 *M* $(NH_4)_2SO_4$. In the case of the UTP limited assay (UL assay) the nucleoside triphosphates added were 0.03 μmol each of ATP, GTP, and CTP and 75 pmol of [^{3}H]UTP.

After addition of the sample, tubes were mixed and incubated at 30° for 10 min. The reactions were terminated by chilling to 0° in an ice–water bath and pipeting the incubation mixture directly onto 2.4-cm diameter DEAE-cellulose filters (Whatman, DE 81). Unincorporated nucleoside triphosphates were freed from the filters by washing with 5% (w/v) Na_2HPO_4 (Sigma) according to the procedure of Litman (1968). Filters were rinsed in water, dried, and counted in a Packard liquid scintillation spectrometer in a 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene toluene based fluor.

Table I: Extraction Summary for Larval RNA Polymerase.

Fraction	Total Activity[a]	Total Protein (mg)	Specific Activity		−fold Purification[b]	% FH Activity
			units/mg	cpm/μg[b]		
FH	3390	625	5.43	8.0	1.00	100.0
F1	1820	810	2.25	0.4	0.05	53.6
F2	577	281	2.05	0.8	0.10	17.0
F3	3200	245	13.05	47.0	6.00	94.3
Ia′		25.5		350	44	
Ib		0.43		5560	700	125.5[c]
II		0.43		7300	900	

[a] One unit of activity is that amount of enzyme which will incorporate 1 pmol of UTP into RNA in 10 min at 30°. [b] Based on the UTP limited assay. [c] Recovery of Ia′ + Ib + II is expressed as the percent of the total F3 activity applied to the column.

Inhibitor assays were done using the UL assay but results were unchanged under nonlimiting conditions. The rifamycin derivatives SV, AF/013, AF/05, and AF/ABDP stock solutions were in 95% ethanol and therefore necessitated controls with just 95% ethanol added to the assay mix. The rifampicin, cordycepin (both from Sigma), and α-amanitin were added in water and appropriate controls were done by adding water to the assay mix. The additional water had no effect on the amount of RNA synthesized, but the additional ethanol did alter enzyme activity. The percentages given in the tables indicate the amount of activity compared to the appropriate control activity.

Protein and Ammonium Sulfate Concentrations. Proteins were precipitated with 10% (w/v) trichloroacetic acid and redissolved in 1 *M* NaOH to eliminate interfering effects due to thiol and sulfate compounds in the buffers. Concentrations were then determined by the method of Lowry et al. (1951) using bovine serum albumin (Sigma, type V) as the standard. All determinations were done in duplicate.

Ammonium sulfate concentrations were determined by diluting 25 μl of sample with 1 ml of doubled distilled, deionized water and comparing the conductivity (0.48 cm cell, Radiometer Conductivity meter) to a standard curve.

α-Amanitin and Rifamycin Derivatives. The α-amanitin was the very generous gift of Dr. T. Wieland (Max Planck Institute, Heidelberg). The rifamycin SV, AF/013, AF/05, and AF/ABDP were the gifts of Drs. R. Reeder and I. B. Dawid (Carnegie Institution of Washington, Department of Embryology, Baltimore).

Results

The Enzymes from Larvae. Enzyme activities and protein analyses from a typical extraction of the RNA polymerases from third instar larvae are compiled in Table I. The enchanced activity of F3 with respect to F2 is probably due to the removal of an inhibitor in the $(NH_4)_2SO_4$ precipitation step, since the supernatant shows an ability to supress the activity of F3 (data not shown).

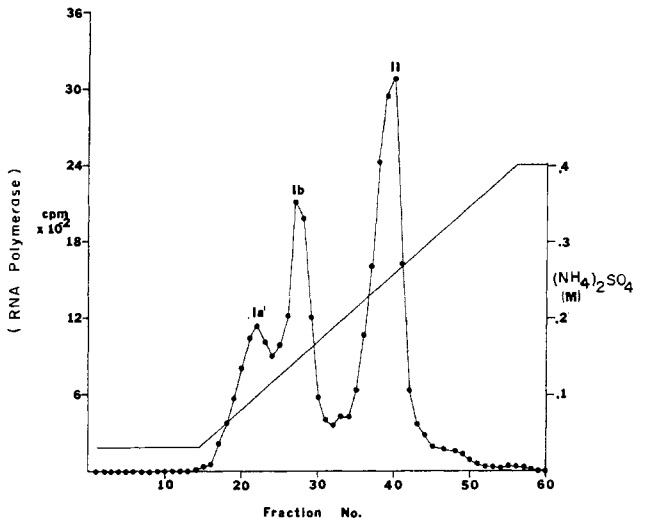

FIGURE 1: Elution profile for enzymes from a larval extraction. F3 RNA polymerase activity (1.6×10^6 cpm) was applied to a DEAE-Sephadex column in 0.03 *M* $(NH_4)_2SO_4$ in buffer C. The column was washed with 10 ml of 0.03 *M* $(NH_4)_2SO_4$ in buffer C, and the enzymes were eluted with a 50-ml linear 0.03–0.4 *M* $(NH_4)_2SO_4$ gradient in buffer C; 1-ml fractions were collected into tubes containing bovine serum albumin and glycerol (final concentration of 0.7 mg/ml of bovine serum albumin and 50% glycerol), and 50-μl aliquots were withdrawn and assayed by the UL assay—all as described in Materials and Methods. The ordinate represents the cpm incorporated by the 50-μl aliquot.

