Effect of Cortisol on the RNA Polymerase System of Rat Thymus[1]

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SUMMARY

The nuclear RNA polymerase system ("aggregate enzyme" of Weiss) isolated from thymus glands of cortisol-treated rats was shown to incorporate ^{3}H-UTP into RNA to a lesser extent than the same system isolated from control animals. The degree of inhibition varied with time after injection and with cortisol dosage and was specific for steroids which cause thymic involution. No difference in nucleotide triphosphatase activity was observed when thymus aggregate enzyme preparations isolated from cortisol-treated rats were compared with identical preparations isolated from control animals. The rate of ^{3}H-UTP incorporation into RNA by thymus aggregate enzyme was found to be dependent on ionic strength. Maximum rates of incorporation and maximum steroid effects were found when NH_4Cl was present in the incubation mixture at a final concentration of 0.75 M. Increasing NH_4Cl concentration in the incubation mixture was found to stimulate control thymus aggregate enzyme to a greater extent than thymus aggregate enzyme isolated from cortisol-treated rats. Cortisol was without effect when added directly to the aggregate enzyme incubation mixture at a final concentration of 10^{-5} M.

INTRODUCTION

The catabolic effects of cortisol on lymphoid tissue have been under intensive study in recent years. Inhibition of protein synthesis has been demonstrated in many systems, such as intact animal (1, 2), cell-free systems from whole animals pretreated with steroid (3, 4), cell culture preparations treated with steroid *in vitro* (5, 6), and cell-free preparations isolated from these cell culture systems (3, 4). More recently it was reported that the decrease in protein synthesis in rat thymus was accompanied by a decrease in the total cytoplasmic particulate RNA of the cell (7). Significant decreases in particulate RNA were seen as early as 6 hr after intramuscular injection of 15 mg cortisol (free alcohol) per kilogram. The greatest effect was on the larger aggregates (polysomes).

Since that time several investigators have reported that cortisol, when added to suspensions of intact lymphoid cells *in vitro*, inhibits incorporation of labeled uridine into RNA (8–10). Pratt *et al.* (10) have pointed out that the inhibition of uridine-^{14}C conversion into acid-insoluble material (RNA) remains constant at both high and low levels of precursor specific activity, whereas the inhibition of deoxycytidine-^{3}H incorporation into DNA, seen with high specific activity precursor, is obliterated when the labeled precursor is diluted with large amounts of the unlabeled compound. The interpretation of these data was that the effect of uridine-^{14}C incorporation was not secondary to dilution of precursor specific activity by an increased intercellular precursor pool. In addition, Nakagawa and White (11) have reported

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an inhibition of labeled UTP uptake by intact nuclei isolated from thymus glands of rats which had been pretreated with cortisol.

This paper presents results of studies on the effects of cortisol on ^{3}H-UTP incorporation into RNA by the "aggregate enzyme" of Weiss (12) isolated from thymus glands of adrenalectomized rats. The influence of ionic strength on the cortisol-induced inhibition of rat thymus aggregate enzyme was also studied.

METHODS

Preparation of aggregate enzyme. Male Sprague-Dawley rats, weighing 80–100 g at the time of sacrifice, were adrenalectomized on arrival and maintained in our animal quarters for 3 days prior to use. Standard laboratory diet and 1% NaCl in the drinking water were available ad libitum up to the time of sacrifice.

