THE ACTION OF OESTRADIOL-17β ON THE RNA POLYMERASES IN THE UTERUS OF THE IMMATURE RABBIT

by

Neil M. Borthwick

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

T. S. Eliot

Little Gidding - Four Quartets.

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ABBREVIATIONS.

In general, abbreviations are as recommended in the Instructions to Authors of the Biochemical Journal, 1972. Additional abbreviations used are:-

DES	:	diethylstilboestrol
DTT	:	dithiothreitol
[Eσ]	:	*E. coli* RNA polymerase holoenzyme
HnRNA	:	heterogeneous nuclear RNA
OE_2	:	oestradiol
poly A	:	polyadenylate
poly d(AT)	:	polydeoxyadenylate thymidylate
SDS	:	sodium dodecyl sulphate
rDNA	:	cistrons for rRNA

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SUMMARY.

1. Several forms of DNA-dependent RNA polymerase have been purified from the uteri of immature rabbits. The isolation procedure involved the extraction of total uterine protein from a whole tissue homogenate using high salt concentrations. The RNA polymerases were partially purified by DEAE-cellulose chromatography and resolved into three species of enzyme which have been designated RNA polymerases A, B and C. These enzymes have been further purified by chromatography on phosphocellulose and by glycerol density gradient sedimentation.

2. The two major species of RNA polymerase, namely A and B, have been extensively characterised. Both enzymes sediment slightly faster than E. coli RNA polymerase in glycerol gradients suggesting a molecular weight in the range of 500,000 - 600,000. RNA polymerase A is more active in low concentrations of salt, although it can utilise both Mg^{++} and Mn^{++} efficiently. RNA polymerase B is more active in high concentrations of salt with Mn^{++} rather than Mg^{++} present as the divalent cation. RNA polymerase A is insensitive to the action of the toxin α-amanitin which specifically inhibits RNA polymerase B at similar concentrations. However, RNA polymerase A is more susceptible to thermal treatment than is RNA polymerase B. The template specificities of both enzymes have also been investigated.

3. A third species of enzyme, designated RNA polymerase C, has been partially purified and characterised. This enzyme may be cytoplasmic in origin or may be 'soluble' with the result that it is leached out readily from the nuclei. RNA polymerase C has some properties similar to those of enzymes A and B and some which are intermediate between the two major enzyme species.

4. Two RNA polymerase activities have been identified in isolated nuclei; one has been equated with RNA polymerase A while the other has been equated with RNA polymerase B.

5. *In vitro* incubation of oestradiol with uteri has shown that the stimulation of RNA polymerase activities in isolated nuclei is only slight when compared with the activities measured in nuclei obtained from uteri treated with oestradiol *in vivo*.

6. When measuring the endogenous RNA polymerase activities of isolated nuclei, prior treatment of the rabbits with oestradiol had a profound effect on the transcriptional capacity. Within 30-45 min after hormone treatment, the activity of RNA polymerase B was considerably increased. This activity decreased towards control levels at 1-2h before exhibiting a second increase of activity at about 3h. From 1h after oestradiol treatment, RNA polymerase A activity in the isolated nuclei was also increased and reached a plateau by about 4h. Both activities have been shown to be sensitive to the action of actinomycin D.

7. Treatment of the animals with α-amanitin prior to oestradiol inhibited the hormone-induced stimulation of RNA polymerase A as well as totally inhibiting RNA polymerase B. However, when α-amanitin was administered after the early enhancement of RNA polymerase B, the oestradiol-induced stimulation of RNA polymerase A was retained.

8. Treatment of the animals with cycloheximide prior to oestradiol did not affect the stimulation of RNA polymerase B but prevented the oestradiol-induced enhancement of RNA polymerase A. However, when cycloheximide treatment was delayed until after the early stimulation of RNA polymerase B, the activity of RNA polymerase A was stimulated.

This suggested that stimulation of RNA polymerase A activity was dependent on protein synthesis subsequent to the hormone-induced stimulation of RNA polymerase B.

9. Since some indications were obtained of an effect of cytoplasm from oestradiol-treated rabbit uteri on the RNA polymerase activity in nuclei, attempts were made to concentrate any such components. It was found that a fraction of the cytoplasm isolated from uteri treated with oestradiol for 30 min was capable of stimulating RNA polymerase A activity in nuclei isolated from control animal uteri.

10. The isolated RNA polymerases from immature rabbit uteri do not show any increase in activity in response to oestradiol irrespective of the time of treatment with hormone. No fractions from cytoplasm treated with hormone have been shown to possess any stimulatory activity for either RNA polymerase A or B. It is possible that the observations of increased RNA polymerase activities in isolated nuclei result from changes in the transcriptional machinery rather than being due to alterations in the RNA polymerases *per se*.

INTRODUCTION

1. Control in Living Systems.

One of the most important problems in biochemistry is the mechanism by which a cell can respond to changes in its external environment by altering the synthesis or activity of certain specific proteins. Even small bacterial viruses, containing as few as three genes, are able to control their development and function (Stavis & August, 1970) and it is not surprising that the first control systems to be elucidated were in viruses and bacteria.

One of the earliest attempts to explain metabolic control in bacteria was a theory of induction and repression of protein synthesis (Jacob & Monod, 1961). It was postulated that a specific repressor protein combined with a particular site on the DNA called the operator thus blocking the transcription of the adjacent gene. Certain molecules called inducers were able to cause the dissociation of this operator-repressor complex, allowing the transcription of previously repressed genes. The work of Gilbert & Müller-Hill (1966; 1967) confirmed these concepts and more recently, *in vitro* systems responding to the regulatory compounds have been developed (deCrombrugghe *et al.*, 1971).

Another control system demonstrated in bacteria was feed back inhibition (Umbarger, 1969). In many of the synthetic pathways of biological compounds products, formed late in the reaction sequence, control the activities of enzymes catalysing earlier reactions. By this method, the cell can prevent further synthesis of all the intermediates and the product of the synthetic pathway.

In multicellular organisms, the term 'control' has much wider implications, embracing processes such as cellular differentiation, organ development and maturation. Very little is known about the interrelationship of individual cells within a population and the manner

in which they influence each other. In addition, the overall coordination of control mechanisms in the whole organism remains poorly understood.

Higher animals possess two major systems by which one tissue can influence the activity of another; the nervous system and the endocrine system. The nervous system transmits information in the form of electrical impulses from the brain to the tissues. By contrast, the endocrine system elicits responses by means of the hormones which are chemical mediators of communication between the different cell types of an organism.

Each hormone produces specific biochemical and physiological changes in its target tissues. There are several different types of hormone and one of these, the steroid hormones, is believed to elicit the response by acting in part in the cell nucleus of their target tissues. Steroid hormones control a wide variety of specific changes in mammals and one of these, maturation, is controlled by androgens in the male and oestrogens and progestins in the female. The mode of action of oestrogens at the molecular level is the subject of this thesis.

2. Biochemistry of the Target Tissue Response to Oestrogens.

Entry of oestradiol into the cells of its target tissue sets in motion a sequence of biochemical events which eventually leads to the physiological changes such as proliferation of the epithelium of the vagina, cervix, endometrium and Fallopian tubes. This section reviews the biochemistry of oestrogen action and, where relevant, compares and contrasts its response with that of other hormones.

2.1. Transport to and Entry into the Target Tissues.

Oestrogens are synthesised continuously in small amounts by the

ovaries and in large amounts by a maturing follicle. They are transported via the blood stream to their target organs, the uterus, the vagina, the mammary glands and the pituitary. Oestrogens spontaneously and reversibly associate with serum proteins to form complexes. Serum albumin is well known for its ability to form associations with many compounds including steroid hormones (Bennhold, 1966), and although the binding is of low affinity, it appears to be important for their transport. Tavernetti et al. (1967) demonstrated the presence of a protein in the β-globulin fraction which bound oestrogens with high affinity. This oestrogen binding protein has been reported in several animal species including cows and man but not in rats, rabbits or dogs (Murphy, 1968). The hormone-protein complex provided a convenient method for the transport of the hormone to the target tissue due to its rapid dissociation.

Little is known about the mechanism of entry of oestrogens into their target tissue cells, but the fact that these cells accumulate the hormone does not in itself imply an active transport mechanism. Milgrom et al. (1972) claimed that oestrogen entry was mediated by a protein and that the process could be inhibited by treatment with sulphydryl blocking agents such as α-iodoacetamide. After this treatment, the amount of oestradiol bound by the cytoplasmic receptor decreased considerably, probably due to damage to the cytoplasmic receptor.

2.2. Target Cell Oestrogen Receptor Proteins.

Numerous recent studies have revealed the presence of specific receptors in oestrogen sensitive and other hormonal target tissues. These receptor proteins appear to be responsible for accumulation of the hormone in the cytoplasm and its transport to the nucleus (Jensen & DeSombre, 1972).

2.2.1. The Cytoplasmic Receptor Protein.

The concept of steroid receptors initially arose from studies involving the administration of tritiated oestradiol to immature rats (Jensen & Jacobson, 1962) when it was shown that only target tissues possessed the ability to retain the hormone against a concentration gradient in the blood. These observations were confirmed by biochemical and autoradiographic methods (Noteboom & Gorski, 1965; Stumpf & Roth, 1966) and it was demonstrated that although much of the accumulated hormone was sited in the nucleus, about 20-30% was present in the cytoplasm. Toft & Gorski (1966) showed that a soluble cytoplasmic protein capable of binding tritiated oestradiol occurred in rat uterus. This protein was considered to be a receptor because significant amounts were only found in oestrogen target tissues and because of the specificity and high binding affinity of the protein for biologically active oestrogens such as oestradiol and the synthetic non-steroidal oestrogen, diethylstilboestrol (DES). The cytoplasmic hormone-protein complex sedimented in sucrose gradients with a value of about 8S when compared with yeast alcohol dehydrogenase (Toft & Gorski, 1966; Rochefort & Baulieu, 1968).

In sucrose gradients with an ionic strength greater than 0.2M KCl, the cytoplasmic 8S receptor was reversibly transformed to a more slowly sedimenting 4S form (Erdos, 1968; Korenman & Rao, 1968; Jensen et al., 1969). An intermediate 6S form has also been reported under physiological ionic conditions (Baulieu et al., 1971). Thus, the reported sedimentation coefficients from various laboratories vary to a considerable extent (Chamness & McGuire, 1972). Stancel et al. (1973a) showed that multiple forms of oestrogen receptors could be produced by concentration dependent aggregation and suggested that the cytoplasmic receptor may exist as the 4S form rather than the 8S entity. With

partially purified calf uterine cytoplasmic complexes, estimates of 200,000 and 5.8 for molecular weight and isoelectric point respectively, were found for the 8S complex compared to 75,000 and 6.4 for the 4S unit (DeSombre et al., 1971). More recently, characterisation of the cytoplasmic receptors which had been purified by affinity chromatography, showed that the 8S form has a molecular weight of approximately 240,000 while the molecular weight of the 4S unit is about 60,000 (Puca et al., 1971; Sica et al., 1973). Once the receptors have been purified to homogeneity, it should be possible to obtain accurate values.

It appears that sedimentation behaviour of receptor proteins can vary in relation to concentration and ionic conditions but sedimentation values are still a useful method for the identification of steroid binding proteins. At present, there is no means of determining the exact size or configuration of a receptor as it exists in vivo.

2.2.2. Transfer of Hormone-Receptor Complex to the Nucleus.

The pioneering work of Gorski et al. (1968) and Jensen et al. (1968) led to the concept that following an oestrogen-induced conformational change in the uterine cytoplasmic receptor protein, this complex was transferred to the nucleus. Jensen et al. (1968) demonstrated a temperature dependent (37°) intracellular transfer of protein-bound oestradiol from the cytoplasm to the nucleus of the rat uterus. About 50% of the nuclear form of the hormone-receptor complex can be extracted by 0.3M KCl and this fraction was found to have a sedimentation coefficient of 5S in sucrose gradients. This complex was only detected in cells which had been exposed to oestradiol. Exposure of isolated nuclei to tritiated oestradiol and cytoplasmic receptor led to an accumulation of a salt extractable 5S complex in the nuclei, whereas no such complex was found when nuclei were incubated with oestradiol alone.

These observations led to the 'two step' hypothesis (Jensen et al., 1968) which suggested that the 5S nuclear complex represented an altered form of the 8S cytoplasmic receptor.

This modification probably occurs in vivo before entry of the 8S receptor into the nucleus. Gorski et al. (1973) suggest that the cytoplasmic binding protein undergoes a conformational change in the cytoplasm after complexing with oestrogen, so permitting it to relocate in the nucleus. This view is supported by the work of Stancel et al. (1973b) who have shown that under denaturing conditions, both cytoplasmic and nuclear forms of the oestrogen receptor sediment at 3.6S suggesting that both forms contain a common subunit which binds oestrogens.

It seems likely that the cytoplasmic receptor functions by delivering the regulatory hormone to the nucleus although an alternative possibility, proposed by Jensen et al. (1971), is that the hormone may function in the transport of the receptor protein to the nucleus where it may act in an analogous manner to a bacterial sigma factor as an inducer of RNA synthesis.

2.3 Binding of Oestradiol to Chromatin

Teng & Hamilton (1968) showed that within 2 min of administration of tritiated oestradiol to ovariectomised rats, the hormone had entered the uterine cell nucleus and was bound to the chromatin. This binding was measured by the radioactivity which was not removed by dialysis at 0^{o} and reached a maximum 8h after hormone treatment.

The function of receptor proteins is not restricted to the transport of hormone to the nucleus. Thus, Steggles et al. (1971a) have demonstrated that receptors are involved in the binding of the hormone to the target cell chromatin. When uterine chromatin is incubated

in vitro with tritiated oestradiol, very little hormone binding is observed but *in vitro* incubation of chromatin with tritiated hormone-receptor complex resulted in significant amounts of complex being retained on the chromatin.

Uterine oestrogen receptors have also been shown to bind to DNA under *in vitro* conditions (King & Gordon, 1972; Toft, 1972). The binding is sufficiently strong to withstand centrifugation of the DNA through sucrose gradients under ionic conditions of up to 0.1M KCl. Using DNA-cellulose chromatography, Toft (1973) has shown that both cytoplasmic and nuclear receptors can bind to DNA. This binding is disrupted by 0.3M KCl and there are probably a limited number of high affinity binding sites or 'acceptor' sites on the DNA for the receptor. King & Gordon (1972) estimated that the number of oestrogen binding sites was 2 sites per 10^7 nucleotides of DNA or about 500 sites per nucleus, while Higgins *et al.* (1973) estimated that the number of 'acceptor' sites was about 4000 per genome in both rat uterus and hepatoma cell.

It appears that the binding site involves other components of chromatin to modify receptor binding to DNA. Chromatin from non-target tissues appear to bind quantitatively less receptor complex than target tissue chromatin (King & Gordon, 1972). Similar results have been reported for androgen receptor interactions with prostate chromatin (Mainwaring & Peterken, 1971; Liao *et al.*, 1973) and progesterone-oviduct cytosol complex with oviduct chromatin (Spelsberg *et al.*, 1971a, b; Stoggles *et al.*, 1971b). By reconstitution of chromatins, Spelsberg *et al.* (1971a) showed that the chromatin lost most of its ability to bind the progesterone-receptor complex if the oviduct non-histone chromosomal proteins were removed. Insertion of oviduct non-histone chromosomal proteins into erythrocyte chromatin endowed a binding capacity to this hybrid chromatin similar to that observed in native oviduct chromatin

(Spelsberg et al., 1972; O'Malley et al., 1972). Additional experiments localised this 'acceptor capacity' to a certain fraction of the total non-histone chromosomal protein of target cell chromatin (Spelsberg et al., 1972). Schrader et al. (1972) demonstrated that only the B subunit of the progesterone receptor had the ability to interact with oviduct non-histone chromosomal proteins.