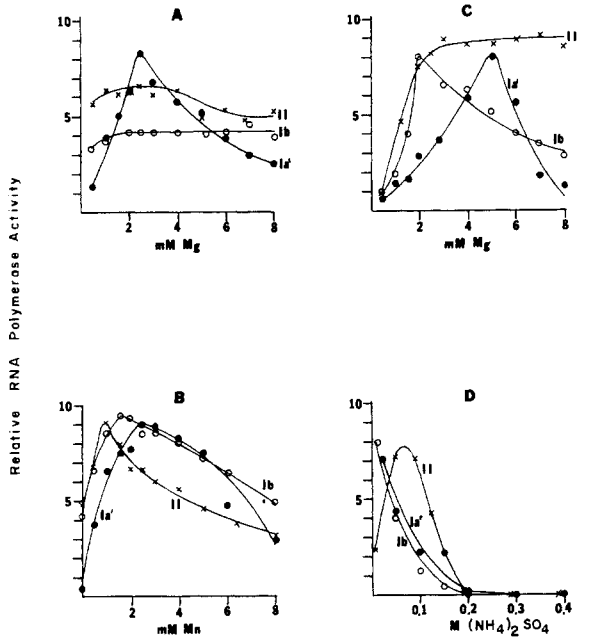

FIGURE 2: Characteristics of the larval enzymes. All assays were done under UTP limiting conditions, but the shapes of the curves remained unchanged under nonlimiting conditions. All data have been normalized and represent the average values obtained from three separate column runs (each column assay done in duplicate): RNA polymerase Ia′ (●), RNA polymerase Ib (○), RNA polymerase II (x). (A) Mg dependence with Mn = 2 m*M* and $(NH_4)_2SO_4$ = 0.05 *M*; (B) Mn dependence with Mg = 1.5 m*M* and $(NH_4)_2SO_4$ = 0.05 *M*; (C) Mg dependence with Mn = 0.0 m*M* and $(NH_4)_2SO_4$ = 0.05 *M*; (D) $(NH_4)_2SO_4$ dependence with Mg = 1.5 m*M* and Mn = 2 m*M*.

Figure 1 shows the elution profile for the larval enzymes from a DEAE-Sephadex column. Enzymes Ia′, Ib, and II elute at 0.095, 0.145, and 0.250 *M* $(NH_4)_2SO_4$. Rechromatography of column fractions representing RNA polymerases Ia′, Ib, and II on DEAE-Sephadex yields the same elution patterns as obtained originally. The only detectable protein peak (A_{280}) is in the washthrough fractions. Both enzymes Ia′ and Ib are completely resistant to the mushroom toxin α-amanitin at concentrations up to 20 μg/ml, while enzyme II is essentially completely inhibited by 0.5 μg/ml.

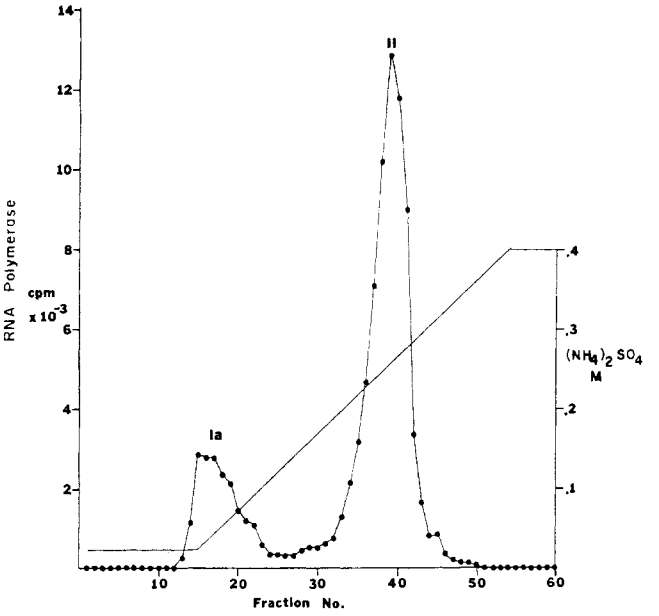

FIGURE 3: Elution profile for embryonic enzymes. F3 RNA polymerase activity (2.3×10^6 cpm) was applied and eluted as described in Figure 1.

Dependence of the three enzyme activities on the concentrations of Mg, Mn, and $(NH_4)_2SO_4$ is shown in Figure 2.

The Enzymes from Embryos. When the extraction procedure used above for third instar larvae is carried out with 12–24-hr embryos and the enzymes are fractionated on DEAE-Sephadex, only two activity peaks result (Figure 3). The yield of activity for extractions performed on embryos is similar to that from larvae (Table I). The first peak (RNA polymerase Ia) is broad and is centered about 0.04 *M* $(NH_4)_2SO_4$. The second peak (RNA polymerase II) is sharp and elutes at 0.25 *M* $(NH_4)_2SO_4$. The Mg and $(NH_4)_2SO_4$ characteristics of enzymes Ia and II from embryos are shown in Figure 4.

