EFFECTS OF ADRENOCORTICAL HORMONES ON
RNA METABOLISM IN RAT KIDNEY AND LIVER

CENTRE FOR NEWFOUNDLAND STUDIES

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RAM K. MISHRA
EFFECTS OF ADRENOCORTICAL HORMONES ON RNA METABOLISM

IN RAT KIDNEY AND LIVER

A Thesis

submitted to the Faculty of Graduate Studies

of Memorial University of Newfoundland

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in

the Department of Biochemistry

by

Rama K. Mishra

June, 1973
The turnover of RNA in various subcellular fractions of kidney and liver of normal, adrenalectomized and adrenalectomized plus adrenocortical hormone treated rats was investigated. The turnover rates were measured by injection of a single dose of $^{14}$C-orotic acid and then following the loss of radioactivity from purified nuclei, mitochondria, rough endoplasmic reticulum, smooth endoplasmic reticulum, free polysomes, total ribosomes and sRNA. Single exponential decay patterns were observed in all the subcellular fractions of both tissues. The half-lives ($t_{1/2}$) of the RNA for above fractions of normal kidneys were 7.4, 5.8, 5.1, 5.0, 5.4, 4.1, and 4.6 days, respectively. The $t_{1/2}$ values for adrenalectomized rat kidneys were 9.2, 7.3, 6.1, 6.0, 5.8, 6.0, and 4.9 days, respectively. The half-lives ($t_{1/2}$) of the RNA of normal rat liver nuclei, mitochondria, rough endoplasmic reticulum, smooth endoplasmic reticulum, polysomes, total ribosomes and sRNA were 8.7, 6.5, 5.4, 6.4, 4.4, 4.9, and 4.8 days, respectively. The corresponding values for adrenalectomized rat livers were 12.0, 8.5, 6.9, 6.5, 5.8, 6.1, and 5.9 days, respectively.

The slower turnover rate of RNA in adrenalectomized rat kidney and liver suggests a slower rate of RNA synthesis (steady state approximation). Daily administration of aldosterone or deoxycorticosterone to adrenalectomized rats restored the turnover rates to normal in kidney and daily administration of corticosterone or hydrocortisone reversed the effects of adrenalectomy in liver indicating a tissue specificity of mineralcorticoids and glucocorticoids.

Aggregate DNA-dependent RNA polymerase activity in nuclei of both tissues was decreased by adrenalectomy and restored by administration...
of aldosterone or deoxycorticosterone in kidney and by corticosterone or hydrocortisone in liver. To further investigate the mechanism of RNA polymerase stimulation by aldosterone in kidney, the polymerases were purified from various groups of rats and activity was determined using different sources of DNA. It was found that the DNA from aldosterone treated rats was transcribed more efficiently than from other sources.

Fractionation of (14C-aldosterone injected) kidney chromatin revealed the presence of radioactivity in the non histone acidic protein and DNA fractions suggesting the possible binding of aldosterone, or aldosterone-receptor complex or metabolite(s) of aldosterone to these chromatin fractions which may result in enhanced template activity of DNA.
ACKNOWLEDGEMENTS

The author expresses his sincere appreciation to Dr. L. A. W. Feltham for his overall supervision and encouragement during the course of this study.

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The help of Mr. S. Andrews (Newfoundland and Labrador Computer Services) in statistical analysis of data on IBM 1130 and 370 computers is gratefully acknowledged.

The author is very grateful to Mr. D. Hall, Mrs. S. Banfield and Mrs. C. P. Mishra for some technical assistance and to Miss M. E. Coleman for skillfully typing the thesis.

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LIST OF ABBREVIATIONS

A Absorbance (optical density)
ACTH Adrenocorticotropic hormone
A260 Absorbancy of 1.0 at 260 nanometers in a 1-cm path
ATP Adenosine - 5' - triphosphate
BSA Bovine serum albumin
C-AMP Adenosine-3', 5'-cyclic monophosphate
CTP Cytidine - 5' - triphosphate
CPM Counts per minute
Cleland's reagent (DTT) Dithiothreitol
DEAE Diethylaminoethyl
DNA Deoxyribonucleic acid
DNase Deoxyribonuclease
dpm Disintegrations per minute
EDTA Ethylene diamine tetraacetic acid
ER Endoplasmic reticulum
GTP Guanosine-5'-triphosphate
k' Apparent turnover constant
mRNA Messenger ribonucleic acid
Medium H Homogenizing medium (0.35 M sucrose, 10 mM Mg2+, 25 mM KCl and 50 mM Tris-HCl, pH 7.4)
Medium H1 (0.32 M sucrose, 1 mM Mg2+, 10 mM Tris-HCl, pH 7)
Medium N1 (0.35 M sucrose, 3 mM CaCl2, 50 mM Tris-HCl, pH 7.2)
Medium N2 (2.4 M sucrose, 3 mM CaCl2, 50 mM Tris-HCl pH 7.2)
Medium B (10 mM Mg2+, 25 mM KCl, 50 mM Tris-HCl, pH 7.5)
NEB-2-buffer (0.2 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl pH 7.4)
Na-K-ATPase sodium, potassium activated adenosine triphosphatase
nm Nanometer (10^-9 meter)
NAD Nicotinamide adenine dinucleotide
Optical density (Absorbance)
p mole pico mole (10^{-12} moles)
RNA Ribonucleic acid
rRNA Ribosomal RNA
RNase Ribonuclease
rpm revolutions per minute
t RNA transfer (soluble) RNA
S svedberg unit 1 x 10^{-13} sec.
SE Standard error
snRNA stable nuclear RNA
TCA Trichloroacetic acid
Tris Tris hydroxymethylaminoethane
TCMHD (50 mM Tris-HCl, pH 7.9, 25% glycerol, 5 mM Mg^{2+}, 0.1 mM EDTA and 0.5 mM Dithiothreitol)
UMP Uridine - 5' - monophosphate
UTP Uridine - 5' - triphosphate

The following trivial names of steroid hormones have been used in this study.

<table>
<thead>
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<th>Trivial Name</th>
<th>IUPAC/IUB^b Complete Identification</th>
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<td>Aldosterone</td>
<td>18, 11 - Hemiacetal of 11β, 21 - dihydroxy - 20 - oxopregn - 4 - en 18 - al</td>
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<td>Corticosterone</td>
<td>11β, 21 - Dihydroxy - 4 - pregnene - 3, 20 - dione</td>
</tr>
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<td>Hydrocortisone</td>
<td>11β, 17α, 21, - Trihydroxy - 4 - pregnene - 3, 20 - dione</td>
</tr>
<tr>
<td>Cortisone</td>
<td>17α, 21 - Dihydroxy - 4 - pregnene - 3, 11, 20 - trione</td>
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<tr>
<td>Deoxycorticosterone</td>
<td>21 - Hydroxy - 4 - pregnene - 3, 20 - dione (that is, the 11-deoxy derivative of corticosterone)</td>
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<th>Compound</th>
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<td>Estradiol - 17β</td>
<td>1, 3, 5 (10) - Estratriene - 3, 17β - diol</td>
</tr>
<tr>
<td>Progesterone</td>
<td>4 - Pregnen - 3, 20 - dione</td>
</tr>
<tr>
<td>Testosterone</td>
<td>17β - Hydroxy - 4 - androsten - 3 - one</td>
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A. INTRODUCTION

Since the operon model for the control of gene expression was proposed by Jacob and Monod (1), work on the mechanism of hormone action has become one of the most popular and challenging areas of research in modern molecular biology. Although a considerable amount of new information has been obtained on the regulation of the lactose operon in *Escherichia coli*, the operon concept is still the simplest model which can be constructed to explain β-galactosidase induction (2). Experimental evidence has accumulated suggesting that hormones regulate growth, differentiation and metabolic activity in target tissues through their effects on both transcriptional and translational processes of protein biosynthesis (3-8). On the basis of biochemical, physiological and histological studies, a model of the molecular pathways involved in the action of several steroid hormones has been proposed (9) comprising (a) formation of a steroid-binding protein (receptor) complex, (b) stimulation of DNA dependent RNA synthesis, (c) RNA mediated protein synthesis and finally, (d) the physiological response. However, despite the amount of information available on steroid hormone effects upon transcriptional and translational steps of protein biosynthesis, only a few attempts have been made to explain how these effects occur. The metabolic role of glucocorticoids and mineralocorticoids is well established in their respective target tissues, but the mode of action at molecular level has not yet been completely established.
Purpose of This Study: This study is concerned with the effects of adrenalectomy and of adrenocortical hormones on RNA turnover and RNA polymerase activity in rat kidney and liver. Several other investigators have studied the incorporation of radioactively labelled nucleotides into total RNA in kidney and liver of adrenalectomized and hormone treated animals (10-16). The interpretation of such isotope incorporation experiments is difficult due to possible differences in precursor transport and pool sizes between the normal, adrenalectomized and adrenalectomized plus hormone treated rats, or possible errors resulting from the study of mixed populations or compartments of RNA within the cell.

In an attempt to circumvent these problems experiments were performed utilizing the turnover method. In a steady-state system the relative rate of incorporation of a radioactively labelled precursor would equal the relative rate of loss and would not depend on absolute incorporation. As long as the rates of synthesis and degradation of the component in question are long with respect to the turnover of precursor pools the measurements are valid. RNAs from various subcellular fractions are known to have turnover half-lives of four days or more (17-18) while radioactivity in precursor pools disappears within twenty-four hours after a single injection (19-21). Thus, turnover measurements can be used to compare RNAs in various subcellular fractions of adrenalectomized and hormone-treated animals. This approach is not applicable to mRNA, which has a half-life estimated at less than one day in most cases (22-23). Thus, no attempt was made to evaluate the turnover rate of this fraction. Aggregate RNA polymerase (nucleoside triphosphate-RNA nucleotidyl transferase, EC2. 7.7.6) activity was also determined in both liver and kidney. Further, to distinguish between the effects of mineralocorticoids on RNA polymerases
and on the DNA template, the enzymes were purified from adrenalectomized
OV. hormone-treated kidneys. The activity was measured using template
dNA from calf-thymus, and from adrenalectomized or mineralocorticoid-
treated rat kidneys.

B. REVIEW OF LITERATURE

(1) MODE OF ACTION OF HORMONES: Hormones regulate growth, differentiation
and metabolic activity in most tissues. They are defined classically as
chemical substances produced by ductless glands usually in trace amounts,
and carried by the systemic circulation to the target tissue on which their
biochemical and physiological effect is produced. Some hormones have a
high degree of specificity in their action on certain cells, others have
more general effects.

Hormones have been classified into three main categories: (i)
simple amino acids derivatives, e.g., histamine, serotonin and catecholamine.
(ii) steroid hormones, (a relatively large group) e.g., corticosterone,
aldosterone and (iii) the largest group, peptides and proteins, e.g., vas-
opressin and insulin.

Hormones may exert their effects on cells at the following
possible sites.

(i) Cell Permeability—The hypothesis that hormones may exert an effect
on transport of inorganic ions and nutrients across cell membranes was
first proposed by Hober (24). Since then a number of hormones have been
shown to affect the rate of transport of nutrients and ions across the
cell membrane. Levine and Goldstein (25) were the first to show the
accelerated transport of sugars across the muscle cell membrane. Christensen
(26) reported that the effect of insulin on cell permeability in muscle
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<td>Na⁺, K⁺</td>
<td>renal transport</td>
<td>decreased</td>
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<td></td>
<td>I⁻</td>
<td>thyroid</td>
<td>increased</td>
<td>(20)</td>
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<td>sugars</td>
<td>adrenal</td>
<td>increased</td>
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<td>adrenal</td>
<td>increased</td>
<td>(22)</td>
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<td>amino acids</td>
<td>renal transport</td>
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<td>(33)</td>
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<td>Aldosterone</td>
<td>Na⁺, K⁺</td>
<td>renal transport</td>
<td>increased</td>
<td>(34)</td>
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<tr>
<td></td>
<td>Na⁺</td>
<td>frog skin, testis bladder, blood vessels, salivary glands and intestine</td>
<td>increased</td>
<td>(35–37)</td>
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<td>renal transport</td>
<td>increased</td>
<td>(40–43)</td>
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<td></td>
<td>amino acids</td>
<td>skeletal muscle, kidney, uterus</td>
<td>increased</td>
<td>(44–45)</td>
</tr>
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<td>Calcitonin</td>
<td>Ca²⁺</td>
<td>bone</td>
<td>increased</td>
<td>(46)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kidney</td>
<td>decreased</td>
<td>(45)</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>amino acids</td>
<td>heart, liver, kidney</td>
<td>increased</td>
<td>(46)</td>
</tr>
<tr>
<td></td>
<td>sugars</td>
<td>renal transport</td>
<td>increased</td>
<td>(57)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>adipose tissue</td>
<td>increased</td>
<td>(36)</td>
</tr>
<tr>
<td>Estrogens</td>
<td>sugars</td>
<td>uterus</td>
<td>increased</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>amino acids</td>
<td>uterine</td>
<td>increased</td>
<td>(19, 49)</td>
</tr>
<tr>
<td></td>
<td>H₂O, Na⁺, K⁺</td>
<td>uterine</td>
<td>increased</td>
<td>(19, 49)</td>
</tr>
<tr>
<td>Glucagon</td>
<td>sugars</td>
<td>adipose tissue</td>
<td>increased</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>diaphragm (in vitro)</td>
<td>increased</td>
<td>(31)</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>amino acids</td>
<td>liver</td>
<td>increased</td>
<td>(51)</td>
</tr>
<tr>
<td></td>
<td>sugars</td>
<td>diaphragm</td>
<td>decreased</td>
<td>(32)</td>
</tr>
<tr>
<td></td>
<td>Ca²⁺</td>
<td>diaphragm</td>
<td>increased</td>
<td>(33)</td>
</tr>
<tr>
<td></td>
<td>PO₄³⁻</td>
<td>diaphragm</td>
<td>decreased</td>
<td>(34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>renal transport</td>
<td>decreased</td>
<td>(35)</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>amino acids</td>
<td>liver, skeletal muscle</td>
<td>increased</td>
<td>(56)</td>
</tr>
<tr>
<td></td>
<td>sugars</td>
<td>heart, diaphragm</td>
<td>increased</td>
<td>(59)</td>
</tr>
<tr>
<td></td>
<td>PO₄³⁻, PO₄⁵⁻</td>
<td>diaphragm, adipose tissue</td>
<td>increased</td>
<td>(57)</td>
</tr>
<tr>
<td>Insulin</td>
<td>amino acids</td>
<td>muscle</td>
<td>increased</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td>sugars</td>
<td>muscle</td>
<td>increased</td>
<td>(25)</td>
</tr>
<tr>
<td></td>
<td>K⁺, PO₄³⁻</td>
<td>muscle (in vitro)</td>
<td>increased</td>
<td>(17)</td>
</tr>
<tr>
<td>Neurohypophyseal hormones</td>
<td>H₂O, Na⁺, K⁺</td>
<td>renal tubule</td>
<td>increased</td>
<td>(58–59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>testis</td>
<td>increased</td>
<td>(58–59)</td>
</tr>
<tr>
<td>Thyroid-stimulating hormones</td>
<td>I⁻</td>
<td>thyroid</td>
<td>increased</td>
<td>(62)</td>
</tr>
<tr>
<td></td>
<td>amino acids</td>
<td>muscle, liver</td>
<td>increased</td>
<td>(33)</td>
</tr>
<tr>
<td></td>
<td>sugars</td>
<td>thyroid tissue</td>
<td>increased</td>
<td>(64)</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>H₂O</td>
<td>mitochondria</td>
<td>increased</td>
<td>(63)</td>
</tr>
</tbody>
</table>

is not restricted to sugars, but also facilitates amino acid permeability in muscle. Insulin also increased accumulation of potassium in muscle, but it is not clear whether this effect is secondary to other actions of the hormone on cell metabolism (27). Estrogens have been demonstrated to modify the transport of a variety of metabolites such as glucose, amino acids and sodium and potassium ions in rat uterus (28). Other hormones, namely glucocorticoids, mineralocorticoids, adrenocorticotrophic hormone, androgens, growth hormone and thyroid hormone respectively, have also been demonstrated to affect the transport of inorganic ions, water, sugars and amino acids in their respective target tissues (29-65). The major in vivo effects of various hormones on transport of various metabolites are briefly summarized in Table 1. The precise mechanism of action of these hormones on transport of metabolites is not known. Nevertheless, it seems that a single hormone usually modifies the transport of not only one but several metabolites at the same time (Table 1). This is particularly true with insulin and the estrogens which affect the transport of sugars, amino acids and inorganic ions. These observations suggest that hormones might modify the general behaviour of the membrane and not simply the transport of specific metabolite.

(iv) Enzyme Activity—D. E. Green (66) proposed a theory that substances functioning in trace amounts in biological systems must act by influencing enzyme systems. However, it is still not possible to account for hormone action in terms of interaction with a specific enzyme(s). The most popular example of an inferred enzymic effect is the stimulation of adenylcyclase by several hormones, shown in Table 2. Most hormones, with the exception of steroid hormones, are associated with alterations in adenylcyclase activity in vivo or in vitro. These lines of evidence strongly suggest a major role of cAMP as a mediator of hormone action, that is hormonal regulation
TABLE II

Some Examples of Hormones Acting on Adenylyl cyclase Activity Present in Target Tissues

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Target Tissue(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>catecholamines</td>
<td>liver, heart</td>
<td>(67)</td>
</tr>
<tr>
<td>catecholamines</td>
<td>brain, skeletal muscle, fat</td>
<td>(68)</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenal gland</td>
<td>(69)</td>
</tr>
<tr>
<td></td>
<td>adipose tissue</td>
<td>(70)</td>
</tr>
<tr>
<td>glucagon</td>
<td>liver</td>
<td>(71)</td>
</tr>
<tr>
<td></td>
<td>heart</td>
<td>(72)</td>
</tr>
<tr>
<td></td>
<td>fat</td>
<td>(73)</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid gland</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td>adipose tissue</td>
<td>(73)</td>
</tr>
<tr>
<td>PTH</td>
<td>bone</td>
<td>(75)</td>
</tr>
<tr>
<td></td>
<td>kidney (cortex)</td>
<td>(76)</td>
</tr>
<tr>
<td>vasopressin</td>
<td>kidney (medulla)</td>
<td>(74)</td>
</tr>
<tr>
<td>MSH</td>
<td>frog skin</td>
<td>(77)</td>
</tr>
</tbody>
</table>

*ACTH: adrenocorticotropic hormone; TSH: thyroid-stimulating hormone; PTH: parathyroid hormone; MSH: melanocyte-stimulating hormone.*
of enzymes levels in vivo. The molecular mechanisms by which hormones affect the adenylcyclase reaction have not yet been resolved.

Liu et al. (78) have recently demonstrated the effect of adrenalectomy and of aldosterone on certain rat kidney mitochondrial enzymes. They have shown that actinomycin D and puromycin administration prior to aldosterone treatment were without effect, suggesting that transcription and translational steps are not involved in the stimulation of mitochondrial oxidative enzymes by aldosterone.

(iii) RNA Synthesis and RNA Polymerase—Since the discovery that regulation of RNA synthesis plays a central role in the process of protein synthesis in microorganisms (1), there have been numerous publications suggesting that animal hormones also regulate the amount of certain cell enzymes and structural proteins through RNA mediators (79). The application of the operon concept to the problem of hormone mechanism of action led to the hormone-gene hypothesis (80). The currently held view is that in target tissue cells hormones may affect the initiation or rate of DNA transcription (81) into mRNA.

(a) Effects on RNA Synthesis: Several investigators have shown that in certain tissues, such as uterus (82), male accessory sex tissue (83), and liver (10-11) all major RNA fractions (ribosomal, messenger, and soluble RNAs) were stimulated by the appropriate steroid hormone. Growth hormone and thyroxine have also been shown to increase all types of RNAs (79).

