CORTISOL EFFECT ON PROTEIN SYNTHESIS AND RNA POLYMERASE ACTIVITY IN RAT THYMUS

by

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DEDICATION

This thesis is dedicated to my wife, Marodean, who provided patience, work, love and understanding; and to my son, William, who provided inspiration and joy. Their conscious and unconscious efforts have been paramount to its completion.

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TABLE OF CONTENTS

Title Page	i
Approval Page	ii
Dedication	iii
Acknowledgments	iv
Table of Contents	v, vi, vii
List of Tables	viii
List of Figures	ix, x, xi
INTRODUCTION	1
Bacterial RNA Polymerase	3
Isolation and Purification of Bacterial RNA Polymerase	3
Stability of Bacterial RNA Polymerase	4
Structure of Bacterial RNA Polymerase--Subunits and Functional Correlations	5
The Binding of Bacterial RNA Polymerase to DNA--Specific vs. Non-specific Binding	8
RNA Synthesis by Bacterial RNA Polymerase	11
Inhibitors of Bacterial RNA Polymerase Activity	15
Eucaryotic RNA Polymerases	16
Isolation, Purification and Stability of Eucaryotic RNA Polymerases	16
Characterization of Eucaryotic RNA Polymerases	20
Factors Altering Eucaryotic RNA Polymerase Activities	22
Inhibitors of Mammalian RNA Polymerase Activities	23
Hormonal Effects in Mammalian Systems	24
Hormonal Regulation of Growth and Differentiation Through RNA Synthesis	24

Cortisol Effects on Transport Phenomena in Thymocytes 27

Cortisol-induced Catabolism in Thymocytes 28

STATEMENT OF THE PROBLEM 35

MATERIALS AND METHODS 37

Animals 37

Chemicals and Drugs 37

Preparation and Extraction of Labelled RNA Polymerase 38

Sucrose-Glycerol Gradient Purification of RNA Polymerase 41

RNA Polymerase Assay System 42

Electrophoretic Examination of RNA Polymerase 43

Determination of Radioactivity 45

DNA Determinations 46

Treatment of Data 47

Thymus Involution and Regeneration 48

RESULTS 50

Thymus Involution and Regeneration 50

Time Course of Protein Precursor Incorporation in Thymocyte Suspension Cultures 56

Examination of Some Gradient Parameters 56

Cortisol Effects on RNA Polymerase Activity and Synthesis 60

Inhibition of ^{3}H-leucine Incorporation in Proteins of Thymus Nuclear Extract After Cortisol Treatment as a Function of Sedimentation Rate 66

Gel Electrophoresis of Proteins Associated with RNA Polymerase Activity at Various Stages of Purification 68

Search for Possible Modifiers of RNAP Activity 79

DISCUSSION

Thymus Involution and Regeneration 82

Cortisol Effects on RNA Polymerase Activity and Synthesis 85

Gel Electrophoresis of Proteins Associated with RNA Polymerase Activity at Various Stages of Purification 90

Search for Possible Modifiers of RNAP Activity 94

SUMMARY AND CONCLUSIONS 100

APPENDIX I 104

APPENDIX II 107

BIBLIOGRAPHY 119

LIST OF TABLES

Table

I. Total Recovery of RNAP Activity on Gradients of Different Composition 59

II. Thymus Weights and RNAP Activity of Nuclear Extracts and ^{3}H-Leucine (^{3}H-Leu) Incorporation into Thymocytes, Nuclei and Nuclear Extracts 62

III. Total Cortisol-treated RNAP Activity and ^{3}H-Leucine Incorporated in Peaks of RNAP Activity for Gradients I and II, as Compared to Nuclear Extracts 67

IV. ^{3}H-Leucine Incorporation vs. Position on Gradient I for Nuclear Extract Proteins Labelled in Vitro Twelve Hours After Treatment in Vivo with Cortisol or Vehicle 69

LIST OF FIGURES

Figure

1. Thymus wet weights (mg. thymus/g. rat) at various times after cortisol and vehicle injection in vivo. 51

2. Incorporation of ^{3}H-uridine and ^{14}C-leucine into thymocytes in vitro at various times after cortisol and vehicle injections in vivo. 52

3. Incorporation of ^{3}H-thymidine and ^{14}C-leucine into thymocytes in vitro at various times after cortisol and vehicle injections in vivo. 53

4. Time course for ^{3}H-leucine and ^{14}C-tryptophan incorporation by thymocyte suspension cultures. 57

5. Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro twelve hours after treatment with cortisol or vehicle in vivo. Experiment No. 3. 64

6. Gradient II fractionation of aggregated fractions of a gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro twelve hours after treatment with cortisol or vehicle in vivo. Experiment No. 3. 65

7. Electrophoretic migration patterns of amido-black staining material in cortisol and vehicle gels of various thymocyte fractions. 71

8. Distribution of amido-black staining material after gel electrophoresis of sucrose-glycerol gradient I purified RNA polymerase. 75

9. Distribution of amido-black staining material after gel electrophoresis of sucrose-glycerol gradient II purified RNA polymerase. 76

10. CPM (^{3}H-leu)/mg DNA present in gels layered with aliquots of fractions from cortisol and vehicle second gradients (GII). 78

11. Gradient I and gradient II fractionations of nuclear extract proteins. Thymocytes were labelled in vitro three hours after treatment with cortisol or vehicle in vivo. Experiment No. 1. 108

Figure

12. Gradient I and gradient II fractionations of nuclear extract proteins. Thymocytes were labelled in vitro three hours after treatment with cortisol or vehicle in vivo. Experiment No. 1. 109

13. Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro three hours after treatment with cortisol or vehicle in vivo. Experiment No. 1. 110

14. Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro six hours after treatment with cortisol or vehicle in vivo. Experiment No. 2. 111

15. Gradient II fractionation of aggregated fractions of a gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro six hours after treatment with cortisol or vehicle in vivo. Experiment No. 2. 112

16. Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro six hours after treatment with cortisol or vehicle in vivo. Experiment No. 2. 113

17. Gradient II fractionation of aggregated fractions of a gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro six hours after treatment with cortisol or vehicle in vivo. Experiment No. 2. 114

18. Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro six hours after treatment with cortisol or vehicle in vivo. Experiment No. 2. 115

19. Gradient I and gradient II fractionations of nuclear extract proteins. Thymocytes were labelled in vitro twelve hours after treatment with cortisol or vehicle in vivo. Experiment No. 3. 116

20. Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro twelve hours after treatment with cortisol or vehicle in vivo. Experiment No. 3. 117

Figure

21. Gradient II fractionation of aggregated fractions of a gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro twelve hours after treatment with cortisol or vehicle in vivo. Experiment No. 3. 118

INTRODUCTION

Inhibition of RNA synthesis in the rat thymus occurs early after cortisol treatment (Gabourel and Aronow, 1962; Makman, Dvorkin and White, 1966; Fox and Gabourel, 1967). Because RNA is an intermediate in the conversion of genetic information into protein molecules, studies to determine how cortisol affects RNA synthesis are warranted. Recent work suggests that cortisol may alter RNA synthesis by an effect on the enzyme RNA polymerase (Gabourel and Fox, 1969, 1971; Nakagawa and White, 1970). An understanding of the enzyme RNA polymerase and the mechanisms which control RNA synthesis is required for an approach to the problem of cortisol-mediated thymus involution. In the next several pages, the scientific literature is reviewed to provide a description of the properties and characteristics of RNA polymerase and some of the mechanisms which affect RNA synthesis.

RNA polymerase (nucleoside triphosphate: RNA nucleotidyltransferase [DNA dependent] EC 2.7.7.6) is an ubiquitous enzyme catalyzing the synthesis of RNA by the DNA dependent sequential reactive addition of nucleoside triphosphates. It has an absolute requirement for all four nucleoside triphosphates, divalent cation and DNA primer for polynucleotide synthesis. The state of RNA polymerase (RNAP) aggregation and its specificity of binding to DNA is strongly influenced by ionic strength, template, substrate, and enzyme concentration.

The specificity of RNA synthesis on DNA by viral and bacterial RNAPs is regulated by a complex interplay of moderating factors (Travers, 1971). In the case of altered transcription specificity in viral and bacterial RNAP, there may be de novo synthesis of a new polymerase or one or more

of its core subunits[1]. Various protein modifiers may be affected which are not part of the core enzyme, but which mediate the attachment of RNAP to DNA (e.g., the initiator sigma). Protein modifiers may be produced which alter initiation specificity by combining with initiators or the core enzyme (Travers, 1971). Protein elements are demonstrable in eukaryotic cells which stabilize, stimulate, or inhibit RNAP activity, and these elements alter transcription specificity in that they change the RNAP preference between native and denatured DNA template (Furth, Nicholson and Austin, 1970).

Additional and interacting mechanisms for control of RNA synthesis may include repressors and positive control elements that combine with DNA or DNA components, and the transfer, acetylation, and methylation of nucleoprotein histones.

In bacterial mutants containing temperature-sensitive RNA polymerase (Igarashi and Yura, 1969), increasing the culture temperature (from 30 to 45° C) blocks the production of all forms of RNA. This would seem to indicate that bacteria contain a single enzyme species. Furthermore, synthesis of all RNA species was inhibited similarly by streptovaricins and a one-step mutation for resistance led to resistance of all species of RNA.

In eukaryotic tissues, however, there are polymerases, chromatographically separable, localized in the nucleoli and in the nucleoplasm (Roeder and Rutter, 1969, 1970; Goldberg and Moon, 1970). These RNAP's apparently mediate, respectively, ribosomal and non-ribosomal RNA

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[1] The core enzyme consists of the subunits α, β and β'; see Structure of Bacterial RNA Polymerase -- Subunits and Functional Correlations.

synthesis. Whether these polymerases differ in basic structure, or in the number and/or nature of initiators and regulatory subunits, is one of the current questions concerning RNAP.

Bacterial and mammalian RNAP's have been found to evidence different rates of transcription on the same DNA template. Thus, E. coli RNAP transcribes rat liver DNA 25 times faster than rat liver polymerase (Jacob, Sajdel and Munro, 1970). This may reflect more complex control mechanisms in the mammalian polymerase forms, e.g., in the recognition of initiation sites, or in the presence or absence of transcription stimulators; or, it may reflect procedural difficulties in extracting a complete, effective mammalian polymerase. Methods of extracting the bacterial RNAP have been the most successful until recently and thus, most of the physical and kinetic studies of RNAP have been done on the bacterial form.

BACTERIAL RNA POLYMERASE

Isolation and Purification of Bacterial RNA Polymerase. Since RNA polymerase was first demonstrated about 12 years ago (Weiss and Gladstone, 1959), a steady evolution of technique for the purification of bacterial RNA polymerase has occurred. These methods have included the use of protamine and/or streptomycin for protein precipitation, DEAE cellulose column purification (Chamberlin and Berg, 1962; Stevens and Henry, 1964; Maitra and Hurwitz, 1967); and the sequential use of DEAE cellulose, ammonium sulfate precipitation, hydroxylapatite and DEAE-Sephadex columns (Tada and Tada, 1970).

Burgess, Travers, Dunn and Bautz (1969) and Burgess (1969) have recently published methods for extracting large amounts of relatively pure E. coli RNAP. These methods included centrifugation, ammonium sulfate precipitation, and purification through DEAE cellulose, phosphocellulose, and Bio-Gel A-1.5 meter columns. The net result was 100 to 300 mg of 98% pure RNAP from a kilogram of E. coli, with an increase in specific activity of 266 and a yield of 56% as compared to crude extracts.

<u>Stability of Bacterial RNA Polymerase</u>. Nakamoto, Fox and Weiss (1964) demonstrated essentially complete stabilization of protein-precipitated, DEAE-cellulose purified, RNAP of M. lysodeikticus for several months in 50% glycerol at -20° C. RNAP that was purified by precipitation, followed by electrophoretic focusing in 40-45% glycerol, was stable for six weeks at 0° C. Dialysis markedly reduced activity, unless against 30-50% glycerol or 1-2 M sucrose. The RNAP was stable at 30° C for several hours, while at 50° C the enzyme lost 20% of its original activity in 20 minutes. At 54° C, five-, ten-, and twenty-minute incubations led to a 29, 49, and 68% loss of the original activity, respectively.

E. coli RNAP showed only about 50% of applied enzyme activity on sucrose gradients centrifuged for six hours, regardless of whether the enzyme was DNA bound or free. Bovine serum albumin (BSA, 250 µg/ml), when added before centrifugation, prevented further loss at low concentrations, but did not prevent the 50% loss of enzyme activity at both high and low concentrations of polymerase (Richardson, 1966). It is

unclear whether this loss of activity was due to denaturation or to the loss of an initiator or stimulator.

Structure of Bacterial RNA Polymerase -- Subunits and Functional Correlations. Travers and Burgess (1969) and Burgess (1969) found that the E. coli RNAP consisted of four protein subunits, α, β, β', σ , in the ratio of 2:1:1:1, for a total molecular weight of approximately 495,000 $\pm$ 50,000 daltons (α mw 39,000 $\pm$ 5%, β 155,000 $\pm$ 10%, β' 165,000 $\pm$ 10%, σ approximately 95,000). There was also a small polypeptide chain (ω) with a molecular weight of approximately 12,000 daltons, which may have been a tightly bound contaminant or an actual portion of the enzyme (Burgess, 1969).

Zillig, Fuchs, Palm, Rabussay and Zechel (1970) found that the RNAP subunits were reversibly dissociable in 6 M urea, but irreversibly dissociable in detergents such as sodium dodecyl sulfate. The authors found that sigma could be collected as an acetone-dried powder after preparative cellogel electrophoresis, and dissolved in 6 M urea at pH 10.5 to 11. Cellogel electrophoresis then suggested sigma to be made up of two subunits, μ and ν, in the ratio 1:2. When β' was treated with heparin, it migrated to the same position on cellogel as β, suggesting β' to be composed of β and a polycation.

The sigma factor controlled initiation (Burgess, Travers, Dunn and Bautz, 1969; Burgess, 1969; Darlix, Sentenac, Ruet and Fromageot, 1969) and appeared to do so with some specificity. At some undefined point after the sigma subunit had initiated transcription, sigma was released from the enzyme-DNA complex and was free for reutilization (Travers and

Burgess, 1969; Travers, 1971). The phosphocellulose purified enzyme did not contain the initiation factor sigma, and was called the core enzyme. Whole enzyme could be obtained by centrifugations on glycerol gradients or by DNA-cellulose column filtration (Burgess, Travers, Dunn and Bautz, 1969).

Core E. coli RNAP (enzyme without the initiator sigma) transcribed calf thymus DNA with about one-half the efficiency of the holoenzyme (complete enzyme consisting of core enzyme and initiator sigma). In contrast, core E. coli RNAP transcribed the viral T4 genome randomly and with very poor efficiency, while the holoenzyme transcribed a specific portion of the T4 genome with great efficiency (Burgess, Travers, Dunn and Bautz, 1969).

When E. coli RNAP was labelled before T4 infection by incubating the bacteria with a radioactive protein precursor, the α, β, and β' subunits were found to be conserved throughout the infection (Travers, 1971) although the migration patterns of α and β' were altered upon electrophoresis (Losick, Shorenstein and Sonenshein, 1970). The E. coli containing sigma was responsible for transcription of only a small fraction of the T4 genes, occurring in the first one to five minutes of infection (Bautz, Bautz and Dunn, 1969). The sigma initiator was lost after the first few minutes of infection (Crouch, Hall and Hager, 1969) and was replaced by a new initiator which was apparently synthesized by the T4 (Travers, 1969). In a covalent reaction that was half completed in two minutes the E. coli RNAP α subunit was modified by the addition of 5' adenylate (Travers, 1971).

This modified E. coli RNAP transcribed a different portion of the T4 genome than that which was transcribed under unaltered host RNAP with the initiator sigma (Travers, 1969). There was a later modification (10-15 minutes after infection) of the β' subunit, with a net increase in negative charge, before 'late' T4 genes were transcribed. Thus, phage-induced changes in the host bacterial RNAP subunits and initiators appeared to direct the transcription of 'early' and 'late' portions of the T4 genome.

There also may be initiators and subunit changes in various phases of bacterial development. RNAP in sporulating B. subtilis does not contain initiator present during the vegetative phase. The sporulation RNAP also has a small β subunit, and will not read genes transcribed during the vegetative phase (Sonenshein and Losick, 1970). The phage ϕe requires the vegetative phase RNAP initiator for effective DNA transcription and thus cannot infect sporulating B. subtilis.

E. coli RNA polymerase without sigma transcribed phage fd DNA well but lost the ability to select the appropriate initiation sites, and both phage strands were copied. Adding sigma factor restored the asymmetric specificity of initiation (Sugiura, Okamoto and Takanami, 1970).

Initiators and subunits (which may be altered to accommodate new initiators) may thus be looked upon as positive and negative control elements which turn on the polymerase in respect to a given operon or group of operons, while turning off the polymerase in respect to other operons. The initiators and subunits may themselves be subject to modification by other elements and effectors. It remains to be determined

whether different initiation factors direct a single core enzyme to produce ribosomal or non-ribosomal RNA on the bacterial genome.

Chelala, Hirschbein and Torres (1971) have presented evidence of active and inactive, interconvertible forms of E. coli RNAP. Conversion of the active to the inactive form was mediated by a protein subunit and required Mg^{++}-ATP. The inactive form contained a covalently bound adenylic acid residue. Conversion from the inactive to an unadenylated active form required Mg^{++}.

The Binding of Bacterial RNA Polymerase to DNA -- Specific vs. Non-Specific Binding. Binding of RNAP to DNA was found to be rapid (even at $0^{o}C$) and reversible. Binding was very sensitive to ionic strength and pH, and did not require Mg^{++} (Richardson, 1966). Non-specific binding was favored by lower ionic strengths, while higher ionic strengths decreased the total binding but increased the specificity of binding and the rate of RNA chain growth. Ionic strengths of 0.35 (NaCl or KCl) prevented effective binding of E. coli RNAP to T7 DNA (Jones and Berg, 1966). Ionic conditions were also important for proper strand (site) selection, and these conditions differed, e.g., between M. lysodeikticus and E. coli (Richardson, 1969). Richardson (1966) reported that no binding occurred when the ionic strength was raised (from 0.1 to 0.5 with KCl) to dissociate the 21S form of E. coli RNAP into 13S forms. Aggregation-disaggregation of RNAP was rapidly reversible as conditions were altered. An ionic strength of 0.5 was very high, however, and lack of binding was probably not a function of the 13S form, but of excessive ionic strength. Thus, Pettijohn and Kamiya (1967) demonstrated that

no attachment of E. coli RNAP to polyoma DNA occurred in a Tris binding buffer (pH 7.8) at ionic strengths of 0.35 or higher.

Smith, Martinez, Ratliff, Williams and Hayes (1967) demonstrated that the state of aggregation of RNAP was DNA dependent and presented evidence that the 13S form was active. The freshly isolated E. coli RNAP existed as an aggregate of 21-24S, or even higher, forms in low ionic strength buffer (0.15 or less), but was dissociated to the 13S form by the presence of short polydeoxynucleotides. Smith et al. (1967) suggested that Richardson's finding of activity of higher forms (1966) was a function of the template used. Smith et al (1967) noted, however, that centrifugation for identification of S value could be misleading if a reaggregation of RNAP molecules occurred in the assay mixture showing enzymatic activity.

Richardson (1969) has since pointed out that the evidence favors the 13S form as the active unit. The 50 binding sites on the T7 DNA molecule represented 1/15 to 1/20 the amount which could be accommodated if linear packing occurred. Electron micrographs confirmed this finding (Slayter and Hall, 1966). Pettijohn and Kamiya (1967) found that low ionic strength (below 0.15-0.18) favored non-specific binding of E. coli RNAP (21S form) to T7 DNA, which was limited only by space on the DNA, and was virtually inactive enzymatically. At higher ionic strengths (0.18 and higher), where optimal enzyme activity occurred (13S form), maximum polymerase binding was much reduced and presumably more specific.

Thus, ionic strength is extremely important in optimizing RNAP activity on any given template and should be determined for each system used. The ionic conditions described are those using bacterial RNAP on

viral DNA and may not optimize the binding and activity of the eukaryotic RNAP on mammalian DNA.

Jones and Berg (1966) found that E. coli RNAP and T7 DNA separately passed through a 0.45 μ Millipore filter, but that the complex was retained. This technique permitted the estimation of 35-70 (average, 50) binding sites for RNAP per mole of T7 DNA. The enzyme-DNA complex was clearly a function of both secondary structure and nucleotide sequence of the nucleic acid. Thus, denatured single-stranded T7 DNA, or dAT, bound approximately 25 times more polymerase than did native helical DNA.

Stead and Jones (1967) studied competition between T7 ^{3}H-DNA and T7 DNA containing a full complement of RNAP. Their experiments determined binding through retention of RNAP-T7 ^{3}H-DNA on Millipore filters, using a Tris-$MgCl_2$ binding buffer (pH 8.0) without nucleoside triphosphates. Accepting an average figure of 50 RNAP binding sites per mole of T7 DNA, the authors demonstrated that RNAP binding was relatively irreversible on only eight of these sites. RNAP binding to the other 42 sites was relatively free dissociable, with differences in site affinity for RNAP as high as eight- to tenfold between the two types of binding. Denaturation increased the number of strongly bound sites approximately fifteen-fold. Perhaps the irreversible sites represent specific sites which initiate RNA synthesis rather than non-specific sites, including those on breaks and ends.

<u>RNA Synthesis by Bacterial RNA Polymerase</u>. RNAP synthesis of RNA on DNA template may be outlined (Goldthwait, Anthony and Wu, 1970) as follows:

Association: DNA + RNA polymerase - $\sigma \rightleftharpoons$ DNA-enzyme-σ

$$\text{DNA-E-}\sigma + \text{XTP}_1 \rightleftharpoons \text{DNA-E}\overset{\sigma}{\text{—}}\text{XTP}_1 + \text{XTP}_2 \rightleftharpoons$$

Initiation: $\text{DNA-E}\overset{\sigma}{\text{—}}\text{XTP}_1\text{--XTP}_2 \rightleftharpoons \text{DNA-E-XTP}_1\text{-XMP}_2 + \text{PP}_i + \sigma$

Polymerization: 1. Migration of polymerase along the DNA template

2. $\text{DNA-E-XTP}_1\text{-XMP}_2 + \text{XTP}_3 + \ldots + \text{XTP}_n \rightleftharpoons$

$\text{DNA-E-XTP}_1\text{-XMP}_2\text{-XMP}_3 \ldots \text{XMP}_n + (\text{PP}_i)_{n-2}$

5' phosphate ⟶ 3' phosphate end

Release: $\text{DNA-E-XTP}_1 \ldots \text{XMP}_n + \overset{*}{\rho} \rightleftharpoons \text{DNA-}\overset{\rho}{\text{E}}\text{-XTP}_1\ldots\text{XMP}_n \rightleftharpoons$

$\text{DNA} + \text{E} + \text{XTP}_1\ldots\text{XMP}_n + \rho$

* rho (ρ) factor of Roberts (1969), see following.

The sigma factor appears to facilitate binding of the first nucleoside triphosphate in such a way as to permit formation of a phosphodiester bond with the incoming second triphosphate, perhaps stabilizing the initiation complex, e.g., by local "melting" of the DNA in order to allow further hydrogen bonding for RNA synthesis between nucleotides and DNA (Bautz, Dunn, Bautz, Schmidt and Mazaitis, 1970).

RNA synthesis in vitro most often starts with a purine residue, initiation occurring at runs of pyrimidine residues in the template (Chamberlin and Berg, 1962; Maitra and Hurwitz, 1965). Richardson (1969) cited evidence and developed an argument for binding of polymerase to runs of cytidine (or thymidine) for orientation of RNAP. Proof of this argument waits on methods of fixing polymerase at the initiation site.

Maitra and Hurwitz (1965) have demonstrated that DNA template denaturation results in an increase in purine ribose 5' β,γ ^{32}P triphosphate incorporation. The 5'β and γ ribose phosphates are only conserved at the initiation of the RNA strand. In RNA chain growth the β and γ phosphates are released in pyrophosphate exchange, with only the α phosphates retained in a phosphate ester bond between nucleotides. Thus, denaturation may increase functional transcription sites. Bremer and Bruner (1968) have shown that denaturation results in a decreased ATP/GTP initiation ratio as compared to native DNA.

Roberts (1969) has isolated a protein (ρ-factor) from E. coli which causes specific termination and release of RNA in vitro. Whether ρ binds to template or polymerase to mediate termination is unknown, but apparently there are specific terminator sequences on the template. Anti-terminator proteins have been postulated (Schmidt, Mazaitis, Kasai and Bautz, 1970) in phage pre-early to early gene replication, in addition to phage-specific initiators.

In low salt conditions where binding occurs within a few seconds, it takes several minutes at 37° C for initiation of 50% of the chains to occur (Maitra and Hurwitz, 1965). It has been suggested that local DNA unwinding may be the rate-limiting step for initiation from double-helical

DNA (Walter, Zillig, Palm and Fuchs, 1967), as both initiation and DNA denaturation are strongly temperature-dependent. The initiation lag is not seen when denatured DNA is used as template, although this may be due to a loss of transcription specificity. It is also possible that initiation rate-limitation is a function of the binding of the first nucleotide or the reaction of the first two nucleotides.

As ionic strength is raised from 0.05 to 0.2 M KCl, the rate of RNA chain growth in vitro is increased from approximately 2.5 to 20 nucleotides per second, and each polymerase molecule is capable of initiating several RNA chains. At lower ionic strengths (0.05 M KCl), RNAP ceases synthesis much more quickly, probably due to combination of RNA with DNA template. RNA is released from this complex slowly, and apparently hinders the initiation of new RNA chains. RNAP complexes with single-stranded DNA are not as stable as those between RNAP and double-stranded DNA. When single-stranded DNA is used as template the RNA's produced are much smaller and new molecules continue to be initiated, even in low salt buffers (Richardson, 1969). If RNA is not released from DNA as a strand is completed, it may hold RNAP on the complex as well.

Millette and Trotter (1970) demonstrated release of RNA chains at low ionic strength, but a failure of reinitiation, suggesting a product inhibition. Many product RNA's inhibit RNA polymerase apparently by combining with a site on the enzyme rather than hybridizing with the DNA primer (Tissieres, Bourgeois and Gros, 1963; Richardson, 1966). Inhibition of RNAP by exogenous RNA requires the addition of RNA before synthesis begins. In a fashion not yet clearly defined, high salt prevents

RNA product or exogenous RNA inhibition of the polymerase and thus provides a rationale for continued RNA synthesis under these conditions (So, Davie, Epstein and Tissiere, 1967; Fuchs, Millette, Zilling and Walter, 1967).

Although there have been reports of increased efficiency of denatured template (Bremer and Bruner, 1968; Jones and Berg, 1966), there have also been many reports of decreased efficiency of denatured template as compared to native template (Fox and Weiss, 1964; Maitra and Hurwitz, 1965; Furth, Rosenberg and Ho, 1967; Furth, Nicholson and Austin, 1970). These contradictory findings may reflect,

(a) different modes of polymerase preparation and/or different forms of polymerase,

(b) the loss of specific RNAP modifiers during preparation of the enzyme,

(c) different assay conditions, and

(d) different template conditions.

Our laboratory has shown (unpublished observations) that crude rat thymus RNAP preparations have a higher activity on native DNA template, while purified RNAP preparations prefer denatured DNA template. This finding suggests the loss with purification of modifier(s) which control the specificity and/or rate of transcription.

Decreased efficiency on denatured template may be due to a specific degradation of the RNA moiety of RNA-DNA hybrids by ribonuclease H (Hausen and Stein, 1970) which is found in all but the purest RNAP preparations. The deoxyribose groups in the hybrid are essential, although, in a fashion not yet understood, double-stranded DNA protects

the RNA chain from ribonuclease degradation. The single-stranded DNA hybrid with RNA leaves the RNA strand susceptible to degradation. Bovine thymus is a good source of ribonuclease H.

Ishihara and Hurwitz (1969) reported that any of the four nucleoside triphosphates will bind to E. coli RNAP without template present, while with template, binding became more specific for purines. Triphosphates continued to bind to RNAP in the presence of thio reagents (p-chlormercuribenzoate, mercuric chloride, N-ethylmaleimide, cyanuric fluoride), but the polymerase would not interact with DNA. Conversely, amino group reagents (β-naphthoquinone-4-sulfonic acid, monochlorotrifluorobenzoquinone) permitted template binding but inhibited interaction with triphosphates. Histidine reagents (diazonium-1-tetrazole, rose bengal; with photooxidation) permitted template and triphosphate binding and pyrophosphate exchange, but blocked chain elongation. Three of the four polymerase activities (template binding, triphosphate binding, pyrophosphate exchange, chain elongation) may thus be chemically discriminated.

Inhibitors of Bacterial RNA Polymerase Activity. Inhibitors of polymerization include antibiotics (actinomycin D, miracil D, aflatoxin, daunomycin and mitramycin), ATP analogues, and polyanions. Inhibitors of initiation which act on the polymerase include high salt concentrations, polynucleotides and polyanions such as heparin (Richardson, 1966). Antibiotics which bind to DNA are effective inhibitors of bacterial and animal polymerases, while antibiotics which bind to the polymerases are more selective.

The antibiotic rifamicin (and synthetic derivatives, such as rifampicin·and streptovaricin) does not alter the DNA binding of RNAP but blocks initiation; once initiation has occurred, synthesis is rather resistant to the antibiotic (Lill, Lill, Sippel and Hartmann, 1970). The core enzyme without sigma binds a single rifampicin molecule (Wehrli and Staehelin, 1970), apparently through such forces as hydrogen bonding rather than chemical bonding. Binding occurs at the β subunit (Rabussay and Zillig, 1969) and has been reported to be lost in resistant strains by a one-step mutation (Knusel, 1968). Mammalian polymerase does not bind, and is not affected by, rifampicin (Wehrli, Nuesch, Knusel and Staehelin, 1968).

EUCARYOTIC RNA POLYMERASES

Isolation, Purification and Stability of Eucaryotic RNA Polymerases. In contrast to bacterial RNAP, eukaryotic RNAP has, until recently, proven to be more labile and therefore more difficult to isolate. This might relate to technical difficulties in isolation and stabilization, or to intrinsic instability, perhaps reflecting an increased number of regulatory axes.