If the embryonic F3 is incubated at 25° for 90 min, the RNA polymerase Ia peak is shifted to a higher DEAE elution point. No significant change in relative amounts of Ia and II activities is observed, nor is there any change in response to Mg, Mn, and $(NH_4)_2SO_4$. The shifted peak is still quite broad but is now centered about 0.08 *M* $(NH_4)_2SO_4$.

A tendency for enzyme Ia to elute at progressively higher ionic strengths was also consistently noted after increasing periods of storage of F3 frozen at −70°. After 3 months storage the mean of the elution profile was at 0.095 *M* $(NH_4)_2SO_4$.

The Enzymes from a Joint Extraction. The difference in elution patterns of the embryonic and larval RNA polymerases suggests that the enzymes are different. To rule out the possibility that these differences arose during the extraction, embryos and larvae were mixed (each representing about 50% of the total activity) and then extracted together. The resulting elution pattern (Figures 5 and 6) is the same as would be obtained by a superposition of patterns arising from embryonic and larval extracts. The agreement between calculated and eluted percent is very good (Table II)

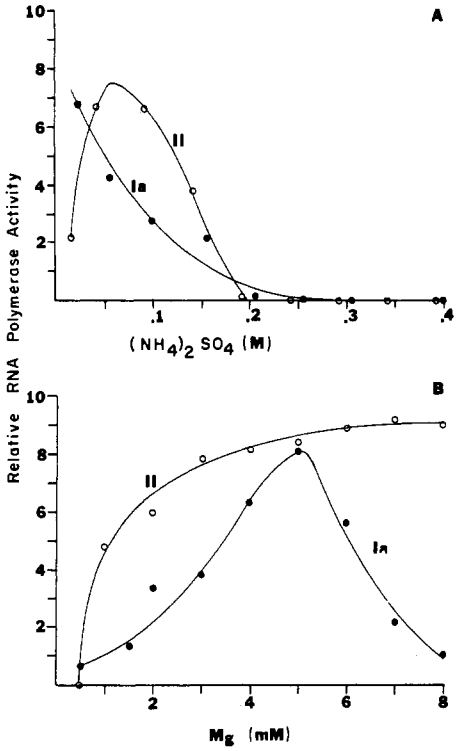

FIGURE 4: Characteristics of the embryonic enzymes. All assays were done under UTP limiting conditions, but the shapes of the curves remained unchanged under nonlimiting conditions. All data have been normalized and represent the average values obtained from two columns (each column assay done in triplicate). (A) $(NH_4)_2SO_4$ dependence with Mg = 1.5 m*M* and Mn = 2.0 m*M*; (B) Mg dependence with $(NH_4)_2SO_4$ = 0.05 *M* and Mn = 0.0 m*M*; RNA polymerase Ia (●); RNA polymerase II (○).

Table II: Enzyme Proportions in the Joint Extraction.[a]

Enzyme	Calcd %	Eluted %
Ia	15.7	14.7
Ia′	5.2	4.2
Ib	8.6	9.8
II	60.3	60.6

[a] Percentages represent either calculated percent of total F3 activity or actual percent of the total eluted activity. Elution peaks were assumed to be symmetrical distributions, and all fractions with 10% of the maximum peak activity were included in the peak.

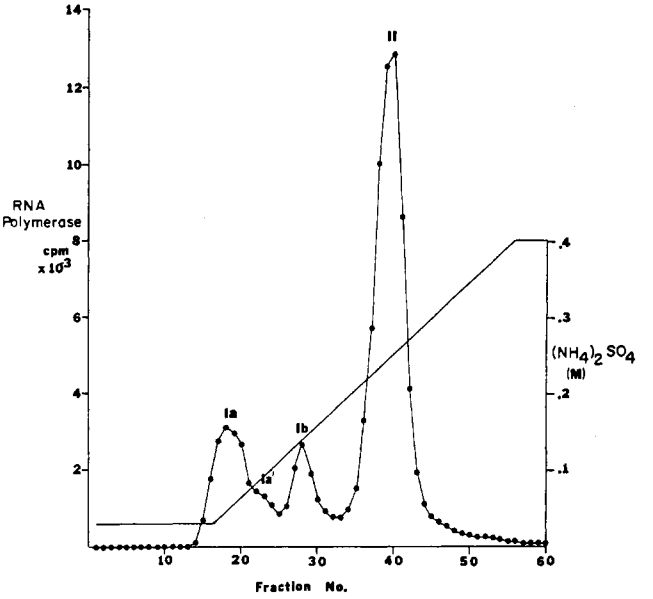

FIGURE 5: Elution profile for joint extraction enzymes. F3 RNA polymerase activity (1.4 × 10^6 cpm) was applied and eluted as described in Figure 1.