Sells and Takahashi (79a) and Jackson & Sells (79b) have demonstrated the effects of growth hormone on increased precursor (orotic acid) incorporation into cytoplasmic mRNA and rRNA. The stimulatory effect of hormones was blocked when the inhibitors of RNA synthesis were used in these studies. This suggests that an effect on the synthesis of mRNA is one of the early events in the mechanism of action of many hormones. Considerable evidence
has accumulated suggesting selective mRNA synthesis in response to hormone action (84-85). Several investigators (86-88) using DNA-RNA hybridization techniques have demonstrated the synthesis of new RNA species (probably mRNA) after hormone administration. These RNA species were rapidly labeled, extractable at high pH and had base composition similar to DNA. Thus, these observations suggest that hormones may activate genes and allow transcription of new species of mRNA, which then code for the synthesis of specific proteins.

(b) RNA Polymerase Activity: RNA polymerases are known to occur in both prokaryotic and eukaryotic cells. In prokaryotes it has been demonstrated that the cell responds to its environment through the use of certain factors which control RNA synthesis. These factors exert precise regulations of initiation (σ factor), termination (ρ factor) and specificity of transcription (δ factor) by RNA polymerase (89-91). Similar RNA polymerase control factors in eukaryotes have also been reported (92-93); however, the significance of these control factors is as yet not clear in eukaryotes. DNA-dependent RNA polymerases have been recently isolated from a variety of eukaryotic tissues such as rat liver (94), rat prostate (95), calf thymus (96), amphibian tissues (97), insect tissue (98) and HeLa cells (99). The typical characteristics of all these enzyme preparations are the existence of multiple forms which differ in template requirements, sensitivity towards α-amanitin, metal ion requirement, ionic strength, sub-unit structure, molecular size and intranuclear localization (94-99).

Hormone-stimulated alterations of RNA polymerase activities have been demonstrated in many eukaryotic tissues. Table 3 summarizes the effects of various hormones on RNA polymerases in several animal tissues. However, the precise mechanism of RNA polymerase stimulation or inhibition by any one of the hormones listed in Table 3 is unknown at this time. It
### TABLE III

**Effect on RNA Polymerase Activity in Various Animal Tissues After Treatment With Hormones**

<table>
<thead>
<tr>
<th>HORMONE</th>
<th>ANIMAL</th>
<th>TISSUE</th>
<th>RNA POLYMERASE I</th>
<th>RNA POLYMERASE II</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth hormone</td>
<td>Hypophysectomized rat</td>
<td>Liver</td>
<td>Increased</td>
<td>Increased</td>
<td>(102, 102a)</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>Thyroidectomized rat</td>
<td>Liver</td>
<td>Increased</td>
<td>Increased</td>
<td>(103)</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>Tadpole (Rana Catesbeiana)</td>
<td>Liver</td>
<td>Increased</td>
<td>Increased</td>
<td>(104)</td>
</tr>
<tr>
<td>Estradiol</td>
<td>Ovariectomized rat</td>
<td>Uterus</td>
<td>Increased</td>
<td>Slightly increased</td>
<td>(132)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Castrated rat</td>
<td>Prostate</td>
<td>Total activity increased*</td>
<td>(105)</td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>Hypophysectomized - Castrated rat</td>
<td>Muscle</td>
<td>Total activity increased*</td>
<td>(79)</td>
<td></td>
</tr>
<tr>
<td>Cortisone</td>
<td>Normal rat</td>
<td>Liver</td>
<td>Increased</td>
<td>No effect</td>
<td>(106)</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Adrenalectomized rat</td>
<td>Liver</td>
<td>Increased</td>
<td>No effect</td>
<td>(100, 107)</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Normal rat</td>
<td>Thymus</td>
<td>Total activity decreased*</td>
<td>(108)</td>
<td></td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Normal rat</td>
<td>Thymus</td>
<td>No effect</td>
<td>Decreased</td>
<td>(109)</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>Adrenalectomized rat</td>
<td>Liver</td>
<td>Increased</td>
<td>No effect</td>
<td>(107)</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>Adrenalectomized rat</td>
<td>Kidney</td>
<td>Increased</td>
<td>Increased</td>
<td>(110-111)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heart</td>
<td></td>
<td></td>
<td>(111)</td>
</tr>
</tbody>
</table>

* Total activity was measured
is postulated that the mechanism of stimulation may work by: (a) an allo-steric interaction between RNA polymerase and the hormone, (b) interaction of the hormone with template or (c) induction of enzyme, its sub-units, or enzyme effectors (100-101).
(iv) Changes in Nuclear Proteins and Chromatin Activity—Since it is widely held that all somatic cells of the adult organism of a given species contain the total genetic information in the DNA template, a central question regarding the mechanism by which selective genetic expression occurs is related to regulation of gene transcription. It is believed that the basic nuclear proteins (histones) might be involved in suppressing DNA transcription by physical attachment to certain loci of DNA (112), and it has been speculated that the histone-DNA association could be under hormonal control. It is not yet clear, however, if histones influence RNA synthesis in nuclei by performing such a "repressor-like" role or whether their main function is to impart rigidity and stability to the intranuclear structures (113-114). The mechanism in vitro DNA-dependent RNA synthesis inhibition probably involves neutralization of the negatively-charged groups of DNA by the lysyl and arginyl residues of basic histones. These electrostatic interactions can be diminished if the charge on the histone is decreased by enzymic phosphorylation, methylation or acetylation of the basic residues (115-117). Acetylation of histones has been shown to be stimulated by a number of steroid hormones. Cortisol, estradiol, and aldosterone have been shown to stimulate acetylation of histones in liver (118), in cell-free extracts of rat uterus (119) and in rat kidney (120), respectively. Insulin has been demonstrated to stimulate phosphorylation of histone fractions in mammary glands during differentiations (121).

O’Malley et al. (122) have recently demonstrated the role of nuclear acidic proteins in steroid (progesterone) action. They have shown that the steroid hormone-binding protein (receptor) complex is transferred to the nuclear compartment where it forms another complex with certain chromosomal "acceptor sites" consisting of DNA and nuclear acidic proteins.
Raynaud-Jammet and Baulieu (129), Mohla et al. (130) and Arnaud et al. (131) have independently shown increased incorporation of labelled precursor into RNA of uterine nuclei incubated in the presence of uterine cytosol (containing receptor) and estradiol but not with hormone alone. Thus, these experiments suggest that the hormone-receptor complex may indeed influence transcriptional events in target cell nuclei. However, the specific roles and relative importance of DNA, receptor, hormone, nuclear acidic proteins, RNA polymerase and the important regulatory factors in gene transcription are not yet known.

Hamilton et al. (132) have demonstrated changes in the RNA-synthesizing capacity of chromatin, isolated from the uterus at various times after administration of estradiol to ovariectomized rats. The template activity of uterine chromatin in vitro was increased 25% over control animals by 30 minutes after administration of hormone. By 8 hours the activity was increased approximately 100% over the controls. Dehnus & Bonner (132) have shown an increase in liver chromatin activity following hydrocortisone administration to adrenalectomized rats. Insulin has also been demonstrated to increase the template activity (28%) of liver chromatin following its administration to diabetic rats, as compared to liver chromatin of untreated diabetic rats (134).

(v) Effects on Protein Synthesis—Hormones may alter the rate of protein biosynthesis.

(a) Induction of Enzymes: Induction of several hepatic enzymes has been demonstrated by glucocorticoid administration (135-138). The most striking changes have been observed in those enzymes involved in gluconeogenesis, such as phosphoenol pyruvate carboxy kinase (135), fructose-1, 6-
diphosphatase (136), serine dehydrase (137) and amino transferases (139-141). Induction by glucocorticoids, of several glycolytic enzymes in liver has also been reported (142). The exact mechanism by which these steroids stimulate enzyme induction is unknown at the present time.

Dexamethasone induced a 5-15 fold increase in the rate of synthesis of tyrosine aminotransferase in cultured rat hepatoma (HTC) cells (143-144). These investigators proposed a "translational repressor" model for dexamethasone induction of aminotransferase. The inducer was suggested to interact directly with a cytoplasmic repressor causing its translocation to nucleus where it could no longer inhibit aminotransferase synthesis. The precise mechanism is, however, still unclear.

(b) Activity or Availability of Ribosomes: Insulin and growth hormone have been shown to alter the activity of ribosomes of liver and muscle in vitro. The amino acid incorporation activity of ribosomes isolated from hypophysectomized rat liver, heart or skeletal muscle was found to be decreased compared to those from normal animals. (145-146) Wool et al. (147) have reported that muscle ribosomes were less active in vitro and contained less polysomes when taken from diabetic rats. Insulin treatment rapidly restored the deficiency. They further hypothesized that insulin forms a "translation factor" by initiating translation of the stable template RNA for that factor. Majumdar et al. (148) have recently reported increased polyphenylalanine synthesis (in presence of poly (U)) by renal cortical ribosomes isolated from mineralocorticoid treated rats.

(c) Availability or Activity of Aminoacyl Transfer RNA Complexes: At the present time it is not known if there are hormonally induced changes in the amount of aminoaclylating enzymes or of t-RNA in the cytosol fraction of tissues. Altman et al. (149), however, have recently reported hydrocortisone-induced changes in leucyl-tRNA as well as in leucyl-tRNA
synthetase activity of rat liver. It is not known if the induced synthetase activity and the tRNA were derived by \textit{de novo} synthesis or by modification of pre-existing molecules drawn from the cellular pool.

(d) Availability or Activity of Messenger RNA: There is evidence (150) that mRNA is transported from nucleus to the cytoplasm as a ribonucleo-protein complex and this provides a potential site where control could be exerted by a hormone. However, no evidence is available on this point.

(2) RECEPTORS AND STEROID HORMONE ACTION: The site of production for most of the steroid hormones is quite different from their site of action. The hormones produced in different ductless glands are carried by systemic circulation to the target tissue where they are retained. In the target tissue the steroid hormones form complexes with specific soluble protein molecules generally called "receptors". Table 3 shows a summary of such "receptor" molecules and some of their properties.

The interaction of a steroid hormone with its binding protein in the cytosol fraction has been suggested to be the first reaction in the tissue's response to the hormone. The hormone-receptor complex thus formed appears to be transported into the cell nucleus and presumably regulates certain nuclear activities including RNA synthesis (122-131). Gopalkrishan and Sadgopal (163) have recently reported the dual role of hydrocortisone receptor in rat liver. That is, the receptor acts as an agent for selective retention of the hormone in the target tissue, and as an activator of the gene(s) in the tissue chromatin.

How the steroid hormone-receptor complex moves to the cell nucleus and how the hormone or hormone-receptor complex leaves the cell nucleus after completion of its function is not known at the present
<table>
<thead>
<tr>
<th>Hormone</th>
<th>Animal tissue</th>
<th>Nuclear Binding</th>
<th>Receptor-Hormone complex &amp; value</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>temperature</td>
<td>cytosol dependent</td>
<td>- KCl</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>rat kidney</td>
<td>+</td>
<td>+</td>
<td>8-9</td>
</tr>
<tr>
<td>Cortisol</td>
<td>rat liver</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>rat liver</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>rat thymus</td>
<td>+</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>rat and mouse</td>
<td></td>
<td></td>
<td>3.5-7</td>
</tr>
<tr>
<td>acetate (glucocorticoid)</td>
<td>thymocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>HTC Hepatoma</td>
<td>+</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>Estradiol</td>
<td>rat uterus</td>
<td>+</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>Progesterone</td>
<td>chick oviduct</td>
<td>+</td>
<td>+</td>
<td>5, 8</td>
</tr>
<tr>
<td>Testosterone</td>
<td>rat prostate</td>
<td>+</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>rat epididymus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat seminal vesicle</td>
<td></td>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>

† Modified and completed to date after Jensen and DesesOMBRE (162)

* Binding shown but sedimentation constants were not determined.
time. O'Malley et al. (Personal Communication) think that transport of
progesterone-receptor complex into the nucleus from the cytoplasm is an
energy dependent process since this process can be blocked by the agents
(dinitrophenol, oligomycin and arsenite) which inhibit the enzymes in-
volved in energy production. Inhibitors of protein synthesis (actinomycin
d, cycloheximide and puromycin) are without effect on this transport
system.

(3) ROLE OF ADRENAL GLANDS AND EFFECTS OF ADRENALECTOMY: The adrenal
glands are composed of two distinct tissues - the adrenal medulla and the
adrenal cortex (zona glomerulosa, zona fasciculata and zona reticularis).
The secretions of the adrenal cortex belong to a class of compounds known
as steroids whereas secretions of adrenal medulla belong to a class of
compounds called catecholamines. The adrenal steroids are further divided
into two main categories - the glucocorticoids and mineralocorticoids, to
identify their two major types of physiological activity. The glucocorti-
coids influence mainly carbohydrate metabolism in the body (primarily in
liver) and the mineralocorticoids play an important role in regulating
sodium and potassium balance.

Bilateral adrenalectomy has been shown to cause a number of
biochemical, physiological and pathological changes in the body and is
fatal in mammalian species unless either sodium chloride or hormonal
therapy is given. The most pronounced metabolic disturbances caused by
bilateral removal of adrenal glands are: (i) decreased sodium retention
which ultimately results in ion and water imbalances arising from water
loss from circulation, (ii) increased erythrocyte count in the blood,
reduced plasma volume, and increase in urea and potassium levels in the
serum, (iii) decreased osmolality of body fluids due to increased sodium loss, (iv) decreased (40%) glucose, triglycerides, and fatty acids absorption in the duodenum (164). The alkaline phosphatase activity of the rat duodenum is also reduced by 50% following adrenalectomy. Ultimately these disturbances result in the death of the animal. These changes are reversed if the adrenalectomized animals are maintained on appropriate doses of mineralocorticoids.

Glucocorticoids have been shown to have profound effects on the thymus. The administration of glucocorticoids results in acute involution of the thymus structure. There is an atrophy of spleen, lymph nodes and thymus. At least one of the sites involved in the action of these steroid hormones is at the level of nuclear RNA synthesis (108-109). A decrease in RNA polymerase activity was also observed in thymocytes following exposure to cortisol in vitro (108). Suppression of DNA synthesis and inhibition of mitosis have also been reported in lymphocytes following cortisol administration (165).

The secretion of glucocorticoids is controlled by ACTH and the glucocorticoids thus produced as a result of ACTH action exert a negative-feedback on the hypothalamus. Consequently the release of corticotropin releasing hormone is inhibited and thus the secretion of ACTH by the adenohypophysis is reduced.

Secretion of mineralocorticoids, especially aldosterone does not, however, depend on ACTH, since it has been shown that in hypophysectomized animals (man and dog) the adrenal cortex continues to secrete aldosterone, albeit at lower than normal rate (166-168). It has been proposed that a decrease in blood volume, pressure or sodium ion concentration acts as a stimulus for the secretion of an enzyme, renin, by the
kidney. This enzyme acts on a circulating $a_2$-globulin (angiotensinogen, formed in liver) to form angiotensin I. The latter is then converted into angiotensin II (an octapeptide) by a second enzyme (converting enzyme). Angiotensin II directly acts on zona glomerulosa to increase aldosterone production (169).

(4) PROPOSED MECHANISM OF ACTION OF MINERALOCORTICOIDS: Mineralocorticoids (mainly aldosterone) have been shown to have effects upon sodium transport in kidney, salivary and sweat glands, the gastrointestinal tract, cardiac muscle and bone (170). The chief effect of mineralocorticoids is on kidney where the reabsorption of sodium and excretion of potassium and hydrogen, ammonium and magnesium ions takes place. Deoxycorticosterone has been demonstrated to stimulate phospholipid synthesis (171), in addition to sodium transport (172). Aldosterone has also been shown to increase transepithelial sodium transport in the urinary bladder of the toad, Bufo marinus.

(a) Effect on Toad Bladder (in vitro): Crabbe in 1961 (173) provided evidence that aldosterone had a direct effect on active sodium transport across the urinary bladder and ventral skin of the toad. Similar results were also reported by McAfee and Locke (174) for frog skin system (effect of cortisol). A latent period of 60-90 minutes in the action of aldosterone on active sodium transport in the isolated toad bladder system was also observed (175). The phenomenon of latent period led to the conclusion that synthesis or activation of a factor is involved in the action of aldosterone. Edelman et al. (13), Williamson (176), Porter et al. (177) and Castles and Williamson (12) suggested that aldosterone regulates
active sodium transport by induction of de novo synthesis of proteins, initiated by stimulation of DNA-dependent RNA synthesis. Hutchinson and Porter (178) have very recently examined the effects of temperature and substrate concentration on uridine incorporation into RNA of toad bladder under the influence of aldosterone. They reported that aldosterone (1.5 hours exposure) produced a significant increase in the incorporation of \( ^3H \)-uridine into heterogenous RNA extracted from purified nuclei, but not in RNA extracted from either cytoplasm or whole cell. However, at a later time there was a significant increase in aldosterone stimulated specific activity of whole cell RNA (3-20 hours after exposure to hormone).

Rousseau and Crabbe (179) however, could not demonstrate any effect of aldosterone on either mRNA synthesis or cytoplasmic RNA turnover in toad bladder. Aldosterone was without effect on protein synthesis also. Therefore, they concluded that the suggestion that the effects of aldosterone on sodium transport in toad bladder involve transcriptional and translational processes remains to be established.

Sharp et al. (180-182) reported that the aldosterone increased sodium transport was accompanied by increased glucose metabolism and utilization of pyruvate and acetoacetate in toad bladder. They have also recently demonstrated that aldosterone decreases \( ^4\)CO\(_2\) evolution from [1\(_{\text{\textsuperscript{14}}}\)C] glucose concomitantly with an increase in sodium transport. They concluded that the hexose monophosphate shunt of glucose metabolism is inhibited by mineralocorticoids. They further showed increased oxygen consumption and increased evolution of \( ^{13}\)CO\(_2\) from [6\(_{\text{\textsuperscript{14}}}\)C] glucose by the hormone, suggesting increased production of energy for stimulation of sodium transport. These effects were, however, abolished when sodium was removed from the bathing medium and consequently no sodium transport
occurred. This implies that the above effects were secondary ones arising from the hormone stimulated Na\textsuperscript+ transport. However, the decreased evolution of $^{14}\text{CO}_2$ from [1 – $^{14}$C] glucose persisted even in the absence of sodium and had similar characteristics with respect to concentration response, steroid specificity and sensitivity to spiroloctone and actinomycin D as did Na\textsuperscript+ transport. The effects of aldosterone on both the decreased evolution of $^{14}\text{CO}_2$ from [1 – $^{14}$C] glucose and increased sodium transport were reproduced by cyclic AMP suggesting the involvement of cyclic AMP in aldosterone action. No change, however, was noticed in tissue concentration of cyclic AMP even after prolonged incubation with hormone. Therefore, the role of cyclic AMP in these phenomena remains questionable.

(b) Effects on Rat Kidney: Williamson (176) reported in his preliminary study that actinomycin D blocked the antinaatriuretic effect but not the natriuretic effect of aldosterone in adrenalectomized rat kidney. Since then several (12, 13, 177) reports have appeared suggesting that the mechanism of action of aldosterone on the rat kidney involves DNA-dependent RNA and protein synthesis. Edelman et al. (13, 151) and Mills et al. (183) have studied the initial interactions of aldosterone with its receptors in the cytosol and nuclei of rat kidney. The mechanism by which the hormone stimulates sodium transport remains to be established. Recently Mishra et al. (110) and Liew et al. (111) have reported simultaneously but independently that there is a stimulatory effect of aldosterone on RNA polymerase in rat kidney. Unfortunately, no direct evidence of the stimulation of specific protein synthesis by aldosterone is available.

Sodium-potassium (Na\textsuperscript+ – K\textsuperscript+) ATPase has been shown to play a role in sodium retention in the kidney. The activity of (Na\textsuperscript+ – K\textsuperscript+) -
ATPase decreases during the first seven days after adrenalectomy (184-186). This decrease in activity of (Na$^+$ - K$^+$) - ATPase in kidney was partly restored by aldosterone in physiological doses. However, this effect could not be demonstrated until after the effect of aldosterone on sodium retention. The recent report of Knox and Sen (188) showing induction of (Na$^+$ - K$^+$) - ATPase protein by aldosterone suggest its role in sodium retention. These effects of aldosterone were not observed in toad bladder (187).

