Adrenocorticotropic Hormone Regulation of Adrenal RNA Polymerases. Stimulation of Nuclear RNA Polymerase III†

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ABSTRACT: In vivo administration of adrenocorticotropic hormone (ACTH) to guinea pigs results in a twofold increase in the synthesis of pre-4S and 5S RNA by adrenal nuclei in vitro. Hormone treatment also results in an increase in the activity of a pool of soluble RNA polymerase III in adrenal nuclei, as well as an increase in the amount of RNA polymerases II and III that can be extracted from adrenal nuclei. The pool of extractable RNA polymerase I is unchanged. A pool of RNA polymerase III that is found in adrenal cytosol is not affected by hormone treatment. DEAE-Sephadex A-25 chromatography of both nuclear and cytoplasmic adrenal RNA polymerases shows that ACTH treatment does not change the elution profiles of RNA polymerases II and III; however, RNA polymerase I from ACTH nuclei appears as two peaks rather than a single peak. Adrenal nuclear RNA polymerase III elutes as a single peak at 0.20 M $(NH_4)_2SO_4$ (peak IIIa); adrenal cytosol RNA polymerase III elutes as a single peak at 0.36 M $(NH_4)_2SO_4$ (peak IIIb). Since in vivo ACTH administration increases only the nuclear RNA polymerase III activity, these results suggest that the two forms of RNA polymerase III are regulated independently and that RNA polymerase IIIa is active in transcribing 4S and 5S RNA genes.

Hormonal and growth stimuli change the rates of synthesis and accumulation of the major classes of cellular RNA (Johnson et al., 1974; Roeder, 1976). When resting fibroblasts are stimulated to grow by addition of serum, increases in nuclear RNA polymerases I (rRNA) and III (pre-4S and 5S RNA) activities occur (Mauck and Green, 1973, 1974); in addition, rRNA and tRNA degradation is decreased (Abelson et al., 1974). Nuclear RNA polymerase II (hnRNA) activity and mRNA stability are unchanged and increased cellular mRNA pools are thought to arise from increased availability of transcribed mRNAs (Abelson et al., 1974; Johnson et al., 1974; Rudland, 1974), although increased transcription of specific mRNAs occurs in response to steroid hormones in appropriate target cells (Harris et al., 1975). Stimulations of nuclear RNA polymerase I, and in some cases nuclear RNA polymerase II, activities are observed in response to a large number of hormonal and growth stimuli (see, for example, Hamilton et al., 1968; Glasser et al., 1972; Pogo, 1972; Mauck and Green, 1973; Jaehning et al., 1975; Fuhrman and Gill, 1975). The response of RNA polymerase III to these stimuli is less well characterized. Estrogen administration to ovariectomized rats results in an increased rate of labeling of uterine 4S and 5S RNA in vivo (Luck and Hamilton, 1975) and an increase in uterine nuclear RNA polymerase III activity assayed in vitro (Webster and Hamilton, 1976). Partial hepatectomy and phytohemagglutinin stimulation of lymphocytes results in increased quantities of RNA polymerase III extractable from target tissue (Jaehning et al., 1975; Yu, 1975).

We have previously shown that in vivo adrenocorticotropic hormone (ACTH[1]) treatment results in a twofold increase in adrenal nuclear RNA polymerase I activity assayed in vitro; this increase in activity occurs without change in the number of RNA polymerase I enzymes engaged in transcription and can be accounted for by an increase in the average rate of RNA chain elongation (Fuhrman and Gill, 1975). Endogenous adrenal nuclear RNA polymerase II activity is increased 50% by ACTH treatment, though activity on added calf thymus DNA is not altered (Fuhrman and Gill, 1974). A twofold increase in adrenal nuclear RNA polymerase III activity assessed by differential α-amanitin sensitivity also occurs (Fuhrman and Gill, 1975).

The experiments described in the present report were undertaken to explore ACTH regulation of RNA polymerase III activity and to compare hormonal effects on endogenous nuclear RNA polymerase activities with enzyme levels assessed by addition of exogenous template to nuclei and by extraction and chromatographic separation of the three forms of RNA polymerase.

Methods

Treatment of Animals and Preparation of Cell Fractions. The methods used in the treatment of guinea pigs and the isolation of adrenal nuclei have been reported (Fuhrman and Gill, 1974, 1975). Purified nuclei were resuspended in TGMED (0.05 M Tris, pH 7.9, at 25 °C, 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol). Postnuclear supernatants in buffer A (0.32 M sucrose, 10 mM Tris, pH 7.4, 5 mM $MgCl_2$, 5 mM dithiothreitol) were centrifuged at 10 000 rpm for 20 min to remove mitochondria; the postmitochondrial supernatant was used directly, or subjected to centrifugation

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[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid; ATP, CTP, GTP, UTP, adenosine, cytidine, guanosine, and uridine triphosphates; DEAE, diethylaminoethyl.

at 50 000 rpm for 90 min to yield microsome and cytosol fractions. Microsomes and mitochondria were resuspended in buffer A; all fractions were stored at −70 °C if not used immediately. Since maximal increases in nuclear RNA polymerase activities occur 14 h following initiation of ACTH treatment, this time point has been used in the present studies. Control and ACTH fractions refer to those isolated from untreated animals and from animals treated for 14 h with ACTH.

RNA Polymerase Assay. RNA polymerase reactions contained 50 mM Tris, pH 7.9, 1.23 mM $MnCl_2$, 1.54 mM $MgCl_2$, 4.6 mM NaF, 2 mM 2-mercaptoethanol, 7.7% glycerol, 0.03 mM EDTA, 1.54 mM dithiothreitol, 50 mM $(NH_4)_2SO_4$, 0.93 mM ATP, and the two other unlabeled nucleoside triphosphates at 0.47 mM. The concentration and specific activity of the radiolabeled nucleoside triphosphate are stated in the text. α-Amanitin was added to a final concentration of either 0.77 μg/ml to inhibit RNA polymerase II or 230 μg/ml to inhibit RNA polymerases II and III. RNA polymerase I is completely resistant to α-amanitin (Schwartz et al., 1974). Enzyme and template sources are indicated in the text. When poly(d(A-T)) template was added to reactions containing nuclei as a source of RNA polymerase enzyme, transcription of endogenous template was suppressed either by adding actinomycin D (30 μg/ml) or by omitting CTP and GTP from the reaction mixture. Though identical results were obtained with both methods, the latter was used in most experiments. Incorporation rates were linear with the enzyme concentrations used. All reactions were initiated by addition of enzyme and were incubated at 30 °C for the times indicated in the text. Unless otherwise stated, reactions were terminated by spotting 50 μl of a 65-μl reaction onto DE81 filter paper disks; the disks were processed as described by Blatti et al. (1970) and counted in 1.5 ml of toluene–Liquifluor (3.8 l.:165 ml).