Many proliferating mammalian tissues contain a readily soluble RNAP (chick embryo, bovine lymphosarcoma, rat testis, HeLa cells). When the relatively mild extraction methods used in proliferating tissues are applied to many essentially non-proliferating tissues (e.g., adult rat liver), little soluble material is obtained. Many studies, therefore, have been carried out on whole nuclei, or on material from an aggregated pellet of lysed nuclei, the "aggregate-enzyme" of Weiss (Weiss, 1960).

Methods have now evolved, however, for the extraction of most of the RNAP of non-proliferating cells into soluble form (Roeder and Rutter, 1969, 1970; Goldberg and Moon, 1970; Jacob, Sajdel and Munro, 1970).

Seifart and Sekeris (1969) reported a method of purifying rat liver nuclei using a detergent. The nuclei were then sonicated and treated with high salt concentrations (0.75 M NaCl), which resulted in the solubilization of 80% of nuclear homogenate RNAP activity. Exogenous DNA was used in all RNAP assays which were used to calculate the amount of RNAP solubilized. Purification on DEAE cellulose, ammonium sulfate precipitation, and isolation on sucrose-glycerol gradients resulted in a 250-fold increase in specific activity, as compared to nuclear homogenate RNAP activity, but with a low yield and extreme lability. A gel electrophoresis separation demonstrated a single major protein peak[(1)]. No stimulation of liver RNAP activity was seen by adding cortisol directly to the RNAP assay mixture, indicating that cortisol did not act directly on this RNAP preparation.

Goldberg, Moon and Rosenau (1969) demonstrated a method of extracting and purifying RNAP from rat liver nuclei using sonication, streptomycin, DEAE-cellulose and phosphocellulose columns, and ultrafiltration. The specific activity rose 2000-fold with a yield of 8% as compared to the soluble RNAP activity of sonicated nuclei. Exogenous mammalian DNA was used in all RNAP assays, and thus the values for crude and purified RNAP activity are comparable as far as template is concerned.

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(1) In our laboratory the Tris-lysine system used by Seifart and Sekeris (1969) proved unsatisfactory. Electrophoresis resulted in many proteins migrating together as a few blurred bands at 25° C or 0° C.

Cunningham, Cho and Stein (1969) solubilized RNAP from rat liver nuclei (80% recovery) and purified it once with DEAE-cellulose to yield 40% of the original nuclear RNAP activity. Specific activity increased 6.4 times, as compared to nuclear RNAP activity, with a yield of 6% of the original soluble proteins. Exogenous calf thymus DNA template was used in all RNAP assays in an effort to make the recovery values comparable. The enzyme in any form other than crude soluble extract (which lost 55% of its original activity in eight weeks at 2° C) was extremely labile. DEAE-cellulose purified and pooled fractions lost 70% of their activity in the first day, and the polymerase was not significantly stabilized by adding DNA, $MnCl_2$, or the four nucleoside triphosphates. In a further study of stability, RNAP was passed through DEAE-cellulose columns and the active fractions were combined with BSA (1 mg/ml) and precipitated with ammonium sulfate. After dissolving in a 30% glycerol, mercaptoethanol, Tris-phosphate buffer, RNAP activity at 2° C on days 1, 2 and 6 was 55%, 45% and 30% of the original, respectively.

Liao, Sagher and Fang (1968) used hypertonic sucrose to retain RNAP in rat liver nuclei, and then reduced the hypertonicity to extract the polymerase by letting the nuclei stand, without rupture of the nuclear membranes. When whole nuclei stood for 30 minutes at 0 to 4° C in a 0.32 M sucrose Tris-$MgCl_2$ buffer (pH 7.5), approximately one-half of the original nuclear RNAP activity, assayed with exogenous calf thymus DNA, was lost to the buffer. This was confirmed by reassay of nuclei after standing for RNAP activity, which demonstrated approximately 50% of the original nuclear activity. Nuclei assayed without added exogenous DNA, however, showed the same RNAP activity before and after standing.

The authors suggested that much of the RNAP may not be functionally bound, or even functionally available, for transcription. They further postulate a 'reserve' or sequestered RNAP which may be affected by hormone treatment. It would be interesting to examine this 'leaky' polymerase for bound inactivators, for different subunit structure, or for different template specificities when compared to the RNAP remaining in nuclei.

Roeder and Rutter (1969, 1970) outlined extraction methods for rat liver RNAP, using sonication, ammonium sulfate precipitation, and DEAE-Sephadex chromatography for retention of 75 to 90% of the original nuclear activity, and separation of nuclear and nucleoplasmic forms of the polymerase. It should be pointed out, however, that their recovery estimates compared nuclear RNAP activity with exogenous calf thymus DNA template, and may be misleading.

Gabourel and Fox (1970, 1971) have defined an extraction procedure for solubilizing RNAP from rat thymus nuclear homogenate, resulting in 70% or greater recovery of initial nuclear activity as judged by the amount of activity remaining in the nuclear lysate pellet after extraction.

Goldberg and Moon (1970) have defined methods for isolation and stabilization of nucleolar and nucleoplasmic bovine thymus RNAP. Streptomycin treatment, ammonium sulfate fractionation, DEAE-cellulose chromatography, and glycerol gradient centrifugation resulted in purification of nucleolar RNAP 400 times, and nucleoplasmic RNAP 1500 times, as compared with nuclear lysate. EDTA treatment was essential, as was glycerol (30%) and thioglycerol (0.05 M) for stability and maximum

activity. These investigators pointed out that RNAP from different species, and also from different tissues within the same species, have different optimal extraction and purification procedures.

Characterization of Eucaryotic RNA Polymerases. Weiss (1960) characterized the rat liver RNAP in the aggregate preparation as DNA-dependent, requiring all four nucleoside triphosphates and a metallic cofactor (Mg^{++} or Mn^{++}), with a pH optimum between eight and nine. Nakagawa and White (1966) and Fox and Gabourel (1967) defined an aggregate enzyme preparation for rat thymus RNAP with similar properties.

Widnell and Tata (1964) recognized the existence of two RNA polymerase activities in rat liver nuclei, and characterized them as producers of ribosomal and DNA-like (non-ribosomal) RNA (Widnell and Tata, 1966). Liao, Sagher, Lin and Fang (1969) centrifuged rat liver RNAP on sucrose gradients. They found a Mg^{++} stimulated RNAP activity at 13S, and a Mn^{++} stimulated activity at 16S. The Mn^{++} stimulated RNAP activity was about ten times greater than the Mg^{++} stimulated RNAP activity. These forms could be separated further by recentrifugation, but only with the loss of considerable activity. The Mn^{++} stimulated RNAP activity was much more active on heat denatured calf thymus DNA than on native DNA, while the Mg^{++} stimulated RNAP activity was much more active on native than denatured DNA.

Roeder and Rutter (1969, 1970) used DEAE-Sephadex chromatography to describe distinct RNAP's from nucleoli (I)[1] and nucleoplasm (II,

[1] Roman numerals were assigned as a designation of the types of RNAP activity found in a particular tissue.

III) of sea urchin, and nucleoli (I) and nucleoplasm (II) of rat liver. In the presence of Mg^{++} and low ionic strength, RNAP activity was located principally in the nucleolus, and nuclei synthesized primarily ribosomal RNA. In the presence of Mn^{++} and high ionic strength, polymerase activity was more diffuse in the nucleoplasm and DNA-like RNA was the major product. These conditions could have reflected differential ionic effects on template, e.g., high salt dissociation of proteins (histones) from either template (Marushige and Bonner, 1966) or from polymerase (Spelsberg and Hnilica, 1969). Roeder and Rutter's case (1969) for the presence of different polymerases is strengthened, however, by their different ion requirements, different ionic strength optimums (I, 0.04 M; II, 0.1 M salt), and different template requirements. There was no evidence of interconversion of the nucleolar and nucleoplasmic forms after freezing and thawing, dilution and concentration, and repeated chromatographic separation.

Roeder and Rutter (1969) pointed out that the RNAP activity generally described (in bacteria, rat liver and testis, bovine lymphosarcoma) fitted their activity II, the soluble, easily extractable, nucleoplasmic enzyme. Disruption of nucleoli by sonication at high ionic strength (0.3 M ammonium sulfate) for solubilization of essentially all the nuclear activity was required to demonstrate activity I.

Furth, Nicholson and Austin (1970) purified a calf thymus RNAP that was stimulated more by Mn^{++} than Mn^{++} and Mg^{++} together. The authors noted that in contrast to the asymmetrical transcription of nicked DNA with E. coli RNAP, the calf thymus RNAP transcribed both strands of double-stranded ØX RF (replicative form) DNA (as shown by

hybridization). The DNA used, however, was a mixture of linear molecules and circular molecules with single-stranded breaks, which makes the conclusions questionable. When calf thymus RNAP transcribed T2 DNA, it did so without the selectivity for early gene transcription conferred by sigma in E. coli polymerase. Addition of bacterial sigma did not stimulate transcription by the mammalian enzyme. A less pure enzyme fraction required native DNA as template, while a more extensively purified template used native or, to a slightly lesser degree, denatured DNA. Ribonuclease H selective degradation of the RNA moiety of DNA-RNA hybrids may be invoked in explanation (Hausen and Stein, 1970). It is also possible, however, that purification of the calf thymus enzyme resulted in the loss of a RNAP subunit or modifier which controlled template preference.

Factors Altering Eucaryotic RNA Polymerase Activities. Stein and Hausen (1970) used DEAE-cellulose columns to isolate a protein factor (factor S) from calf thymus, which stimulated a purified preparation of nucleoplasmic RNAP from the same tissue. The purified calf thymus RNAP without factor S transcribed denatured DNA more efficiently than native DNA, while in the presence of factor S, native DNA was preferred. The presence of factor S also resulted in a change in the salt concentration required for optimum RNAP activity (from 0.2 to 0.02 M KCl). Factor S also altered the enzyme kinetics of ^{3}H-UMP incorporation by RNAP. RNAP in the presence of S required a period of five minutes to develop maximal stimulation of enzymatic activity, while without S, enzymatic activity reached a lower, constant level almost immediately.

Factor S stimulated calf thymus RNAP activity on native template at any time after initiation, but did not stimulate RNAP activity on denatured DNA template. Factor S did not stimulate E. coli RNAP, and would not substitute for sigma in E. coli RNAP. Thus, factor S seemed to be specific for both the enzyme and the template used. The loss of factor S could provide an explanation for the loss of activity seen in many mammalian RNAP purification methods.

Goldberg and Moon (1970) found a phosphocellulose separable protein factor in nucleoplasmic bovine thymus RNAP which was necessary for enzyme stability and also stimulated the enzyme activity fourfold. A comparable factor for nucleolar RNAP could not be isolated. The nucleolar RNAP was very unstable, however, suggesting that a stabilizing factor may have been lost.

Mondal, Mandal and Biswas (1970) isolated two RNAP's and three other protein fractions from chromosomal acidic proteins of coconut nuclei. One protein fraction stimulated both polymerase activities, while addition of another was inhibitory.

<u>Inhibitors of Mammalian RNA Polymerase Activities</u>. Stirpe and Fiume (1967) demonstrated that α-amanitin selectively depressed rat liver nucleoplasmic RNAP, while nucleolar RNAP was only slightly affected. This was in contrast to a selective inhibition of nucleolar RNAP activity seen with aflatoxin B and low doses of actinomycin D (Drews, 1969). Jacob, Sajdel and Munro (1970) showed that the effects of α-amanitin were due to a direct combination with the mammalian polymerase molecule. Alpha-amanitin did not combine with, or affect, the bacterial enzyme,

nor did it interfere specifically with initiation. Novello and Stirpe (1970) used α-amanitin for a simultaneous assay of nucleoplasmic and nucleolar RNAP, and have shown shifts in their relative concentrations in rat liver with growth and differentiation.

Salicylate (3 mM) specifically inhibited rat liver nucleoplasmic RNAP in a fashion similar to α-amanitin (Janakidevi and Smith, 1970). Straat, Ts'o and Bollum (1968) reported an inhibition of M. lysodeikticus RNAP by ethanol in concentrations as low as 0.5% in reactions directed by single-stranded homopolymers. When native calf thymus DNA was used as template, however, concentrations up to 5% were without effect.

HORMONAL EFFECTS IN MAMMALIAN SYSTEMS

Hormonal Regulation of Growth and Differentiation Through RNA Synthesis. Hormones have widespread effects in mammals, and a single hormone may have many 'target organs'. Anabolic effects include stimulation of metabolism, stimulation of cell growth, mediation of biochemical differentiation, and the induction of specific proteins in differentiated cells (O'Malley, 1971). Control of growth, differentiation and induction is believed to be mediated through the disposition of all types of RNA which, in turn, are responsible for the synthesis of proteins. A large body of experimental evidence has accumulated which suggests that RNA synthesis is a primary site of hormonal action for many anabolic processes. Hormonal increases of protein synthetic ability have been shown to be preceded by increases in tritiated uridylic acid (^{3}H-UMP) incorporation, which is used to measure increases

in RNA synthesis (Tata, 1966). These effects appear to be mediated through the combination of hormones with specific receptor molecules (Jensen, Suzuki, Kawashima, Stumpf, Jungblut and DeSombre, 1968; O'Malley, 1971).

A good model of hormonal action is provided by examining the effects of estrogen. Estrogen increases the growth and mitotic activity of the myometrium, endometrium, and tissues in the breast. Within two to ten minutes after injection of estrogen in vivo, there is a 40% increase in ^{3}H-UMP incorporated into RNA in nuclei isolated from the rat uterus. This effect becomes maximal within 20 to 40 minutes, and is followed by increases in protein synthesis (O'Malley, 1971). Estrogen effects in the liver of the ovarectomized rat are expressed within 20 minutes as a maximum stimulation of incorporation of ^{3}H-UMP into rapidly labelled nuclear RNA in isolated nuclei. One hour after injection there is an increase in Mg^{++} stimulated RNA synthesis (nucleolar) in liver nuclei, and this is followed at four hours by an increase in the incorporation of labelled precursors into the cytoplasmic proteins of liver cell microsomes (Tata, 1966).

In many mammalian tissues, RNA synthesis (measured as ^{3}H-UMP incorporation into RNA) increases under hormonal stimuli, and nucleolar and nucleoplasmic RNA synthesis respond differently. The rate of RNA synthesis is increased in rat uterus nuclei with estradiol; in rat liver nuclei, with growth hormone, cortisone, testosterone and estradiol; and in rat prostate cell nuclei with testosterone (Tata, 1966). Each of these experiments examined the incorporation of ^{3}H-UMP by isolated nuclei after administration of hormone in vivo. Growth hormone, triiodothyronine,

and testosterone stimulated rat liver growth by increasing Mg++ activated (nucleolar) RNA synthesis in rat liver nuclei (Tata, 1966). Growth hormone increased only Mg++ stimulated RNA synthesis, whereas triiodothyronine and testosterone increased Mg++ stimulated RNA synthesis first, and then increased Mn++ activated (nucleoplasmic) RNA synthesis.

Increases in incorporation of ^{3}H-UMP into RNA of chick oviduct nuclei after estrogen or progesterone injection in vivo, decreased or disappeared in a high ionic strength assay (McGuire and O'Malley, 1968). An increase in ionic strength would tend to optimize conditions for nucleoplasmic RNA synthesis and nucleoplasmic RNAP. These conditions would not provide optimum conditions, however, for nucleolar RNA synthesis and nucleolar RNAP, and it is reasonable to expect masking of the hormonal effect. These experiments made use of endogenous DNA template, and it is also possible that high salt conditions dissociated histones from the DNA, resulting in an altered template activity.

Halkerston, Scully, Feinstein and Hechter (1965) showed that the anabolic effects of cortisol in liver could be blocked by doses of actinomycin D which did not completely inhibit RNA synthesis. This finding is consistent with the findings that steroid-mediated anabolic processes in liver are accomplished through nucleolar RNA synthesis (Tata, 1966), and that low doses of actinomycin D are selectively inhibitory for nucleolar RNA synthesis (Drews, 1969).

Thus, in many cases, anabolic processes seem to be initially or completely mediated through increases in nucleolar RNA synthesis. The

hormonal effects described would be consistent with effects on nucleolar and nucleoplasmic RNAP's, and many authors describe alterations in the rate of ^{3}H-UMP incorporation into cells or nuclei as changes in RNAP activity. Hormonally induced changes in ^{3}H-UMP incorporation occurring at the tissue, cellular, or nuclear level, however, cannot be described as effects on the activity of the enzyme RNAP without closer examination of the system.

Cortisol Effects on Transport Phenomena in Thymocytes. The earliest reported effects of cortisol on thymus cell metabolism have been inhibition of glucose uptake and/or glucose phosphorylation (Munck, 1968). Young (1969) suggested that inhibition of protein and RNA synthesis in rat thymus after cortisol injection might be secondary to decreased ATP production resulting from the inhibition of glucose metabolism. Drews and Wagner (1970), however, presented data which indicate that the cortisol effects on RNA synthesis are not secondary to effects on glucose metabolism. They demonstrated that when thymocytes were incubated in the absence of glucose, there was an impairment of ^{3}H-uridine phosphorylation within 15 to 20 minutes, but that 60 minutes was required for inhibition of RNA synthesis. Varying the concentration of glucose from 0.1 to 1.0 mg/ml had little effect on RNA synthesis. In contrast, prednisolone (10 mg/kg; equivalent to 50 mg/kg cortisol) caused a synchronous fall in both uridine phosphorylation and RNA synthesis. These effects were detectable 30 minutes after prednisolone injection, and increased in magnitude with time. In addition, prednisolone inhibited ^{3}H-uridine phosphorylation when RNA synthesis was completely stopped by actinomycin D or by reduction of the incubation

temperature to 0° C. Nuclei from prednisolone-treated cells, depleted of phosphorylation capacity by Triton X-100, still displayed an impairment of ^{3}H-ATP utilization when compared with controls. Thus, decreases in precursor phosphorylation, while an early steroid effect, appeared distinct from the impairment of RNA synthesis.

Makman, Nakagawa and White (1967) and Makman, Dvorkin and White (1968) have also demonstrated a cortisol-mediated fall in the transport of nucleic acid and protein precursors into the thymus cell by analyzing the soluble precursor pool. Cortisol (1×10^{-6} M), incubated with thymocytes for four hours, decreased the uptake of ^{3}H-uridine and ^{14}C-glycine. Actinomycin D (4 µg/ml) or puromycin (2.5×10^{-5} M), when present throughout the four-hour incubation, decreased the uptake of ^{3}H-uridine and ^{14}C-glycine, but prevented further effects on uptake by cortisol. Cortisol effects on precursor uptake were sufficient to explain the decreased TCA-soluble amino acid and RNA precursor pools, but the impairment of RNA synthesis appeared separable. Progesterone (1×10^{-5} M) also inhibited α-aminoisobutyric acid and uridine transport into thymocytes (to a lesser extent than cortisol), but had no effects on the uridine incorporation into RNA. Additional evidence of a binary site of action was found in the conservation of cortisol effects on RNA synthesis in nuclei and aggregate enzyme, where precursor pools were added to the assay mixture at equal, defined levels.

Cortisol-induced Catabolism in Thymocytes. Cortisol, while causing specific anabolic effects in liver, is primarily a catabolic hormone in most tissues of the body. Lymphoid tissue such as the thymus gland has

been used extensively to study catabolic effects. Cortisol-mediated thymus involution has been described in detail by Dougherty and White (1943, 1945). Since that time, inhibition of protein, RNA, and DNA synthesis have been clearly shown as early glucocorticoid effects in the thymus (Gabourel and Aronow, 1962; Makman, Dvorkin and White, 1966). Recent reports indicate that glucocorticoid catabolic effects may be receptor-mediated (Munck and Brinck-Johnson, 1968; Hackney, Gross, Aronow and Pratt, 1971; Mosher, Young and Munck, 1971; Kirkpatrick, Milholland and Rosen, 1971).

Gabourel and Aronow (1962) demonstrated selective effects of glucocorticoids on the growth of mouse lymphoma ML-388 cells in vitro, and correlated these effects with a diminution of the cellular protein, DNA, RNA, and amino acid pool sizes. The authors also demonstrated an inhibition of protein synthesis with no effects on protein catabolism. Cortisol inhibition of precursor incorporation into thymus RNA and protein was demonstrated in vivo by Feigelson in 1964. Pena, Dvorkin and White (1964, 1965) sacrificed rats three hours after cortisol injection, and reported decreases in the ability of thymus nuclei to incorporate labelled UMP into RNA, and in the ability of cell-free thymus homogenates to incorporate amino acids.

Gabourel and Comstock (1964) demonstrated that microsomes from mouse lymphoma ML-388 cells exposed in culture to cortisol for twelve hours were impaired in amino acid incorporating ability. The same effect was also apparent in thymocytes isolated from rats injected with cortisol twelve hours previously. Cortisol added directly to the microsomal system was without effect. Cell sap from cortisol-treated

cells added to control microsomes did not alter control ^{3}H-leu incorporation. Cell sap from control cells did not alter the cortisol-mediated decrease in ^{3}H-leu incorporation. These results suggested that the biochemical lesion induced by cortisol was in the particulate fraction, and that cortisol did not act directly on the preformed microsomal system.

Gabourel and Fox (1965) noted that cortisol (15 mg/kg) decreased the total amount of particulate RNA by six hours, a time at which the gland weights were unaffected. Sucrose gradient analysis showed that all ribosomal fractions were affected -- monomer, dimer, and polysomal, with the most pronounced effects being on the larger aggregates (polysomes). The data provided a physical basis for the assumption that decreased ^{3}H-leu incorporation (decreased protein synthesis) might be secondary to a decreased synthesis of RNA.

Makman, Dvorkin and White (1966) used thymocyte suspensions prepared from rats pretreated with cortisol (50 mg/kg) to demonstrate a 23% and 54% inhibition of uridine incorporation at 40 minutes and three hours after cortisol, respectively; and a 16% and 18% inhibition of leucine incorporation at one and three hours after cortisol. When 1×10^{-6} M cortisol was added to thymocyte suspensions, inhibition of uridine incorporation began at one and one-half hours later and increased to 40% in four hours. At four hours after cortisol, leucine incorporation was inhibited 17%.

Nakagawa and White (1966) reported a decrease in RNA synthesis by nuclei of thymocytes isolated from rats which had been treated with cortisol (50 mg/kg). Decreased RNA synthesis was significant (34%)

at three hours after treatment. Standard deviations were not given, however, and the ranges were enormous. Fox and Gabourel (1967) found a decrease in the ability of thymus aggregate enzyme (a sedimentable complex obtained from lysed nuclei containing repressed DNA template and endogenous RNA polymerase) to incorporate ^{3}H-UMP into RNA as early as three hours after cortisol (50 mg/kg). The effect was dose and time dependent, and six and twelve hours after treatment there was a 54% and 62% inhibition of ^{3}H-UMP incorporation, respectively. They also showed that the effect was not secondary to a decreased availability of nucleoside triphosphate precursors.

After injecting cortisol (50 mg/kg) into rats three hours prior to sacrifice, Nakagawa and White (1967) demonstrated that the ability of both thymic nuclei and aggregate enzyme to incorporate ^{3}H-UMP was reduced approximately 25%. Cortisol effects were seen regardless of the RNA precursor used or its level in the assay, indicating that the reduction of RNA synthesis was not a characteristic of a particular precursor or due to alterations in pool size. Acid and alkaline RNase activities were not significantly altered, suggesting that cortisol reduction of RNA synthesis was not due to increased RNA breakdown. DNase II activity of thymus homogenates, and nucleoside triphosphatase of thymus nuclei, were questionably activated, suggesting that one could rule out cortisol effects on RNA synthesis due to increased breakdown of RNA precursors or DNA. These results were in close agreement with those reported by Fox and Gabourel (1967).

Pratt, Edelman and Aronow (1967) found a 37% inhibition of uridine incorporation into RNA by thymocytes isolated from rats given cortisol

three hours earlier. The inhibition was not a function of changes in precursor pool size. These investigators also observed inhibition of labelled deoxycytidine incorporation into DNA three hours after cortisol injection. This effect was obliterated, however, when the specific activity of the deoxycytidine was decreased, suggesting that the decrease in DNA synthesis was secondary to an altered precursor pool size. The effect on uridine incorporation was not affected by altering the specific activity of the labeled uridine.

Brunkhorst (1968) outlined the effects of cortisol (20 mg/kg) on ^{14}C-leu incorporation in rabbit thymus nuclei and microsomal fractions. Effects were observed at four hours, and progressive from four to twelve hours. Inhibition of the microsomal fraction always exceeded that for intact nuclei. (Nuclei at four, six, and twelve hours showed 16.2 $\pm$ 3.9%, 32.1 $\pm$ 2.4%, and 44.0 $\pm$ 1.6% inhibition, while microsomes showed 43.4 $\pm$ 6.7%, 52.2 $\pm$ 2.9%, and 61.8 $\pm$ 6.8% inhibition.)

Gabourel and Fox (1969) reported that cortisol did not alter chromatin template activity when assayed in the presence of added E. coli RNAP. They also showed that E. coli RNAP added to aggregate enzyme preparations from cortisol-treated rats could reverse the cortisol induced inhibition of RNA synthesis. These authors suggested that cortisol somehow decreased the amount or activity of the enzyme RNAP in lymphoid tissue.

Makman, Nakagawa, Dvorkin and White (1970) studied the effects of cortisol and inhibitors of protein and RNA synthesis on rat thymocytes in vitro. After incubation with puromycin (2.5×10^{-5} M), and cortisol (1×10^{-6} M) for six hours, the inhibitory effect of cortisol on the

nuclear synthesis of RNA was diminished, but still present. Puromycin at this concentration stopped 80% of thymocyte ^{14}C-leu incorporation within ten minutes, and caused 85% inhibition in three hours. Aggregate enzyme prepared from thymocytes which had undergone incubation with cortisol and puromycin or cycloheximide (2.5×10^{-5} M) also showed a cortisol-mediated reduction of ^{3}H-UMP incorporation. Thus, continued protein synthesis was not required for at least part of the inhibitory effect of cortisol on RNA synthesis, and the authors suggested that the synthesis of RNAP molecules was not a factor in the manifestation of cortisol effects. Sucrose gradient fractionation of total RNA from cortisol-treated cells labelled with ^{3}H-UMP indicated no differential effects on a particular RNA species.

Nakagawa and White (1970) studied the effects of cortisol in vivo on the rat thymus aggregate RNAP system. Failure to eliminate the steroid reduction of ^{3}H-UMP incorporation by saturating the aggregate enzyme with calf thymus DNA argued against cortisol alteration of aggregate enzyme template activity, and argued for an altered or decreased RNAP. Base ratios differed for RNA synthesized when the aggregate enzyme was stimulated with Mg^{++} or Mn^{++}, and for RNA synthesized by aggregate enzyme or added E. coli RNAP. The base ratios in thymus aggregate preparations treated with cortisol, however, did not differ from controls. The simultaneous addition of E. coli RNAP and optimal ammonium sulfate eliminated the cortisol effect. Although data were not presented, the authors stated that no significant differences were found in the base compositions of RNA synthesized by the aggregate enzyme from control or cortisol injected rats

in the presence of optimal Mg++, Mn++, or ammonium sulfate. This finding suggests that cortisol does not affect a particular class of RNA, e.g., nucleolar as compared to nucleoplasmic, and thus not a particular RNAP.

Gabourel and Fox (1970, 1971) provided further evidence that cortisol (50 mg/kg) did not alter the maximum template activity of thymus gland chromatin isolated from steroid-treated rats when the chromatin was assayed in the presence of E. coli RNAP. They also showed that cortisol reduced the amount of soluble RNAP activity extractable from thymus nuclei, and the magnitude of the reduction was comparable to reduced RNAP activity in thymus nuclei or aggregate enzyme preparations from cortisol treated rats. Additional studies on reversal of cortisol effects on aggregate enzyme by added E. coli RNA polymerase showed that at low ionic strength (no NH_4Cl added to the enzyme assay), E. coli RNAP added to cortisol-treated thymus aggregate enzyme preparations eliminated the cortisol reduction of RNA synthesis. At higher ionic strengths (o.3 M NH_4Cl added to the assay), however, E. coli RNAP added to cortisol-treated thymus aggregate enzyme preparations caused a 'paradoxical' stimulation of RNA synthesis over control preparations. If the assumption is made that E. coli RNAP is more efficient than the endogenous RNAP, then the presence of thymus RNAP could limit access of E. coli RNAP to the template. When RNAP activity from control thymus aggregate enzyme preparations was partially extracted, added E. coli RNAP also stimulated RNA synthesis over unextracted control values. Gabourel and Fox again

suggested that cortisol caused a reduction of the amount, availability, or activity of the endogenous thymic RNAP.

STATEMENT OF THE PROBLEM

Many reports have demonstrated that cortisol inhibits RNA synthesis in lymphoid tissue by interfering with the final polymerization of nucleoside triphosphates into RNA (Gabourel and Aronow, 1962; Pena, Dvorkin and White, 1964, 1965; Nakagawa and White, 1966, 1967; Fox and Gabourel, 1967; Gabourel and Fox, 1965, 1969, 1971). This effect occurs independent of changes in precursor pool size due to altered membrane transport (Pratt, Edelmann and Aronow, 1967; Makman, Nakagawa and White, 1967; Makman, Dvorkin and White, 1968). This effect also occurs independent of changes in precursor phosphorylation due to decreases in glucose uptake or phosphorylation (Drews and Wagner, 1970). Since RNA is a key intermediate in the process of converting genetic information into protein molecules, and plays an important role in the control of cell growth, our efforts have been directed to learning how cortisol inhibits RNA synthesis.

Gabourel and Fox (1971) demonstrated that thymus nuclei, isolated from rats twelve hours after injection with cortisol, contained less extractable, soluble RNA polymerase than identical preparations from control animals. This observation, coupled with the absence of cortisol effects on chromatin template activity (Gabourel and Fox, 1969, 1971), led to the suggestion that cortisol may inhibit RNA synthesis by decreasing the amount, activity, or availability

of RNA polymerase. The cortisol-mediated decrease in extractable RNA polymerase might arise from several sources, i.e. decreased synthesis, increased breakdown, redistribution within the cell, or inactivation of the enzyme by modifiers. Fox and Gabourel have shown (1970, unpublished observations) that redistribution of RNA polymerase from the thymocyte nucleus to the cytoplasm does occur in cortisol treatment, but that the magnitude of the effect is insufficient to explain the nuclear decrease in RNA polymerase activity.