The rats were injected intramuscularly with cortisol[2] (15 or 50 mg/kg) or an equal volume of the appropriate vehicle[3]; 3, 6, or 12 hours later they were sacrificed by decapitation and the thymus glands were rapidly excised. This and all subsequent operations were carried out in the cold (2°–4°). The aggregate enzyme was isolated by modifications of the methods of Busch *et al.* (13) and Ramuz *et al.* (14). Thymus glands of from three to six animals were combined for each preparation. The thymic tissue was minced, suspended in 20 ml of a solution containing 0.003 M $MgCl_2$, 0.01 M tris(hydroxymethyl)aminoethane acetic acid) (Tris), pH 6.4, in a Dounce homogenizer with a few strokes of the A- (loose-fitting) pestle, and allowed to stand in ice for 10 min. The preparation was then homogenized with ten additional strokes of the A-pestle and sucrose was added to make the solution 0.2 M. The resulting suspension of nuclei was centrifuged at 200 *g* for 10 min. The nuclei were washed twice with a solution containing 0.2 M sucrose, 0.003 M $MgCl_2$, and 0.01 M Tris, pH 6.4.[4] After the last centrifugation, the nuclei were suspended in 15 ml of 0.05 M Tris, pH 8.0, in a Dounce homogenizer with a few strokes of the B- (tight-fitting) pestle and allowed to stand 15–20 min. The preparation was then homogenized with an additional 10–15 strokes of the B-pestle, and the nuclear lysate was centrifuged in the Spinco, Model L, ultracentrifuge at 70,000 *g* for 15 min. The pellet was resuspended in a volume of 0.05 M Tris, pH 8.0, calculated to give 60 mg wet weight of the pellet per milliliter of the suspension. The preparation was homogenized in a Dounce homogenizer (B-pestle), filtered through three layers of gauze, and stored in ice. This suspension was used as aggregate enzyme in the incubation mixture. Assays were always completed within 48 hr after isolation of enzyme.

Chemical analysis. Aliquots of the enzyme preparation were taken for chemical analysis. An equal volume of cold 12% perchloric acid was added to the sample; the resulting mixture was centrifuged. The precipitate was resuspended in 6% perchloric acid, heated at 90° for 20 min, cooled in ice, and centrifuged. The pellet was used for determination of protein nitrogen by the method of Oyama and Eagle (15). The supernatant was used for determination of ribose by the orcinol method (16) and for the determination of deoxyribose by the method of Burton (17). Micromoles of assayed deoxyribose were converted to micrograms of DNA by assuming an average molecular weight of 327 for the deoxyribonucleotides. For enzymes isolated from rat thymus 12 hr after treatment, the ratio of micrograms of protein nitrogen to micrograms of DNA was 0.64 $\pm$ 0.03[5] for vehicle-treated (control), and 0.66 $\pm$ 0.05 for cortisol-treated. The ratio of millimicromoles of ribose to micrograms of DNA for the same preparations was 0.44 $\pm$ 0.06 for vehicle-treated (control), and 0.41 $\pm$ 0.04 for cortisol-treated. There was no significant difference between

[2] Cortef®, The Upjohn Company.

[3] Vehicle Number 100, The Upjohn Company. Each cubic centimeter contains carboxymethylcellulose 5 mg, polysorbate 4 mg, sodium chloride 9 mg, and benzyl alcohol 9 mg.

[4] Electron micrographs of nuclei prepared by this method showed very little cytoplasmic material adhering to the nuclear membrane.

[5] $\pm$ standard error of the mean for eleven separate enzymes.

cortisol-treated and control values in either case. On a weight basis[6] the proportion of protein to RNA to DNA for rat thymus aggregate enzyme was 4.07:0.14:1.00.

Incorporation of ^{3}H-UTP. The incubation mixture, modified from Goldberg (18), contained 50 μmoles of Tris, 1.5 μmoles of $MnCl_2$, 5 μmoles of 2-mercaptoethylamine, 10 μmoles of NaF, 1.0 μmole each of ATP,[7] CTP, and GTP, aggregate enzyme, and 5 μC ^{3}H-UTP (0.003 μmole). The ionic strength of the incubation mixture was 0.094, and this was increased by adding NH_4Cl. The final volume of each incubation sample was 0.50 ml and the pH was 8.0. Replicate samples were prepared and incubated in a water bath at 37° for 15 min. At the end of the incubation period the samples were transferred to ice and 0.03 ml of a cold 40 mg/ml bovine serum albumin solution was added immediately, followed by 2 ml cold 6% trichloroacetic acid. The samples were washed three times by centrifugation at 15,000 *g* for 5 min and resuspension in 2 ml of cold 6% trichloroacetic acid. They were then filtered onto 0.45 μ Millipore filters and washed with three additional 2-ml portions of cold 6% trichloroacetic acid. The filters were placed in vials and dried, 10 ml of toluene scintillation solution [4 g of 2,5-diphenyloxazole, 0.1 g of 2,2-p-phenylenebis(5 phenyloxazole) per liter of toluene] was added, and the radioactivity was counted in a Packard TriCarb scintillation spectrometer. After subtraction of the zero time values,[8] the counts per minute were converted to micromicromoles of ^{3}H-UTP incorporated on the basis of a 15% counting efficiency.[9]

[6] Calculated on the basis of 16% nitrogen in protein and an average molecular weight for the ribonucleotides of 339.

[7] Source of chemicals used in these experiments was as follows: ATP, CTP, and GTP, A grades, Calbiochem; uridine 5-triphosphate-^{3}H, tetralithium (^{3}H-UTP labeled in the pyrimidine base, specific activity 1.6 C/mmole), Schwarz BioResearch, Inc.; deoxyribonuclease (DNase) and ribonuclease (RNase), Sigma Chemical Co.; actinomycin D, National Cancer Institute.

[8] Zero time samples were prepared by adding ^{3}H-UTP to iced, nonincubated mixtures immediately followed by serum albumin and 6% trichloroacetic acid as described. Zero time values (± standard deviation) averaged 59 ± 12 counts per minute or 0.11 ± 0.02 μμmole of UTP.

RESULTS

Aggregate enzyme incorporates radioactivity from ^{3}H-UTP into an acid-insoluble product which has the properties of RNA (Table 1). The data presented also show that the omission of a single triphosphate (CTP) abolishes incorporation of the label, and that incorporation is blocked by actinomycin D or pretreatment with DNase.

TABLE 1

Characteristics of the rat thymus aggregate enzyme system

NH_4Cl was added to the incubation system (see Methods) to a concentration of 0.75 M. Aggregate enzyme DNA was present in the amount of 3.9 μg per incubation.

Conditions	μμmoles ^{3}H-UTP/mg DNA
Control	50
Minus CTP	<1
Actinomycin D, 10 μg	<1
Preincubate enzyme with 10 μg DNase for 30 min at 37°	<1
Treat product with 1 μg RNase[a] for 30 min at 37°	<1
Treat product with 0.5 M NaOH[b] for 15 hr at 37°	2
Treat product with 1 N $HClO_4$[b] for 20 min at 90°	<1

[a] The product was centrifuged, and the pellet was dissolved in 0.2 M Tris, pH 7.5. After treatment with RNase, cold trichloroacetic acid was added to a concentration of 6% and the precipitate was treated as described in the Methods.

[b] The product was centrifuged; the pellet was resuspended and treated as indicated in Table 1. After treatment, cold trichloroacetic acid was added to a concentration of 6% and the precipitate was treated as described in the Methods.

The kinetics for ^{3}H-UTP incorporation by aggregate enzyme are shown in Fig. 1. Incorporation of radioactivity was linear over a 15-min interval for aggregate preparations isolated from thymus glands of both control and cortisol-treated rats.

[9] Counting efficiency was determined with ^{3}H-UTP absorbed on a filter containing materials precipitated from an unlabeled incubation sample.

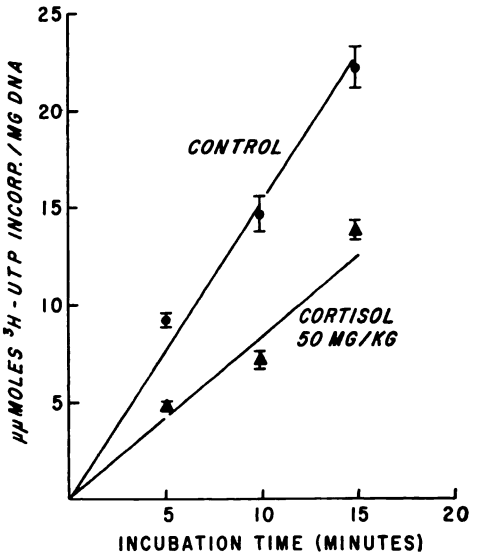

FIG. 1. *Kinetics of ³H-UTP incorporation*

Incorporation of ³H-UTP by rat thymus aggregate enzyme 12 hr after treatment with vehicle (control) or cortisol (50 mg/kg). NH_4Cl was added to the incubation system (see Methods) to a concentration of 0.75 M. The control assays contained 1.26 μg of aggregate DNA; and the cortisol-treated, 1.28 μg of aggregate DNA. The standard error of the mean for three separate assays is indicated by the vertical bars.

Incorporation of radioactivity in this system was found to be directly proportional to the amount of aggregate enzyme up to about 40 μg aggregate DNA per incubation tube (Fig. 2). In order to assure that aggregate enzyme was a limiting component of the system, incubations were usually performed with less than 10 μg aggregate DNA per tube and never more than 20 μg per tube.

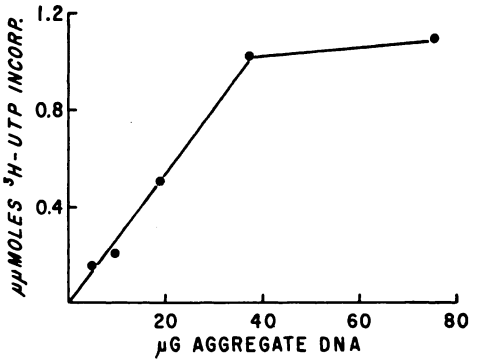

FIG. 2. *Dependency of ³H-UTP incorporation on the amount of aggregate enzyme added to the incorporation system*

NH_4Cl was added to the incubation system (see Methods) to a concentration of 0.75 M.

Since the activity of aggregate enzyme was known to be affected by ionic strength (18), the effect of ionic strength on ³H-UTP incorporation by rat thymus aggregate enzyme was studied (Fig. 3). Activity of this preparation was found to be maximal when NH_4Cl was present in the incubation mixture at a final concentration of 0.75 M (total ionic strength =

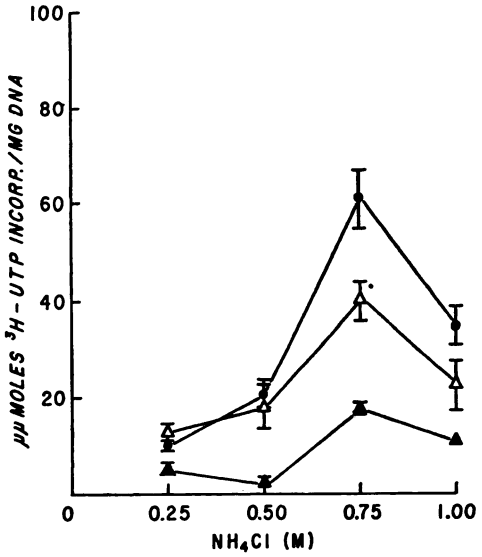

FIG. 3. *Effect of NH_4Cl on the incorporation of ³H-UTP by rat thymus aggregate enzyme*

³H-UTP incorporation was examined 12 hr after treatment with vehicle (●), 15 mg/kg cortisol (△) or 50 mg/kg cortisol (▲). NH_4Cl was added to the incubation system (see Methods) to give the concentrations indicated in the figure. The standard error of the mean is indicated by the vertical bars. The *t* test of significance between control (vehicle) and treated groups gave the following *P* values:

NH_4Cl (M)	15 mg/kg	50 mg/kg
1.0	>0.10	—*
0.75	<0.05	<0.01
0.50	>0.10	<0.05
0.25	>0.10	<0.05

* One enzyme preparation only.

0.84). Figure 3 also shows the effect of NH_4Cl on the incorporation of ^{3}H-UTP by aggregate enzyme prepared from rat thymus 12 hr after injection with cortisol, 15 mg/kg or 50 mg/kg. The curves are similar in all cases, with maximum rate of incorporation occurring at 0.75 M NH_4Cl. The inhibitory effect of cortisol at a dose of 50 mg/kg is significant at all ionic strengths studied, but inhibition at a dose of 15 mg/kg is significant only at 0.75 M NH_4Cl.

adrenalectomy[10] on arrival and maintenance in our animal quarters for 3 days prior to injection with steroid. No attempt was made to determine the exact cause of this variation since each experiment was performed as a paired comparison between control (vehicle-treated) and cortisol-treated rats. These paired comparisons gave consistent results for any dose of cortisol for a given time after administration. The results of Table 2, however, are presented as averages of value for all experiments

TABLE 2

Effect of cortisol on incorporation of ^{3}H-UTP by aggregate enzyme

NH_4Cl was added to the incubation mixture (see Methods) to a concentration of 0.75 M.

Treatment	Time after treatment (hours)	n[a]	$\mu\mu$moles ^{3}H-UTP incorporated per mg DNA[b]	Percent inhibition	P[c]
Vehicle	3	3	39 ± 6		
Cortisol, 15 mg/kg	3	3	37 ± 6	5	>0.10
Vehicle	6	6	35 ± 3		
Cortisol, 15 mg/kg	6	6	28 ± 1	20	<0.01
Vehicle	6	2	28 ± 1		
Cortisol, 50 mg/kg	6	2	13 ± 0.3	54	<0.01
Vehicle	12	9	61 ± 6		
Cortisol, 15 mg/kg	12	9	40 ± 6	34	<0.05
Vehicle	12	2	48 ± 7		
Cortisol, 50 mg/kg	12	2	18 ± 1	62	<0.05

[a] Number of aggregate enzyme preparations studied.

[b] Average value ± standard error of the mean.

[c] t test for significance between cortisol and vehicle-treated.

Table 2 presents results of studies on the effect of dose and time after administration of cortisol on incorporation of ^{3}H-UTP by rat thymus aggregate enzyme in the presence of 0.75 M NH_4Cl. There was no significant effect of cortisol at a 15 mg/kg dose 3 hr after treatment, but by 6 hr this dose decreased activity by 20%, and by 12 hr it was decreased 34%. Six hours after treatment with a dose of 50 mg/kg, cortisol inhibited incorporation of the labeled UTP by 54%, and this inhibition increased to 62% by 12 hr. The variation in incorporation seen in the data for control animals in Table 2 is most likely attributable to variation between groups. The experiments were carried out over many months, and standard conditions consisted only of performed in each group. The effect of cortisol treatment on rat thymus aggregate enzyme activity is quite clear from the averaged data even without taking advantage of the paired design of the experiments.

Table 3 demonstrates the specificity of the inhibitory effect of cortisol. The effect of cortisol (15 mg/kg) was compared with equal doses of Reichstein's S and dexamethasone 12 hr after treatment. Dexamethasone

[10] Adrenalectomy was performed to reduce variation due to endogenous release of adrenal steroids by nonspecific stress factors which could not be adequately controlled. Similar results were obtained in nonadrenalectomized rats, but the incorporation of ^{3}H-UTP was more variable in control (vehicle-treated) animals.

TABLE 3
The effect of various steroids on ^{3}H-UTP incorporation by aggregate enzyme

Aggregate enzyme was isolated from thymus glands of rats 12 hours after injection with vehicle or steroid. NH_4Cl was added to the incubation system (see Methods) to a concentration of 0.75 M.

Treatment	μμmoles ^{3}H-UTP incorporated/mg DNA[a]	Percent inhibition
Vehicle	40 ± 1	—
Dexamethasone, 15 mg/kg	17 ± 2	58
Reichstein's S, 15 mg/kg	40 ± 2	0
Cortisol, 15 mg/kg	26 ± 3	35

[a] Value ± standard error of mean for four separate assays.

was found to be almost twice as effective as cortisol in inhibiting rat thymus aggregate enzyme, while Reichstein's S was found to be inactive.

Since easy measurement of label incorporation by aggregate enzyme required having ^{3}H-UTP present at a high specific activity, it was necessary to use low amounts of chemical UTP in the incubation medium (see Methods). The possibility existed, therefore, that the cortisol-induced inhibition of ^{3}H-UTP incorporation by thymus aggregate enzyme could be the result of a stimulation of nucleotide triphosphatase activity. To examine this possibility, the nucleotide triphosphatase activity of thymus aggregate enzyme preparations obtained from rats sacrificed 12 hr after injection with vehicle or cortisol (50 mg/kg) was determined. Table 4 shows there is no significant increase in triphosphatase activity after cortisol treatment with either ATP or UTP as substrate.

Table 5 shows the results of treatment *in vitro* of aggregate enzyme with cortisol. Cortisol at 10^{-5} M final concentration did not inhibit ^{3}H-UTP incorporation. The

TABLE 4
Effect of cortisol on aggregate enzyme triphosphatase activity

Rat thymus aggregate enzyme prepared 12 hr after treatment with vehicle (control) or cortisol (50 mg/kg). Triphosphatase activity determined by the method for adenosine triphosphatase (30). Assays were run in duplicate with ATP and in triplicate with UTP. Replicate values agreed within ±5% in all cases. Cortisol-induced inhibition of ^{3}H-UTP incorporation for these preparations (as compared to control) was 68% for experiment I and 55% experiment II.

Experiment	Substrate	μg P liberated/μg DNA		
		Control	Cortisol, 50 mg/kg	$\frac{\text{Cortisol}}{\text{control}} \times 100$
I	ATP	0.603	0.697	115
	UTP	0.328	0.385	117
II	ATP	0.619	0.648	105
	UTP	0.302	0.297	98

TABLE 5
Effect of cortisol added in vitro on rat thymus aggregate enzyme

NH_4Cl was added to the incubation system to a concentration of 0.75 M.

Treatment[a]	μμmoles ^{3}H-UTP incorporated	μμmoles ^{3}H-UTP/mg DNA[b]
None	0.296	67 ± 4
10^{-5} M cortisol	0.282	64 ± 3
Incubate with vehicle, 1 hour, 37°	0.201	45 ± 2
Incubate with 10^{-5} M cortisol, 1 hour, 37°	0.208	47 ± 4

[a] Cortisol (Calbiochem Hydrocortisone, A Grade) in 0.05% ethanol. Vehicle 0.05% ethanol.
[b] Value ± standard error of the mean for three separate assays.

results also show that preincubation at 37° for 1 hr in the presence of vehicle causes a partial loss of activity in the preparation, but that cortisol (10^{-5} M) has no further effect.

DISCUSSION

Aggregate enzyme isolated from thymus glands of cortisol-treated rats has been shown to have a decreased ability to incorporate ^{3}H-UTP into RNA as compared to an identical preparation isolated from control animals. The degree of inhibition increases with dose and with time after a fixed dose of cortisol (Table 2). These results are compatible with the data of Nakagawa and White (11), who have reported inhibition of UTP uptake by intact nuclei isolated from thymus glands of rats pretreated with cortisol. These investigators reported that cortisol (50 mg/kg) decreased UTP incorporation 15% by 0.5 hr, 23% by 1 hr, and 34% by 3 hr after injection. If these results are plotted and extrapolated to 6 hr, a value is obtained which is very close to the 54% inhibition reported here (Table 2) 6 hr after the same dose of cortisol (50 mg/kg).

Examination of nucleotide triphosphatase levels failed to show any significant increase in the rate of hydrolysis of either ATP or UTP by aggregate enzyme obtained from thymus glands of rats pretreated with cortisol (50 mg/kg) 12 hr before sacrifice. Since steroid-induced inhibition of ^{3}H-UTP incorporation was 55% and 68% for the two enzymes tested, it is unlikely that the 15% increase in nucleotide triphosphatase activity seen in one preparation (Table 4) could explain the steroid effect, even if it were significant. A similar conclusion was reached by Nakagawa and White (11), who showed that excess nucleotide triphosphates added to their incubation system of intact thymus nuclei failed to reverse the steroid effect. In addition Peña *et al.* (8) have shown that RNase and nuclear DNase activities of rat thymocytes are not altered by cortisol treatment (50 mg/kg).

Two other steroids (Reichstein's S and dexamethasone) were examined for their ability to inhibit ^{3}H-UTP incorporation by rat thymus aggregate enzyme. Reichstein's S is known to be devoid of thymolytic activity (19, 20) while dexamethasone is known to be a more potent thymolytic steroid than cortisol (21). The results in Table 3 show that the same rank order is obtained in the aggregate system, and support the idea that this effect is limited to those steroids which possess thymolytic activity.

Addition of cortisol (10^{-5} M) to the incorporation system did not significantly decrease ^{3}H-UTP incorporation of aggregate enzyme isolated from thymus glands of untreated rats (Table 5). It appears than that cortisol does not exert a direct effect on the preformed aggregate RNA polymerase system, but rather acts indirectly—perhaps on the formation of the active complex.

These above results, coupled with those of other laboratories (9–11) suggest inhibition of RNA synthesis as an early steroid action on lymphoid tissue. The mechanism by which cortisol inhibits RNA synthesis in this system is not yet clear but appears to be associated with the final polymerization of nucleotides into RNA.

In contrast to the early effect on RNA synthesis seen only at low ionic strength with anabolic steroids such as testosterone (22–24), estradiol (25, 26), and cortisone (27) on the liver, the early catabolic effects of cortisol on rat thymus are best seen at high ionic strength (Fig. 3). As predicted from the previous work of Goldberg (18), ^{3}H-UTP incorporation by rat thymus aggregate enzyme is enhanced by increasing the ionic strength of the incubation mixture. Although interpretation of the ionic strength effect is somewhat uncertain, the recent work of Huang *et al.* (28) and Marushige and Bonner (29) suggests that the stimulation of RNA synthesis seen with increasing salt concentration in the incubation mixture is due to the removal of "inhibitory" proteins (histones) from template DNA. If their explanation is correct, the fact that less stimulation is obtained with increasing NH_4Cl concentration in thymus aggregate preparations obtained from steroid-treated rats suggests

that steroid may enhance the affinity of "inhibitory" proteins for DNA.

Other considerations, however, make alternative possibilities apparent. For example, it was found that *Escherichia coli* RNA polymerase[11] (2.5 units) added to the complete aggregate enzyme incubation system under conditions where "inhibitory" proteins were tightly bound to template DNA (no NH_4Cl added) increased the rate of ^{3}H-UTP incorporation per μg DNA more than 800% (Gabourel and Fox, unpublished). This suggests that RNA polymerase may be the limiting component of the aggregate enzyme preparation, rather than the amount of active DNA template. Thus, cortisol might inhibit ^{3}H-UTP incorporation by reducing the amount or activity of RNA polymerase in the aggregate preparation.

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[11] Obtained from Biopolymer, Inc., Changebridge Road, Pinebrook, New Jersey. Reported specific activity 2000 units/mg.