NUCLEIC ACID METABOLISM OF AN ESTROGEN DEPENDENT ADRENAL CORTICAL TUMOR

by

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We accept this thesis as conforming to the required standard.

The University of British Columbia
December, 1968
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ABSTRACT

The work in this thesis consisted of initial experiments designed to elucidate the role of hormones in a hormonal dependent tumor. Various aspects of nucleic acid synthesis in a hormone dependent tumor in the presence (growing) and absence (regressing) of the hormone were studied.

The rates of nucleic acid synthesis were studied in whole animals by injecting radioactive formate and allowing the animal to incorporate radioactivity for various periods of time. Nucleic acids were extracted by PAS, phenol procedure and separated on a MAK column.

Labelling of all species of nucleic acid was decreased in regressing tumors.

In order to determine whether estrogen is acting directly on cells or at some indirect physiological level; the ability of cells from growing and regressing tumor to synthesize nucleic acids in vitro was determined. Results of experiments with these cell suspensions demonstrate that cells from the regressing tumor had a decreased ability to synthesize nucleic acids relative to growing tumor. The rate of DNA synthesis was decreased somewhat more than RNA.

In preliminary experiments the activity of DNA dependent DNA polymerase and RNA polymerase from regressing tumor was compared with the same enzyme in growing tumor. The specific activity of both RNA and DNA polymerase was decreased in the regressing tumor.
In target tissue like uterus stimulation with estradiol results in an increased rate of synthesis of several species of RNA. In the tumor system used in these preliminary experiments, stimulation with estrogens has a greater effect on the synthesis of DNA than RNA.
ACKNOWLEDGEMENTS

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<td>Adreno cortical trophic hormone</td>
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<tr>
<td>ATP</td>
<td>5' triphosphate of adenosine</td>
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<td>dATP</td>
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<td>AMP</td>
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<td>Messenger RNA</td>
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<td>sRNA</td>
<td>'Soluble RNA'</td>
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<td>UTP</td>
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INTRODUCTION

A. Induction of tumors by hormone imbalance

Hormonal imbalance has been implicated as a cause of tumorigenesis in various organs of the endocrine system. In some cases tumorigenesis seems to result from abnormally high amounts of hormone, other tumors have resulted from removal of normal stimulus. In this laboratory tumors of the adrenal cortex were developed in rats subjected to prolonged exposure to high levels of estrogens. This is an unusual tumor in that most adrenal tumors arise because of decrease in estrogen content.

Endocrine carcinogenesis became a major aspect of cancer research after Lacassagne (1) first demonstrated that the naturally occurring steroid hormone estrone was implicated in the cause of breast cancer in mice. This led gradually to general recognition that hormones are significant factors not only in the aetiology, but also in the prevention and treatment of cancer.

There are, broadly speaking, two different types of hormonal cancers which occur in animals and man. To describe these tumor types the terms "hormone dependent" and "hormone-independent" were first used by Huggins and Scott (2) to denote those neoplasms which depend upon hormones for their maintenance or those which originate by hormonal stimulus and later become autonomous. Dependent growths are often composed of cells which have been stimulated to proliferate because of hormonal imbalance, but in some instances they can be induced
by extrinsic agents such as irradiations, carcinogenic hydrocarbons or hormones in combination with irradiation.

Noble (3) has written a comprehensive review on the relationship between endocrine tumors and hormones.

Prolonged exposure of high levels of estrogen have been found to produce a variety of tumors of endocrine tissue.

Tumor induction in the anterior pituitary of mice and rats was produced by prolonged stimulation with estrogen (4,5,6). Adenomas of the intermediate lobe of the pituitary have been observed rarely in rats, but more frequently in hamsters, after estrogen treatment. Tumors of the cervix and vagina have been reported in many strains of mice following prolonged stimulus with estrogen (12,13,14,15,16). The effects of treatment with estrogens on mammary tumor induction in mice have been studied extensively and reviewed by Shim Kim (17). The prolonged administration of estrogens to rats has been followed by a high incidence of mammary multiple carcinomas (18,19); some showed metastases.

Interstitial cell tumors have been produced in mice and rats that have been exposed to estrogens for prolonged periods (20,21). Fibromas of the epididymis have been described in the hamster following prolonged treatment with estrogen plus androgen. Regression of the tumors followed withdrawal of therapy (22). In contrast a number of tumors have arisen by decreased estrogen stimulus.

Ovarian tumors have been readily produced in gonadectomized mice and rats by transplanting the ovary into tissues from which the venous return enters the portal system, and
the liver inactivates the estrogenic hormones (23,24).

Rats treated for a year or more with growth hormone tend to develop tumors of the adrenal medulla. Tumors of the adrenal cortex have developed in certain strains of mice, rats, guinea pigs and hamsters gonadectomized early in life. They were frequently associated with mammary tumor development. Wholley (25) in extensive studies with tumor bearing mice, found that transplanted tumors grew equally well in normal and gonadectomized mice. Most adrenal tumors described in the literature have arisen from decreased levels of estrogens or gonadectomy. These tumors showed evidence of abnormal hormone production. Estrogenic, androgenic and corticoid effects were produced by such lesions (25,26). The induction of adrenocortical tumors by gonadectomy could be prevented by the administration of stilbestrol and other estrogens or androgens, or by hypophysectomy, but not by the administration of cortisone. Secretion of sex hormone resumed when hypophysectomized rats bearing such neoplasms were given the gonadotrope, whereas ACTH was ineffective in restoring secretory activity of the adenomas.

Franks and Chesterman (27) found hyperplasia and adenoma of the adrenal cortex in hamsters treated with stilboestrol. Gardner (28) has reported that some strains of intact mice acquiring pituitary adenomas after treatment with estrogens frequently develop adrenal tumors. Noble (29) has recently reported an estrogen dependent unilateral adrenal cortical carcinoma in an inbred strain of hooded female rats
that were stimulated continuously for 5-8 months with estrone pellets. This strain of rats normally shows about a 1% incidence of spontaneous adrenal tumors but after estrone treatment the incidence rises to 25%. The normal adrenals seem predisposed to become carcinomas; often there are adenomas present. The carcinomas appear to arise from the fascicular zone. They are highly anaplastic with mitotic figures. The transplanted tumors arise over a 4 month period with a 95% take, some are hemorrhagic and necrotic. This is apparently the only reported instance of production of tumors of the adrenal cortex by prolonged treatment with excess estrogen, in contrast with gonadectomy or decreased estrogen. In addition this tumor is hormone dependent, in contrast with hormone independent tumors arising from treatment with low levels of estrogens.

The mechanism by which estrogen or other steroid hormones act to induce the neoplastic state is still unknown, in fact it is not known even how steroids effect their proliferative processes in normal target tissues. Lazarev (30) postulated that only those hormones which possess the property of stimulating proliferative processes can cause the development of tumors. Before these hormones can exert their carcinogenic action, several conditions are necessary, notably: 1) an increase in their level; 2) their continuous action; and 3) their prolonged action. Lazarev (30,35) in a hypothesis for the mechanism of the carcinogenic action of hormones proposes that hormones can stimulate proliferation and cause de-differentiation of cells. The carcinogenic effect arises only
after the prolonged and continuous action of hormones. The cells under this stimuli become hyperplastic, then precancerous, and finally a tumor develops.

Because so little is known about hormone-induced tumor genesis, some investigators feel an understanding may develop through a study of the mechanisms by which steroid hormones produce their normal physiological effects in target organs.

B. The effect of estrogen treatment on normal target tissue

Model systems like mammary gland, ovaries, uterus, testes, and prostate, have been used to investigate the role of hormones in control of their target tissues. The effect of estrogen on the hormonal control of rat uterus is the most investigated system to date.

The physiological effects of estrogen in the mammalian uterus are mediated by increases in the synthesis of RNA and protein (31-34). It was clear that an acceleration of synthesis of ribosomal RNA and of ribosomes was an essential feature in the early action of estrogen in its target organ (35-37). It is also known that the growth of the estrogen-deficient uterus was induced by treatment in utero with RNA extracted from the organ stimulated by the hormone (33,34). The role of messenger RNA is not determined yet. Furthermore it was also uncertain whether, for early estrogen action, genetic transcription was truly stimulated prior to enhancement of cytoplasmic genetic translation.

The general topics deal with the synthesis of RNA and protein in the nucleus, the binding in vivo of tritiated 17β-estradiol to chromatin and the latter's template activity
assayed in vitro. Equally important in the effects of estradiol on target tissue is the transport of RNA from nucleus to cytoplasm, the formation of polyribosomes and the variation in the incorporation of amino acids by the polyribosomes as assayed in the cell-free system.