Several investigators (189-191) have reported effects of aldosterone on the kidney mitochondrial enzymes. Liljeroof and Hall (192) found that mitochondrial preparation from adrenalectomized rat liver showed partial uncoupling of oxidative phosphorylation. Administration of aldosterone to these animals reversed the effects of adrenalectomy on P/O ratio to normal values.
C. EXPERIMENTAL DESIGN

(i) Theory: It seems obvious that RNA metabolism is affected by steroid hormones in their respective target tissues, but no clear mechanistic picture has yet emerged. Moreover earlier studies of total cellular RNA and isotope incorporation have not been reliable for reasons already discussed in Section A (problems of precursor pool sizes, transport, and the study of a mixture of all types of RNA). Experimental approaches have therefore been adopted which will overcome these problems and yield an understanding of the mechanism of stimulation of RNA synthesis by steroid hormones. The following experimental questions have been asked in this study.

1. What is the rate of RNA turnover in various subcellular fractions of kidney and liver in normal, sham operated and adrenalectomized rats?

2. Is the turnover rate of RNA affected in adrenal deficient animals? If so, in all of the fractions or in some of the subcellular fractions?

3. Are the differences in turnover rates reversible in adrenal-depleted animals by the administration of adrenocortical steroid hormones? What is the specificity of mineralocorticoids and glucocorticoids in kidney and liver respectively, as to the effects on RNA turnover?

4. What are the effects of adrenalectomy and of adrenocortical hormones on the RNA polymerase system of kidney and liver?

5. Are mineralocorticoid hormones affect the RNA polymerase system of rat kidney?
(ii) **Turnover Approach:** Studies of the metabolic stability of macromolecules with the use of radioactive tracers can be undertaken in two ways:

a. Follow the rate of incorporation of labeled precursors into the macromolecule.

b. Follow the rate of degradation of labeled macromolecule after labeling to some maximum specific activity.

In the present experiments the latter approach (commonly called the "turnover approach") is followed, since the interpretation of results is less difficult than in the case of incorporation experiments. This is because turnover results do not depend on knowledge of precursor transport rates and precursor pool sizes. If the rates of synthesis and degradation of the compound being studied are much longer than the turnover time of compounds in the precursor pools, absolute values for the "steady state" rates of degradation can be obtained.

(iii) **Kinetic Equations:** If an isotopically labeled precursor of RNA is injected into an animal, it rapidly enters the cell, and is incorporated into RNA. If the precursor is cleared rapidly (assumption discussed later), then the RNA synthesized will quickly reach some maximum specific activity. If no more labeled precursor is available the specific activity will decrease as RNA is degraded (193-195).

The newly labeled RNA \((x^*)\) will be mixed with the unlabeled RNA \((x)\) of the system. The specific activity would be defined as

\[
A = \frac{x^*}{x + x^*}
\]

or

\[
A = -\frac{x^*}{x \text{ total}}
\]

where \(A\) is specific activity.
which is actually what is being measured and expressed as dpm/A_{260} units.

If the loss of x from a particular subcellular compartment is considered to be random (whatever the mechanism is, it does not distinguish between x and x*) and first order, then the differential equation for the rate of loss of A would be

\[- \frac{dA}{dt} = k'A\]

where \( k' \) is an apparent decay or fractional turnover constant. The integrated form of this equation between the limits of \( t = 0 \) and some time \( t \) is:

\[ A_{t} = A_{o} e^{-k't} \quad (2) \]

where \( A_{t} \) = specific activity at time \( t \) and
\( A_{o} \) = specific activity at time zero.

If one plots the natural logarithm of specific activity against time \( t \), a straight line is obtained with the slope \(-k'\).

If the pool of A is composed of two different kinds of A's, that is, \( A_{1} \) and \( A_{2} \), then each with \( k'\)'s greatly different will generate a single exponential curve.
However, a plot of the experimental data would show a composite curve such as:

The composite equation for the total specific activity in the compartment would be the sum of the individual exponentials.

\[ A_{\text{total}} = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}. \]  
(ref. 193)
MATERIALS AND METHODS

A. Materials

I. Chemicals and Biochemicals

1. Ribonucleoside triphosphates (ATP, GTP, CTP, UTP), disodium salts were obtained from Sigma Chem. Co. Stock solutions were prepared in 0.01 M Tris-HCl (pH 7.5) and small aliquots stored at -20°C.

2. 3H-Ribonucleoside 5'-Triphosphate (3H-UTP), tetradsodium salt, was purchased from New England Nuclear, Canada, Ltd. Specific activity was 22.2 Ci/mM. Excess ethanol was removed by a gentle stream of nitrogen gas and the labeled nucleotides were stored in 0.01 M Tris-HCl (pH 7.5) at -20°C for periods of up to two weeks before use.

3. 14C-6-Orotate acid hydrate, specific activity 60.8 mCi/mM, was obtained from New England Nuclear. The solutions were prepared in deionized water immediately before injections.

4. 3H-4-Aldosterone, specific activity 32 Ci/mM, and 14C-4-Aldosterone, specific activity 55 mCi/mM, were obtained from New England Nuclear. Dilutions were made up with Tris-HCl (pH 7.4) immediately prior to use.

5. 14C-Toluene (Sp. Act. 7 x 10^5 dpm/ml) and Tritiated water (Sp. Act. 1 mCi/ml) were supplied by New England Nuclear.

6. Spirolactone, hydrocortisone, corticosterone, deoxycorticosterone and unlabeled aldosterone were obtained from Sigma Chem. Co. Steroid solutions were prepared in ethanol containing 0.9% NaCl prior to use.

7. NAD, NADP, NADH, NADPH and cytochrome c reductase type I (pig heart) were obtained from Sigma Chem. Co. Solutions were prepared in deionized water immediately before use.
8. Ribonuclease A, 5 times recrystallized from bovine pancreas
was purchased from Sigma Chem. Co. Stock solutions of 2 mg/ml were pre-
apared in 0.01 M sodium citrate (pH 5) and heated at 90°C for 10 minutes
prior to use.

9. Deoxyribonuclease I, electrophoretically purified, was from
Worthington Biochemical Co.

10. Pyruvate Kinase type II (rabbit muscle) was supplied by
Sigma Chem. Co. The enzyme was obtained as a suspension in 2.2 M ammonium
sulfate. The specific activity was 350 - 500 E.U./mg. Enzyme was
desalted by centrifuging the aliquots at 10,000 x g for 20 minutes
and the pellets resuspended in 0.01M Tris-HCl (pH 7.4) just before use.

11. Phosphoenol pyruvate (tri sodium salt ) was obtained from
Sigma Chem. Co. Stock solutions of 2 mg/ml were prepared in 0.01 M Tris-
HCl (pH 7.4) and stored at -20°C.

12. Calf-Thymus DNA type I, was supplied by Sigma Chem. Co.
Stock solutions (1-5 mg/ml) in 0.01M Tris-HCl (pH 8.5) were prepared
fresh every week and kept at 0-4°C.

13. Ribonucleic acid from Torula yeast was obtained from Sigma
Chem. Co. Stock solutions (0.5-1 mg/ml) were prepared in deionized water.

14. Bovine serum albumin was obtained from Sigma Chem. Co.

15. α-Amanitin was purchased from Henely and Co., New York.

16. Rifampicin was a generous gift from Professor Silvestri
(gruppo Lepetit Italy).

17. Actinomycin D from Streptomyces chrysomalus, dithiothreitol
(Cleland's reagent) and spermine tetrahydrochloride were supplied by
Sigma Chem. Co.
18. Ammonium sulfate (Mann enzyme grade) and sucrose (density grade, ribonuclease free, crystalline, ultrapure) were obtained from Mann Research Laboratories.

19. Polyvinyl sulfate (ribonuclease inhibitor) was obtained from Eastman Kodak Co.

20. DEAE-Sephadex A-25 and Sephadex G-25 (medium) were supplied by Pharmacia Fine Chemicals, Inc., Montreal, Quebec.

21. 1, 4-bis [2-5 (Phenyl oxazoly)] benzene (POPOP), 2, 5-diphenyl oxazole (PPO), were obtained from Nuclear Associates, Inc., New York.

22. All other routinely used chemicals were obtained from general stock.

II. Animals: Female Wistar strain rats (80-150g) were used throughout these studies and were obtained from Canadian Breeding Laboratories or from the animal unit, Faculty of Medicine, Memorial University of Newfoundland, St. John's. Bilateral adrenalectomy* was performed through the dorsal route, using diethyl ether as an anaesthetic. The adrenalectomized animals were maintained on 0.9% sodium chloride and purina laboratory chow ad libitum and used for experiments 3 - 4 days after operations. Normal and sham operated animals were treated in the same way except that they were supplied with tap water. Aldosterone (5 μg/100g) and deoxycorticosterone (100 μg/100g) treated rats were also maintained on tap water. The experimental animals were maintained at 25°C.

*Completeness of adrenalectomy was checked both visually and by offering tap water.
B. Methods

I. Animal Groups: (a) For RNA turnover studies the rats were divided into three groups (14 each): sham operated, adrenalectomized and normal. Each animal was injected intraperitoneally with 4 μCi/100g body wt. of 14C-orotic acid. The adrenalectomized animals were maintained on 0.9% NaCl. Sham operated and normal animals were given tap water. One animal from each group was sacrificed by cervical dislocation at 1, 2, 3, 5, 8, 10, 13, 16, 19, 21, 25, 28, 32 and 36 day(s) after isotope administration. The kidneys and livers were very quickly excised and plunged into ice-cold saline (0.9% NaCl) washed and stored at -20°C until further processing. Approximately 90-120 seconds elapsed between killing of the rat and freezing of the excised tissues.

(b) To study the effects of adrenocorticoids on RNA turnover, the animals were divided into 5 groups (14 each): 1) adrenalectomized, 2) adrenalectomized plus aldosterone treated (5 μg/100g body wt.) 3) adrenalectomized plus deoxycorticosterone treated (100 μg/100g), 4) adrenalectomized plus corticosterone treated (2 mg/100g) and 5) adrenalectomized plus hydrocortisone treated (2 mg/100g). All the animals in the above groups were injected intraperitoneally with 4 μCi of 14C-orotic acid/100g body wt. The hormones were injected immediately after isotope administration and daily thereafter until the last experimental day (36 day). Adrenalectomized group 1) served as a control and received equivalent volumes of 0.9% NaCl. One animal from each group was sacrificed at days, 1, 2, 3, 5, 8, 10, 13, 16, 19, 21, 25, 28, 32 and 36. The animals were generally sacrificed between 9 and 11 a.m. to minimize diurnal variation. Tissues were removed as described above and stored at
-20 °C until further processing. The hormones were injected in ethanol or ethanol - 0.9% NaCl mixture.

(c) The animals for RNA polymerase experiments were divided into 6 groups (4-6 animals each): 1) normal, 2) adrenalectomized, 3) adrenalectomized plus aldosterone treated (5 μg/100g), 4) adrenalectomized plus deoxycorticosterone treated (100 μg/100g), 5) adrenalectomized plus corticosterone treated (2 mg/100g) and 6) adrenalectomized plus hydrocortisone treated (2 mg/100g). The hormones were injected intraperitoneally in ethanol - 0.9% NaCl mixture 3 hours before killing the rats. The livers and kidneys were removed and the nuclei were prepared immediately (without freezing) by the one step procedure of Busch et al. (196) and aggregate RNA polymerase activity was determined.

II. Preparation of Subcellular Fractions: The tissue fractionation scheme is shown in Fig. 1 and is based on techniques of Whitaker et al. (197), Von Hungen et al. (198) and Bloemendal et al. (199). The tissues were homogenized in 4.0 ml ice-cold homogenizing medium (0.35 M sucrose, 10 mM MgCl₂, 25 mM KCl and 50 mM Tris-HCl, pH 7.4 at 25°C) per gram wet-weight of tissue. A teflon-glass homogenizer previously cooled to 0°C was used. Eight to ten strokes at 800 rev./minute were applied and the subsequent operations were carried out at 2-4°C. One μg/ml of polyvinyl sulfate was mixed with the total homogenate to inhibit ribonuclease and the mixture centrifuged at 1000 x g for 10 minutes in a Lourdes refrigerated centrifuge. The nuclear pellet (P₁) was suspended in medium H₁ (0.32 M sucrose, 1 mM MgCl₂, 10 mM, Tris-HCl, pH 7 at 25°C) and frozen at -20°, and supernatant S₁ was centrifuged at 15,000 x g for 20 minutes to sediment the crude mitochondrial fraction (P₂).
A FLOW SCHEME FOR TISSUE FRACTIONATION

TISSUE
  Homogenized in Homogenizing Medium.

HOMOGENATE
  1000 x g, 10 min.

\[ \text{P}_1 \]
\[ \text{S}_1 \]

(Crude Nuclei)
Further Purified

\[ \text{P}_2 \]
\[ \text{S}_2 \]

(Crude Mitochondria) (Ribosomes, sRNA)
Resuspended in Med. H.
10,000 x g / 20 min. twice.

\[ \text{P}_3 \]

Supt. (discard)

\[5 \text{ ml. } 0.8M \]
\[5 \text{ ml. } 1.1M \]
\[5 \text{ ml. } 1.2M \]
\[5 \text{ ml. } 1.5M \]
\[5 \text{ ml. } 2.0M \]

Centrifuged at 131,000 x g for 80 minutes.

\[ \text{S}_2 \]
Ribosomes and sRNA

10 ml + 1% Triton x 100 (Final Concentration)
Centrifuged at 144,000 x g / 2 hr.

pellet
(Total ribosomes)
Further Purified

supt.
Discard

10 ml of 1.5M sRNA
Smooth E R
10 ml of 2 M Rough E R
Polysomes

FIGURE 1.
Preparation of Mitochondria: The pellet (P₂) was resuspended in medium H₁ and centrifuged at 10,000 x g for 20 minutes. This step was repeated once more and then P₂ was resuspended in 5 ml of medium H₁. The mitochondria were further purified by layering this suspension over a discontinuous sucrose density gradient consisting of 5 ml each of 2M, 1.5M, 1.2M, 1.1M and 0.8M sucrose. The gradients were then centrifuged at 131,000 x g (average) for 80 minutes in Beckman Model L-3-50 preparative ultracentrifuge, using Rotor 8 S.W. 27. The schematic representation of mitochondria obtained from liver is shown in Fig. 2. The mitochondria sedimented to the 1.5M layer of sucrose. The contaminated polysomes and rough ER penetrated the 1.5M sucrose layer and sedimented to the bottom of the tube. Lysosomes and other membrane fragments were sedimented at various sucrose layers. The mitochondria and other layers were removed by means of a syringe and collected separately. The mitochondria were resuspended in cold distilled water and washed by centrifugation.

Preparation and Purification of Total Ribosomes: The total ribosomes were prepared by the method of Henzies et al. (18).

To 10 ml of post-mitochondrial supernatant (S₂), Triton-X-100 was added to the final concentration of 1%. Triton-X-100 acts as a detergent to free the membrane ribosomes. The resulting solution was centrifuged at 144,000 x g (average) for 2 hrs. in a Beckman Model L-3-50 preparative ultracentrifuge using Rotor #40. The supernatant was removed with a pasteur pipette and discarded.

The surface of the total ribosomal pellet from the centrifugation at 144,000 x g was gently rinsed without resuspension with 5 ml of homogenizing medium. Three ml of Medium B (10 mM Mg²⁺, 25 mM KCl, 50 mM...
FIGURE 2. SCHEMATIC REPRESENTATION OF PURE MITOCHONDRIA

FIGURE 3. SCHEMATIC REPRESENTATION OF ROUGH ER, SMOOTH ER, POLYSOMES AND SRNA
Tris-HCl, pH 7.5) and three drops of polyvinyl sulfate solution (10 µg/ml) were added and the pellet allowed to soften overnight at 0°C. The ribosomes were then gently resuspended and transferred with rinses to a second centrifuge tube. The suspension was centrifuged at 10,000 x g for 10 minutes to remove debris, and the resulting pellet was extracted once more with 2 ml of Medium B for recovery of trapped ribosomes. The combined supernatants constituted the crude ribosomes fraction. Sedimentation analysis (Beckman Model E, 20°C) of this fraction showed one major peak at about 80 S as well as a small amount of ribosomal subunits (Fig. 4). The presence of protein contaminants was indicated by low \( A_{260}/A_{230} \) ratios (1.2-1.4).

To minimize contamination by soluble RNA and protein, the ribosomes were precipitated by adding an equal volume of 0.14M Mg\(^2+\) and the pellet resuspended in 2 ml of NEB-2-buffer (0.2 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 7.4). The above steps were repeated (usually 2 to 3 times) until \( A_{260}/A_{230} \) ratios of 1.6-1.7 were obtained. Sedimentation analysis of ribosome preparations of this purity usually showed only two major peaks: 60 S and 45 S (Fig. 5). When Mg\(^2+\) is added to 5-10 mM in excess of the EDTA present, an 80 S peak appears indicating some reassociation or aggregation. This constituted the total ribosomal preparation.

Preparation of Polysomes, Rough Endoplasmic Reticulum, Smooth Endoplasmic Reticulum, and sRNA: A 15 ml aliquot of post-mitochondrial supernatant (S \(_2\)) was carefully layered over a discontinuous sucrose density gradient consisting of 10 ml of 2M sucrose in Medium B, and 10 ml of 1.5M sucrose in Medium B and centrifuged at 75,000 x g (average) for 18 hrs.
FIGURE 4. ANALYTICAL ULTRACENTRIFUGATION PATTERN OF TOTAL RIBOSOMES OF LIVER.

The ribosomes were in medium B, pH 7.5. Picture taken at 4 min after reaching speed of 40,000 rpm, temperature 20°C. The schlieren angle was 60°. Direction of sedimentation is from left to right.
FIGURE 5. ANALYTICAL ULTRACENTRIFUGATION PATTERN OF PURE TOTAL RIBOSOMAL PREPARATION FROM LIVER

The ribosomes were in NEE-2 buffer, pH 7.4. Picture taken at 4 min after reaching speed of 45,000 rpm, temperature 20°C. The schlieren angle was 60°. Direction of sedimentation is from left to right.
(Rotor 50) a Beckman Model L-3-50 preparative ultracentrifuge.

The schematic representation of fractions obtained from liver (S₂o) after centrifugation through a discontinuous sucrose density gradient (2M, 1.5M) in Beckman Model L-3-50 preparative ultracentrifuge is shown in Fig. 3.

The fractions after centrifugation were separated by Buchler Densi-flow apparatus. To minimize the cross contamination the upper and bottom portion of each fraction was discarded. The rough ER and smooth ER fractions were pelleted at 15,000 x g by resuspending in Medium B and centrifugation.

Preparation of Nuclei: The crude nuclei (P₁) were further purified by the method of Chauveau et al. (200). The nuclear fraction was purified by thawing the pellet (P₁), resuspending it in Medium N₁ (0.35 M sucrose, 3 mM CaCl₂, 50 mM Tris-HCl pH 7.2) underlayed with equal volume of Medium N₂ and centrifuging it for 15 minutes at 1500 x g in a Sorvall refrigerated centrifuge. The supernatant was discarded and the pellet was resuspended in Medium N₂ (2.4 M sucrose, 3 mM CaCl₂, 50 mM Tris-HCl pH 7.2) by gentle homogenization and centrifuged for 1 hr. at 50,000 x g (average) in Beckman Model L-3-50 preparative ultracentrifuge using Rotor 40. The supernatant was discarded and the resulting white pellet was suspended in distilled water and represents the nuclear fraction. The nuclei were examined by phase contrast microscopy (using Carl Zeiss Photomicroscope) and appeared to be free of contaminations as shown in Figs. 6 and 7.

III. Marker Enzymes: In order to estimate the purity of various rat liver subcellular fractions, the following specific marker enzymes were assayed in isolated fractions: NADPH-cytochrome c reductase for rough
Liver nuclei were isolated as described in Methods, section II. Suspensions in medium H₁ were examined under phase contrast optics at 800X magnification with Zeiss standard RA microscope.
FIGURE 7. ISOLATED NUCLEI FROM RAT KIDNEY.

Kidney nuclei were isolated as described in Methods, section II. Suspensions in medium H₁ were examined under phase contrast optics at 800X magnification with Zeiss standard RA microscope.
ER and smooth ER (201) and succinate-dehydrogenase and glutamate dehydrogenase for mitochondria (202-203).