From these various experiments, it seems that both DNA and a non-histone chromosomal protein fraction play an important role in the formation of 'acceptor sites' for progesterone receptor in chick oviduct chromatin and dihydrotestosterone receptor in prostatic chromatin. Although less information is available on the binding of oestradiol to uterine chromatin, King et al. (1969) have reported the presence of an acidic protein from rat liver which binds tritiated oestradiol in vivo and Alberga et al. (1971) have described a non-histone chromosomal protein from rat uterus with a high binding affinity for oestradiol.

Therefore, it seems probable that the binding of the hormone-receptor complex to the genome may be of major importance to the understanding of steroid hormone action.

2.4. Effect of Oestradiol on the Chemical Composition of Chromatin.

Teng & Hamilton (1968) showed that in rat uteri the RNA to DNA ratio increased by 17% within 15 min of oestradiol treatment and the protein to DNA ratio increased by 35% 2-4h after hormone administration. This stimulation of protein synthesis did not appear to involve the histones (Barker, 1971; Glasser et al.,1972) but a rapid stimulation in the synthesis of non-histone chromosomal protein has been observed by a number of workers in the uterus of both rats and mice (Teng & Hamilton, 1969; Smith et al., 1970; Glasser et al., 1972). Some evidence indicated that a specific fraction of these acidic proteins was

synthesised in response to the hormone (Teng & Hamilton, 1970) and Barker (1971) demonstrated, associated with uterine histone F_{III}, the presence of an acidic protein whose synthesis was stimulated within 45 min of oestrogen adminstration.

2.5. Effect of Oestrogen on Chromatin Template Activity.

Administration of oestradiol to ovariectomised rats has been shown to give rise to an increase in chromatin template activity (Barker & Warren, 1966; Warren & Barker, 1967; Teng & Hamilton, 1968) when this is measured using exogenous E. coli RNA polymerase rather than endogenous enzyme. The limitations of such an approach were discussed by Dati & Maurer (1971) who recorded an oestrogen-induced increase in chromatin template activity of rat uterus but a decrease in mouse uterine chromatin template activity.

Increased template activity in chromatin isolated from rabbit endometrium has been demonstrated by Church & McCarthy (1970) using the endogenous enzyme. Within 2h of hormone treatment, activity rose by 500% and the addition of E. coli RNA polymerase was found to have no effect on the amount of RNA synthesised. The change in template activity led to an increase in the rate of RNA synthesis in vivo.

Glasser et al. (1972) also showed an increase in rat uterine chromatin template activity using endogenous RNA polymerase which was demonstrable by 30 min after hormone treatment, was maximal at 1h and remained constant for a further 4h before falling back to control levels by 8h. This increase in template activity could be attributed to either an increased availability of transcribable DNA or an increase in the amount of RNA synthesised due to activation of the RNA polymerase.

2.6. Effect of Oestrogen on RNA Synthesis.

One of the most clearly established biochemical responses to oestradiol is the stimulation of RNA synthesis in target tissues. Aizawa & Mueller (1961) showed an increase in the measurable uterine RNA content 6h after hormone treatment and measurements of RNA synthesis performed using techniques of isotope incorporation, indicated a much earlier onset (Hamilton, 1964). By far the most extensive changes in RNA synthesis arising from oestrogen administration was the increased synthesis of rRNA which preceded the overall hypertrophy and hyperplasia of the uterus. This has been observed both at the level of the synthesis of rRNA and its precursors (Billing et al., 1969a; Knowler & Smellie, 1971) and in the production of increased numbers of ribosomes (Hamilton et al., 1968a).

Oestrogen also stimulated the uterine ribosomes to aggregate into polysomes (Teng & Hamilton, 1967) and these polysomes showed an increased incorporation of amino acids into protein, this stimulation being enhanced by 30% and 100% at 2h and 8-12h respectively after oestradiol treatment. Continued presence of hormone was found to be essential for the maintenance of polysome levels.

Evidence is accumulating that this stimulation of synthesis of rRNA may be preceded by, and possibly dependent upon, mRNA synthesis. Initial studies on the base composition of rapidly labelled nuclear RNA suggested that it was ribosomal-like (Hamilton et al., 1968a) but evidence that the early oestrogen stimulation was of mRNA rather than rRNA came from inhibition studies. Notides & Gorski (1966) demonstrated the induction of a specific protein in rat uterus 30 min after oestradiol treatment and showed that production of this protein was inhibited by actinomycin D (DeAngelo & Gorski, 1970). More recently, the presence of high molecular weight RNA has been observed 30-45 min after oestradiol

injection to immature rats (Knowler & Smellie, 1971; Luck & Hamilton, 1972). This RNA was shown to have all the characteristics of heterogeneous nuclear RNA (HnRNA) and oestrogen-stimulated increases in incorporation of labelled precursor were found in RNA species with a wide range of molecular weights (Knowler & Smellie, 1973). A mRNA precursor function has been proposed for HnRNA (Darnell et al., 1971a; 1973).

When these results are analysed together with the recent demonstration that oestrogen specifically stimulated the synthesis of mRNA coding for ovalbumin in the chick oviduct (Rosenfeld et al., 1972), it seems highly likely that an early stimulation of uterine RNA synthesis might also be necessary in the final hormone action. Knowler & Smellie (1973) have suggested that stimulated mRNA synthesis may be necessary for the synthesis of a small number of uterine proteins which in turn initiate the increased production of ribosomes.

2.7. Effect of Oestrogen on DNA-Dependent RNA Polymerase.

It is well documented that RNA polymerase activity is enhanced after oestradiol treatment. Gorski (1964) first characterised RNA polymerase activity in nuclei isolated from immature rat uteri and showed elevated levels of enzyme activity in rats treated with oestradiol for 1-4h. Hamilton et al. (1965) distinguished two distinct types of RNA polymerase in nuclei from ovariectomised rat uteri. One type, which was stimulated by Mg^{++}, appeared to catalyse the synthesis of rRNA in the nucleolus while the other synthesised a more DNA-like RNA in the nucleoplasm in the presence of Mn^{++} and 0.4M ammonium sulphate. The enzyme that responded to Mg^{++} ions showed increased activity 1h after oestradiol treatment but no alteration in the Mn^{++}/ammonium sulphate-stimulated activity was observed in the first 12h of hormone treatment

(Hamilton et al., 1965; 1968a). Nicolette et al. (1968) also found elevated RNA polymerase activity in nuclei isolated from uteri treated for 4h with oestradiol in vivo. Gorski et al. (1965) observed that inhibition of protein synthesis in vivo prevented the oestrogen-induced stimulation of RNA synthesis.

Barry & Gorski (1971) investigated the location of newly incorporated nucleotides in rat uterine RNA in order to determine whether these were incorporated into the 3' end or internal positions. They found that oestradiol stimulated incorporation only into the internal positions of the RNA and concluded that the increased RNA polymerase activity observed, was a consequence of stimulation in the rate of RNA chain elongation within 1h but did not affect the number of growing chains. This was interpreted as indicating that oestradiol did not stimulate the initiation of transcription of additional template.

In 1969, Roeder & Rutter (1969) succeeded in isolating multiple forms of RNA polymerase from rat liver. The nucleolar form, RNA polymerase I, was believed to synthesise rRNA while the nucleoplasmic enzyme, RNA polymerase II, catalysed the synthesis of high molecular weight heterodisperse RNA.

The nomenclature of mammalian nuclear RNA polymerases has not been uniform because different investigators have adopted various criteria in enzyme classification. The RNA polymerases I and II (Roeder & Rutter, 1970), which are the major species of nuclear RNA polymerases found in most tissues examined, have also been designated RNA polymerases A and B respectively (Chambon et al., 1970). The latter nomenclature will be used throughout the remainder of this thesis.

Few studies have succeeded yielding data in vitro which could be related to the in vivo oestrogen situation. The first in vitro effect of oestradiol on uterine RNA polymerase was demonstrated by Raynaud-Jammet & Baulieu (1969). Oestradiol was incubated with calf uterine

cytoplasm to form the receptor complex. This step was essential in order to show increased RNA polymerase activity in uterine nuclei which were later added to the cytoplasm. Similar observations were made by Arnaud et al. (1971a) who suggested that only the 4S form (5S in their determinations) of the oestradiol-receptor complex would stimulate RNA synthesis in vitro by acting on RNA polymerase A. They later proposed that phosphorylation of the 4S complex and the RNA polymerase greatly enhanced the ability of the complex to stimulate RNA synthesis (Arnaud et al., 1971b).

Mohla et al. (1972) also reported stimulation of uterine RNA polymerase activity in vitro. This enhancement was effected only by oestradiol-receptor complex in which the receptor binding unit had been transformed from the native 4S form to a modified 5S form. Jensen et al. (1973) showed that the transformed receptor protein, which was not complexed with the hormone, also stimulated RNA synthesis in uterine nuclei. These results were used to support their hypothesis that receptor transformation is an important step in oestrogen action. They suggested that one of the biochemical functions of oestradiol may be to induce this transformation of the receptor to an active form that can enter the nucleus, bind to acceptor molecules and initiate RNA synthesis.

2.8. Effect of Oestrogen on Protein Synthesis.

The proteins synthesised in response to oestradiol fall into two groups. Firstly, there appears to be a few partially characterised species produced early in the hormone-elicited sequence of biochemical events, which may be essential for the observed changes in ribosomal and transfer RNA synthesis. Secondly, there is a later increase in total protein synthesis following the stimulation of RNA synthesis. These proteins play important structural and functional roles in the

hormone differentiated target tissue.

2.8.1. Early Proteins.

Early changes in the synthesis of uterine proteins were detected by Notides & Gorski (1966) following the incorporation of labelled amino acids into protein. They found an oestrogen-stimulated peak of precursor incorporation within 30 min of hormone treatment. This fraction was termed 'induced protein' and its synthesis was shown to be dependent on RNA synthesis (DeAngelo & Gorski, 1970). Barnea & Gorski (1970) demonstrated that the protein was synthesised de novo and Mayol & Thayer (1970) indicated that a group of acidic proteins, synthesised early in the oestrogenic response, might contain the same protein. This protein can also be induced in vitro by exposure of immature uteri to 1nM oestradiol for 60 min (Wira & Baulieu, 1971) and the 'induced protein' synthesis could also be suppressed by addition of actinomycin D to the incubation medium. Katzenellenbogen & Gorski (1972) compared 'induced proteins' synthesised in vivo and in vitro and found them to be identical.

Recently, Iacobelli et al. (1973) have begun a preliminary characterisation of 'induced protein' from rat uterus. SDS-polyacrylamide gel electrophoresis revealed a homogeneous polypeptide chain with a molecular weight of 45,000. The acidic nature of the 'induced protein' was indicated by an isoelectric point of 4.7 and a ratio of acidic to basic amino acid residues of 1.66. At present, no physiological role has been ascribed for the 'induced protein'. It has been suggested that it may function in gene expression leading to increased RNA synthesis. Thus, it would fulfil the role of a 'key intermediary protein' (KIP) (Baulieu et al., 1972). Unfortunately for this hypothesis 'induced protein' has not yet been shown to be present in

the nucleus.

2.8.2. Later Proteins.

2h after oestradiol administration, there is an increase in the synthesis of total uterine protein which is manifested by 12h in measurable increases in the total protein content (Means & Hamilton, 1966b). However, the uterus has not proved to be as amenable to the study of later protein synthesis as other oestrogen responsive tissues. Although the structural proteins involved in hormone-induced differentiation of the uterus will obviously be synthesised, there does not appear to be a readily detectable single protein species whose synthesis has been followed. Other systems exist for this study.

Oestrogens and progesterone also stimulate the growth of chick oviduct and cause the formation of glands for the secretion of egg white proteins (Kohler et al., 1968). O'Malley et al. (1967) showed that treatment of chicks with 5mg of DES daily caused increased synthesis of ovalbumin and lysozyme. If, after 12-18 days of DES treatment progesterone was given, the synthesis of avidin began within 6h. Thus, it appears that oestrogen can induce the synthesis of some oviduct proteins and prime the tissue for the synthesis of others.

Another readily detected product of oestrogen action is phosvitin, a protein containing many phosphorylated serine residues. This protein is normally produced in the livers of laying fowl (Heald & McLachlan, 1963) but its synthesis was stimulated in cockerel liver 24h after oestrogen administration (Greengard et al., 1965). It must be emphasised that these proteins are merely examples of oestrogen and progesterone-induced proteins whose synthesis can be readily followed, and that at this stage in the hormone response the tissue is synthesising many proteins necessary for differentiation.

2.9. Effect of Oestrogen on DNA Synthesis and Cell Division.

Kaye et al. (1972) demonstrated a stimulation of DNA synthesis in immature rat uterus 24h after a single injection of oestradiol. This enhancement was also age dependent. The epithelial, stromal and myometrial cells all displayed a wave of cell division with a peak of mitotic activity between 24 and 28h after hormone adminstration. Lee (1972) also showed stimulation of DNA synthesis in mouse uterus responding to continuous oestrogen treatment after 2-3 days. This activity fell to control levels on day 4 and 5 and was followed by a second wave of activity about a week later. However, DNA synthesis, mitosis and cell division are late responses to oestradiol compared with the other metabolic parameters covered in this review.

2.10. Effect of Oestrogen on Water Imbibition.

In the rat uterus there appears to be at least one hormone-induced response which can be separated from the others covered in earlier sections of this review; this is the increased uptake of water. Oestradiol causes an imbibition of water and ions in the rat uterus detectable as increased tissue wet weight 2h after hormone treatment (Mueller et al., 1958; Billing et al., 1969b). This induction of water uptake was inhibited by cortisol which had no effect on the enhancement of RNA and protein synthesis (Spaziani & Szego, 1958; Nicolette & Gorski, 1964). After oestradiol injection, uterine histamine levels decreased (Spaziani & Szego, 1958) and it was later shown that histamine imitated the oestrogenic uterine response of water uptake and anti-histamines inhibited it (Spaziani & Szego, 1959). Neither histamines nor anti-histamines had any effect on oestradiol-stimulated RNA synthesis (Hamilton et al., 1968a). This clearly

distinguished the imbibition of water by the uterus from the other responses.

3. DNA - Dependent RNA Polymerases.

The above review has shown that oestradiol can stimulate the cellular synthetic processes in target tissues. It is still unknown how these events are controlled. Most of the present theories envisage either a transcriptional or a translational control of protein synthesis. Since oestradiol stimulates production of both rRNA and HnRNA, it is possible that the hormone elicits its response by altering the chromatin template, the RNA polymerases or a combination of both. Before describing the results obtained in attempting to determine which mechanism occurs in the immature rabbit uterus, it is important to review the present knowledge of both bacterial and mammalian RNA polymerases.

3.1. Bacterial RNA Polymerase.

Most of the work on bacterial RNA polymerase has been with the *E. coli* enzyme. The structure and control of this enzyme has been the topic of several reviews (Richardson, 1969; Burgess, 1971; Travers, 1971), and only some of the details are presented here.

3.1.1. Subunit Structure of *E. coli* RNA Polymerase.

E. coli RNA polymerase is a large complex enzyme composed of several polypeptide subunits. The subunits have been designated β', β, σ, α, and ω in order of decreasing molecular weight. The values obtained for the molecular weights of the subunits are shown in Table I.

In 'core' enzyme, the stoichiometry of the subunits was $\alpha_2 \beta' \beta$

(Burgess, 1969) while in the active enzyme complex (holoenzyme), the stoichiometry appeared to be $\alpha_2\beta\beta'\sigma\omega_{0.5-2}$ (Travers & Burgess, 1969) giving an overall molecular weight of 490,000.

No function has been attributed to the α and ω subunits but β' has been shown to be essential for the binding of RNA polymerase to the DNA template (Zillig et al., 1970). Rabussay & Zillig (1969) demonstrated that the β subunit interacted with rifampicin, an inhibitor of bacterial RNA synthesis.