In this thesis I propose to study cortisol-mediated rat thymus involution. In particular, I want to study the effects of cortisol on the synthesis of RNA polymerase and to compare the time action curve for this effect with those for reduction in extractable RNA polymerase activity and the inhibition of total cell (and total nuclear) protein synthesis. Such data will determine whether the decrease in extractable (soluble) RNA polymerase can explain the early inhibitory effects of cortisol on RNA and protein synthesis.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 60-65 gm were purchased from Simonsen Laboratories, Gilroy, California. Animals were adrenalectomized under ether anesthesia upon arrival, and maintained for three to five days on a standard laboratory diet and drinking water containing 1% NaCl.

Chemicals and Drugs. Cortisol (Cortef^{R}, 50 mg/cc in Vehicle No. 100), and Vehicle No. 100[(1)], were purchased from Upjohn. Unlabelled cytidine triphosphate (CTP), guanosine triphosphate (GTP), uridine triphosphate (UTP), and adenosine triphosphate (ATP) (all Grade A); calf thymus DNA (Grade A); dithiothreitol (DTT); bovine serum albumin (BSA), fraction V or electrophoretically pure; and D-deoxyribose were all obtained from Calbiochem. The ^{3}H-UTP (17.1 to 25.0 C/mM in 50% ethanol as the tetralithium salt), 6-^{3}H-thymidine (10 C/mM), ^{3}H-uridine (26.1 C/mM), ^{14}C-DL-tryptophan-2-ring (26 mC/mM), L-Leucine-1-^{14}C (26.2 mC/mM), and 4,5-^{3}H-L-leucine (20 C/mM or 54 to 58 C/mM in 0.01 N HCl) were all purchased from Schwarz Bioresearch. Eagle's Minimum Essential Medium[(1)] for Suspension Cultures (MEM) was obtained as packets (No. F-14) of powdered material from GibCo, or prepared from stocks. Calf serum was also purchased from GibCo. Tris (hydroxymethyl) aminomethane (Tris) was obtained from Sigma. Photo-Flo 200, N,N,N',N'-tetramethylethylenediamine (TEMED), acrylamide, and

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(1) See Appendix I.

N,N'-methylenebisacrylamide were purchased from Eastman Kodak. Analytical reagent grade toluene was obtained from Mallinckrodt. Soluene^R solubilizer, 2,5 diphenyloxazole (PPO), 1,4-bis-(2[5-phenyloxazolyl])-benzene (POPOP) were purchased from Packard Instrument Corporation. Bio-Solv BBS-3^R solubilizer was obtained from Beckman Instrument Corporation.

Preparation and Extraction of Labelled RNA Polymerase. For each experiment, fourteen rats were injected with cortisol (50 mg/kg in Vehicle No. 100), and ten with an equivalent volume of Vehicle No. 100[(1)]. The rats were sacrificed by decapitation three, six, and twelve hours after treatment, and the thymus glands excised, weighed, and minced. Sacrifice and subsequent procedures through incubation were carried out at 37° C. Cells of the minced glands were dispersed in 30 ml of MEM[(1)] by three slow strokes of a loosely-fitting pestle (Size A) in a Dounce homogenizer. The cell suspension was filtered through gauze, allowed to stand for 15 minutes and refiltered into 350-400 ml MEM. One-tenth ml aliquots were removed and diluted to ten ml with normal saline containing 1.0% formaldehyde. One ml of this suspension was then diluted to ten ml for the determination of cell number. Cell counts were done on duplicate samples using the Coulter Electronic Particle Counter, Model A (current setting 7, threshold 30). The cell counts were then used to approximate equal numbers of cells in each incubation flask.

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(1) See Appendix I.

After a 10 to 20 minute equilibration of the cell suspension under Carbogen (5% CO_2, 95% air) in a shaking water bath at 37° C, thymocytes were labelled by adding 200 µC of 4,5-^{3}H-L-leucine (^{3}H-leu) to each flask. The final concentration of L-leucine was 1 x 10^{-5} M. Incubations were continued for four hours and the flasks were flushed with Carbogen every hour. Incorporation was essentially linear over the incubation period.

After four hour's incubation with ^{3}H-leu, 100 ml of ice-cold modified MEM[(1)] containing 1 x 10^{-3} M non-radioactive leucine was added to each of the flasks, and they were transferred to an ice-water slurry and permitted to cool for 15 to 30 minutes. All subsequent operations were performed in the cold (0 to 4° C). Cells were harvested in the Sorvall RC2-B by centrifugation in the SS-34 rotor for ten minutes at 2200 rpm (630 x g_{max}). Supernatants were decanted and discarded, and the cells washed with 20 ml ice-cold MEM and resedimented.

Nuclei were obtained and purified by suspending the washed cells in 12 ml 2.0 M sucrose containing 0.05 M Tris, 0.025 M KCL, and 0.005 M $MgCl_2$, pH 6.4 (2.0 M sucrose TKM). The cells were homogenized with 20 to 25 strokes of the A pestle in a 15 ml Dounce homogenizer and checked by light microscopy for the presence of nuclei and the absence of whole cells. Nuclei were then purified by the Blobel-Potter (1966) modification of the Chaveau method (1956). Cell homogenates were diluted to 23.0 ml with 2.0 M sucrose

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(1) See Appendix I.

TKM, and 21.0 ml were underlaid with ten ml of 2.3 M sucrose containing the same salts and buffer (2.3 M sucrose TKM). The remaining 2.0 ml were used for assays of DNA and ^{3}H-leu content. Cell lysates in sucrose were centrifuged for one hour in the Beckman, SW 25.1 at 22,000 rpm (70,000 x g_{max}). Cellular debris and cytoplasm remained in the 2.0 M sucrose TKM or at the interface, while nuclei sedimented to the bottom of the tube.

Nuclei were harvested from the bottom of the tube and lysed by homogenization in a 0.005 M DTT, 0.05 M Tris buffer containing 0.025 M KCl and 0.1 M NH_4Cl adjusted to pH 8.0 with concentrated HCl. This buffer is referred to as DTT-Tris, pH 8.0. Homogenization was accomplished by 15 strokes of a Teflon pestle held in an electric drill, driven at a setting of 100 on a Superior Electric Powerstat, Type 116.

The nuclear homogenate was then incubated for ten minutes at 25° C, a procedure resulting in the solubilization of approximately 70% of the original nuclear RNAP activity (Gabourel and Fox, 1971). The extraction procedure solubilized a specific fraction of ^{3}H-leu labelled nuclear proteins, and would not be expected to solubilize basic proteins such as histones. Glycerol was then added to the incubated nuclear lysate to a final concentration of 15% to 18% (w/v). The nuclear lysate was centrifuged for 30 minutes at 50,000 rpm (280,000 x g_{max}) in an International Ultracentrifuge, Model B-60, using a 405 rotor. The supernatant contained a high level of RNAP activity, and was referred to as nuclear extract.

The nuclear sediment was discarded. Analysis of DNA recoveries at each step did not show any significant differences between cortisol and vehicle preparations, indicating that there was no preferential loss of DNA with cortisol treatment.

Sucrose-Glycerol Gradient Purification of RNA Polymerase. One ml aliquots of the nuclear extracts were layered onto eleven ml linear 5% to 20% sucrose (w/w) gradients containing 20% glycerol (w/v) and 100 µg/ml BSA in DTT-Tris, pH 8.0. This gradient was the first of two sequential centrifugations used for the purification of RNAP, and is referred to as gradient I (GI). The gradients were centrifuged for 24 hours at -5° C in the International B-60, SB-283, at 40,000 rpm (270,000 x g_{max}). The gradients were fractionated into 25 to 30 twelve-drop fractions. Each tube in which a twelve-drop fraction was collected contained 0.35 ml of DTT-Tris, pH 8.0, containing 15% glycerol and 1.0 mg/ml BSA. Gradients were prepared and collected with the aid of a Buchler Auto Densi-Flow using the Buchler Polystatic Pump.

Aliquots (50 to 100 µl) of each fraction were added directly to counting vials with 0.5 ml Soluene solubilizer. Fifteen ml of toluene scientillation solution [1] was then added and ^{3}H-leu determined (see Determination of Radioactivity). Similar aliquots were assayed for RNAP activity by the method of Chamberlin-Berg

(1) See Appendix I.

(1962; see RNA Polymerase Assay System) with excess calf thymus DNA as template (Gabourel and Fox, 1971).

The two to five fractions containing peak enzyme activity were combined and one to one and one-half ml applied to linear 7.5% to 20% sucrose gradients containing the same ingredients as GI. This gradient, the second of two centrifugations used for the purification of RNAP, is referred to as gradient II (GII). The gradients were centrifuged for 32 hours under the same conditions as those for GI, and then fractionated and analyzed for ^{3}H-leu and RNAP activity as in GI.

RNA Polymerase Assay System. RNAP assays were performed according to the method of Chamberlin and Berg (1962). The incorporation of ^{3}H-UMP (from ^{3}H-UTP) into cold acid insoluble macromolecular material was used as an assay of RNAP activity. Each assay tube contained: Tris, 10 µmoles; MgCl, 1.0 µmole; $MnCl_2$, 0.25 µmole; ATP, CTP, GTP, and UTP, each 0.1 µmole; 2-mercaptoethanol, 0.3 µmole; ^{3}H-UTP, 5 µC; excess calf thymus DNA (50 µg) dissolved in glass distilled water (GDW); RNAP (nuclear extract or gradient fraction), 50 or 100 µl; and GDW, 100 or 50 µl depending upon the amount of RNAP material used. Total volume was 0.255 ml, pH 8.0, and ionic strength was 0.045.

Incubation mixtures were prepared in the cold with ^{3}H-UTP added last. The assays were then transferred to a 37° C shaking water bath and incubated for 15 minutes. Incubations were terminated by removing the assay tubes and placing them in an ice-water slurry. One-half ml of carrier (1.0 mg/ml BSA in 100 µM/ml $Na_4P_2O_7$)

was added immediately to each tube, and macromolecular material was precipitated by the addition of one ml 12% trichloroacetic acid (TCA). The mixture was vigorously agitated, and 1.0 ml 6% TCA was added. The tubes were placed in ice for 10 minutes, and centrifuged in the Sorvall SE-12 at 12,000 rpm for 10 minutes. The pellets were dissolved in one ml 0.1 N NaOH, reprecipitated twice, and then taken up in 0.5 ml Soluene for counting in toluene scintillation solution. RNAP assays of nuclear extract and GI aggregated fractions were done in triplicate, while gradient RNAP assays were single determinations.

Simultaneous zero time incubations for each assay were carried out with the addition of ^{3}H-UTP after incubation and chilling to 0° C. Immediately following ^{3}H-UTP addition, carrier and TCA were added and the sample was carried through the procedure described above. Zero time values were then subtracted from each RNAP assay. Zero time subtractions corrected for background, for non-specific ^{3}H binding to proteins, and for ^{3}H-leu incorporated into the proteins of the assayed fraction.

Electrophoretic Examination of RNA Polymerase. A Tris-lysine buffered gel electrophoresis system similar to that described by Seifart and Sekeris (1969) led to unsatisfactory gel polymerization and blurred protein band formation. Tris-glycine gels which were prepared and which underwent electrophoresis at 0° C with 20% glycerol and 0.05 M DTT also led to heavy blurring of the protein bands.

The gel electrophoresis system which gave the best results consisted of 5% acrylamide gel columns which were prepared and which underwent electrophoresis with a Tris-glycine buffer system[1]. The resolving pH at 25° C was 9.6, and at 0° C was 10.3. Gels were prepared in 5 mm (i.d.) by 100 mm pyrex tubes. The tubes were placed in concentrated HNO_3 for at least 30 minutes, and then sequentially washed in tap, deionized, and glass distilled water. The tubes were then rinsed in a 0.5% solution of Photo-Flo 200 and dried in the oven. The Photo-Flo coat prevented the gels from sticking to the sides of the tubes.

Gels prepared at 25° C hardened within 30 minutes, and 200 to 500 µl of protein solution was applied and elctrophoresis begun within one to one and one-half hours later. Gels underwent electrophoresis at 2 mA/tube (100 V) for three or seven hours at 25° C. Gels prepared in the cold room (0 to 4° C) were allowed to harden for 16 hours. Protein solutions were then applied and underwent electrophoresis at 2 mA/tube (150 V) for three or seven hours at 0° C. Gels which underwent electrophoresis at 25° C were cooled by circulating cold tap water, those at 0° by circulating ice water. Electrophoresis was performed with the Buchler 3-1014A Voltage and Current Regulated DC Power Supply.

Bromphenol blue (0.001%) was used as a visible marker for the migration of the upper buffer boundary, and a three hour

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(1) See Appendix I.

electrophoresis left the bromphenol ring one to three mm from the bottom of the gel. The gels were easily removed after placing them in an ice water slurry for a few minutes, and then carefully separating the gel from the tube with a thin wire. Gels were stained with 0.5% amido black in 7% acetic acid (HAc) for 30 minutes, then rinsed and destained overnight in 7% HAc in the Hoefer Diffusion Destaining Unit. Gels were stored in 7% HAc and scanned in the visual range (540 mμ) with the Beckman DU Spectrophotometer, Model No. 2400, using the Gilford Linear Transport System.

In some cases, replicates of scanned gels were frozen on dry ice and sliced into discs 1.5 mm thick with a slicer made by bolting together thin, double-edged razor blades separated by precisely machined washers. Two gel slices were combined and depolymerized by heating to 70-90° C for 30 minutes in 30% H_2O_2. The depolymerized gel slices in water (breakdown product of peroxide formed by heating) were then dissolved in toluene scintillation solution containing 15% Bio-Solv BBS-3 for counting. BBS-3 is a solubilizer used for counting samples containing a relatively high water content. The ^{3}H-leu counting efficiency was generally 70% of the efficiency of counting soluene dissolved ^{3}H-leu in toluene scintillation solution.

Determination of Radioactivity. Duplicate or triplicate aliquots (0.5 to 1.0 ml) for assay of ^{3}H-leu incorporation were removed from thymocyte and nuclear suspensions after incubation with ^{3}H-leu. Proteins were precipitated with 6% TCA, sedimented at 12,000 rpm, and then washed with 6% TCA and resedimented twice. The resulting pellets were dissolved in 0.5 to 1.0 ml of Soluene

and counted in 15 ml toluene scintillation solution. Tritiated leucine determinations of nuclear extract and aggregated fractions from gradients (50 to 100 µl) were done in duplicate, while single determinations (50 to 100 µl) were done to determine the distribution of ^{3}H-leu in sucrose-glycerol gradients. All ^{3}H-leu assays were corrected for background.

The method for preparing samples for the determination of ^{3}H-UMP incorporation as an assay for RNAP activity was previously described (see RNA Polymerase Assay System). At least triplicate ten minute counts of tritium were made in the Packard Tricarb Scintillation Spectrometer, with Automatic External Standardization used to correct for quenching. Corrections for quenching were rarely necessary and when necessary were minor. The counting efficiency for tritium was consistently close to 27%.

DNA Determinations. Duplicate aliquots of thymocytes, nuclei, and nuclear lysates before and after incubation were precipitated with 6% perchloric acid (PCA). The precipitated material was centrifuged at 12,000 rpm for ten minutes in the Sorvall SS-34 (70,000 x g_{max}), then resuspended and recentrifuged once, and the pellets frozen. No later than the next day, the pellets were suspended in two ml of 6% PCA and incubated at 70° C for 20 minutes (Burton, 1968). The material was chilled in ice for at least ten minutes, centrifuged at 12,000 rpm, and the supernatant frozen for DNA analysis. The next day, DNA assays were prepared in triplicate using the diphenylamine reaction described by Burton (1956). Standards were prepared with D-deoxyribose.

Treatment of Data. The total amount of DNA present in nuclear lysate before the extraction of RNAP (or occasionally in nuclei, with almost identical results), was used as the basis for normalizing RNAP activity and ^{3}H-leu incorporation in the nuclear extracts and gradient fractions. The ratios of DNA present in steroid and vehicle at this stage were generally similar to thymocyte DNA ratios. RNAP activity was expressed as cpm ^{3}H-UMP incorporated per 100 µg DNA present in the nuclear lysate prior to extraction. Similarly, ^{3}H-leu incorporation was expressed as cpm ^{3}H-leu incorporated per 100 µg nuclear lysate DNA. The amount of radioactivity contained in each fraction of GI was divided by the amount of nuclear lysate DNA from which the layered volume of nuclear extract was derived. The gradient distribution plots thus had axes of fraction number and total cpm/100 µg DNA (equivalents of nuclear extract layered onto the gradient). When the peak enzyme fractions of GI were aggregated and relayered onto GII, the fraction of the total enzyme peak removed was used to calculate a new value for DNA equivalents of nuclear extract layered onto GII. When calculating ^{3}H-leu cpm/100 µg DNA incorporated in whole cell or nuclear fractions, the DNA present in that fraction was used.

Plots of RNAP activity and ^{3}H-leu activity were cut out and weighed to provide ratios of the total activity under RNAP peaks. Recoveries of enzyme activity on GI were generally 20% to 50% of that present in the layered nuclear extract. Paired tubes of preparations from cortisol-and vehicle-treated rats (gradients prepared one after the other, nuclear extracts layered one after

the other, tubes centrifuged together, and most critically, assayed together or frozen together for later assay together) generally showed very similar recoveries. The final ratios of total enzyme activities were corrected to identical recoveries as percent of original nuclear extract material layered.

Total recoveries of RNAP activity from GII centrifugations were generally 65% to 90%, and final ratios of total activities were also corrected to identical recoveries. Total recoveries of ^{3}H-leu on both gradients were generally very close to 100% and corrections were not made.

Thymus Involution and Regeneration. In the first of two experiments performed to study the time course of thymus involution and regeneration after a single injection of cortisol, 24 male rats were adrenalectomized and maintained as described previously. Twelve animals were injected intramuscularly with cortisol, and twelve with vehicle. Two steroid- and two vehicle-treated rats were then sacrificed at various times after injection (12 to 72 hours), and thymocyte cell suspensions prepared as previously desdescribed. The second experiment contained larger numbers of animals, and extended over longer time periods (36 to 192 hours) with greater numbers of steroid-treated animals sacrificed as involution progressed.

From a flask of thymocytes pooled from all glands in the sample, triplicate aliquots were removed for cell counts and DNA analyses. On the basis of cell number, approximately equal aliquots (1.0 to 2.5 x 10^{7} cells) were placed in separate 10 ml incubation

flasks containing MEM, supplemented with thymidine, uridine and leucine. For labelling, 10 μC 6-^{3}H-thymidine and 0.5 μC L-leucine-1-^{14}C or 5 μC 5-^{3}H-uridine and 0.5 μC L-leucine-1-^{14}C were added to the flasks. Final supplement concentrations in MEM were: thymidine, 5×10^{-6} M; uridine, 5×10^{-6} M; and leucine, 1×10^{-5} M. Cells were incubated at 37° in a shaking water bath for 30 minutes under Carbogen. Zero times were determined by adding radioactive precursors to steroid- and vehicle-treated cells after incubation and cooling to 0° C.

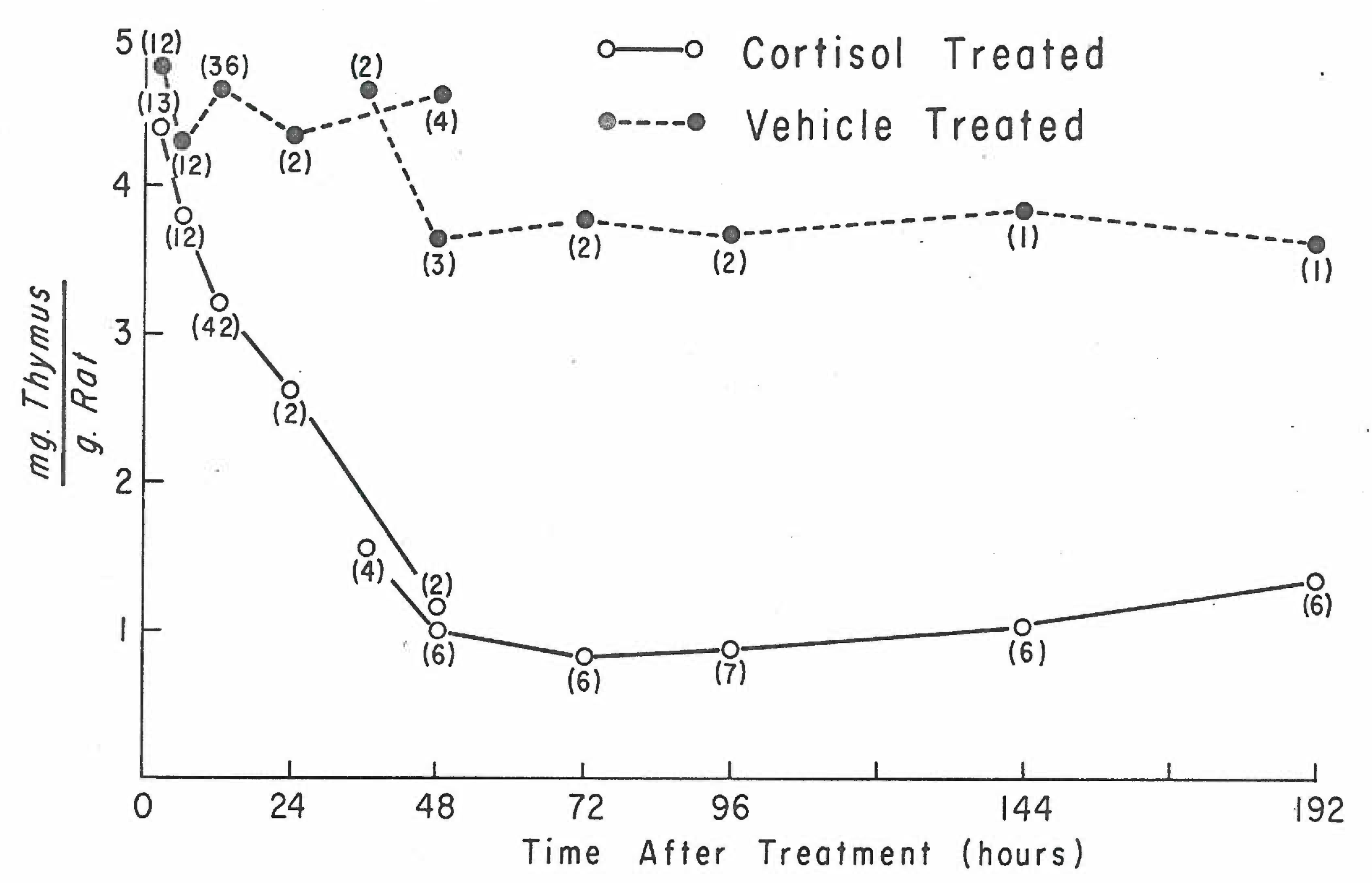
Incubations were terminated and cells collected as previously described. Cells were washed twice in MEM containing non-radioactive precursors at 100 times the levels used in the incubations. The cells were precipitated in 6% TCA, washed twice with 6% TCA, and then dissolved in Soluene and counted in toluene scintillation solution. Efficiencies for ^{3}H were approximately 27%; for ^{14}C, approximately 90%. Zero time corrected incorporations were plotted as cpm/μg DNA incorporated into cold acid insoluble material.

RESULTS

Thymus Involution and Regeneration. Figure I depicts the effects on wet thymus weights of 50 mg/kg cortisol or vehicle given to rats at various times before sacrifice. There was a marked fall in thymus weights after cortisol treatment that became evident as early as three hours after injection. Thymus weights continued to decrease with time and reached maximum involution 72 hours after treatment. Some indication of gland weight recovery was seen at 96-144 hours, and continued slowly through the last post-injection time examined (192 hours).

Figures 2 and 3 demonstrate the effects of cortisol and vehicle given in vivo on the incorporation of labelled precursors into cold acid insoluble material of intact thymocytes in vitro. Incorporation of ^{3}H-uridine (Figure 2) seemed to be maximally inhibited 12-24 hours after steroid treatment; maximum inhibition of ^{3}H-thymidine incorporation (Figure 3) appeared to occur a little later (24-36 hours). A clear separation of these effects, however, could not be made from these data. Gabourel and Comstock (1964) found that it was not possible to separate the time sequence of cortisol inhibition of incorporation of precursors of RNA, DNA, and protein into mouse lymphoma ML-388 cells at four and six hours after treatment, and that question was not reexamined in this experiment.

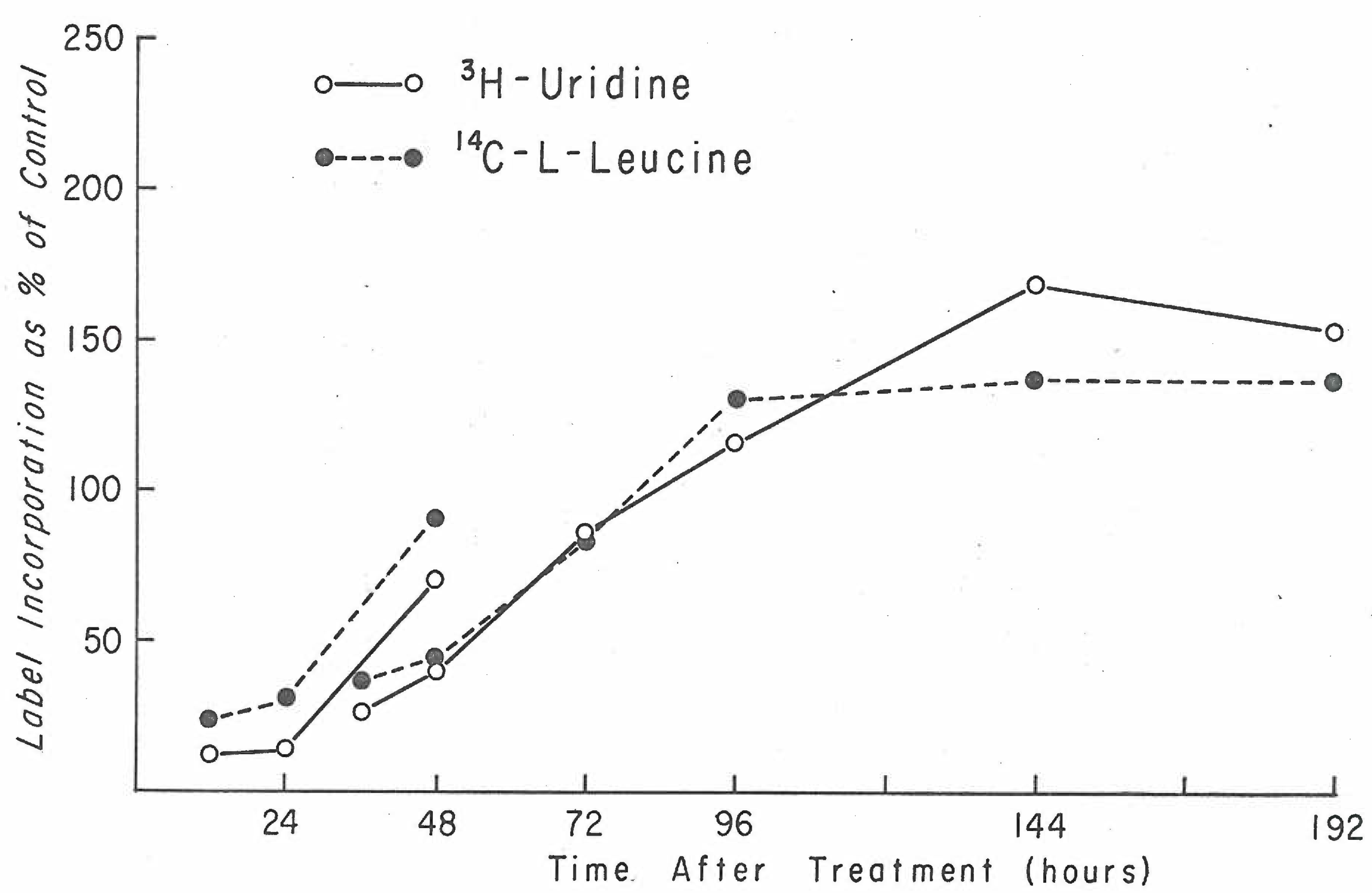
There was a much clearer separation of effects on precursor incorporation during regeneration. ^{3}H-uridine incorporation (Figure 2) began to increase as early as 48 hours after cortisol treatment, and increased beyond control incorporations between 72



Cortisol Treated
Vehicle Treated
mg. Thymus / g. Rat
Time After Treatment (hours)
0
24
48
72
96
144
192
1
2
3
4
5

Figure I: Thymus wet weights (mg. thymus/ g. rat) at various times after cortisol and vehicle injection in vivo.

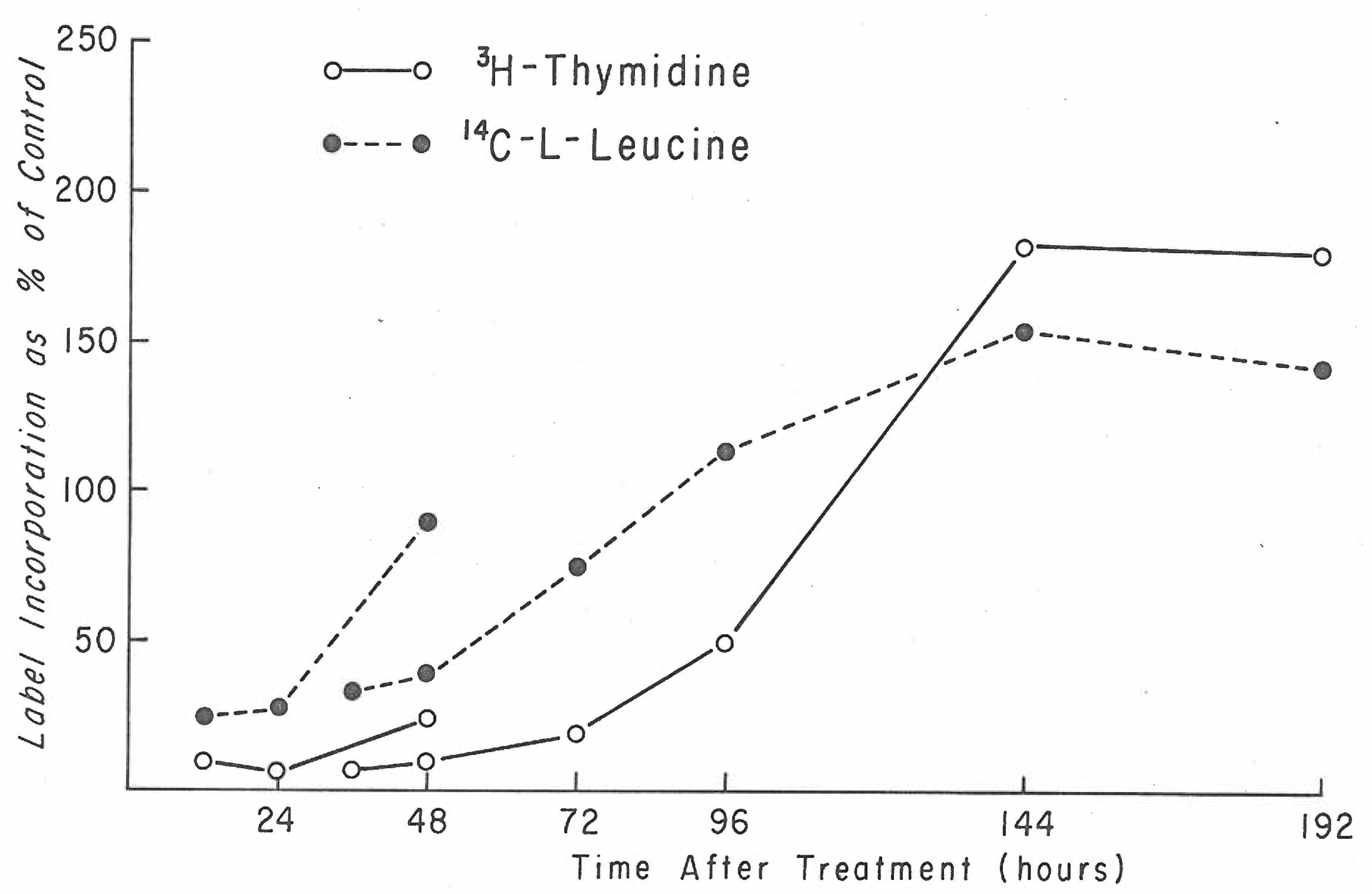
The number of rats used in each weight determination is indicated in parentheses. The sum of thymus weights (mg.) was divided by the sum of animal weights (g.) to obtain each point. Two separate experiments are plotted together as discontinuous lines for cortisol and vehicle treatments. The three, six, and twelve hour times include a large number of animals which were used to prepare labelled RNA polymerase.