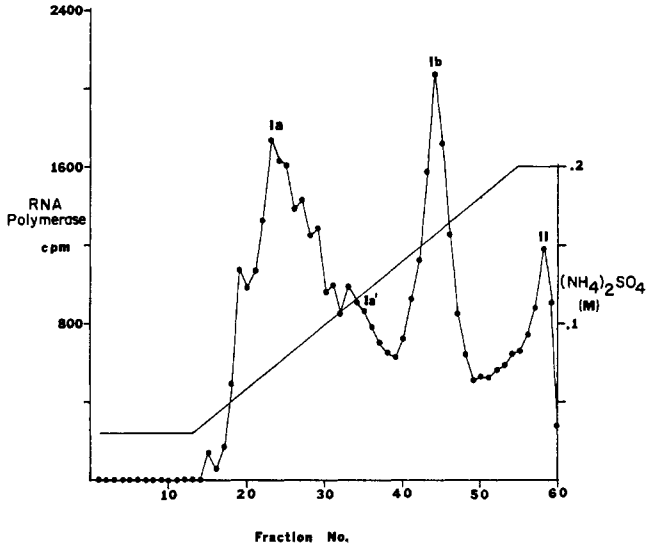

FIGURE 6: High resolution elution profile for joint extraction enzymes. The sample (1.3 × 10^6 cpm of F3 RNA polymerase activity) was applied and collected as described in Figure 1. The enzymes were eluted with a linear $(NH_4)_2SO_4$ gradient from 0.02 to 0.20 *M* $(NH_4)_2SO_4$ in buffer C. Aliquots of 50 μl were assayed under UTP limiting assay conditions.

indicating that all four species of enzyme (Ia, Ia′, Ib, and II) are inherently different.

Enzyme Sensitivities to Various Inhibitors. In order to learn more about the interrelationships among the various enzyme species the effect of various inhibitors on the enzyme activities was investigated. The data from such a study are shown in Table III. The characteristics of larval and embryonic enzymes II were indistinguishable as were those of embryonic enzyme Ia and larval enzyme Ia′. Enzymes Ia and Ia′ are 90% resistant to rifampicin, rifamycin SV, and cordycepin (3′-deoxyadenosine) and actually show enhanced activity in the presence of α-amanitin. Enzyme Ib is essentially completely inhibited by rifampicin (5 μg/ml) and rifamycin SV (11 μg/ml), but is not greatly affected by either α-amanitin or cordycepin. Enzyme II is approximately 80% resistant to rifampicin, rifamycin SV, and cordycepin (the only enzyme affected by this drug), but is greatly affected by α-amanitin. It is interesting to note that the presence of NP40 (see below) increases the resistance of enzymes Ib and II to rifamycin AF/013 and AF/05, while having very little influence on the effects of other inhibitors.

Some inhibitor experiments were done using embryonic F3 with and without α-amanitin to determine enzyme II and enzyme Ia drug sensitivities. Both enzymes in F3 showed enhanced resistance to all inhibitors (the maximum inhibition in both cases was by AF/013 and AF/05 which inhibited less than 30% of the activity), and the F3 was completely resistant to rifampicin and rifamycin SV. (Rifampicin and rifamycin SV inhibition of larval F3 was approximately equivalent to the Ib content of the F3.) As with the fractionated enzymes, ethanol controls were done in these inhibitor assays. In the presence of α-amanitin, there is a large enhancement of activity equivalent to a 50% stim-

Table III: The Effect of Inhibitors on the RNA Polymerases.[a]

Inhibitor		Ia (Ia') + NP40		Ib		Ib + NP40		II		II + NP40	
Type	Concn	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%
None		558	100.0	760	100.0	1598	100.0	1039	100.0	1713	100.0
Ethanol	1.1%	585	104.8	813	105.8	1641	102.7	1125	108.3	1792	104.6
Ethanol	2.2%	666	119.3	863	112.4	1546	97.9	1035	99.6	1860	108.6
Ethanol	4.4%	563	100.9	672	87.5	1344	84.1	807	77.7	1591	92.9
Rifampicin	5 μg/ml	478	85.7	90	11.7	66	4.1	886	85.3	1509	88.1
Rifampicin	10 μg/ml	483	86.5	84	11.0	62	3.9	846	81.4	1552	90.6
Rifampicin	20 μg/ml	492	88.2	68	8.8	43	2.7	803	77.3	1473	86.0
Rifamycin SV	11 μg/ml	523	89.4	83	10.2	77	4.7	880	78.2	1443	80.5
Rifamycin SV	22 μg/ml	586	88.0	75	8.7	45	2.9	849	82.0	1369	73.6
Rifamycin SV	44 μg/ml	471	83.7	67	9.9	28	2.1	767	95.0	1238	77.8
Rifamycin AF/013	11 μg/ml	381	65.1	7	0.8	843	51.4	72	6.4	1091	60.9
Rifamycin AF/013	22 μg/ml	305	45.8	7	0.8	552	35.3	30	2.9	809	43.5
Rifamycin AF/013	44 μg/ml	224	39.8	1	0.1	270	20.1	8	1.0	485	30.5
Rifamycin AF/05	11 μg/ml	417	71.3	39	4.8	771	47.0	61	5.4	1085	60.5
Rifamycin AF/05	22 μg/ml	355	53.3	16	1.9	536	34.3	65	6.3	459	24.7
Rifamycin AF/05	44 μg/ml	223	39.6	5	0.7	300	22.3	48	6.0	304	19.1
Rifamycin AF/ABDP	11 μg/ml	481	82.3	76	9.3	433	26.4	707	62.8	1376	76.8
Rifamycin AF/ABDP	22 μg/ml	458	68.7	78	9.0	338	21.6	615	59.4	1066	57.3
Rifamycin AF/ABDP	44 μg/ml	468	83.1	80	11.9	184	13.7	475	58.9	802	50.4
Cordycepin	11 μg/ml	523	93.7	655	86.2	1595	99.8	828	79.7	1559	91.0
Cordycepin	22 μg/ml	530	95.0	695	91.4	1553	97.2	830	79.9	1391	81.2
Cordycepin	44 μg/ml	508	91.0	738	97.1	1475	92.3	837	80.6	1252	73.1
α-Amanitin	0.25 μg/ml	610	109.3	735	96.7	1627	101.8	103	9.9	154	9.0
α-Amanitin	0.50 μg/ml	623	111.7	762	100.3	1569	98.2	69	6.6	134	7.8
α-Amanitin	1.00 μg/ml	641	114.8	766	100.8	1627	101.8	80	7.7	84	4.9