The ratios of total amounts of nuclear RNA to DNA and of protein to DNA exhibit only minor decreases after ovariectomy, and only slight increases during the estrous phase but the amounts of RNA and protein in the cytoplasm of an uterine cell from an ovariectomized animal are much lower than in normal uterine cells (38). Thus the observation that in the uterus the concentration of cytoplasmic RNA, but not of nuclear RNA, is altered markedly by ovariectomy, estrous cycle, or hormone treatment constitutes one line of evidence that estrogen in some way regulates nuclear synthesis of RNA, its transport to, and its accumulation in, the cytoplasm. Much of the rapidly labeled RNA or nuclear RNA synthesized in higher organisms under hormonal stimulus is ribosomal precursor RNA particles. Hamilton (38) showed that there was a 500% increase in the incorporation of tritiated uridine into uterine nuclear RNA 20 minutes after hormone administration to an ovariectomized rat. Sedimentation studies have shown that the label is incorporated into 28s and 18s R-RNA or its precursor (39-42). Studies indicate that there is no accumulation of RNA for any length of time after hormone administration. This suggests that the additional nuclear RNA synthesized in response to the hormone is either turned over into
the cytoplasm (43) or degraded in the nucleus (44). In some isotopic studies the hormone stimulated synthesis of RNA was studied simultaneously in the nucleus and cytoplasm (45-47). Short pulses revealed a hormonal effect only on nuclear RNA, but longer exposure to the isotope indicated an accelerated synthesis of all types of RNA. The enzyme responsible for RNA formation is a DNA dependent RNA polymerase. The activity of this enzyme can be altered by varying the ionic strength of the medium. Hamilton (38) has shown that there are two different RNA polymerase activities; one sensitive to (NH₄)₂SO₄ and the other not. It has been suggested that the stimulatory effect of high salt concentration on RNA polymerase activity is due to dissociation of nucleo protein complexes with consequent enhancement of template activity. Widrell has found that (NH₄)₂SO₄ shifts the type of RNA being synthesized from primarily ribosomal to DNA-like in base composition and that hormonal effects which occur in the absence or presence of (NH₄)₂SO₄ reflect the effects of these hormones on the synthesis of R-RNA and DNA-like RNA, respectively.

The fraction of RNA polymerase activity insensitive to (NH₄)₂SO₄ was stimulated 2-4 hours after a single injection of estradiol but the sensitive fraction was not stimulated until 24 hours later (38). Thus it seems that the hormonal stimulation of target tissue nuclear RNA polymerase is initially restricted to the synthesis of the ribosomal type of RNA and only at later times, or not at all, will there be an
effect that can be interpreted as the synthesis of DNA-like RNA. Hamilton (38) has shown that estrogen administration to an ovariectomized rat increases the template activity of uterine chromatin by 26% at 30 minutes after hormone treatment and at 8 hours this activity had increased 300% over the control. The ratio of RNA to DNA of the chromatin showed a significant increase after only 15 minutes of estrogen action in vivo. From 12 to 24 hours the ratio of protein to DNA in the chromatin also increased. There was a parallel decrease in the ratio of histone to DNA. The physiological implication of these findings is not clear yet, but the increase in chromatin RNA may be a prerequisite for hormonal stimulation of the template activity of the organ's chromatin.

Direct evidence that hormones selectively control the transcription of mRNA is lacking. Barondes (48) and others (49) have found that the template activity of RNA extracted from nuclei or ribosomes can be estimated by its ability to stimulate amino acid incorporation into protein in a cell free system. Results have indicated that at least part of the additional RNA synthesized under hormonal stimulation is mRNA. Although evidence for the transcription of mRNA molecules coding for specific proteins in higher organisms has yet to come; some interesting situations do exist. Gorski (50) has shown the induction of the synthesis of a single protein occurred within 30 minutes after estrogen treatment and prior to the overall stimulation of protein synthesis. The synthesis of this estrogen-induced protein was still demonstrable but at lowered levels in the presence of actinomycin D suggesting
that either its synthesis is under translational control or that RNA synthesis in the specific gene locus for this protein is not effectively suppressed by actinomycin D. This may mean that there is a stable mRNA present in the cytoplasm that is activated by estrogen. In an in vivo pulse experiment estrogen depressed the incorporation, 30-90 minutes after injection, of radioactive amino acids into proteins of different cell organelles (38,51). This depression may be due to a drain on ATP supply. Observations on the ability of polysomes to function in a cell free protein synthesizing system lead to the conclusion that an essential feature of the early action of estrogen in the uterus is the appearance in the cytoplasm, of new polyribosomes, having amino acid incorporation properties different from the old ones.

Jensen (52-54) Maurer and Chalkley (55) have described properties of estradiol receptors found both in the cytoplasm and nucleus of target tissues. They suggest that the estrogen binds to a protein component of chromatin.

In summary the early action of 17β-estradiol on uterine cells appears to be (i) binding of the hormone to the chromatin in the nucleus, (ii) stimulation of synthesis of chromosomal and ribosomal RNA in conjunction with chromosomal and nucleolar activity, (iii) an acceleration of the rate of formation of ribosomal precursor particles; (iv) an acceleration of transport of the ribosomal precursor particles with attached messenger RNA to the cytoplasm, and (v) an accumulation of new polyribosomes in the cytoplasm having different
amino acid incorporating properties compared to those of the old ones.

C. **Stimulation of normal adrenal tissue with estrogens**

It is difficult to assess the function of estrogen in tumorigenesis or its effect on normal adrenal tissue. The administration of estrogen stimulates growth of the adrenal glands, and ACTH production by the pituitary, but causes a reduction in the content of cholesterol, ascorbic acid, corticoids and glucose metabolites in mice and rats (56-60). All these changes, being similar to those produced by ACTH, have been interpreted as a manifestation of increased activity in the adrenal cortex. These effects do not occur in the hypophysectomized rat; subsequently the presence of the pituitary gland is indispensable for the response of the adrenal cortex to estrogen treatment. The stimulation of adrenal growth and protein synthesis by ACTH has been found to be largely dependent on changes in RNA metabolism mediated by RNA polymerase (61). It would seem then that estradiol stimulates the pituitary to release ACTH which stimulates RNA synthesis. Estrogen may also have a direct effect on the adrenal. The work described in this thesis is a comparison of the role of estrogen in maintaining growth of the adrenal tumor tissue, with the role of estrogen in target tissue.

Initial studies were designed to compare nucleic acid metabolism in growing tumors with tumors that have stopped growing, by evidence of a decreased size, as a result of removal of the source of estrogen, in an attempt to assess
the function of the estrogen in metabolism of the adrenal tumor.

The work described in this thesis involves three different types of experiments:

1) Pelleted and unpelleted tumor bearing animals were exposed in vivo to radioactive nucleic acid precursors, to compare the abilities of tumors and other tissues to synthesize nucleic acids. The nucleic acids were extracted with paramino salicylate, phenol, mixture and fractionated on a methylated albumin kieselguhr column.

2) The possibility exists that the hormone may be exerting its action by altering some parameter in the whole animal and not directly effecting the tumor cells. In order to eliminate this consideration, in vitro experiments were designed using a variety of radioactive precursors to examine the rate of formation of RNA and DNA species in the cells from the tumor.

3) In preliminary experiments the nucleic acid precursor supply and the activities of certain enzymes (i.e. RNA polymerase and DNA polymerase) were compared in tumor tissue from pelleted and unpelleted animals. Estrogen appears to stimulate the rate of RNA and DNA synthesis in tumor tissue.
A. Materials and Methods

1) Treatment of Animals

An inbred strain of female hooded rats was used in these experiments. The animals were fed purina laboratory pellets and water ad libitum. The tumor passage was affected by injecting cells subcutaneously in the neck region of pelleted animals. Tumor regression was recorded by changes in total body weight and tumor measurement. The depelleted animals used in all experiments were allowed to regress for long periods of time (14 days) in order that large changes in metabolic rates may be seen.

Radioactive precursors were injected intraperitoneally; and the animals killed by stunning in ether and then decapitating. The tissues were removed rapidly, frozen in ethanol-CO₂ and stored at -20°C.

2) Radioactivity

(a) Source of chemicals - Brays liquid scintillator (62). The reagents used in preparing the liquid scintillator described in this thesis were obtained from the following sources: p-dioxane (purified), ethylene glycol, absolute methanol, naphthalene (recrystallized) from Fisher Scientific Co., 2,5 di-phenyl oxazole (PPO) and 14 di-2-(5-phenyl oxazolyl) benzene (POPOP) from Pilot Chemicals Inc.

The composition of the liquid scintillator is as follows: naphthalene 60 gm, (PPO) 4 gm, POPOP 0-2 gm, methanol (absolute) 100 ml, ethylene glycol 20 ml and p-dioxane to make 1 liter.
(b) **Chemical determinations of DNA and RNA**

The amounts of these materials in fractions obtained by chromatography on MAK columns and the amounts in total tissue extracts were determined spectrophotometrically by the di-phenylamine and orcinol color reactions respectively (63,64).

3) **Extraction of Nucleic Acids**

(a) **Source of chemicals**

The reagents used in the extraction procedure were obtained from the following sources: sodium paramino salicylate (K & K Laboratories, California), 90% liquid phenol, m-Cresol, 8-OH quinoline, bentonite, Naphthalene 1,5 disulphonate all were purchased from Fisher Scientific.

(b) **Method**

In the paramino salicylate procedure (65-68) described by Kirby, weighed samples of tissue were homogenized in 15 volumes of 6% paramino salicylate (17 mg/ml Bentonite) at 21,000 r.p.m. in an ice cooled Serval homogenizer. Fifteen volumes of 90% phenol, 10% m-cresol, 0.1% 8 OH quinoline were added to the mixture, which was shaken for 1 hr at room temperature on a water bath shaker. The two phase system was centrifuged at 1000 xg, 0°C, for 1 hr in a Universal refrigerated centrifuge. The aqueous layer was removed by pasteur pipette, the interface and phenol layer was re-extracted with 5 volumes of 6% paramino salicylate as described above. The pooled aqueous layers were extracted three times with ethyl ether to remove residual phenol and the
ether was removed through evaporation in a stream of nitrogen. The nucleic acids were precipitated from solution by the addition of two to four volumes of ice-cooled 95% ethanol followed by storage overnight at 0°C.

(c) Disulphonate, paramino salicylate method (65-68)

Weighed samples of tissue were homogenized in 15 volumes of 0.5% naphthalene, 1-5 disulphonate, shaken with 15 volumes 90% phenol, 10% m-cresol, 0.1% 8-hydroxy quinoline and the aqueous layer withdrawn. The interface and phenol layer were re-extracted with 15 volumes each of 6% paramino salicylate, 0.7 mg/ml bentonite, and 90% phenol, 10% m-cresol, 0.1% 8-hydroxy quinoline.

4) Preparation of acid soluble fraction and separation of RNA and DNA (69)

Weighed amounts of tissue were homogenized in cold 0.7 N perchloric acid, in a Serval homogenizer, and centrifuged for 10 minutes at 1000 xg in a refrigerated centrifuged set at 0°C. The supernatant was saved and enough 5 N KOH added to remove the perchlorate ion; the pH was adjusted to 6.0. This constitutes the acid soluble fraction.

The acid insoluble precipitate was extracted once with each of acetone, ethanol, ethanol:chloroform (1:1), ethanol: ether (3:1) and ether; each supernatant was discarded and the final precipitate was distributed over the tubes to dry. Nucleic acids were obtained from the dry material by extracting with hot 10% NaCl at pH 7.4, and precipitating overnight with
2-4 volumes of ice cooled 95% ethanol.

The nucleic acid precipitate was collected by centrifugation, dissolved in 1 volume of 0.3 N KOH/gm tissue and incubated at 37°C for 18-22 hrs. The DNA was precipitated by acidification with 0.2 volumes of cold 3 N HCl. Aliquots of the supernatant and the precipitate were used for determinations of radioactivity, ribose and DNA.

5) Preparation of cell suspensions

Tumors were removed from animals, minced with scissors and homogenized several times in Robinson's Media (70) with a loose-fitting glass hand homogenizer. Connective tissue was removed by filtering through 2 layers of gauze. Aliquots of the cell suspension were then added to the incubation flasks, which contained Robinson's Medium (70); a buffered salts plus glucose media, made 10% with respect to horse serum; and radioactive precursors. The incubations were stopped by placing the flasks on ice. The nucleic acids were extracted by homogenizing the cell suspension in an equal volume of 6% paramino salicylate plus bentonite, with a small potter homogenizer. The mixture was shaken for 1 hr at room temperature with an equal volume of 90% phenol, 10% m-cresol. The aqueous layer was separated from the phenol layer by centrifugation and treated as previously described to obtain RNA and DNA fractions.

6) Chromatography of nucleic acids on MAK columns

(a) Source of chemicals

Kieselguhr was purchased in the grade sold as "Hyflo Supercel" by the Johns-Manville Products Corporation, New York
City. "Fraction V" bovine serum albumin powder, "for microbiological use" was purchased from the Armour Laboratories, Chicago. Methylated albumin was purchased from the Worthington Biochemical Corporation.

(b) **Preparation of methylated albumin** (71)

Five gm of albumin were suspended in 500 mls of absolute methyl alcohol and 4.2 mls of 12 N hydrochloric acid were added. The mixture was allowed to stand in the dark for 3 days or more with occasional shaking. The precipitate was collected and washed twice with both methyl alcohol and anhydrous ether. Most of the ether was evaporated in the air and then in vacuo over KOH pellets. The material was reduced to a powder and stored over KOH. The methylated protein was used in the form of a 1% solution in water.

(c) **Preparation of MAK**

The fines were removed from the Kieselguhr by making a slurry with 0.2 M buffered saline and repeatedly decanting the lighter material.

A suspension of 20 gm of Kieselguhr in 100 mls of 0.1 M buffered saline was boiled (to expel air) and cooled. Five ml of 1% esterified albumin were added. The mixture was stirred and an additional 20 mls of saline added. This suspension is methylated albumin Kieselguhr or MAK. The MAK must be prepared fresh each day or the column will lose its capacity.

Buffered salt solutions were prepared to contain 0.05 M phosphate buffer (\(\text{Na}_2\text{HPO}_4\) and \(\text{KH}_2\text{PO}_4\)), appropriate to give a final pH of 6.7. The solutions were preserved by addition of a few drops of chloroform to each bottle.
(d) Preparation of MAK columns

The chromatographic columns have an inner joint with a sealed-in fritted glass disc. Both 1 cm and 2 cm diameter columns were used with varying amounts of MAK. The MAK was poured directly into the column, the excess buffered saline was driven down to the level of the packed material by applying 4 lbs pressure with nitrogen. The columns were washed with 10 volumes of buffered saline to 1 volume of MAK.

Samples were added to the column in 0.2 M NaCl .05 M phosphate buffer, pH 6.7 and allowed to soak into column until the first fraction of ultraviolet absorbing material at 265 μm was eluted with the starting buffer. Material was then eluted from the column with a linear salt gradient pumped onto the column with a Beckman solvent pump (Model 746), at 2 mls/minute. Fractions were collected on a Gilson automatic fraction collector, scanned for ultraviolet light absorbance at 265 μm and radioactivity. After the gradient was finished more ultraviolet absorbing material at 265 μm was eluted with 1.5 M NH₄OH. In order to prevent degradation, the 1.5 N NH₄OH was neutralized with concentrated HCl as soon as it came off the column. This technique was later replaced by eluting with 0.2% sodium dodecyl sulphate in 0.4 M buffered NaCl, sometimes this was followed by 1.5 N NH₄OH treatment.

7) DNA and RNA polymerase assays

(a) Source of chemicals

The reagents used in these incubations were obtained
from the following sources: C\(^14\) UTP(u) New England Nuclear, UTP, CTP, GTP, ATP, Schwarz Bioresearch Inc., New York, dithiothreitol, Sigma Chemical Co., St. Louis, 8-C\(^14\) dATP, dATP, dCTP, TTP, dGTP, Schwarz Bioresearch Inc., New York.

(b) DNA polymerase preparation (72)

Weighed amounts of minced tissue were suspended in 3 volumes of 50 M Tris buffer containing 5 mM MgCl\(_2\) and 1 mM dithiothreitol at pH 7.5. The mixture was then homogenized in a Serval homogenizer at 16,000 r.p.m. for 60-90 seconds; all operations were carried out on ice. The homogenate was spun in a cellulose nitrate centrifuge tube for 20 minutes at 30,000 g, 0°C in a Spinco centrifuge Model L-40. The supernatant was filtered through several layers of gauze into fresh cellulose nitrate tubes and spun at 105,000 g, 0°C for 1 hour, the supernatant was again filtered through gauze and an aliquot set aside for protein determination. Aliquots of the supernatant were now added to the incubation tubes. Each tube contained 0.1 μc 8-\(^14\)C-dATP (28 μmole), 12.2 μmole dATP, 80 μmole of each of dGTP, dCTP and TTP, 1 μmole dithiothreitol, 1 μmole MgCl\(_2\), Tris buffer pH 7.8 to final concentration of 50 mM in a total volume of 0.5 ml, 25 μg denatured calf thymus DNA and 1 μmole ATP. The incubation was carried on at 37°C in a water bath.

(c) RNA polymerase preparation (73-76)

Weighed tissue was minced and then suspended in 10 volumes 0.25 M sucrose containing 1 mM MgCl\(_2\) and 3 mM CaCl\(_2\).
The suspension was homogenized for 3 seconds in an ice cooled Serval homogenizer, followed by twelve strokes on a large Potter-Elvehjem homogenizer. The homogenate was filtered through nylon and spun for 10 minutes at 1000 g, 0°C, in an International refrigerated centrifuge. The supernatant was discarded, pellets were taken up in a small amount of 0.25 M sucrose (1 mM MgCl$_2$ and 3 mM CaCl$_2$) and layered over 10 volumes of 0.32 M sucrose (1 mM MgCl$_2$ and 3 mM CaCl$_2$) in 12 ml centrifuge tubes and spun at 1000 g, 0°C for 10 minutes. The supernatant was discarded, the pellets taken up in a small volume of 0.25 M sucrose (1 mM MgCl$_2$ and 3 mM CaCl$_2$) and layered over 9.0 mls of 1.6 M sucrose with 1 mM MgCl$_2$ and 3 mM CaCl$_2$ in cellulose nitrate tubes and spun at 100,000 g, 0°C for 40 minutes. The supernatant was discarded and the nuclear pellets resuspended in 0.25 M sucrose in 12 ml centrifuge tubes, spun at 1000 g, 0°C for 10 minutes. This last step was repeated and the final nuclear pellet was taken up in 1/10 of the original diluting volume.

Aliquots of the suspension of nuclei were now added to the standard incubation mix. Each tube contained 0.1 μc $^{14}$C UTP (0.2857 μmoles), 20 μmoles UTP, 160 μmoles each of CTP, GTP, ATP, 1 μmole MnCl$_2$, 4 μmoles MgCl$_2$, 1 μmole dithiothreitol and Tris-maleate buffer pH 7.8 made up to a final concentration of 50 mM in 0.5 ml total volume.

(d) Post incubation procedures for RNA and DNA polymerase assays

The reactions were terminated by placing tubes on ice.
The mixture was transferred by pasteur pipette to chilled 12 ml centrifuge tubes, each containing 0.4 ml of cold 7% HClO₄. A solution containing 500 µg of calf thymus DNA was added to each tube as a rinse and then transferred to the HClO₄ acid tubes and mixed. The tubes were spun at 1000 g, 0°C, for 10 minutes; the supernatants were set aside for counting and the precipitates resuspended in 6 mls of 1% HClO₄. The mixture was spun at 1000 g, 0°C, for 10 minutes and resuspended in 3 mls of 1% HClO₄, spun and supernatants pooled. The final precipitate was dissolved in 1.5 ml of 0.2 N NH₄OH and counted.

B. Results

1) Comparison of extraction methods

Different methods for extracting nucleic acid species were investigated to establish a convenient, quantitative technique suitable for routine use.

The naphthalene disulphonate-phenol treatment liberates sRNA and r-RNA into the aqueous phase. When the remaining interface and phenol layer are re-extracted with paramino salicylate and phenol, the aqueous layer contains mRNA and DNA. Direct extraction with paramino salicylate and phenol solubilizes the total nucleic acid fraction into the aqueous phase. Several experiments were done to compare the efficiency of these methods to that of a standard salt extraction.

In this comparative study weighed samples of rat liver were extracted with Na disulphonate, paramino salicylate,
phenol or with paramino salicylate phenol mixture as described in the methods. The results of these experiments are shown in Table I. It is necessary to re-extract the interface and phenol layer several times to effect complete solubilization of all nucleic acid fractions.

The extracts were shaken on a water bath for 1 hr at room temperature; one paramino salicylate extract was stirred for 1 hr with a magnetic stirrer.

Extraction of tissues with paramino salicylate is more efficient and convenient than with Na disulphonate; especially in regard to solubilization of DNA. Stirring appears crucial in these experiments.

In the experiment whose results are shown in Table II 4 male hooded rats were injected with 25 $\mu$curies of C$^{14}$ Na formate (3.76 mc/min). The animals were killed 4 1/2 hours later by stunning with ether and then decapitated. The tissues were removed, frozen in dry ice and ethanol. Weighed samples were extracted with paramino salicylate, phenol or by a standard salt extraction, acid precipitation method (69).

The total amounts of nucleic acids extracted with the standard salt extraction method were less than that extracted by paramino salicylate; but the specific activities were higher. The salt extraction method may be liberating nucleic acid species whose rate of synthesis is different. The data indicates that the paramino salicylate method is a more convenient quantitative and routine procedure for extracting
<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of Exts.</th>
<th>Disulphonate (H₂O bath)</th>
<th>PAS (H₂O bath)</th>
<th>PAS &amp; Magnetic Stirrer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µg RNA /gm</td>
<td>µg DNA /gm</td>
<td>µg RNA /gm</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
<td>7504.8</td>
<td>25.0</td>
<td>7835.2</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>1359.36</td>
<td>12.5</td>
<td>1543.44</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>118.0</td>
<td>35.0</td>
<td>410.17</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>15.0</td>
<td>0.0</td>
<td>87.23</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>8983.66</td>
<td>72.5</td>
<td>9876.04</td>
</tr>
</tbody>
</table>
### TABLE II. C\(^{14}\) sodium formate incorporation into nucleic acids of thymus, spleen and liver tissues. Extraction of nucleic acids from these tissues by standard salt treatment or paramino salicylate phenol.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Standard Salt Extraction</th>
<th>PAS Extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg RNA/gm</td>
<td>µg DNA/gm</td>
</tr>
<tr>
<td>Thymus</td>
<td>-</td>
<td>28,305.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>2000</td>
<td>9,940</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16m</td>
<td>6020</td>
<td>380</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56m</td>
<td>6810</td>
<td>318.8</td>
</tr>
</tbody>
</table>
nucleic acids from all tissues; then the standard salt extraction method or disulphonate plus paramino salicylate one. One disadvantage with the paramino salicylate phenol extraction procedure is the presence of contaminating polysaccharides. When the total nucleic acid fraction was extracted with paramino salicylate and divided into RNA and DNA with 0.3 N KOH an insoluble residue remained behind.

This residue was hydrolyzed with 0.6 N HCl in a boiling water bath for 2 1/2 hours. The hydrolysate was spotted on a chromatogram (Fig. 1) which was developed in an ascending solvent (isopropanol water 4:1) for 30 hours. Material in the hydrolysate ran with the same Rf value as glucose standard; this was interpreted as the contaminating material being glycogen. In subsequent experiments polysaccharides were removed from the total nucleic fraction in 0.2 M NaCl, 05 M phosphate buffer by centrifugation. An alternative to this method was the removal of polysaccharides by digestion with α amylase, this treatment did not alter the spectra of a total nucleic acid fraction, as will be seen later.

2) Standardization of the MAK column

(a) Chromatography of standards

Figure 2 depicts a typical elution pattern of ultraviolet absorbing material at 265 μ obtained, when a total nucleic acid fraction which was extracted by the paramino salicylate phenol cresol method, was chromatographed on a MAK column. The peaks were identified by comparison with standards,
Figure 1. Identification of contaminating material found in nucleic acid fractions extracted from tissues with equal volumes of 6% p-aminosalicylate and 90% phenol 10% m-cresol. The contaminating material was hydrolyzed in 0.6 N HCl in a boiling water bath for 2½ hours. The hydrolysate was spotted on a chromatogram, developed in an ascending solvent (isopropanol water 4:1) for 30 hours, and detected by dipping the chromatogram first into a saturated AgNO₃ Acetone solution and then into a 0.5% NaOH 100% ethanol solution.
Figure 2. A typical elution profile on a 10 ml MAK column of ultraviolet absorbing material, extracted from growing tumor tissue with equal volumes of 6% p-aminosalicylate and 90% phenol - 10% m-cresol.

1.0 mg of nucleic acids put on column in 0.2 m NaCl (.05 m phosphate buffer, pH 6.7) - flow rate 2 mls per minute- 5.0 mI fractions collected.

1. Gradient started 0.4 m NaCl -- 1.5 NaCl (0.05 m phosphate buffer, pH 6.7 500 mls)
2. 1.5 m NH₄OH
sugar determinations and degradation with enzymes. Figures 3(a), (b) and (c) are elution patterns of ultraviolet absorbing material of standard sRNA, calf thymus DNA and rat liver R-RNA (77). sRNA was eluted from the column in a stepwise manner with 0.4 M NaCl, 0.05 M phosphate buffer pH 6.7, DNA at 0.6 M and R-RNA with 0.8 M. The same results were obtained when elution was done with a linear salt gradient. R-RNA was prepared by extracting a total nucleic acid fraction twice with cold 3 M-sodium acetate pH 6.0. This removed DNA, sRNA and glycogen into the supernatant.

(b) Color determinations of nucleic acid fractions

After a total nucleic acid sample was fractionated on a MAK column by eluting with a linear salt gradient, aliquots from each tube were assayed for radioactivity on a scintillation spectrometer. Contents of the tubes within an individual nucleic acid fraction were pooled and dialyzed against distilled H₂O at 4°C. The eluate was lyophilized on a thermovac freeze dryer (Model FD2). The residue containing DNA was dissolved in a small amount of 0.1 N NH₄OH and the diphenylamine reaction (63) performed on an aliquot. Similarly the residue containing RNA was dissolved in water and the orcinol color determination performed (64). Color determinations indicated that peaks No. (a) degradation peak, (b), (c), (d), (e) eluated with 1.5 M NH₄OH or 0.2% SDS in 0.4 M buffered saline all were ribose containing peaks; the diphenylamine color determination of these peaks showed very little contamination with DNA when compared with color development.
Figure 3 (a). Ultraviolet absorption pattern of Standard yeast sRNA, eluted from a 4.0 ml MAK column.

0.400 mg of sRNA put on column in 0.2 m NaCl (0.05 m phosphate buffer, pH 6.7) - flow rate 2 mls per minute
- 5.0 ml fractions collected.

1: 0.4 m NaCl (0.05 m phosphate buffer, pH 6.7)
Figure 3 (b). Ultraviolet absorption pattern of Standard calf thymus DNA, eluted from a 4.0 ml MAK column. 0.400 mg of DNA put on column in 0.2 m NaCl (.05 m phosphate buffer, pH 6.7) - flow rate 2 mls per minute - 5.0 ml fractions collected.

1. - 0.6 m NaCl (0.05 m phosphate buffer, pH 6.7)
Figure 3 (c). Ultraviolet absorption pattern of rat liver r-RNA, eluted from a 4.0 ml MAK column. The r-RNA was prepared by extracting a total nucleic acid preparation twice with cold 3 m sodium acetate, pH 6.0

0.400 mg of r-RNA put on column in 0.2 m NaCl (.05 m phosphate buffer, pH 6.7) - flow rate 2 mls per minute

- 5.0 ml fractions collected.

1. - 0.8 m NaCl (0.05 phosphate buffer, pH 6.7)
2. - 1.2 m NaCl (0.05 phosphate buffer, pH 6.7)
3. - 1.5 m NH₄OH
in the DNA peak.

(c) **Digestion with degradative enzymes**

Figure 4(a) shows an elution pattern of ultraviolet absorbing material at 265 μm, in a total nucleic acid extract from pelleted tumor bearing animals after digestion with pancreatic RNase (Worthington Biochemical Corp). R-RNA was the only fraction removed by this treatment. This digestion was performed in 0.2 M NaCl, 0.05 M phosphate pH 6.7, these conditions may have been too mild for enzymatic degradation of sRNA. Figure 4(b) shows an elution pattern of ultraviolet absorbing material at 265 μm from a total nucleic acid extract of tumor tissue from a pelleted animal after digestion with pancreatic DNase (Worthington Biochemical Corp.). The enzyme degraded only the DNA fraction. Figure 4(c) shows an elution pattern of ultraviolet absorbing material at 265 μm from a total nucleic extract of tumor tissue from a pelleted animal that was digested with α-amylase; the nucleic acid species are not affected by this enzyme. It was concluded that the fractions eluted from the MAK column with increasing salt concentrations were the degradation peak, sRNA, xRNA, DNA, R-RNA and yRNA. xRNA may be similar to 5 sRNA, reported in the literature (84). Some authors have considered yRNA rapidly labelled RNA.

Separation of total nucleic acid species by chromatography on MAK columns is a fast, efficient, routine procedure. The columns take five minutes to prepare and approximately one
Figure 4 (a). Elution pattern from a 4 ml MAK column, of ultraviolet absorbing material in a total nucleic acid extract after digestion with pancreatic Rnase.

0.400 mg of nucleic acids put on column in 0.2 m NaCl (.05 m phosphate buffer, pH 6.7) - flow rate 2 mls per minute - 5.0 ml fractions collected.

1. - 0.2 m NaCl (0.05 m phosphate buffer, pH 6.7)
2. - Gradient started 0.4 m -- 1.5 m NaCl (0.05 m phosphate buffer, pH 6.7 500 mls)
3. - 1.5 m NH₄OH
Figure 4 (b). Elution pattern from a 4 ml MAK column of ultraviolet absorbing material in a total nucleic acid extract after digestion with pancreatic Dnase.

0.400 mg of nucleic acids put on column in 0.2 m NaCl (.05 m phosphate buffer, pH 6.7) - flow rate 2 mls per minute - 5.0 ml fractions collected.

1. Gradient started 0.4 m -- 1.5 m NaCl (0.05 m phosphate buffer, pH 6.7 500 mls)
2. - 0.2% SDS (Sodium dodecyl sulphate) in 0.4 m NaCl (0.05 m phosphate buffer, pH 6.7)
3. - 1.5 m NH₄OH
Figure 4 (c). Elution pattern from a 4 ml MAK column, of ultraviolet absorbing material in a total nucleic acid extract after digestion with α-amylase.

0.400 mg of nucleic acids put on column in 0.2 m NaCl (0.05 m phosphate buffer, pH 6.7) - flow rate 2 mls per minute. 5.0 ml fractions collected.

1. 0.2 m NaCl (0.05 m phosphate buffer, pH 6.7)
2. Gradient started 0.4 m -- 1.5 m NaCl (0.05 phosphate buffer, pH 6.7 500 mls)
hour to equilibrate, depending on the size of the column.

The nucleic acid fractions can be eluted from the larger columns (10 ml MAK) in 3 hours, whereas the smaller columns (4 ml MAK) take only one and half hours.

The development of routine extraction and separation methods has been discussed. These methods were used to analyze different nucleic acid species under a variety of biological conditions.

3) _In vivo_ experiments

(a) _Choice of precursors_

2-C¹¹ glycine, 6-C¹⁴-orotate and C¹⁴-uridine (U) were used as radioactive precursors to study rates of nucleic acid synthesis _in vivo_. These precursors labelled the RNA and DNA fractions of normal tissues; but did not label those of the tumor sufficiently to produce significant counts in each fraction after spreading of the sample by elution from a MAK column. The nucleic acid species of pelleted and unpelleted tumor bearing animals were labelled by incorporation for various periods of time of 100 μcuries C¹¹ Na formate (3.76 mc/mmole) injected intraperitoneally.

The data presented in Table III illustrates the differences in labelling of nucleic acids in tissues of pelleted tumor bearing animals that have been injected with 100 μcuries C¹¹ Na formate 4 and 10 hrs previously.

(b) C¹⁴ sodium formate incorporation into RNA and DNA

After pelleted tumor bearing animals were exposed to 100 μcuries C¹¹ Na formate (3.76 mc/mmole) for 4 hrs. The synthetic activity of tumor DNA was only one half of that seen for
## TABLE III. Comparison of C\textsuperscript{14} formate incorporation into nucleic acid fractions of various tissues of estrone pelleted animals.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ug RNA 1 gm tiss</th>
<th>ug DNA 1 gm tiss</th>
<th>Deg</th>
<th>sRNA 100</th>
<th>xRNA</th>
<th>DNA</th>
<th>R-RNA</th>
<th>yRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver 4 hr pulse pelleted</td>
<td>12,692.4</td>
<td>605.0</td>
<td>280</td>
<td>1,660</td>
<td>310</td>
<td>240</td>
<td>110</td>
<td>210</td>
</tr>
<tr>
<td>Liver 10 hr pelleted</td>
<td>12,160.1</td>
<td>2,655.0</td>
<td>210</td>
<td>220</td>
<td>170</td>
<td>416</td>
<td>70</td>
<td>110</td>
</tr>
<tr>
<td>Tumor 4 hr pelleted</td>
<td>5,117.7</td>
<td>2,520.0</td>
<td>50</td>
<td>420</td>
<td>540</td>
<td>696</td>
<td>70</td>
<td>250</td>
</tr>
<tr>
<td>Tumor 10 hr pelleted</td>
<td>11,574.4</td>
<td>680.0</td>
<td>1,490</td>
<td>370</td>
<td>1,000</td>
<td>4,752</td>
<td>4,860</td>
<td>200</td>
</tr>
<tr>
<td>Spleen 4 hr pelleted</td>
<td>10,205.58</td>
<td>4,560.0</td>
<td>230</td>
<td>650</td>
<td>210</td>
<td>2,384</td>
<td>4,600</td>
<td>260</td>
</tr>
<tr>
<td>Spleen 10 hr pelleted</td>
<td>12,894.4</td>
<td>3,193.4</td>
<td>20,460</td>
<td>11,950</td>
<td>3,950</td>
<td>232</td>
<td>12,480</td>
<td>1,760</td>
</tr>
</tbody>
</table>
liver and spleen DNA. Under these labelling conditions the degree of labelling of RNA species from tumor, liver and spleen tissues were all similar except the spleen R-RNA fraction was labelled thirty times faster than the R-RNA species of tumor and liver.

After exposure of the pelleted tumor-bearing animal to 100 μcuries C\textsuperscript{14} Na formate for 10 hrs. The synthetic activity of tumor DNA fraction was fifty to eighty times that found for liver and spleen DNA. The rates of labelling liver and spleen DNA actually decreased from their 4 hr levels. This large increase in the specific activity of tumor DNA may be an expression of the length of time that is necessary for the radioactive isotope to reach maximum concentration in the tumor cells. It may take tumor tissue longer to accumulate a critical concentration of the isotope because it is farther from the site of injection than the other tissues. If this difference in the rate of synthesizing tumor DNA is due to supply, then tumor tissue from pelleted animals is dividing very rapidly.

After exposure to 100 μcuries of C\textsuperscript{14} Na formate (3.76 mc/m mole) for 10 hours the rate of labelling RNA species of liver tissue had declined from the 4 hr level, but the specific activities of the RNA fractions of tumor and spleen tissues were increased greatly over their 4 hr levels, especially the degradation, sRNA and xRNA fractions.

There is one major difference between tumor and spleen tissue and this is illustrated at the 10 hr labelling period. The rate of labelling the RNA fractions of both tissues and
tumor DNA increases with the length of exposure to the isotope; but the rate of labelling spleen DNA decreases after exposure to radioactive formate for 4 hrs. This preliminary evidence indicates that the cells of tumor tissue are small and rapidly dividing when under hormonal control. The data summarized in Table IV is a comparison of the ability of growing and regressing tumor tissue to incorporate radioactive precursors into total RNA and DNA. The nucleic acid fractions were extracted by the standard salt extraction method from tumor tissue of pelleted and unpelleted female hooded rats that had been subjected to 100 μcuries C¹¹ Na formate (3.76 mc/mmole) for 4 and 10 hours. The rate of labelling of total RNA and DNA fractions were reduced more than 50% in depelleted animals. This decrease was observed after exposure to the isotope for both intervals. The data from these preliminary experiments indicate that the synthesis of both RNA and DNA in tumor tissue is under hormonal control. In order to determine if the synthesis of any particular species of RNA or DNA was preferentially affected by the presence of the hormone the nucleic acids of growing and regressing tumor tissue were extracted and separated. The data summarized in Table V is a comparison of the ability of growing and regressing tumor tissue to synthesize different species of RNA and DNA.

The nucleic acid fractions were extracted by the p-amino salicylate method from tumor tissue of pelleted and unpelleted
TABLE IV. C\(^{14}\) sodium formate labelling of RNA and DNA from growing and regressing tumor tissue in vivo.

<table>
<thead>
<tr>
<th>Conditions of Incubation (hr)</th>
<th>C(^{14}) Precursor</th>
<th>DNA CPM/(\gamma)DNA</th>
<th>RNA CPM/(\gamma)ribose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growing</td>
<td>4</td>
<td>formate</td>
<td>4.719</td>
</tr>
<tr>
<td>Growing</td>
<td>10</td>
<td>&quot;</td>
<td>5.725</td>
</tr>
<tr>
<td>Regressing</td>
<td>4</td>
<td>&quot;</td>
<td>1.472</td>
</tr>
<tr>
<td>Regressing</td>
<td>10</td>
<td>&quot;</td>
<td>2.047</td>
</tr>
</tbody>
</table>
female hooded rats that had been subjected to 100 μcuries of C\textsuperscript{\text{14}} Na formate for 4, 10, and 16 hrs. The nucleic acid species were separated by chromatography on a 10 ml MAK column, and the fractions eluted with a linear salt gradient (0.4 M NaCl with .05 M PO\textsubscript{4}, pH 6.7 → 1.5 M NaCl). All RNA and DNA containing fractions extracted from tumor tissue of depelleted female hooded rats were labelled at a slower rate than those extracted from tumor tissue of pelleted animals. Some species are more highly labelled than others regardless of the length of time the animal is exposed to the isotope (i.e. degradation fraction, sRNA, xRNA, DNA, R-RNA and yRNA) S.A.'s 19.8, 10.4, 5.34, 6.0, 2.17 and 20.2 respectively for 4 hrs exposure - pelleted animal. The data summarized in Table III illustrated that exposure of the tumor bearing animal for 10 hrs to radioactive Na formate labelled all nucleic acid species optimally; this observation is again borne out in the experiments summarized in Table V.

(c) The effect of hormone removal on the synthesis of individual nucleic acids

If a comparison is made of the effect of hormone removal on the individual nucleic acid species labelled after exposure of the animal to isotope for 10 hrs the specific activity of the DNA containing fraction was reduced 15 fold. The specific activities of all other fractions containing nucleic acids was reduced 4-6 fold. If other exposure times are considered the pattern of reduction in labelling of a
TABLE V. The effect of hormone removal on the synthesis of individual nucleic acid species of tumor tissue.

<table>
<thead>
<tr>
<th>Description</th>
<th>Pulse Time 100 µc C&lt;sup&gt;14&lt;/sup&gt; formate</th>
<th>Degradation</th>
<th>4S</th>
<th>xRNA</th>
<th>DNA</th>
<th>R-RNA</th>
<th>yRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growing tumor</td>
<td>4 hrs</td>
<td>19.8</td>
<td>10.4</td>
<td>5.34</td>
<td>6.0</td>
<td>2.17</td>
<td>20.2</td>
</tr>
<tr>
<td>Depelleted Animal</td>
<td>4 hrs</td>
<td>11.38</td>
<td>8.38</td>
<td>7.76</td>
<td>1.06</td>
<td>9.02</td>
<td>5.66</td>
</tr>
<tr>
<td>Growing tumor</td>
<td>10 hrs</td>
<td>32.15</td>
<td>35.9</td>
<td>12.84</td>
<td>31.2</td>
<td>76.0</td>
<td>42.8</td>
</tr>
<tr>
<td>Depelleted Animal</td>
<td>10 hrs</td>
<td>6.82</td>
<td>9.85</td>
<td>-</td>
<td>2.54</td>
<td>12.75</td>
<td>9.63</td>
</tr>
<tr>
<td>Growing tumor</td>
<td>16 hrs</td>
<td>19.4</td>
<td>18.2</td>
<td>-</td>
<td>10.26</td>
<td>20.73</td>
<td>8.7</td>
</tr>
<tr>
<td>Depelleted Animal</td>
<td>16 hrs</td>
<td>12.68</td>
<td>1.8</td>
<td>4.47</td>
<td>3.45</td>
<td>3.28</td>
<td>2.86</td>
</tr>
</tbody>
</table>
nucleic acid species is not altered drastically.

When a visual comparison was made of the elution patterns of ultraviolet absorbing material at 265 μ from growing and regressing tumor tissue the R-RNA fraction appeared to be quantitatively reduced in size in the regressing tissue more than the other fractions. This disproportionate reduction of the R-RNA fraction was further seen when the total amounts of RNA for each fraction were compared. Despite the implication that R-RNA synthesis in tumor tissue is preferentially controlled by the presence of estrogens the rate of labelling of the DNA containing fraction is reduced to a greater degree in the depelleted animal exposed to C\textsuperscript{14} Na formate for 10 hrs. than the R-RNA fraction.

The preliminary evidence thus far presented seems to indicate that hormone removal affects the rate of synthesis of DNA to a greater degree than the rate of synthesis of RNA in tumor tissue. Growth then of the adrenal tumor, as seen by an increase in size and weight of the tumor in pelleted animals, may be brought about by controlling the size a tumor cell obtains and the rate it divides.

4) Precursor supply in growing and regressing tumors

The precursor supply in growing and regressing adrenal tumors was examined in preliminary experiments to determine if the supply of nucleotides was limiting in the regressing tumor compared to the growing tumor; and might cause a decreased rate of synthesis of RNA and DNA. Weighed amounts of tumor
tissue from pelleted and unpelleted animals that were exposed for 4 and 10 hrs to 100 μcuries C\textsuperscript{14}Na formate were extracted with cold 0.7 N HClO\textsubscript{4} acid to obtain an acid soluble fraction. This fraction was chromatographed on a 1 x 3 cm DEAE cellulose column in the bicarbonate form. The nucleosides, bases and unused label were eluted quantitatively from the column with 0.005 M NH\textsubscript{4}HCO\textsubscript{3} pH 8.6; the nucleotide fraction was eluted from the column with 1 M NH\textsubscript{4}HCO\textsubscript{3} pH 8.6. The data summarized in Table VI are the results of many experiments in which the total amount of radioactivity in the nucleotide fraction was examined. The amount of radioactivity/gm of tissue in the nucleotide fraction of depelleted tumor bearing animals was less than in animals with growing tumors; after exposure to 100 μcuries of C\textsuperscript{14} Na formate (4.01 mc/mmole) for 4 and 10 hours. The microscopic and macroscopic morphology of growing and regressing adrenal tumors shows a higher preponderance of connective tissue in the regressing than in the growing tumors. Preliminary evidence suggests then that hormone removal causes a reduction in cell division and growth, with the net effect of fewer cells/gm regressing tumor. If there were fewer cells/gm tissue then there would be less radioactivity in the nucleotide fraction and total amounts of RNA and DNA would be reduced. In order to eliminate this and eliminate the possibility that estrogen is acting at a site other than within the cell in vitro experiments were performed.