250
200
150
100
50
Label Incorporation as % of Control
³H - Uridine
¹⁴C - L - Leucine
24
48
72
96
144
192
Time After Treatment (hours)

Figure 2: Incorporation of ^{3}H-uridine and ^{14}C-leucine into thymocytes in vitro at various times after cortisol and vehicle injections in vivo.

Approximately 2 x 10^{6} cells were incubated with 10 µC ^{3}H-uridine and 0.5 µC ^{14}C-leucine for 30 minutes in MEM at 37° C under Carbogen. Incorporations of ^{3}H-uridine and ^{14}C-leucine are plotted as separate curves. Averages of duplicate or triplicate incubations are graphed as percent of control. Two separate experiments are plotted together as discontinuous lines for cortisol and vehicle treatments. Glands used to prepare cell suspensions provided the weights shown in Figure 1. Control ^{3}H-uridine incorporation (cpm/µg DNA) declined linearly from 110 at 12 hours to 70 at 48 hours, then increased linearly to 110 at 144 hours and declined to 95 at 192 hours. Control ^{14}C-leucine incorporation (cpm/µg DNA) declined linearly from 34 at 12 hours to 25 at 48 hours, then increased linearly to 35 at 192 hours. While control variations were large, they appeared to follow a distinct pattern. Replicate control ^{3}H-uridine and ^{14}C-leucine incorporations both agreed with $\pm$ 5%.



^{3}H-Thymidine
^{14}C-L-Leucine
Label Incorporation as % of Control
250
200
150
100
50
24
48
72
96
144
192
Time After Treatment (hours)

Figure 3: Incorporation of ^{3}H-thymidine and ^{14}C-leucine into thymocytes in vitro at various times after cortisol and vehicle injections in vivo.

Approximately 2 x 10^{6} cells were incubated with 10 µC ^{3}H-thymidine and 0.5 µC ^{14}C-leucine for 30 minutes in MEM at 37° C under Carbogen. Incorporations of ^{3}H-thymidine and ^{14}C-leucine are plotted as separate curves. Duplicate or triplicate incubations are graphed as percent of control. Two separate experiments are plotted together as discontinuous lines for cortisol and vehicle treatments. Glands used to prepare cell suspensions provided the weights shown in Figure 1. Control ^{3}H-thymidine incorporation (cpm/µg DNA) declined linearly from 175 at 12 hours to 130 at 72 hours; whereupon it increased linearly to 225 at 192 hours. Control ^{14}C-leucine incorporation (cpm/µg DNA) declined linearly from 35 to 12 hours to 25 at 48 hours, then increased linearly to 37 at 192 hours. Thus, while control variations were large, they appeared to follow a distinct pattern. Replicate control ^{3}H-thymidine incorporations agreed within $\pm$ 3%, while replicate control ^{14}C-leucine incorporations agreed within $\pm$ 5%.

and 96 hours after treatment. The incorporation of ^{14}C-leucine into protein responded similarly (Figures 2 and 3). Increases in ^{3}H-thymidine began at 48 hours, became evident at 72 hours, and increased beyond control values between 96 and 120 hours after cortisol treatment. Stimulation of ^{3}H-uridine and ^{14}C-leucine incorporations preceded ^{3}H-thymidine incorporation. Marked stimulation of all incorporations preceded any appreciable increases in gland weight.

Distinctive alterations in cell populations and gland structure during involution and regeneration were observed. The normal rat thymus (and the gland observed three hours after cortisol treatment) was creamy in color and had two firm, discrete, lobes. The glands were easy to remove and the cells were easy to disperse. Light microscopy (LM) of glands dispersed in a loose-fitting Dounce homogenizer showed round cells with identifiable nuclei, with very little debris present. There was a mixed cell population of small, intermediate, and large thymocytes, with a clear preponderance of small forms, and a few cells of elongated shape which were probably reticular. This description holds for all vehicle treated glands, except for an occational gland (less than 5%) that appeared to have hemorrhaged, either with a clear infusion of red blood cells, or with a grayish, spotty appearance suggestive of previous injury. These glands were always discarded in preparing thymocyte suspensions.

Six hours after cortisol treatment, thymus glands were still creamy in color, although the borders and bilobular structure were less distinct. LM showed some cellular debris in cell suspensions prepared in a loose-fitting Dounce homogenizer. Twelve hours after

treatment, the glands were cream colored, but noticeably smaller in size. The borders were indistinct and the gland often fragmented into two or three pieces upon removal. LM showed increasing cellular debris in the cell suspensions, seemingly at the expense of the small thymocyte population.

Thirty-six hours after cortisol, glands were markedly smaller and assumed a whitish cast. Glands were difficult to remove, with LM showing a large amount of debris in the cell suspensions. There was now a clear shift in cell size, with intermediate to large thymocytes remaining.

At 48 hours after cortisol, the whitish glands were difficult to find and yielded very few cells. Many cells appeared clumped together on what may have been extracellular DNA. Large amounts of debris were present. Seventy-two hours after cortisol, the whitish glands were difficult to find, extract, clean, and homogenize. There was a large amount of debris, although no obvious clumping of cells. LM showed that the gland structure was now primarily reticular, populated by a few large thymocytes that may have functioned as stem cells to repopulate the largely empty reticular matrix. The cortisol-treated glands appeared decimated in contrast to vehicle control glands. Ninety-six hours after steroid, the very small glands were still difficult to process with a very large amount of debris present.

At 144 hours post-cortisol, the glands began to recover. The cream color returned, although the glands still appeared 'shattered' and were difficult to clean. Finally, at 192 hours post-cortisol,

thymus glands began to look normal again, although small in size. The glands were creamy in color, firm, and more sharply defined, and easier to remove and disperse. Small thymocytes were again present.

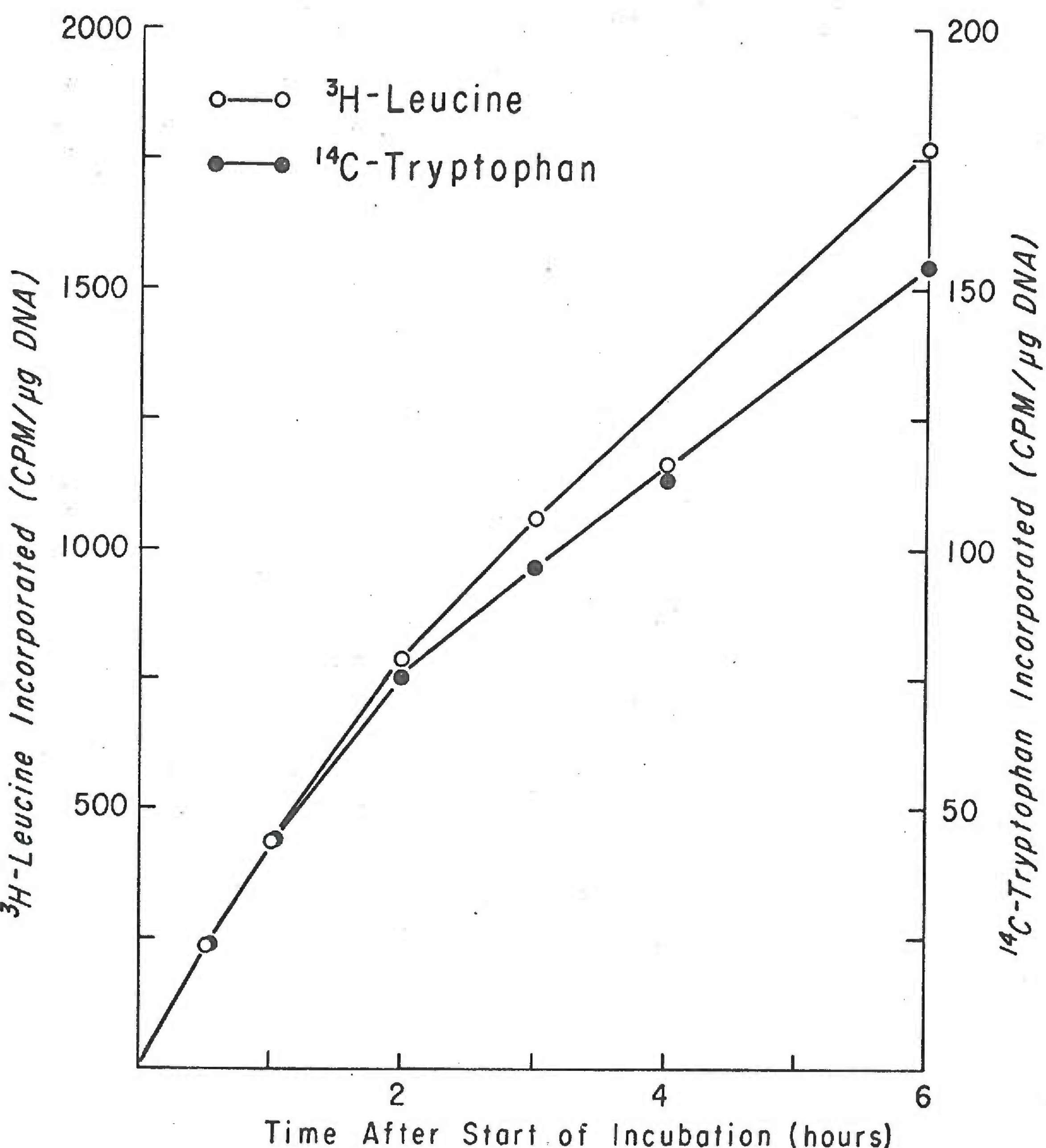
Time Course of Protein Precursor Incorporation in Thymocyte Suspension Cultures. In order to study the effects of cortisol on ^{3}H-leu incorporation into thymocyte proteins in general, and RNAP in particular, it was necessary to determine that the rate of incorporation was constant throughout the labelling period. Preliminary experiments indicated that ^{3}H-leu incubation times of about four hours were necessary to adequately label proteins sedimenting with the RNAP activity in sucrose-glycerol gradients.

Figure 4 shows ^{3}H-leucine and ^{14}C-tryptophan accumulation at various times up to six hours after label was added to thymocyte cell suspensions under standard incubation conditions. The rate of incorporation was essentially linear for the entire six hour period. In all subsequent experiments in which RNAP was labelled with ^{3}H-leu, a four hour incubation period was used under the same conditions.

Examination of Some Gradient Parameters. Initial purifications were attempted using linear 20-50% glycerol gradients in DTT-Tris, pH 8.0 for the first of two sequential centrifugations used for the purification of RNAP (Gradient I or GI). Gradient II (GII), the second of two sequential centrifugations used for the purification of RNAP, used linear 25-50% glycerol gradients in DTT-Tris, pH 8.0.

Figure 4: Time course for ^{3}H-leucine and ^{14}C-tryptophan incorporation by thymocyte suspension cultures.

Six rats were injected with vehicle and sacrificed twelve hours later to provide two equal cell suspensions of 3×10^{9} cells in 100 ml MEM. To one flask was added 200 µC ^{3}H-4,5-L-leucine, and to the other 10 µC ^{14}C-DL-tryptophan-2-ring. Nonradioactive leucine was present in MEM at 1×10^{-5} M, tryptophan at 2×10^{-7} M. Incubations were performed in a shaking water bath at 37° C under Carbogen. At appropriate intervals, five or ten ml aliquots were removed for assay of label incorporated and DNA present. The results are expressed as cpm/µg DNA vs hours after start of incubation.



2000
1500
1000
500
200
150
100
50
³H-Leucine
¹⁴C-Tryptophan
3H-Leucine Incorporated (CPM/µg DNA)
14C-Tryptophan Incorporated (CPM/µg DNA)
2
4
6
Time After Start of Incubation (hours)

Twelve to fourteen hour centrifugations were performed at 5° C. Recoveries were found to be very poor; on the order of 10% for GI, and ranging from 0-10% for GII.

An attempt was then made to examine some gradient parameters as they affected the efficiency of recovery of RNAP. Linear sucrose gradients (5-20%) were prepared in 10% and 20% glycerol, with and without NH_4Cl. It was necessary to store RNAP in at least 20% glycerol for stability at -20° C. The NH_4Cl was used to increase the ionic strength of the gradient. Single gradients were run for 16 hours at -5° C, fractionated, and assayed in the standard Chamberlin-Berg system (see Methods) for RNAP activity. The results are shown in Table I. Combining fractions from the most active gradient, and reapplying an aliquot to a second gradient of the same composition (5-20% sucrose, 20% glycerol in DTT-Tris, pH 8.0), and centrifuging under the same conditions a second time (GII), led to the loss of all RNAP activity.

Bovine serum albumin (BSA) is a component of the commercially supplied bacterial RNAP, added to stabilize the enzyme presumably by preventing RNAP denaturation on the walls of the test tube. It seemed wise, therefore, to incorporate BSA into the gradients for stabilization of the mammalian RNAP activity. BSA (100 µg/ml) was added to the gradient material, and the first gradients (GI) were fractionated into tubes containing approximately equal volumes (0.35 ml) of DTT-Tris, pH 8.0, containing BSA 1.0 mg/ml. Recoveries on GI centrifuged for 24 hours were 20% to 50% of the original RNAP activity applied. Furthermore, the RNAP activity was maintained

TABLE I

Total Recovery of RNAP Activity on Gradients of Different Composition[1]

Gradient Composition	% Recovery of Applied RNAP Activity
Linear 5-20% sucrose containing:	
20% glycerol in DTT-Tris, pH 8.0[2]	86.0
20% glycerol in DTT-Tris, pH 8.0, without 0.1 M NH_4Cl	88.0
10% glycerol in DTT-Tris, pH 8.0	81.4
10% glycerol in DTT-Tris, pH 8.0, without 0.1 M NH_4Cl	47.9
10% glycerol, 0.01 M NH_4Cl, 0.01 M $MgCl_2$, 0.25 M EDTA, and 0.001 M 2-mercaptoethanol in 0.067 M Tris-HCl, pH 7.9[3]	18.7

[1] Single gradients centrifuged sixteen hours at -5° C.

[2] DTT-Tris buffer, pH 8.0, see Methods and Materials.

[3] Gradient material used by Seifart and Sekeris (1969).

throughout GII centrifugations to the extent of 65% to 90% of the RNAP activity applied from GI. Table I suggests the recovery of a higher fraction of layered RNAP activity on gradients without BSA (86% to 88%) than on gradients containing BSA (20% to 50%). Gradients without BSA, however, were centrifuged for shorter periods of time (16 hours) than the gradients containing BSA (24 hours). When fractions of gradients without BSA were aggregated and centrifuged for 16 hours for a second time, all RNAP activity was lost. In contrast, centrifugation of gradients containing BSA led to the loss of very little RNAP activity (10% to 35%).

In one experiment, BSA was added to the buffer in which nuclei were lysed. No additional recovery of RNAP activity was found, and it was assumed that the proteins present in the nuclear lysate functioned to stabilize RNAP activity until application to the gradient. In another experiment, BSA at the level of 250 µg/ml was added to a series of gradients, without improvement of recovery, indicating that 100 µg/ml supplied as much stabilization as the higher concentration. In all subsequent gradients, BSA (100 µg/ml) was added to all gradient material, and GI was fractionated into tubes containing approximately equal volumes (0.35 ml) of DTT-Tris, pH 8.0, containing BSA 1.0 mg/ml.

<u>Cortisol Effects on RNA Polymerase Activity and Synthesis</u>. Thymocyte cell suspensions obtained from rats at varying times after injection with cortisol or vehicle were incubated for four hours with ^{3}H-leu. RNAP was extracted from the purified nuclei of these

cells, and the ^{3}H-labelled proteins in this extract were then subjected to two sequential gradient centrifugations to obtain RNAP as outlined in Materials and Methods.

Table II provides comparative data of cortisol effects on thymus weight, RNAP activity extractable from thymus nuclei, and ^{3}H-leu incorporation into proteins of nuclear extract, whole nuclei, and whole cells. Measurements were made on preparations obtained from rats sacrificed three, six, and twelve hours after injection with cortisol (50 mg/kg) or an equivalent volume of vehicle. RNAP activity decreased as a function of time after cortisol treatment, and wet thymus weight decreased in a parallel fashion. The reduction in incorporation of ^{3}H-leu into the proteins of the nuclear extract, nuclei, and thymocytes was greater at three, six, and twelve hours after cortisol treatment than the corresponding decreases in RNAP activity. After cortisol treatment, decreases in incorporation of ^{3}H-leu into total cell protein consistently led decreases in incorporation into the proteins of the nuclear extract, but led incorporation into total nuclear protein only at three hours. At twelve hours the decrease in ^{3}H-leu incorporation into thymocytes and nuclei was slightly less than incorporation into nuclear extract. Decreases in ^{3}H-leu incorporation into whole nuclei exceeded those in nuclear extract at times beyond three hours.

In an earlier experiment, 1 µC ^{14}C-DL-tryptophan-ring-2 (26.7 mC/mM) was added to two incubation flasks containing 1×10^{8} cells. Each thymocyte suspension was prepared from two rats which had received cortisol (50 mg/kg) or vehicle twelve hours earlier.

TABLE II

Thymus Weights and RNAP Activity of Nuclear Extracts and

^{3}H-Leucine (^{3}H-Leu) Incorporation into Thymocytes, Nuclei and Nuclear Extract.

Hours after Cortisol Treatment (No.)	mg Thymus / gram Rat (% of Control)	RNA Polymerase Activity(1) (% of Control)	^{3}H-Leu Incorporation(2) (% of Control)		
		Nuclear Extract	Nuclear Extract	Nuclei	Cells
3 (1)	92.2	102.4	89.6	98.0	72.7
6 (1)	89.8	86.7	71.5	61.0	59.6
12 (4) ± S.E.M.	71.2 ± 2.8	63.9 ± 3.4	54.3 ± 2.0	36.3 ± 1.4	41.3 ± 1.8

(1) CPM ^{3}H-UMP incorporated/100 µg DNA. Figures shown at three and six hours are means of triplicate assays from one experiment. Figures shown at twelve hours are means of triplicate assays from each of four experiments, ± standard error of the mean for all twelve assays. All data were zero time corrected.

(2) CPM ^{3}H-Leu incorporated/100 µg DNA. Figures shown at three and six hours are means of duplicate determinations from one experiment. Figures shown at twelve hours are means of duplicate determinations from each of four experiments, ± standard error of the mean for all eight assays. All data are background corrected.

Cells were incubated in MEM containing 1 x 10^{-7} M nonradioactive tryptophan for 30 minutes at 37° C under Carbogen. The cells were collected by centrifugation, washed, treated with cold 6% TCA and the cold acid insoluble material counted by dissolving in Soluene^{R} and then adding the mixture to toluene scintillation solution. The ratio of cortisol/vehicle (cpm/µg DNA) x 100 was 51.3%.

Figure 5 shows the distribution of RNAP activity and ^{3}H-leu labelled proteins in a typical gradient I fractionation of nuclear extract from cortisol- and vehicle-treated rats. Nuclear extracts were obtained from thymocytes labelled twelve hours after rats were injected with cortisol or vehicle. Dotted lines represent the distribution of ^{3}H-leu labelled proteins, and the solid lines depict the location of RNAP activity. Cortisol decreased ^{3}H-leu incorporation into all protein fractions, including those migrating with RNAP activity. The GI fractions containing the highest level of RNAP activity were relayered onto GII. GII corrected any GI overloading, and sharpened the RNAP peaks.

Figure 6 shows a typical gradient II purification of RNAP. The RNAP material applied to GII consisted of the combined fractions of gradient I (Figure 5) containing RNAP activity. The cortisol reduction of RNAP activity is still seen, accompanied by reductions of ^{3}H-leu incorporation into all fractions, including those associated with RNAP activity. Figures 5 and 6 were chosen to illustrate these results; the gradients from other experiments and replicate gradients from the experiment shown in Figures 5 and 6 are shown in Figures 11 through 21 (Appendix II).

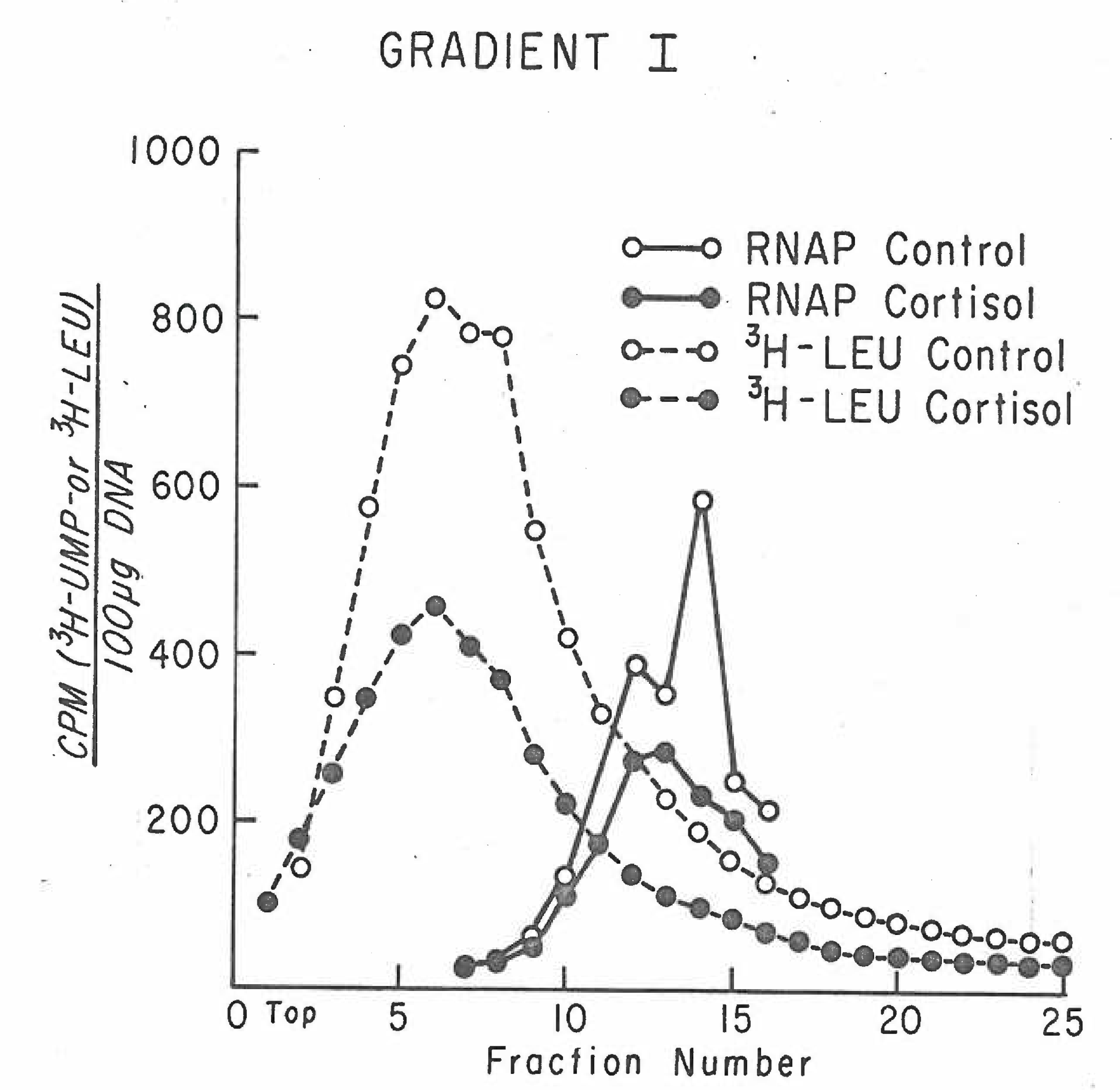

GRADIENT I
1000
800
600
400
200
CPM (³H-UMP-or ³H-LEU) / 100μg DNA
RNAP Control
RNAP Cortisol
³H-LEU Control
³H-LEU Cortisol
0 Top
5
10
15
20
25
Fraction Number

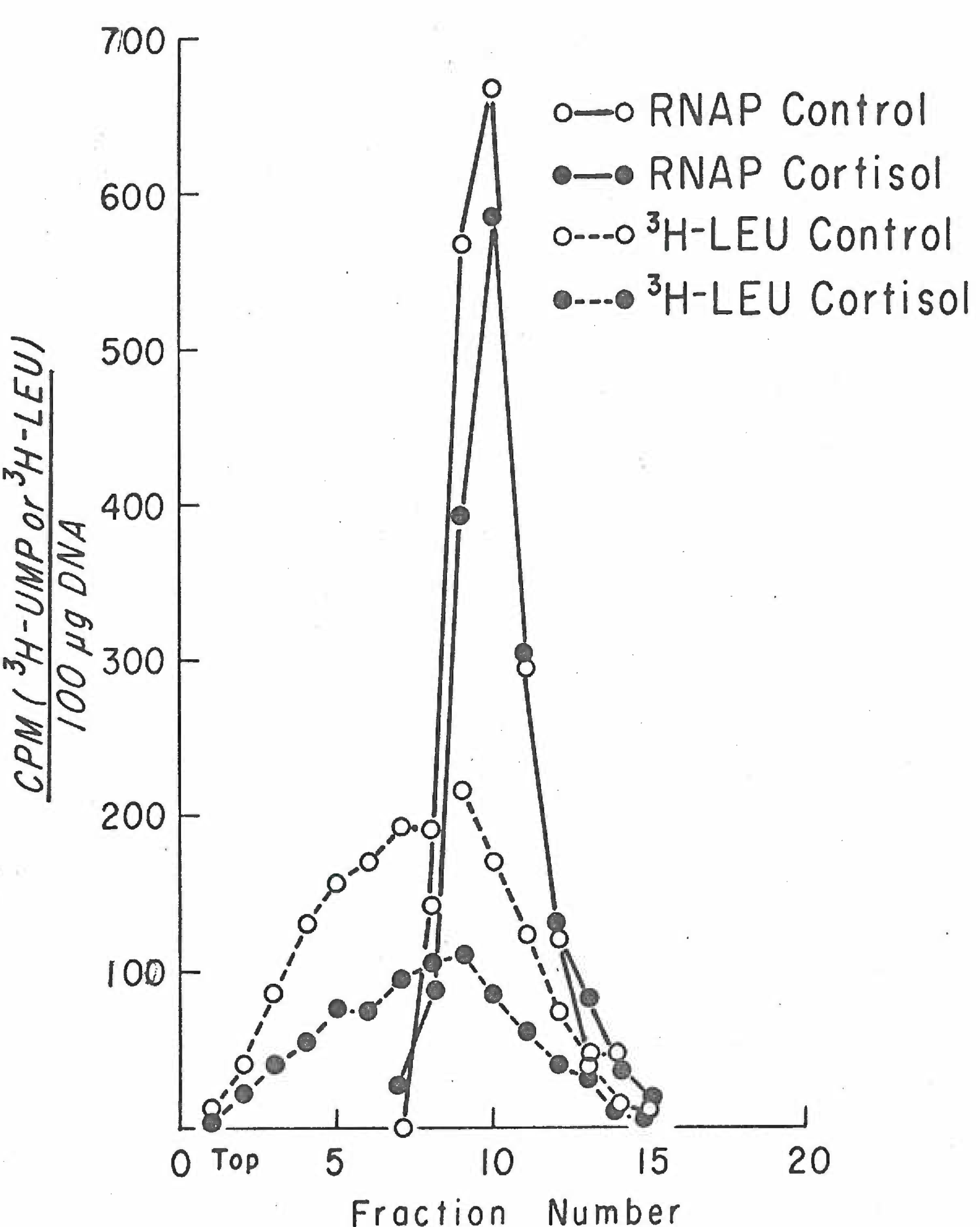
Figure 5: Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro twelve hours after treatment with cortisol or vehicle in vivo. Experiment No. 3.

This figure demonstrates a typical GI fractionation of nuclear extract proteins. One ml aliquots of the nuclear extracts were layered over linear 5% to 20% sucrose gradients containing 20% glycerol and 100 µg/ml BSA in DTT-Tris, pH 8.0, and centrifuged for 24 hours at -5° at 270,000 x g_{max}. The distribution of RNAP activity and ^{3}H-leu incorporation is shown for cortisol and vehicle treatments. Figures 19-A and 20 (Appendix II) show replicate gradients from Experiment 3, and centrifugation of the gradients shown in this figure occurred at the same time as the gradients shown in 19-A. Percentage recoveries of nuclear extract RNAP activity applied to the gradients were: cortisol 37.8%, vehicle 42.8%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GI RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 9 to 16, vehicle fractions 9 to 16. The final value of toal cortisol/vehicle x 100 for GI RNAP activity when cortisol and vehicle gradients were corrected to equivalent recoveries was: 74.0%.