Extraction and Urea–Polyacrylamide Gel Analysis of RNA Synthesized in Vitro. RNA polymerase reactions (0.325 ml) containing nuclei, 0.77 μg/ml of α-amanitin, and 40 μCi of [^{3}H]UTP (13–17 Ci/mmol) or 40 μCi of [^{3}H]GTP (12 Ci/mmol) were incubated at 30 °C for various times. Reactions were terminated by the addition of 25 μl of 1% sodium dodecyl sulfate and cooling. Carrier yeast tRNA, 2 OD_{260} units, 500–1000 cpm of marker *Dictyostelium discoidum* 4S and 5S [^{32}P]RNA (kindly provided by Dr. Richard Firtel), and 15 μl of a solution of pancreatic DNase (Worthington: 2.5 mg/ml in 10% glycerol, 50 mM Tris, pH 7.4, 0.5 mM $CaCl_2$) were added; this mixture was incubated at 30 °C for 1.5 min. RNA was then isolated as described previously (Fuhrman and Gill, 1975), except that the proteinase K step was omitted. After ethanol precipitation, the RNA was dissolved in 1 M LiCl and reprecipitated with the addition of 3 volumes of 95% ethanol. The DNase treatment was necessary for quantitative recovery of the RNA and the second ethanol precipitation from 1 M LiCl was necessary to adequately desalt for subsequent gel analysis of the RNA. The dried RNA pellet was dissolved in 100 μl of 7 M urea, 10% sucrose, 0.01% bromophenol blue, 0.01% phenol red, and subjected to urea–polyacrylamide gel electrophoresis (Heyden et al., 1972). Gels (9 × 0.6 cm) were composed of 10% (w/v) acrylamide, 0.067% bisacrylamide, 0.05% TEMED, 0.75% ammonium persulfate, 7 M urea, 1 mM EDTA, 0.04 M sodium acetate, pH 5.0; the running buffer was 7 M urea, 1 mM EDTA, 0.04 M sodium acetate, pH 5.0. Large ribosomal RNA precursors are excluded from these 10% gels, facilitating analysis of the pre-4S and 5S RNA synthesized by adrenal nuclei. Samples were electrophoresed at 4 mA/gel until the phenol red marker was 1–2 cm from the end of the gel. Gels were cut into 1.1-mm slices with an egg-slicer apparatus; slices were incubated overnight at 50 °C in 5 ml of toluene–Liquifluor–NCS (3.8 l.:165 ml:145 ml) and counted at a ^{3}H counting efficiency of 30%. A gel containing only the ^{32}P marker was included for calculation of recovery of [^{3}H]RNA in the gels containing both [^{3}H]RNA synthesized in vitro and *D. discoidum* [^{32}P]RNA markers. The mobility (relative to phenol red) of *D. discoidum* 4S RNA was slightly less than that of both *Escherichia coli* and Kreb's ascites 4S RNA (R_f = 0.84 for *D. discoidum* 4S RNA compared to R_f = 0.89 for *E. coli* and ascites 4S RNA). The reason for this difference is unclear; however, after conversion, guinea pig adrenal nuclear pre-4S RNA has the same mobility as ascites 4S RNA.

In Vitro Conversion of Pre-4S to 4S RNA. [^{3}H]RNA synthesized in vitro in a 20-min reaction was prepared as described above and dissolved in H_2O. A cytoplasmic extract containing converting enzyme was prepared from mouse liver as described by Mowshowitz (1970) (1 mouse liver to 12 ml of hypotonic buffer (10 mM Tris, pH 7.4, 1.5 mM $MgCl_2$, 10 mM NaCl, 0.25 mg/ml of dithiothreitol)). The incubation mixture for conversion contained: 250 μl of [^{3}H]RNA, 0.5 ml of 0.5 M Tris, pH 9.0, 0.1 ml of 2.5 mg/ml of dithiothreitol, 0.2 ml of 4 M ammonium acetate, 1 ml of cytoplasmic extract, and 1 ml of hypotonic buffer. Mock incubations contained 2 ml of hypotonic buffer. Reactions were incubated for 60 min at 37 °C; the RNA was then reisolated and subjected to urea–polyacrylamide gel electrophoresis. Recovery of marker [^{32}P]RNA carried through the entire procedure ranged from 40–60%, and the electrophoretic profile of the 4S and 5S [^{32}P]RNA was not altered.

Solubilization and DEAE-Sephadex Chromatography of Adrenal RNA Polymerases. Nuclear extracts in TGMED + 0.05 M $(NH_4)_2SO_4$ were prepared as described by Schwartz et al. (1974). Postmitochondrial extracts were prepared similarly. Soluble cytoplasm in buffer A was diluted 1:1 with TGMED + 0.1 M $(NH_4)_2SO_4$. All were subjected to chromatography on DEAE-Sephadex A-25 columns (bed volume = 8 ml, 1 cm × 10 cm) equilibrated with TGMED + 0.05 M $(NH_4)_2SO_4$ and eluted with a 38-ml linear gradient of 0.05–0.4 M $(NH_4)_2SO_4$ in TGMED; fractions of 1 ml were collected and 20-μl aliquots were assayed for RNA polymerase activity. No additional $(NH_4)_2SO_4$ was added to these assays.