[a] Assays were done under UTP limiting conditions (UL assay). The rifamycin SV, rifamycin AF/013, rifamycin AF/05, and rifamycin AF/ABDP were added from ethanol stock solutions making the assay mix 1.1, 2.2, or 4.4% in ethanol. Percentages of control activities given for these inhibitors are calculated based on ethanol controls (see Materials and Methods). The rifampicin, cordycepin, and α-amanitin were added from water stock solutions. Percentages for these inhibitors are based on the non-ethanol control activity. The values given for Ib and II represent the average of four independent determinations, those for Ia and Ia' represent two determinations from each of Ia and Ia' (all assays were done in triplicate). NP40 was present as 0.2% (v/v) in the sample.

ulation of enzyme II activity. Enzyme Ia, although not stimulated by ethanol to any great extent, shows a remarkable resistance to it, keeping greater than 15% of its activity in the presence of 35% ethanol.

The Origin of RNA Polymerase Ib. By all criteria examined except for point of elution, embryonic enzyme Ia appears the same as larval enzyme Ia', and embryonic and larval enzymes II appear to be the same. The question then arises as to the origin of enzyme Ib which is found in larvae but not in embryos.

In an attempt to learn whether Ib was the result of proteolytic action on another RNA polymerase, extractions were carried out in the presence of $PhCH_2SO_2F$ (phenylmethanesulfonyl fluoride), a potent protease inhibitor. This resulted in a fivefold reduction in the amount of Ia', while the amounts of Ib and II remained unaltered.

The rifampicin sensitivity of Ib suggested bacterial or mitochondrial contamination, since prokaryotic and mitochondrial RNA polymerases (but not eukaryotic RNA polymerases) are sensitive to this drug. To investigate the possibility of bacterial contamination, larvae without microbial contaminants were obtained by growth under sterile conditions and an extraction was performed as usual. The resulting elution profile was indistinguishable from that obtained from larvae grown under nonsterile conditions, suggesting that the polymerase activities observed are not of microbial origin.

To check for the presence of mitochondrial RNA polymerases, which have molecular weights of about 50,000 (see Discussion), larval F3 was sedimented into a sucrose–glycerol gradient. Since eukaryotic RNA polymerases have molecular weights of approximately 500,000, any mitochondrial RNA polymerase contaminant should be readily resolvable. The RNA polymerase profiles for gradients run at two different $(NH_4)_2SO_4$ concentrations are shown in Figure 7. There is no evidence in either gradient of a slowly sedimenting peak. When applied to a DEAE-Sephadex column, the enzyme peak from the sucrose–glycerol gradient gives the normal elution profile (Ia', Ib, and II activities).

Enzyme Stability. The *Drosophila* RNA polymerases are highly unstable, especially after column chromatography. Fraction 3 may be stored 1–2 months at −70° without significant loss of activity, but storage in liquid nitrogen (−196°) leads to a 70% loss of activity in about 1 week. After column chromatography, the most successful short term storage conditions were found to be 50% glycerol (v/v) and greater than 0.5 mg/ml of protein at −20° (unfrozen). After 24 hr, enzyme II has 80% of its original activity, while enzymes Ia' and Ib both have 100%. By 48 hr, however, all three enzymes are down to 10% of their original activity. If the column fractions (at 50% glycerol and greater than 0.5 mg/ml of protein) are frozen in liquid nitrogen, 70% of the activity is lost within 30 hr, but remains constant at 30% survival for at least 6 days. The surviving fraction of the original enzyme activity is greater when bovine serum albumin is added as a carrier protein than when lysozyme is added.

The enzymes apparently interact with glass, since column chromatography in glass columns failed to show any significant eluting activity. In the plastic colums routinely used, 100% recovery could be expected. The enzymes also interact with dialysis tubing, since any attempt at dialysis (even for periods as short as 30 min) resulted in a loss of 80–90% of the activity.