Figure 6: Gradient II fractionation of combined fractions of a gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro twelve hours after treatment with cortisol or vehicle in vivo. Experiment No. 3.

This figure demonstrates a typical GII centrifugation of combined fractions of labelled proteins associated with GI RNAP activity. Gradient I (Figure 5) fractions combined and recentrifuged on GII were: cortisol fractions 11 to 14, vehicle fractions 11 to 14 inclusively. One ml aliquots were layered over linear 7.5% to 20% sucrose gradients containing 20% glycerol and 100 µg/ml BSA in DTT-Tris, pH 8.0, and centrifuged for 32 hours at -5° C at 270,000 x g_{max}. The distribution of RNAP activity and ^{3}H-leu incorporation is shown for both cortisol and vehicle treatments. Figures 19-B and 21 (Appendix II) show replicate gradients from Experiment 3, and centrifugation of the gradents shown in this figure occurred at the same time as those shown in Figure 19-B. Percentage recoveries of GI RNAP activity applied to the gradients were: cortisol 87.7%, vehicle 75.9%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GII RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 7 to 13, vehicle fractions 7 to 13 inclusively. The final value of total cortisol/ vehicle x 100 for GII RNAP activity when cortisol and vehicle gradients were corrected to equivalent recoveries was: 76.1%.

GRADIENT II



replicate gradients from the experiment shown in Figures 5 and 6 are shown in Figures 11 through 21 (Appendix II).

Table III compares cortisol treated RNAP activity and ^{3}H-leu incorporation as percent of vehicle control for nuclear extracts and gradients I and II. Data for this table was calculated by determining the total areas of the peaks of RNAP activity and ^{3}H-leu migrating with RNAP activity from Figures 5 and 6, and Figures 11 through 21 in Appendix II. Cortisol-mediated reductions of RNAP activity, and ^{3}H-leu incorporation into protein migrating with RNAP activity, were still evident after gradient II purification. The gradient II ratios of cortisol/vehicle x 100 for RNAP activity remained similar to those in gradient I with the exception of the six hour values. The ratios of cortisol/vehicle x 100 for ^{3}H-leu incorporation in specific peaks under the enzyme activity peaks in the second gradients were similar to the incorporation ratios of ^{3}H-leu in the areas under the enzyme peaks in the first gradients. The ratios of both of these ^{3}H-leu incorporations were essentially the same as the ratios of cortisol/vehicle x 100 for ^{3}H-leu incorporation in nuclear extracts, with the exception of the six hour values.

Inhibition of ^{3}H-Leucine Incorporation in Proteins of Thymus Nuclear Extract After Cortisol Treatment as a Function of Sedimentation Rate. In one experiment, we contrasted ^{3}H-leu incorporation with position on gradient I for all proteins of the nuclear extract.

TABLE III

Total Cortisol-Treated RNAP Activity and ^{3}H-Leucine Incorporated in Peaks of RNAP Activity for Gradients I and II, as Compared to Nuclear Extracts

Hrs after Cortisol Treatment (No.)	Nuclear Extract		Gradient I (No.)		Gradient II (No.)	
	RNAP (% of Control)	^{3}H-Leu (% of Control)	RNAP (% of Control)	^{3}H-Leu (% of Control)	RNAP (% of Control)	^{3}H-Leu (% of Control)
3 (1)	102.4 (1)	89.6 (1)	108.3 ± 8.0 (3)	94.3 (1)	92.9 ± 4.5 (2)	84.8 ± 6.9 (2)
6 (1)	86.7 (1)	71.5 (1)	85.7 ± 1.2 (3)	62.0 (1)	97.4 ± 0.9 (2)	58.2 ± 1.8 (2)
12 (3)	65.2 ± 4.3 (3)	54.1 ± 2.6 (3)	68.9 ± 3.0 (5)	51.8 (1)	73.6 ± 2.4 (5)	48.7 ± 1.4 (5)

Fourteen rats received cortisol and twelve rats received vehicle twelve hours before sacrifice. Thymocytes were labelled for four hours under standard conditions, and a nuclear extract prepared. Identical fractions from three gradients of exactly equal length were combined for both cortisol and vehicle treatments, and aliquots were removed for ^{3}H-leu determinations. Table IV shows the results, with cortisol ^{3}H-leu incorporation expressed as percent of vehicle control for each combined fraction. The decrease in protein synthesis which occurs in involution does not appear to occur equally throughout the proteins of the nuclear extract. Incorporation of ^{3}H-leu into larger proteins appeared to be inhibited to a greater degree than incorporation into smaller proteins.

Gel Electrophoresis of Proteins Associated With RNA Polymerase Activity at Various Stages of Purification. Disc gel electrophoresis was used to separate the proteins of the nuclear supernatants [(1)], nuclear extracts, and aggregates of fractions containing RNAP activity from GI and GII. These fractions were prepared from thymocytes obtained from rats twelve hours after injection with cortisol or vehicle and labelled in vitro with ^{3}H-leu. The gradients and acrylamide gels of material derived from thymocytes of cortisol and vehicle treated rats are referred to as cortisol and vehicle gradients and gels.

Individual GI fractions were combined with identical individual GI fractions from two other replicate gradients of equal length,

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(1) The 2.3 M sucrose TKM buffer through which nuclei were centrifuged for purification, containing primarily cytoplasmic proteins.

TABLE IV

^{3}H-Leucine Incorporation vs. Position on Gradient I for Nuclear Extract Proteins Labelled in Vitro Twelve Hours After Treatment in Vivo with Cortisol or Vehicle[1].

Combined Fractions	Cortisol/Vehicle x 100 (cpm ^{3}H-leu/100 μg DNA)
1-5 (top)	77.5%
6-11	56.5%
12-17	36.6%
18-23	28.1%

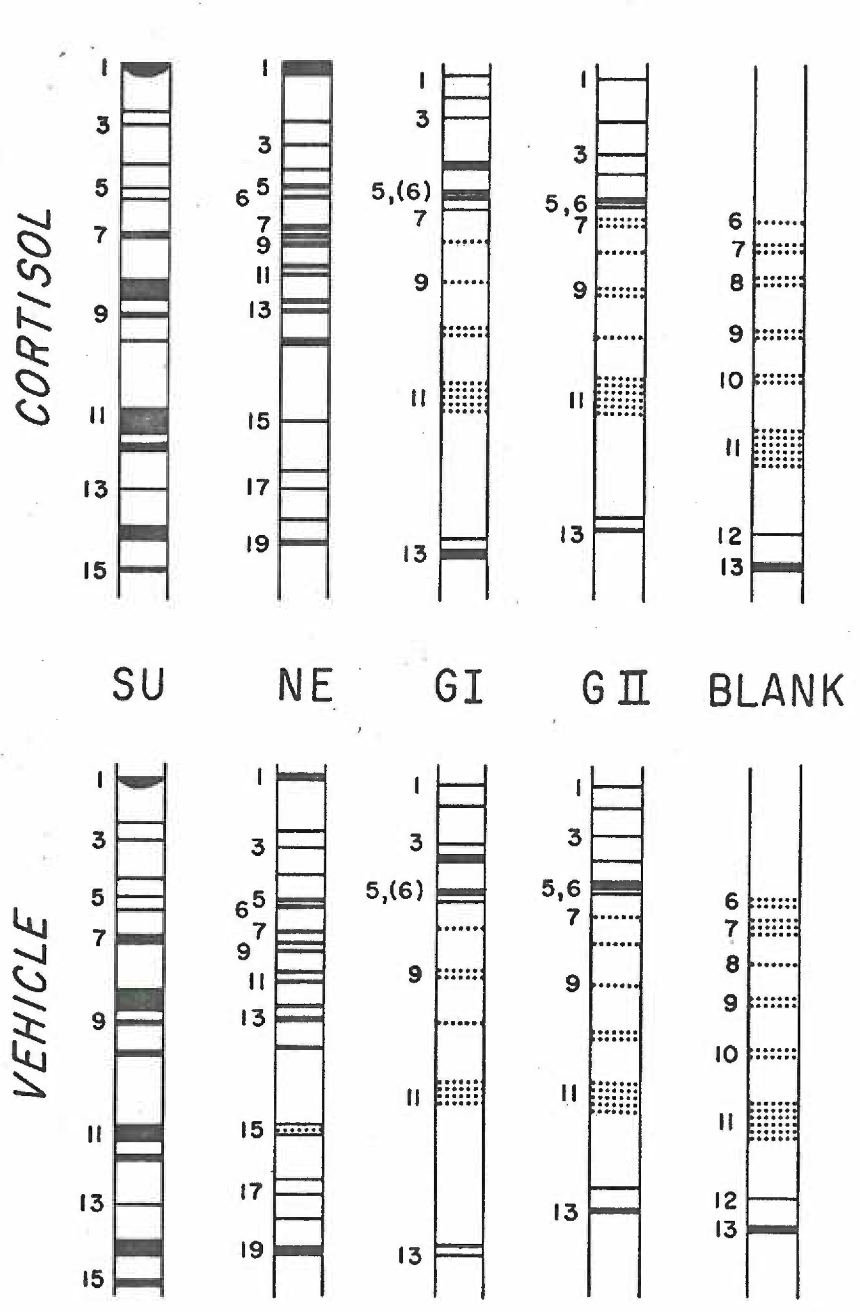
(1) Identical fractions from three gradients of equal length from one experiment were combined to provide each combined fraction for cortisol and vehicle.

resulting in a single composite gradient with three times the volume of the original individual gradients. The composite gradient was assayed for RNAP activity, and the five fractions containing the greatest RNAP activity were combined for electrophoretic examination of GI proteins, and for recentrifugation to obtain GII fractions. These GII fractions were combined in a similar fashion, the composite gradient assayed for RNAP activity, and the three or four fractions containing the greatest RNAP activity were combined to obtain a protein solution for the electrophoretic examination of GII. Electrophoretic examination was done on protein solutions composed of several pooled fractions, and is thus consistent with all our calculations using peaks of RNAP activity and ^{3}H-leu activity. Gels were stained with amido-black, examined visually, and scanned at 540 mμ with a Gilford Gel Scanner attached to a Beckman DU Spectrophotometer as described in Materials and Methods.

When amido-black stained gels that had undergone electrophoresis for three hours were examined visually, it was noted (Figure 7) that nuclear supernatant proteins (15 bands) differed from nuclear extract proteins (19 bands). Gradient I gels differed in the position of some bands from GII gels. Successive GI and GII gels each had thirteen bands, but eight of these were present as contaminants in a gel layered with material from a sucrose-glycerol-buffer (Figure 7, Blank, bands 6 through 13) and were ignored. Band No. 11 was the electrophoretically pure BSA.

Figure 7: Electrophoretic migration patterns of amido-black staining material in cortisol and vehicle gels of various thymocyte fractions.

Sucrose TKM supernatants (SU), nuclear extracts (NE), gradient I (GI), gradient II (GII), and sucrose-glycerol-buffer blank (BLANK) gels are shown for cortisol and vehicle treatments. SU gels were layered with 100 µl, NE, GI and GII gels with 200 µl, and BLANK gels with 400 µl of solution. Cortisol and vehicle gels for each fraction are compared in the vertical direction. Gels (5%) underwent electrophoresis at 2 mA/tube for three hours at room temperature. Gels were stained and destained as described in the text and in Materials and Methods. Gels were 68 mm in lenght and 1 mm in diameter. Gel band intensities are indicated by light and dark lines and by band width. In gels SU, NE, GI and GII, bands are numbered sequentially from the top of the gel. The bands in the BLANK gel are numbered according to their position in preceeding gels. Gradient I gel bands 5 and 6 overlap. Graidient II gel bands 5 and 6 are more clearly distinct, although they remain partially overlapping.


CORTISOL
VEHICLE
SU
NE
GI
GII
BLANK
1
3
5
5,(6)
5,6
6
7
8
9
10
11
12
13
15
17
19

Gel penetration of high molecular weight proteins is a complex function of ionic strength, pH, protein charge and aggregation. Ornstein[1] has shown penetration, however, of 7% gels by proteins with molecular weights as high as 850,000 to 1,300,000. RNAP is a large protein and should appear as a band near the top of the 5% gel, even if it forms a dimeric aggregate.

There is leakage of a fractional amount of RNAP into the sucrose TKM nuclear supernatants, with the retention of most of the RNAP in nuclei. Thus, the RNAP band should be minimally present in supernatants, while clearly present in extracts. Procedures which act to purify and conserve RNAP should clarify and preserve the RNAP band.

Two clear bands appeared near the top of both cortisol and vehicle gels which were minimally present in sucrose TKM nuclear supernatants, while much more clearly present in nuclear extracts (Figure 7, bands 5 and 6). Gradient centrifugations resulted in the retention of only one band (Figure 7, band 5). When contaminant bands were ignored in GI and GII gels, a sharp, main band (Figure 7, band 5) remained along with four light and indistinct bands migrating above band 5 (Figure 7, GI and GII, bands 1 through 4).

Spectroscopic scanning of GI gels led to fewer identifiable peaks (bands). In areas containing several bands and a high background

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(1) Ornstein, L., Disc Electrophoresis. I. Background and Theory. Ann. N. Y. Acad. Sci., 1964. 121 (2), 321-349.

Spectroscopic scanning of GI gels led to fewer identifiable peaks (bands). In areas containing several bands and a high background stain, the bands did not always appear clearly as peaks on the spectrogram. Thus, supernatant gels contained twelve identifiable peaks, nuclear extract gels, eleven, and GI and GII gels, nine each (not shown). Blank gradient gels showed seven peaks plus BSA. Generally, peak positions were similar between cortisol and vehicle gels. In all cases except the nuclear extract, however, the cortisol gel contained less amido-black staining material. Furthermore, there were often quantitative differences from peak to peak in the relative decrease of amido-black staining material found in cortisol gels as compared to vehicle gels. A sharp, consistent peak near the top of all of the gels and above the contaminant peaks was identified as band 5 in Figure 7. Above this sharp peak, a few minor peaks were identified.

Gels layered with GI and GII protein fractions were then subjected to electrophoresis for seven hours to eliminate the contaminant bands. This placed the band thought to be RNAP in the lower half of the gel, and permitted a better observation of the indistinct bands closer to the top of the gel. Gradient I gels (Figure 8) were layered with 200 µl of protein solution from five aggregated fractions from both vehicle and cortisol gradients. Gradient II gels (Figure 9) were layered with 500 µl of solution from three aggregated fractions from the cortisol gradients, and 400 µl of solution from four aggregated fractions from the vehicle gradients. The differences in the number of fractions aggregated reflected an attempt to examine

approximately equal protein levels. In Figures 8 and 9, the optical density of a gel layered with 400 µl of sucrose-glycerol-buffer blank is indicated as background. No corrections were made in Figures 8 and 9 for the relative amounts of material layered onto the gels or the relative amounts of DNA used to provide nuclear extracts.

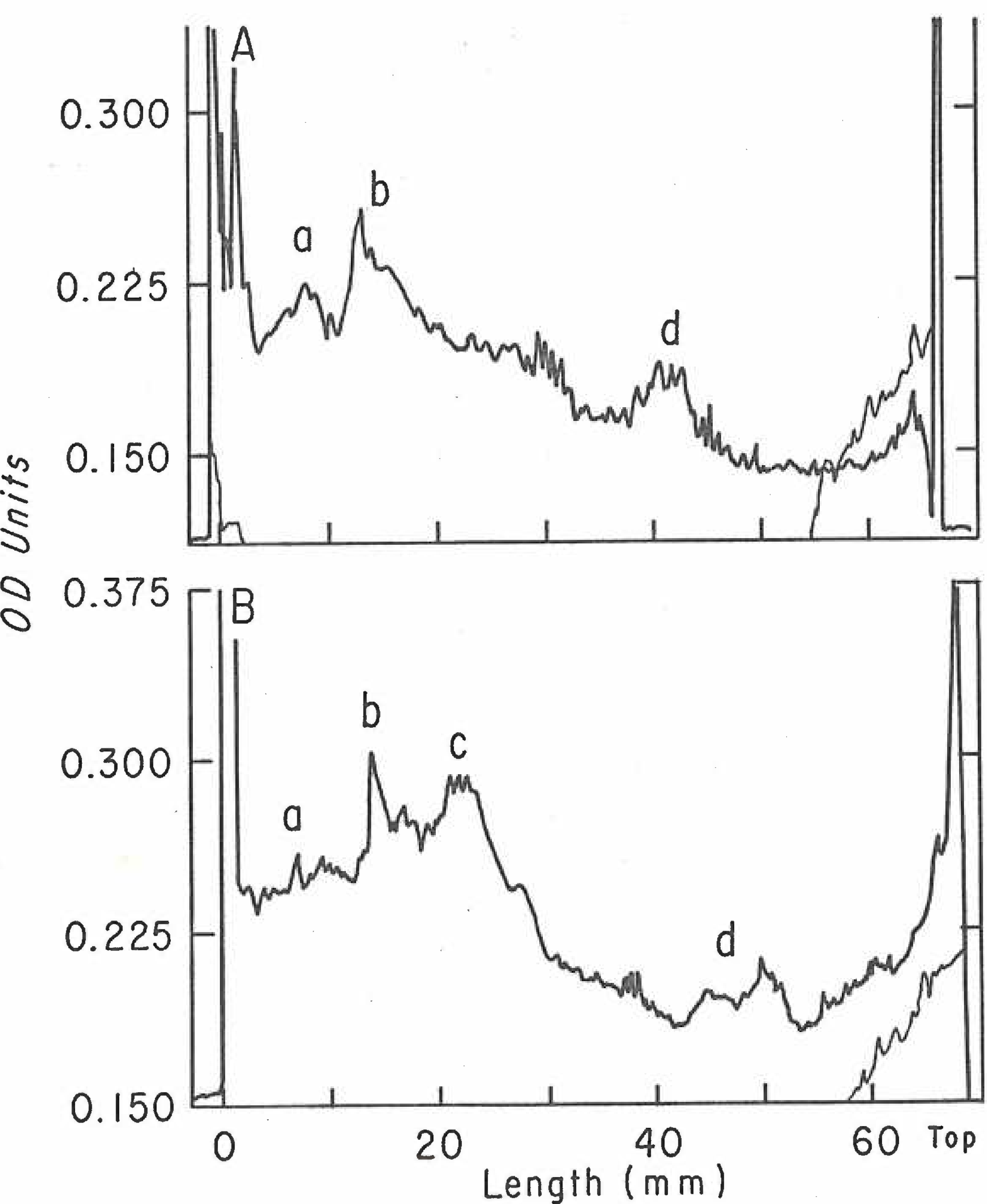
Visually, the main band (presumably band 5, Figure 7) moved the predicted distance into the lower half of the gel and remained distinct. It appeared to be the only band in both cortisol and vehicle gels of GI and GII protein solutions. The minor bands were no longer distinct, but the gels contained instead what appeared to be a very diffuse stain throughout the lower half of the gel.

Spectroscopic examination showed that the vehicle GI solution (Figure 8-B) contained four identifiable peaks ("a", "b", "c", and "d"), with the relatively sharp peak "b" the only visible band. While peaks "a" and "b" were positioned similarly in both cortisol and vehicle gels, cortisol peak "d" (Figure 8-A) appeared shifted to the left, i.e., its molecular weight appeared to have decreased. Peak "c" of the vehicle gel was absent from the cortisol gel, suggesting a specific cortisol-mediated protein deletion. The vehicle gel contained more amido-black staining material.

Gradient II (Figure 9) demonstrated similarly positioned peaks "a" and "b" for both cortisol (A) and vehicle (B) gels. These two peaks were the same peaks found in GI gels (peaks "a" and "b" in Figures 8-A and 8-B). Cortisol gels were layered with 500 µl, vehicle gels with 400 µl, and blank gels with 400 µl of gradient material. The increased OD of cortisol GII solution above the

Figure 8: Distribution of amido-black staining material after gel electrophoresis of sucrose gradient I purified RNA polymerase.

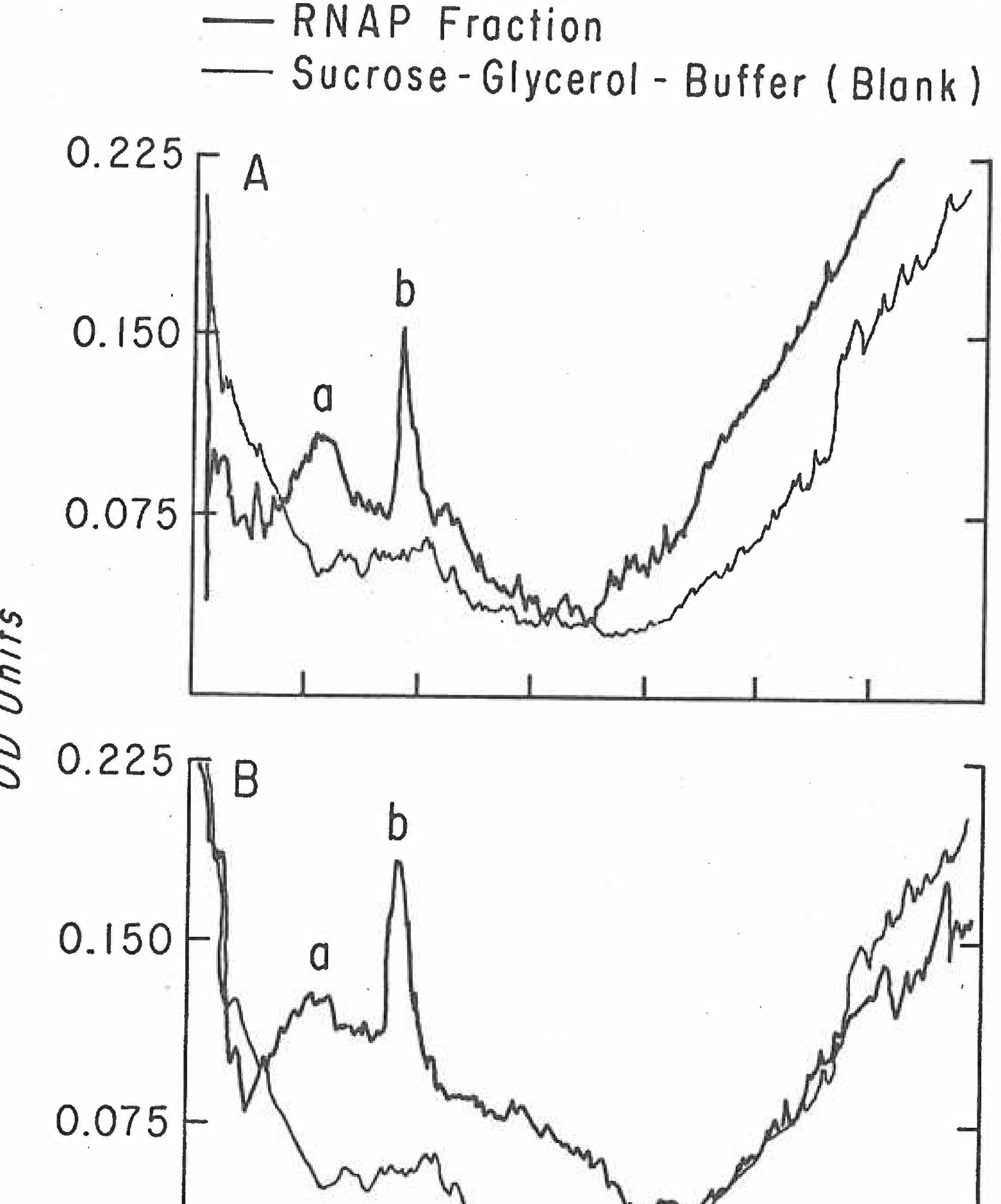
Gradient I centrifugations were made of nuclear extract proteins labelled in vitro twelve hours after treatment in vivo with cortisol or vehicle. Gels (5%) were layered with 200 µl of protein solution from five aggregated fractions from both cortisol (A) and vehicle (B) gradients. Gels underwent electrophoresis for seven hours at room temperature under standard conditions (see Materials and Methods). Gels were stained with 0.5% amido-black in 7% HAc for 30 minutes, and destained overnight in 7% HAc in the Hoefer Diffusion Destainer Unit. Gels were stored in 7% HAc and scanned at 540 mµ using the Gilford Linear Transport System on the Beckman DU Spectrophotometer. A sucrose-glycerol-buffer blank was scanned for background and is included although it is only visible at the top of the gel.



RNAP Fraction
Sucrose - Glycerol - Buffer (Blank)
A
a
b
d
0.300
0.225
0.150
OD Units
B
a
b
c
d
0.375
0.300
0.225
0.150
0
20
40
60
Top
Length (mm)

Figure 9: Distribution of amido-black staining material after gel electrophoresis of sucrose-glycerol gradient II purified RNA polymerase.

Gradient II centrifugations were made of aliquots of five aggregated fractions of Gradient I which contained high RNAP activity. The solution layered onto the gradients also underwent electrophoresis (Figure 8). Gels (5%) were layered with 500 µl of solution from three aggregated fractions from cortisol gradients (A), and 400 µl of solution from four aggregated fractions from vehicle gradients (B). Gels underwent electrophoresis for seven hours at room temperature under standard conditions (see Materials and Methods). Gels were stained, destained, and scanned as in Figure 7. A sucrose-glycerol-buffer blank was scanned for background.


RNAP Fraction
Sucrose-Glycerol-Buffer (Blank)
0.225
A
b
0.150
a
0.075
OD Units
0.225
B
b
0.150
a
0.075
0
20
40
60
Top
Length (mm)

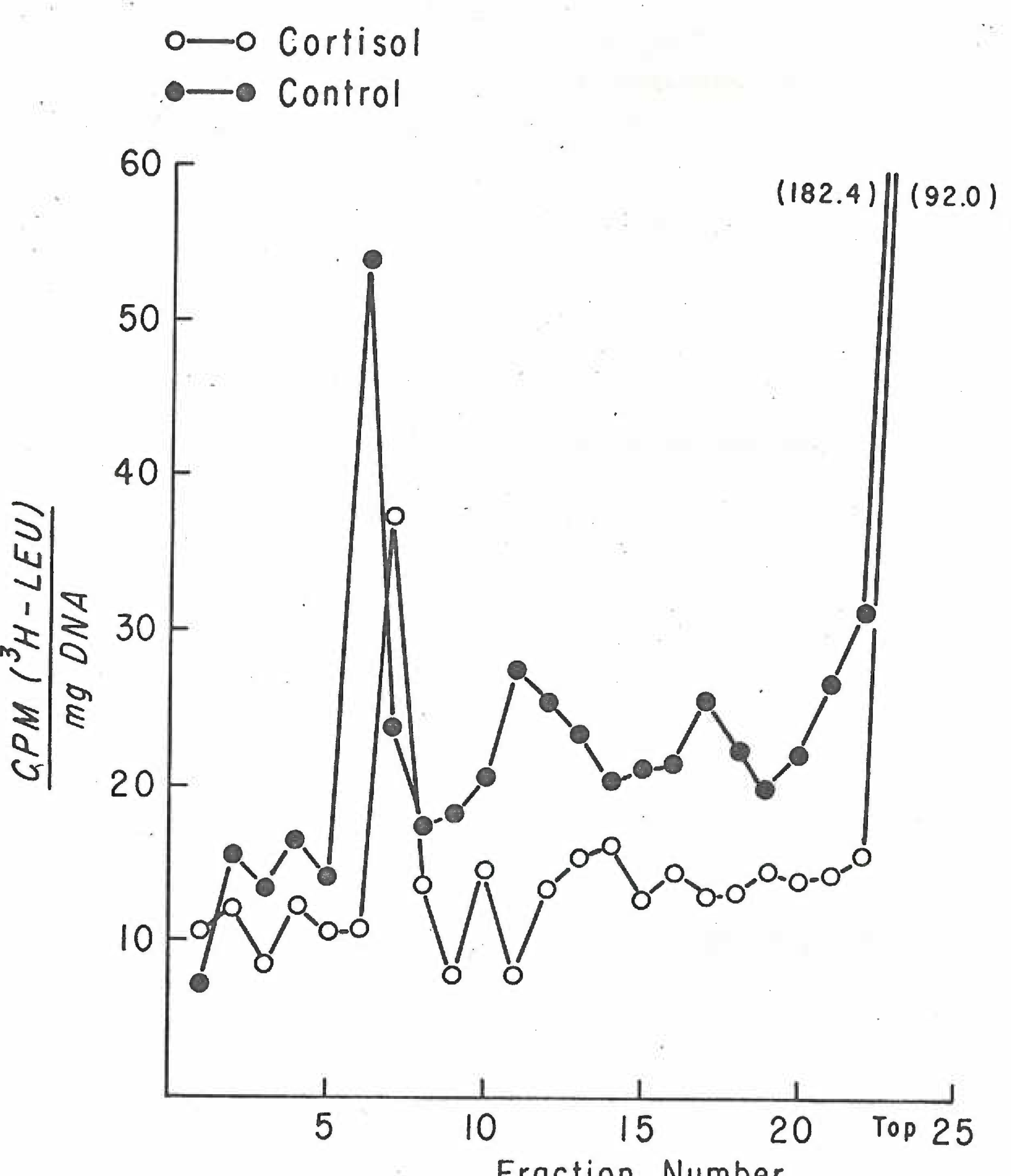
blank in the top 20 mm of the gel in Figure 9-A can be accounted for by the increased volume of solution layered onto the gel (500 µl vs. 400 µl). When the blanks are subracted, there are increases in amido-black staining material in the vehicle gel in spite of the increased volume of material layered onto the cortisol gel. In both cases, sharply defined peak "b" was the only peak visible.

The amounts of amido-black staining material were compared by tracing the peaks shown in Figures 9-A and 9-B onto graph paper and cutting out the peaks minus the sucrose-glycerol-buffer blank areas. Those areas which were compared from Figure 9 were: total areas (cortisol, Figure 9-A, 8 to 31 mm; vehicle, Figure 9-B, 7 to 17 mm), and the sharp "b" peaks (cortisol, Figure 9-A, 17 to 22 mm; vehicle, Figure 9-B, 17 to 22 mm). In all calculations, corrections were made for the amount of gradient solution layered onto the gel. The resultant ratios of (cortisol/vehicle amido-black staining material minus blank) x 100 were: total area, 30.4%; peak "a", 42.8%; and peak "b", 37.4%.

Replicate gels from the same electrophoretic run shown in Figures 9-A and 9-B were frozen on dry ice and sliced into 1.5 mm discs. Two discs were combined, and the gels depolymerized by heating in concentrated H_2O_2. The ^{3}H-leu activity of bands in the gels (Figure 10) was counted by dissolving the depolymerized gels in toluene scintillation solution containing 15% Bio-Solv BBS-3 (see Materials and Methods). The peaks of ^{3}H-leu activity shown in Figure 10 were corrected for differences in the amounts layered

Figure 10: CPM (^{3}H-leu)/mg DNA present in gels layered with aliquots of fractions from cortisol and vehicle second gradients (GII).