DNA and Protein Determinations. DNA concentrations were determined by the ethidium bromide method of LePecq and Paoletti (1966) using calf thymus DNA as a standard. Protein was removed from samples by adding NaCl to a concentration of 1 M, followed by extraction with chloroform–isoamyl alcohol (24:1, v/v), prior to DNA measurement. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results

Synthesis of Pre-4S and 5S RNA by Adrenal Nuclei in Vitro. RNA polymerase III, the enzyme catalyzing pre-4S and 5S RNA synthesis, is resistant to low but sensitive to high concentrations of the mushroom toxin, α-amanitin (Weinmann and Roeder, 1974). Approximately 10% of the RNA synthesizing activity observed in adrenal nuclei assayed at low concentrations of α-amanitin (0.77 μg/ml) is inhibited by high concentrations (230 μg/ml) of the toxin (Fuhrman and Gill, 1975). In order to characterize this adrenal nuclear activity and to analyze the effects of in vivo ACTH administration on this activity, the RNA synthesized in vitro by adrenal nuclei

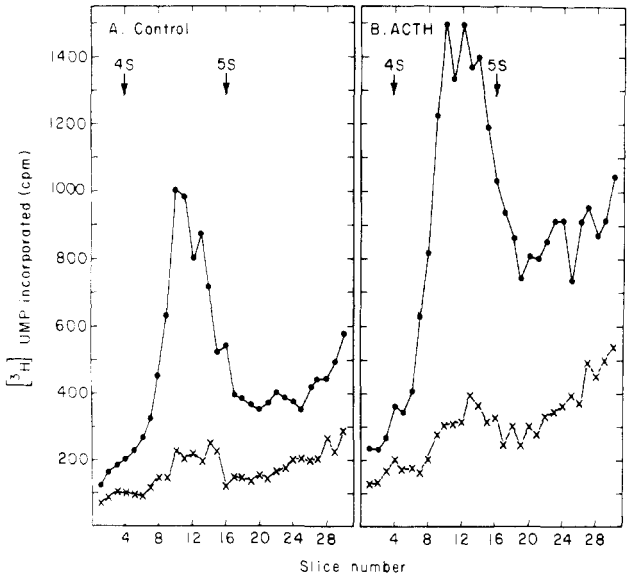

FIGURE 1: Urea-polyacrylamide gel analysis of low-molecular-weight RNA synthesized in vitro by adrenal nuclei. RNA synthesized in vitro by adrenal nuclei from control and ACTH-treated guinea pigs was isolated and analyzed by urea-polyacrylamide gel electrophoresis as described under Methods. Reactions, which contained 8.3 μg of nuclear DNA from control adrenals and 8.6 μg of nuclear DNA from ACTH adrenals, were incubated for 20 min at 30 °C. Data shown have been corrected for recovery of ^{32}P markers included in the extraction procedure; recoveries ranged from 65–75%. The position noted for the 4S marker is that for ascites 4S RNA; the 5S RNA position is that of *D. discoidum* 5S [^{32}P] RNA. The phenol red dye marker is in slice number 2. (●), From incubations containing 0.77 μg/ml of α-amanitin; (X), from incubations containing 230 μg/ml of α-amanitin.

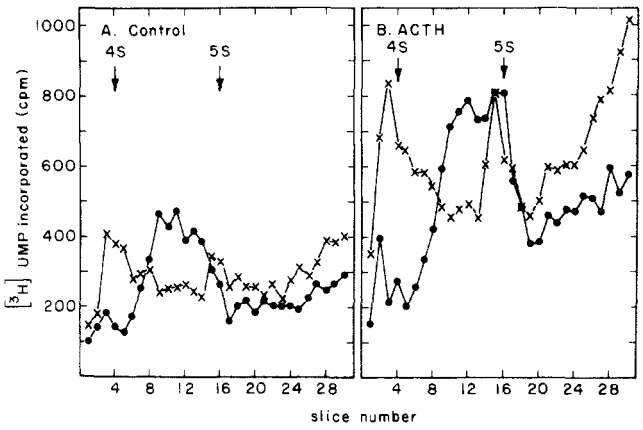

FIGURE 2: In vitro conversion of pre-4S RNA to 4S RNA. [^{3}H]UMP-labeled RNA, synthesized by adrenal nuclei, was isolated, incubated with mouse liver cytosol converting enzyme, reisolated, and analyzed on urea-polyacrylamide gels as described under Methods. RNA polymerase reactions, which contained 2.9 μg of nuclear DNA from control adrenals, 3.6 μg of nuclear DNA from ACTH-treated adrenals, and 0.77 μg/ml of α-amanitin, were incubated for 20 min at 30 °C. Data shown has been corrected for recovery of ^{32}P markers included in the extraction and incubation procedures. (●), Control; (X), incubated with mouse liver cytosol converting enzyme.

from control and ACTH-treated guinea pigs was analyzed and quantitated by urea-polyacrylamide gel electrophoresis. As shown in Figure 1, both control and ACTH adrenal nuclei actively synthesize RNA which migrates as a broad peak between 4S and 5S marker RNAs. The synthesis of this RNA is inhibited more than 90% by the inclusion of 230 μg/ml of α-amanitin in the RNA polymerase reaction. Several RNA species that migrate more slowly than 5S RNA are synthesized under these same conditions.

Transfer RNA is synthesized in vivo as a larger precursor RNA (pre-4S RNA), which is processed to 4S RNA in the cytoplasm (Chipchase and Birnstiel, 1963; Mowshowitz, 1970; McReynolds and Penman, 1974). Pre-4S RNA synthesized by adrenal nuclei in vitro can be converted to 4S RNA by incubation with a cytoplasmic converting enzyme from mouse liver (Figure 2). Thus, adrenal nuclei are active in the synthesis of pre-4S and 5S RNA; the synthesis of these RNAs is sensitive to high concentrations of α-amanitin, suggesting that RNA polymerase III is involved in their synthesis.

Quantitation of pre-4S and 5S RNA synthesized by both control and ACTH adrenal nuclei shows that the synthesis of these RNAs is increased twofold by ACTH treatment. Data derived from seven experiments using three different nuclear preparations were compiled; the activity in control nuclei was 33.3 ± 9.8 fmol of UMP incorporated per μg of nuclear RNA and the activity in ACTH nuclei was 72.1 ± 9.0 fmol of UMP incorporated per μg of nuclear RNA ($p < 0.01$). Urea polyacrylamide gel analysis of nonconverted RNA does not resolve pre-4S and 5S RNA, and the total synthesis of these RNA species has been summed in collecting the data given above. However, after treatment with converting enzyme, the 4S RNA is well resolved from 5S RNA and it is clear that the synthesis of 5S RNA, as well as pre-4S RNA, is increased by ACTH (Figure 2).

The identity of the RNA which migrates more slowly than the 5S RNA marker is not clear. The synthesis of this RNA is resistant to low but sensitive to high concentrations of α-amanitin, similar to the synthesis of pre-4S and 5S RNA. Aggregation appears unlikely in 7 M urea, since the marker [^{32}P]RNAs which are coelectrophoresed with in vitro [^{3}H] RNA show no evidence of aggregation. This RNA resembles products of nuclear RNA polymerase III, which others have reported as larger than 5S (Marzluff et al., 1975; Udvardy and Seifart, 1976). The synthesis of these RNA products of adrenal nuclear RNA polymerase III are also increased by in vivo ACTH administration (Figure 1).