In recent experiments, we have found that the addition of the nonionic detergent NP40 has a remarkable effect on the stability of the enzymes after column fractionation. The column enzymes with 50% glycerol, 1 mg/ml of bovine serum albumin, and 0.2% NP40 have been stored for over 1 month at −70° without significant loss of activity. The presence of NP40 also causes two interesting side effects: (1) RNA polymerase activity is enhanced, especially enzyme II where the activity is up to two times greater than in the absence of NP40; (2) enzyme resistance to the rifamycin derivatives AF/013 and AF/05 is increased when NP40 is present. The NP40 does not help enzyme survival during dialysis (there still is more than a 90% activity loss in 30 min), nor does it have any effect on RNA polymerase resistance to cordycepin, α-amanitin, rifampicin, rifamycin SV, or rifamycin AF/ABDP.

Discussion

The breadth and shape of the embryonic enzyme Ia peak suggest a high degree of heterogeneity in the enzyme. Since the Mg and $(NH_4)_2SO_4$ characteristics of the column fractions eluting at different points on the enzyme Ia peak are the same, however, it seems likely that we are dealing with the same enzyme species (as defined by $(NH_4)_2SO_4$ and Mg) having altered DEAE affinities. The distribution of enzyme Ia can be skewed toward higher DEAE binding affinities (by 25° incubation or storage at −70°) without any change in total enzyme Ia activity, implying that there is some conversion of the early eluting Ia to a later eluting Ia activity. None of the other enzymes observed (Ia′, Ib, II) showed any indication of heterogeneity nor any tendency toward changing elution properties.

RNA polymerase II from third instar larvae is indistinguishable from the embryonic RNA polymerase II. It has the same elution profile, the same $(NH_4)_2SO_4$, Mg, Mn, and inhibitor characteristics, is α-amanitin sensitive at low inhibitor concentrations, and coelutes with embryonic RNA polymerase II in the column from the joint extraction. One may conclude, therefore, that embryonic and larval RNA polymerases II are the same enzyme and this activity corresponds to the RNA polymerase II activities found in other eukaryotes.

The broad peak designated Ia in embryos must correspond to a group of closely related enzymes. They resemble each other as well as the Ia′ enzyme from larvae in having the same Mg, Mn, $(NH_4)_2SO_4$, and inhibitor characteristics. Enzymes Ia and Ia′ differ only in their elution profiles, the former eluting as a broad peak at 0.04 *M* $(NH_4)_2SO_4$ and the latter as a sharp peak at 0.095 *M* $(NH_4)_2SO_4$. Since both Ia and Ia′ are seen in the joint extraction, they must result from inherent differences in the enzymes and not differences arising as artifacts of extraction. Storage of enzyme Ia′ in larval F3 does not cause any change in its characteristics, while storage of enzyme Ia in embryonic F3 causes its elution profile to approach that of larval Ia′. It seems likely, therefore, that larval enzyme Ia′ corresponds to embryonic enzyme Ia that has been modified.

RNA polymerases have been extracted from other eukaryotes at different developmental stages, and although changes in relative enzyme proportions have been observed, the number of enzyme species remains the same (Roeder et al., 1970; Roeder and Rutter, 1970a). Roeder and Rutter (1970a) studied the changing amounts of the three sea urchin RNA polymerases during early embryonic development. The level of enzyme I per cell remained nearly constant, while the level of RNA polymerases II and III per cell decreased severalfold. The elution patterns for the enzymes from embryos changed with development only in the relative quantities of RNA polymerases I, II, and III; the points of elution for the three enzymes remained unchanged. It is interesting to note that sea urchin RNA polymerase I showed considerable heterogeneity at each of the four stages of development examined.

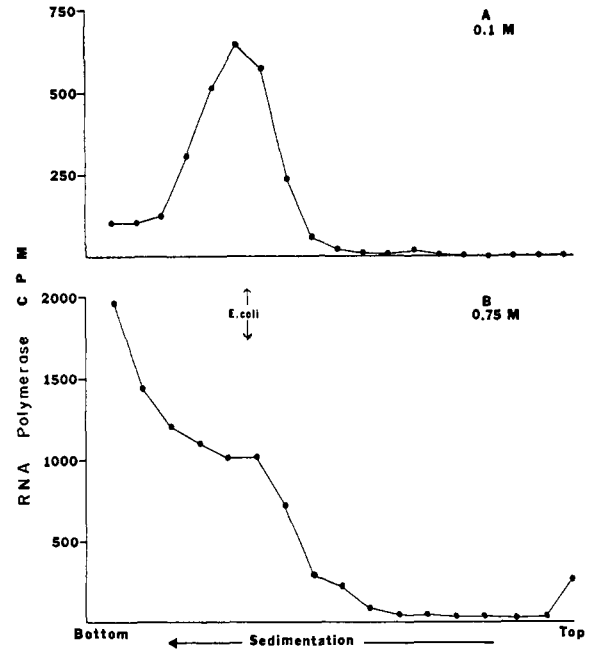

FIGURE 7: Sedimentation of larval F3 RNA polymerases; 0.3-ml samples of F3 (at the indicated salt concentration) were applied to 4.7-ml 0–15% sucrose–20% glycerol gradients as described in Materials and Methods. Centrifugation was for 13 hr at 40,000 rpm in a SW50L rotor at 2°. Fractions were collected and assayed for RNA polymerase activity by the UTP limiting assay. *E. coli* RNA polymerase was run in a parallel tube to serve as a marker. (A) F3 applied at 0.1 *M* ammonium sulfate; (B) F3 applied at 0.75 *M* ammonium sulfate.