Gels (5%) were layered with 500 µl of solution from three aggregated GII fractions from cortisol gradients, and 400 µl of solution from four aggregated GII fractions from vehicle gradients. Gels underwent electrophoresis for seven hours at room temperature under standard conditions (see Materials and Methods). The gels shown in this figure are replicates of those shown in Figure 9. The gels were sliced and depolymerized for counting ^{3}H-leu activity as described in Materials and Methods. When corrections were made for efficiency, the recovery of ^{3}H-leu layered onto the gels exceeded 90%. The figures shown are derived from means of at least four ten minute counts and are background subracted. The peak control (vehicle) fraction six contained 260 net counts per ten minutes (about twice background). The 1.5 ml of GII material layered onto the gel was derived from 481.9 gamma of nuclear DNA. CPM/mg DNA was calculated at 54.0, and similar calculations for the cortisol peak fraction seven yielded a value of 37.3. The obvious peaks between fractions 5 and 10 each contained approximately 10% of the total counts in the gels. While there are suggestions of other peaks closer to the top of the gels, they correspond to no obvious peaks of amido-black staining material. The ^{3}H-leu material through the upper portion of the gel may represent tailing or background variation.


Cortisol
Control
60
50
40
30
20
10
$\frac{CPM\ (^3H\text{-}LEU)}{mg\ DNA}$
(182.4)
(92.0)
5
10
15
20
Top 25
Fraction Number

onto the gels and compared. The ratios of background subtracted cortisol/vehicle x 100 ^{3}H-leu activity were: total (cortisol and vehicle fractions 1 to 22), 50.4%, and specific peaks (cortisol fractions 6 to 8; vehicle fractions 5 to 9), 52.3%. The positions of the ^{3}H-leu peaks in Figure 9 were coincident with the protein peaks "b" in Figures 9-A and 9-B. Figure 10 fractions 1 to 5 were coincident with protein peaks "a" in Figures 9-A and 9-B. These fractions contain no distinctive ^{3}H-leu peaks. The background subtracted cortisol/vehicle x 100 ^{3}H-leu ratio for fractions 1 to 5 was 78.3%.

Several attempts were made to examine the gel for RNAP activity. Gels which underwent electrophoresis at 25° C and 0° C were sliced, and the individual discs assayed intact, crushed, or allowed to stand in the RNAP assay buffer for various lengths of time before assays were performed on the buffer. In each case, the results were negative, and the area of RNAP could not be enzymatically defined.

<u>Search For Possible Modifiers of RNAP Activity</u>. The consistent and substantial loss of enzymatic activity on GI, and the less dramatic loss on GII, led us to hypothesize the loss of an activator or initiator for RNAP on GI. This hypothesis would be consistent with the presence of activation, stabilization, and/or initiation factors found in eukaryotic RNAP systems (Stein and Hausen, 1970; Goldberg and Moon, 1970; Mondal, Mandal, and Biswas, 1970).

Approximately three weeks after freezing the GI shown in Figure 5, composite fractions were prepared by combining fractions 1 to 4 and fractions 5 to 8 for both cortisol and vehicle gradients. These fractions were chosen under the assumption that any modifiers present were lighter than RNAP. Assays were performed for RNAP activity in these composite fractions. In both gradients, substantial RNAP activity was found in fraction 9, and therefore composite fractions were not prepared with gradient material beyond fraction 8. The previously aggregated and frozen fractions containing peak enzymatic activity (cortisol fractions 12 to 15; vehicle fractions 10 to 13) were then assayed in triplicate, corrected by one zero time assay. The activity of the RNAP in cortisol fractions 12 to 15 and vehicle fractions 10 to 13, had declined to 51% and 58% of the original RNAP activity respectively. The fractions containing peak enzymatic activity were then assayed in combination with aliquots of the composite fractions added to the assay mixture. In each experiment, 100 µl of the composite fractions was added to 50 µl of the RNAP material. RNAP assays were done in duplicate and corrected for zero times and the slight amount of RNAP activity present in the composite fractions. Addition of three of the composite fractions to RNAP (cortisol fractions 1 to 4 and cortisol fractions 5 to 8 added to cortisol fractions 12 to 15; vehicle fractions 1 to 4 added to vehicle fractions 10 to 13) led to a loss of activity (5% to 12%). Addition of the fourth composite fraction (vehicle fractions 5 to 8 added to vehicle fractions 10 to 13) led to an increase in activity (13%). No definitive alteration in enzymatic activity was seen.

In one experiment, GI fractions were prepared from thymocyte nuclear extracts obtained from rats injected with cortisol or vehicle six hours previously. The fractions were frozen at -20° C with an equivalent volume of DTT-Tris, pH 8.0 buffer containing 1.0 mg/ml BSA. When the gradient fractions were assayed one month later, cortisol was found to have 24.1% and vehicle 23.5% of the RNAP activity originally layered onto the gradient. The RNAP activity of two sets of replicate gradients assayed directly after fractionation without storage were: cortisol 21.3% and 21.6%; vehicle 18.0% and 23.5% of the RNAP activity originally layered onto the gradients. These values are essentially the same, indicating no further loss of activity upon storage for one month after centrifugation. An assay of crude nuclear extract RNAP activity remaining after storage at -20° C for one month yielded values approaching the fraction of activity found in the gradients immediately after fractionation (cortisol, 37.7%; vehicle, 29.2%). These data suggest the loss of an RNAP activator, initiator, or stabilizer at a rapid rate with centrifugation, and at a slower rate with storage as nuclear extract.

DISCUSSION

Thymus Involution and Regeneration. I could not separate the time sequence of inhibition of incorporation of precursors of RNA, DNA and protein into cold-acid insoluble material of thymocytes during the course of involution. However, marked inhibitory effects on the incorporation of ^{3}H-uridine and ^{14}C-leucine were apparent early after a single injection of cortisol. In the later regenerative phase, increase in uridine and leucine incorporation into cold-acid insoluble material occurred before an increase was seen in thymidine incorporation. Increases in the incorporation of RNA, DNA and protein precursors occurred before significant increases in gland weight were observed. Cortisol at 50 mg/kg led to severe thymic involution, but the glands began to recover, as detected by increased biosynthetic activity, as early as 48 hours after cortisol treatment.

There was a shift in cell populations with time after cortisol treatment, an effect previously described by Dougherty and White (1943, 1945). Cortisol destroyed primarily small thymocytes, leaving a reticular matrix containing intermediate to large thymocytes. Daniels, Ritzmann and Levin (1968) have stated that the large and intermediate thymocytes may be the precursors of small thymocytes. The destruction of small thymocytes may represent a physiological event (e.g. in the release of 'lymphopoietin', a factor believed to stimulate a general lymphopoiesis in the lymphoid organs; Defendi and Metcalf, 1964). Endogenous glucocorticoids may play a role in thymocyte regulation. Thus, the marked thymic involution seen with

cortisol may represent an exaggerated response which ordinarily occurs with adrenal stimulation and the release of endogenous glucocorticoids. Cortisol may act to arrest stem cell division, in which case involution could represent the physiological destruction of small thymocytes, which results in gland shrinkage because the destroyed cells are not replaced.

It is unclear whether regeneration of the thymus gland after irradiation or glucocorticoid treatment occurs via repopulation by remaining stem cells, or whether the gland is repopulated by cells from other lymphoid organs such as lymph nodes (Wolstenholme and Porter, 1966). We must consider, therefore, that cortisol treatment may alter cellular biosynthetic processes through enzymes such as RNAP, or it may select, or partially select, a particular thymocyte cell population. This cell population may contain an RNAP with enzyme kinetics differing from that evidenced by RNAP from an unchallenged thymocyte population. Small thymocytes may be differentiated in the fulfillment of an unknown function, perhaps lytic secretion, whose RNA synthesis may be primarily mediated through nucleoplasmic RNAP. Our laboratory has shown that nucleoplasmic RNAP is much more sensitive to cortisol than nucleolar RNAP (Fox, Gauslaa and Gabourel, 1971, manuscript in preparation), and this could provide an explanation of how small thymocytes are destroyed. Cortisol may thus select a population of stem cells whose primary role is division and whose rapid growth is mediated primarily by nucleolar RNAP. The RNAP characteristics of the selected cell

population might then be interpreted as a cortisol-mediated inhibition of thymocyte RNAP.

Although different cell types were observed only on the basis of size, no attempts were made to separate different cell types. To further investigate the possibility of altered cell populations, thymocytes should be separated according to size via sieving, mild cell centrifugation, cell column chromatography, or other techniques. There are undoubtedly many cell types present, which may be identified and separated according to various criteria. The RNAP response to cortisol may then be studied in various thymocyte populations which contain a predominance of a single cell type.

Thymocytes could also be cloned out and maintained in culture to determine glucocorticoid sensitivity. These experiments should provide the answer to questions such as whether larger thymocytes are progenitors of small thymocytes. Cortisol may select a steroid resistant cell population. Experiments could be performed which would examine whether the cortisol involuted and regenerated thymus is as susceptible to a second cortisol challenge as the first. Cells of severely involuted thymus glands could also be cloned to investigate this possibility, which could have a clear bearing on malignant lymphoid cell line steroid resistance. If there are differences in steroid susceptibility of cell types or regenerated thymocytes, we may then pose the question of whether these effects are mediated by differences in RNAP core structure or in RNAP modifiers.

Cortisol Effects on RNA Polymerase Activity and Synthesis. Thymus nuclei, isolated from rats six and twelve hours after cortisol treatment, contained less soluble, extractable RNAP activity than identical preparations from control animals (Table II). These findings confirm the data of Gabourel and Fox (1970, 1971) and Nakagawa and White (1971), who have demonstrated decreases in extractable thymus RNA polymerase activity after cortisol treatment.

The soluble RNAP found in the nuclear extract has undergone a considerable purification as compared to the RNAP in whole cells, intact nuclei, or aggregate enzyme preparations. Extraction of nuclei was performed at pH 8.0, and would not be expected to solubilize basic proteins such as histones. DNA also remained in the sedimentable fraction after extraction, and the soluble RNAP required addition of exogenous DNA template for activity. The assay system used to measure RNAP activity contained excess purified calf thymus DNA and nucleoside triphosphate precursors. Thus, cortisol-induced changes in the rate of RNA synthesis by this system cannot be attributed to membrane effects, changes in precursor pool sizes, changes in template activity, or other unknown factors present in less purified systems.

Table II contrasts cortisol effects on the RNAP activity of nuclear extracts with effects on ^{3}H-leu incorporation into thymocyte proteins. Three hours after cortisol treatment, soluble, extractable RNAP activity has not been affected. There was, however, a dimunition of ^{3}H-leu incorporation into whole cell proteins (27%) and, to a lesser degree, into nuclear extracts proteins (10%).

Six hours after cortisol treatment, RNAP activity was decreased slightly (13%), whereas incorporation of ^{3}H-leu into whole cell, whole nuclei, and nuclear extract proteins was decreased to a greater degree (40%, 39% and 28% respectively). Twelve hours after cortisol treatment, RNAP activity was significantly reduced (36%), but ^{3}H-leu incorporation into whole nuclear, whole cell, and nuclear extract proteins was still depressed to a greater extent (64%, 59% and 46% respectively). The reduction in ^{3}H-leu incorporation into whole cell protein consistently exceeded that into nuclear extract protein, including RNAP, at all times studied. The ^{3}H-leu incorporation into proteins of intact nuclei was also reduced to a greater extent than into proteins of nuclear extract six and twelve hours after cortisol injection, but not at three hours.

Table III compares RNAP activity and ^{3}H-leu incorporation for nuclear extract and two sequential gradient purifications of proteins associated with RNAP activity. The cortisol-mediated reduction of RNAP activity and ^{3}H-leu incorporation seen in nuclear extracts was evident after the two centrifugations, and was similar to that seen in nuclear extracts. With a few exceptions, the ratio of cortisol/ vehicle x 100 for ^{3}H-leu incorporation in specific peaks under the RNAP activity peaks in GII was similar to the same ratio in GI and nuclear extracts. As will be discussed in the following section on electrophoresis, the ^{3}H-leu activity in the GII RNAP peak was contributed by a single protein. Thus, in the spectrum of inhibition of protein synthesis, proteins associated with RNAP activity do not appear to be affected as early or to as great an extent

as many other proteins. The cortisol-mediated decrease in ^{3}H-leu incorporation into nuclear extract proteins (Table II), and the proteins associated with RNAP activity through two sequential gradient fractionations (Table III), always precedes and exceeds decreases in soluble RNAP activity. It is possible, therefore, that the decrease in RNAP activity seen in these experiments may result from a decreased synthesis of the enzyme. Although the decrease in soluble RNAP activity seen at six and twelve hours after cortisol treatment may be linked to a decrease in RNAP synthesis, the early effects (three hour) of cortisol on protein synthesis can not easily be attributed to a decrease in the amount of extractable, soluble RNAP activity.

The time course for decreases in thymocyte ^{3}H-leu incorporation at various times after cortisol treatment (Table II) agrees with previous work on the inhibition of protein synthesis by cortisol in thymocytes and in microsomal preparations (Gabourel and Comstock, 1964; Makman, Dvorkin and White, 1966; Brunkhorst, 1968). Earlier and more dramatic decreases have been shown to occur for ^{3}H-uridine incorporation into whole thymocytes and for ^{3}H-UMP into aggregate enzyme preprations. Makman, Dvorkin and White (1966) reported a 54% inhibition of ^{3}H-uridine incorporation into thymocyte suspensions in vitro three hours after cortisol injection in vivo. Similar effects were seen in thymocytes treated with cortisol in vitro. Fox and Gabourel (1967) found a 54% decrease in ^{3}H-UMP incorporation into thymocyte aggregate enzyme preparations six hours after cortisol treatment in vivo, which increased to 62% by twelve hours. Nakagawa and White (1967) reported a 25% reduction in ^{3}H-UMP incorporation into

thymic nuclei and aggregate enzyme three hours after cortisol injection in vivo. These data make it difficult to associate the decrease in soluble RNAP activity or RNAP synthesis with the early decreases in uridine incorporation by whole cells or UMP incorporation by aggregate preparations.

It is tempting to conclude that the later (six and twelve hour) decreases in ^{3}H-UMP incorporation reported for our soluble extracts are due to a true decrease in RNAP activity, since pool sizes, template and other factors were controlled. If early cortisol-induced thymus involution is mediated through RNAP, however, it must act through the synthetic or allosteric alteration of some unknown modifier of RNAP activity. If this modifier is lost in the extraction procedure, then it would not be reflected in an early reduction in RNAP activity in the nuclear extracts or gradient purified RNAP.

We have demonstrated that the synthesis of proteins associated with soluble RNAP activity do not mediate the early (three hour) decreases in protein synthesis seen in thymus involution. This is not to say that synthesis of RNAP may not play an important role in involution. Figures 1, 2 and 3 show that the course of involution requires several days, and it may be significant that the loss of gland weight becomes marked only after extractable RNAP activity has been depressed. Thus, the nucleoplasmic RNAP may play an important role by biologically amplifying primary involutionary events which are not yet understood.

A single experiment was performed measuring cortisol-induced inhibition of ^{14}C-tryptophan incorporation into thymocytes treated in vivo with cortisol twelve hours previously. The incubation period of 30 minutes led to a ratio of cortisol/vehicle x 100 for ^{14}C-tryptophan incorporation of 51.3, which is close to the same ratio of ^{3}H-leu incorporation seen with a four hour incubation (41.3, Table II). This finding suggests that cortisol inhibition of incorporation of protein precursors is independent of the precursor used. It also suggests that cortisol inhibition of incorporation of protein precursors is essentially independent of incubation after sacrifice of the animals. Related to this finding is the report of Makman, Dvorkin and White (1968) that washing cortisol out of thymus cell suspensions did not alter the degree of existing inhibition. A further incubation of three hours in a cortisol-free medium did not alter the degree of inhibition.

Table IV compares the inhibition of ^{3}H-leu incorporation into the proteins with their position on GI. Decreases in ^{3}H-leu incorporation appeared to occur as a function of molecular weight, with inhibition greatestin the larger proteins and least in the smaller proteins. Gabourel and Fox (1965) reported that while cortisol affected all particulate fractions of RNA, the most pronounced effects occurred on the larger aggregates (polysomes). Cortisol effects which affect larger molecular weight RNA might also be expected to affect larger molecular weight proteins. Cortisol thus could affect the synthesis of various proteins differently.

There is a selective cortisol-mediated induction of rat liver proteins, e.g., tyrosine transaminase (Kenney and Flora, 1961), and our data suggests that involution may be mediated by selective effects on specific thymic proteins. The specific induction of rat liver proteins by cortisol is mediated primarily through nucleolar RNAP (Tata, 1966), while our laboratory has shown that cortisol-mediated involution decreases the nucleoplasmic RNAP activity in the rat thymus.

Gel Electrophoresis of Proteins Associated With RNA Polymerase Activity at Various Stages of Purification. Electrophoresis established that a specific fraction of nuclear proteins was selected through the RNAP extraction and purification procedures. In almost every case, portions of cortisol gels contained less amido-black staining material per mg DNA than the corresponding portions of vehicle gels. This finding is consistent with the marked decrease in ^{3}H-leu incorporation found in thymocyte fractions from rats treated with cortisol twelve hours previously (Table II), and indicates that these decreases may be physically correlated with a diminished quantity of protein per mg DNA or per cell. There were differences from band to band in the relative decreases in amido-black staining material found in cortisol gels when compared to material found in vehicle gels. These differences may reflect different rates of protein turn-over, but are not evidence in themselves for selective cortisol effects on protein synthesis.

Proteins differ in their abilities to absorb stains such as amido-black, and the amount of stain absorbed is only quantitatively comparable when the same proteins are examined. To compare peaks on the gel scans, therefore, is to make the assumption that the compared peaks contain the same protein(s). What was seen in the gel scan may not be an accurate quantitative assessment of the amount of protein present, however, because some material invariably remained on the top of the gel and could not be quantitated because of optical distortion at the edge of the gel. Retention of material at the top of the gel could occur through retention of material of sufficiently high molecular weight that it does not penetrate the gel, through precipitation of very basic proteins under the basic conditions of the electrophoresis, or through protein denaturation and/or aggregation. Retention of material on the upper surface of the gel can be measured, however, in Figure 10, as retained radioactivity. In this instance, the ratio of cortisol/vehicle x 100 for ^{3}H-leu activity retained on the upper surface of the gel (50.0%) is very similar to the same ratio on other portions of the gel. This finding suggests a specific phenomena, such as the retention of high molecular weight or basic proteins.

While proteins on cortisol and vehicle gels generally migrated to similar positions, there was a cortisol-mediated shift of one protein peak to what appeared to be a lighter molecular weight (Figure 8-A, peak "d"). In Figure 8-A, there also seemed to be a specific

cortisol-induced deletion (peak "c"). If peak "c" is deleted, then cortisol may selectively cause its destruction or in some other way alter its state (e.g., by conformational changes) so that it no longer appears in the gel under the experimental conditions used. These findings suggest that cortisol may selectively affect the synthesis or disposition of specific proteins or perhaps even specific protein subunits, with a resultant altered electrophoretic migration pattern. This finding lends credence, a priori, to the hypothesis that the synthesis of proteins such as RNAP or RNAP modifiers may be selectively depressed.

If RNAP activity is directly correlated with the amount of RNAP molecule present, then I would expect the cortisol-mediated decrease in RNAP to approximate the decrease in amido-black staining material. This would not be the case, however, if a modifier of RNAP was altered by cortisol rather than the synthesis of the entire core enzyme. The decrease in RNAP activity would approximate the decrease in ^{3}H-leu incorporation into RNAP protein only if the enzyme turned over rapidly, i.e., if a large fraction of the total pool was labelled under the conditions of the experiment.

Visual examination of gels after electrophoresis of peak RNAP fractions from GII indicated a single distinct band of amido-black staining material with areas of diffuse staining above and below it. This suggested that the bulk of the material associated with RNAP activity was a single protein. When the gel was scanned with a spectrophotometer, however, two peaks were discernable (peaks "a" and "b" in Figure 9), indicating the presence of two protein entities.

The ratios of cortisol/vehicle x 100 for amido-black staining material minus blank (from Figure 9) were: total 30.4%, peak "a" 42.8%, peak "b" 37.4%. The ratios of cortisol/vehicle x 100 for ^{3}H-leu activity (from Figure 10) were: total 50.4%, and specific peaks 52.3%. The latter value matches the ratio of cortisol/vehicle x 100 for ^{3}H-leu activity for the area of GII RNAP activity (Table III, twelve hours after cortisol treatment), which is 48.7 $\pm$ 1.4%. The ratiosof cortisol/vehicle x 100 for RNAP activities (Table III, twelve hours after cortisol treatment) were: nuclear extract 65.2 $\pm$ 4.3, GI 68.9 $\pm$ 3.0, and GII 73.6 $\pm$ 2.4. Thus, the reduction of protein synthesis in the specific portion of the gradient containing RNAP by approximately 50% leads to a 30% reduction in RNAP activity (presumably the total RNAP pool). These data are consistent with the partial turn-over of the thymocyte enzyme RNAP during the twelve hour cortisol treatment in vivo and the four hour labelling period in vitro.

The retention and clarification of the "b" peaks in procedures which conserve and purify RNAP would be consistent with the suggestion that peak "b" is RNAP. Peak "b" was the only peak which contained appreciable radioactivity after labelling with ^{3}H-leu (Figure 10). This suggests that peak "a", which migrated with the protein thought to be RNAP in GII, turned over at a very slow rate. The "a" peak might then represent a minority protein contaminant. I cannot conclude this with certainty, however, since I could not enzymatically

define the area of RNAP on the gels[1]. If the peak "a" is RNAP, then I must conclude that synthesis of the enzyme RNAP is even more resistant to cortisol than I have indicated in the previous discussion.

Search for Possible Modifiers of RNAP Activity. Gradient I recoveries were 20% to 50% of the layered nuclear extract RNAP activity. In contrast, Cunningham, Cho and Steiner (1969) solubilized RNAP from rat liver nuclei and obtained a 40% yield after a single purification through DEAE-cellulose. This loss is very close to our own rate of decay of rat thymus RNAP during the initial sucrose-glycerol gradient fractionation, and fits Richardson's observations (1966) of a 50% activity loss of bacterial RNAP on sucrose gradients. All of these gradients, including our own, contained BSA. In our experiments, no further loss of RNAP activity occurred in gradient I purified preparations during storage at -20° C for periods up to one month. These observations suggest that the initial loss of RNAP activity seen after a gradient purification may be the result of the loss of a modifier (stimulator) of RNAP activity.

It would seem that either an activator or initiator, when added back to the fractions containing RNAP activity, could stimulate the enzyme. Assay of material in the upper portion of gradient I, however, failed to detect the presence of any activator or initiator under the

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[1] J. C. Bagshaw and J. W. Drysdale reported methods at the June, 1971 San Francisco Biochemistry Society Meetings of assaying mouse kidney and liver RNAP after isoelectric focusing in 4% acrylamide gels (Isoelectric Focusing of Mouse RNA Polymerase, Fed. Proc., 30, 1971. 1302Abs.).

conditions of the standard RNAP assay. It should be pointed out, however, that this experiment was performed with gradients which had been stored at -20° C for three weeks after fractionation. It is possible that a modifier of RNAP activity is not stable under these conditions. Consistent with this suggestion is the fact that RNAP in nuclear extract has been found to be less stable than gradient purified preparations during storage at -20° C. The nuclear extracts used in these experiments lost about 50% of their original activity during the three weeks of storage. Gradient I purified RNAP stabilized with BSA lost little RNAP activity in the course of three weeks, although its original activity consisted of only approximately 20% of the original nuclear extract activity.

The disruption of a functional relationship between the enzyme RNAP and a modifier, and their separation by centrifugation, does not necessarily mean that adding the modifier back would result in the reformation of the same functional relationship. Nonetheless, Goldberg and Moon (1970), and Stein and Hausen (1970) have demonstrated that the addition of separable protein fractions back to the eukaryotic RNAP may result in dramatic modifications of its activity, both in regard to stability and activation.

Our laboratory has also shown a shift in template specificity after RNAP is purified on GI (Gabourel and Fox, 1971, unpublished observations). The nuclear extract RNAP has greater activity on native than on denatured template, while the purified RNAP has greater activity on denatured than on native template. Although this effect could be due to the loss of ribonuclease H on the gradient, which specifically

degrades RNA formed on the denatured template (Hausen and Stein, 1970) it suggests the loss of a component which controls template specificity. This component could also act as a stabilizer. Stein and Hausen (1970) isolated a protein factor S from bovine thymus which stimulated calf thymus RNAP, changed the concentration of salt for optimum activity, and also changed the enzyme kinetics. With factor S, RNAP preferred native template, while without it, RNAP was more active on denatured template. Goldberg and Moon (1970) found a bovine thymus RNAP factor which not only stimulated RNAP activity, but was necessary for its stability. The loss of a similar component could explain our loss of activity on GI and the change in template specificity seen with purification.

I have demonstrated that involution of the rat thymus gland cannot be explained as an early cortisol-mediated effect on the activity of soluble, extractable RNAP. Since it was probable that the extraction method used extracted primarily nucleoplasmic RNAP, it is possible that other RNAP forms may account for the early cortisol-mediated decrease in ^{3}H-UMP incorporation seen in thymocytes (Makman, Dvorkin and White, 1966), thymocyte nuclei (Nakagawa and White, 1967), and thymocyte aggregate enzyme preparations (Fox and Gabourel, 1967; Nakagawa and White, 1967). Our laboratory has shown, however, that thymus nucleoplasmic RNAP is the form affected by cortisol, and not the nucleolar form extracted to a smaller extent by our extraction procedure (Fox, Gauslaa and Gabourel, 1971, manuscript in preparation).

Early cortisol-induced decreases in RNA synthesis by whole cells and aggregate preparations may include effects on precursor uptake and phosphorylation, pool sizes, template and unknown cellular factors. My experiments isolated the enzyme RNAP and measured the rate of inhibition of the enzyme itself.

Attempts were made to measure ^{3}H-leu incorporation into RNAP itself. While significant purification of the enzyme was accomplished, failure to demonstrate enzyme activity on the gels prevented a precise quantitation of ^{3}H-leu incorporation. Although a decrease in incorporation of ^{3}H-leu occurs in the particular protein factions containing nucleoplasmic RNAP, this decrease does not appear to be an early or selective decrease in that it occurs at the same time or later and less dramatically than decreases in other cell proteins. When amido-black staining or ^{3}H-leu incorporation was examined for all of the protein factions seen on the gels, it was found that cortisol did not selectively inhibit their synthesis. Significant decreases in RNAP activity occur only after large decreases in ^{3}H-leu incorporation into proteins have occurred. Although the decreases in soluble RNAP activity seen at six and twelve hours after cortisol treatment may be linked to a decrease in RNAP synthesis, these effects are not observed early enough to account for the effects seen three hours after cortisol treatment.

If early cortisol-induced thymus involution is mediated through RNAP, it may act through the synthetic or allosteric alteration of a modifier of RNAP activity. However, extractable RNAP activity was not significantly affected until twelve hours after cortisol treatment, and therefore it is unlikely that such a modifier appeared in this preparation.

Halkerston, Scully, Feinstein and Hechter (1965) found that actinomycin D (1 mg/kg) given to rats in vivo stopped 50% of the RNA synthesis in rat thymus, but did not cause involution. Actinomycin D did not prevent cortisol involution of the thymus, although Drews (1969) has shown that even relatively low doses lead to selective inhibition of Mg^{++} stimulated RNA synthesis (i.e., nucleolar). These data suggest that cortisol-mediated involution is not mediated through an effect on nucleolar RNAP. I would expect, however, that α-amanitin or salicylates, specific inhibitors of nucleoplasmic RNAP, might cause thymic involution.

Nicolette and Mueller (1966) showed that inhibition of protein synthesis in vitro by cycloheximide (25 μg/ml) prevented the estrogen stimulation of ^{3}H-UMP incorporation into rat uterus RNA. The authors suggested that synthesis of a rapidly turning over protein was involved in the estrogen stimulation of ^{3}H-UMP incorporation. Gorski and Morgan (1967) demonstrated that cycloheximide (100 μg/20-25 day old rat) or puromycin (5 mg/rat given twice, an hour apart) reversed estrogen stimulation of ^{3}H-UMP incorporation into rat uterus RNA once it had begun. It is difficult to derive conclusions from experiments using exceedingly toxic antibiotics and which may potentially have multiple sites of action, but these experiments suggest that estrogen stimulation of ^{3}H-UMP incorporation into RNA requires the continued synthesis of protein(s).

Shelton and Allfrey (1970) have shown that synthesis of a specific nuclear acidic protein is enhanced in rat liver cells by

the injection of cortisol. This effect becomes evident between two and three hours after cortisol injection. These findings may indicate interference with specific proteins which are RNAP modifiers or mediators of growth in some other fashion. It is possible that involution occurs in a similar fashion, with the synthesis of specific inhibitors or by the deletion of specific, rapidly turning over RNAP modifiers. I have provided data suggesting the alteration, deletion and differential inhibition of various protein fractions after cortisol in the thymus.

Evidence is accumulating for the presence of moderating subunits and factors on eukaryotic as well as bacterial RNAP's. These subunits may represent initiators, stimulators, stabilizers, inactivators, inhibitors, or releasers. The synthesis and disposition of these regulators opens a new realm of exploration in growth and differentiation, as well as sites of drug action on these processes.

SUMMARY AND CONCLUSIONS

Cortisol at 50 mg/kg given to rats IM led to severe thymus gland involution. Thymus wet weights were significantly decreased within twelve hours, reached their smallest size by 72 hours, and only began to recover very slowly from 96 through 144 hours after cortisol treatment. Thymic involution was characterized by dramatic decreases in DNA, RNA and protein precursor incorporation into whole thymocytes. Maximum decreases occurred within 12 hours after cortisol treatment. Separation of the time course of inhibition for the various precursors could not be made at the times studied. Alterations of cell populations were seen after cortisol treatment, with destruction of the small thymocytes and the retention of intermediate to large thymocytes in a reticular matrix. It was suggested that the involuted thymus could contain a selected thymocyte population whose RNAP characteristics are interpreted as cortisol-mediated decreases in RNAP activity.