**NADPH-cytochrome c Reductase**: Activity was assayed by measuring the reduction of cytochrome c at 550 nm (201). The reaction mixture contained 50 mM Tris-HCl pH 7.4, 0.1 mM NADPH, 50 μM cytochrome c and subcellular fraction containing approximately 1 mg protein in total volume of 3 ml. One unit is defined as the amount of enzyme which causes a change of 0.01 A₅₅₀ per minute.

**Succinate Dehydrogenase**: Activity was determined by measuring the reduction of the dye iodosetetrazolium at 490 nm (202). The reaction mixture contained potassium phosphate buffer, 0.25 M pH 7.4, sodium succinate 0.25 M, iodosetetrazolium 0.5% - 0.01 M EDTA and subcellular fraction containing approximately 0.5-1 mg protein in total volume of 1 ml. One unit is defined as the amount of enzyme which causes a change of 1 A₄₉₀ per 15 minutes.

**Glutamate Dehydrogenase**: Activity was determined by measuring the oxidation of NADH at 340 nm (203). The reaction mixture contained triethanolamine buffer, 50 mM pH 7.4, NADH 0.19 mM, ADP 0.95 mM, ammonium sulfate 50 mM and subcellular fraction representing 0.5 mg protein in total volume of 3 ml. The mixture was incubated for 15 minutes at 37°C and blank was measured. 10 mM of 2-oxoglutarate was mixed and the actual rate was measured. One unit of enzyme is that amount which causes a change of 1 A₃₄₀ per minute.

**IV. Sucrose Density Gradient Analysis of RNA from Subcellular Fractions**: RNA from subcellular fractions was extracted by the cold phenol-sodium dodecyl sulphate method (204) 24 hours after injection of ¹⁴C-ornic
acid. The frozen fractions (polysomes, ribosomes, rough ER and smooth ER) were suspended in 10 mM sodium acetate buffer pH 5.1. To this solution was added sodium dodecyl sulphate (final concentration 1%) and cold redistilled phenol (2 vol. of a water saturated solution to 1 vol. of suspension). The mixture was centrifuged at 16,000 x g for 30 minutes in a Lourdes refrigerated centrifuge. After centrifugation the aqueous upper phase was removed with a pasteur pipette and stored on ice. Equal volume of acetate buffer (pH 5.1) was added to the remaining underlayer and the mixture was recentrifuged for 15 minutes. The aqueous phase was re-extracted with sodium dodecyl sulphate and phenol as above. The aqueous phases were pooled and the RNA was precipitated with 2 vol. of cold 95% ethanol and collected by centrifugation. The RNA was dissolved in a solution containing 5 mM Tris-HCl, pH 7.2, 50 mM NaCl and 1 mM EDTA.

The resulting solution was layered over a linear 5 to 20% sucrose gradient containing 5 mM Tris-HCl pH 7.2 and 50 mM NaCl and centrifuged (SW 27 rotor) at 27,000 rpm for 16 hours at 0° in a Beckman Model L-3-50 preparative ultracentrifuge. The gradients were analyzed by upward displacement with 40% sucrose and the absorbance of the gradient at 260 nm monitored using an ISCO Model D automatic density gradient fractionator. Fractions of 0.8 - 1 ml were collected directly into scintillation vials for radioactivity determination.

(v) Isolation of DNA from Nuclei and Mitochondria: DNA from nuclei and mitochondria was isolated and purified by the methods of Drews and Brauer- man (86); (205) and Gross et al. (206), with the following modifications. Tissue nuclei were suspended in a buffer solution (0.1 M Tris-HCl, pH 9.5, 0.5% sodium dodecyl sulphate) 4 times the original weight of tissue.
The viscous suspension was stirred with an equal volume of water-saturated phenol for 60 minutes at 0-4°C. The resulting mixture was centrifuged at 10,000 x g in a Lourdes refrigerated centrifuge and the aqueous overlay was separated with a pasteur pipette. This aqueous phase was re-extracted with phenol and the DNA was precipitated by addition of 0.1 volume of 10% NaCl and 3 vol. of cold ethanol. The fibrous precipitate was freeze-dried and dissolved in 0.1 M NaCl and the resulting solution was incubated at 37° for 30 minutes with RNase (20 µg/ml). The enzyme was then removed by extracting with phenol. The aqueous phase was further deproteinized by chloroform-methyl alcohol (6:1, v/v) treatment. The DNA was again precipitated with ethanol, dried and stored at -20°C.

Mitochondrial samples were thawed and solubilized in cold 1% sodium deoxycholate. After standing for 10 minutes at 0-4°C, DNA was precipitated with cold perchloric acid (final concentration, 0.5 M). The precipitate was vigorously dispersed with a Vortex mixer and collected by centrifugation at 1000 x g for 5 minutes. The pellet was extracted three times more with cold 0.5 M perchloric acid, and then further re-extracted successively at room temperature with 95% ethanol, ethanol-chloroform (3:1, v/v), and ethanol-ether (3:1, v/v). The pellet was dissolved in 3.0 ml of 0.3 M KOH and incubated at 40°C for 2 hours to hydrolyze RNA. After cooling the sample to 0-4°C sufficient cold 2 M perchloric acid was added to neutralize the KOH and bring the acid concentration to 0.5 M. After vigorous shaking the precipitate was extracted twice more with 0.5 M perchloric acid. The pellet was resuspended in 2 ml of 0.5 M perchloric acid, incubated at 90°C for 15 minutes, cooled and centrifuged as described above. The supernatant was removed, stored at -20°C and used for radioactivity determination.
(vi) Preparation of Histones and Nonhistone Proteins from Kidney Nuclear Fraction: Kidney chromatin, histones and nonhistone (acidic proteins) were prepared by a modification of Paul and Gilmour (207), and Späth and HaIlica method (208). The procedure is shown by a flow sheet in Fig. 8.

(vii) Aggregate RNA Polymerase Assay: Both Mg\(^{2+}\) and Mn\(^{2+}/(NH_4)_2SO_4\)-activated RNA polymerase (forms I and II, respectively) reactions were studied in isolated nuclei.

The incubation mixture for the Mg\(^{2+}\) activated RNA polymerase reaction contained (in a final volume of 0.5 ml): 50 \(\mu\)moles Tris-HCl (pH 8.2 at 25°C); 4 \(\mu\)moles Cleland's reagent; 5 \(\mu\)moles MgCl\(_2\); 0.05 \(\mu\)moles each of GTP, CTP and UTP, 1.0 \(\mu\)mole ATP, 2 \(\mu\)Ci \(^3\)H-UTP and nuclear suspension representing 50-200 \(\mu\)g DNA. The reaction mixture for Mn\(^{2+}/(NH_4)_2SO_4\)-activated polymerase was similar to Mg\(^{2+}\)-containing assay mixture, except that 2 \(\mu\)moles of MnCl\(_2\) and 250 \(\mu\)moles of \((NH_4)_2SO_4\) pH 7.5 were substituted for MgCl\(_2\).

In both cases the reaction was initiated by the addition of nuclei. After incubation for 15 minutes at 37°C, the reaction was terminated by placing the tubes in chipped ice, followed by immediate addition of 5 ml of 10% (v/v) trichloroacetic acid. The acid-insoluble material was collected on Whatman GF/C filters, which were then washed three times with 10 ml of 5% trichloroacetic acid containing 0.05M sodium pyrophosphate, and once with ethanol-ether 3:1. The filters were dried under an infra-red lamp and radioactivity was counted in a liquid scintillation counter using 10 ml of Bray's solution (222).
Fractionation of Kidney Nuclear Fraction

(All steps carried out at 0-8°C)

**Kidney Nuclei**

1. **Wash twice with 0.15M NaCl, 0.01M Tris-HCl (pH 7.5) using centrifugation (10,000 x g for 15 min.).**
   - **Pellet**

2. **Suspend in distilled water and centrifuge gently (1000 x g for 10 min.).**
   - **Pellet**

3. **Resuspend in water and centrifuge gently (1000 x g for 10 min.).**
   - **Chromatin**

   **Extract twice with 0.2M H2SO4**
   - **Pellet**
   - **Supernatant (Histones)**

   **(DNA-non-histone proteins)**

4. **Extract with 0.1M NaOH (15,000 x g for 10 min.).**
   - **Pellet**
   - **Supernatant**

   **(DNA)**

   **(Non-histone acidic proteins)**

**Figure 8.**
(viii) Quantitative Solubilisation and Purification of RNA Polymerases from Rat Kidney: To distinguish the effects of aldosterone on template DNA or RNA polymerases, RNA polymerases were quantitatively solubilized and purified from normal, adrenalectomized, and adrenalectomized plus aldosterone treated rats. The techniques of Sajde et al. (100) and Roeder and Rutter (209) were used with some modifications. Tissue nuclei were isolated by the one step procedure of Busch et al. (196). The nuclei were then suspended in 100 mM Tris-HCl buffer pH 8.9 containing 25 mM KCl, 4 mM magnesium acetate, and 1 mM Ciealand's reagent (1.5 ml of buffer/ g. wet wt. of tissue). The nuclei were lysed by homogenization using a teflon-glass homogenizer. The lysed nuclei were then incubated for 20 minutes at 30°C with gentle shaking. Glycerol was added (final conc. 30% v/v) and the shaking continued for another 5 minutes. The homogenous mixture was then centrifuged at 100,000 x g for 30 minutes at 4°C in a Beckman Model L-3-50 preparative ultracentrifuge using rotor 50. The supernatant obtained after centrifugation was filtered through four layers of cheese cloth which was previously washed with buffer. The filtered supernatant contained 80-90% of RNA polymerase activity and no DNA. The solubilized enzyme was precipitated with ammonium sulfate (0.4 g/ml) and stirred at 4°C for 1 hour. The mixture was then centrifuged at 105,000 x g for 1 hour. The precipitate was suspended in 50 mM Tris-HCl, pH 7.9, 25% (v/v) glycerol 5 mM MgCl₂, 0.1 mM EDTA and 0.5 mM Cleland's reagent (TGME) and dialyzed for 8 hours against the same buffer. The dialysate was subjected to DEAE-A 25 Sephadex chromatography as follows:

DEAE-A 25 Sephadex was suspended in 30 volumes (w/v) of distilled water, titrated to pH 7.8 with 1 N Tris-Hydroxide and allowed to swell for 24 hours. The slurry was then washed 3 times with 30 volumes
of 0.5 M ammonium sulfate (pH 7.9) by settling and decantation procedure, and equilibrated with TGME buffer containing 0.05 M ammonium sulfate.

Columns of 0.9 x 15 cm (Sephadex column K 9/15) were packed with the final slurry and a further 5 bed volumes of TGME containing 0.05 M ammonium sulphate was allowed to run through. Solubilized enzyme preparations containing 4-10 mg/ml protein were placed on the column and eluted with linear ammonium sulfate gradients (0.1 to 0.5 M) in TGME buffer. The flow rates were approximately 0.4 ml/cm²/min. The enzyme preparations were passed through (0.6 x 25 cm) Sephadex G-25 (medium) equilibrated with 0.05 M Tris-HCl, pH 7.9, 25% glycerol (v/v), 0.5 mM Cleland's reagent. This procedure removed the metal ion (Mg²⁺) and most of the salt. In experiments where it was necessary to concentrate the enzyme activity, the fractions in each peak were combined and concentrated by pressure ultrafiltration through Diaflow UM-20 E membranes.

Assay for Solubilized RNA Polymerase: The standard assay mixture for polymerase I reaction contained the following reagents in total volume of 500 ul.

50 μmoles Tris-HCl pH 7.9
4 μmoles Cleland's reagent
0.05 μmoles each of, ATP, GTP, CTP, and UTP
2 μCi ³²P-UTP (ethanol-free, .04 Ci/m M)
5 μmoles MgCl₂
10 μmoles phosphoenol pyruvate
20 μg pyruvate kinase (20 ul desalted enzyme suspension)
50 μg Calf-thyus DNA (Native)
50 μl of enzyme solution
The reaction mixture for polymerase II was similar to I, except that 2 μmoles \( \text{MnCl}_2 \) and 250 μmoles of \((\text{NH}_4)_2\text{SO}_4\) were substituted for \( \text{MgCl}_2 \). The rest of the procedure was similar to that described for aggregate RNA polymerase assay in section VII.

(iii) Analytical Methods: Proteins were determined by the method of Lowry et al. (210) using bovine serum albumin as a standard. DNA was estimated by the method of Burton (211) using Calf-thymus DNA as a standard and RNA by the method of Schneider (212) using yeast RNA as a standard.

(a) Isolation of RNA and Determination of Radioactivity: The RNA from each subcellular fraction was isolated by a modification of the Schmidt-Thannhauser method (213) as described by Menzies, et al. (18). The RNA content was determined by both ultraviolet absorption and the colorimetric orcinol method (212). Aliquots were counted in an Intertechnique liquid scintillation counter using Triton-X-100/toluene scintillation fluid. (0.1 g of 1, 4-bis [2-(5 phenyl oxazoly)] benzene, 5.0 g of 2, 5-diphenyl oxazole, 330 ml of Triton-X-100 and 667 ml of toluene per liter). The count rate was corrected to dpm by internal standardization with \(^{14}\text{C}-\text{toluene}.\) The counting efficiency was 85-90%. Specific activity is expressed as dpm per \( A_{260} \) unit; that is, an absorbancy of 1.0 at 260μm in a 1-cm light path. The total RNA content of various fractions was not determined because of the difficulty of obtaining absolutely pure fraction in 100% yield. The objective of the experiment (to study turnover rates) can, however be accomplished by determination of specific activity and does not require knowledge of the RNA content of the fraction.

(iv) Statistical Analysis of the Data: The RNA turnover data were subjected to statistical analysis for linear regression. All calculations were done on an IBM 1130 computer, or IBM 370 computer. Two types of
tests to distinguish differences in slopes ($k'$) were applied as outlined by Steel and Torrie (24) (1960, page No. 171 and 173). The values of the 95% confidence limits on $k'$ are shown in the Tables VII-VIII. This value is the one most commonly found in the literature and allows a rough test of significance at a glance. However, where differences between slopes were suggested by the data, the more rigorous t-test was applied.
EXPERIMENTAL RESULTS

(1) Purity of Fractions: The degree of cross contamination among rough ER, smooth ER and mitochondria in both kidney and liver was determined from the distribution of the marker enzymes (Tables V and VI). It was found that cross contamination between the mitochondria and the rough ER and smooth ER respectively was less than 1%, but the possibility of contamination between rough ER and smooth ER could not be ruled out.

Bloemendal et al. (199), however, have shown by electronmicroscopic studies that rough ER and smooth ER prepared by this technique are not cross contaminated. The results with marker enzymes are in agreement with reports in the literature (215-216). The purity of total ribosomes was determined by model E-analytical ultracentrifuge studies as described in Methods. Purified polysomes showed two major peaks (monomers and dimers) and minor peaks (trimers, tetramers, etc.) on linear 10-34% sucrose density gradients (Figs. 9 and 10).

(2) Characteristics of RNAs on Sucrose Density Gradients: RNA from rat liver ribosomes, free polysomes and rough ER of normal and adrenalectomized rats showed a typical well-defined sedimentation pattern of 28S, 18S and 5S peaks (Figs. 11 and 12). RNA from the smooth ER, unlike rough ER showed the sedimentation pattern of 28S, 18S, 11S and 4S peaks (Fig. 13). RNA from kidney fractions showed a similar pattern except smooth ER, which showed 28S, 18S, 9S and 4S peaks (Figs. 14-16). The 9S and 11S species of RNA are not present in the ribosomes or rough ER and it seems that it does not arise from 28S or 18S RNA by degradation since its specific activity (counts/min/mg RNA) after 24 hours of labeling differed from other RNA species. This type of RNA in smooth ER has also been reported by King and Fitschen (217). The precise nature and function of this RNA species is unknown.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>NADPH-cytochrome c reductase</th>
<th>Succinate dehydrogenase</th>
<th>Glutamate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total units</td>
<td>% recovered</td>
<td>units/mg protein</td>
</tr>
<tr>
<td>Total homogenate</td>
<td>160.00 ± 23.00</td>
<td>100.00</td>
<td>0.42</td>
</tr>
<tr>
<td>Purified mitochondria</td>
<td>0.31 ± 0.04</td>
<td>0.19</td>
<td>0.01</td>
</tr>
<tr>
<td>Purified Rough ER</td>
<td>47.00 ± 4.00</td>
<td>29.00</td>
<td>18.00</td>
</tr>
<tr>
<td>Purified Smooth ER</td>
<td>36.00 ± 11.00</td>
<td>22.00</td>
<td>16.00</td>
</tr>
</tbody>
</table>

Preparation of fractions, assays and units are described in "Methods." (Section III). Each value is the average of 6 replicate experiments ± SE (assays were performed in duplicate).

* nd - not detectable
<table>
<thead>
<tr>
<th>Fraction</th>
<th>NADPH-cytochrome c reductase</th>
<th>Succinate dehydrogenase</th>
<th>Glutamate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total units</td>
<td>% recovered</td>
<td>units/mg protein</td>
</tr>
<tr>
<td>Total homogenate</td>
<td>600.00 ± 41.00</td>
<td>100.00</td>
<td>0.690</td>
</tr>
<tr>
<td>Purified mitochondria</td>
<td>0.25 ± 0.08</td>
<td>0.04</td>
<td>0.006</td>
</tr>
<tr>
<td>Purified Rough ER</td>
<td>200.00 ± 12.00</td>
<td>33.30</td>
<td>40.000</td>
</tr>
<tr>
<td>Purified Smooth ER</td>
<td>125.00 ± 56.00</td>
<td>20.80</td>
<td>31.200</td>
</tr>
</tbody>
</table>

Preparation of fractions, assays and units are described in "Methods." (Section III) Each value is the average of 6 replicate experiments ± SE (assays were performed in duplicate).

* nd - not detectable
FIGURE 9. SUCROSE DENSITY GRADIENT PROFILES OF POLYSOMES OF NORMAL RAT KIDNEY.

Two ml of polysome suspension containing a total of 25 optical density units were layered over 30 ml linear (10-34%) sucrose density gradient containing 5 mM Tris-HCl, pH 7.4, 25 mM KCl and 5 mM MgCl₂. The tubes were centrifuged at 27,000 rpm for 2½ hours at 0°C in a SW 27 rotor of the Spinco preparative ultracentrifuge. At the end of the run, the A_{260} of the gradient was monitored continuously from the top using the ISCO automatic gradient analyzer. T, top; B, bottom.
FIGURE 10. SUCROSE DENSITY GRADIENT PROFILE OF POLYSOMES OF ADRENATECTOMIZED RAT LIVER.

Two ml of polysomal suspension containing a total of 30 optical density units were layered over 30 ml linear (10-34%) sucrose density gradient containing 5 mM Tris-HCl, pH 7.4, 25 mM KCl and 5 mM MgCl₂. The tubes were centrifuged at 27,000 rpm for 2½ hours at 0°C in a SW 27 rotor of the Spinco preparative ultracentrifuge. At the end of the run, the A₂₆₀ of the gradient was monitored continuously from the top using the ISCO automatic gradient analyzer. T, top. B, bottom.
FIGURE 11. SUCROSE DENSITY GRADIENT PROFILES OF RAT LIVER TOTAL RIBOSOMAL RNA LABELED IN VIVO.

The RNA from ribosomal fractions of normal and adrenalectomized rats were isolated by cold phenol sodium dodecyl sulfate method and layered over a linear (5-20%) sucrose density gradient containing 5 mM Tris, pH 7.2, 50 mM NaCl and 1 mM EDTA and centrifuged at 27,000 rpm for 16 hours at 0°C. Approximately 40 optical density units were put on the gradient. For details, see "Methods" section IV. T, top. B, bottom.

Normal rats: Absorbance

Counts per minute

Adrenalectomized rats: Absorbance

Counts per minute
FIGURE 12. DENSITY GRADIENT PROFILES OF ROUGH ENDOPLASMIC RETICULUM RNA OF ADRENALECTOMIZED RAT LIVER LABELED IN VIVO.

The RNA was isolated after 24 hours of labeling by cold phenol sodium dodecyl sulfate method and layered over sucrose density gradient containing 50 mM NaCl, 1 mM EDTA and 50 mM Tris-HCl, pH 7.2 and centrifuged at 27,000 rpm for 16 hours at 0°C. Approximately 35 optical density were put on the gradients. For details see "Methods" section IV. T, top, B, bottom.
FIGURE 13: SUCROSE DENSITY GRADIENT PROFILES OF SMOOTH ENDOPLASMIC RETICULUM RNA LABELED in vivo.