3.1.2. Sigma Factor.

The holoenzyme can be separated into sigma factor and 'core' enzyme by phosphocellulose chromatography (Burgess & Travers, 1970). The 'core' enzyme contained the catalytic site for RNA synthesis but its ability to transcribe native E. coli DNA was greatly reduced. Sigma factor possessed no synthetic activity itself but readily combined with 'core' enzyme to stimulate RNA synthesis. This stimulation was due to an increase in the number of RNA chains initiated rather than chain elongation (Travers & Burgess, 1969). Sigma is believed to stimulate initiation at certain specific sites, called promoter regions, on the DNA and to be released from holoenzyme after initiation (Berg et al., 1969). The degree to which initiation was stimulated depended on the type and quality of DNA used as a template. Stimulation was higher with intact double-stranded DNA which was poorly transcribed by 'core' enzyme. The introduction of single or double-stranded breaks in the template increased the ability of 'core' enzyme to begin transcription (Vogt, 1969).

RNA synthesis initiated on bacteriophage DNA in the presence of sigma factor, was restricted to the sites utilised in vivo immediately after infection, while in the absence of sigma, initiation occurred on

all regions of the genome. This observation was demonstrated by hybridisation of the RNA product to separated DNA strands from bacteriophage T4 (Bautz et al., 1969) and bacteriophage T7 (Summers & Siegel, 1969). Sugiura et al. (1970) showed the initiation of RNA synthesis by sigma factor at specific sites on fd replicative form DNA. Holoenzyme initiated three RNA chains of discreet size from one DNA strand while 'core' enzyme transcribed both DNA strands to produce a heterogeneous mixture of RNA molecules.

Thus, the sigma factor is of prime importance in the selection of initiation sites.

3.1.3. Psi Factor.

Up to 40% of the RNA synthesised in rapidly growing E. coli was shown to be rRNA (Kennel, 1968). However, when E. coli DNA was utilised as a template for holoenzyme in vitro, less than 0.2% of the total RNA synthesised was rRNA (Travers et al., 1970a). This was interpreted as meaning that another factor was required to initiate rRNA synthesis. A low molecular weight protein designated psi was isolated and shown to stimulate transcription of E. coli DNA by holoenzyme but not by 'core' enzyme (Travers et al., 1970a). The nucleotide guanosine tetraphosphate, ppGpp, prevented this specific stimulation by psi factor (Travers et al., 1970b). The appearance of this nucleotide in vivo has been correlated with inhibition of RNA synthesis (Cashel & Gallant, 1969). It has been proposed that synthesis of rRNA may be subject to positive control with psi factor as the control element permitting the RNA polymerase to initiate transcription specifically at otherwise inaccessible promoter sites of rRNA genes.

In complete contrast, Haseltine (1972) reported that the psi stimulation of transcription of E. coli DNA in vitro was entirely non-

specific and even in the absence of psi factor 7-14% of the RNA synthesised *in vitro* was rRNA. This controversy has still to be resolved.

Travers (1973) proposed a hypothesis for the control of rRNA synthesis in *E. coli*. He suggested that the RNA polymerase could exist in two initiation conformations $(E\sigma)_s$ and $(E\sigma)_m$ which were in equilibrium. In the absence of psi factor, $(E\sigma)_m$ was the predominant form which specifically initiated RNA synthesis at the relaxed form of mRNA promoters (Travers *et al.*, 1973). The role of psi was thought to displace the equilibrium between the two conformations in favour of $(E\sigma)_s$ and to stabilise this conformation.

Psi factor has been equated with the protein synthesis elongation factors TuTs (Blumenthal *et al.*, 1972). Travers & Buckland (1973) showed that in crude extracts of *E. coli* there were three peaks of RNA polymerase activity at 16S, 21S and 27S in sucrose gradients. Only the 16S and 27S forms synthesised rRNA efficiently and were inhibited by ppGpp and GDP. These workers concluded that non-transcribing RNA polymerases *in vivo* did not appear to exist as free holoenzyme but rather in a complex association with other regulatory proteins.

3.1.4. Rho Factor.

In addition to factors which are specific for initiation of transcription, there also appear to be several ways in which termination of RNA synthesis can occur. One mechanism is mediated by a protein factor called rho which has a molecular weight of 200,000 and which depresses RNA synthesis without affecting chain initiation (Roberts, 1969).

Thus, it is apparent that control of transcription in bacteria requires the presence of specific protein factors.

3.2. Mammalian RNA Polymerases.

DNA-dependent RNA polymerase was first identified in rat liver nuclei by Weiss (1960). The enzyme proved difficult to solubilise and most early studies were conducted using whole nuclei or chromatin. Widnell & Tata (1964, 1966) showed that when nuclei were incubated in a low ionic strength medium a predominantly GC rich, ribosomal-like RNA was synthesised, whereas in high ionic strength medium the RNA produced was more DNA-like in base composition. In low ionic strength conditions RNA was mainly synthesised in the nucleolus, while in high ionic strength extranucleolar synthesis of RNA was predominant (Maul & Hamilton, 1967; Pogo et al., 1967). These studies suggested that animal cell nuclei contained at least two forms of RNA polymerase. Another indication of the presence of multiple forms of mammalian RNA polymerases came from the observations of Stirpe & Fiume (1967) who found that α-amanitin, a toxin from the toadstool Amanita phalloides, specifically inhibited RNA synthesis catalysed under high ionic strength conditions in mouse liver nuclei.

The first isolation of multiple forms of nuclear RNA polymerases was presented by Roeder & Rutter (1969) working with developing sea urchin embryos and rat liver. The isolation procedure involved the sonication of nuclei in high salt conditions and stabilisation of the isolated enzymes by high concentrations (up to 30%) of glycerol. The same workers found that one activity, RNA polymerase A, was located in the nucleolus and was resistant to the action of α-amanitin *in vitro* while the α-amanitin-sensitive activity, RNA polymerase B, was predominantly sited in the nucleoplasm (Roeder & Rutter, 1970b).

At present, the existence of multiple forms of nuclear RNA polymerases has been demonstrated in a wide variety of tissues and cell types including rat liver (Blatti et al., 1970; Jacob et al.,

1970a), calf thymus (Chambon et al., 1970; Ked er et al., 1972), bovine lymphoid tissue (Furth & Austin, 1970), . ventral prostate (Mainwaring et al., 1971), Xenopus laevis embryos (Roeder, 1974), HeLa cells (Sugden & Sambrook, 1970) and KB cells (Keller & Goor, 1970). In addition to nuclear RNA polymerases, RNA polymerase activities have been detected in rat liver mitochondria (Reid & Parsons, 1971) and rat liver cytoplasm (Seifart et al., 1972). Multiplicity of RNA polymerases has also been demonstrated in lower eukaryotes such as the aquatic fungus, Blastocladiella emersonii (Horgen & Griffin, 1971) and yeast (Ponta et al., 1972) as well as in some higher plants including coconuts (Mondal et al., 1972) and maize (Strain et al., 1971).

Part of the work presented in this thesis describes the isolation and characterisation of multiple forms of RNA polymerase from immature rabbit uterus.

3.2.1. Nuclear Localisation of RNA Polymerases.

Roeder & Rutter (1970) established that RNA polymerase A was of nucleolar origin. Purified preparations of nucleoli contained very little RNA polymerase B activity. RNA polymerase A from rat liver nuclei could be separated into two species called AI and AII (Chesterton & Butterworth, 1971a) but only RNA polymerase AI was obtained from calf thymus (Gissinger & Chambon, 1972).

RNA polymerase B is believed to function in the nucleoplasm and can be separated into two species designated BI and BII from calf thymus (Kedinger & Chambon, 1972) and rat liver (Weaver et al., 1971).

3.3. Properties of Nuclear RNA Polymerases.

3.3.1. Subunit Structure and Molecular Weight.

Sedimentation rates in glycerol gradients of 14.5-15.5S have been obtained for calf thymus and rat liver RNA polymerase B suggesting a molecular weight of 500,000-600,000 (Mandel & Chambon, 1971). Initial studies using SDS-acrylamide gel electrophoresis suggested that RNA polymerase B contained three major subunits with molecular weights of 215,000, 185,000 and 150,000 from calf thymus (Chambon et al., 1970) and 200,000, 180,000 and 160,000 from rat liver (Chesterton & Butterworth, 1971b). Weaver et al. (1971) analysed the subunit structure of rat liver RNA polymerase B and found two forms of the enzyme each having three subunits of molecular weight 150,000, 35,000 and 25,000 and a fourth subunit which, in one form had a molecular weight of 190,000, while the other form was 170,000. They suggested that one enzyme was the proteolytic product of the other. Using highly purified preparations of calf thymus RNA polymerases BI and BII, Kedinger & Chambon (1972) established the subunit structures of the enzymes as shown in Table I. No conversion of one form to the other could be detected by 'ageing' of enzyme at room temperature.

Thus, the molecular weight of calf thymus RNA polymerase BI was calculated to be approximately 510,000 and RNA polymerase BII was about 475,000. These molecular weights were similar to the value of 495,000 estimated as the molecular weight of E. coli RNA polymerase (Burgess, 1971).

Similarly, Gissinger & Chambon (1972) demonstrated that the structure of calf thymus RNA polymerase AI was as shown in Table I. This subunit pattern accounted for a molecular weight of 550,000 for the active enzyme.

TABLE I.

Subunit Structures of RNA Polymerases.

Calf thymus BI			Calf thymus BII			Calf thymus AI			E. coli holoenzyme		
Sub-unit	M.Wt.	M.R.	Sub-unit	M.Wt.	M.R.	Sub-unit	M.Wt.	M.R.	Sub-unit	M.Wt.	M.R.
B1	214,000	1	B2	180,000	1	A1	200,000	1	β'	160,000	1
B3	140,000	1	B3	140,000	1	A2	126,000	1	β	150,000	1
B4	34,000	1-2	B4	34,000	1-2	A3	51,000	1	σ	90,000	1
B5	25,000	2	B5	25,000	2	A4	44,000	1	α	40,000	2
B6	16,500	3-4	B6	16,500	3-4	A5	25,000	2	ω	12,000	0.5
						A6	16,500	2			-2
Kedinger & Chambon (1972)						Gissinger & Chambon (1972)			Burgess (1969)		

M.R. = Molar Ratio.

3.3.2. Effect of Metal Ion Concentration and Ionic Strength.

The isolated calf thymus RNA polymerases exhibited different optima for divalent metal cations and ionic strength. RNA polymerase A utilised Mn^{++} and Mg^{++} equally efficiently and there was only a slight stimulation of activity with increasing ionic strength (Chambon et al., 1970). Conversely, RNA polymerase B was stimulated in high ionic strength conditions, while Mn^{++} was shown to be a better activator than Mg^{++}. Similar results were obtained for the rat liver RNA polymerases (Roeder & Rutter, 1970b).

3.3.3. Effect of α-Amanitin In Vitro.

α-Amanitin, a bicyclic octapeptide isolated from the toadstool Amanita phalloides (Weiland, 1968), was first shown to inhibit RNA synthesis by Stirpe & Fiume (1967). Its selective effect on RNA polymerase activity at high ionic strengths contrasted with actinomycin D and similar inhibitors which block RNA synthesis by binding to the DNA template, suggesting that α-amanitin might act on the RNA polymerase itself. This was shown to be the case (Kedinger et al., 1970; Lindell et al., 1970). The toxin selectively inhibited RNA polymerase B at concentrations as low as 3×10^{-8}M, whereas RNA polymerase A was unaffected even at much higher levels. Chambon et al. (1970) showed that one molecule of α-amanitin was bound per molecule of RNA polymerase B.

Further studies on the action of the toxin revealed that it inhibited RNA synthesis after initiation, presumably at the level of chain elongation (Kedinger et al., 1970; Novello et al., 1970).

3.3.4. Effect of α-Amanitin *in Vivo*.

Experiments using α-amanitin *in vitro* indicated that the toxin could be used to inhibit specifically RNA polymerase B activity *in vivo*. Stirpe & Fiume (1967) showed a decrease in precursor incorporation into mouse liver nuclear RNA in animals treated *in vivo* with α-amanitin. However, administration of α-amanitin to rats resulted in the depression of rRNA synthesis as well as non-rRNA synthesis within 1h of treatment (Jacob *et al.*, 1970b; Niessing *et al.*, 1970). Synthesis of all species of nuclear RNA remained blocked for several hours (Tata *et al.*, 1972).

3.3.5. Thermal Sensitivity of RNA Polymerases.

Shields & Tata (1973) showed that the isolated RNA polymerases from rat liver exhibited different thermal sensitivities, with RNA polymerase A being more labile to thermal treatment than RNA polymerase B. Preincubation of either whole nuclei or isolated enzymes for 15 min at 45° caused the loss of up to 90% of RNA polymerase A activity, while RNA polymerase B only lost about 30% of its activity in controls.

3.3.6. Template Specificity.

The demonstration that bacterial RNA polymerase binds to the template and initiates transcription at specific initiation sites led to a search for similar sites in higher cell types. This has been made difficult by the complexity of the eukaryotic genome and the difficulty in isolating the DNA without large numbers of artificial initiation sites in the form of single-strand 'nicks'. Most workers have to choose between poorly preserved natural templates or artificial ones such as synthetic polymers or viral DNAs. Recently, more success has

been achieved in preparing high molecular weight DNA (Gross-Bellard et al., 1973).

In experiments where natural templates have been used in in vitro assays, most workers have employed calf thymus DNA although Jacob et al. (1970a) showed that rat liver DNA was a better template for rat liver nuclear RNA polymerases. In general, native DNA was a superior template for RNA polymerase A from various sources in vitro and denatured DNA was a more efficient template for RNA polymerase B.

Studies with synthetic polynucleotides have indicated that templates composed of pyrimidine nucleotides were more readily transcribed by both A and B enzymes than templates composed of purine nucleotides (Blatti et al., 1970). Bacteriophage T4 DNA was shown to be a poor template for mammalian RNA polymerases (Gniazdowski et al., 1970), even in the presence of E. coli sigma factor which stimulated the activity of E. coli RNA polymerase (Burgess et al., 1969). The lack of transcription of phage DNA by either RNA polymerase AI or B indicated that mammalian RNA polymerases could only recognise specific initiation sites present on the DNA.

Meilhac & Chambon (1973) investigated the possibility that different RNA polymerases might initiate at specific sites on calf thymus DNA. They used the rifampicin derivative AF/013 which inhibits initiation but not elongation of RNA synthesis by mammalian RNA polymerases (Meilhac et al., 1972), and found that pre-incubation of enzyme and template before the addition of the inhibitor conferred resistance to the derivative. Different AF/013 resistant sites were found for calf thymus AI, calf thymus B and E. coli holoenzyme RNA polymerases. A similar result was obtained by Butterworth et al. (1971) who showed that rat liver RNA polymerase B transcribed rat liver chromatin more efficiently than Micrococcus lysodeikticus RNA polymerase. They suggested that the two enzymes bound to different sites

on the chromatin DNA making it virtually impossible to obtain a meaningful transcription of mammalian DNA or chromatin with a bacterial RNA polymerase.

Mandel & Chambon (1974a) found that transcription of SV40 form I DNA was dependent on the presence of Mn^{++}, irrespective of the type of RNA polymerase used. Both form II and form III of SV40 DNA were poorly transcribed by calf thymus RNA polymerase AI and B suggesting that both enzymes could not easily initiate RNA synthesis on intact double-stranded DNAs. Hossenlopp et al. (1974) suggested that the stable complex between SV40 DNA form I and mammalian RNA polymerases was due to binding to unpaired regions present in the superhelical DNA.

3.3.7. Stimulatory Factor Requirements.

Factors influencing the activity of E. coli RNA polymerase and their role in controlling gene transcription have already been described in section 3.1. Bacterial sigma factor had no effect in vitro on mammalian RNA polymerases (Chambon et al., 1970) but several workers have now shown the presence of mammalian protein factors which stimulate RNA synthesis.