Measurement of Soluble Nuclear RNA Polymerase Activity Using Poly(d(A-T)) Template. Several reports have noted the existence of a soluble pool of RNA polymerase associated with highly purified nuclei (Yu and Feigelson, 1971; Lampert and Feigelson, 1974; Yu, 1974, 1975). This pool of RNA polymerase can be assayed using an exogenously added template, such as poly(d(A-T)) or poly(dC) and actinomycin D to suppress endogenous RNA polymerase activity. Alternatively, this activity can be assayed using poly(d(A-T)) template and UTP and ATP as sole nucleoside triphosphates. Equivalent results are obtained with both methods.

The time course of [^{3}H]UTP incorporation by adrenal nuclei in the presence of 0.77 μg/ml of α-amanitin is shown in Figure 3A. In nuclei (broken lines), the accumulation of [^{3}H]UMP in RNA is linear for only 6–8 min. This endogenous RNA polymerase activity is completely inhibited by actinomycin D. When nuclei are incubated with actinomycin D and poly(d(A-T)) (solid lines), accumulation of [^{3}H]UMP in RNA is linear for at least 30 min. In both cases, ACTH nuclei are more active in the synthesis of RNA (Figure 3B); however, the endogenous activity, which represents approximately 90% RNA polymerase I and 10% RNA polymerase III (Fuhrman and Gill, 1975), is increased more than the soluble activity.

Transcription of the poly(d(A-T)) template in adrenal nuclei is due primarily to RNA polymerase III. As shown in Figure 4, the major RNA polymerase activity transcribing

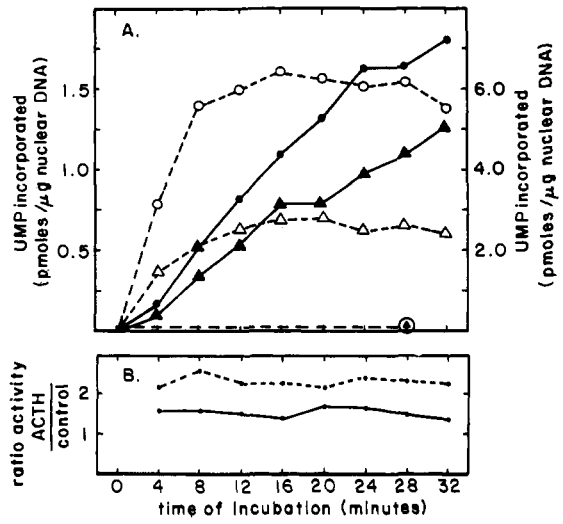

FIGURE 3: Effect of in vivo ACTH administration on in vitro adrenal nuclear RNA polymerase activity. (A) Control and ACTH adrenal nuclei were incubated at 30 °C in 250-μl reactions containing 0.77 μg/ml of α-amanitin, 20.3 μM [^{3}H]UTP (0.76 Ci/mmol), 0.47 mM CTP and GTP, 0.93 mM ATP, 23.2 μg of DNA from control nuclei, and 21 μg of DNA from ACTH nuclei, as described under Methods. Where indicated, reactions also contained 38.5 μg/ml of poly(d(A-T)) and 30 μg/ml of actinomycin D. Portions (30 μl) were removed at the times indicated, spotted onto DE81 filter paper disks, and processed as described under Methods. (Δ- - -Δ), Control nuclei, endogenous activity; (O- - -O), ACTH nuclei, endogenous activity; (- - -▲), control and ACTH nuclei, plus actinomycin D; (▲—▲), control nuclei, plus actinomycin D and poly(d(A-T)); (●—●), ACTH nuclei, plus actinomycin D and poly(d(A-T)). The scale for endogenous activity is on the left side of the graph; for poly(d(A-T)) dependent activity, on the right side. (B) The ratio of RNA polymerase activity in ACTH nuclei compared to activity in control nuclei is shown. (•- - -•), endogenous activity; (•—•), plus actinomycin D and poly(d(A-T)).

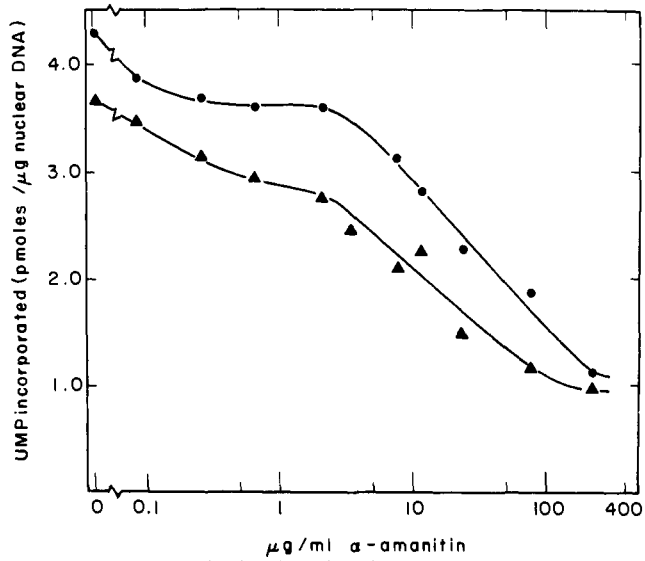

FIGURE 4: α-Amanitin titration of nuclear RNA polymerase activity transcribing poly(d(A-T)) template. Control and ACTH nuclei were incubated at 30 °C for 20 min in 65-μl reaction mixtures containing 7.7 μg/ml of poly(d(A-T)), 8.9 μM [^{3}H]UTP (1.71 Ci/mmol), 0.93 mM ATP, and the concentrations of α-amanitin indicated. Reactions contained 0.77 μg of DNA from control nuclei and 1.06 μg of DNA from ACTH nuclei. Each point shown is the average of duplicate assays. Values for the three RNA polymerases (pmol of UMP/μg of nuclear DNA) were calculated from differential α-amanitin sensitivity: control I, 0.99; control II, 0.85; control III, 1.80; ACTH I, 1.13; ACTH II, 0.70; ACTH III, 2.50. (▲), control; (●), ACTH.

poly(d(A-T)) is resistant to low but sensitive to high concentrations of α-amanitin. The three RNA polymerases are also distinguishable in nuclei by their response to divalent cations (Figure 5). All three nuclear soluble RNA polymerases prefer Mn^{2+} to Mg^{2+}. RNA polymerase III transcription of poly(d(A-T)) template demonstrates a sharp Mn^{2+} optima at approximately 1 mM with severe inhibition at 6 mM. Similar optima are observed for solubilized enzymes separated by DEAE-Sephadex chromatography (data not shown). Schwartz et al. (1974) have reported that solubilized RNA polymerase III preferentially transcribes a poly(d(A-T)) template; this is also true for adrenal RNA polymerase III (see Table II below). Although the amount of RNA polymerase III activity may be amplified by the use of the poly(d(A-T)) template, it is clear from Figures 4 and 5 that the activities of RNA polymerases I and II are unchanged by ACTH treatment, while the activity of RNA polymerase III measured with the poly(d(A-T)) template is significantly increased (Table I).