The RNA from smooth ER fractions of normal rat liver was isolated by cold phenol sodium dodecyl sulfate method and layered over a linear (5–20%) sucrose density gradient containing 5 mM Tris, pH 7.2 and 50 mM NaCl and 1 mM EDTA and centrifuged at 27,000 rpm for 16 hours at 0°C. Approximately 30 optical density units were applied on the gradient. For details, see "Methods" section IV. T, top; B, bottom.
FIGURE 14. SUCROSE DENSITY GRADIENT PROFILES OF KIDNEY TOTAL RIBOSOMAL RNA LABELED IN VIVO.

The RNA from ribosomal fractions of normal and adrenalectomized rats was isolated after 24 hours of injections by cold phenol sodium dodecyl sulfate method and layered over a linear (5-20%) sucrose density gradient containing 5 mM Tris-HCl, pH 7.2, 50 mM NaCl and 1 mM EDTA and centrifuged at 27,000 rpm for 16 hours at 0°C. Approximately 30 optical density units were put on the gradients. For details see "Methods" section IV. T, top; B, bottom.

Normal rat kidney: Absorbance - Absorbance - - - - - - -
Counts per minute -

Adrenalectomized rat kidney: Absorbance - - - - - - -
Counts per minute -

Counts per minute -
FIGURE 15. SUCROSE DENSITY GRADIENT PROFILES OF NORMAL RAT KIDNEY ROUGH ENDOPLASMIC RETICULUM RNA LABELED in vivo.

The RNA was isolated after 24 hours of injection by cold phenol sodium dodecyl sulfate method and layered over a linear (5-20%) sucrose density gradients containing 5 mM Tris-HCl, pH 7.2, 50 mM NaCl and 1 mM EDTA and spun at 27,000 rpm for 16 hours at 0°C. Approximately 25 optical density units were applied on the gradient. For details see "Methods" section IV. T, top. E, bottom.

Absorbance

Counts per minute
FIGURE 16. DENSITY GRADIENT PROFILES OF SMOOTH ENDOPOLASMIC RETICULUM RNA OF NORMAL RAT KIDNEY LABELED in vivo.

The RNA was isolated after 24 hours of injections by cold phenol sodium dodecyl sulfate method and layered after sucrose density gradient containing 5 mM Tris-HCl, pH 7.2, 50 mM NaCl and 1 mM EDTA and centrifuged at 27,000 rpm for 16 hours at 0°C. Approximately 20 optical density units were put on the gradients. The sedimentation constants of 9-10 peak was confirmed by model E Analytical ultra centrifuge run at 20°C. T, top. B, bottom.

Absorbance

Counts per minute
In all cases, the large 28S accounted for more than 50% of the total RNA, the 18S represented about 35% and about 15% of RNA was of low molecular weight suggesting no RNA degradation during isolation.

(2) Effect of Adrenalectomy on RNA Turnover in Kidney and Liver:

(i) Patterns of RNA Turnover: The turnover rates of RNAs in the various subcellular fractions, namely nuclei, mitochondria, rough ER, smooth ER, free polysomes, total ribosomes and sRNA (low molecular weight RNA) of normal and adrenalectomized kidney and liver are shown graphically in Figs. 17 and 18 and in Tables VII and VIII. The logarithm of the specific activity (dpm/\(\lambda_{260}\)) is linear with time in all of the plots. The pattern conforms to a random degradation model in all the subcellular fractions and is consistent with a homogeneous compartment with respect to degradation rate. Thus, all of the patterns fit the single exponential removal model:

\[
A_t = A_0 e^{-k't} \quad (As \ described \ in \ Experimental \ design)
\]

Where \(A_t\) and \(A_0\) are the specific activities at time \(t\) and zero respectively. In all cases \(k'\) represents the apparent decay constant which takes into account some possible reutilization of labeled precursor (195). The possibility of substantial reutilization, however, can be eliminated for the following reasons:

(a) Almost no free labeled pyrimidine nucleotides were detected one week after initial injection of \(14C\)-orotic acid. Some of the label found in this pool was associated with various nucleotide cofactors such as uridine diphosphate glucose etc. (218)

(b) It is possible that label may be reincorporated through paths other than pyrimidine nucleotides. That is, after degradation of the pyrimidine ring, label may also be recycled through purine
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Animal Group</th>
<th>( k' )</th>
<th>( \pm 95% ) confidence limit</th>
<th>Initial specific activity</th>
<th>Half-life (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>Normal</td>
<td>0.094</td>
<td>( \pm 0.0107 )</td>
<td>2737</td>
<td>7.41</td>
</tr>
<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.079</td>
<td>( \pm 0.0089 )</td>
<td>2647</td>
<td>9.23</td>
</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>0.092</td>
<td>( \pm 0.0075 )</td>
<td>2580</td>
<td>7.32</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Normal</td>
<td>0.112</td>
<td>( \pm 0.0097 )</td>
<td>2994</td>
<td>5.63</td>
</tr>
<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.094</td>
<td>( \pm 0.0098 )</td>
<td>2647</td>
<td>7.32</td>
</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>0.114</td>
<td>( \pm 0.0089 )</td>
<td>2940</td>
<td>5.78</td>
</tr>
<tr>
<td>Rough ER</td>
<td>Normal</td>
<td>0.113</td>
<td>( \pm 0.0111 )</td>
<td>3576</td>
<td>5.13</td>
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<td></td>
<td>Adrenalectomized</td>
<td>0.100</td>
<td>( \pm 0.0108 )</td>
<td>2686</td>
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</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>0.142</td>
<td>( \pm 0.0974 )</td>
<td>2740</td>
<td>4.98</td>
</tr>
<tr>
<td>Smooth ER</td>
<td>Normal</td>
<td>0.137</td>
<td>( \pm 0.0098 )</td>
<td>2930</td>
<td>5.03</td>
</tr>
<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.114</td>
<td>( \pm 0.0196 )</td>
<td>2562</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Polyribosomes</td>
<td>Normal</td>
<td>0.126</td>
<td>( \pm 0.0093 )</td>
<td>3090</td>
<td>5.67</td>
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<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.119</td>
<td>( \pm 0.0073 )</td>
<td>2785</td>
<td>5.81</td>
</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>0.128</td>
<td>( \pm 0.0045 )</td>
<td>3940</td>
<td>5.52</td>
</tr>
<tr>
<td>Total RNAs</td>
<td>Normal</td>
<td>0.172</td>
<td>( \pm 0.0080 )</td>
<td>4824</td>
<td>4.11</td>
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<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.114</td>
<td>( \pm 0.0138 )</td>
<td>4143</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>sRNA</td>
<td>Normal</td>
<td>0.340</td>
<td>( \pm 0.0034 )</td>
<td>3222</td>
<td>4.60</td>
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<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.140</td>
<td>( \pm 0.0155 )</td>
<td>2721</td>
<td>4.93</td>
</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

\( A_0 \) is the specific activity at time zero \((A_0 = A_k/k')\) where \( A_k \) is the specific activity at time \( t \).

\( \dagger \) Significant differences \((p < 0.05)\) as compared with normal

\( ** \) na – not analyzed
FIGURE 17. DECAY OF THE SPECIFIC RADIOACTIVITY OF RNA FROM NORMAL AND ADRENALECTOMIZED RAT KIDNEY.

Each data point represents an individual animal. The regression lines were calculated using all of the points. The following fractions were isolated and specific activity determined as described in "Methods" section X.

a. nuclear RNA; b. mitochondrial RNA; c. rough endoplasmic RNA;
d. smooth endoplasmic RNA; e. polysomal RNA; f. total ribosomal RNA; g. soluble RNA.

NOR, normal; ADX, adrenalectomized; ER, endoplasmic reticulum.
TABLE VIII
Effect of Adrenalectomy on RNA Turnover in Various
Subcellular Fractions of rat Liver

The apparent decay constants (k') and their 95% confidence limits were calculated as described by Steel & Terrie (214) using IBM-370 computer.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Animal Group</th>
<th>k'</th>
<th>± 95% confidence limit</th>
<th>Initial specific activity A₀*</th>
<th>Half-life (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>Normal</td>
<td>0.079</td>
<td>± 0.0056</td>
<td>826</td>
<td>8.69</td>
</tr>
<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.057†</td>
<td>± 0.0036</td>
<td>612</td>
<td>12.02</td>
</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>na**</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Normal</td>
<td>0.106</td>
<td>± 0.0041</td>
<td>2390</td>
<td>6.51</td>
</tr>
<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.086†</td>
<td>± 0.0038</td>
<td>1652</td>
<td>8.50</td>
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<tr>
<td></td>
<td>Sham Oper.</td>
<td>0.104</td>
<td>± 0.0042</td>
<td>2450</td>
<td>6.45</td>
</tr>
<tr>
<td>Rough ER</td>
<td>Normal</td>
<td>0.127</td>
<td>± 0.0056</td>
<td>5708</td>
<td>5.24</td>
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<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.109†</td>
<td>± 0.0042</td>
<td>3222</td>
<td>6.88</td>
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<tr>
<td></td>
<td>Sham Oper.</td>
<td>0.129</td>
<td>± 0.0067</td>
<td>5760</td>
<td>5.31</td>
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<tr>
<td>Smooth ER</td>
<td>Normal</td>
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<td>± 0.0067</td>
<td>3254</td>
<td>6.36</td>
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<td>± 0.0045</td>
<td>2093</td>
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<td></td>
<td>Sham Oper.</td>
<td>0.107</td>
<td>± 0.0032</td>
<td>2157</td>
<td>6.12</td>
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<td>Polysoes</td>
<td>Normal</td>
<td>0.157</td>
<td>± 0.0014†</td>
<td>3233</td>
<td>4.61</td>
</tr>
<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.115†</td>
<td>± 0.0060</td>
<td>3039</td>
<td>5.86</td>
</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>0.149</td>
<td>± 0.0036</td>
<td>3290</td>
<td>4.62</td>
</tr>
<tr>
<td>Total Ribosomes</td>
<td>Normal</td>
<td>0.140</td>
<td>± 0.0046</td>
<td>4321</td>
<td>4.92</td>
</tr>
<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.114†</td>
<td>± 0.0063</td>
<td>2411</td>
<td>6.05</td>
</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>0.141</td>
<td>± 0.0031</td>
<td>4250</td>
<td>5.0</td>
</tr>
<tr>
<td>sRNA</td>
<td>Normal</td>
<td>0.145</td>
<td>± 0.0089</td>
<td>3141</td>
<td>4.76</td>
</tr>
<tr>
<td></td>
<td>Adrenalectomized</td>
<td>0.116</td>
<td>± 0.0036</td>
<td>1967</td>
<td>5.92</td>
</tr>
<tr>
<td></td>
<td>Sham Oper.</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

* A₀ is the initial or time-zero specific radioactivity calculated from the equation

A₀ = A₀e⁻kt

** na not analyzed
† statistically significant differences (p < 0.05) as compared with normal
‡‡ (p < .1 but > 0.05)
FIGURE 18. DECAY OF SPECIFIC RADIOACTIVITY OF RNA FROM NORMAL AND
ADRENALECTOMIZED RAT LIVER.

Each data point represents an individual animal. The
regression lines were calculated using all of the points. The follow-
ing fractions were isolated and specific activity determined as described
in "Methods" section X.

a. nuclear RNA; b. mitochondrial RNA; c. rough endoplasmic reticulum
RNA; d. smooth endoplasmic reticulum RNA; e. polysomal RNA;
f. total ribosomal RNA; g. soluble RNA.

NOR, normal; ADX, adrenalectomized; ER, endoplasmic reticulum.
nucleotides such as adenosine monophosphate and guanosine monophosphate. Mannies et al. (19), however, could not detect label in any of these nucleotides; therefore, this possibility would seem unlikely.

(c) Bucher & Swaffield (21) have presented evidence that the nucleotide pool (cytidylic and uridylic acids) turn over rapidly. A turnover half-life of the order of minutes was reported by these authors. When this is compared to the half-lives for most fractions evaluated in this study, it is clear that recycling is negligible.

(d) Reutilization of label via DNA in nuclear and mitochondrial RNA is possible but in view of the above arguments and in consideration of the number of reaction steps involved in the conversion of deoxyribonucleotides to ribonucleic acid precursors this possibility is unlikely. Additionally, no label in DNA isolated from liver and kidney nuclei and mitochondria was detected suggesting reutilization is negligible.

(e) Tissue samples (stored at -20°C) were fractionated and analyzed within 50-60 days. The possibility of differential hydrolysis of labeled RNA during storage at -20°C as a result of endogenous ribonuclease activity was ruled out by the observation that the specific activities (dpm/μg unit) of various subcellular fractions from normal and adrenalectomized rat kidneys, prepared after 24 hours and after 3 months of storage at -20°C, varied less than ± 3% in all RNA fractions from both the groups (Table IX).

The apparent decay constant (k') and its 95% confidence limit, the zero time or initial specific activity (A0) and the turnover half-life (t1/2) for each fraction have been summarized in Tables VII and VIII.
### TABLE IX

**Effects of Storage of Kidneys at -20° on Specific Activity (DPM/A<sub>260</sub> Units)**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Normal Animals</th>
<th></th>
<th>Adrenalectomized Animals</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours of</td>
<td>3 month of</td>
<td>24 hours of</td>
<td>3 months of</td>
</tr>
<tr>
<td></td>
<td>storage at -20°</td>
<td>storage at -20°</td>
<td>storage at -20°</td>
<td>storage at -20°</td>
</tr>
<tr>
<td>Nuclei</td>
<td>1650 ± 15 (3)</td>
<td>1630 ± 28 (3)</td>
<td>1150 ± 14 (3)</td>
<td>1170 ± 30 (4)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>900 ± 26 (3)</td>
<td>925 ± 48 (3)</td>
<td>865 ± 21 (3)</td>
<td>885 ± 39 (3)</td>
</tr>
<tr>
<td>Rough ER</td>
<td>2762 ± 24 (4)</td>
<td>2700 ± 51 (4)</td>
<td>2600 ± 64 (4)</td>
<td>2640 ± 95 (4)</td>
</tr>
<tr>
<td>Smooth ER</td>
<td>1251 ± 16 (4)</td>
<td>1261 ± 17 (4)</td>
<td>925 ± 18 (4)</td>
<td>900 ± 74 (4)</td>
</tr>
<tr>
<td>Polysomes</td>
<td>2100 ± 30 (3)</td>
<td>2080 ± 16 (3)</td>
<td>1790 ± 34 (3)</td>
<td>1730 ± 89 (3)</td>
</tr>
<tr>
<td>Total Ribosomes</td>
<td>2700 ± 78 (4)</td>
<td>2675 ± 29 (4)</td>
<td>2430 ± 90 (4)</td>
<td>2390 ± 55 (4)</td>
</tr>
<tr>
<td>sRNA</td>
<td>750 ± 21 (3)</td>
<td>731 ± 18 (3)</td>
<td>586 ± 28 (3)</td>
<td>560 ± 22 (3)</td>
</tr>
</tbody>
</table>

Number of experiments is given in parentheses.

± Standard error
(ii) **Effects of Adrenalectomy:** Tables VII and VIII and Figures 17 and 18 reveal that adrenalectomy significantly decreased the turnover rates of RNAs in nuclei, mitochondria and total ribosomes of kidney and nuclei, mitochondria, rough ER and free polysomes of liver respectively. Other fractions in both tissues showed similar tendencies, although in these cases the changes were not statistically significant. It is also apparent from the Tables VII and VIII that sham-operated animals showed similar turnover rates to those of normal intact ones.

Slower turnover rates in adrenalectomized animals suggest a slower synthesis of RNA (steady-state approximation). This is also consistent with the low initial specific activity ($A_0$) which reflects the initial net synthesis of RNA (Tables VII and VIII).

(4) **Effects of Adrenocortical Hormones on RNA Turnover in Kidney and Liver:** Daily injections of appropriate adrenocortical hormones reversed the effects on RNA turnover rates in all the subcellular fractions (Tables X and XI and Figs. 19 and 20). It is seen from Table X that the effects on turnover rates were reversed in kidney by the daily injections of aldosterone (5 µg/100 g body wt.), or deoxycorticosterone (100 µg/100 g body wt.), however, daily injections of corticosterone (the rat's main glucocorticoid) (2 mg/100 g) or hydrocortisone (2 mg/100 g body wt.) were without effects. These results suggest that the effects on turnover rates in rat kidney are specific to mineralocorticoids.

Table XI shows that daily injections of corticosterone and hydrocortisone reversed the effects of adrenal depletion on RNA turnover in liver and daily injections of aldosterone were without effects. This also suggests the tissue specific effects of glucocorticoids in liver.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Animal group</th>
<th>$k'$</th>
<th>95% confidence limit</th>
<th>Initial specific activity $A_0$</th>
<th>Half-life (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>ADX + NaCl</td>
<td>0.073</td>
<td>± 0.0054</td>
<td>1274</td>
<td>9.53</td>
</tr>
<tr>
<td></td>
<td>ADX + Cortico</td>
<td>0.058</td>
<td>± 0.0132</td>
<td>1816</td>
<td>9.62</td>
</tr>
<tr>
<td></td>
<td>ADX + DOC</td>
<td>0.089</td>
<td>± 0.0032</td>
<td>2600</td>
<td>7.63</td>
</tr>
<tr>
<td></td>
<td>ADX + AG</td>
<td>-0.110</td>
<td>± 0.0051</td>
<td>3085</td>
<td>6.25</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>ADX + NaCl</td>
<td>0.088</td>
<td>± 0.0076</td>
<td>2678</td>
<td>7.82</td>
</tr>
<tr>
<td></td>
<td>ADX + Cortico</td>
<td>0.089</td>
<td>± 0.0074</td>
<td>2694</td>
<td>7.79</td>
</tr>
<tr>
<td></td>
<td>ADX + DOC</td>
<td>0.123</td>
<td>± 0.0107</td>
<td>3079</td>
<td>6.11</td>
</tr>
<tr>
<td></td>
<td>ADX + AG</td>
<td>-0.125</td>
<td>± 0.0120</td>
<td>5567</td>
<td>5.51</td>
</tr>
<tr>
<td>Rough ER</td>
<td>ADX + NaCl</td>
<td>0.103</td>
<td>± 0.0056</td>
<td>3452</td>
<td>6.72</td>
</tr>
<tr>
<td></td>
<td>ADX + Cortico</td>
<td>0.104</td>
<td>± 0.0073</td>
<td>3572</td>
<td>6.65</td>
</tr>
<tr>
<td></td>
<td>ADX + DOC</td>
<td>0.138</td>
<td>± 0.0079</td>
<td>4117</td>
<td>4.99</td>
</tr>
<tr>
<td></td>
<td>ADX + AG</td>
<td>-0.165</td>
<td>± 0.0177</td>
<td>4445</td>
<td>4.18</td>
</tr>
<tr>
<td>Smooth ER</td>
<td>ADX + NaCl</td>
<td>0.103</td>
<td>± 0.0097</td>
<td>2682</td>
<td>6.70</td>
</tr>
<tr>
<td></td>
<td>ADX + Cortico</td>
<td>0.104</td>
<td>± 0.0097</td>
<td>2683</td>
<td>6.56</td>
</tr>
<tr>
<td></td>
<td>ADX + DOC</td>
<td>0.136</td>
<td>± 0.0120</td>
<td>2870</td>
<td>5.09</td>
</tr>
<tr>
<td></td>
<td>ADX + AG</td>
<td>-0.165</td>
<td>± 0.0177</td>
<td>4345</td>
<td>4.18</td>
</tr>
<tr>
<td>Polysomes</td>
<td>ADX + NaCl</td>
<td>-0.119</td>
<td>± 0.0500</td>
<td>2211</td>
<td>5.79</td>
</tr>
<tr>
<td></td>
<td>ADX + Cortico</td>
<td>-0.123</td>
<td>± 0.0108</td>
<td>2311</td>
<td>5.63</td>
</tr>
<tr>
<td></td>
<td>ADX + DOC</td>
<td>-0.169</td>
<td>± 0.0093</td>
<td>6003</td>
<td>4.64</td>
</tr>
<tr>
<td></td>
<td>ADX + AG</td>
<td>-0.202</td>
<td>± 0.0070</td>
<td>7715</td>
<td>3.41</td>
</tr>
<tr>
<td>Total ribosomes</td>
<td>ADX + NaCl</td>
<td>-0.110</td>
<td>± 0.0050</td>
<td>3314</td>
<td>6.37</td>
</tr>
<tr>
<td></td>
<td>ADX + Cortico</td>
<td>-0.116</td>
<td>± 0.0086</td>
<td>3640</td>
<td>6.19</td>
</tr>
<tr>
<td></td>
<td>ADX + DOC</td>
<td>-0.184</td>
<td>± 0.0110</td>
<td>3944</td>
<td>4.21</td>
</tr>
<tr>
<td></td>
<td>ADX + AG</td>
<td>-0.213</td>
<td>± 0.0108</td>
<td>6553</td>
<td>3.10</td>
</tr>
<tr>
<td>sRNA</td>
<td>ADX + NaCl</td>
<td>-0.161</td>
<td>± 0.0043</td>
<td>2809</td>
<td>4.91</td>
</tr>
<tr>
<td></td>
<td>ADX + Cortico</td>
<td>-0.145</td>
<td>± 0.0036</td>
<td>3118</td>
<td>4.73</td>
</tr>
<tr>
<td></td>
<td>ADX + DOC</td>
<td>0.135</td>
<td>± 0.0051</td>
<td>3910</td>
<td>5.13</td>
</tr>
<tr>
<td></td>
<td>ADX + AG</td>
<td>-0.124</td>
<td>± 0.0097</td>
<td>5450</td>
<td>3.96</td>
</tr>
</tbody>
</table>

ADX = Adrenalectomized
Cortico = Corticosterone
DOC = Deoxycorticosterone
AG = Aldosterone
FIGURE 19. DECAY OF SPECIFIC RADIOACTIVITY OF RNA FROM ADRENALECTOMIZED
PLUS HORMONE TREATED RAT KIDNEYS.