Increases in incorporation of ^{3}H-uridine and ^{14}C-leucine into whole thymocytes occurred by 48 hours after cortisol treatment. Incorporation passed control values between 72 and 96 hours after treatment. Increases in incorporation of ^{3}H-thymidine into whole thymocytes began between 48 to 72 hours, and passed control values between 96 and 120 hours after cortisol. Incorporation of ^{3}H-uridine, ^{3}H-thymidine, and ^{14}C-leucine then remained elevated at approximately 150% of control values through the last time period examined (192 hours). Thymus gland regeneration begins with increased protein and RNA precursor incorporation before increasing DNA precursor incorporation.

The incorporation of ^{3}H-leu into various thymic protein fractions was studied by exposing thymocyte cell suspensions to the label for four hours in vitro after cortisol or vehicle treatments in vivo. Cortisol-mediated decreases in protein precursor incorporation were not a function of the protein precursor used nor the length of time of incubation with the precursor in vitro. Respective ^{3}H-leu incorporations 3, 6 and 12 hours after cortisol treatment were: whole cells 73%, 60% and 41%; whole nuclei 98%, 61% and 36%; and nuclear extracts 90%, 72% and 54% of control values. Decreases in ^{3}H-leu incorporation occurred early and to a greater degree in fractions other than the fraction containing RNAP (nuclear extract), suggesting that RNAP was not selectively inhibited by cortisol.

Soluble, extractable, thymic RNAP activities in nuclear extracts at 3, 6 and 12 hours after cortisol treatment and ^{3}H-leu labelling were: 102%, 87% and 64% of control values respectively. Significant decreases in soluble RNAP activity did not occur until 12 hours after cortisol treatment. The reduction of incorporation of ^{3}H-leu into all thymocyte fractions consistently preceded and exceeded decreases in soluble, extractable RNAP activity. Although the decreases in soluble RNAP activity seen at 6 and 12 hours after cortisol treatment may be linked to a decrease in RNAP synthesis, the decrease in extractable RNAP activity did not occur early enough to explain the inhibition of protein synthesis seen 3 hours after cortisol treatment. Thus, cortisol effects on extractable RNAP activity cannot account for the primary involutionary events in the rat thymus. Decreases in RNAP

activity could account for early decreases in ^{3}H-leu incorporation only if a modifier of RNAP activity was lost in extraction of the enzyme.

Nuclear extract RNAP was purified by two sequential sucrose-glycerol gradient centrifugations. Cortisol-induced decreases in RNAP activity were evident after both centrifugations. On gradient I, 20% to 50% of the layered activity was recovered, while on gradient II, 65% to 90% of the layered activity was recovered. An RNAP modifier (stimulator) hypothesized to be lost on gradient I was not detected when composite fractions of lighter proteins were added to fractions containing RNAP activity.

Examination of ^{3}H-leu incorporation into various fractions on gradient I suggested that inhibition occurred differently throughout the proteins of the gradient. Greater inhibition occurred in the larger proteins than in the smaller proteins. This finding suggested that cortisol could alter the synthesis of specific proteins such as modifiers of RNAP activity or other proteins which may act to initiate involution.

The peak of RNAP from gradient I was relayered and centrifuged on gradient II. Comparison of cortisol and vehicle peaks for RNAP and ^{3}H-leu activity demonstrated that activities in gradient I and nuclear extracts were similar to final gradient II activities.

Electrophoretic examination of control gradient I fractions containing RNAP activity demonstrated four protein peaks, while after cortisol treatment only three peaks were found. There was a

cortisol-mediated protein peak deletion and a protein peak modification. Electrophoretic examination of gradient II fractions containing RNAP activity demonstrated two protein peaks. One peak coincided with a sharp peak of ^{3}H-leu activity while the other peak was virtually unlabelled. Attempts to enzymatically define the gel area of RNAP activity were unsucessful, and thus I could not quantitate the level of ^{3}H-leu incorporation into RNAP. The decreases in ^{3}H-leu incorporation into specific proteins associated with gradient I and gradient II RNAP activities occurred to a lesser degree than decreases in other thymocyte proteins. Cortisol treatment did not appear to selectively inhibit the ^{3}H-leu incorporation into protein bands observed on the gels. This applied to all proteins to the extent that they were purified on the sucrose-glycerol gradients.

The cortisol-mediated decreases in RNAP activity seen in extractable RNAP occur later than inhibition of RNA synthesis in whole thymocytes, thymocyte nuclei, and aggregate preparations. It was proposed that we have measured the true rate of decrease of RNAP activity without earlier intervening effects on template, on precursor uptake and phosphorylation, and on pool sizes.

A model for growth and involution was proposed which suggested that growth is mediated through a sequence of events which affect nucleolar RNAP, while involution is mediated through events affecting nucleoplasmic RNAP.

APPENDIX I.

1. Vehicle No. 100

Upjohn Vehicle No. 100 contained, per liter of sterile water: Sodium carboxymethylcellulose, 10 mg; Sodium chloride, 18 mg; polysorbate 80, 8 mg; and benzyl alcohol, 18 mg.

2. Eagle's Minimum Essential Medium for Suspension Cultures

For thymocyte incubations, Eagle's Minimum Essential Medium for Suspension Cultures (MEM), was prepared fresh from stocks for each experiment (Eagle, 1969). MEM is an Earle's Balanced Salt Solution containing the essential amino acids, glucose, sodium bicarbonate, and phenol red. Non-radioactive L-leucine was added so that the final concentration with added 4,5-^{3}H-L-leucine was 1×10^{-5} M. MEM also contains choline chloride, 1.0 mg; folic acid, 1.0 mg; inositol, 2.0 mg; niacinamide, 1.0 mg; D-Ca-panthothenate, 1.0 mg; pyridoxal HCl, 1.0 mg; riboflavin, 0.1 mg; and thiamine HCl, 1.0 mg. To each liter of MEM was added:

L-serine, 2.102 gm; Na pyruvate, 11.010 gm; Na penicillin G, 5.0 gm; and streptomycin sulfate, 5.0 gm.

MEM was diluted to 940 ml with glass distilled water and sterilized by filtration through a Millipore Type HA filter, pore size 0.45 microns. Sixty ml of calf serum were added under sterile conditions.

Modified MEM, the medium used for chilling and washing cells, was prepared from commercial GibCo packets (No. F-14) of MEM to which 2.2 gm of sodium bicarbonate was added and, after filtration, 60 ml of calf serum. Additional non-radioactive leucine was added to the level of 1 x 10^{-3} M. In MEM for chilling and washing cells, serine, pyruvate, penicillin, and streptomycin were not added.

3. Scintillation Solution

Scintillation solution contained, per liter of toluene (Mallinckrodt, analytical reagent grade): PPO, 4.0 gm; POPOP, 0.1 gm.

4. Disc Gel Electrophoresis - 5% Acrylamide Gel with Tris-glycine Buffer[1].

The gel electrophoresis system consisted of the following ingredients in the proportions - A:C:P:L - 1:2:4:1.

A: Tris, 36.6 gm; TEMED, 0.46 ml at 25° C or 0.80 ml at 0° C; 1 N HCl, approximately 38.0 ml, to a pH of 8.9; GDW to 100 ml and filter.

C: Acrylamide, (4)(X) gm where X equals % gel desired, e.g., for 5% use (4)(5) = 20 gm; N,N'-Methylenebisacrylamide, 0.8(4)(X)/30 gm; GDW to 100 ml and filter.

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[1] Personal communication from Dr. Eric Shooter and students (1969), Dept. of Genetics, Stanford University.

P: $(NH_4)_2S_2O_8$, 0.14 gm at 25° C or 0.25 gm at 0° C. GDW to 100 mls).

L: GDW.

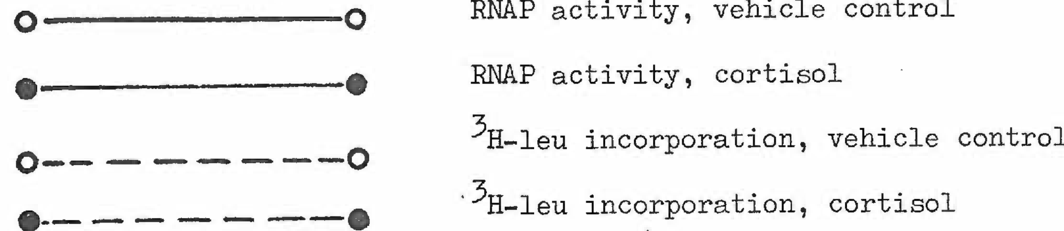
Reservoir buffers contained, per liter, in GDW:

Upper Buffer: Tris 6.32 gm; glycine, 3.94 gm; pH 8.9

Lower Buffer: Tris 12.1 gm; 1 N HCl, 50 ml; pH 8.1

APPENDIX II.

Graphs used in the calculation of values for Table III are given in Figures 5 and 6 of the Results Section and Figures 11 to 21 in this section. The gradient parameters and calculation methods have been cited in Materials and Methods (Treatment of Data) and in the legends of Figures 5 and 6 of the Results Section. In all figures, the following representations are used:



Graphs are not shown for two experiments from which data was derived for the effects of twelve hour cortisol treatment shown in Table III. In these experiments, three GI's of equal length were combined to provide a single gradient of increased volume. The position of the RNAP activity was predicted according to the length of the composite gradient, and was confirmed by RNAP assay. Fractions containing RNAP activity were combined and recentrifuged to provide several GII gradients, which were also combined to form an composite gradient for RNAP assay and ^{3}H-leu determinations. The plots of each of these composite gradients (not shown) closely resembled the individual gradients shown in Figures 5 and 6 of the Results Section, and Figures 19, 20 and 21 of this section.

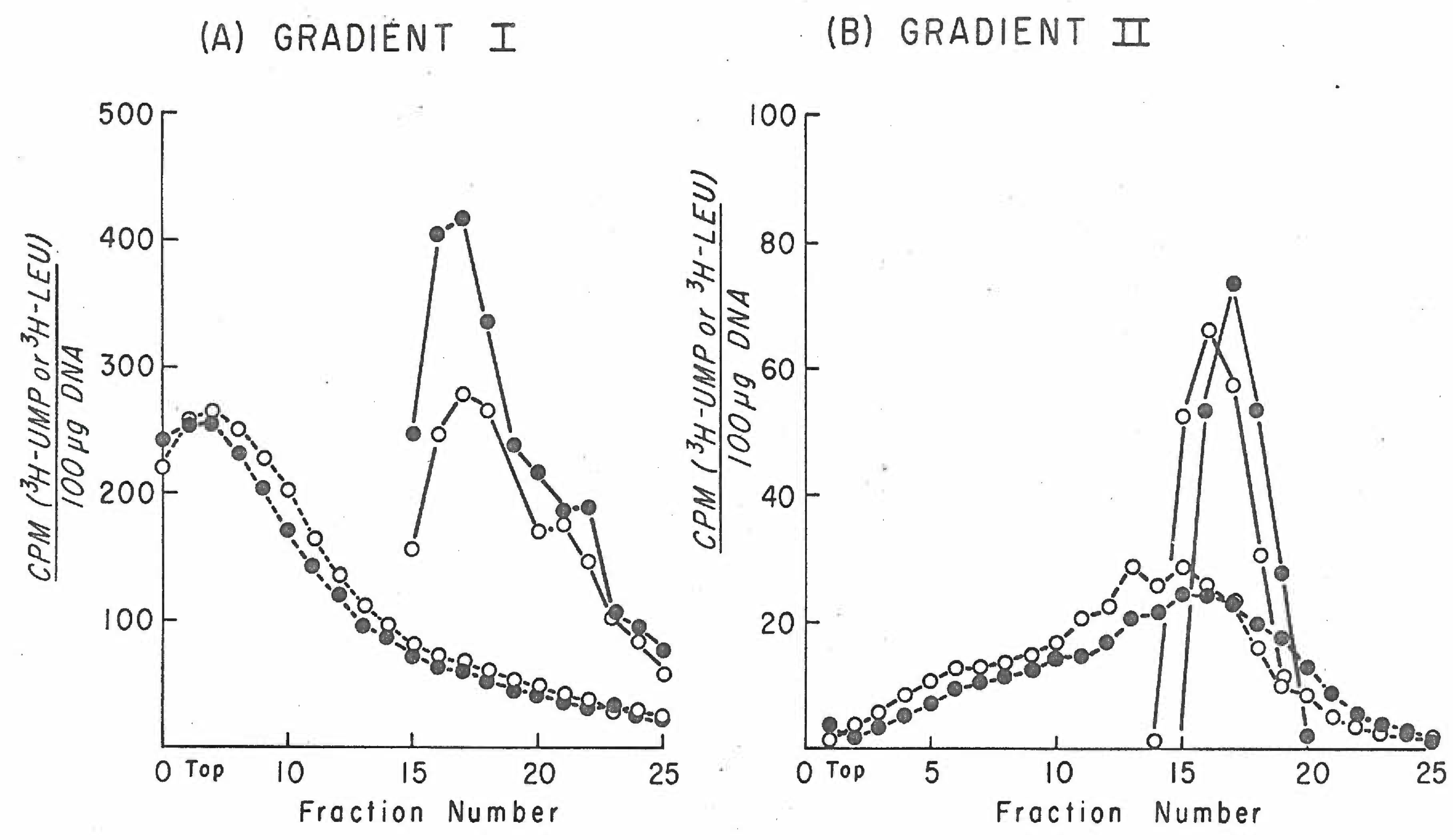

(A) GRADIENT I
(B) GRADIENT II
CPM (^{3}H-UMP or ^{3}H-LEU) / 100 μg DNA
500
400
300
200
100
80
60
40
20
0 Top
5
10
15
20
25
Fraction Number

Figure 11: Gradient I and gradient II fractionations of nuclear extract proteins. Thymocytes were labelled in vitro three hours after treatment with cortisol or vehicle in vivo. Experiment No. 1.

Both GI and GII RNAP activities and ^{3}H-leu incorporations are shown for cortisol and vehicle treatments. (A) Gradient I. Percentage recoveries of nuclear extract RNAP activity applied to GI were: cortisol 79.3%, vehicle 55.0%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GI RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 15 to 25, vehicle fractions 15 to 25. The final value of total cortisol/vehicle x 100 for GI RNAP activity corrected to equivalent recoveries was 92.5%. (B) Gradient II. Gradient I (A) fractions combined and recentrifuged on GII were: cortisol fractions 15 to 19, vehicle fractions 16 to 20 inclusively. Percentage recoveries of GI RNAP activity applied to GII were not calculated. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GII RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 15 to 20, vehicle fractions 14 to 19. The final value of total cortisol/vehicle x 100 for GII RNAP activity (uncorrected) was: 99.3%.

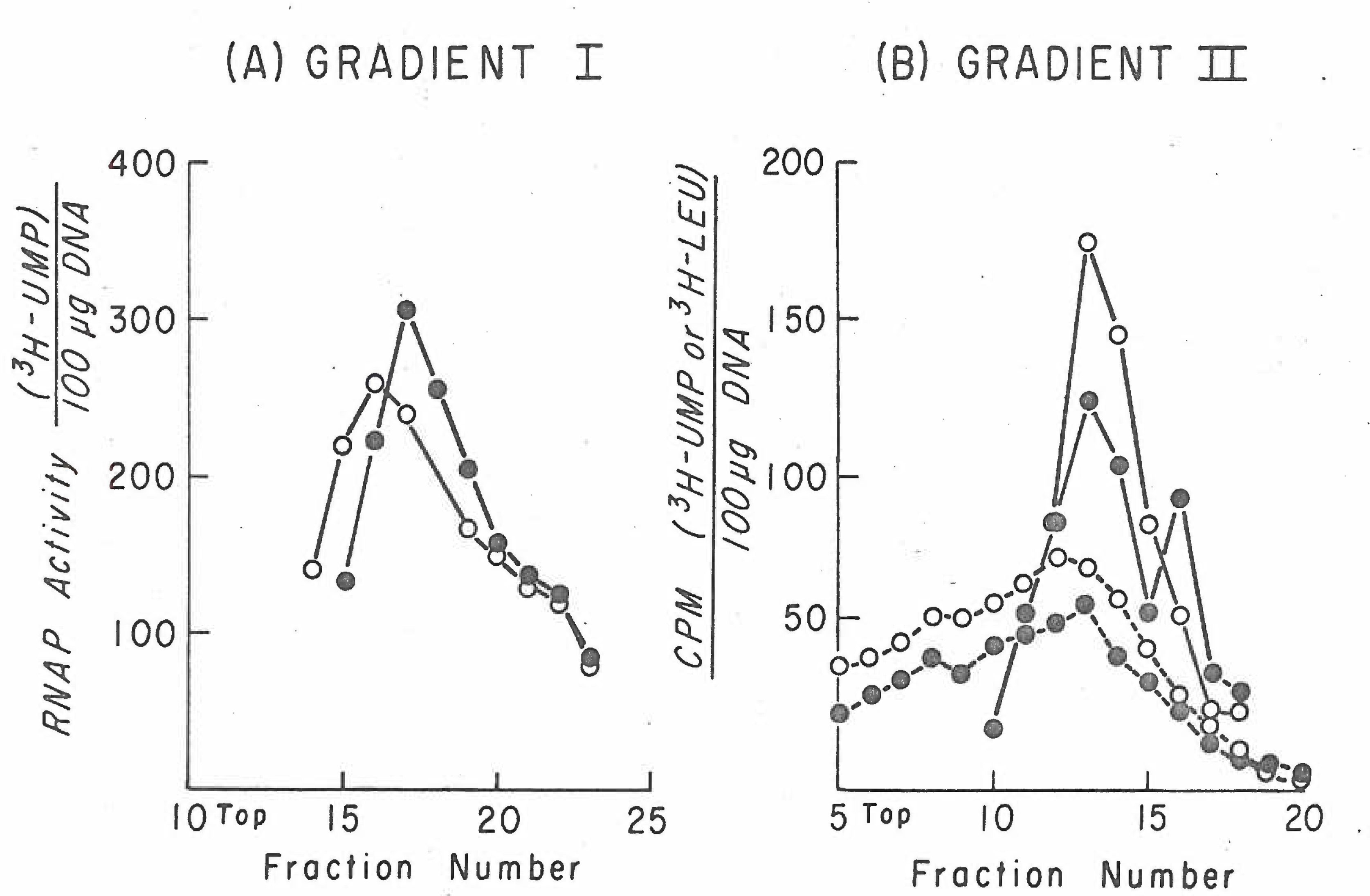

(A) GRADIENT I
(B) GRADIENT II
RNAP Activity (^{3}H-UMP) / 100 μg DNA
400
300
200
100
10 Top
15
20
25
Fraction Number
CPM (^{3}H-UMP or ^{3}H-LEU) / 100 μg DNA
200
150
100
50
5 Top
10
15
20
Fraction Number

Figure 12: Gradient I and gradient II fractionations of nuclear extract proteins. Thymocytes were labelled in vitro three hours after treatment with cortisol or vehicle in vivo. Experiment No. 1.

Gradients shown in this figure are replicates of those shown in Figure 11, but were centrifuged thirteen days later. (A) Gradient I. Gradient I RNAP activity is shown for cortisol and vehicle treatments. Percentage recoveries of nuclear extract RNAP activity applied to GI were: cortisol 44.4%, vehicle 50.8%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GI RNAP activity in Table III were: cortisol fractions 15 to 23, vehicle fractions 14 to 23. The final value of total cortisol/vehicle x 100 for GI RNAP activity corrected to equivalent recoveries was: 106.3%. (B) Gradient II. Gradient I (A) fractions combined and recentrifuged on GII were: cortisol fractions 16 to 19, vehicle fractions 15 to 18 inclusively. Gradient II RNAP activity and ^{3}H-leu incorporation are shown for cortisol and vehicle treatments. Percentage recoveries of GI RNAP activity applied to GII were not calculated. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GII RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 10 to 17, vehicle fractions 11 to 17. The final value of total cortisol/vehicle x 100 for GII RNAP activity (uncorrected) was: 86.5%.

Figure 13: Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro three hours after treatment with cortisol or vehicle in vivo. Experiment No. 1.

Gradient I RNAP activity is shown for cortisol and vehicle treatments. Gradients shown in this figure are replicates of those shown in Figures 11-A and 12-A, and were centrifuged with the gradients shown in 12-A. Percentage recoveries of nuclear extract RNAP activity applied to GI were: cortisol 38.8%, vehicle 41.5%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GI RNAP activity in Table III were: cortisol fractions 15 to 20, vehicle fractions 15 to 20. The final value of total cortisol/vehicle x 100 for GI RNAP activity corrected to equivalent recoveries was: 126.1%.

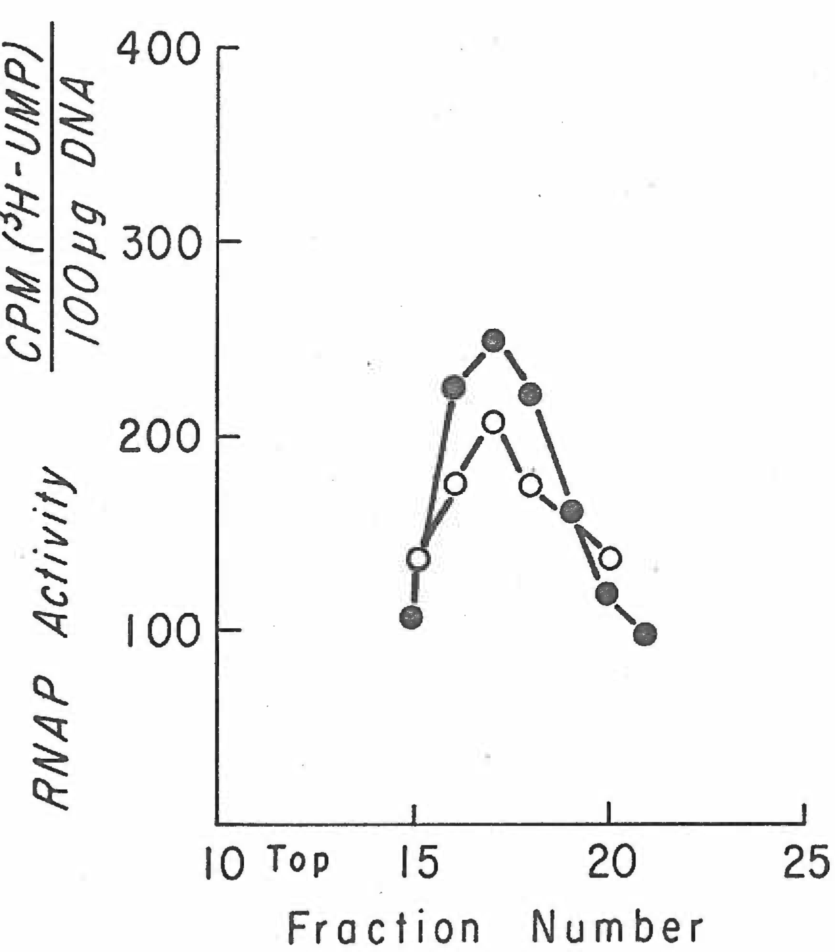

GRADIENT I
RNAP Activity CPM (³H-UMP) / 100 µg DNA
400
300
200
100
10 Top
15
20
25
Fraction Number

Figure 14: Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro six hours after treatment with cortisol or vehicle in vivo. Experiment No. 2.

(A) Gradient I. Gradient I RNAP activity and ^{3}H-leu incorporation are shown for cortisol and vehicle treatments. Percentage recoveries of nuclear extract RNAP activity applied to GI were: cortisol 21.3%, vehicle 18.0%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GI RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 10 to 20, vehicle fractions 10 to 20. The final value of cortisol/vehicle x 100 for GI RNAP activity corrected to equivalent recoveries was 87.4%.

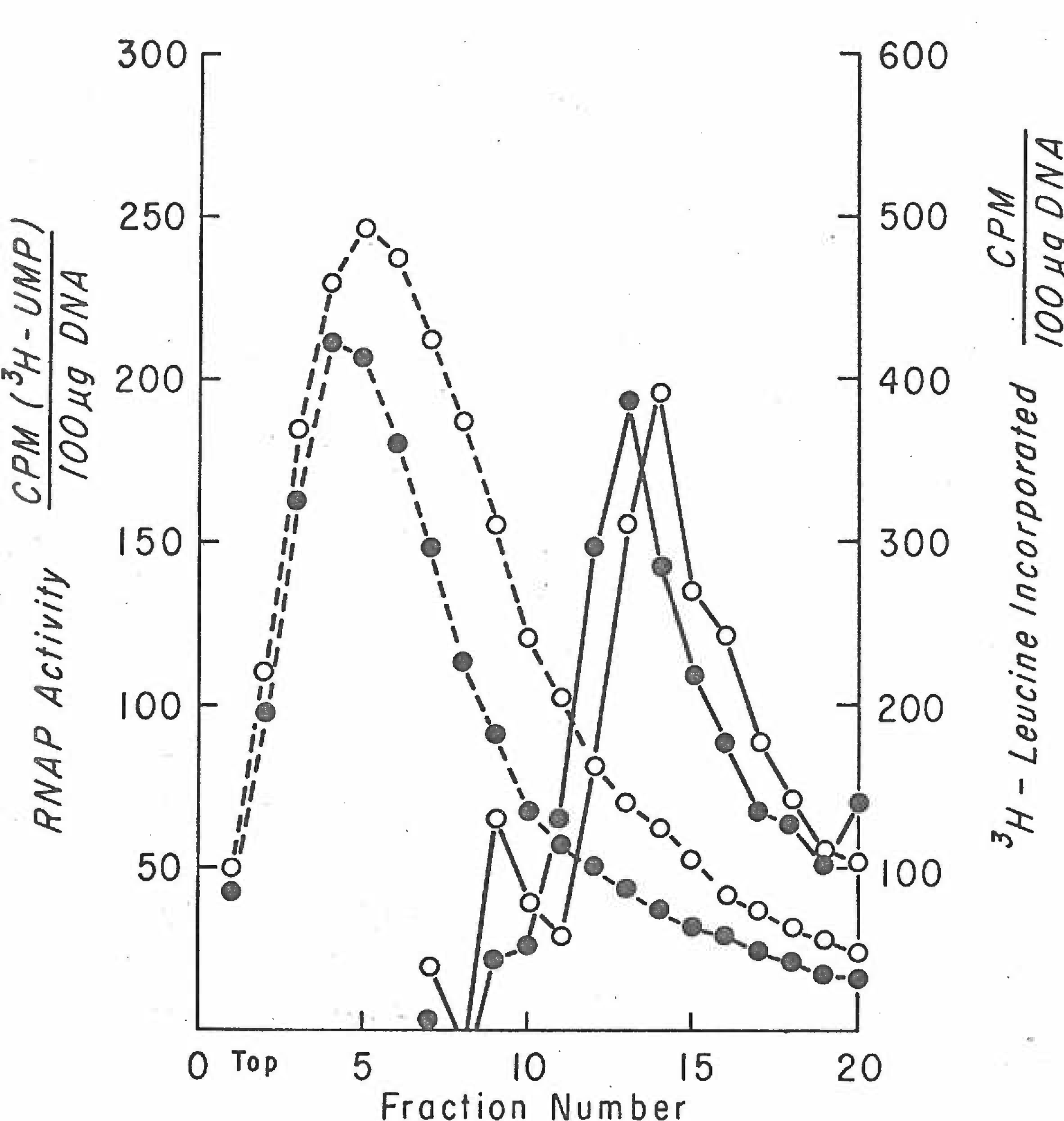

(A) GRADIENT I
300
250
200
150
100
50
600
500
400
300
200
100
RNAP Activity $\frac{CPM\ (^3H\text{-}UMP)}{100\ \mu g\ DNA}$
^3H - Leucine Incorporated $\frac{CPM}{100\ \mu g\ DNA}$
0
Top
5
10
15
20
Fraction Number

Figure 15: Gradient II fractionation of combined fractions of a gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro six hours after treatment with cortisol or vehicle in vivo. Experiment No. 2.

(B) Gradient II. Gradient I (Figure 14) fractions combined and recentrifuged to provide GII were: cortisol fractions 12 to 16, vehicle fractions 12 to 16 inclusively. Gradient II RNAP activity and ^{3}H-leu incorporation are shown for cortisol and vehicle treatments. Percentage recoveries of GI RNAP activity applied to GII were: cortisol 76.1%, vehicle 77.8%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GII RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 9 to 16, vehicle fractions 9 to 16. The final value of cortisol/vehicle x 100 for GII RNAP activity corrected to equivalent recoveries was: 98.6%.

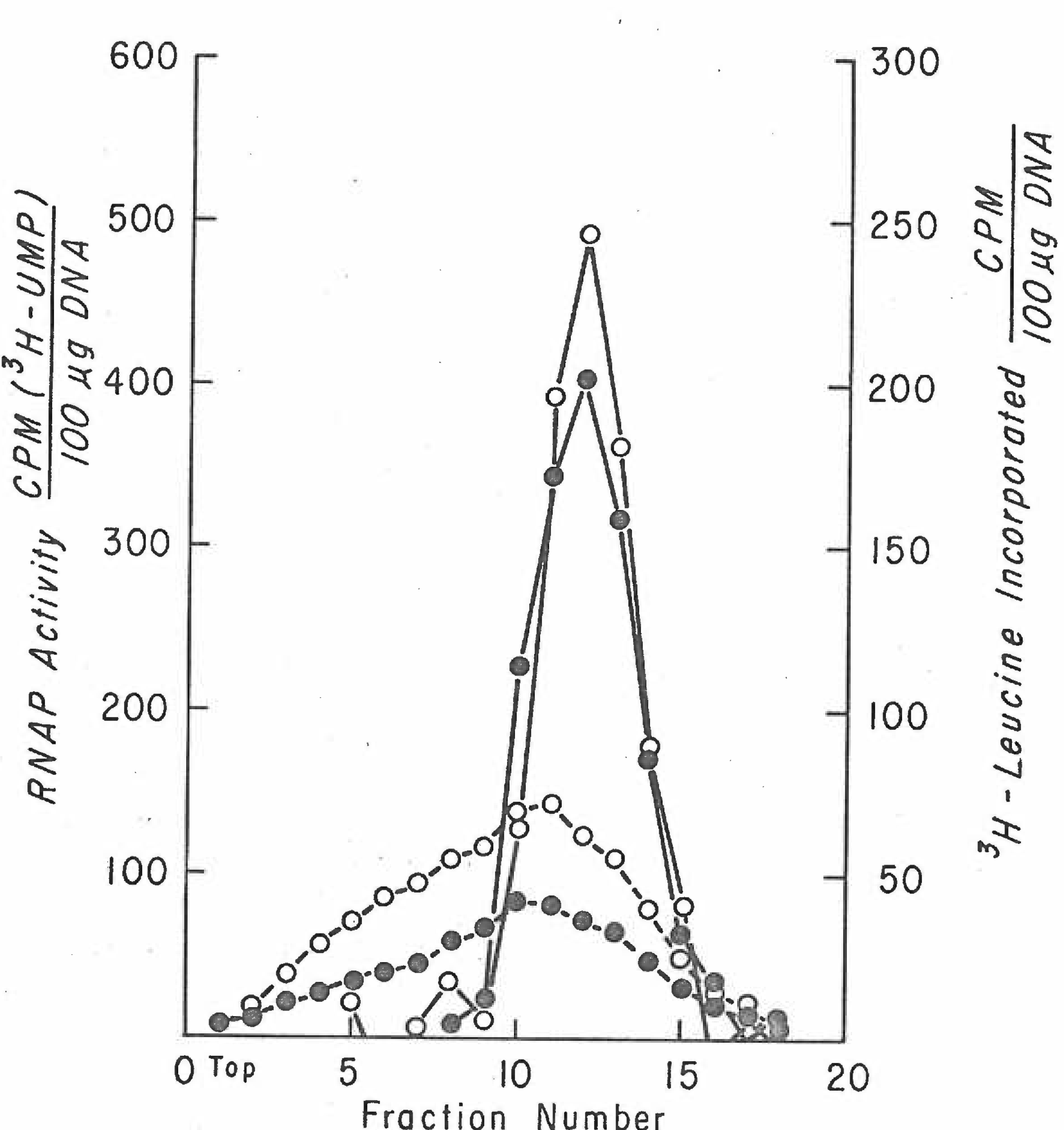

(B) GRADIENT II
RNAP Activity CPM (³H-UMP) / 100 μg DNA
³H-Leucine Incorporated CPM / 100 μg DNA
600
500
400
300
200
100
300
250
200
150
100
50
0 Top
5
10
15
20
Fraction Number

Figure 16: Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro six hours after treatment with cortisol or vehicle in vivo. Experiment No. 2.