A factor which stimulated RNA polymerase activity tenfold in vitro has been isolated from calf thymus (Stein & Hausen, 1970a; 1970b). Factor activity was present in a small group of proteins with a molecular weight range of 20,000-25,000 (Hameister et al., 1973). The factor sensitive step was not the binding of the RNA polymerase to the DNA and initiation did not seem to be positively affected. This factor has been shown to form a complex with the RNA polymerase (Stein & Hausen, 1970) although it may also form a complex with the DNA (Hameister et al., 1973).

A protein factor from rat liver has also been described (Seifart,

1970; Seifart et al., 1973). The protein had a molecular weight of 30,000 and enhanced the transcription of native rat liver DNA by homologous RNA polymerase B. This factor had no effect on initiation but the average chain length of the RNA product was increased by its presence (Seifart et al., 1973). This suggested that the protein operated by acting in some unknown manner on chain elongation.

3.3.8. Products of RNA Polymerases.

The products of nuclear RNA polymerases were examined in intact HeLa cell nuclei by Zylber & Penman (1971) who found that RNA polymerase A was capable of synthesising partially completed precursor molecules of rRNA. RNA polymerase B was shown to synthesise giant heterogeneous nuclear RNA. However, it is difficult to obtain chromatin or DNA templates which are not affected by single-strand 'nicks' at which RNA synthesis could be non-specifically initiated. This makes it difficult to characterise the products of the RNA polymerases.

Maryanka & Gould (1973) found that rat liver RNA polymerase B synthesised high molecular weight RNA from a rat liver chromatin template. This RNA had sedimentation coefficient values of between 18S and 45S which was considerably less than the reported values for HnRNA (Darnell et al., 1971a).

The products of rat liver RNA polymerases A and B on a homologous DNA template were examined by nearest neighbour frequency analysis (Smuckler & Tata, 1972). The RNA product of RNA polymerase A had sequences richer in GC than that formed by RNA polymerase B, suggesting that enzyme A preferentially transcribed ribosomal cistrons while enzyme B formed an RNA with a more DNA-like base composition. Mandel & Chambon (1974b) studied the RNA synthesised by calf thymus RNA polymerases A and B using SV40 form I DNA as template. They found .

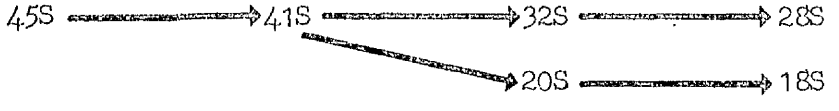
labelled ATP and CTP at the 5' terminus of the RNAs synthesised indicating that there may be at least two initiation sites for each enzyme. The maximum size of the product synthesised by RNA polymerase A was larger than the SV40 genome while the RNA polymerase B product had a maximum size (18-20S). In contrast to E. coli holoenzyme, which transcribed asymmetrically the SV40 genome, animal RNA polymerases exhibited symmetric transcription.

4. The Synthesis of Mammalian RNA.

In conclusion to the above section on the products of mammalian RNA polymerases, a brief review of the current knowledge of mammalian RNA synthesis is presented.

4.1. The Synthesis of Ribosomal RNA.

In the eukaryotic cell, ribosomal RNA is synthesised in the form of a giant precursor molecule, containing the sequences of both 28S and 18S RNA, together with long stretches of non-ribosomal RNA. Synthesis of rRNA has been shown to occur in the nucleolus (Penman et al., 1966) and is probably catalysed by RNA polymerase A which is also located in the nucleolus (Roeder & Rutter, 1970). The giant precursor molecule, which appears in nucleolar RNA after a short pulse of radioactive precursors, sediments at 45S in sucrose gradients and matures to give rise to the individual 28S and 18S ribosomal RNAs through the following intermediates (Maden, 1971) :



Ribosomal RNA is methylated with the substitution mainly being at the 2' position of the ribose molecule rather than on the bases themselves. Methylation appears to occur during the actual synthesis of 45S RNA (Greenberg & Penman, 1966), with the exception of one substitution which occurs after transcription (Zimmerman, 1968).

Relatively little is known of the non-ribosomal RNA sequences of 45S RNA except that comparison with mature rRNA indicates that they have a high GC base content and are unmethylated (Amaldi & Attardi, 1968; Willems et al., 1968).

The ribosomal 28S and 18S RNA species occur in the 60S and 40S ribosomal subunits respectively. The ribosomal precursor can be extracted from the nucleolus in the form of ribonucleoprotein particles sedimenting at 80S and 55S respectively (Warner & Soeiro, 1967). The 55S particle contains 32S RNA, 5S RNA and ribosomal proteins, while the 80S particle contains both 45S RNA, 5S RNA and ribosomal proteins.

4.2. The Synthesis of Messenger RNA.

The manner in which higher animals synthesise mRNA has been the subject of much investigation and it now appears that mRNA is also derived from larger RNA precursor molecules which are modified after transcription. These molecules have been designated HnRNA and are probably synthesised by RNA polymerase B. The mRNA precursors form a fraction of these HnRNA molecules whose base composition is similar to that of DNA. The evidence that HnRNA is a precursor for mRNA has come from recent studies concerning sequence similarities between the two types of molecule.

Firstly, it was found that cells infected with SV40 virus were shown to have HnRNA which contained regions complementary to viral DNA but which were larger than virus-specific polysomal mRNA (Lindberg &

Darnell, 1970). HnRNA molecules containing virus-specific sequences were heterogeneous in size and also contained host cell sequences, whereas virus-specific mRNA had a discreet size and lacked host cell sequences.

Further evidence that the heterogeneous nuclear species is a messenger precursor came with the finding that both species contain a poly A sequence about 200 nucleotides long (Darnell et al., 1971b) which is part of the polynucleotide chain and not dissociable by treatment with dimethylsulphoxide. Digestion of HnRNA and mRNA with an exonuclease requiring a free 3'OH group removes the poly A from both types of molecule suggesting that the poly A is sited at the 3' terminus of the polynucleotide (Molloy et al., 1972). It appears that poly A is added in the nucleus by a post-transcriptional mechanism as treatment with actinomycin D does not inhibit its synthesis (Darnell et al., 1973). It is probable that poly A is synthesised by the stepwise addition of individual adenylate residues to pre-existing HnRNA molecules.

After 1-2 min treatment with ^{3}H-adenosine, more than 95% of the poly A is found in HeLa cell nuclei as part of the HnRNA implying that the nucleus is the most important site for the synthesis of poly A. Most of the poly A seems to be transferred to the cytoplasm although some turnover may take place in the nucleus.

Penman et al. (1970) found that cordycepin inhibited the appearance of labelled RNA in polysome derived mRNA but had little effect on HnRNA synthesis. The cytoplasmic appearance of newly synthesised RNA in polyribosomes was blocked by both actinomycin D and cordycepin (Adesnik et al., 1972) suggesting that post-transcriptional addition of poly A must proceed for the conversion of HnRNA to mRNA to occur.

Thus, it seems likely that HnRNA is the precursor for mRNA although the exact mechanism by which this takes place has still to be elucidated.

The aim of the project is to investigate the nature of the response of the immature uterus to oestradiol with particular reference to the RNA polymerases and the control of rRNA synthesis in an attempt to determine whether the enzymes themselves are altered by the action of the hormone in the tissue.

MATERIALS AND METHODS.

1. Materials.

1.1. Hormone, Enzyme and Inhibitors.

Oestradiol-17β, pancreatic ribonuclease (RNase), deoxyribonuclease (DNase I), cycloheximide and heparin were all obtained from Sigma Chemical Co. (London) Ltd., Kingston upon Thames, Surrey. Solutions of RNase were heated at 85° for 20 min before use to inactivate latent deoxyribonuclease activity.

Actinomycin D and Pronase were the products of Calbiochem, Los Angeles, California. Pronase solution was autodigested at 37° for 2h to destroy latent nuclease activity.

α-Amanitin was obtained from both Calbiochem and C. H. Boehringer Sohn, 6507, Ingelheim am Rhein, West Germany, and rifampicin from the Boehringer Corporation (London) Ltd., Ealing, London.

E. coli RNA polymerase holoenzyme was a generous gift of Mr. E. Robertson of this department.

1.2. Nucleic Acids.

Highly polymerised calf thymus DNA was the product of Sigma Chemical Co. Ltd., London and poly d(AT) was obtained from Boehringer Corporation, London.

Tritiated SV40 form I DNA was the generous gift of Dr. Martin White of this department. Unlabelled 28S RNA and ^{32}P-labelled 28S RNA from HeLa cells were the generous gift of Dr. B. E. H. Maden also of this department.

1.3. Reagents for Column Chromatography.

1.3.1. Diethylaminoethyl cellulose (DEAE-cellulose)

DE52 cellulose, pre-swollen, was obtained from Whatman Biochemicals Ltd., Maidstone, Kent. The substituted cellulose was stirred into 15 volumes of 0.5M HCl and left to stand for 1h. The supernatant liquid was removed and the cellulose was washed with distilled water until the pH of the suspension was 4. The ion exchanger was then stirred into 15 volumes of 0.5M NaOH and left to stand for another hour. The supernatant was decanted and the DEAE-cellulose washed with distilled water until the pH was near neutrality. The ion exchanger was equilibrated with buffer by stirring one volume of DEAE-cellulose into 20 volumes of buffer overnight. The suspension was degassed and stored at 4° with the addition of 0.02% (v/v) chloroform to prevent bacterial growth.

1.3.2. Carboxymethyl cellulose (CM-cellulose).

CM52 cellulose was obtained from Whatman Biochemicals Ltd., Kent. The pretreatment for CM-cellulose was identical to that for DEAE-cellulose except that the first treatment was with 0.5M NaOH and after washing with distilled water to pH 8, the second treatment was with 0.5M HCl. Degassing was not carried out on CM-cellulose and the ion exchanger was stored at 4° also with the addition of 0.02% (v/v) chloroform.

1.3.3. Phosphocellulose.

P11 was the product of Whatman Biochemicals and was washed with

0.5M HCl and 0.5M NaOH as for DEAE-cellulose. After removal of 'fines', the ion exchanger was equilibrated with the appropriate buffer and stored at 4^{o} in the presence of 0.02% (v/v) chloroform.

1.4. Dialysis Tubing.

Dialysis was carried out in Visking tubing obtained from the Scientific Instrument Centre Ltd., 1 Leeke St., London, W.C.1. The tubing was boiled for 30 min in 0.05M NaOH containing 0.25% (w/v) EDTA to remove impurities. The tubing was then washed thoroughly with distilled water and stored in 0.5% (w/v) EDTA. Before use, the tubing was washed 5 times with distilled water.

1.5. Reagents for In Vitro Incubations.

Eagle's medium (Glasgow modification) (Busby et al., 1964) containing 100µg/ml of streptomycin, 100 units of penicillin and 0.002% (w/v) phenol red was the medium in which excised uteri were incubated in vitro.

1.6. Radioisotopes and Reagents for Scintillation Counting.

All radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks. at the following specific activities:

[5-^{3}H] UTP, 1C$_{i}$/mmol; [4-^{14}C] UTP, 43mC$_{i}$/mmol;

[8-^{3}H] GTP, 1.8C$_{i}$/mmol; [5-methyl-^{3}H] dTTP, 40C$_{i}$/mmol;

[5-^{3}H] uridine, 28C$_{i}$/mmol; [8-^{3}H] guanosine, 16C$_{i}$/mmol;

[4,5-^{3}H] L-lysine, 5.5C$_{i}$/mmol; [4,5-^{3}H] L-leucine, 1C$_{i}$/mmol;

[2,4,6,7-^{3}H] oestradiol-17β, 85C$_{i}$/mmol.

Kieselguhr was obtained from Koch-Light Laboratories, Colnbrook, Bucks. Toluene scintillator was 0.5% (w/v) PPO (2,5 diphenyloxazole, Koch-Light) in toluene and in some experiments was mixed 3:2 (v/v) with 2-methoxyethanol. Dioxan scintillator was 0.7% (w/v) PPO and 10% (w/v) naphthalene in scintillation grade dioxan (Koch-Light). 1M hyamine hydroxide in methanol was obtained from Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh.

1.7. Buffers.

TGMED buffer was 0.05M tris-HCl pH 7.9, 5mM $MgCl_2$, 0.1mM EDTA, 0.5mM DTT and 25% (v/v) glycerol.

TGMED(15) buffer was identical to TGMED except 15% (v/v) glycerol was used instead of 25%.

TGED buffer was 0.05M tris-HCl pH 7.9, 0.1mM EDTA, 0.5mM DTT and 25% (v/v) glycerol.

TKM buffer was 50 mM tris-HCl pH 7.4, 25mM KCl and 5mM $MgCl_2$.

TGED(50) buffer was identical to TGED except 50% (v/v) glycerol was present instead of 25%.

PGED buffer was 10mM potassium phosphate pH 7.5, 0.1mM EDTA, 0.5mM DTT and 15% (v/v) glycerol.

1.8. Miscellaneous.

ATP, CTP, GTP, UTP, dATP, dCTP, dGTP and dTTP were obtained from Sigma Chem. Co. Ltd., London.

Whatman No. 1 and 3MM filter discs were the product of Whatman Biochemicals Ltd., Kent.

Ammonium sulphate used was Enzyme grade, specially low in heavy metals from BDH Chemicals Ltd., Poole, Dorset. All other chemicals were Analar grade where possible and were usually obtained from BDH.

2. Biological Methods.

2.1. Experimental Animals.

Female Dutch rabbits were obtained from Hyline Rabbit Farms, Northwich, Cheshire and were fed ad libitum on diet SG1 in pellet form obtained from Oxoid Ltd., Southwark Bridge Rd., London, S.E.1. The animals were used when 7-8 weeks old within a weight range of 1.2-1.4 kg. In some experiments adult animals (6-8 months old) were used.

The animals were killed by cervical dislocation and the excised uteri were dissected free of fat and connective tissue before further treatment.

2.2. Administration of Hormone, Inhibitors and Radioisotopes to Experimental Animals.

2.2.1. Hormone.

Oestradiol-17β was solubilised by the method of Roberts & Szego (1947) using methyl red as indicator. The hormone was injected intravenously through the lateral ear vein in 0.9% saline containing 2.5% (v/v) ethanol. Control animals received saline-ethanol alone.

2.2.2. Inhibitors.

Actinomycin D, α-amanitin and cycloheximide were all injected intraperitoneally in saline. Control animals received saline alone.

2.2.3. Radioisotopes.

Tritiated oestradiol was injected intravenously as in section 2.2.1.

All other radioisotopes were injected intraperitoneally and control animals received the appropriate carrier alone.

2.3. In Vitro Incubations.

The uteri of three immature animals were incubated in 10 ml conical flasks under an atmosphere of 95% O_2/5% CO_2 at 37° in a shaking water bath. The incubation was carried out in 5 ml of Eagle's medium containing 1nM oestradiol. After incubation, the uteri were washed twice with cold saline and blotted dry before further treatment.

2.4. Preparation of Acid-Soluble and Acid-Insoluble Fractions of Whole Uteri.

This method was a modification of that of Billing et al., (1969a). Excised uteri were washed with cold saline, finely minced and homogenised in 10 ml of ice cold distilled water using a Teflon-glass homogeniser. An equal volume of 20% (w/v) trichloroacetic acid was added to the homogenate and the mixture was centrifuged for 10 min at 800xg. The supernatant was retained and the pellet washed with 5 ml of 5% (w/v) trichloroacetic acid. The washings were added to the supernatant and this constituted the acid-soluble fraction.