Each data point represents an individual animal. The regression lines were calculated using all of the points. The following fractions were isolated and specific activity determined as described in "Methods" section X.

a. nuclear RNA; b. mitochondrial RNA; c. rough endoplasmic reticulum RNA; d. smooth endoplasmic RNA; e. polysomal RNA; f. total ribosomal RNA; g. soluble RNA.

ADX + A0. Adrenalectomized plus aldosterone treated.

ADX + DOC. Adrenalectomized plus deoxycorticosterone treated.

ER. Endoplasmic reticulum.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Animal group</th>
<th>( k' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>ADX + NaCl</td>
<td>0.056 ± 0.0039</td>
</tr>
<tr>
<td></td>
<td>ADX + corticosterone</td>
<td>-0.067 ± 0.0026</td>
</tr>
<tr>
<td></td>
<td>ADX + hydrocortisone</td>
<td>-0.111 ± 0.0049</td>
</tr>
<tr>
<td></td>
<td>ADX + aldosterone</td>
<td>-0.056 ± 0.0027</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>ADX + NaCl</td>
<td>0.087 ± 0.0038</td>
</tr>
<tr>
<td></td>
<td>ADX + corticosterone</td>
<td>0.119 ± 0.0062</td>
</tr>
<tr>
<td></td>
<td>ADX + hydrocortisone</td>
<td>-0.133 ± 0.0034</td>
</tr>
<tr>
<td></td>
<td>ADX + aldosterone</td>
<td>0.066 ± 0.0037</td>
</tr>
<tr>
<td>Rough ER</td>
<td>ADX + NaCl</td>
<td>-0.124 ± 0.0038</td>
</tr>
<tr>
<td></td>
<td>ADX + corticosterone</td>
<td>-0.163 ± 0.0063</td>
</tr>
<tr>
<td></td>
<td>ADX + hydrocortisone</td>
<td>0.185 ± 0.0038</td>
</tr>
<tr>
<td></td>
<td>ADX + aldosterone</td>
<td>-0.101 ± 0.0045</td>
</tr>
<tr>
<td>Smooth ER</td>
<td>ADX + NaCl</td>
<td>-0.104 ± 0.0046</td>
</tr>
<tr>
<td></td>
<td>ADX + corticosterone</td>
<td>-0.124 ± 0.0037</td>
</tr>
<tr>
<td></td>
<td>ADX + hydrocortisone</td>
<td>-0.124 ± 0.0035</td>
</tr>
<tr>
<td></td>
<td>ADX + aldosterone</td>
<td>-0.107 ± 0.0047</td>
</tr>
<tr>
<td>Polysomes</td>
<td>ADX + NaCl</td>
<td>-0.135 ± 0.0043</td>
</tr>
<tr>
<td></td>
<td>ADX + corticosterone</td>
<td>-0.157 ± 0.0070</td>
</tr>
<tr>
<td></td>
<td>ADX + hydrocortisone</td>
<td>-0.175 ± 0.0033</td>
</tr>
<tr>
<td></td>
<td>ADX + aldosterone</td>
<td>-0.119 ± 0.0061</td>
</tr>
<tr>
<td>Total Ribosomes</td>
<td>ADX + NaCl</td>
<td>-0.103 ± 0.0068</td>
</tr>
<tr>
<td></td>
<td>ADX + corticosterone</td>
<td>-0.170 ± 0.0080</td>
</tr>
<tr>
<td></td>
<td>ADX + hydrocortisone</td>
<td>-0.168 ± 0.0026</td>
</tr>
<tr>
<td></td>
<td>ADX + aldosterone</td>
<td>-0.112 ± 0.0064</td>
</tr>
<tr>
<td>rRNA</td>
<td>ADX + NaCl</td>
<td>-0.116 ± 0.0044</td>
</tr>
<tr>
<td></td>
<td>ADX + corticosterone</td>
<td>-0.175 ± 0.0033</td>
</tr>
<tr>
<td></td>
<td>ADX + hydrocortisone</td>
<td>-0.226 ± 0.0076</td>
</tr>
<tr>
<td></td>
<td>ADX + aldosterone</td>
<td>-0.117 ± 0.0057</td>
</tr>
</tbody>
</table>

ADX = Adrenalectomized.

\( A_0 \) is the initial or time-zero specific radioactivity calculated from the equation:

\[
A_t = A_0 e^{-k't}
\]
FIGURE 20. DECAY OF SPECIFIC RADIOACTIVITY OF RNA FROM ADRENALECTOMIZED PLUS HORMONE TREATED RAT LIVERS.

Each data point represents an individual animal. The regression lines were calculated using all of the points. The following fractions were isolated and specific activity determined as described in "Methods" section X.

a. nuclear RNA; b. mitochondrial RNA; c. rough endoplasmic RNA; d. smooth endoplasmic reticulum RNA; e. polysomal RNA; f. total ribosomal RNA; g. soluble RNA.

ADX + Cortic. Adrenalectomized plus corticosterone treated.

ADX + Hyco. Adrenalectomized plus hydrocortisone treated.

ER. Endoplasmic reticulum.
(5) Aggregate RNA polymerase Activity: Most of the RNA polymerase activity is firmly bound to the DNA template and corresponds to an insoluble complex which is called the "aggregate enzyme." The assay is generally performed using whole nuclear fraction which contains both the enzyme and the template.

(2) Characteristics of RNA polymerase Reaction in vitro.

The characteristics of RNA polymerase assay using rat kidney and liver nuclei are shown in Tables XII and XIII respectively. It is apparent that the reaction is dependent on DNA as template, divalent cations, and ribonucleoside triphosphates ATP, GTP, UTP and CTP. Actinomycin D strongly inhibited both polymerase reactions in kidney and liver. Rifampicin which is a potent inhibitor of bacterial RNA polymerase (219) and of DNA synthesis in isolated rat liver mitochondria had no effect on either of the polymerases reactions in both kidney and liver. It is seen from the Tables XII and XIII that inclusion of α-amanitin in the assay system abolished the activity of RNA polymerase II in both kidney and liver. A third RNA polymerase (RNA polymerase III) has been reported in nuclei from rat liver and sea urchin (220) but solubilization and chromatography of kidney RNA polymerase on DEAE Sephadex reveals only trace amounts of polymerase III (see section on purified RNA polymerase, page 85). It is hard to predict, however, which assay system (I or II) will include the RNA polymerase III activity.

The data presented in Tables XII and XIII demonstrate that inclusion of RNase in the assay systems results in decreased activity suggesting that the product is RNA. Furthermore, when the product at the end of the incubation period is treated with 0.2 M KOH for 2 hours
### TABLE XII

Characteristics of Incorporation of $^3$H-UMP into RNA in Isolated Kidney Nuclei of Normal Rats

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>$^3$H-UMP incorporated</th>
<th>% of complete system</th>
<th>$^3$H-UMP incorporated</th>
<th>% of complete system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg$^{2+}$ dependent</td>
<td>system</td>
<td>Mn$^{2+}$ (NH$_4$)$_2$SO$_4$ dependent</td>
<td>system</td>
</tr>
<tr>
<td>Complete system</td>
<td>2470 ± 317</td>
<td>100</td>
<td>2950 ± 416</td>
<td>100</td>
</tr>
<tr>
<td>-ATP, -GTP</td>
<td>215 ± 78</td>
<td>8.6</td>
<td>264 ± 47</td>
<td>9</td>
</tr>
<tr>
<td>-ATP, GTP, CTP</td>
<td>185 ± 34</td>
<td>6</td>
<td>185 ± 16</td>
<td>6</td>
</tr>
<tr>
<td>+DNase (500 µg)</td>
<td>460 ± 78</td>
<td>18.5</td>
<td>510 ± 102</td>
<td>17</td>
</tr>
<tr>
<td>+RNase (100 µg)</td>
<td>374 ± 42</td>
<td>15</td>
<td>464 ± 84</td>
<td>15.6</td>
</tr>
<tr>
<td>+Actinomycin D (10 µg)</td>
<td>110 ± 28</td>
<td>4.4</td>
<td>140 ± 35</td>
<td>5</td>
</tr>
<tr>
<td>+α-Amanitin (2 µg)</td>
<td>2430 ± 390</td>
<td>98</td>
<td>80 ± 12</td>
<td>2.7</td>
</tr>
<tr>
<td>+Rifampcin (200 µg)</td>
<td>2475 ± 280</td>
<td>100</td>
<td>3100 ± 435</td>
<td>104</td>
</tr>
</tbody>
</table>

Each value is an average of 4 replicate experiments ± SE. (Assays were performed in triplicates as described in Methods, Section VII.)
TABLE XIII

Characteristics of Incorporation of \(^{3}H\)-UMP
Into RNA in Isolated Liver Nuclei of
Normal Rats

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>(^{3}H)-UMP incorporated Mg(^{2+}) dependent (p moles/mg DNA)</th>
<th>% of complete system</th>
<th>(^{3}H)-UMP incorporated (Mn^{2+}(NH_{4})<em>{2}SO</em>{4}) dependent (p moles/mg DNA)</th>
<th>% of complete system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>2985 ± 228</td>
<td>100%</td>
<td>3460 ± 284</td>
<td>100%</td>
</tr>
<tr>
<td>Minus GTP, minus ATP, minus CTP</td>
<td>210 ± 17</td>
<td>7.0</td>
<td>245 ± 37</td>
<td>7.1</td>
</tr>
<tr>
<td>Plus RNase A (100 µg)</td>
<td>470 ± 19</td>
<td>15.7</td>
<td>550 ± 46</td>
<td>16.2</td>
</tr>
<tr>
<td>Plus DNase (500 µg)</td>
<td>597 ± 23</td>
<td>20</td>
<td>795 ± 76</td>
<td>23</td>
</tr>
<tr>
<td>Plus Actinomycin D (10 µg)</td>
<td>160 ± 21</td>
<td>5.4</td>
<td>190 ± 40</td>
<td>5.5</td>
</tr>
<tr>
<td>Plus a-Amanitin (2 µg)</td>
<td>2953 ± 172</td>
<td>98.9</td>
<td>37 ± 7</td>
<td>1.1</td>
</tr>
<tr>
<td>Plus Rifampicin (200 µg)</td>
<td>2910 ± 240</td>
<td>97.5</td>
<td>3300 ± 280</td>
<td>95.4</td>
</tr>
</tbody>
</table>

Each value is an average of 4 replicate experiments ± SE (assays were performed in triplicate as described in methods section VII.)
at 37°C hydrolysis of the product resulted suggesting that the product was RNA.

Tables XII and XIII also reveal that in both kidney and liver the \( \text{Pm}^{2+}/(\text{NH}_4)_2\text{SO}_4 \) dependent assay system showed higher activity than the \( \text{Mg}^{2+} \) dependent that is, RNA polymerase II shows more activity than I.

(b) Effects of Adrenalectomy on RNA polymerase system:

The kinetics of precursor incorporation by both RNA polymerases in normal and adrenalectomized kidney and liver (and also hormone treated groups) is shown in Figs. 21 and 23. In all cases the incorporation is linear for 10 to 15 minutes. The nonlinearity of the reaction after 15-20 minutes may be due to substrate depletion, product inhibition, or destruction of enzyme or its subunits.

Adrenalectomy caused a significant decrease in both RNA polymerase activities in kidney. Enzyme I showed about 64% decrease in its activity and enzyme II showed about 36% decrease in activity (Table XIV). This decrease in activity leveled off within 5-6 days of adrenalectomy (Fig. 22). Adrenalectomy also caused a marked decrease in activity in liver. RNA polymerase I showed a 63% decrease in the activity following adrenalectomy. Polymerase II, however, did not show any significant change in activity (Fig. 23 and Table XV). The decrease in the activity leveled off within 4-6 days after operations (Fig. 24).

(c) Effects of Adreno cortical Hormones on the RNA polymerase System:

Administration of aldosterone (5 g/100g body wt.) or deoxycorticosterone (100 g/100g body wt.) 3 hours before killing the rats resulted in twofold or greater stimulation of both polymerases in
<table>
<thead>
<tr>
<th>Animals</th>
<th>RNA polymerase Activity</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>polymerase I (mg²⁺ dependent)</td>
<td>% Activity</td>
<td>polymerase II (mn²⁺/(NH₄)₂SO₄ dependent)</td>
<td>% Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenalectomized</td>
<td>1563 ± 130</td>
<td>100</td>
<td>2293 ± 246</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>2557 ± 174</td>
<td>164*</td>
<td>3121 ± 167</td>
<td>136*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenalectomized plus aldosterone treated (5 µg/100g)</td>
<td>1380 ± 374</td>
<td>280*</td>
<td>4583 ± 247</td>
<td>200*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenalectomized plus deoxycorticosterone treated (100 µg/100g)</td>
<td>3654 ± 312</td>
<td>234*</td>
<td>3853 ± 216</td>
<td>168*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenalectomized plus corticosterone treated (2 mg/100g)</td>
<td>1643 ± 104</td>
<td>104</td>
<td>2460 ± 305</td>
<td>107</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenalectomized plus hydrocortisone treated (2 mg/100g)</td>
<td>1735 ± 193</td>
<td>11.0</td>
<td>1875 ± 204</td>
<td>82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value is an average of four replicate experiments ± SE (assays were performed in duplicate or triplicate).

† The RNA polymerase activity of adrenalectomized group is set at 100% and the activity in other groups is expressed as percentage of that value.

Hormones were injected 3 hrs. before killing the rats and assays were performed as described in Methods (Section VII).

* Statistically significant difference (p < 0.01) as compared with adrenalectomized.
FIGURE 21. KINETICS OF THE RNA POLYMERASE REACTION IN ISOLATED NUCLEI OF RAT KIDNEY OF VARIOUS HORMONE TREATED ANIMALS.

The activities of RNA polymerase I and II were determined as described in "Methods" section VII.

Each data point represents the mean of 4 replicate experiments. Assays were performed in duplicate or triplicate. Duplicate assays varied less than ± 2%.

Normal

Adrenalectomized

Adrenalectomized plus aldosterone treated (5 µg/100g body wt.)

Adrenalectomized plus deoxycorticosterone treated (100 µg/100g body wt.)

Adrenalectomized plus corticosterone treated (2 mg/100g body wt.)

The hormones were injected in ethanol-0.9% NaCl mixture three hours before killing the rats.
FIGURE 24  KINETICS OF KIDNEY RNA POLYMERASE REACTION AFTER ADRENALECTOMY (DAYS).

Each data point represents the mean of 4 replicate experiments (assays were performed in duplicate or triplicate as described in "Methods" section VII). Duplicate assays were varied less than ± 22.

RNA polymerase I

RNA polymerase II
kidney (Fig. 21 and Table XIV). This stimulation continued throughout the incubation period. Deoxycorticosterone, although injected in a dose 20 x than the aldosterone was less effective in stimulating either polymerase reactions than aldosterone. Administration of hydrocortisone or corticosterone was without effect in kidney suggesting that the effects are mineralocorticoid specific. This type of observation on corticosterone was also made by Liew et al. (111).

Table XV and Fig. 23 reveal that administration of hydrocortisone or corticosterone stimulated RNA polymerase I in liver but polymerase II did not show any significant change in activity. Injection of aldosterone or deoxycorticosterone were without effect in liver again suggesting the tissue specificity of glucocorticoids or mineralocorticoids. The observations on effects of hydrocortisone on RNA polymerase I are consistent with the results reported by other investigators (100, 221). The data for corticosterone did not appear to be available in the literature.

(d) Time Course of RNA Polymerase Stimulation by aldosterone in Kidney and by Corticosterone in Liver:

Figs. 25 and 26 illustrate the time course of the RNA polymerase reaction after appropriate steroid hormone administration. It is apparent from the Fig. 25 that aldosterone had its maximum effect after 2.5 hours of injections on both polymerases in kidney and there is virtually no stimulation of either enzyme during the first hour. This is also consistent with the observations of Liew et al. (111) who reported no stimulation of RNA polymerase during the first two hours of hormone administration. This latent period of at least
TABLE XV

The Effects of Adrenalectomy and Adrenocortical Hormones on RNA Polymerase Activity in Rat Liver

<table>
<thead>
<tr>
<th>Animals</th>
<th>RNA polymerase activity (pmoles of $^3$H-UMP incorporated/mg/DNA/15 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polymerase I $^{2+}$ dependent $%$ RNA</td>
</tr>
<tr>
<td>Adrenalectomized</td>
<td>1650 ± 107</td>
</tr>
<tr>
<td>Normal</td>
<td>2700 ± 230</td>
</tr>
<tr>
<td>Adrenalectomized + corticosterone</td>
<td>3400 ± 205</td>
</tr>
<tr>
<td>Adrenalectomized + hydrocortisone</td>
<td>3500 ± 264</td>
</tr>
<tr>
<td>Adrenalectomized + aldosterone</td>
<td>1670 ± 140</td>
</tr>
<tr>
<td>Adrenalectomized + deoxycorticosterone</td>
<td>1830 ± 214</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE of 4 replicate experiments (each assay performed in triplicate as described in Methods (section VII). The hormones were injected 3 hours before killing the rats.

*Statistically significant differences (P<0.01) compared with adrenalectomized group.
†The polymerase activity of adrenalectomized animals is set at 100% and the activity in other groups is expressed as a percentage of that value.
FIGURE 23. KINETICS OF RNA POLYMERASE REACTION IN ISOLATED NUCLEI OF RAT LIVER OF VARIOUS HORMONE TREATED ANIMALS.

The activities of polymerase I(A) and II(B) were determined as described in "Methods" section VII.

Each data point represents the mean of 4 replicate experiments. Assays were performed in duplicate or triplicate. Duplicate assays varied less than ± 2%.

Normal

Adrenalectomized

Adrenalectomized plus hydrocortisone treated (2 mg/100g body wt.)

Adrenalectomized plus corticosterone treated (2 mg/100g body wt.)

The hormones were injected in ethanol-NaCl mixture three hours before killing the animals.
FIGURE 24. KINETICS OF LIVER DNA POLYMERASE REACTION AFTER ADRENALECTOMY (DAYS).

Each data point represents the mean of four replicate experiments (assays were performed in duplicate as described in "Methods" section VII). Duplicate assays varied less than ± 2%.
Figure 25. Effect of Aldosterone on time course of $^3$H-UMP incorporation into RNA of isolated kidney nuclei.