In *Xenopus laevis* embryos, Roeder et al. (1970) found that the relative levels of enzymes I and II remained unchanged during development. Therefore, the appearance of enzyme Ib with development in *Drosophila* is unique. This fact, together with the rifampicin and rifamycin SV sensitivity of Ib, suggested that Ib might not be a true nuclear RNA polymerase but rather a bacterial or mitochondrial enzyme.

Since the sterile and nonsterile DEAE-Sephadex elution profiles are indistinguishable, bacterial contamination can be ruled out. The sedimentation profile of the larval F3 RNA polymerases in sucrose-glycerol gradients indicates a molecular weight on the order of 500,000 in both 0.1 and 0.75 *M* $(NH_4)_2SO_4$. Mitochondrial RNA polymerases have been extracted from several organisms (South and Mahler, 1968; Kuntzel and Schafer, 1971; Horgen and Griffin, 1971; Tsai et al., 1971; Scragg, 1971; Gadaleta et al., 1970; Reid and Parsons, 1971; Wu and Dawid, 1972) and, in general, their molecular weights have been found to be about 50,000 under the salt conditions used above. This argues against Ib being of mitochondrial origin.

Following the procedures used in nuclear RNA polymerase extractions for the extraction of mitochondrial RNA polymerase gives very poor yields since mitochondrial RNA polymerases are probably membrane bound (see Wu and Dawid, 1972). It seems unlikely that the extraction proce-

dure used in this study would solubilize much mitochondrial RNA polymerase activity. Therefore, the fact that there is almost as much Ib as II in the larval extractions argues against enzyme Ib being of mitochondrial origin. Furthermore, since the ratio of mitochondria to nuclei is higher in embryos than in adults (I. B. Dawid, personal communication), if there is a contaminating mitochondrial RNA polymerase activity one would expect it to appear in embryos only, or perhaps in both embryos and larvae; one would not expect mitochondrial RNA polymerase to appear in larval extractions and not in embryonic extractions. Overall, then, it seems quite improbable (though not impossible) that Ib is derived from mitochondria.

There have been three other reports on RNA polymerases from *Drosophila*. Natori et al. (1973) prepared RNA polymerase from *Drosophila* tissue culture cells and larval imaginal discs. They found only one RNA polymerase from each tissue and concluded that this was RNA polymerase II since it was sensitive to α-amantin. Their extraction procedure differed from the one presented here in several respects and these differences probably account for the absence of any enzyme I.[2] It seems unlikely that those tissues are missing an enzyme I, since all other eukaryotes examined have been shown to contain an RNA polymerase I, and *Drosophila* larvae (this paper) and embryos (Phillips and Forrest, 1973; this paper) contain an enzyme I.

While this work was in progress, Phillips and Forrest (1973) reported the extraction of RNA polymerases from 12- to 24-hr embryos of *Drosophila melanogaster*. They found two enzymes (I and II) that have the same Mg, Mn, and $(NH_4)_2SO_4$ characteristics as reported here for embryonic enzymes Ia and II. Their purification procedure makes use of DEAE-cellulose instead of DEAE-Sephadex as used in this investigation, which can cause some difference. For example, if our larval F3 is eluted from DEAE-cellulose instead of DEAE-Sephadex as is usually done, the enzymes Ia′ and Ib elute as a single peak—this single peak can then be resolved into Ia′ and Ib by passage through DEAE-Sephadex. Of particular interest is their use of phosphocellulose column chromatography in their purification procedure. Direct chromatography of the crude soluble enzymes (F2) on DEAE-cellulose results in two activity peaks as mentioned. Each of these separate peaks, when passed through phosphocellulose, yielded a single peak. If the chromatographic order was reversed, however, four peaks of activity resulted (i.e., F2 eluted as a single peak from phosphocellulose; this single peak, when applied to DEAE-cellulose, eluted as four peaks). They have designated these four peaks Ia, Ib, IIa, and IIb on the basis of the α-amanitin sensitivity of the last two peaks and their points of elution. No further characterization of these four species was carried out.

Meilhac et al. (1972) have thoroughly studied the effects of many inhibitors on calf thymus enzymes I and II. The AF/013, AF/05, and AF/ABDP have the same effect on calf thymus enzymes I and II as they do on *Drosophila* enzymes Ia (and Ia′) and II, respectively. The calf thymus enzymes were essentially unaffected by rifampicin concentrations as high as 400 μg/ml, while *Drosophila* enzymes Ia (and Ia′) and II were inhibited by about 20% at 20 μg/ml, and *Drosophila* enzyme Ib was more than 95% inhibited by 5 μg/ml. Adman et al. (1972) found that rifamycin SV had no effect on any yeast RNA polymerases (Ia, Ib, II, and III) at concentrations of 100 μg/ml; Strain et al. (1971) found that a concentration of rifamycin SV of 14 μg/ml had no affect on Maize RNA polymerase II, but inhibited RNA polymerase I by 14%.

Three unique features have emerged from this study of the RNA polymerases of *Drosophila melanogaster:* (1) an enzyme species (embryonic enzyme Ia) has been shown to change its DEAE binding affinity during development (to larval RNA polymerase Ia′) implying a biological modification; (2) a new enzyme species (Ib) is seen to emerge during development; and (3) this new enzyme, Ib, is sensitive to rifampicin and rifamycin SV, is not bacterial, and probably not mitochondrial.