Class III RNA polymerase has been observed in cytosol as well as in nuclear fractions from mouse plasmocytoma and rat liver cells (Seifart et al., 1972; Schwartz et al., 1974; Seifart and Benecke, 1975). Class III RNA polymerase is present also in adrenal cytosol (Table I). Total RNA polymerase III activity in cytosol is approximately the same as that present in nuclei. Little activity is present in the microsomal fraction. ACTH treatment significantly increases the total pool of soluble nuclear RNA polymerase III without altering the total cytosol-associated RNA polymerase III activity.

Solubilization and Chromatographic Separation of Adrenal RNA Polymerases. In order to analyze total adrenal

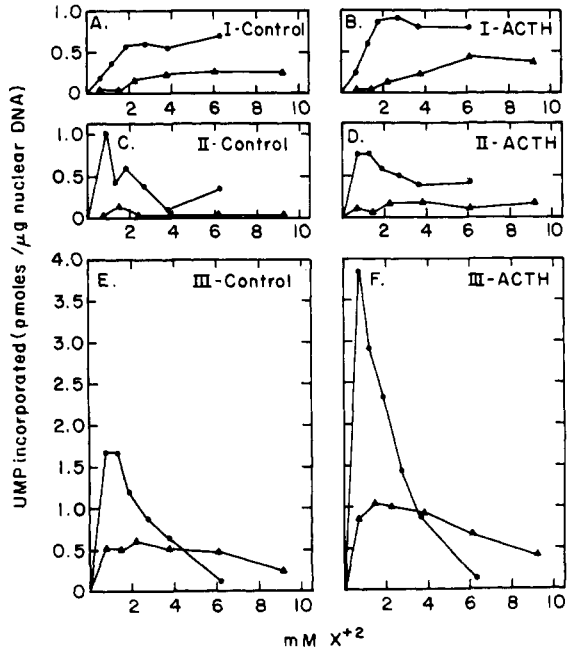

FIGURE 5: The response of adrenal nuclear RNA polymerase activities to varying concentrations of Mn^{2+} and Mg^{2+}. Nuclei in TGMED were centrifuged and resuspended in the same buffer without $MgCl_2$. Reactions (65 μl) containing 7.7 μg/ml of poly(d(A-T)), 8.5 μM [^{3}H]UTP (1.81 Ci/mmol), 0.93 mM ATP, 1.2 μg of DNA from control nuclei, 1.1 μg of DNA from ACTH nuclei, and the indicated concentrations of $MgCl_2$ and $MnCl_2$ were incubated for 20 min at 30 °C. The activity of the three RNA polymerases was determined as described under Methods. (●), With $MnCl_2$; (▲), with $MgCl_2$.

nuclear RNA polymerase activities, the enzymes were extracted from adrenal nuclei and assayed using both calf thymus DNA and poly(d(A-T)) templates. While loss of RNA polymerase activity during preparation of the extracts cannot be excluded, the results obtained are consistent with other data

TABLE I: Nuclear Soluble and Cytoplasmic RNA Polymerase III.[a]

	Control	ACTH	
(A) UMP incorporated (pmol/μg of nuclear DNA)			
Nuclei	2.19 ± 0.05	3.79 ± 0.06	$p < 0.01$
(B) Total UMP incorporated (pmol/10 glands)			
Nuclei	1567 ± 27	2340 ± 76	$p < 0.05$
Cytosol	2159 ± 449	2274 ± 265	$p > 0.70$
Microsomes	329	389	

[a] RNA polymerase reactions (65 μl), containing 7.7 μg/ml of poly(d(A-T)), 0.93 mM ATP, and 16 μM [^{3}H]UTP (0.94 Ci/mmol), were incubated for 20 min at 30 °C. α-Amanitin (0.77 μg/ml or 230 μg/ml) was added to the assays and RNA polymerase III activity was computed as the difference in activity at the two concentrations of α-amanitin. Incorporation of [^{3}H]UTP in the absence of poly(d(A-T)) was negligible except in the microsomal fraction, where incorporation in the absence of poly(d(A-T)) was one-third that in the presence of poly(d(A-T)). In all cases, the non-poly(d(A-T))-dependent incorporation has been subtracted. Data shown for total nuclear RNA polymerase III has been corrected for recovery of total homogenate DNA.

as noted below. No residual RNA polymerase activity was detectable in the chromatin pellets after polymerase solubilization. As shown in Table II, the amount of extractable RNA polymerase I activity is unaltered by ACTH treatment, while the amounts of extractable RNA polymerase II and III activities are increased approximately twofold. This result is observed when calf thymus DNA or poly(d(A-T)) are used as templates. RNA polymerase I preferentially transcribes calf thymus DNA, whereas RNA polymerases II and III preferentially transcribe poly(d(A-T)) at the salt concentration used. The finding of equal quantities of solubilized RNA polymerase I activity agrees with previous experiments which indicated that, although the activity of adrenal nuclear RNA polymerase I is increased about twofold by ACTH administration, the number of RNA polymerase I molecules engaged in transcription remains constant (Fuhrman and Gill, 1975). Though the soluble pool of RNA polymerase II in nuclei assayed with poly(d(A-T)) (Figures 4 and 5) and calf thymus DNA (Fuhrman and Gill, 1974) is not changed by ACTH treatment, the endogenous nuclear activity of RNA polymerase II is increased 50% (Fuhrman and Gill, 1974). This suggests that the increase in total extractable RNA polymerase II activity primarily reflects the endogenous template-bound enzyme. The increased quantity of total extractable RNA polymerase III activity parallels both the increased nuclear synthesis of pre-4S and 5S RNA and the increased nuclear soluble RNA polymerase III measured on poly(d(A-T)) template (Table I).