Aldosterone (5 µg/100g body wt.) was injected and the rats were sacrificed at 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 14 hours after hormone administration. Each data point represents the mean of 2 replicate experiments. Assays were performed in duplicate as described in "Methods" section VII.

RNA polymerase I  RNA polymerase II
FIGURE 26. EFFECT OF CORTICOSTERONE (2 mg/100g) ON TIME COURSE OF $^3$H-UMP INCORPORATION INTO RNA OF ISOLATED RAT LIVER NUCLEI.

Corticosterone (2 mg/100g body wt.) was injected and the animals were sacrificed at 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 14 hours after hormone administration. Each data point represents the mean of two replicate experiments. Assays were performed in duplicate as described in "Methods" section VII.

RNA polymerase I

RNA polymerase II
one hour was also observed by Pamgnari et al. (16) for RNA synthesis and sodium retention. The significance of this latent period is unknown. It is possible, however, that time course of formation of steroid-protein complex in kidney may be a slower process than other tissues and thus, a latent period of one hour may be required for gene activation.

The situation for corticosterone in liver is different. It is seen from the Fig. 25, that corticosterone had an effect on RNA polymerase I as early as 30 minutes after administration.

RNA polymerase II was not affected significantly at any time during the time course.

In both tissues the effect of the appropriate hormone persisted for at least 10 hours.

(e) Effect of addition of hormone on RNA polymerase in assay system (in vitro):

When aldosterone or deoxycorticosterone were added directly to the assay mixture, no stimulation of either enzyme was observed in kidney nuclei (Table XVI). Similar observation was made with corticosterone in liver nuclei (Table XVII). In view of these observations, it is suggested that an appropriate hormone forms a receptor-complex in the cytoplasm which in turn moves to the nuclei and stimulates RNA polymerase. Such receptor molecule may have been lost during isolation of nuclei or it may be present in an insignificant amount.

(6) Purification of RNA polymerase from kidney and effects of Aldosterone:

The results of steroid hormone stimulation of RNA polymerase activity in kidney or liver (Tables XIV and XV) can be interpreted in terms of either template or enzyme alterations. In order to discover the effects
### TABLE XVI

**Effect of Aldosterone* on RNA Polymerase Activity in Kidney (in vitro)**

<table>
<thead>
<tr>
<th>Animals</th>
<th>RNA polymerase activity (p moles of $^3$H-UMP incorporated/mg DNA/15 min)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polymerase I Mg$^{2+}$ dependent</td>
<td>% Activity$^+$</td>
<td>Polymerase II Mn$^{2+}$/($\text{NH}_4$)$_2$SO$_4$ dependent</td>
<td>% Activity$^+$</td>
<td></td>
</tr>
<tr>
<td>Adrenalectomized</td>
<td>$1640 \pm 135$</td>
<td>100</td>
<td>$2360 \pm 204$</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>$2765 \pm 186$</td>
<td>167</td>
<td>$3190 \pm 276$</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>Adrenalectomized + aldosterone (2 μg/assay)</td>
<td>$1590 \pm 146$</td>
<td>97</td>
<td>$2310 \pm 193$</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Adrenalectomized + aldosterone (5 μg/assay)</td>
<td>$1680 \pm 176$</td>
<td>102</td>
<td>$2420 \pm 214$</td>
<td>103</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as a mean ± SE of 5 replicate experiments (assay were performed in duplicate).

* Aldosterone was directly added to the assay system.

$^+$ The polymerase activity of adrenalectomized group is set at 100% and the activity in other fractions is expressed as percentage of that value.
TABLE XVII

Effect of Corticosterone* on RNA polymerase Activity
in Liver (in Vitro)

<table>
<thead>
<tr>
<th>Animals</th>
<th>RNA polymerase activity (p moles of $^3$H-UMP incorporated/mg DNA/15min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polymerase I</td>
</tr>
<tr>
<td></td>
<td>$^{2+}$ dependent</td>
</tr>
<tr>
<td>Adrenalectomized</td>
<td>$1710 \pm 135$</td>
</tr>
<tr>
<td>Normal</td>
<td>$2785 \pm 194$</td>
</tr>
<tr>
<td>Adrenalectomized + corticosterone (100µg/Assay)</td>
<td>$1690 \pm 78$</td>
</tr>
<tr>
<td>Adrenalectomized + corticosterone (1000µg/Assay)</td>
<td>$1730 \pm 107$</td>
</tr>
</tbody>
</table>

Values are given as Mean ± SE of 5 replicate experiments (Assays were performed in duplicate).

* The hormone was added directly to the assay system.

† The polymerase activity of adrenalectomized animals is set at 100% and the activity in other groups is expressed as a percentage of that value.
of aldosterone on enzyme or DNA template, the RNA polymerases were pur-
ified from kidney nuclei of normal adrenalectomized or adrenalectomized
plus aldosterone treated animals.

Fig. 27 illustrates the pattern of purified RNA polymerase from
rat kidney. It is apparent that kidney contains the multiple forms of
DNA dependent RNA polymerase, namely IA, IB, II and an extremely small
amount of polymerase III. No claim has been made for their specific lo-
calization in the nuclei of rat kidney. Roeder and Rutter (94), however,
have reported their localization in nucleoli and nucleoplasm respectively
(IA, IB in the nucleolus and II in the nuclear sap) in rat liver.

Fig. 27 reveals that there is no direct effect of adrenal hormone de-
pletion on either of the polymerases in kidney. It is seen from Fig. 27
and Table XVIII that purified RNA polymerases (IA, IB, and II) from adren-
alectomized, normal and adrenalectomized plus aldosterone injected animals
showed no apparent differences in activity when assays were performed
using calf-thymus DNA. Table XVIII also reveals that approximately the
same percentage activity of RNA polymerases from all the three groups of
animals (calf-thymus DNA template) were recovered. These observations
suggest that aldosterone probably acts on template in kidney rather than
on RNA polymerase.

To test this hypothesis, the polymerases were purified from
another group of animals (adrenalectomized and adrenalectomized plus
aldosterone treated) and the activity was measured using DNA from various
sources as shown in Table XIX. It is observed from the Table XIX that
DNA from aldosterone treated rats acted as a better template. All three
purified enzymes (IA, IB and II) showed increases in activity when template
was used from the aldosterone treated animals. These observations suggest
Nuclei were isolated from normal, adrenalectomized and adrenalectomized plus aldosterone treated animals. The solubilized enzyme preparation after dialysis (see "Methods" section VIII) was applied to 0.6 x 15 cm column packed with DEAE-Sephadex A-25 and equilibrated with 0.05 M (NH₄)₂SO₄ in TCMED. The enzyme activities were eluted with linear gradients of (NH₄)₂SO₄ (in TCMED). One ml fractions were collected and 50 µl aliquots assayed. The activity is expressed as p moles of ³²P-UMP incorporated into RNA/Mg/DNA/15 min.

↑ Indicates assay system for polymerase II was used.

NORMAL □——□
ADRENALECTOMIZED ●——●
ADRENALECTOMIZED PLUS ALDOSTERONE TREATED ▲——▲
TABLE XVIII

Solubilization and Purification of RNA Polymerases
From Normal, Adrenalectomized and Adrenalectomized
Plus Hormone Treated Animals

<table>
<thead>
<tr>
<th>RNA polymerase activity</th>
<th>p moles (3H-UMP) incorporated/mg/DNA/15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals</td>
<td>Solubilized</td>
</tr>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>ADX</td>
<td>1870</td>
</tr>
<tr>
<td>Normal</td>
<td>1900</td>
</tr>
<tr>
<td>ADX plus Aldosterone treated†</td>
<td>2060</td>
</tr>
</tbody>
</table>

Kidneys were pooled from three animals and RNA polymerases were solubilized, purified and assayed using 50 µg native calf-thymus DNA as described in Methods (Section VIII). Each value is an average of two experiments.

* The activity of adrenalectomized animals is set at 100% and the activity in other groups is expressed as a percentage of that value.

ADX - adrenalectomized
† Aldosterone (5 µg/100g) was injected 3 hrs. before killing the rats.
**TABLE XIX**

**Effect of DNA (Source) on Purified EMA Polymerase Activity of Rat Kidney**

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Source of DNA</th>
<th>1&lt;sub&gt;A&lt;/sub&gt;</th>
<th>% Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1&lt;sub&gt;B&lt;/sub&gt;</th>
<th>% Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>II</th>
<th>% Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenalectomized plus aldosterone treated</td>
<td>Adrenalectomized rac kidney</td>
<td>626 ± 86</td>
<td>100.0</td>
<td>720 ± 95</td>
<td>100.0</td>
<td>780 ± 90</td>
<td>100.0</td>
</tr>
<tr>
<td>Adrenalectomized plus aldosterone treated</td>
<td>Calf-thymus</td>
<td>618 ± 74</td>
<td>98.8</td>
<td>685 ± 104</td>
<td>95.9</td>
<td>810 ± 110</td>
<td>105.6</td>
</tr>
<tr>
<td>Adrenalectomized plus aldosterone treated</td>
<td>Adrenalectomized plus aldosterone treated</td>
<td>1,280 ± 115</td>
<td>204.5</td>
<td>1,360 ± 164</td>
<td>190.5</td>
<td>1,342 ± 182</td>
<td>197.4</td>
</tr>
<tr>
<td>Adrenalectomized plus aldosterone treated</td>
<td>Adrenalectomized plus corticosterone treated</td>
<td>645 ± 62</td>
<td>103.0</td>
<td>780 ± 68</td>
<td>109.2</td>
<td>845 ± 105</td>
<td>108.2</td>
</tr>
<tr>
<td>Normal</td>
<td>Adrenalectomized</td>
<td>850 ± 89</td>
<td>92.5</td>
<td>530 ± 42</td>
<td>74.2</td>
<td>715 ± 88</td>
<td>91.5</td>
</tr>
<tr>
<td>Adrenalectomized</td>
<td>Adrenalectomized</td>
<td>610 ± 57</td>
<td>97.6</td>
<td>715 ± 80</td>
<td>100.1</td>
<td>865 ± 50</td>
<td>110.7</td>
</tr>
<tr>
<td>Adrenalectomized plus aldosterone treated plus 2 µg a Amanitin</td>
<td>Adrenalectomized plus aldosterone treated</td>
<td>1,310 ± 142</td>
<td>209.6</td>
<td>1,375 ± 105</td>
<td>192.0</td>
<td>115 ± 42</td>
<td>14.7</td>
</tr>
</tbody>
</table>

Each value is an average of four replicate ± SE (with respect to DNA source) experiments. Assays were performed in triplicate using 50 µg of native DNA from designated sources as described in Methods in (Section VIII).

<sup>a</sup>The activity of first group (RNA polymerase from ADX + aldosterone treated and DNA from adrenalectomized animals) is set at 100% and the activity in other groups is expressed as a percentage of that value.
that there is a definite specific effect of aldosterone on DNA or on some of its components (regulatory factors, histones or non-histone acidic proteins). This is further underlined by the observation that kidney DNA from corticosterone treated animals acted only as efficiently as calf-thymus DNA (Table XIX).

To explain the increased template activity due to aldosterone treatment four main possibilities can be ascribed:

(a) Hormone may be causing some chemical changes in the DNA.

(b) Hormone may be causing some changes in physical properties of the DNA molecule.

(c) There may be direct binding between hormone (or hormone-receptor complex) and DNA. And probably this DNA-bound-hormone complex survives all the treatments that it received during the isolation procedure and acts as a better template.

(d) The hormone (or hormone-receptor complex) may be modifying the acidic proteins or histones or some other regulatory factors that regulate template activation.

Attempts were made in this study to test some of these possibilities.

The possibility (b) of a simple physical change ( nicking) was tested by preparing the DNA from combined kidneys of adrenalectomized and adrenalectomized plus aldosterone treated rats. When RNA polymerase was assayed using this DNA, an intermediate activity was observed (Table XX). Therefore, the possibility of greater " nicking" occurring in the DNA molecule of aldosterone treated animals than adrenalectomized animals during work up procedure can be ruled out. Thus, the simple physical change ( nicking) is certainly not involved in template activation. Melting
### TABLE XX

**Effect of DNA (on RNA polymerase) prepared from adrenalectomized and adrenalectomized plus aldosterone treated animals.**

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Source of DNA</th>
<th>IA</th>
<th>% of ADX</th>
<th>IB</th>
<th>% of ADX</th>
<th>II</th>
<th>% of ADX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenalectomized</td>
<td>Adrenalectomized and adrenalectomized plus aldosterone treated</td>
<td>878</td>
<td>141</td>
<td>1145</td>
<td>160</td>
<td>1380</td>
<td>179.4</td>
</tr>
</tbody>
</table>

Each value is an average of two experiments. Kidneys from adrenalectomized and adrenalectomized plus aldosterone treated animals were combined and homogenized together and DNA was isolated as described in Methods (Section V). Assays were performed using 50 μg of native DNA. The values for adrenalectomized (ADX) control are shown in Table XIX.
profiles and other physical properties of the hormone treated DNA were not studied.

To gain some more information about possibilities (c) and (d), experiments were performed to check the location or distribution (in DNA, histones or non-histone acidic proteins) of $^{14}$C-aldosterone in kidney, spleen, and liver.

Table XXI shows the percentage distribution of $^{14}$C-aldosterone in various fractions of kidney, spleen and liver. It is observed that kidney nuclear DNA contained approximately 2% of the injected radioactivity. When this is compared with liver and spleen it becomes apparent that aldosterone binds specifically to the kidney nuclear DNA.

The protein content of purified DNA was 140 µg/mg DNA. Table XXII shows the quantitative amino acid composition of the protein contaminating DNA. It is not clear which types of nuclear proteins (histones or non-histones) are contaminating the DNA, since acid hydrolysis converts the glutamine and asparagine into glutamic and aspartic acid and therefore a clear distinction can not be made. To overcome some of these problems more rigorous experiments involving fractionation of chromatin into DNA, histones, and non-histone acidic proteins were performed. The final preparation of DNA was treated with pronase to remove any contaminating proteins. Proteins in this preparation were not detectable by the Lowry et al. method (210).

The results of $^{14}$C-aldosterone distribution in adrenalectomized rat kidney chromatin fractions are illustrated in Table XXIII. It is evident that purified DNA and non-histone acidic proteins contained most of the radioactive aldosterone 45 minutes after injections. Simultaneous administration of spironolactone (20 µg/100g body weight) or deoxycorticosterone
TABLE XXI

Distribution of $^{14}$C-aldosterone in Subcellular Fractions of Adrenalectomized Rat Kidney, Liver and Spleen.

<table>
<thead>
<tr>
<th></th>
<th>Homogenate</th>
<th>Cytosol</th>
<th>Mitochondria</th>
<th>Nuclei</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney (5)</td>
<td>14</td>
<td>10</td>
<td>0.40</td>
<td>2.4</td>
<td>1.66</td>
</tr>
<tr>
<td>Liver (3)</td>
<td>19</td>
<td>17</td>
<td>0.10</td>
<td>1.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Spleen (2)</td>
<td>9</td>
<td>7</td>
<td>0.05</td>
<td>0.8</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Number of experiments are shown in parenthesis. Each value is an average of 5, 3, 2 experiments respectively. 5 µg/100g of $^{14}$C-aldosterone (1 µCi) injected intraperitoneally 45 minutes before killing the rats. The tissues were fractionated and DNA was isolated as described in Methods (section V). An aliquot of a fraction in question was solubilized in protocol (New England Nuclear Solubilizer) and radioactivity was determined.

* 1% is equivalent to 19,800 dpm.
TABLE XXII

Amino Acids (quantitative) Analysis of Proteins Contaminating Kidney DNA

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>µg/µg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>0.17</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.09</td>
</tr>
<tr>
<td>Serine</td>
<td>0.16</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.22</td>
</tr>
<tr>
<td>Proline</td>
<td>0.16</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.27</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.24</td>
</tr>
<tr>
<td>Valine</td>
<td>0.05</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.06</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.06</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.16</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.04</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.16</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.12</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.03</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.00</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.00</td>
</tr>
</tbody>
</table>

2 µg DNA was hydrolyzed in 2 ml of 6N HCl for 24 hours at 110°C under vacuum. The acid was removed under vacuum deaspiration with NaOH and the remaining sediment was reconstituted with sodium citrate buffer (pH 2.2). The sample was run on a Beckman Model 121 amino acid analyzer utilizing spherical sulfonated polystyrene DVB cross linked resin. (Method of J. V. Benson and J. A. Paterson, (1965) Analytical Biochemistry, 13, 265-289).
### TABLE XXIII

**Distribution of $^{14}$C-Aldosterone in Adrenalectomized Kidney Chromatin Fractions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$^{14}$C-Aldosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(dpm/kidney pair)</td>
</tr>
<tr>
<td>Whole nuclei</td>
<td>$31,200 \pm 1,105$</td>
</tr>
<tr>
<td>Chromatin</td>
<td>$27,360 \pm 956$</td>
</tr>
<tr>
<td>Histones</td>
<td>$1,620 \pm 312$</td>
</tr>
<tr>
<td>Non-histone acidic proteins</td>
<td>$16,200 \pm 578$</td>
</tr>
<tr>
<td>Purified DNA (pronase treated)</td>
<td>$6,900 \pm 152$</td>
</tr>
</tbody>
</table>

1 µCi of $^{14}$C-aldosterone (5 µg/100g) was injected into adrenalectomized rats 45 minutes before sacrifice. Both kidneys were fractionated as described in Methods Section VI and radioactivity was determined. Each value is an average of 4 experiments ± SE.
(100 µg/100g body wt.) resulted in a decreased recovery of radioactivity from the adrenalectomized rat kidney chromatin fractions. Table XXIV shows the % inhibition of aldosterone binding to DNA and acidic proteins by spirolactone and deoxycorticosterone.

Two main speculations can be presented to explain these results.

(a) Kidney nuclear DNA acts as an acceptor for aldosterone or aldosterone-receptor complex and specific non-histone acidic protein(s) determines the specificity in target tissues.

(b) Both non-histone acidic protein(s) and DNA play an active role in forming the template-hormone complex.

These observations make it impossible to distinguish between these two speculations.
TABLE XXIV

Inhibition of Radioactive (\(^{14}C\)) Aldosterone

Binding to Various Chromatin Fractions of
Adrenalectomized Rat Kidney

<table>
<thead>
<tr>
<th>Fraction</th>
<th>No inhibitor dpm/kidney pair</th>
<th>200 µg/100g spirolactone dpm/kidney pair</th>
<th>% Inhibition</th>
<th>200 µg/100g deoxycorticosterone dpm/kidney pair</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole chromatin</td>
<td>34,800 ± 887</td>
<td>13,976 ± 754</td>
<td>60</td>
<td>20,880 ± 1,012</td>
<td>40</td>
</tr>
<tr>
<td>Histones</td>
<td>2,070 ± 719</td>
<td>1,860 ± 174</td>
<td></td>
<td>2,142 ± 514</td>
<td></td>
</tr>
<tr>
<td>Non-histone acidic proteins</td>
<td>20,850 ± 685</td>
<td>9,798 ± 346</td>
<td>53</td>
<td>10,800 ± 754</td>
<td>50</td>
</tr>
<tr>
<td>DNA (purified &amp; pronase treated)</td>
<td>7,200 ± 635</td>
<td>2,376 ± 245</td>
<td>65</td>
<td>3,636 ± 305</td>
<td>47</td>
</tr>
<tr>
<td>RNA</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

1 µCI of \(^{14}C\)-aldosterone (5 µg/100g) was injected into adrenalectomized rats 45 minutes before sacrifice. Spirolactone or deoxycorticosterone was injected immediately before the labeled aldosterone injection. Each value is an average of 4 experiments ± SE.
DISCUSSION

Significance of random removal model, apparent turnover constant ($k'$) and initial specific activity ($A_0$): In turnover experiments performed in this study, a random removal model was assumed as described in Experimental Design (Section C). The turnover data fit this model well in that a plot of the logarithm of the specific activity against time was linear in all experiments.

The turnover constant ($k'$) determined from the slope of the regression curve (Figs. 17-20) reflects an average degradation rate of mRNA for the entire group of rats and the 95% confidence limit suggests it is a reliable average. In most of the experiments (Figs. 17-18 and Tables VII and VIII) ... the 95% confidence limit deviates less than 5-10% of the calculated $k'$. It is therefore possible to detect significant differences between RNA turnover rates which vary by as little as this amount.