The pellet was suspended in a small volume of 5% (w/v) trichloroacetic acid and mixed with 2 ml of 2% (w/v) kieselguhr in 5% (w/v) trichloroacetic acid. An additional 2 ml of kieselguhr suspension was added to a Millipore filtration unit and the kieselguhr collected as a pad on a 2.5 cm Whatman No. 1 filter disc. The acid-insoluble pellet bound to kieselguhr was collected as a second layer on this pad and washed three times with 10 ml of 5% (w/v) trichloroacetic acid, once with 10 ml of absolute alcohol and twice with 10 ml of diethyl

ether. The pad was extracted with 0.5 ml of 1M hyamine hydroxide for 20 min at 60° and the radioactivity measured by scintillation counting in toluene scintillator with tritium counting efficiency of 16-18%. The radioactivity of 0.5 ml aliquots of the acid-soluble fraction was counted in 10 ml of dioxan scintillator with tritium counting efficiency of 18-21%.

Protein and DNA determination were carried out on the acid-insoluble material after resuspension of the pellet in 5% (w/v) trichloroacetic acid.

3. Preparation of Subcellular Fractions.

3.1. Isolation of Nuclei.

This method is a modification of that of Blobel & Potter (1966).

Excised uteri were finely chopped and homogenised in 5-10 volumes of 0.25M sucrose in TKM buffer. Homogenisation was carried out in an Ultra-Turrax homogeniser run at 60V for 40s. The homogenate was filtered through four layers of muslin cloth and the filtrate was mixed with two volumes of 2.3M sucrose in TKM buffer to bring the final sucrose concentration to 1.6M. This solution was underlaid with 10 ml of 2.4M sucrose in TKM buffer in a centrifuge tube. Centrifugation was carried out in a Spinco SW27 rotor run at 27,000 rpm for 90 min. The pellet, which contained purified nuclei, was resuspended in a small volume (1-2ml) of TGED buffer.

3.1.1. Preparation of Acid-insoluble Fractions from Nuclei.

One volume of ice cold 20% (w/v) trichloroacetic acid was added to an equal volume of resuspended nuclei in TGED buffer. The mixture was

centrifuged for 15 min at 800xg and the supernatant discarded. The pellet was resuspended in 5 ml of 10% (w/v) trichloroacetic acid and recentrifuged for 15 min at 800xg. This washing was repeated once more and the final pellet resuspended in distilled water and the pH adjusted to 7 with 1M NaOH. Aliquots were used to estimate DNA, RNA and protein concentrations.

3.2. Preparation of Uterine Cytoplasm.

Uteri were homogenised as for the preparation of nuclei in section 3.1. The filtered homogenate was centrifuged at 800xg for 15 min and the supernatant was then recentrifuged in a Spinco SW40 rotor at 105,000xg for 60 min. The pellet was discarded and the supernatant used as a soluble cytoplasmic preparation.

3.3. Electron Microscopy.

Examination of nuclei was carried out by the personnel, under the direction of Dr. H. Elder, of the Electron Microscopy Unit of the Physiology Department of this University. Samples supplied as pellets were fixed in gluteraldehyde-osmium tetroxide, embedded in plastic and cut sections were stained with lead and uranium for visualisation in the electron beam of an A.E.I. EM6B electron microscope.

4. Enzyme Assays.

4.1. RNA Polymerase.

4.1.1. Assay for RNA polymerase activity in nuclei.

RNA polymerase activity was assayed in nuclei in either high salt

to measure RNA polymerase B activity or in low salt +α-amanitin to measure RNA polymerase A activity.

The reaction mixture contained in a volume of 0.05 ml, 0.075 µmol each of ATP, CTP and GTP, 0.005 µmol ^{3}H-UTP (0.5 µC$_i$), 1.25 µmol tris-HCl pH 7.9, 0.5 µmol 2-mercaptoethanol and 5% (v/v) glycerol.

Under low salt conditions, the same volume of reaction mixture also contained 0.2 µmol $MgCl_2$ and 1 µg α-amanitin. Assays carried out in high salt conditions contained 0.2 µmol $MnCl_2$ and 20 µmol ammonium sulphate in the same volume of reaction mixture.

0.05 ml of a suspension of nuclei in TGED buffer containing about 25 µgDNA were added to the reaction mixture at 0°, and this mixture was incubated at 37° for 30 min. The reaction was stopped by cooling the incubated samples to 0°. 0.05 ml of the incubation mixture was spotted on a 2.5 cm Whatman No. 1 filter disc and washed for 30 min with occasional stirring in 10% (w/v) trichloroacetic acid containing 1% (w/v) tetrasodium pyrophosphate. The filters were washed twice more with 5% (w/v) trichloroacetic acid containing 1% (w/v) tetrasodium pyrophosphate, twice with absolute alcohol, once with alcohol:ether (1:1) and once with ether. They were then dried in a stream of air and the radioactivity solubilised by the addition of 0.5 ml of 1M hyamine hydroxide and incubation at 60° for 20 min. The radioactivity was measured by scintillation counting in toluene scintillator with tritium counting efficiency of 20-24%.

4.1.2. Assay for solubilised RNA polymerase activity.

The reaction mixture contained in a volume of 0.025 ml, 0.075 µmol each of ATP, CTP and GTP, 0.005 µmol ^{3}H-UTP (0.5 µC$_i$), 1.25 µmol tris-HCl pH 7.9, 0.5 µmol 2-mercaptoethanol, 5% (v/v) glycerol, 0.2 µmol $MnCl_2$ and 10 µg DNA template. In some experiments, ^{3}H-UTP was replaced by

0.5 μC_i of ^{14}C-UTP, and in other experiments by 0.5 μC_i of ^{3}H-GTP.

0.05 ml of the polymerase preparation was added to the reaction mixture at 0° and incubated at 37° for 30 min. After incubation, the radioactivity in each sample was determined as described in section 4.1.1.

4.2. DNA Polymerase.

This assay was based on the method of Shepherd & Keir (1966). Samples (0.1 ml) were incubated at 37° for 60 min in a total volume of 0.25 ml containing 4 µmol tris-HCl pH 7.5, 1.5 µmol $MgCl_2$, 15 µmol KCl, 0.1 µmol EDTA, 3 µmol 2-mercaptoethanol, 100 µg calf thymus DNA and 0.05 µmol each of dATP, dCTP and dGTP and (^{3}H-methyl) dTTP (0.5 μC_i).

The reaction was terminated by the addition of 0.05 ml of 2M NaOH and the samples were reincubated at 37° for another 60 min. Portions (0.1 ml) were spotted onto Whatman 3MM filter discs (2.5 cm diameter) which were then washed six times in 5% (w/v) trichloroacetic acid containing 1% (w/v) tetrasodium pyrophosphate. After washing with absolute alcohol and ether, the filters were dried in air. The DNA was dissolved by heating for 20 min at 60° after the addition of 0.5 ml of 1M hyamine hydroxide. Radioactivity was determined after the addition of toluene scintillator.

4.3. Ribonuclease Assay.

3 x 10^6 cpm of ^{32}P-labelled 28S rRNA from HeLa cells (5 µg) was incubated in the standard RNA polymerase assay mixture for 30 min at 37°. After incubation, the mixture was heated to 70° for 30s, quickly cooled and layered on a 10-30% (w/v) sucrose gradient in LETS buffer (0.01M tris-HCl pH 7.4, 0.1M LiCl, 0.1% (w/v) SDS and 1mM EDTA). After

centrifugation in a Spinco SW56 rotor run at 49,000 rpm for 2.5h at 18°, fractions were collected on Whatman No. 1 filter discs and air dried. The radioactivity was measured in toluene scintillator. The presence of RNA sedimenting more slowly than a marker of untreated RNA indicated the presence of RNase activity in the RNA polymerase preparation.

4.4. Deoxyribonuclease Assay.

The sample was incubated for 30 min at 37° in the standard RNA polymerase assay mixture with unlabelled UTP and 5×10^5 cpm of SV40 ^{3}H-DNA (5 µg). After incubation, the reaction was terminated by heating to 70° for 30s, followed by rapid cooling to 0°. 0.1 ml was layered on a 4 ml gradient of CsCl containing 0.1 mg/ml ethidium bromide (ρ=1.42). After centrifugation for 6h at 35,000 rpm at 20° in a Spinco SW50.1 rotor fractions were collected and spotted on Whatman No. 1 filter discs. The filters were washed in 10% (w/v) trichloroacetic acid, absolute alcohol and ether, air dried and the radioactivity determined by counting in toluene scintillator. The presence of DNA sedimenting more slowly than a marker of untreated DNA indicated the presence of DNase activity in the preparation.

5. Chemical Measurements.

5.1. Protein Determination.

Protein concentrations were determined by the method of Bramhall et al. (1969). 0.05 ml of sample was spotted on a Whatman No. 1 filter disc and the filters placed in 7.5% (w/v) trichloroacetic acid and heated at 80° for 30 min. After washing three times with 7.5% (w/v) trichloroacetic acid at room temperature, the filters were washed with

alcohol:ether (1:1), ether and finally dried in a stream of air.

The dry filters were stained with Xylene Brilliant Cyanin G (E. Gurr Ltd., London, S.W.14) at a concentration of 10 mg/ml in 7% (v/v) acetic acid for 15 min at 50°. After removal of the stain, the filters were washed with 7% (v/v) acetic acid until the blank was colourless. The filters were dried in air.

The blue colour was displaced from the filters by the addition of 5 ml of methanol:distilled water:0.88 ammonia (66:34:1) to each filter in a test tube. After mixing, the optical density of the samples was read at 610 nm. A standard curve was constructed using bovine serum albumin as standard over a concentration range of 0-1 mg/ml.

5.2. DNA Determination.

DNA was measured by the method of Burton (1956). Samples were mixed with an equal volume of 0.5M perchloric acid and heated for 15 min at 70°.

The Burton reagent was prepared freshly before use by mixing 0.1 ml of acetaldehyde solution (16 mg/ml) with 20 ml of diphenylamine solution (1.5 mg in 100 ml of glacial acetic acid and 1.5 ml of concentrated sulphuric acid). 1 ml of sample was mixed with 2 ml of Burton reagent and left to stand overnight at room temperature in the dark. The absorbance was read to 600 nm and a calibration curve, using calf thymus DNA as standard, was constructed over a concentration range of 0-200 µg/ml.

5.3. RNA Determination.

RNA was measured by the method of Kerr & Seraidarian (1945). The orcinol reagent was 60 mg orcinol in 10 ml of 0.02% (w/v) $FeCl_3$ in .

concentrated HCl.

3 ml of orcinol reagent was mixed with an equal volume of RNA sample and heated for 30 min at 95°. The mixture was cooled to room temperature and the absorbance read at 665 nm.

6. Preparation of DNA from Rabbit Liver.

This method was based on that of Kay et al. (1952). All steps were carried out at 0-4°.

20g of rabbit liver were finely minced and homogenised in ice cold 0.9% (w/v) NaCl in 0.01M trisodium citrate (20 ml per 5g tissue). The homogenate was filtered through four thicknesses of muslin and centrifuged for 30 min at 800xg. The supernatant was discarded and the pellet rehomogenised and recentrifuged.

The pellet was then homogenised for 3 min in 0.9% (w/v) NaCl in 0.01M trisodium citrate (100 ml per 5g tissue), placed in a beaker and stirred mechanically. 5% (w/v) SDS in 45% (v/v) aqueous ethanol was added (9 ml per 100 ml of suspension) with thorough stirring which was continued for 2h. Solid NaCl was added to a final concentration of 1M (5.5g per 109 ml of suspension). The suspension was centrifuged for 30 min at 10,000 rpm in an M.S.E. 8 x 50 ml rotor. The supernatant was carefully removed and an equal volume of 95% (v/v) aqueous ethanol was added with continuous stirring. A gelatinous precipitate formed which slowly dehydrated into a white fibrous mass which was spooled from the suspension on a glass rod. A second alcohol precipitation was performed on the spooled material and the DNA was then dissolved in 0.01M tris-HCl pH 7.5.

6.1. Determination of the Molecular Weight of DNA.

Molecular weight determinations of the rabbit liver DNA prepared by the above method were kindly performed by Dr. R. Eason of this department using the method of Studier (1965). Samples were centrifuged in a Spinco Model E ultracentrifuge at neutral pH to determine the average molecular weight of double-stranded DNA and at alkaline pH to determine the molecular weight of single-stranded DNA.

7. Extraction of Multiple Forms of RNA Polymerases.

All procedures were carried out at 0-4° unless otherwise specified. The method was based on that of Sugden & Keller (1973).

Immature rabbit uteri were homogenised in 5-10 volumes of 10mM tris-HCl pH 7.9, 1mM EDTA and 5mM DTT. Homogenisation was carried out in an Ultra-Turrax homogeniser run at 60V for 45s and followed by a second homogenisation of 5-10 strokes in a Teflon-glass homogeniser run at half-maximum speed. The homogenate was filtered through four layers of muslin cloth and the filtrate adjusted to final concentrations of 25mM tris-HCl pH 7.9, 0.5mM EDTA, 3.5mM DTT, 5mM $MgCl_2$, 10% (w/v) sucrose, 20% (v/v) glycerol and 0.4M ammonium sulphate. The ammonium sulphate, which was added last, caused lysis of any intact nuclei. The viscous lysate was heated at 35° for 1h and then cooled at 0° for 8h. After this salt extraction, the suspension was centrifuged in a Spinco 50Ti rotor for 10h at 40,000 rpm to remove cellular debris.

7.2. Ammonium Sulphate Fractionation.

The concentration of ammonium sulphate in the supernatant of the high speed centrifugation was adjusted to 100% saturation by the slow

addition of 0.38g of solid ammonium sulphate per ml of supernatant, 0.1 ml of 1M NaOH per 10g of ammonium sulphate added and 0.1 ml of 0.1M DTT per 100 ml volume of solution. The mixture was stirred mechanically for 6h at 0° and the precipitate collected by centrifugation in a Spinco SW27 rotor for 1h at 27,000 rpm. The pellet was resuspended in a small volume of TGMED buffer and dialysed overnight against 100 volumes of TGMED buffer containing 0.08M ammonium sulphate.

7.3. DEAE-Cellulose Chromatography.

After dialysis overnight, the solution was clarified by centrifugation in a Spinco 50Ti rotor for 1h at 45,000 rpm. The supernatant was passed on to a 12 x 0.9 cm column of DEAE-cellulose which had previously been equilibrated with TGMED buffer containing 0.08M ammonium sulphate. After sample application, the column was washed with 5 column volumes of 0.08M ammonium sulphate, 5 column volumes of 0.14M ammonium sulphate and finally a linear gradient of 10 column volumes of 0.14M-0.4M ammonium sulphate all in TGMED buffer. Fractions (2 ml) were collected and assayed for RNA polymerase activity and protein content.

7.4. Phosphocellulose Chromatography.

The fractions containing RNA polymerase activity after DEAE-cellulose chromatography were pooled and dialysed against 0.08M ammonium sulphate in TGED buffer for 6h at 4°. After dialysis, the solution was passed on to a 10 x 0.9 cm column of phosphocellulose which had been equilibrated with 0.08M ammonium sulphate in TGED buffer. The column was washed with 3 column volumes each of 0.08M, 0.15M, 0.2M, 0.3M and 0.4M ammonium sulphate all in TGED buffer. Fractions (2 ml) were

collected and assayed for RNA polymerase activity.

7.5. Glycerol Density Gradient Sedimentation.

The fractions containing RNA polymerase activity were pooled after phosphocellulose chromatography and dialysed against TGMED(15) buffer overnight. Glycerol gradient centrifugation was carried out by layering 0.5 ml of sample on 4.5 ml of a 15-30% (v/v) glycerol gradient containing 50mM tris-HCl pH 7.9, 0.1mM EDTA, 1mM DTT, 50mM ammonium sulphate and 0.5 mg/ml lysozyme. Centrifugation was carried out in a Spinco SW65 rotor run at 65,000 rpm for 4.5h at 0^{o}. 0.2 ml fractions were collected by piercing the bottom of the centrifuge tube.