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[2] In a recent report (Adoutte et al., 1974), *Drosophila* RNA polymerases were extracted by two methods. One method yields a single RNA polymerase activity (sensitive to α-amanitin), while the other method yields two activities. It is difficult to evaluate these experiments, however, since no activity is detectable until after DEAE column chromatography, and some RNA polymerase activity may have been lost prior to this step.

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Conformers, Dimers, and Anticodon Complexes of $tRNA^{Glu_2}$ (*Escherichia coli*)†

J. Eisinger* and N. Gross‡

ABSTRACT: The existence of two conformers of $tRNA^{Glu_2}$ (*Escherichia coli*) is demonstrated. The two conformers, n and d, are prepared by incubation at moderate temperatures in Mg^{2+} containing and Mg^{2+} free buffer, respectively, the d conformer having a vanishing amino acid acceptance and an electrophoretic mobility 10% larger than the n conformer. The structure and accessibility of the anticodon loop is shown to be the same for both conformers by gel electrophoresis studies of the anticodon–anticodon complex formed with $tRNA^{Phe}$. The $tRNA^{Glu_2}$–$tRNA^{Phe}$ complex has an equilibrium constant of about 10^7 M^{-1} at 0° and an enthalpy of binding of −20 kcal M^{-1} and these parameters are the same for $tRNA^{Phe}$ (*E. coli*) and $tRNA^{Phe}$ (yeast). Both the n → d and the d → n conversion rates have activation energies of the order of 60 kcal M^{-1}. The d conformer is shown to form a dimer in the presence of Mg^{2+} ions, the association and dissociation rates having activation energies of 14 and 43 kcal M^{-1}, respectively. The d-$tRNA^{Glu_2}$ dimer can form a tetrameric complex with two $tRNA^{Phe}$ molecules. These results are discussed in relation to the 3-Å $tRNA^{Phe}$ (yeast) structure and it is suggested that the two conformers of $tRNA^{Glu_2}$ differ primarily in their tertiary interactions.

Although X-ray diffraction studies of crystalline tRNA are now becoming available (Robertus et al., 1974; Suddath et al., 1974; Kim et al., 1974a), it is clear that an understanding of the interactions and conformations of tRNA during protein synthesis requires information about the *solution* structure of these molecules. Recently published evidence (Prinz et al., 1974) shows that organic additives used in growing tRNA crystals cause structural changes in tRNA and has served to emphasize the continued need for the development of solution structure probes. Structural studies of tRNA in solution have previously made use of comparisons of stable tRNA conformers (Lindahl et al., 1966, 1967; Gartland and Sueoka, 1966; Ishida and Sueoka, 1968; Muench, 1969; Adams et al., 1967; Webb and Fresco, 1973) and investigations of tRNA dimers (Loehr and Keller, 1968; Yang et al., 1972) and complexes between tRNA with complementary anticodons (Eisinger, 1971; Eisinger and Gross, 1974; Grosjean et al., 1973). The present paper applies these techniques to $tRNA^{Glu_2}$ (*Escherichia coli*). Ionic strength dependent conformational transitions between "native" (i.e., capable of aminoacylation) and "denatured" states have been observed for unfractionated tRNA as well as for purified species (Adams et al., 1967; Webb and Fresco, 1973). While stable conformers may exist for most tRNA species, the conformer transition kinetics permit the study of only a few of these, the best known one to date being $tRNA^{Leu_3}$ (yeast) (Kowalski et al., 1971).

The present study constitutes an attempt to gain insight into the solution structure of $tRNA^{Glu_2}$ (*E. coli*) whose stable conformers were discovered by D. M. Crothers and M. Bina-Stein (private communication). The present paper reports results of experiments designed to determine (1) the kinetics of forming the two conformers, (2) the kinetics of dimerization of one of these conformers, (3) the thermodynamic properties of the complex formation between $tRNA^{Glu_2}$ and $tRNA^{Phe}$, two tRNAs whose anticodons have base complementarity, and (4) the character of the tertiary structures of the two $tRNA^{Glu_2}$ conformers and of the dimer.

Materials and Methods

tRNA. $tRNA^{Glu_2}$ (*E. coli*) (Boehringer Mannheim Corporation) was dissolved in sterile distilled water to a desired concentration. For the native conformer (n-tRNA) the tRNA was dialyzed in the cold against sterile 0.1 *M* cacodylate buffer (pH 7.0) and 1 m*M* $MgCl_2$ (n buffer) for 3 hr, and then in fresh buffer overnight. Just before use, the tRNA was heated at 42° for 5 min to ensure conversion of any denatured tRNA to the native conformer.

To produce the denatured species (d-tRNA), the tRNA was dialyzed in the cold against sterile 0.1 *M* cacodylate buffer (pH 7.0) and 0.01 *M* EDTA for 3 hr, and then overnight in 0.1 *M* cacodylate buffer (pH 7.0) and 1 m*M* EDTA (d buffer). Alternatively, the same d conformer can

† From Bell Laboratories, Murray Hill, New Jersey 07974. *Received February 18, 1975.*

‡ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut.