Effects of Ammonium Sulfate, Growth Hormone, and Testosterone Propionate on Ribonucleic Acid Polymerase and Chromatin Activities in Rat Skeletal Muscle*

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ABSTRACT: Single injections of testosterone propionate, growth hormone, or both hormones stimulated activity of aggregate ribonucleic acid (RNA) polymerase from femoral muscles of hormone-deficient rats; combined injection produced an additive increase. The stimulatory effect of testosterone was masked when assays were done with ammonium sulfate in the medium, whereas that of growth hormone was unaffected by $(NH_4)_2SO_4$. Assays of the priming activity of muscle chromatin were performed using RNA polymerase from *Escherichia coli*. Administration of testosterone propionate increased priming activity compared to that of controls; treatment with growth hormone did not stimulate template efficiency of chromatin although activity of aggregate RNA polymerase was elevated. Exposure of chromatin to 0.3 M ammonium sulfate during preparation caused an increase in its priming activity and masked increases in priming activity of chromatin resulting from treatment of castrates with androgen. We interpret these data to mean that testosterone, but not growth hormone, stimulates RNA synthesis in muscle by increasing priming efficiency of deoxyribonucleic acid (DNA). Growth hormone may act by directly affecting RNA polymerase. These differential effects could explain the known additivity of the two hormones in increasing both the body weight gain and the nitrogen retention.

Hormonal regulation of growth has received considerable attention for many years. Recently a number of anabolic hormones have been shown to stimulate protein synthesis in "target" tissues as a consequence of prior enhancement of RNA synthesis. Despite the difficulty in ascribing a single mode of action to a variety of agents such as androgens, thyroxine, insulin, and growth hormone, the evidence for a common effect on synthesis of macromolecules is considerable (Korner, 1965).

Additivity of growth-promoting hormones is an intriguing phenomenon. It has been shown that additivity in increasing body weight gain and nitrogen retention occurred when growth hormone and testosterone were administered in combination (Li, 1953; Kochakian, 1960). Additive responses were observed with levels producing maximal effects individually, indicating that the hormones acted by different mechanisms. We have previously reported that stimulation of protein synthesis in muscle by these agents is also additive (Florini and Breuer, 1966), but these studies gave no indication of differences in mode of action. The data of Widnell and Tata (1966) showing additive stimulation of RNA polymerase activity in liver by thyroid hormone and growth hormone or testosterone propionate also provided no evidence for differences in action to explain the observed additivity.

This report describes results of assays of aggregate RNA polymerase and chromatin activities from rat skeletal muscle. We find that testosterone propionate and growth hormone both stimulate RNA polymerase activity while only the androgen enhances the priming efficiency of DNA. These results, in addition to data obtained with assays of muscle RNA polymerase in the presence of ammonium sulfate, indicate that the two hormones stimulate RNA synthesis in muscle by different mechanisms.

Materials and Methods

[^{14}C]UTP[1] (sp act. 18.4 mc/mmole) was purchased from Schwarz Bio-Research. Testosterone and testosterone propionate were from Mann Research Laboratories. Calf thymus DNA, five-times crystallized ribonuclease, and porcine growth hormone were obtained from the California Corp. for Biochemical Research. Desoxyribonuclease was from Worthington Biochemicals; frozen *Escherichia coli* strain B (midlog phase) was purchased from General Biochemicals.

Isolation of Nuclei from Skeletal Muscle. Our procedure is similar to that reported by Edelman *et al.* (1965). Femoral muscles pooled from 6 ot 12 male rats were the starting material. All operations subsequent to sacrifice of the animals were performed at

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[1] Abbreviations used: TCA, trichloroacetic acid; UMP, uridine monophosphate; UTP, uridine triphosphate.

2–4°. Muscle pieces, dissected free of fat, were ground in an electric meat grinder, weighed (50–80 g/preparation), and homogenized in a Vir-Tis "45" homogenizer with two volumes of medium consisting of 0.25 M sucrose, 0.025 M KCl, 0.01 M $MgCl_2$, and 0.05 M Tris–HCl, pH 7.6.

The homogenate was centrifuged for 15 min at 700*g*; the supernatant was discarded and the sediment was rehomogenized. This suspension was filtered by squeezing through prewetted cotton flannel. Homogenization and filtration were repeated, and the pooled filtrates were centrifuged at 12,000*g* for 10 min.

Pellets so obtained were resuspended in medium and layered over 1.0 M sucrose; tubes were centrifuged for 30 min at 40,000*g* in an angle-head rotor in the Servall SS-3 centrifuge. Samples of the final nuclear pellets were routinely examined in the microscope. Aliquots were stained directly on slides using 0.1% crystal violet in 5% acetic acid. Nuclei were of a characteristic oval shape, 20–40 μ in length. Some myofibrillar debris was always present, but the nuclei were essentially free of adherent cytoplasmic contamination.

Isolation of Aggregate RNA Polymerase. The method of preparation of aggregate enzyme was essentially that of Weiss (1960). Aggregates were separated from small molecules and nuclear debris by repeated washing and centrifugation in 0.05 M Tris–HCl, pH 7.4. Washed aggregate was resuspended in 0.05 M Tris–HCl, pH 8.3 (3 ml/50 g of muscle), by vigorous homogenization in an all-glass Kontes homogenizer. Enzyme suspensions were stored overnight in an ice bath at 0° prior to assay; activity decreased by 80% after 2-week storage at 0°.

Preparation of Chromatin. Chromatin was prepared from nuclei using the procedure of Marushige and Bonner (1966). Chromatin exposed to ammonium sulfate (final concentration 0.3 M) was mixed with a saturated solution of $(NH_4)_2SO_4$ prior to the centrifugation through 1.7 M sucrose which also contained 0.3 M ammonium sulfate; subsequent steps were done as described in the procedure of Marushige and Bonner (1966). DNA content of chromatin and aggregate preparations was determined by a modification of the method of Burton (1956) using salmon sperm DNA as a standard.

Protocol for Hormone Experiments. Male Sprague–Dawley rats of the Carworth Farms CFN strain were used for all studies involving castration and androgen administration. Animals were bilaterally castrated under ether anesthesia and maintained at least 1 week after operation and prior to experiment. Hypophysectomized Sprague–Dawley rats were obtained from Charles River Breeding Laboratories. These rats were kept at least 2 weeks before use; rats gaining over 10-g body weight were not used. Food and water were available at all times. Groups of 6–12 animals were used in all studies; aggregate enzyme and chromatin were prepared from nuclei obtained from femoral muscles pooled from all rats in each group.

Testosterone propionate was made up as a microcrystalline suspension in 0.5% sodium carboxymethylcellulose. Porcine growth hormone was dissolved in 0.9% saline with the aid of a few drops of 1 N NaOH. Actinomycin D was freshly dissolved in a minimum amount of ethanol and diluted with saline prior to use. All injections were given intraperitoneally in volumes of 0.2 ml.

Each experiment involved a control and two or three treated groups. Preparations of aggregates and chromatin were made on the same day using the same equipment such as homogenizers and centrifuges. Care was taken to standardize preparative procedures involving homogenizations so that accurate comparisons could be made within each experiment.

Assay of Aggregate RNA Polymerase. Incubations were conducted under air at 37° in a water bath with shaking at 120 cycles/min. Reaction mixtures were as specified in the legends to tables; when high ionic strength was desired, 0.05 ml of a saturated (0°) solution of $(NH_4)_2SO_4$ was added to a volume of 0.70 ml, giving a final sulfate concentration of 0.3 M. Enzyme preparations containing 60–150 μg of DNA were added last to initiate the reaction.

Incubations were stopped after 10 min by the addition of 0.5 ml of a saturated solution of sodium pyrophosphate. Precipitates were washed once with cold 15% TCA[1]–0.1 M $Na_4P_2O_7$, twice with cold 15% TCA, and three times with cold 95% ethanol. The RNA in the dried precipitates was converted to acid-soluble nucleotides using 25 μg/ml of ribonuclease in 0.05 M Tris–HCl, pH 7.4; precipitates were resuspended in this solution and incubated for 2 hr at 37°, according to the method of Gorski (1964). RNA concentration was determined on the supernatant after acidification by measurement of absorbance at 260 mμ and conversion to micrograms of RNA per milliliter by multiplying absorbance by 33.16 as described by DeDeken-Grenson and DeDeken (1959).

Radioactivity of RNA nucleotides was measured in a Packard Tri-Carb. Samples were placed in vials containing 20 ml of polyether phosphor (Davidson and Feigelson, 1957). Counting efficiency was determined by addition of internal standard and recounting; disintegrations per minute were calculated for all samples. Results are expressed as micromicromoles of labeled UMP incorporated per milligram of DNA incubated. As the DNA:RNA ratio of aggregate preparations was found to be constant in all experiments (10:1, DNA:RNA), total disintegrations per minute incorporated per microgram of RNA isolated was calculated, converted to total disintegrations per minute per microgram of DNA incubated, and micromicromoles of labeled UMP per microgram of DNA were calculated from the known specific activity of the radioactive UTP. This procedure was used to correct for the small losses encountered during washing of precipitates.

Assay of Chromatin. Priming activity of chromatin preparations was assayed using *E. coli* RNA polymerase (fraction 3) prepared according to Chamberlain and Berg (1962). Incubation conditions were identical

with those used by Chamberlain and Berg, except that the concentrations of enzyme (52 μg/tube) and primer (up to 2 μg/tube) were adjusted to make primer the limiting component. After 10-min incubation at 37°, the reaction was stopped by addition of 5 ml of ice-cold 10% TCA; acid-insoluble material was collected on Millipore filters. Filters were washed with at least 50 ml of cold 10% TCA, dried, and placed in counting vials with 1.0 ml of 88% formic acid to dissolve precipitates. After 15–30 min at room temperature, 10 ml of a 1:1 mixture of toluene:methyl cellosolve containing 4 mg/ml of 2,5-bis[2-(5-*t*-butylbenzoxazolyl)]thiophene was added; vials were shaken until the filters dissolved completely.[2] Samples were counted in a Packard Tri-Carb; counting efficiencies were determined by the internal standard method and results were converted to millimicromoles of labeled UMP incorporated per microgram of DNA incubated. Incorporation by the enzyme preparation in the absence of added primer was subtracted from all results; it amounted to 2–5 μμmoles/tube in these experiments. The same enzyme preparation was used for all of the experiments to be reported. Small aliquots were stored in a liquid nitrogen freezer and thawed prior to use; they were diluted with the enzyme diluent described by Chamberlain and Berg (1962).

Results

Characterization of the Aggregate Enzyme. Incorporation of labeled UMP into ribonuclease-sensitive material was proportional to the amount of radioactive UTP incubated in the range from 0.42 to 11 mμmoles used in these experiments. Addition of unlabeled UTP had no effect on incorporation except for dilution of the precursor. Incorporation was linear for 10 min; when ammonium sulfate was present in the medium, incorporation proceeded at a greater rate and was linear for 30 min. The extent of the reaction was proportional to the amount of aggregate incubated up to at least 150 μg of DNA/tube.

Optimal concentrations of all cofactors and ions were determined; they were similar to those reported for enzymes from other mammalian tissues. The only exception was an absence of a requirement for a thiol reagent in the muscle system, as shown in Table I; similar results were obtained using reduced glutathione and cysteine. Other characteristics are shown in Table I. Inhibition of activity by DNase and actinomycin D, absolute requirement for all nucleoside triphosphates, and release of all incorporated radioactivity by ribonuclease provided evidence that DNA-dependent RNA synthesis occurred.

Effects of Treatment of Castrates with Testosterone Propionate. Table II shows the results of studies in which a single injection of testosterone propionate was given to castrates at various times prior to sacrifice.

[2] We are grateful to Miss P. Cavicchi (University of Chicago) for introducing us to this technique.

TABLE I: Characteristics of the Aggregate RNA Polymerase from Rat Skeletal Muscle.

Expt	Additions	Incorp[a]
I	None	125
	10 μg of actinomycin D	43
	50 μg DNase	36
	Omit ATP, CTP, GTP	0
	1.5 μmoles of 2-mercaptoethanol	69
	3.0 μmoles of 2-mercaptoethanol	65
	6.0 μmoles of 2-mercaptoethanol	50
II	None	72
	Omit ATP	0
	Omit CTP	19
	Omit GTP	0

[a] Micromicromoles of labeled UMP incorporated per milligram of DNA incubated. All tubes contained in a final volume of 0.75 ml: 50 μmoles of Tris–HCl, pH 8.3; 50 μmoles of KCl; 40 μmoles of NaF; 7.0 μmoles of $MnCl_2$ in expt I and 5.0 μmoles of $MgCl_2$ in expt II; 1.0 μmole of ATP; 0.4 μmole each of CTP and GTP; 11 mμmoles of UTP containing 0.2 μc of ^{14}C; 0.05 ml of a saturated solution of $(NH_4)_2SO_4$ in expt I; enzyme containing 70 μg of DNA. Incubations (in duplicate) and sample preparations were as described under methods.

Aggregates were isolated from pooled femoral muscle of eight rats per group. Activity was elevated rapidly after treatment; a 2.7-fold increase was seen at 12 hr. When assays were performed using $(NH_4)_2SO_4$, the hormone effect was masked; enzymes from control and treated rats were stimulated to approximately the same high level of activity. In preliminary experiments it was observed that greater elevation of activity by ammonium sulfate occurred when Mn^{2+} was used in place of Mg^{2+} as the divalent cation, similar to data reported by Widnell and Tata (1964a) for the enzyme from rat liver. It was therefore desirable to test the masking effect of high ionic strength with Mn^{2+} and Mg^{2+} separately. The data in Table II show that complete masking of stimulation by treatment with testosterone occurred at high ionic strength regardless of the divalent cation present and that stimulation of enzyme activity by androgen was observed in assays with either cation.

Further evidence that stimulation of RNA polymerase activity by testosterone was the result of an effect on DNA-dependent RNA synthesis was obtained in experiments in which actinomycin D was injected together with androgen in castrates. Animals were sacrificed 3, 8, 16, and 18 hr after injection. In all cases, the stimulation of aggregate RNA polymerase activity by testosterone was completely blocked by administration of 250 μg/kg of actinomycin D.

Additivity of Testosterone Propionate and Growth

TABLE II: Effects of Testosterone Propionate in Castrated Rats on Activity of Aggregate RNA Polymerase from Skeletal Muscle Assayed at Low or High Ionic Strength with Mn^{2+} or Mg^{2+}.

Expt	Time of Injection (hr before sacrifice)	Divalent Cation	μg of DNA Incubated/ Tube	Incorporation[a]	
				$-(NH_4)_2SO_4$	$+(NH_4)_2SO_4$
I	0	Mn^{2+}	105	19	183
	12	Mn^{2+}	105	52	164
	20	Mn^{2+}	105	42	134
	26	Mn^{2+}	105	38	167
II	0	Mg^{2+}	85	45	135
	8	Mg^{2+}	85	83	121
	18	Mg^{2+}	85	85	121
	24	Mg^{2+}	85	87	125

[a] Micromicromoles of labeled UMP incorporated per milligrams of DNA incubated. Treated rats received 100 μg of testosterone propionate by intraperitoneal injection at the specified times; controls were injected with vehicle. Enzymes isolated from femoral muscles pooled from six to ten rats per group were assayed utilizing amounts specified by content of DNA. Reaction mixtures were as described in the legend to Table I. Incubations (in duplicate) and sample preparations were as described under Methods.

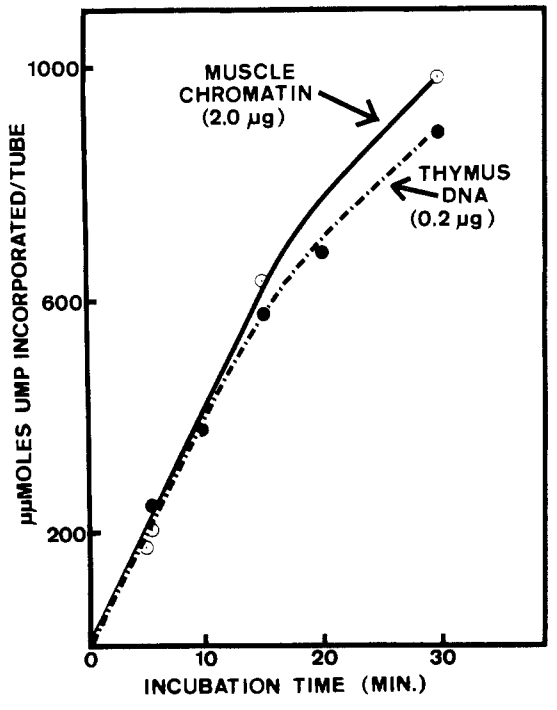

FIGURE 1: Time course of incorporation of labeled UMP into RNA directed by muscle chromatin or thymus DNA as source of template, with *E. coli* RNA polymerase. Reaction mixtures (0.25 ml) were identical with those of Chamberlain and Berg (1962) using 52 μg of bacterial polymerase (fraction 3) and amounts of DNA specified. Incubation conditions and sample preparations were as described under Methods. Note that ten times as much chromatin as DNA was incubated per tube.

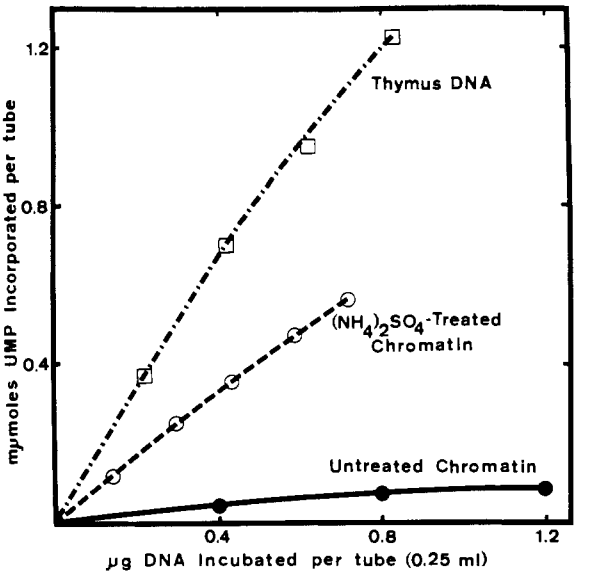

FIGURE 2: Effect of exposure to ammonium sulfate on template activity of chromatin from rat skeletal muscle. Incubation conditions and sample preparations were as described in the legend to Figure 1 and under Methods.

Hormone in Stimulating RNA Polymerase Activity. Castrated rats were injected with testosterone propionate, growth hormone, or both, at levels found to produce maximal stimulation of aggregate enzyme activity. Groups of six rats were sacrificed 16 hr after injection, and aggregates isolated from nuclei of muscle pooled from each group. The results of assays done with and

TABLE III: Additivity of Growth Hormone and Testosterone Propionate in Stimulating the Activity of Aggregate RNA Polymerase from Muscle of Castrated Rats.

Treatment	Incorporation[a]		Relative Activity (treated:control)	
	$-(NH_4)_2SO_4$	$+(NH_4)_2SO_4$	$-(NH_4)_2SO_4$	$+(NH_4)_2SO_4$
Saline	75	140	1.00	1.00
100 μg of testosterone propionate	117	121	1.56	0.86
200 μg of growth hormone	121	272	1.61	1.94
100 μg of testosterone propionate + 200 μg of growth hormone	177	284	2.36	2.03

[a] Micromicromoles of labeled UMP incorporated per milligram of DNA incubated. Groups of six castrates were injected intraperitoneally with the specified hormones 16 hr prior to sacrifice; controls received saline. Aggregate enzymes isolated from pooled femoral muscles of each group were assayed in reaction mixtures identical with those in the legend to Table I. Mg^{2+} (5 μmoles/tube) was the divalent cation used, and enzymes containing 150 μg of DNA were incubated in each tube. Incubations (in duplicate) and sample preparations were as described under Methods.

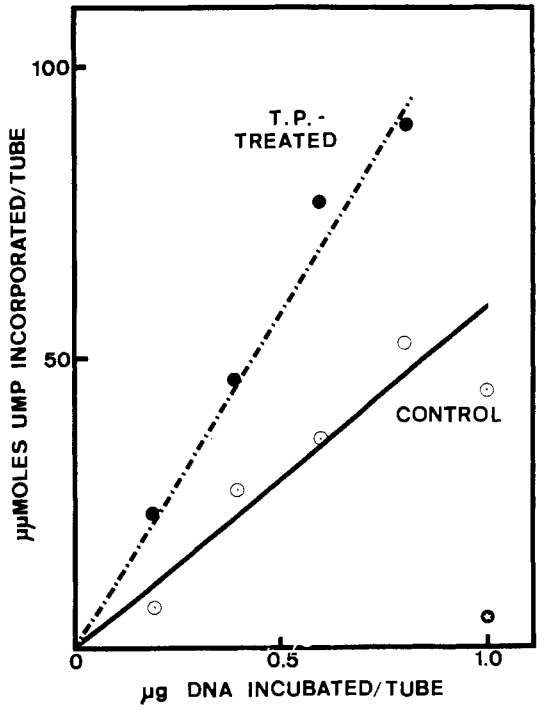

FIGURE 3: Increase of priming activity of chromatin from muscle of castrated rats after a single injection of testosterone propionate. Experimental details were as described in the Results; incubation conditions and sample preparations were as described in the legend to Figure 1 and under Methods.

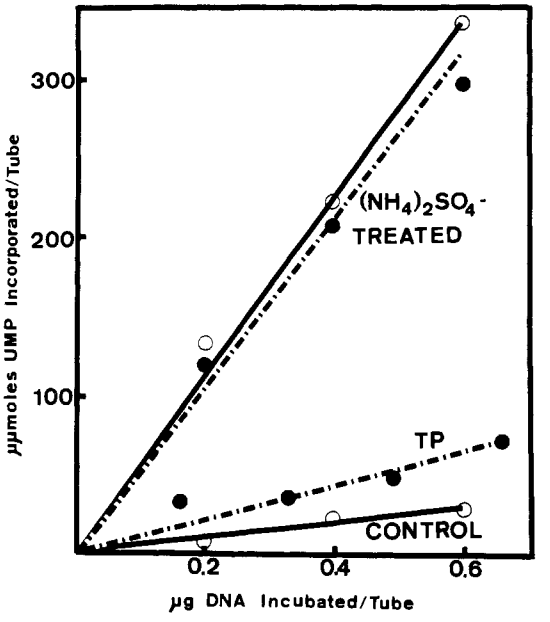

FIGURE 4: Effect of exposure to ammonium sulfate on template activity of chromatin from muscle of testosterone propionate treated and control castrates. Experimental details were as described in the Results; incubation conditions and sample preparations were as described in the legend to Figure 1 and under Methods.

without ammonium sulfate are shown in Table III. Administration of androgen increased activity of aggregate preparations; the effect was again masked by $(NH_4)_2SO_4$. The effect of growth hormone in stimulating aggregate activity was evident regardless of the presence or absence of ammonium sulfate. This result has been observed in many studies using growth hormone treated rats, and in assays of aggregate enzyme using either Mn^{2+} or Mg^{2+}. Treatment *in vivo* with a combination of both hormones produced a clearly additive stimulation of activity; again, assays done at high ionic strength showed no masking of hormonal stimulation.

Characteristics of Chromatin-Primed RNA Synthesis. The results shown in Table III indicated to us that

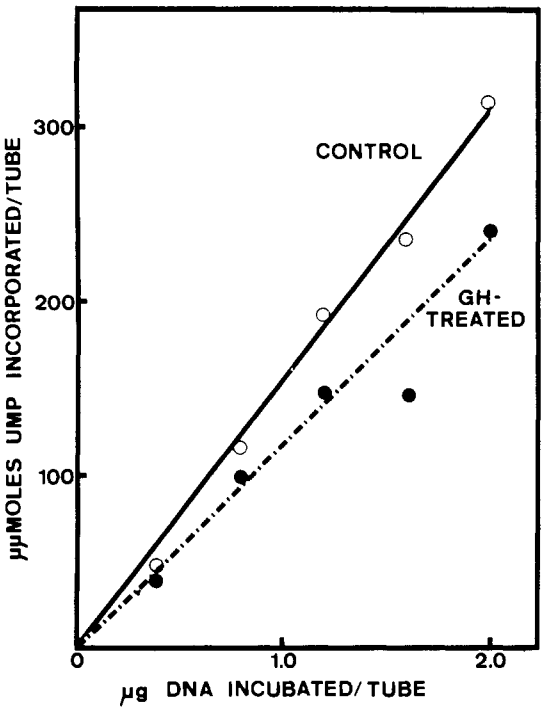

FIGURE 5: Priming activity of chromatin from muscle of hypophysectomized rats given growth hormone. Experimental details were as described under Results; incubation conditions and sample preparations were as described in the legend to Figure 1 and under Methods.

the two hormones act by different mechanisms, and that assays of priming activity of chromatin might provide greater insight into these mechanisms.

Using calf thymus DNA as primer for the polymerase from *E. coli*, incorporation of radioactivity from [^{14}C]UTP into RNA was proportional to either DNA or enzyme concentration, was inhibited by actinomycin D, required all nucleoside triphosphates, and was linear for 30 min. The chromatin-primed reaction exhibited similar properties. Our incubations were done with an excess of enzyme and triphosphates; the reaction was linear with amount of primer added (Figure 1). Although the priming activity of muscle chromatin (per microgram of DNA) was much lower than that of thymus DNA, the time course was identical up to 30 min using either primer; to obtain equal incorporation, one-tenth as much DNA as chromatin was incubated in this experiment.

Effects of Ammonium Sulfate on Activity of Muscle Chromatin. Exposure of chromatin to 0.3 M $(NH_4)_2SO_4$ during the isolation procedure caused a striking increase in the template activity of the preparation (Figure 2). The activity of ammonium sulfate treated chromatin was 7.4 times that of untreated chromatin, while that of thymus DNA was 15 times as active as untreated chromatin. Visual inspection of the pellet obtained after centrifugation of crude chromatin through 1.7 M sucrose containing 0.3 M ammonium sulfate showed a clean, translucent pellet obviously containing less protein than preparations not exposed to the salt. Addition of ammonium sulfate to the assay medium caused a 90% inhibition of RNA synthesis when either untreated chromatin or thymus DNA was used as primer. Thus it seems unlikely that the increased priming activity of chromatin exposed to $(NH_4)_2SO_4$ resulted from entrapment of the salt during isolation.

Effects of Testosterone Propionate and Growth Hormone on Activity of Chromatin from Muscle. Groups of 12 castrated rats were given a single injection of 2 mg/kg of testosterone propionate or 0.2 ml of 0.9% saline and sacrificed 18 hr later. Nuclei were isolated from pooled femoral muscles; each preparation was divided into two portions. One was used for preparation of aggregate enzyme and the remainder was utilized for isolation of chromatin. Results of assays of priming activity are shown in Figure 3. Chromatin from muscle of castrates given androgen exhibited greater activity than that of controls. Endogenous RNA polymerase activity of the chromatin preparations was low; it is indicated by the point in the lower right portion of the figure. Figure 4 illustrates the results of a study in which chromatin from muscle of castrates injected with either testosterone propionate or saline was treated with ammonium sulfate as described in the Methods section. It is evident that exposure to 0.3 M $(NH_4)_2SO_4$ masks the androgen-induced increase in priming activity; both preparations are equally as active as a template for the bacterial polymerase.

Groups of 12 hypophysectomized rats were given a single injection of porcine growth hormone (2 mg/kg) or of saline and were sacrificed 18 hr later. As in the previous experiments, aggregates and chromatin were isolated from muscle of both groups. Results of the assays of chromatin are shown in Figure 5. No increase was seen in template activity of chromatin from muscle of treated rats; in this study and in three similar experiments, the activity of chromatin from growth hormone treated rats has consistently been slightly lower than that of the controls.

Table IV shows the correlation obtained in these studies between priming activity of chromatin and of aggregate enzyme assayed with and without $(NH_4)_2SO_4$. The stimulatory effect of testosterone was not observed when assays were done at high ionic strength; that produced by growth hormone was not masked by high salt, as shown previously (Table III). Although both hormones stimulated RNA synthesis by aggregate preparations, only testosterone increased template activity of chromatin.

We observed a threefold difference in the template efficiency of chromatin from hypophysectomized and castrated rats; however, the activity of these preparations has been consistent from one experiment to another. It was surprising that hypophysectomy increased the priming activity of chromatin; we have been reluctant to assign great significance to the differences because rats from different suppliers were used. Nevertheless, in a recent study utilizing castrated rats from Charles River Laboratories, the activity of

TABLE IV: Correlation between Chromatin and Aggregate RNA Polymerase Activities from Muscle of Rats Given Growth Hormone or Testosterone Propionate.

Condition of Rats	Injections	Aggregate Enzyme Activity[a]		Chromatin Act.[a]
		$-(NH_4)_2SO_4$	$+(NH_4)_2SO_4$	
Hypophysectomized	Saline	48	174	178,000
Hypophysectomized	Growth hormone	71	270	136,000
	Treated:control	1.48	1.55	0.76
Castrated	Saline	37	231	59,000
Castrated	Testosterone propionate	89	291	117,000
	Treated:control	2.41	1.26	1.98

[a] Micromicromoles of labeled UMP incorporated per milligram of DNA incubated. Groups of 12 castrated or hypophysectomized rats were injected with 2 mg/kg of the appropriate hormone and sacrificed 18 hr later. Aggregate enzymes and chromatin were prepared from pooled femoral muscles. Incubation mixtures and details of the assay procedures were as described in legends to Table I, Figure 1, and Methods.

TABLE V: Effect of Muscle Chromatin Preparations on RNA Synthesis with Thymus DNA as Primer.[a]

Chromatin Added (μg of DNA/tube)	Source of Chromatin	$\mu\mu$moles of UMP Incorporated into RNA/Tube				
		Calculated				
		Primed DNA	Primed Chromatin	Total	Obsd	Calcd (%)
None		1473	—	1473	1473	100
0.5	Control	1473	27	1500	1319	87.9
0.3	Control	1473	16	1489	1316	88.4
0.1	Control	1473	5	1478	1458	98.6
0.42	TP treated	1473	43	1516	1325	87.4
0.25	TP treated	1473	26	1499	1494	99.6
0.08	TP treated	1473	8	1481	1424	96.1

[a] Incubation tubes contained the constituents listed under Figure 1 as well as 2.0 μg of thymus DNA in a final volume of 0.25 ml. The chromatin samples used were those described in Figure 2.

chromatin was on the same order as that from castrated and normal Carworth Farms animals.

It has been reported that protein associated with DNA must be removed from chromatin preparations for expression of activity as a template for RNA synthesis (Marushige and Bonner, 1966). In order to show that the lowered activity of chromatin from muscle of untreated castrates was not due to incomplete removal of these proteins or to the presence of a soluble inhibitor, the experiments summarized in Table V were performed. Chromatin from muscle of control and androgen-treated rats was added to reaction mixtures containing calf thymus DNA and *E. coli* polymerase. No inhibition could be attributed to the various preparations of chromatin; incorporation directed by the mixed primers was additive. This type of study was done also with aggregate enzyme preparations with similar results.

Growth hormone and testosterone were tested for effects *in vitro* on template activity of chromatin and on aggregate enzyme activity. Preparations were isolated from muscle of hormone-deficient rats and assayed in the presence of 0.1–10 μg/ml of the appropriate hormone. No stimulatory effects were observed, and slight inhibition was seen at higher concentrations of the hormones.

Discussion

The data presented in this and a previous paper (Florini and Breuer, 1966) provide evidence that both testosterone propionate and growth hormone stimulate the activity of RNA polymerase in rat skeletal muscle. The activity of the aggregate enzyme in this tissue is

low, approximately one-fortieth that of similar preparations from liver (C. B. Breuer, 1966, unpublished observations). The same order of activity was observed with intact muscle nuclei, indicating that no activity was lost during preparation of the aggregate. The low level of RNA polymerase in muscle is similar to that observed in uterine tissue (Gorski, 1964) and is consistent with the reduced RNA content in muscle compared to actively metabolizing tissues such as liver (Devi *et al.*, 1963). The vastly greater activity of chromatin from muscle assayed with bacterial RNA polymerase (Table IV) indicates that the amount of muscle RNA polymerase is limiting the extent of RNA synthesis, *i.e.*, only a small portion of the derepressed DNA is being transcribed at a given time in this tissue.

We have also shown that administration of androgen to castrates increased the priming activity of chromatin; exposure of muscle chromatin to 0.3 M ammonium sulfate during preparation masked differences in activity between samples from control and treated rats by elevating activities to the same high level. This is directly analogous to masking by $(NH_4)_2SO_4$ of androgen-induced increases in aggregate enzyme activity (Tables II and IV). The degree of masking observed in these studies ranged from 74 to 100%, indicating that although the predominant effect of high salt was to disguise the stimulatory action of testosterone propionate, other lesser effects might be involved. Similar results have been reported for a variety of other hormonally responsive systems (Gorski, 1964; Pegg and Korner, 1965; Liao *et al.*, 1965; Ramuz *et al.*, 1965; Barnabei *et al.*, 1966). Interpretation of the salt effect is thus particularly important because the mechanism of action of these hormones *in vivo* may be similar to the effect of high salt *in vitro*, as suggested by Pegg and Korner (1965). Ammonium sulfate may act by removing inhibitory proteins from DNA, in analogy with the effects of 4 M CsCl on protein content and priming activity of chromatin (Marushige and Bonner, 1966). However, the concentration of salt used in assays of aggregate enzyme is much lower than that employed to deproteinize DNA. It is also possible that ammonium sulfate affects the polymerase molecule, nucleases, or the binding of substrates. The salt may influence the physical configuration of DNA, thus affecting chain initiation or the read-out efficiency of RNA polymerase. If is therefore not suprising that some disagreement exists over interpretation of the effects of ammonium sulfate (Goldberg, 1961; Widnell and Tata, 1964b; Pegg and Korner, 1965; Chambon *et al.*, 1965). Our results make clear that the stimulatory effects of ammonium sulfate can be localized in the primer portion of aggregate enzyme preparations. The only reported effects of added $(NH_4)_2SO_4$ or other constituents of aggregate preparations have been inhibitory (Chambon *et al.*, 1965), and the results of our intermixing experiments (Table V) demonstrate that the salt effect is not due to influences on inhibitors in either chromatin or aggregate preparations. Whether stimulation of priming activity can be attributed to removal of inhibitory proteins or to changes in the structure of the primer cannot be decided on the basis of our data; the demonstrated deproteinizing action of high salts (Marushige and Bonner, 1966) makes the former interpretation more likely.

Although priming activity of muscle chromatin was enhanced by treatment of rats with testosterone propionate, administration of growth hormone did not produce this effect even though increases in the activity of aggregate enzyme were observed. This indicates that the action of growth hormone in stimulating RNA synthesis in muscle does not involve substantial activation of the DNA primer. Growth hormone may then affect RNA polymerase by increasing the synthesis or activity of the enzyme; further evidence is necessary to confirm this supposition.

Evidence is accumulating to show that several hormones stimulate RNA synthesis by influencing the priming efficiency of DNA. This is observed either as an increase in priming activity of chromatin (Dahmus and Bonner, 1965; Kim and Cohen, 1966) or as stimulation of RNA polymerase activity detectable in assays done at low ionic strength. The effects of administration of testosterone propionate on chromatin and aggregate enzyme activities in muscle provide another instance of this mode of action. We have shown that growth hormone does not stimulate RNA synthesis in muscle by this mechanism. The differential actions of the two hormones can explain their known additivity in promoting body weight gain and nitrogen retention; a stimulation of priming activity of chromatin and of RNA polymerase activity would act as a concerted mechanism allowing greater effects on RNA synthesis than either action by itself. There is a considerable lag period prior to the effects we observe on RNA synthesis, indicating that the primary biochemical response of muscle to androgen and to growth hormone may involve other sites within the cell. However, the observations that the two hormones do affect RNA synthesis differently does provide a basis whereby additivity of anabolic activities can occur.

In addition, we have demonstrated that the effect of ammonium sulfate in increasing the activity of aggregate enzyme preparations can be traced to an action on the primer portion of the complex. Since treatment with $(NH_4)_2SO_4$ of chromatin from muscle of castrates given androgen abolishes the increase in priming activity over that of controls, further evidence is not at hand showing that one action of this hormone is to mimic the effects of high salt, *i.e.*, to increase the efficiency of DNA as a template for RNA polymerase.

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Flavoenzyme Models. I. Flavin Free-Radical Formation in the Reduced Nicotinamide–Adenine Dinucleotide–Flavin Mononucleotide System*

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ABSTRACT: The mode of production of flavin free radical was investigated in a reduced pyridine nucleotide dehydrogenase model system. Reaction kinetics were followed optically at 340, 445, 570, and 900 nm and by electron paramagnetic resonance spectrometry, using an anaerobic stopped-flow apparatus. The identity of the 570-nm absorbing species and the flavin free radical is confirmed by kinetics and a molar absorption coefficient of 8000–10,000 cm^{-1} M^{-1} is obtained. A comparison of the various biological analogs of the coenzymes was also made. It was found that flavin–adenine dinucleotide (FAD) has an enhanced rate of flavin free-radical formation as compared with flavin mononucleotide (FMN). The direct formation of flavin free radical *via* a single electron transfer from reduced nicotinamide–adenine dinucleotide (NADH) to FMN is ruled out. A mechanism which satisfactorily accounts for all of the data is presented.

Flavoenzymes often function as mediators between one-electron- and two-electron-transfer processes. Free-radical signals have been observed in a number of flavoenzymes and the flavin free radical has been invoked as the species responsible (Beinert and Palmer, 1965). Inasmuch as the experimental difficulties encountered in attempts to elucidate the mechanism of reaction of flavoenzymes are formidable, it was felt that an examination of a model system might lead to new insight into mechanisms of reaction.

The model chosen for this study was a reaction system

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