5) In vitro experiments

In order to establish that the hormone was not exerting
TABLE VI. Distribution of radioactivity in the acid soluble fraction of growing and regressing tumor tissue.

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Description and Labelling Time</th>
<th>Radioactivity in Nucleotide Fraction C.P.M./gm of Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na formate</td>
<td>Growing tumor 4 hrs</td>
<td>19,105</td>
</tr>
<tr>
<td>Na formate</td>
<td>Growing tumor 4 hrs</td>
<td>20,129</td>
</tr>
<tr>
<td>Na formate</td>
<td>Tumor from depelleted animal 4 hrs</td>
<td>12,701</td>
</tr>
<tr>
<td>Na formate</td>
<td>Tumor from depelleted animal 4 hrs</td>
<td>15,228</td>
</tr>
<tr>
<td>Na formate</td>
<td>Growing tumor 10 hrs</td>
<td>18,354</td>
</tr>
<tr>
<td>Na formate</td>
<td>Growing tumor 10 hrs</td>
<td>17,632</td>
</tr>
<tr>
<td>Na formate</td>
<td>Tumor from depelleted</td>
<td>9,720</td>
</tr>
</tbody>
</table>
its stimulatory action indirectly by altering other parameters in the whole animal instead of acting directly at the cell level. Single cell suspensions were prepared from growing and regressing tumors, and aliquots were incubated in Robinson's media (10% with respect to horse serum) with radioactive precursors for periods up to 2 hours. Under these conditions 2-Cl4 TdR and C14 Na formate were incorporated linearly into the DNA fraction over a period of 2 hours. C14 Na formate, 8-C14 hypoxanthine, C14-uridine (U) and 6-C14-orotate incorporation into RNA was completed within 30 minutes.

The data summarized in Table VII is a comparison of the ability of cells from growing and regressing tumor tissue to synthesize RNA and DNA, labelled with C14-2-uridine (495 mc/mmole) and 2-C14-TdR (54.5 mc/mmole) respectively. The tumor was allowed to regress for 14 days, at this time the rate of formation of DNA was reduced but the rate of RNA synthesis was not in Experiment II and III. Tumor lines may vary in their synthetic activities. Table VII, Experiment I shows a comparison of RNA labelled with C14-uridine (U) 6-C14-orotate, and DNA labelled with 2-C14-TdR, by tumor cells from pelleted and unpelleted animals. The rate of synthesis of RNA and DNA in this tumor line depends on the presence of the hormone. Any influence by cell numbers on the ability of cells to synthesize in vitro RNA and DNA has been eliminated by examining the rate of synthesis of these molecules.

6) Comparison of DNA polymerase activity in growing and regressing tumors
TABLE VII. \( ^{14}C \) Uridine and \( ^{14}C \) TdR labelling of RNA and DNA by cell suspensions prepared from growing and regressing tumors.

<table>
<thead>
<tr>
<th>Type of Tumor</th>
<th>Precursor</th>
<th>S.A. of DNA</th>
<th>S.A. of RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td>2-( ^{14}C ) TdR</td>
<td>6-C( ^{14}C ) orotate</td>
<td>6-C( ^{14}C ) orotate</td>
</tr>
<tr>
<td>Growing tumor male (6A-8) unpelleted male 14 days</td>
<td>no incorporation</td>
<td></td>
<td>61.8</td>
</tr>
<tr>
<td>Growing tumor male depelleted female 14 days</td>
<td>2-( ^{14}C ) TdR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growing tumor female (ovarectomized) (3A-8) depelleted female 14 days</td>
<td>3.755</td>
<td>1.530</td>
<td>0.3542</td>
</tr>
<tr>
<td>Average of 4 determinations</td>
<td>49.606</td>
<td>62.876</td>
<td>50.967</td>
</tr>
</tbody>
</table>
Although the method used (72) to solubilize DNA polymerase produces a crude homogenate, the enzymatic activity of this fraction has requirements similar to various purified mammalian enzymes (72-76). Figure 5(a) is an enzyme concentration curve for DNA polymerase. The incorporation of $8-C^{14}\text{dATP}$ into acid insoluble counts by DNA polymerases from both growing and regressing tumor tissue was dependent on protein concentration. Figure 5(b) shows a time curve for DNA polymerase. The incorporation of $8-C^{14}\text{dATP}$ into acid insoluble counts by DNA polymerases from regressing and growing tumor tissue was linear for 8 and 15 minutes respectively, with optimum amounts of enzyme. The activity of the enzyme from regressing tumor is lower under all conditions. The response to increasing amounts of primer DNA is similar in regressing tumor to growing tumor.

The results in Table VIII indicate that on the basis of specific activity measurements there is proportionately 50% less activity in the fraction from the regressing tumor. These results agree with earlier findings; the affect of removing the hormone from tumor bearing animals appears to be directed more at the level of DNA synthesis than RNA synthesis.

7) RNA polymerase activity in growing and regressing tumors

The experiments reported here are of a very preliminary nature.
Figure 5 (a). Activity of DNA polymerase in homogenates of growing and regressing tumor tissue. Incorporation of $^{14}$C d ATP into DNA - Effect of protein concentration. Each tube contained 0.1 μc $^{14}$C d ATP (28 μ moles), 12.2 μ moles d ATP, 80 μ moles of each of d GTP, d CTP and TTP, 1 μ mole dithiothreitol, 1 μ mole MgCl$_2$, Tris buffer pH 7.8 to final concentration of 50 m Molar in a total volume of 0.5 mls, 25 μg denatured calf thymus DNA and 1 μ mole ATP. The incubation was carried on at 37°C in a water bath.
Figure 5 (b). Incorporation of $^4\text{C} \text{d ATP}$ into DNA by cell free systems at different times. Each tube contained 0.1 μc of $^4\text{C} \text{d ATP}$ (28 m μ moles), 12.2 m μ moles d ATP, 80 m μ moles of each of d GTP, d CTP and TTP, 1 μ mole dithiothreitol, 1 μ mole MgCl$_2$, Tris buffer pH 7.8 to final concentration of 50 m Molar in a total volume of 0.5 ml, 25 μg denatured calf thymus DNA and 1 μ mole ATP. The incubation was carried on at 37°C in a water bath.
TABLE VII  The DNA polymerase activity from the 105,000 g supernatant from cell homogenates of growing and regressing tumor tissue.

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>m μmoles of C¹⁴dAMP incorporated /mg Protein/30' incub.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growing</td>
</tr>
<tr>
<td>Med. ENZ 15' incub. at 37°C</td>
<td>0.07292</td>
</tr>
<tr>
<td>-denatured DNA</td>
<td>0.02358</td>
</tr>
<tr>
<td>+undenatured DNA (25 μg)</td>
<td>0.09126</td>
</tr>
<tr>
<td>+low amt. den. DNA (10 μg)</td>
<td>0.05828</td>
</tr>
<tr>
<td>+high amt. den. DNA (75 μg)</td>
<td>0.1672</td>
</tr>
</tbody>
</table>
The rate of incorporation of C\textsuperscript{14}UTP (U) into acid insoluble counts was not linear after 3 minutes incubation with RNA polymerases from both growing and regressing tumor tissue. Degradative enzymes may be responsible for some of this decrease in synthetic activity. The activity seems to reach a plateau after a very short time interval, for this reason we were not able to establish an adequate time curve. If a comparison is made at any one time interval between RNA polymerases from growing and regressing tumor tissue a difference can be seen. RNA polymerases from growing and regressing tumor tissue showed a greater affinity for Mn\textsuperscript{++} than Mg\textsuperscript{++}, both enzymes are stimulated by (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, their results are seen in Table IX. After 7 minutes incubation time when the activities had plateaued a difference in activity can be seen between RNA polymerases of growing and regressing tumor tissue. Table IX illustrates that in most cases the enzyme from regressing tissue incorporates C\textsuperscript{14} UTP (U) into acid insoluble counts at a lower rate than the enzyme from growing tumor tissue.

In summary, the hormone appears to be stimulating the synthesis of DNA and to a lesser degree the synthesis of RNA by altering the synthetic abilities of DNA dependent, DNA and RNA polymerases. This level of hormonal stimulation of adrenal tumor tissue is observed both \textit{in vivo} and \textit{in vitro}.