7.6. CM-Cellulose Chromatography.

The protein, which did not bind to the DEAE-cellulose during the purification of the RNA polymerases, was dialysed overnight against PGED buffer at 4^{o} and then passed on to a 10 x 0.9 cm column of CM-cellulose which was equilibrated with PGED buffer. The column was washed with 5 column volumes of PGED buffer followed by a linear gradient of 10 column volumes of PGED containing 0 to 0.4M KCl. Fractions (1 ml) were collected and assayed for stimulatory activity in the presence of both RNA polymerase A and B.

Similarly, cytoplasms from oestradiol-treated and control rabbit uteri prepared as described in section 3.2. were dialysed overnight against PGED buffer and passed on to a CM-cellulose column which was washed with buffer as described above.

RESULTS

RESULTS.

1.1. Purification of Multiple Forms of DNA-Dependent RNA Polymerase from Rabbit Uterus.

In the initial studies on the purification of uterine RNA polymerases, adult animals were used so as to provide a larger wet weight of starting material from which the RNA polymerases could be extracted. Once these methods had been developed using adult uteri, they were applied to extract the multiple forms of RNA polymerase from immature rabbit uteri starting with a wet weight of 2-3g. The results presented in the sections on purification and characterisation of the enzymes are applicable for both adult and immature animals.

1.2. Extraction of Uterine RNA Polymerases.

Most of the established methods for the isolation of mammalian nuclear RNA polymerases involve the sonication of a nuclear preparation in media with an ionic strength greater than 0.3M ammonium sulphate (Roeder & Rutter, 1969; Chambon *et al.*, 1970). Early attempts to isolate the uterine RNA polymerases by these standard methods failed mainly because of the shortage of material in preparations of uterine nuclei. However, Sugden & Keller (1973) developed a method for the extraction of multiple forms of RNA polymerases from whole homogenates of KB cells which avoided the preparation of either crude or purified nuclear fractions before the extraction of the enzymes. The method described in this section was based on this procedure and was employed to isolate the RNA polymerases from a whole tissue homogenate of uteri.

10g of uteri in the case of adult animals or 2-3g of uteri in the case of immature animals were homogenised in 10 volumes of 10mM tris-HCl pH 7.9, 1mM EDTA and 5mM DTT as described in the Methods section. The

homogenate was adjusted to final concentrations of 25mM tris-HCl pH 7.9, 0.5mM EDTA, 3.5mM DTT, 5mM $MgCl_2$, 10% (w/v) sucrose, 20% (v/v) glycerol and 0.4M ammonium sulphate. Glycerol was essential in concentrations of 20% (v/v) upwards in order to stabilise the RNA polymerases after they had been solubilised. The ammonium sulphate was added last and caused lysis of any intact nuclei present in the homogenate. As a result of this addition, the homogenate became very viscous.

The suspension was allowed to stand for 8h at 0° in the presence of high salt so as to facilitate the dissociation of protein and nucleic acid in the chromatin. After this period it was centrifuged at 105,000xg for 10h to remove any insoluble material such as cellular debris, and the resulting supernatant containing both protein and nucleic acids was fractionated with ammonium sulphate as described in the Methods section. As a result of this procedure all the RNA polymerase activity was recovered in the precipitated protein while over 85% of the measurable DNA content remained in the supernatant fraction which was discarded.

The pellet was resuspended in a small volume of TGMED and dialysed against 100 volumes of TGMED containing 0.08M ammonium sulphate to reduce the salt concentration. After dialysis, the solution appeared very cloudy because the contaminating DNA became insoluble and this precipitate was removed by centrifugation. The supernatant fraction which contained between 10 and 15mg of protein per gram of initial tissue was then fractionated further by chromatography on columns of DEAE-cellulose.

1.3. DEAE-Cellulose Chromatography.

The clarified supernatant fraction derived from the high salt extract was passed on to a DEAE-cellulose column equilibrated with TGMED buffer containing 0.08M ammonium sulphate, and chromatography was carried out as described in the Methods section. Fractions (2 ml) were

collected and assayed for RNA polymerase activity and protein content. A representative elution profile is shown in Fig. 1. The bulk of the cellular protein eluted in the column wash and three distinct peaks of RNA polymerase activity were obtained. The first peak of activity was eluted with the bulk of the cellular protein in the column wash and was designated RNA polymerase C. The second peak, eluted from the column by 0.14M ammonium sulphate, was insensitive to the action of α-amanitin added to the RNA polymerase assay, and was designated RNA polymerase A, while the third peak of RNA polymerase activity, which was eluted by 0.22 - 0.25M ammonium sulphate, was sensitive to the action of α-amanitin in the assay and was designated RNA polymerase B (Fig. 1).

In some experiments the original homogenate was centrifuged for 15 min at 800xg immediately after filtration and a crude nuclear preparation was obtained. This nuclear pellet was treated in a similar manner to the homogenate by extraction in high salt concentrations followed by DEAE-cellulose chromatography. The results of this procedure are shown in Fig. 2, which demonstrates the presence of the two principal peaks of RNA polymerase A and B alone suggesting that the peak of RNA polymerase C activity obtained in experiments with whole homogenates was either 'soluble' or of cytoplasmic origin.

The purification and characterisation of the RNA polymerases A and B are contained in the following sections and the properties of RNA polymerase C are presented at a later stage in this thesis.

1.4. Phosphocellulose Chromatography.

1.4.1. RNA Polymerase A.

The fractions containing RNA polymerase A activity after DEAE-cellulose chromatography were pooled and dialysed against TGED buffer

FIG. 1: Multiple forms of DNA-dependent RNA polymerases from Rabbit Uterus separated on DEAE-cellulose.

Immature uteri were homgenised and the total protein extracted by salt as described in the Methods section. 80-100mg of uterine protein was passed on to a DEAE-cellulose column which was developed with an ammonium sulphate gradient as described in the Methods section. Each fraction was assayed for RNA polymerase activity and protein content in the column buffer.

RNA polymerase activity

RNA polymerase activity in presence of 1 µg α-amanitin

Absorbance at 610nm

Salt concentration.

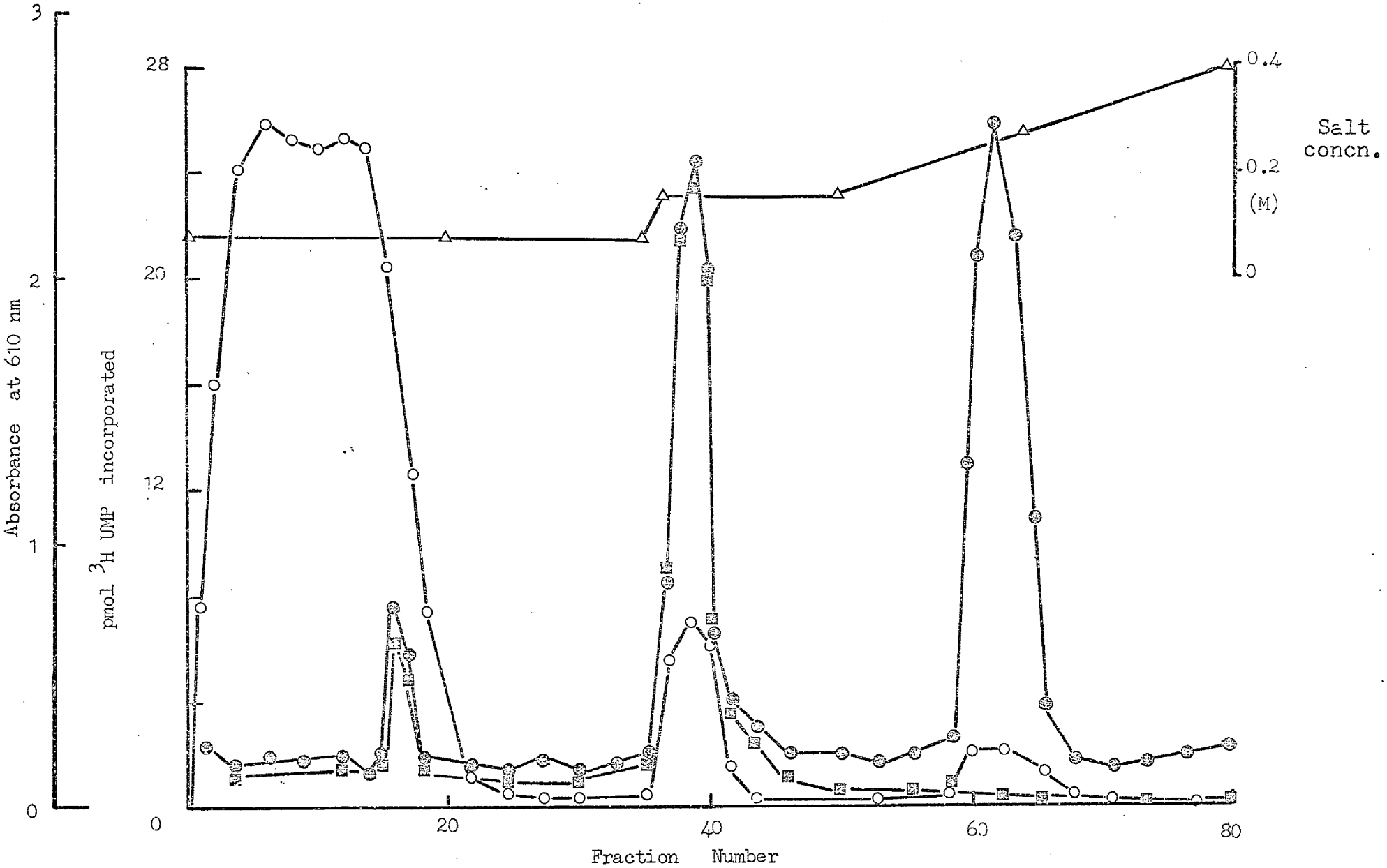

Absorbance at 610 nm
pmol ^{3}H UMP incorporated
Salt concn. (M)
Fraction Number
0
1
2
3
12
20
28
0.2
0.4
40
60
80

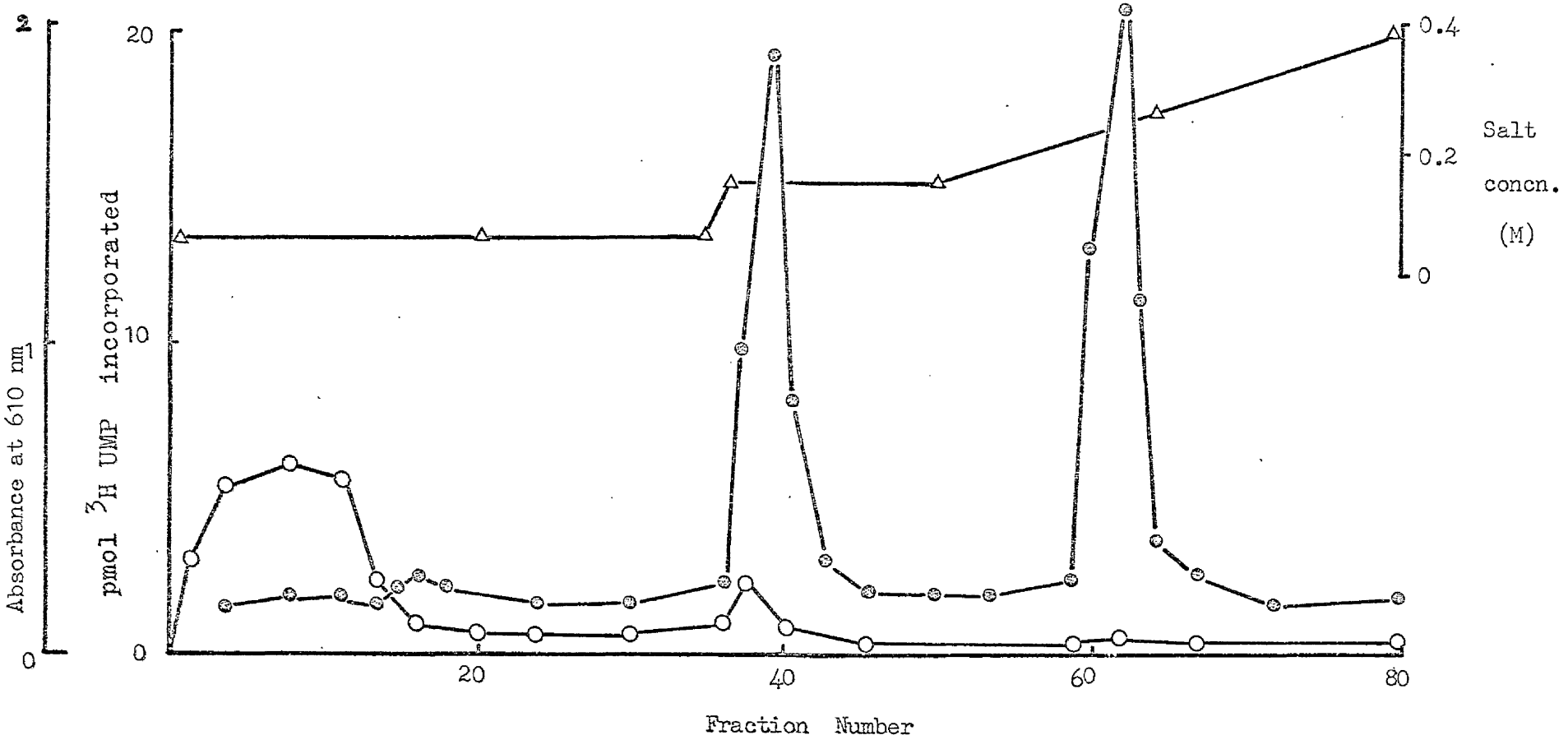
FIG. 2: Multiple forms of DNA-dependent RNA polymerases from uterine nuclei separated on DEAE-cellulose.

Uterine nuclei were prepared and the total nuclear protein extracted as described in the Methods section.
10mg of protein was passed on to a DEAE-cellulose column and chromatography effected as described in the Methods section. Each fraction was assayed in column buffer in duplicate for RNA polymerase activity and protein content.

●——● RNA polymerase activity

o——o Absorbance at 610nm

△——△ Salt concentration.


Absorbance at 610 nm
2
1
0
pmol ^{3}H UMP incorporated
20
10
0
0.4
0.2
0
Salt
concn.
(M)
20
40
60
80
Fraction Number

containing 0.08M ammonium sulphate for 6h at 4°. After dialysis, chromatography on phosphocellulose was effected as described in the Methods section. Fractions (2 ml) were collected and assayed for RNA polymerase activity and protein content (Fig. 3a). RNA polymerase A was eluted by 0.3M ammonium sulphate.

1.4.2. RNA Polymerase B.

The fractions containing RNA polymerase B activity after DEAE-cellulose were pooled and dialysed against 0.08M ammonium sulphate in TGED buffer for 6h at 4°. After dialysis, phosphocellulose chromatography was carried out as above. Fractions (2 ml) were collected and assayed for RNA polymerase activity and protein content (Fig. 3b). RNA polymerase B activity was eluted by 0.3M ammonium sulphate.

After phosphocellulose chromatography, the concentration of protein in the fractions containing each RNA polymerase activity was very low. In order to prevent loss of enzyme activity at low protein concentrations, lysozyme was added to the pooled fractions at a final concentration of 0.5 mg/ml. Lysozyme was used as a stabilising protein for the RNA polymerases in preference to bovine serum albumin because it was free from RNase activity unlike commercial preparations of serum albumin.

1.5. Glycerol Density Gradient Sedimentation.

1.5.1. RNA Polymerase A.

The pooled fractions containing RNA polymerase A activity after phosphocellulose chromatography and addition of lysozyme were dialysed overnight against TGMED(15) buffer and centrifuged in glycerol gradients as described in the Methods section. Fractions (0.2 ml) were collected

FIG. 3: Purification of Uterine RNA Polymerases A and B on Phosphocellulose.

a) The pooled fractions containing RNA polymerase A after DEAE-cellulose chromatography were passed on to a phospho-cellulose column which was developed with an ammonium sulphate gradient as described in the Methods section.
Each fraction was assayed in column buffer in duplicate for RNA polymerase activity.