(A) Gradient I. Gradient I RNAP activity is shown for cortisol and vehicle treatments. These gradients are replicates of those shown in Figure 14, and were centrifuged at the same time. Percentage recoveries of nuclear extract RNAP activity applied to GI were: cortisol 24.1%, vehicle 23.6%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GI RNAP activity in Table III were: cortisol fractions 10 to 18, vehicle fractions 10 to 18. The final value of cortisol/vehicle x 100 for GI RNAP activity corrected to equivalent recoveries was: 82.9%.

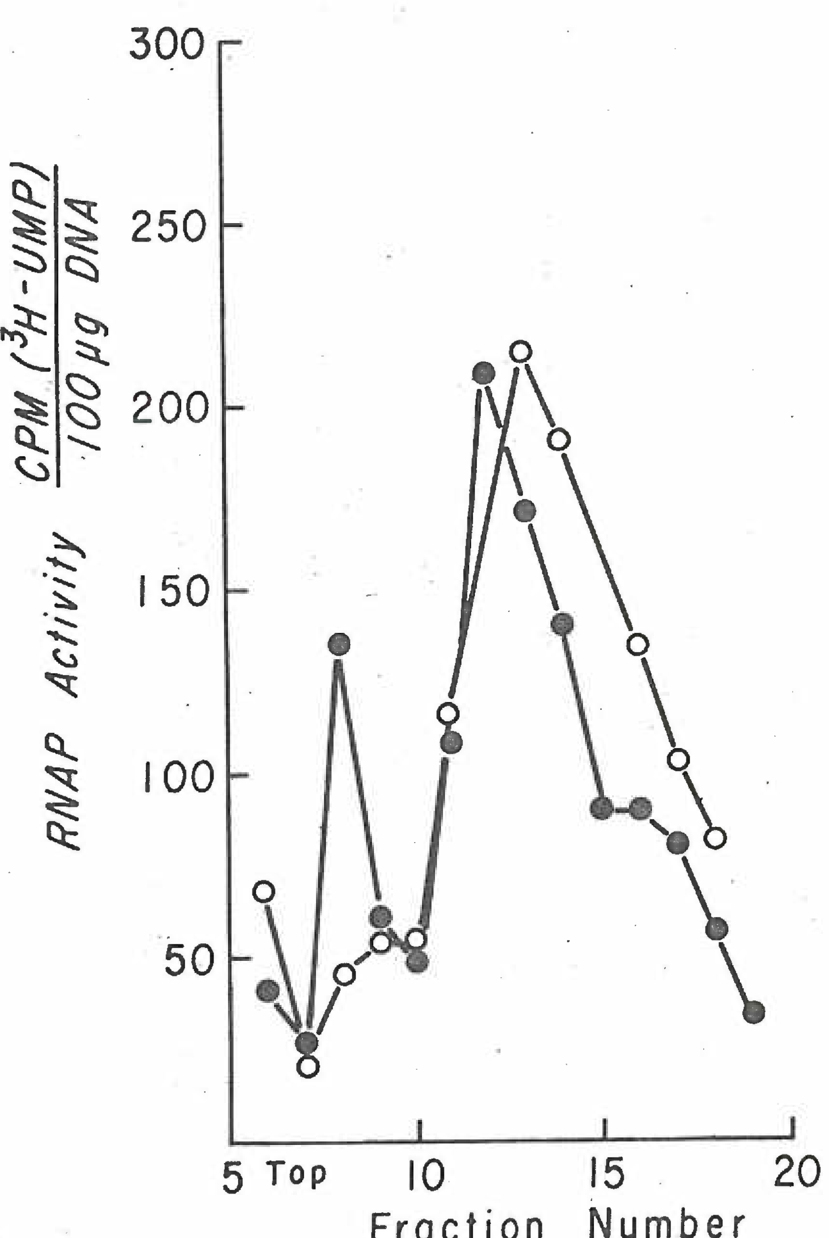

(A) GRADIENT I
RNAP Activity CPM (³H-UMP) / 100 µg DNA
300
250
200
150
100
50
5 Top
10
15
20
Fraction Number

Figure 17: Gradient II fractionation of combined fractions of a gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro six hours after treatment with cortisol or vehicle in vivo. Experiment No. 2.

(B) Gradient II. Gradient I (Figure 16) fractions combined and recentrifuged to provide GII were: cortisol fractions 12 to 14, vehicle fractions 12 to 14 inclusively. Gradient II RNAP activity and ^{3}H-leu incorporation are shown for cortisol and vehicle treatments. These gradients are replicates of those shown in Figure 15, and were centrifuged at the same time. Percentage recoveries of GI RNAP activity applied to GII were: cortisol 74.6%, vehicle 61.3%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GII RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 10 to 17, vehicle fractions 10 to 15. The final value of cortisol/vehicle x 100 for GII RNAP activity corrected to equivalent recoveries was: 96.1%.

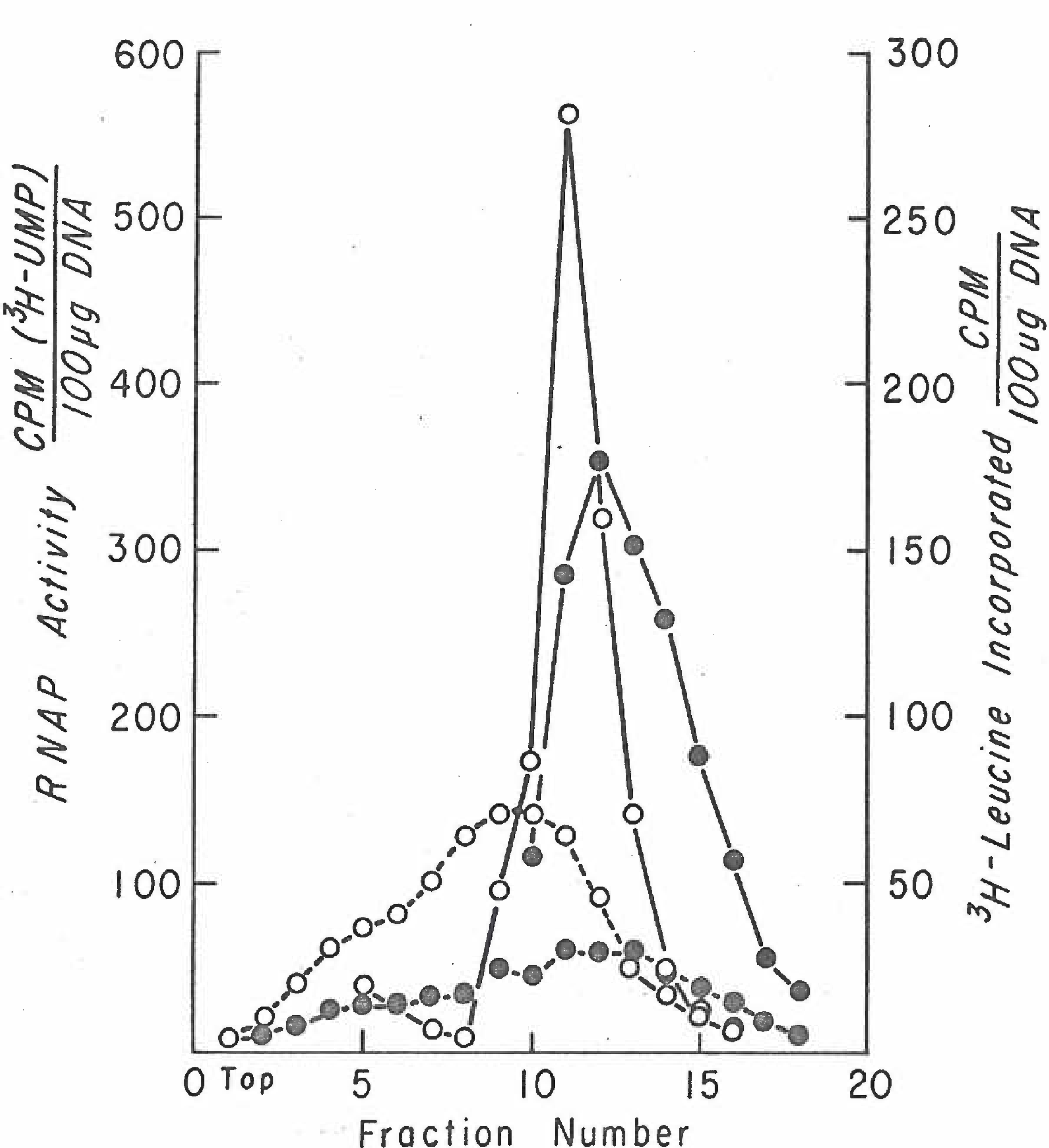
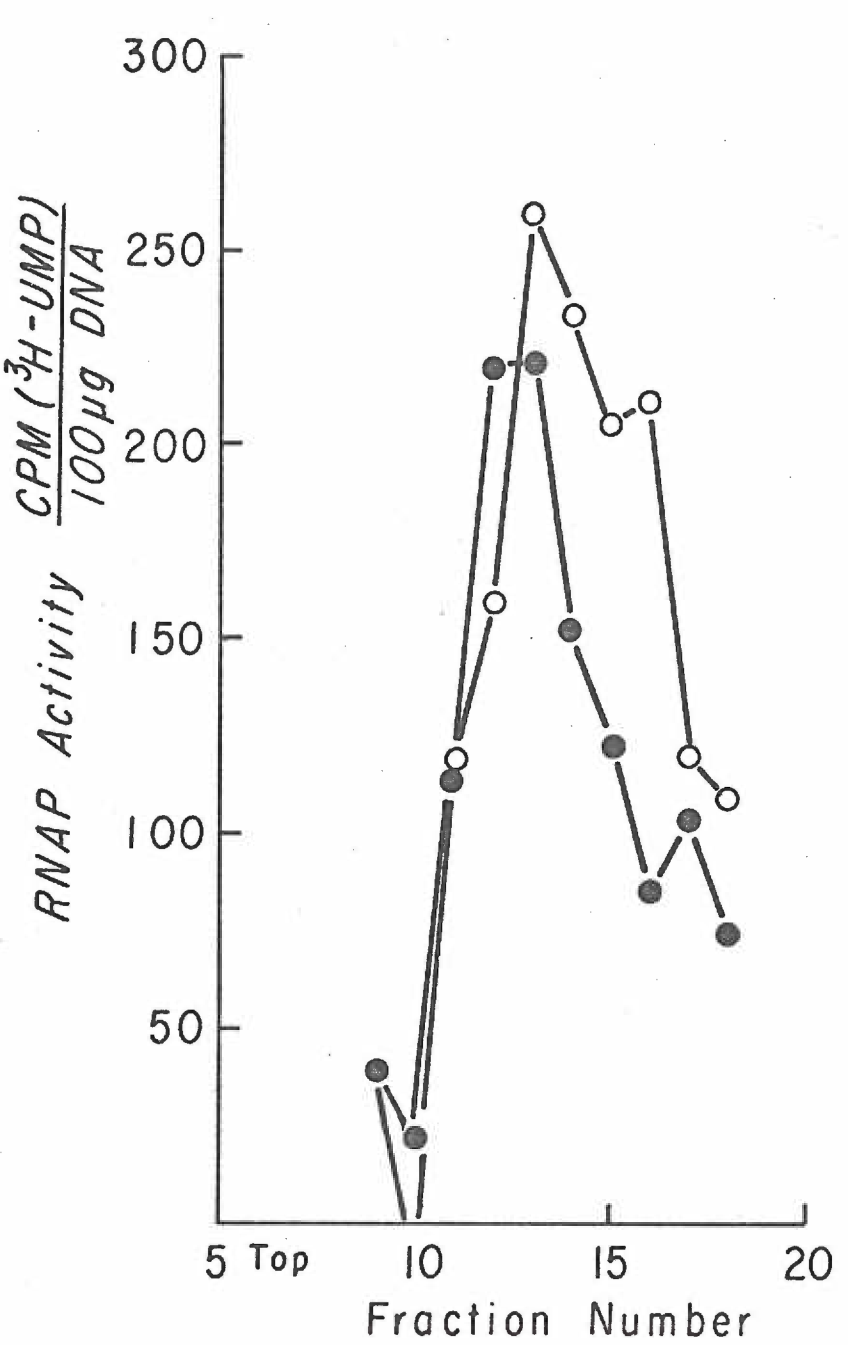

(B) GRADIENT II
600
500
400
300
200
100
0
300
250
200
150
100
50
RNAP Activity CPM (³H-UMP) / 100µg DNA
³H-Leucine Incorporated CPM / 100ug DNA
Top
5
10
15
20
Fraction Number

Figure 18: Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro six hours after treatment with cortisol or vehicle in vivo. Experiment No. 2.

Gradient I RNAP activity is shown for cortisol and vehicle treatments. These gradients are replicates of those shown in Figures 14 and 16, and were centrifuged at the same time. After fractionation the gradients were immediately frozen. In contrast to the usual RNAP assay on the day of fractionation or one day later, these gradients were assayed one month later. Percentage recoveries of nuclear extract RNAP activity applied to GI were: cortisol 21.6%, vehicle 23.5%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GI RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 10 to 18, vehicle fractions 10 to 18. The final value of cortisol/vehicle x 100 for GI RNAP activity corrected to equivalent recoveries was: 86.7%.



GRADIENT I
RNAP Activity $\frac{CPM\ (^3H\text{-}UMP)}{100\mu g\ DNA}$
300
250
200
150
100
50
5 Top
10
15
20
Fraction Number

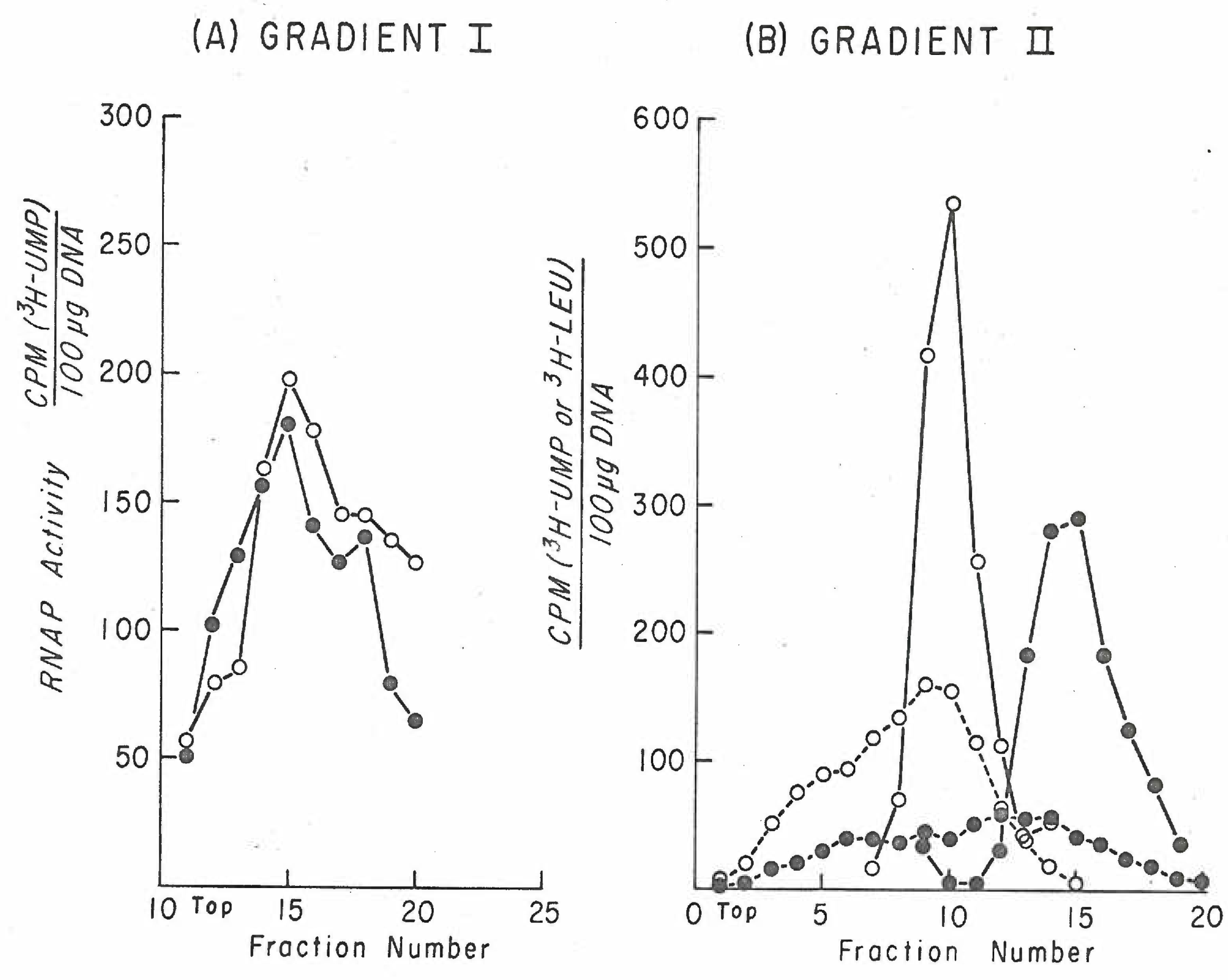

(A) GRADIENT I
(B) GRADIENT II
RNAP Activity
CPM (3H-UMP) / 100 µg DNA
CPM (3H-UMP or 3H-LEU) / 100 µg DNA
300
250
200
150
100
50
600
500
400
300
200
100
10 Top 15 20 25
0 Top 5 10 15 20
Fraction Number
Fraction Number

Figure 19: Gradient I and gradient II fractionations of nuclear extract proteins. Thymocytes were labelled in vitro twelve hours after treatment with cortisol or vehicle in vivo. Experiment No. 3.

(A) Gradient I. Gradient I RNAP activity is shown for cortisol and vehicle treatments. These gradients are replicates of those shown in Figure 5 of the Results Section, and were centrifuged at the same time. Percentage recoveries of nuclear extract RNAP activity applied to GI were: cortisol 26.0%, vehicle 24.6%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GI RNAP activity in Table III were: cortisol fractions 11 to 20, vehicle fractions 11 to 20. The final value of total cortisol/vehicle x 100 for GI RNAP activity corrected to equivalent recoveries was: 70.7%. (B) Gradient II. Gradient I (A) fractions combined and recentrifuged on GII were: cortisol fractions 13 to 16, vehicle fractions 14 to 17 inclusively. Gradient II RNAP activity and ^{3}H-leu incorporation are shown for cortisol and vehicle treatments. These gradients are replicates of those shown in Figure 6 of the Results Section, and were centrifuged at the same time. Percentage recoveries of GI RNAP activity applied to GII were: cortisol 86.6%, vehicle 73.1%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GII RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 12 to 19, vehicle fractions 8 to 13. The differences in position of cortisol and vehicle RNAP activity shown in (B) reflect differences in the lengths of the fractionated gradients. The final value of total cortisol/vehicle x 100 for GII RNAP activity corrected to equivalent recoveries was: 64.0%.

Figure 20: Gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro twelve hours after treatment with cortisol or vehicle in vivo. Experiment No. 3.

(A) Gradient I. Gradient I RNAP activity is shown for cortisol and vehicle treatments. The gradients shown in this figure were centrifuged one day later than the replicates shown in Figure 5 of the Results Section and Figure 19-A of this section. Percentage recoveries of nuclear extract RNAP activity applied to GI were: cortisol 47.3%, vehicle 47.5%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GI RNAP activity in Table III were: cortisol fractions 9 to 17, vehicle fractions 9 to 17. The final value of total cortisol/vehicle x 100 for GI RNAP activity corrected to equivalent recoveries was: 76.1%.

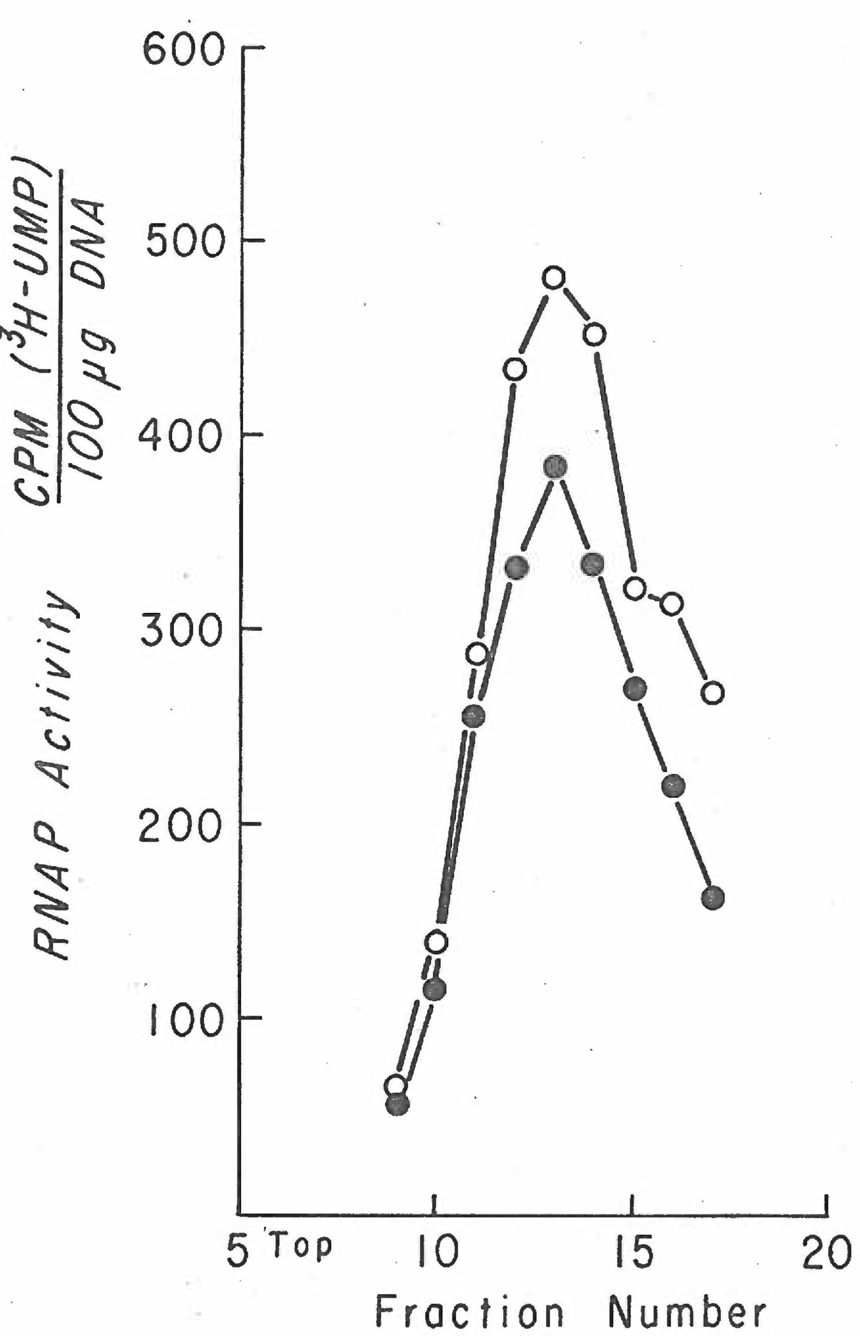
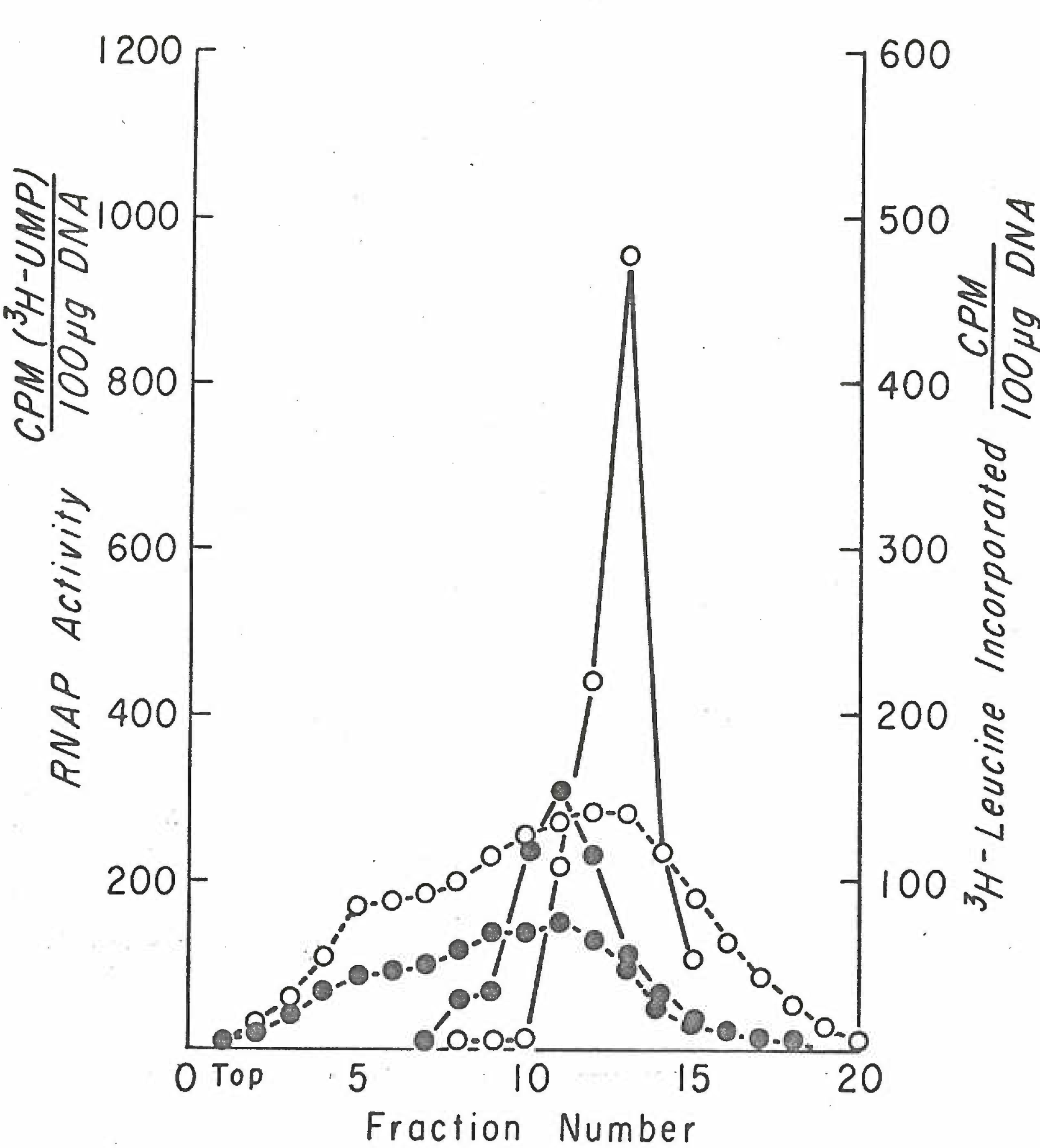

(A) GRADIENT I
RNAP Activity $\frac{CPM\ (^3H\text{-}UMP)}{100\ \mu g\ DNA}$
600
500
400
300
200
100
5 Top
10
15
20
Fraction Number

Figure 21: Gradient II fractionation of combined fractions of a gradient I fractionation of nuclear extract proteins. Thymocytes were labelled in vitro twelve hours after treatment with cortisol or vehicle in vivo. Experiment No. 3.

(B) Gradient II. Gradient I (Figure 20) fractions combined and recentrifuged on GII were: cortisol fractions 12 to 15, vehicle fractions 12 to 15 inclusively. Gradient II RNAP activity and ^{3}H-leu incorporation are shown for cortisol and vehicle treatments. The gradients shown in this figure were centrifuged three days later than the replicate gradients shown in Figure 6 of the Results Section and Figure 19-B of this section. Percentage recoveries of GI RNAP activity applied to GII were: cortisol 66.7%, vehicle 86.3%. Portions of the gradients used in calculating ratios of cortisol/vehicle x 100 for GII RNAP activity and ^{3}H-leu incorporation in Table III were: cortisol fractions 9 to 14, vehicle fractions 10 to 15. The final value of cortisol/vehicle x 100 for GII RNAP activity corrected to equivalent recoveries was: 74.6%.


(B) GRADIENT II
1200
1000
800
600
400
200
600
500
400
300
200
100
RNAP Activity CPM (³H-UMP) / 100μg DNA
³H-Leucine Incorporated CPM / 100μg DNA
0 Top
5
10
15
20
Fraction Number

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