C. Discussion

The paramino salicylate method of extracting nucleic acids proved to be convenient, and the recovery of DNA and
TABLE IX  A comparison of the activity of nuclear RNA polymerases in growing and regres­sing tumor tissue.

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>M μmoles of C^{14} UMP incorporated /mg DNA/30'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growing</td>
</tr>
<tr>
<td>Med. amt. of Nuclei; 7' incub. at 37° C</td>
<td>15.5</td>
</tr>
<tr>
<td>-Mg + Mn 0.5 μm</td>
<td>16.85</td>
</tr>
<tr>
<td>+ (NH₄)₂SO₄</td>
<td>35.95</td>
</tr>
<tr>
<td>+ Mn 1.0 μm</td>
<td>16.45</td>
</tr>
<tr>
<td>+ Mn 2.0 μm</td>
<td>14.95</td>
</tr>
<tr>
<td>+ Mn 4.0 μm</td>
<td>14.29</td>
</tr>
<tr>
<td>-MN + Mg 1.0 μm</td>
<td>12.25</td>
</tr>
<tr>
<td>+ Mg 8.0 μm</td>
<td>18.86</td>
</tr>
</tbody>
</table>
RNA is more efficient than with the disulphonate paramino salicylate treatment or standard salt extraction.

The milder treatment of extracting nucleic acids with paramino salicylate and denaturing the proteins with a phenol-cresol mixture has the disadvantage of extracting glycogen as well as the nucleic acids. Although there is no conclusive evidence of complete removal of polysaccharides by their relative insolubility in buffered saline solutions or by enzymatic digestion, that which may remain does not appear to interfere with separation of nucleic acids by column chromatography.

The elution patterns observed in the present study with methylated albumin Kieselguhr columns are consistent with that reported in the literature.

Chromatography on columns of methylated bovine serum albumin absorbed on Kieselguhr has proved a very useful tool to fractionate nucleic acids. The separation of the broad classes of RNA and DNA is clear, and recovery of undenatured nucleic acids is virtually quantitative. Philipson (78) extracted nucleic acids from HeLa cells and chromatographed them on a MAK column; he reported a degradation, sRNA, DNA and R-RNA peaks. Ellem (74,80) and Yoshikawa (81) have found that a large part of rapidly synthesized RNA from L cells is bound too tenaciously to the MAK column to be eluted by a salt gradient; it could be eluted either with 1.5 M NH₄OH or 1.5 M NaCl at an elevated temperature (90°C).
Many other authors (82-94) have extracted nucleic acids from plant and animal tissues and chromatographed them on Mandel and Hershey's 3 layered columns (82) or Sueoka's (83) one layered column and obtained results similar to those reported here. Fraser (84) recently has reported a 5S microsome RNA fraction that is eluted from the MAK column close to our x-RNA fraction.

The nucleic acid species eluted from the MAK columns in this project were identified in the following order and manner. sRNA fraction by sugar determination and column chromatography of standard sRNA. The DNA component was identified by these two methods and degradation with DNase I which was (RNase free). The R-RNA fraction was examined by all three methods. Some authors omit the degradation fraction when reporting their results, this relatively rapidly labelled species may contain oligoribonucleotides that are insensitive to RNase digestion under the present incubation conditions. The sRNA species was RNase insensitive while R-RNA was RNase sensitive. It was felt that the incubation conditions employed were not drastic enough to effect hydrolysis of the helical areas in the sRNA molecule. Many authors refer to the material eluted with 1.5 M NH₄OH or 0.2% sodium dodecyl sulphate in buffered 0.4 M NaCl as rapidly labelled DNA-like RNA, or mRNA; (95,96) others refer to this peak as single stranded DNA. (97). RNase digestion and color determinations of material in this fraction do not tend to substantiate these
theories. mRNA is degraded in 5 minutes with RNase at 0°C, while the yRNA was RNase insensitive and did not contain DNA, as shown by color reaction and enzymatic degradation; the specific activity of yRNA was higher than R-RNA but similar to sRNA and the degradation fraction. Further investigations with different isolation methods may help to identify the degradation, xRNA and yRNA peaks. For the present purposes the MAK column appears suitable for differentiating the general species of nucleic acids.

On comparing tumor, liver and spleen tissues from pelleted animals, exposed for 4 hrs to radioactive Na formate, it appears that tumor tissues are synthesizing nucleic acids quite slowly; but on comparing synthesis after 10 hrs exposure to the isotope, nucleic acid synthesis compared to other tissues appears to have reached an optimum. This may be due to the remoteness of the tumor from the site of injection or this time is taken to label the precursor pool of tumor tissue and hence label RNA and DNA maximally.

When optical density scans of ultraviolet absorbing material at 265 mμ from growing and regressing tumor tissue were examined the R-RNA fraction from regressing tumor appeared diminished visually and spectrophotometrically more than other fractions.

One explanation for these observations may be that the numbers of cells/gm of regressing tumor tissue are less than in the growing tumor; both microscopic and macroscopic observations tend to support this.
Precursor supply may be limited. Nucleotide supply is ATP dependent, the formation, transportation, or utilization of this energy source may be affected when the estrone pellet is removed. These observations can be explained very easily if there are fewer cells/gm of tissue in the regressing tumor and hence less RNA and DNA.

However, specific activities which are a better reflection of nucleic acid synthesis show that all fractions in regressing tumors are being labelled at a slower rate than in growing tumor. The rate of labelling of the DNA fraction is reduced to a greater degree than the other species. In order to eliminate any effect that fewer cells/gm of tissue may have and the possibility that the hormone may be affecting the animal at an organ level and not intracellularly, in vitro experiments utilizing a variety of precursors were performed. The ability of growing and regressing tumor tissue to synthesize nucleic acids was compared. In some tumor lines both RNA and DNA synthesis was decreased after tumor bearing rats were depelleted for 14 days whereas in other lines just DNA synthesis was decreased. These observations in vitro supplement the data obtained in whole animal experiments, hormone removal affects the synthesis of RNA and DNA (i.e. RNA and DNA polymerase).

The activity of key enzymes in the synthesis or degradation of nucleic acids may be affected by the removal of the hormonal stimulus. Some very preliminary experiments were done to compare activities of RNA, DNA polymerases in growing
and regressing tumor tissue. These enzymes were not purified, because a comparison between the enzyme activity in growing and regressing tumor tissue was all that was desired.

The activity of DNA polymerase from growing and regressing tumor tissue is dependent on protein concentration. The enzyme of regressing tumor tissue is not so dependent on protein concentration for its activity as the enzyme in growing tumor tissue; this may reflect the presence of degradative enzymes. Both enzymes prefer denatured DNA as a primer in preference to native DNA. When the estrone pellet was removed from tumor bearing animals the activity of DNA dependent DNA polymerase in tumor tissue was reduced 50%.

The nuclear RNA polymerases from growing and regressing tumor tissue both show preference for Mn$^{++}$ over Mg$^{++}$, and are stimulated by (NH$_4$)$_2$SO$_4$. One of the main difficulties in this preliminary experiment was that the activity of RNA polymerase was not linear after 3 minutes incubation time. If one compares the specific activity of RNA polymerase from growing tumor with that of regressing tumor after a plateau in activity is reached, the enzyme from growing tumor has a higher specific activity. These results complement those obtained in whole animal and in vitro experiments.

The total amount of radioactivity per gram of tissue in the nucleotide fraction of regressing tumor is less than in the nucleotide fraction of growing tumor.

In summary, we have studied the general nucleic acid metabolism of an estrogen dependent adrenal cortical tumor. The tumors regress in size and synthetic activity after the
estrone pellet has been removed. If the pellet is not replaced the tumor will regress to a small nodule and stay in this inactive form until again stimulated by estrogens. Experiments in which the RNA and DNA synthesis were measured under a variety of conditions (in vivo, acid soluble, in vitro DNA and RNA polymerase experiments) indicate that the synthesis of all species of nucleic acid are reduced upon hormone removal. The synthesis of DNA is reduced more than the synthesis of RNA, more work is needed before conclusions can be drawn as to the action of estrogens in this tumor system.

Some interesting experiments that should be performed might include looking at the effect of hormone removal on RNA metabolism after short time intervals, i.e. 4-5 hours, the effect of injecting 17β estradiol into animals or the addition of 17β estradiol to in vitro cell incubations, to see if a reversal of the decrease in RNA and DNA metabolism of regressing tumors is possible.

In conclusion then, the stimulation of RNA and DNA synthesis in this hormone dependent adrenal cortical tumor with estrogens, is a different type of response than that seen in target tissues like uterus, where only RNA synthesis is stimulated when the animal is treated with 17β estradiol.
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