The use of "apparent" instead of an "absolute" turnover constant ($k'$ vs. $k$) takes into consideration the possible reutilization of labeled precursor (195). That is, the shape of the decay or turnover curve will not be changed by the reutilization of the radioactive precursor component after it is released from the macromolecule by degradation. Only the slope of the curve will change and thus the rate constant, $k$, will be modified. Another factor that may contribute to an error in the estimation of $k$ is the persistence of label in the precursor pool. If the turnover rate of precursor pool is not short as compared to the macromolecule studied, both the initial precursor pool and contributions to this pool by degradation will continually feed label back into the macromolecule. This, however, was discussed earlier (page 60) and it has been suggested
that the problem of recycling is negligible, and it would therefore appear that the apparent turnover constant \( k' \) is a good estimate of its "absolute" counterpart, \( k \).

The initial specific activity, \( A_0 \) (Tables VII-VIII, X and XI) or the zero time intercept obtained in all experiments by calculations from the regression equations, may reflect the initial net synthesis of RNA as well as indicating the portion of total RNA synthesis concerned with a particular sub-cellular fraction. This excludes, of course, any rapidly turning-over RNA such as certain mRNAs that have half-lives shorter than the earliest times of sampling, or too short to be detected by these experiments.

**RNA turnover in various subcellular fractions of normal rat kidney and liver:** The turnover rates of total ribosomal, polysomal, and sRNA of normal rat kidney and liver observed in this study are about the same as those reported in the literature (17, 18, 223, 224). Ribosomes of both tissues, although differing slightly (differences in \( T_{1/2} \) = 1 day) in kidney, had about the same turnover rate as polysomes. Both fractions also showed identical RNA patterns, that is, 28S, 18S and 5S on linear sucrose density gradients (Figs. 1 and 14). These results are consistent with those of Loeb et al. (225), Manganiello and Phillips (226) and Moule and Delhumeau de Mongay (227) and suggest that there are not intrinsic differences in the patterns of RNA synthesis and degradation between the ribosomes found in these two topographical states in the cell.

The turnover rates of 28S and 18S subunits have been reported to be identical (223) to those of total ribosomal RNA and are in agreement with the turnover rates obtained in this study.
The turnover rates of RNA from rough ER, smooth ER and mitochondria are new values obtained in this study for normal rat kidney and liver and there are no literature values available for these fractions. However, protein turnover rates in rough ER and smooth ER are about the same (228-229) as those of RNA reported in this study. The patterns of RNA on sucrose density gradients from rough ER of both tissues is similar to that of ribosomal RNA. It is therefore suggested that rough ER does not contain any other type of RNA than ribosomal. The turnover half-life of RNA from rough ER, as expected, was similar to that of ribosomal RNA. However, RNA from smooth ER of both tissues, while showing half-lives similar to those of ribosomal and rough ER RNA showed sedimentation patterns on sucrose density gradients with extra peaks of 11S and 9S in liver and kidney, (Figs. 13, 16 and 12) respectively. Such a type of RNA in smooth ER has also been reported by other investigators (217, 230). These authors have also suggested that this membrane RNA is probably related to stable cytoplasmic messenger RNA. The precise functional role of this RNA is unknown.

Total mitochondrial RNA of normal kidney and liver showed half-lives ranging between 5.9 and 6.5 days. No attempt was made to fractionate the various RNA species (tRNA, mRNA, and rRNA) known to be present in the mitochondria (231-233). The difference between the half-lives of polysomal and mitochondrial RNA in both tissues are not highly significant. Possible cross contamination, however, is ruled out since mitochondria will not penetrate the 1.5 M sucrose layer (Fig. 2) whereas both polysomes and rough endoplasmic reticulum will. Furthermore electron micrographs of brain mitochondria prepared by sedimentation to a bottom layer of 1.2 M sucrose (198) showed very little contamination by rough
ER and none by polysomes. In the present study, the provision of 1.5 and 2.0 M sucrose layers should have allowed these contaminants to pass on through leaving pure mitochondria at the interphase of 1.2 and 1.5 M sucrose. The possibility of other membraneous contaminants was not excluded, but the RNA contribution of these is minimal.

The single exponential (Fig.17-18) pattern seen in both tissues has been reported for a variety of other mitochondrial fractions (proteins, DNA and phospholipids) although the half-lives are not similar to that of RNA. That is, liver mitochondrial proteins, lipids and DNA have been found to turn over with half-lives ranging from 8.5 to 11 days by most investigators (206, 234-236). Swick et al. (237), have argued that most of the reported results on the turnover of mitochondrial fractions are clouded by the problem of reutilization especially in the experiments where the essential amino acid leucine has been used as a source of radioactive precursor. Additionally, the values found in this study for RNA compare favourably with some protein components previously reported by Beattie et al. (235a) but not with others (234-236), or DNA turnover values (206).

From the above, it would seem that while certain "core" components of the mitochondrion turn over as a unit, other components do not. Thus, as more mitochondrial components are studied, more diversity is detected which weakens the unit turnover concept (Fletcher & Sanadi (236)).

In both kidney and liver, nuclear RNA showed longer half-lives (Figs.17-43) than any other type of RNA examined in this study. In fact, this particular class of nuclear RNA is more stable than most cytoplasmic RNAs. It is quite likely that the turnover experiments performed in this study would not have detected the rapidly turning-over heterogenous nuclear
RNA but would rather be a measure of the stable monodisperse nuclear RNA (snRNAs). This monodisperse nuclear RNA consists of seven discrete species of RNA ranging in size from 100 to 180 nucleotides. The nucleotides are extensively methylated. (238-240). The functions of snRNAs are completely unknown at the present time. Rein and Penman (241) have suggested that these RNAs probably perform some "general" function in the cell nucleus, perhaps similar to the structural role of rRNA in the ribosome (242).

Failure to observe a rapidly turning over component in nuclear RNA, however, is inconsistent with the result of Yoshikawa et al. (243) who suggested that two species of RNA, q₁ and q₂, are rapidly synthesized in the nucleus. q₁ had the size of 40S and base composition similar to rRNA whereas the q₂ was 50S and its base composition resembled DNA. These authors (243) hypothesized that q₁ was the precursor of rRNA and q₂ was assumed to be mRNA. It is possible that these species of RNA may have been lost during the centrifugation period. The differences in turnover rate of nuclear RNA on one hand and snRNA, ribosomal RNA and polysomal RNA on the other hand suggest that nuclear RNA has some intranuclear function.

Effects of adrenalectomy and of adrenocortical hormones on RNA turnover in kidney and liver: The data presented in this thesis (Tables VII-VIII and Figs. 17-18) reveal that RNA of nuclei, mitochondria and total ribosomes in kidney and nuclear, mitochondrial, rough ER and polysomal RNA in liver respectively showed significantly (P < 0.05) decreased rates following adrenalectomy. Rough ER, smooth ER, polysomes and snRNA in kidney and smooth ER, ribosomes and snRNA in liver respectively also showed the same apparent trend, although in these cases the changes were not statistically
significant. This slower rate of RNA turnover reflects a lowered rate of RNA synthesis since both the theoretical maximum or initial specific activity (obtained by extrapolation) and the activity of RNA polymerases are also decreased after adrenalectomy. These changes are not attributable to cellular turnover since it has been shown that little or no cellular turnover occurs in kidney and liver (244).

The administration of appropriate steroid hormones restored these changes in both kidney and liver. The daily injections of corticosterone (rat's main glucocorticoid) or hydrocortisone reversed both the turnover half-life (t½) and initial specific activity in various subcellular fractions of rat liver. Daily injections of aldosterone were without effects in liver. Several other investigators (3-5, 10-11) have also established that glucocorticoids augment the incorporation of radioactivity from labeled precursors into hepatic RNA and it is quite possible that increased radioactivity in RNA truly represents an increased RNA synthesis. The increased turnover rates of RNA observed in this study are consistent with these observations. That is, if the synthesis and degradation of RNA in various subcellular fractions are in steady-state then the increased degradation rate observed in glucocorticoid treated animals reflect increased RNA synthesis.

Alternatively Ottolenghi and Barnabel (245) have reported that cortisone treated rats showed a decreased rate of microsomal RNA turnover in rat liver. The discrepancy between their results and the results obtained in this study for hydrocortisone and corticosterone may be due to (a) differences in the dose of hormone used, (b) sex of the rats, (c) experimental methods employed and (d) the use of cortisone in place of hydrocortisone or corticosterone.

It is suggested that steady-state conditions existed in the adrenalectomized and normal animals. Under non-steady-state conditions
one might expect faster turnover rates in adrenalectomized rats. (This is because total RNA has been shown to be decreased following adrenalectomy (15). The results obtained in the present study are contrary to this, showing a slower turnover in adrenalectomized animals. Any error would therefore result in an underestimation of the differences between turnover rates in normal and adrenalectomized animals.

The mineralocorticoids (aldosterone and deoxycorticosterone) reversed the effects of adrenalectomy in various subcellular fractions of rat kidney. Daily injections of corticosterone were without effect.

It is suggested from the above observations that the effects on RNA turnover in liver are glucocorticoid specific and in the kidney mineralocorticoid specific. This is also in agreement with the results of RNA polymerase obtained in this study and by other investigators (111). The data for aldosterone are consistent with other reports which showed increased RNA synthesis in rat kidney after hormone administration in adrenalectomized animals (13, 15). It is also of interest to note that aldosterone has been shown to increase ribonuclease activity in rat kidney (148).

Ichii and Ikeda (246) found that adrenalectomy decreased turnover of proteins in mouse liver and also spleen and the effects were reversed by daily injection of hydrocortisone. These effects on protein turnover are probably a reflection of decreased RNA turnover.

It is conceivable that steroid hormones may directly cause changes in mitochondrial RNA synthesis and turnover, since some of the glucocorticoids have been found in mitochondria (247). This could also explain the changes in mitochondrial protein turnover due to adrenalectomy and glucocorticoid administration (246). The present turnover results are consistent with these observations.
Sekeris and Lang (248) have shown the stimulation of messenger as well as ribosomal and transfer RNA by administration of hydrocortisone in rat liver. Since messenger RNA has a half-life estimated less than one day, which is considerably shorter than the half-lives of other RNAs observed in this study, it is unlikely that it contributes to the observed turnover patterns.

Effect of adrenalectomy and of adrenocortical hormones on aggregate RNA polymerase in liver and kidney: The mammalian RNA polymerase is still generally assayed as an "aggregate" enzyme (preparation consisting of the polymerase tightly bound to deoxyribonucleoprotein complex). The rat kidney and liver nuclei used in this study constitute a physiologically relevant and excellent model system for studying the mechanism of steroid hormone action as well as the regulation of polymerase activity in mammalian cell nuclei. Two types of RNA polymerase activity have been reported in the literature (132), one requiring the presence of Mg$^{2+}$ and the other Mn$^{2+}/\text{NH}_4\text{SO}_4$. Both types of reactions have been studied in this report. The Mg$^{2+}$ stimulated RNA polymerase (polymerase I) has been primarily shown to stimulate ribosomal type (AU$_{GC} < 0.8$) RNA (249). The Mn$^{2+}/\text{NH}_4\text{SO}_4$ stimulated RNA polymerase (polymerase II) has been primarily shown to synthesize DNA like (AU$_{GC} \rightarrow 1$) RNA (249). The results of this study have demonstrated that adrenocortical hormones do affect the activity of RNA polymerases in kidney and liver. Hormone-stimulated alterations of RNA polymerase activities have also been demonstrated in other eukaryotic organisms and rat tissues (summarized in Table 3 in the Review of Literature page 9).

The activity of polymerase I decreased significantly in liver following adrenalectomy whereas polymerase II was not affected significantly. Injection of corticosterone or hydrocortisone stimulated the polymerase I
to a maximum level twice that of the control activity of this enzyme, 2-4 hours after hormone treatment. The activity was maintained well above control levels at least up to 10 hours after hormone administration (Fig. 26). The differences between RNA polymerase activity I and II suggest that the regulation of nuclear plasmic enzyme (polymerase II) may be independent of nucleolar polymerase (enzyme I). The regulatory system for polymerase I may be more sensitive to adrenalectomy and glucocorticoid treatment in rat liver. The results obtained for the hydrocortisone effect in liver are in agreement with literature reports showing significantly more stimulation of polymerase I than II by glucocorticoids in rat liver (100, 101, 106, 221). Another report (250) suggests the sequential stimulation of polymerase I and polymerase II in rat liver by hydrocortisone administration.

Both mineralocorticoids (aldosterone and deoxycorticosterone) were without effect on the RNA polymerase system in rat liver. Aldosterone did cause some stimulation (but not significant) of RNA polymerase II in liver. In the light of currently held views about steroid hormone action, these observation may be explained by the presence of specific "acceptor" sites on liver chromatin for the homologous-hormone complex which enhances the activity for RNA synthesis. It has been shown (153, 163) that liver contains most of the glucocorticoid-binding (receptor) proteins and no receptor for aldosterone or deoxycorticosterone, and thus the first-step, formation of steroid-hormone complex in liver may be limiting for mineralocorticoids. On the other hand Sejdel and Jacob (100) have suggested that hydrocortisone is an allosteric regulator of polymerase I in liver. Their evidence for this, however, is not convincing, and their data could be equally well explained by the above hypothesis.
The effect of mineralocorticoids on RNA polymerase in kidney: The dose for mineralocorticoids was in the physiological range (251-253) that is, 5 μg/100g body wt. for aldosterone and 100 μg/100g body wt. for deoxycorticosterone.

Unlike liver where only polymerase I was affected, both polymerases (I and II) showed significant (p < 0.01) decreases in activity following adrenalectomy. Administration of aldosterone or deoxycorticosterone reversed these effects of adrenalectomy in kidney. The increase in the activity of both enzymes suggests that the mechanism of ribosomal as well as messenger RNA synthesis is stimulated by aldosterone in kidney. Simultaneous stimulation after a lag period of at least 1.5 hour (Fig. 26) was observed for both polymerases. Administration of corticosterone was without effect on either of the polymerases and it is interesting to note that hydrocortisone has a somewhat inhibitory effect on polymerase II in kidney as shown in Table XIV. Hydrocortisone also inhibits RNA polymerase I and II in thymus (108). An inhibitory effect has also been reported on precursor incorporation into RNA of spleen, thymus (254) and kidney (255). These reports and the results obtained in this study for kidney present an example of a steroid hormone which has opposite effects on RNA polymerase and RNA synthesis.

As shown in Table XVI and Fig. 26 the effect of aldosterone on the rat kidney polymerase systems shows two noteworthy characteristics (a) a lag period of 1-2 hours and (b) no effect on the RNA polymerase in vitro system (aldosterone added directly in the assay system). These observations suggest two possibilities. First, it is likely that aldosterone has an effect at some other cellular level before RNA polymerase activity is stimulated. This is possibly a slow formation of aldosterone-receptor complex
in the cytoplasm of the cell. Secondly, the time required for gene activation may vary from tissue to tissue.

It is not known at this time whether the stimulation of RNA polymerase by aldosterone in kidney is mediated via changes in the activity of RNA polymerase associated with a specific DNA template or whether it is mediated via changes in the availability or structure (chemical or physical modifications) of specific DNA template (genes). To explore this question the enzymes were purified from hormone treated animals (Table XVIII) and the activity was measured against several different sources of DNA templates. It is seen from the Table XIX that DNA from aldosterone treated animals was transcribed more efficiently by the kidney polymerases than DNA from untreated adrenalectomized or adrenalectomized plus corticosterone treated animals. This suggests specific effects of a potent mineralocorticoid on rat kidney DNA. The increased template activity of DNA from aldosterone treated animals was not simply due to the presence of nucleases which could result in nicking occurring during the isolation procedure. This is shown by the observation that template activity was intermediate when the DNA was prepared from the combined rat kidneys (adrenalectomized and adrenalectomized plus aldosterone treated) (Table XX). A more likely possibility is that the isolated DNA is still associated with nuclear proteins. The acid hydrolysatate of DNA revealed the presence of amino acids (Table XXII) and therefore the possibility of histones or other proteins contaminating DNA was likely. This problem has been explored, at least in part, by studying the binding or distribution of \(^{14}\)C-aldosterone in renal chromatin fractions. The results presented in Tables XXI and XXII suggest the involvement of DNA as well as acidic proteins in binding of labeled aldosterone in rat kidney. This is further substantiated by the observations of Table XXIV.
which illustrates that simultaneous administration of spirolactone or deoxy-
corticosterone caused an inhibition of $^{14}$C-aldosterone binding to chromatin
components. These compounds have also been shown to cause a marked inhibi-
tion of formation of aldosterone-receptor (protein) complex in rat kidney
cytosol fraction (151, 183).

Three possible explanations can be presented for these results.
(Table XXIII and XXIV).
(a) Non-histone acidic protein(s) is (are) the acceptor for hormone
or hormone-receptor complex.
(b) DNA is the acceptor and the non-histone acidic proteins play a
"passive role" in determining the specificity of regions of the
target tissue genome which are accessible to aldosterone in kidney.
(c) Both renal DNA and non-histone acidic proteins play a positive
role in forming the acceptor site for aldosterone.

It is impossible, however, to distinguish between these explana-
tions. In the light of currently held views (9, 163) for the steroid hor-
mone action, the last possibility is the most likely.

Hypothetical model for aldosterone action in kidney: Based on the results
obtained in this study for effects of aldosterone in kidney, and the large
body of evidence which shows that steroid hormones first combine with the
cytoplasmic receptor proteins almost immediately upon entering the target
tissue cell, a hypothetical working model for the action of aldosterone in
rat kidney is proposed in Fig. 28.

The characteristics of this model are similar in some respects
to that proposed by Edelman et al. in 1964 (256). The model predicts that
the first step (I) is the formation of aldosterone receptor complex in
cytoplasm of the kidney epithelial cell. This complex is then (Fig. 28,
Step II) transferred to the nuclear compartment of the cell and there it
forms another complex (Step III) with DNA or acidic proteins (or both) and
FIGURE 28. HYPOTHETICAL MODEL FOR MECHANISM OF ACTION OF ALDOSTERONE IN KIDNEY.

Aldosterone (AO) enters the cell and forms a complex (ABP) with aldosterone binding proteins (BP). The complex then moves to the cell nucleus and forms another complex with DNA or acidic proteins (AP) or both. This results in increased template activity of DNA. mRNA and ribosomal RNA are synthesized by RNA polymerase II and I. A protein factor (F) is synthesized under the influence of specific mRNA which directly or indirectly acts on sodium retention process in the kidney epithelial cell.
results in template activation. RNA polymerase II transcribed DNA of this activated genome and hormone specific mRNA is produced (Step IV). At the same time ribosomal RNA is also synthesized by RNA polymerase I. Later on the specific mRNA codes for synthesis of a protein factor (Step V) (there is also evidence that puromycin or cycloheximide inhibits sodium retention (13) and aldosterone increases \(^{3}H\)-leucine incorporation in microsomal proteins in rat kidney (16)). This protein factor in turn acts on sodium retention in the kidney epithelial cell. The biochemistry of aldosterone action at this step, however, becomes vague and controversial.
CONCLUSIONS AND PROBLEMS FOR THE FUTURE

Experimental results obtained in this study indicate that RNA turnover and the RNA polymerase system is regulated by glucocorticoids in liver and by mineralocorticoids in kidney. The decreased turnover rate of RNA and decreased RNA polymerase activity following adrenalectomy suggest that RNA synthesis is one of the sites involved in the mechanism of action of adrenocortical hormones in mammalian systems. Furthermore, it has also been shown in this study that aldosterone specifically increases template activity of DNA in kidney. It is not possible to draw firm conclusions, however, about the mechanism of template stimulation and therefore, future efforts must be directed towards understanding of this mechanism. The problems in the future may be concerned with changes (chemical or physical) that may be caused by aldosterone in kidney DNA, de novo synthesis, processing and degradation of aldosterone specific mRNA molecules and the involvement of protein factors in sodium retention in kidney epithelial cells. Further studies should also be concerned with the effects of the aldosterone-receptor complex on chromatin in the in vitro system.
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