DEAE-Sephadex A-25 chromatography of solubilized adrenal nuclear RNA polymerases is shown in Figure 6. In addition to confirming the results noted in Table II, chromatography shows that, after ACTH treatment, a second form of RNA polymerase I appears (peak Ia). RNA polymerase I from control nuclei elutes as a single peak at 0.16–0.18 M $(NH_4)_2SO_4$ (peak Ib), while RNA polymerase I from ACTH nuclei elutes as two peaks with the additional peak eluting at 0.14–0.15 M $(NH_4)_2SO_4$. There is no change in the elution profile of either RNA polymerase II or III after ACTH treatment. RNA polymerase II elutes as a single broad peak between 0.24 and 0.27 M $(NH_4)_2SO_4$, and RNA polymerase III elutes as a single peak at 0.20 M $(NH_4)_2SO_4$. No additional RNA polymerase III activity is eluted at higher salt concentrations.

Two chromatographic forms of RNA polymerase III have

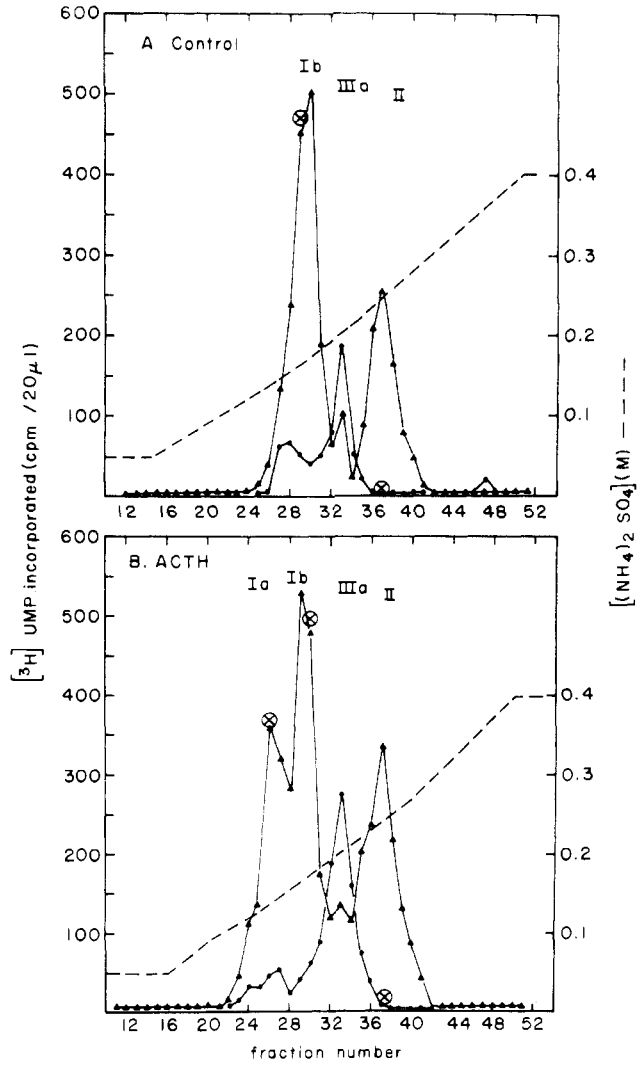

FIGURE 6: DEAE-Sephadex A-25 chromatography of solubilized adrenal nuclear RNA polymerases. Extracts were prepared from purified nuclei, chromatographed, and assayed as described under Methods. These data represent activities extracted from control nuclei containing 194 μg of DNA and ACTH nuclei containing 257 μg of DNA. The activity noted as IIIa was inhibited 90% by 230 μg/ml of α-amanitin. Assays were performed immediately, as the enzymes were dilute and proved to be unstable to dialysis and storage at −70 °C. When assays were performed immediately, 100–150% of the activity of each form of polymerase applied to the column was recovered. (▲), Activity with calf thymus DNA (10 μg/assay); (⊗), activity with calf thymus DNA (10 μg/assay) and 0.77 μg/ml of α-amanitin; (●), activity with 7.7 μg/ml of poly(d(A-T)) and 0.77 μg/ml of α-amanitin.

been described in mouse plasmocytoma cells (Schwartz et al., 1974). The early eluting form, IIIa, is found predominately in nuclei, while the later eluting form, IIIb, is found predominately in cytosols; whole cell homogenates show both forms (Schwartz et al., 1974). RNA polymerase III from purified adrenal nuclei is eluted from DEAE-Sephadex at 0.20 M $(NH_4)_2SO_4$ (IIIa), while the RNA polymerase III, which is observed in adrenal cytosol, is eluted at 0.34–0.38 M $(NH_4)_2SO_4$ (IIIb) (Figure 7). Little, if any, RNA polymerase IIIa is present in adrenal cytosol. As indicated in Table I, ACTH administration has no effect on the amount of cytosol-associated RNA polymerase III, while adrenal nuclear RNA polymerase III increased twofold (Figure 1 and Table II). This suggests that ACTH treatment specifically induces the activity of RNA polymerase IIIa, and that this form of the enzyme catalyzes the pre-4S and 5S RNA synthesis observed

TABLE II: RNA Polymerases Extracted from Adrenal Nuclei.[a]

	UMP Incorporated (pmol/20 min)					
	RNA polymerase I		RNA polymerase II		RNA polymerase III	
	Control	ACTH	Control	ACTH	Control	ACTH
Calf thymus DNA	753	729	141	303	155	303
Poly(d(A-T))	354	299	457	805	702	1,259
Ratio activity (calf thymus DNA/ poly(d(A-T)))	2.13	2.44	0.31	0.38	0.22	0.24

[a] Nuclei purified from 10 adrenal glands were pooled, extracts were prepared, and the solubilized fraction, after DNA removal, was assayed as described under Methods. Reactions containing either 10 μg of calf thymus DNA or 0.5 μg of poly(d(A-T)) were incubated for 20 min at 30 °C. The three RNA polymerases were quantitated in assays containing 0.0, 0.77, or 230 μg/ml of α-amanitin. The $(NH_4)_2SO_4$ concentration (50 mM) in the assays is optimal for solubilized RNA polymerases I and III; however, RNA polymerase II activity measured on calf thymus DNA is underestimated about 50% (Fuhrman and Gill, 1974). These data represent activities extracted from control nuclei containing 464 μg of DNA and ACTH nuclei containing 514 μg of DNA.