●——● RNA polymerase activity

○——○ Absorbance at 610nm

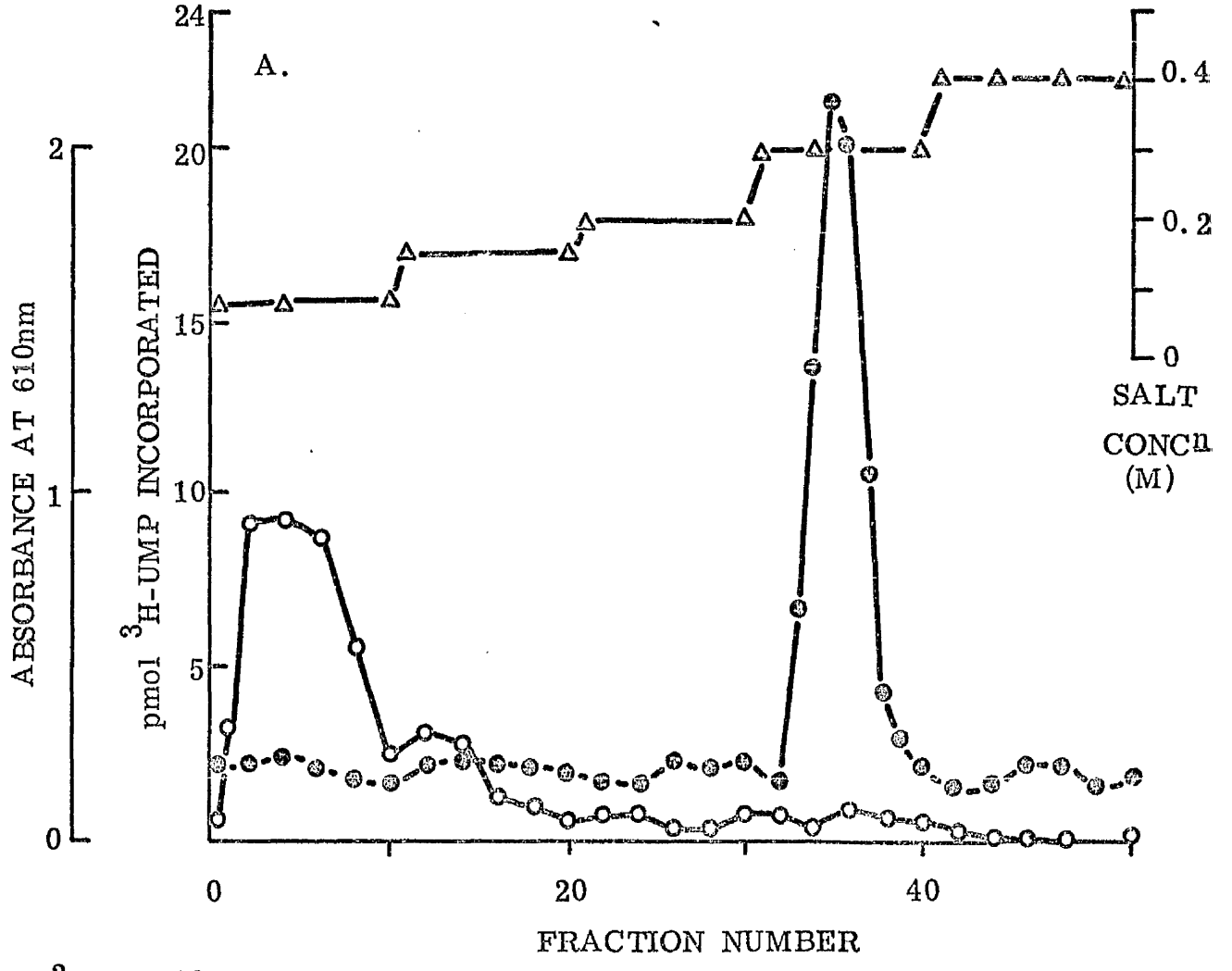
△——△ Salt concentration.

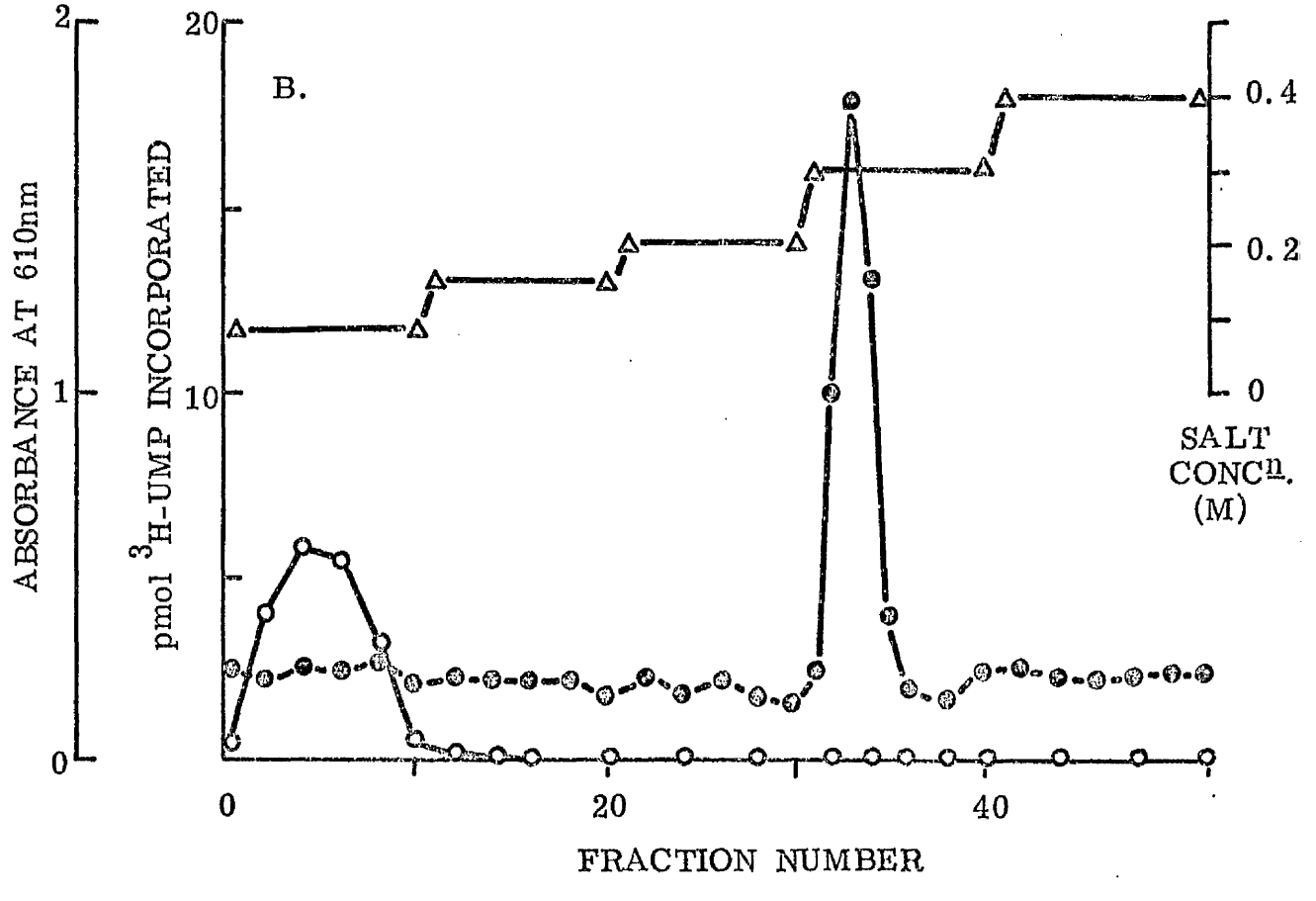
b) The pooled fractions containing RNA polymerase B after DEAE-cellulose chromatography were passed on to a phospho-cellulose column which was developed with an ammonium sulphate gradient as described in the Methods section.
Each fraction was assayed in column buffer in duplicate for RNA polymerase activity.

●——● RNA polymerase activity

○——○ Absorbance at 610nm

△——△ Salt concentration.


A.
ABSORBANCE AT 610nm
pmol ^{3}H-UMP INCORPORATED
SALT CONCn (M)
FRACTION NUMBER
0
20
40
0.4
0.2
0
24
20
15
10
5
2
1


B.
ABSORBANCE AT 610nm
pmol ^{3}H-UMP INCORPORATED
SALT CONCn. (M)
FRACTION NUMBER
0
20
40
0.4
0.2
0
20
10
2
1

by piercing the bottom of each tube and the RNA polymerase activity and protein content (Fig. 4a) were measured. RNA polymerase A was obtained as a well defined peak of activity from the gradient.

1.5.2. RNA Polymerase B.

The pooled fractions containing RNA polymerase B activity after treatment on phosphocellulose were also dialysed overnight against TGMED(15) buffer and glycerol gradient sedimentation carried out as above. The results of this procedure are shown in Fig. 4b and again give rise to a clear cut peak of activity.

1.6. Storage of Enzymes.

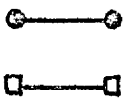
After density gradient centrifugation, the fractions containing enzyme A and B activity were made up to 50% (v/v) glycerol, dialysed against TGED(50) overnight and stored in TGED(50) buffer containing 0.5 mg/ml lysozyme. Storage of partially purified RNA polymerase fractions at -20° in TGED buffer caused total loss of activity of both enzymes within 1-2 days. However, enzyme samples stored at -20° in 50% (v/v) glycerol and at a protein concentration of 0.5 mg/ml remained active for several weeks.

1.7. Enzyme Activity Units.

A unit of RNA polymerase activity is defined as the amount of enzyme which catalyses the incorporation of 1 pmol of UMP into acid-insoluble material per mg protein in 30 min. It is difficult to obtain accurate values for the specific activities at different stages of the purification because of the very small amount of protein present in the fractions,

FIG. 4: Purification of Uterine RNA Polymerases A and B by Glycerol Density Gradient Sedimentation.

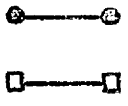
a) The pooled and dialysed fractions containing RNA polymerase A after phosphocellulose chromatography were layered on a 15-30% glycerol gradient containing 0.5mg/ml lysozyme as described in the Methods section.
Each fraction was assayed in duplicate for RNA polymerase activity and protein content.



RNA polymerase activity

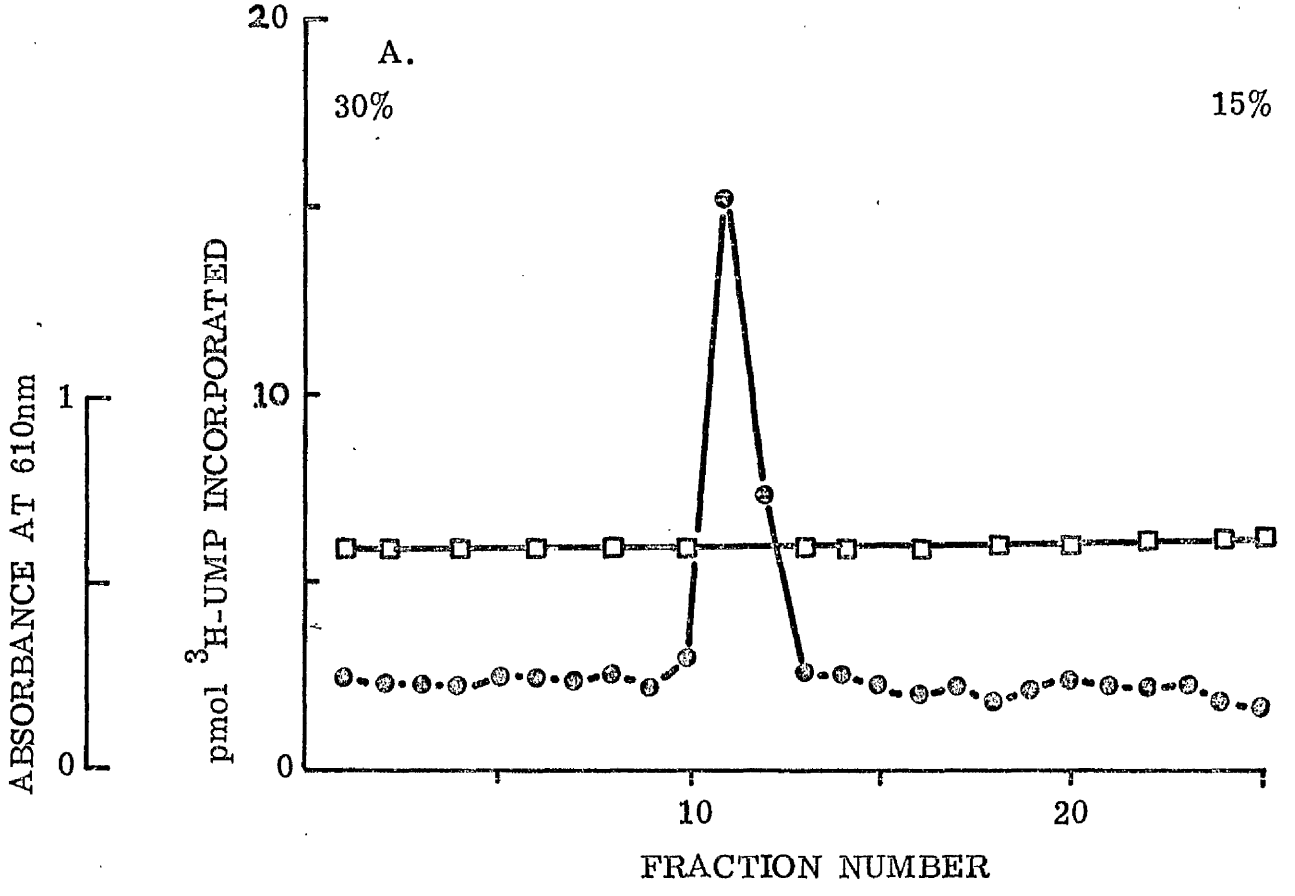
Absorbance at 610nm.