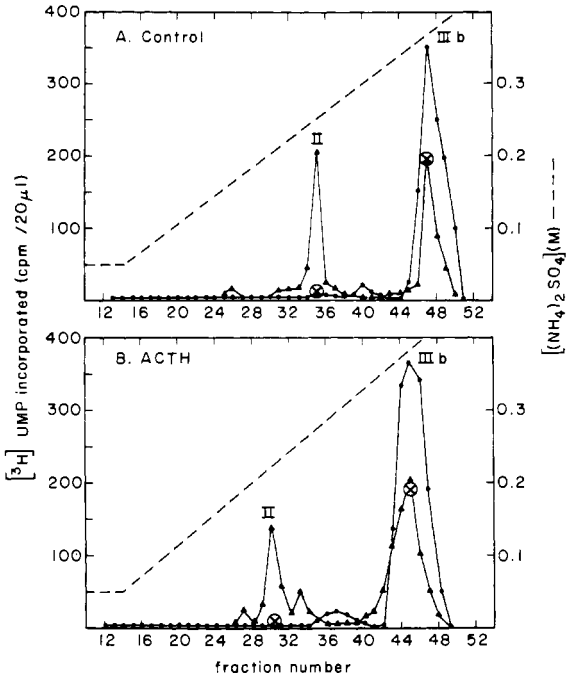

FIGURE 7: DEAE-Sephadex A-25 chromatography of adrenal cytosol RNA polymerases. Adrenal cytosols were prepared, chromatographed, and assayed as described under Methods. The activity noted as IIIb was inhibited >90% by 230 μg/ml of α-amanitin. (▲), Activity with calf thymus DNA (10 μg/assay); (⊗), activity with calf thymus DNA (10 μg/assay) and 0.77 μg/ml of α-amanitin; (●), activity with 7.7 μg/ml of poly(d(A-T)) and 0.77 μg/ml of α-amanitin.

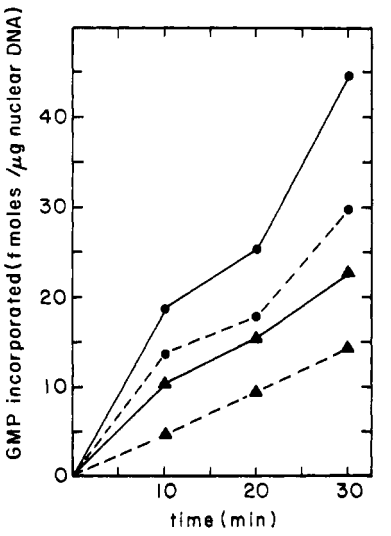

FIGURE 8: Effect of poly(d(A-T)) on accumulation of pre-4S and 5S RNA. [^{3}H]GMP-labeled RNA synthesis in vitro by adrenal nuclei was isolated and analyzed by urea-polyacrylamide gel electrophoresis as described under Methods. Reactions (0.325 ml) contained 0.77 μg/ml of α-amanitin and 15 μg of DNA from control nuclei or 12.6 μg of DNA from ACTH nuclei. Poly(d(A-T)) (8.4 μg/assay) was added where indicated. Synthesis of pre-4S and 5S RNA was quantitated as described in the legend of Table I. (▲-▲), control minus poly(d(A-T)); (▲- - -▲), control plus poly(d(A-T)); (●-●), ACTH minus poly(d(A-T)); (●- - -●), ACTH plus poly(d(A-T)).

in adrenal nuclei. The differing forms of RNA polymerase III in nuclei and cytosol provide evidence that the nuclear soluble pool of RNA polymerase III is not derived from cytoplasmic contamination, although leakage of RNA polymerase IIIb from nuclei during the initial isolation procedures is not excluded as both forms of RNA polymerase III are found in manually dissected oocyte nuclei (Roeder, 1974), in purified HeLa nuclei (Weil and Blatti, 1976), in crude plasmocytoma nuclei (Schwartz et al., 1974), and in crude adrenal nuclei (data not shown). The small amount of RNA polymerase II observed in cytosol elutes at the same position as nuclear RNA polymerase II.

Source of Nuclear Soluble RNA Polymerase III. If the pool of soluble RNA polymerase III which transcribes added poly(d(A-T)) template is distinct from the pool of RNA polymerase III transcribing endogenous template, the addition of poly(d(A-T)) should not affect nuclear synthesis of pre-4S and 5S RNA. To evaluate this possibility, [^{3}H]GTP was used to label RNA transcribed on endogenous template in the presence and absence of poly(d(A-T)) and the pre-4S and 5S RNA products quantitated by urea-polyacrylamide gel electrophoresis. As shown in Figure 8, accumulation of label in pre-4S and 5S RNA is linear with or without poly(d(A-T)) addition. Inclusion of poly(d(A-T)), however, inhibits the synthesis of pre-4S and 5S RNA by 40–50%.

These data suggest that the nuclear soluble pool of RNA polymerase III is not distinct but is derived from RNA polymerase III molecules that would ordinarily initiate transcription on the endogenous template, but which can initiate on and transcribe from poly(d(A-T)) when that template is included in the reaction. Several laboratories have shown that new pre-4S and 5S RNAs are initiated during in vitro incubation of nuclei or chromatin (McReynolds and Penman, 1974; Marzluff et al., 1974; Marzluff and Huang, 1975; Udvardy and Seifart, 1976); whether this represents recycling RNA

polymerase III or a preexisting free pool of enzyme is not clear. RNA polymerase III that is already initiated on its endogenous template would continue to elongate RNA chains, whether or not poly(d(A-T)) is present.

Discussion

The increase in pre-4S and 5S RNA synthesis observed in adrenal nuclei derived from guinea pigs treated in vivo with ACTH is accompanied by an increase both in a nuclear pool of RNA polymerase III active in transcribing added poly(d(A-T)) template and in extractable RNA polymerase III. Though changes in the endogenous chromatin template may occur in response to ACTH, these results suggest that ACTH administration increases the amount and/or activity of nuclear RNA polymerase III. The increase in RNA polymerase III reflects an increase in form IIIa; form IIIb, which is observed in adrenal cytosol, is unchanged. Studies of purified RNA polymerases IIIa and IIIb from mouse plasmocytoma cells indicate that the forms differ in the molecular weight of one small subunit (32 000, IIIa vs. 33 000, IIIb) (Sklar and Roeder, 1976). It is not known whether this difference arises from posttranslational modification of a common protein or from the two subunits being two separate gene products; there are also no data concerning the interconvertibility of the two forms of RNA polymerase III. Since ACTH administration increases nuclear RNA polymerase IIIa activity without affecting cytosol-associated IIIb activity, preferential control of form IIIa is suggested. A similar result was observed by Yu (1975) in regenerating rat liver; however, phytohemagglutinin stimulation of lymphocytes resulted in the stimulation of both forms of RNA polymerase III (Jaehning et al., 1975).

Data shown in Figure 8 suggests that nuclear soluble RNA polymerase IIIa is derived from a pool of RNA polymerase III that would, in the absence of poly(d(A-T)), transcribe pre-4S and 5S RNA genes. In contrast, Lampert and Feigelson (1974) and Yu (1974) have reported that inclusion of poly(d(A-T)) in an RNA polymerase reaction which contains low concentrations of α-amanitin and nuclei or nucleoli as an enzyme source did not affect incorporation of [^{3}H]GTP into RNA. This is also true for adrenal nuclei incubated in 0.77 μg/ml of α-amanitin (data not shown). Since RNA polymerase III represents only 10% of the combined RNA polymerase I and III activities measured in nuclei incubated in low concentrations of α-amanitin (Fuhrman and Gill, 1975), the 50% decrease in RNA polymerase III catalyzed synthesis of pre-4S and 5S RNA observed in the present studies would have been difficult to detect (50% $\times$ 10% = 5%). Direct quantitation of the pre-4S and 5S RNA products of RNA polymerase III is required to observe the poly(d(A-T)) inhibition of RNA polymerase III transcription of chromatin template. The apparent lack of effects of poly(d(A-T)) on total RNA polymerase I (90%) plus III (10%) activity argues against nonspecific effects of the added poly(d(A-T)).

Quantitation of total extractable RNA polymerase I and II activities agrees with findings in studies of nuclear RNA polymerase activity (Fuhrman and Gill, 1974, 1975). Though ACTH administration results in increased rRNA content (Farese and Reddy, 1963) and in increased nuclear synthesis of rRNA precursors, the number of RNA polymerase I enzymes engaged in transcription is not changed, while the rate of RNA chain elongation is doubled (Fuhrman and Gill, 1975). Total extractable RNA polymerase I activity is not altered by ACTH (Table II); however, the DEAE-Sephadex chromatographic profile shows the appearance of an early eluting form designated Ia. Two forms of RNA polymerase I have been observed in mouse plasmocytoma (Schwartz and Roeder, 1974), calf thymus (Gissinger and Chambon, 1975), and rat liver (Muramatsu et al., 1974); these forms differ by the presence (Ia) or absence (Ib) of the third largest subunit (molecular weight 61 000 for mouse plasmocytoma RNA polymerase I; Schwartz and Roeder, 1974). Since endogenous RNA polymerase I activity is increased in ACTH nuclei without a change in extractable activity assayed on calf thymus DNA or poly(d(A-T)) templates, one must consider the possibility that form Ia is preferentially able to transcribe its endogenous chromatin template. The increase in extractable RNA polymerase II activity parallels the increased endogenous activity of the enzyme (Fuhrman and Gill, 1974).

Although ACTH administration is associated with adrenal cortical hypertrophy and ACTH removal with adrenal cortical atrophy (for review, see Garren et al., 1971; Gill, 1972), recent results suggest that these growth effects may be indirect. Addition of ACTH to normal adrenal cells (Ramachandran and Suyama, 1975) or functional adrenal tumor cells in tissue culture (Masui and Garren, 1971) inhibits DNA synthesis and cell division. In the latter case, however, ACTH does not block the increase in RNA and protein content produced by serum addition to quiescent cells (Weidman and Gill, 1976). The patterns of RNA polymerase control evident in the present studies reflect a generalized cellular-growth response analogous to the changes occurring in resting fibroblasts stimulated to grow by addition of serum (Johnson et al., 1974; Rubin and Koide, 1975). The mechanisms through which ACTH initiates this growth response in adrenocortical cells remain unknown.

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Hemocyanin from the Australian Freshwater Crayfish *Cherax destructor.* Studies of Two Different Monomers and Their Participation in the Formation of Multiple Hexamers†

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ABSTRACT: The molecular weights of four electrophoretic components of the 17S constituent of the freshwater crayfish *Cherax destructor* have been determined by gel electrophoresis. All four have the same molecular weight, about 470 000, and are therefore hexamers of the 5S constituent of molecular weight 70 000–80 000. The hexamers are stable at pH 7.8 but dissociate at pH 10, upon removal of calcium ions, into the two monomers M_1 and M_2. These monomers are slowly broken down at pH 10, apparently by an enzyme present in serum. The lability thus demonstrated provides a possible explanation for the findings, by some previous investigators, of fragments suggesting that the subunit molecular weight of arthropod hemocyanins was considerably less than 75 000. Evidence for differences in the lability and proteolytic cleavage pattern between M_2 and M_1 is presented. Studies at pH 7.8 in the presence of calcium ions demonstrate that both M_1 and M_2 can form hexamers, the one formed by M_1 having the highest negative charge and that by M_2 the lowest. The proportions of M_1 and M_2 evidently vary in individual crayfish, the variability accounting for the observation that the number of hexamer bands visible on polyacrylamide gels ranges from four to the maximum of seven possible from combinations of two different monomers. The four intense hexamer bands always seen with pooled *Cherax* serum samples are satisfactorily accounted for by the observed ratio of M_1 and M_2 of about 3:1 in such samples. Amino acid compositions are given for serum, M_1, the mixture of hexamers containing M_1 and M_2, and the dimer M_3', a component not involved in the formation of hexamers. Tryptic peptide maps of different hexamers enriched in either M_1 or M_2 are also presented and discussed.

In a previous communication (Murray and Jeffrey, 1974), it was shown that hemocyanin from the crustacean *Cherax destructor* may be regarded as a typical arthropod hemocyanin with respect to the copper content and the molecular weight of the monomer, the types of aggregates it forms, and the dependence of the aggregation upon pH and divalent metal ions. Two different monomeric forms, denoted M_1 and M_2, were seen on polyacrylamide gels at pH 10 and, it appeared, were

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