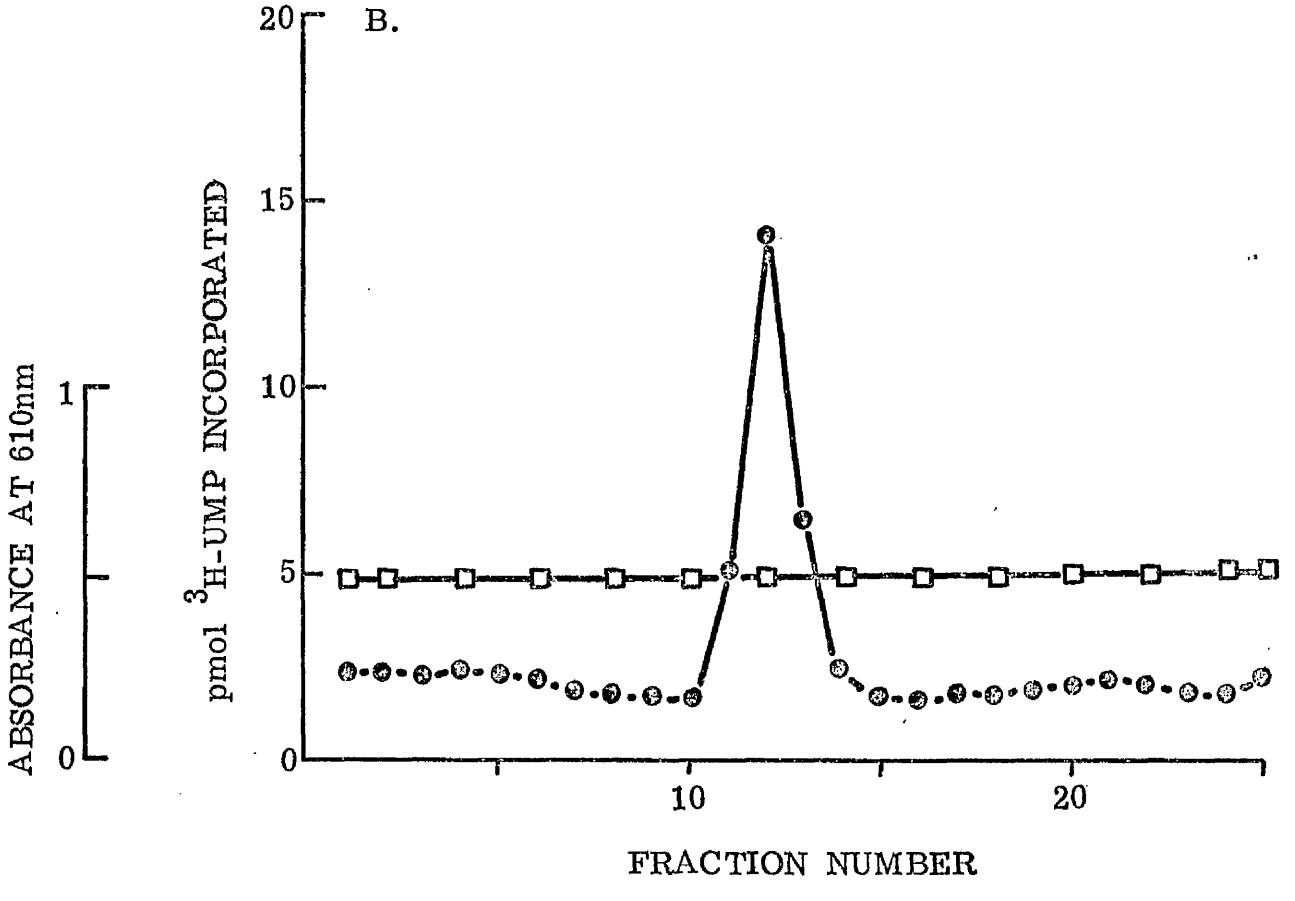
b) The pooled and dialysed fractions containing RNA polymerase B after phosphocellulose chromatography were layered on a 15-30% glycerol gradient containing 0.5mg/ml lysozyme as described in the Methods section.
Each fraction was assayed in duplicate for RNA polymerase activity and protein content.



RNA polymerase activity

Absorbance at 610nm.


A.
30%
15%
20
10
0
1
0
ABSORBANCE AT 610nm
pmol ^{3}H-UMP INCORPORATED
10
20
FRACTION NUMBER


B.
20
15
10
5
0
1
0
ABSORBANCE AT 610nm
pmol ^{3}H-UMP INCORPORATED
10
20
FRACTION NUMBER

and the degree of purification achieved at each step is therefore approximate.

2. Characterisation of Uterine RNA Polymerases A and B.

In all the experiments in this section, the enzyme samples used were partially purified preparations of uterine RNA polymerases A and B in TGED(50) buffer containing 0.5 mg/ml lysozyme.

2.1. Cation Requirements.

The metal ion requirements of the two uterine RNA polymerases are shown in Fig. 5. It is evident that RNA polymerase A can utilise efficiently both Mg^{++} and Mn^{++} but that the concentration of Mn^{++} ions was quite critical (Fig. 5a). The optimal concentrations were 8mM for Mg^{++} and 3mM for Mn^{++}. On the other hand, Mn^{++} is seen to be a better activator for RNA polymerase B than Mg^{++} with an optimal concentration of 3mM (Fig. 5b). Again the concentration of Mn^{++} ions was more critical.

2.2. Salt Requirements.

The effect of the addition of salt to the RNA polymerase assays is shown in Fig. 6. Increasing salt concentrations, irrespective of whether ammonium sulphate or KCl was used, did not affect the activity of RNA polymerase A until above concentrations of 20mM with Mg^{++} and 60mM with Mn^{++} when a marked inhibition takes place. The optimal ammonium sulphate concentration was 10mM in the presence of 8mM Mg^{++} and 40mM in the presence of 3mM Mn^{++} (Fig. 6a).

It is evident from Fig. 6b, however, that an increase in the ionic

FIG. 5: The Ionic Requirements for Uterine RNA polymerases A and B.

a) 30 units of RNA polymerase A in TGED buffer was assayed for RNA polymerase activity in increasing concentrations of Mg^{++} ions and Mn^{++} ions.

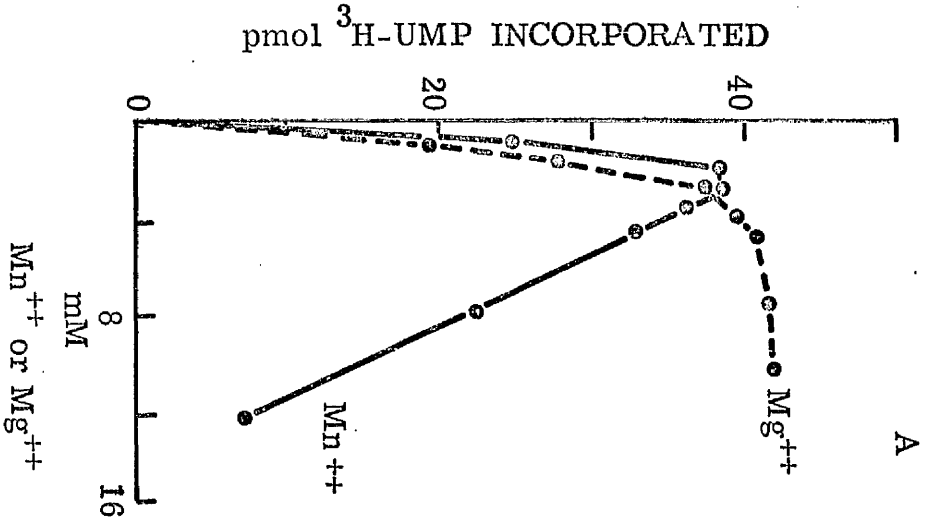
●——● Activity in presence of Mn^{++}

●- - -● Activity in presence of Mg^{++}

b) 30 units of RNA polymerase B in TGED buffer was assayed for RNA polymerase activity in increasing concentrations of Mg^{++} ions and Mn^{++} ions.

●——● Activity in presence of Mn^{++}

●- - -● Activity in presence of Mg^{++}


pmol ^{3}H-UMP INCORPORATED
0
20
40
0
8
16
mM
Mn^{++} or Mg^{++}
Mn^{++}
Mg^{++}
A

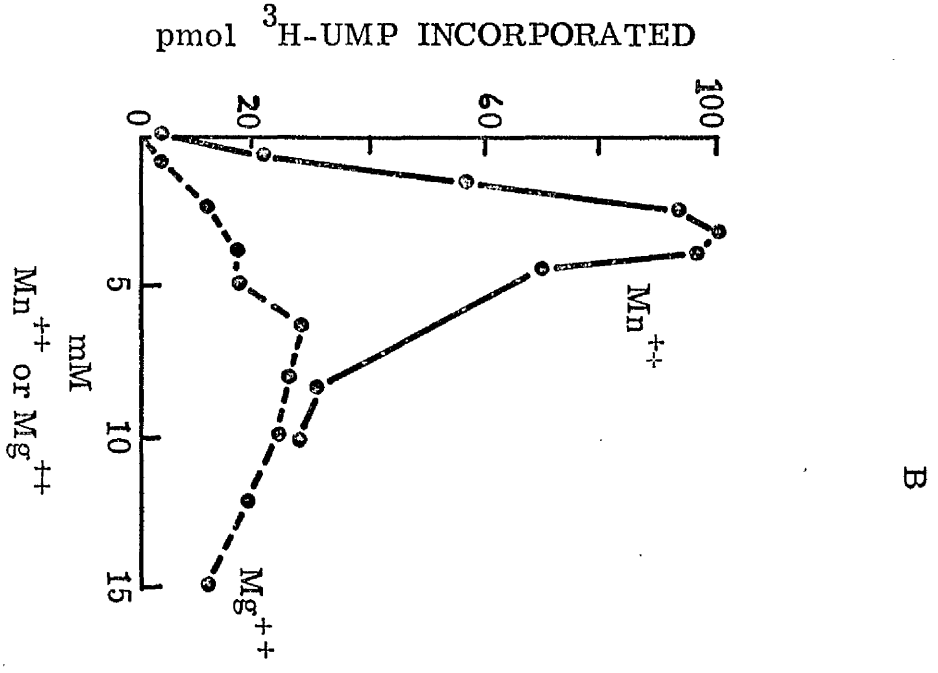
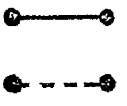

pmol ^{3}H-UMP INCORPORATED
0
20
60
100
0
5
10
15
mM
Mn^{++} or Mg^{++}
Mn^{++}
Mg^{++}
B

FIG. 6: The Salt Requirements for Uterine RNA polymerases A and B.

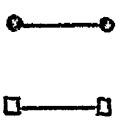
a) 40 units of RNA polymerase A in TGED buffer was assayed for RNA polymerase activity in duplicate in the presence of 8mM Mg^{++} and 3mM Mn^{++} in increasing concentrations of ammonium sulphate.



Activity in presence of 3mM Mn^{++}

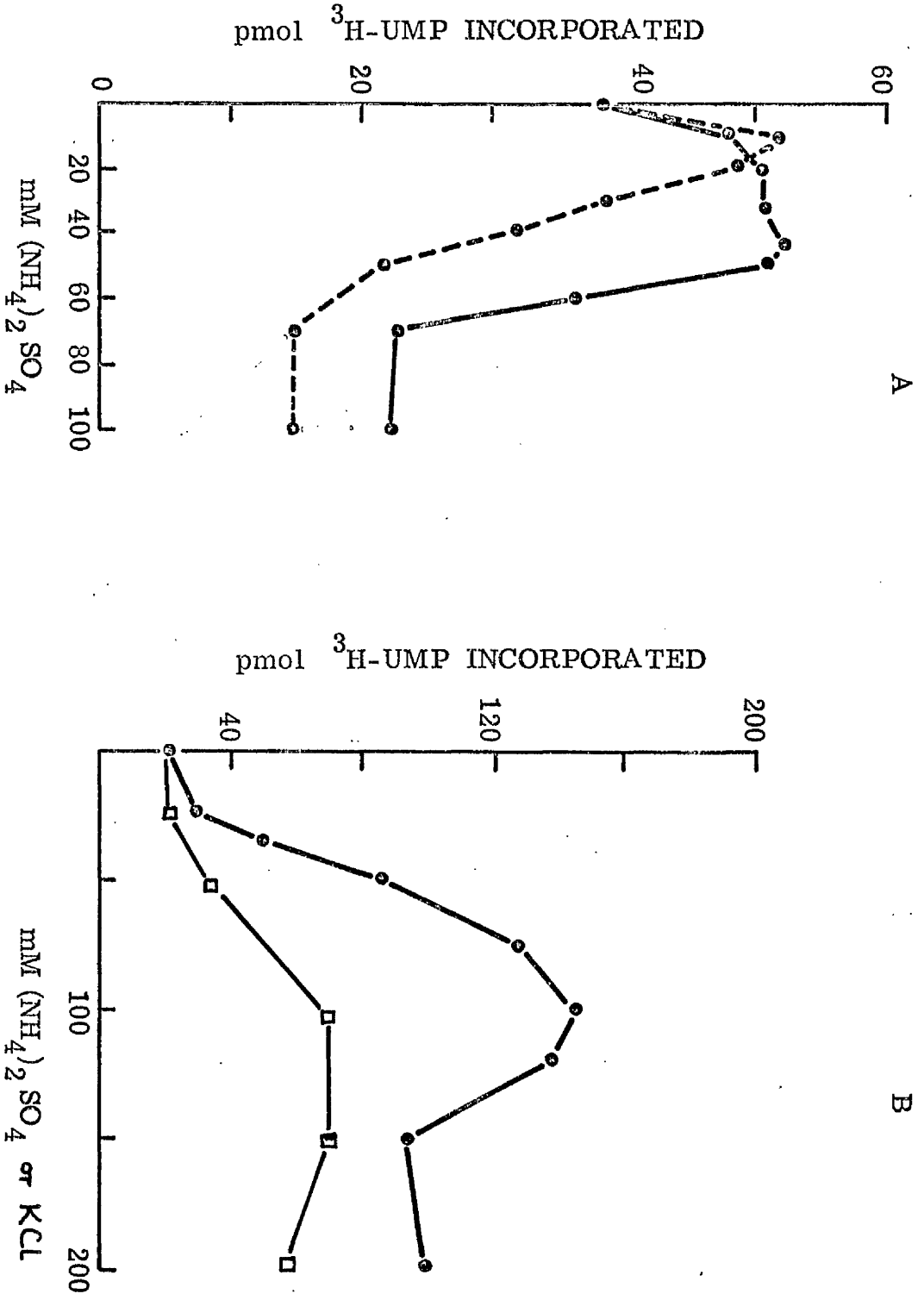
Activity in presence of 8mM Mg^{++}

b) 40 units of RNA polymerase B in TGED buffer was assayed for RNA polymerase activity in duplicate in the presence of 3mM Mn^{++} in increasing concentrations of ammonium sulphate and KCl.



Activity in presence of $(NH_4)_2\ SO_4$

Activity in presence of KCl.


pmol ^{3}H-UMP INCORPORATED
0
20
40
60
mM $(NH_4)_2SO_4$
20
40
60
80
100
A
pmol ^{3}H-UMP INCORPORATED
40
120
200
mM $(NH_4)_2SO_4$ or KCL
100
200
B

strength of the incubation medium greatly enhances RNA polymerase B activity. Ammonium sulphate was more effective than KCl in stimulating RNA synthesis and the optimal concentration was found to be 100mM.

In subsequent experiments on isolated uterine RNA polymerases, the enzymes were assayed under their respective optimal salt and ionic conditions unless stated otherwise.

2.3. Sensitivity to α-Amanitin.

The effect of adding the toxin, α-amanitin, to RNA polymerase assays using the solubilised RNA polymerase preparations is shown in Fig. 7. RNA polymerase B was specifically inhibited at very low levels of the toxin, a concentration of 0.01 μg per assay (0.05 ml) being sufficient to inhibit enzyme B activity by up to 80%, while concentrations of greater than 1 μg per assay completely inhibited enzyme B activity. RNA polymerase A was entirely unaffected by the toxin even at concentrations of 10 μg per assay.

2.4. Thermal Sensitivity.

Fig. 8 shows the effect of incubating the solubilised RNA polymerases at various temperatures for 15 min prior to measuring their activities. RNA polymerase A is very heat labile and more than 75% of its activity is lost by pre-treatment at 45° for 15 min. By contrast, RNA polymerase B is much less sensitive to thermal inactivation, only 20% of its activity being lost after incubation at 45° for 15 min.

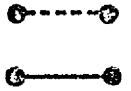
2.5. Substrate Requirements.

The nucleotide requirements for RNA synthesis catalysed by both

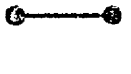
FIG. 7: The Effect of α-Amanitin In Vitro on Uterine RNA polymerases A and B.

20 units of RNA polymerase A or B in TGED buffer were incubated under standard assay conditions as described in the Methods section in the presence of increasing concentrations of α-amanitin. Results are expressed as a percentage of the activity measured in the absence of α-amanitin.

RNA polymerase A in the absence of α-amanitin incorporated 18.6 p mol UMP into RNA in 30 min.
RNA polymerase B in the absence of α-amanitin incorporated 23.0 p mol UMP into RNA in 30 min.



RNA polymerase A activity



RNA polymerase B activity.

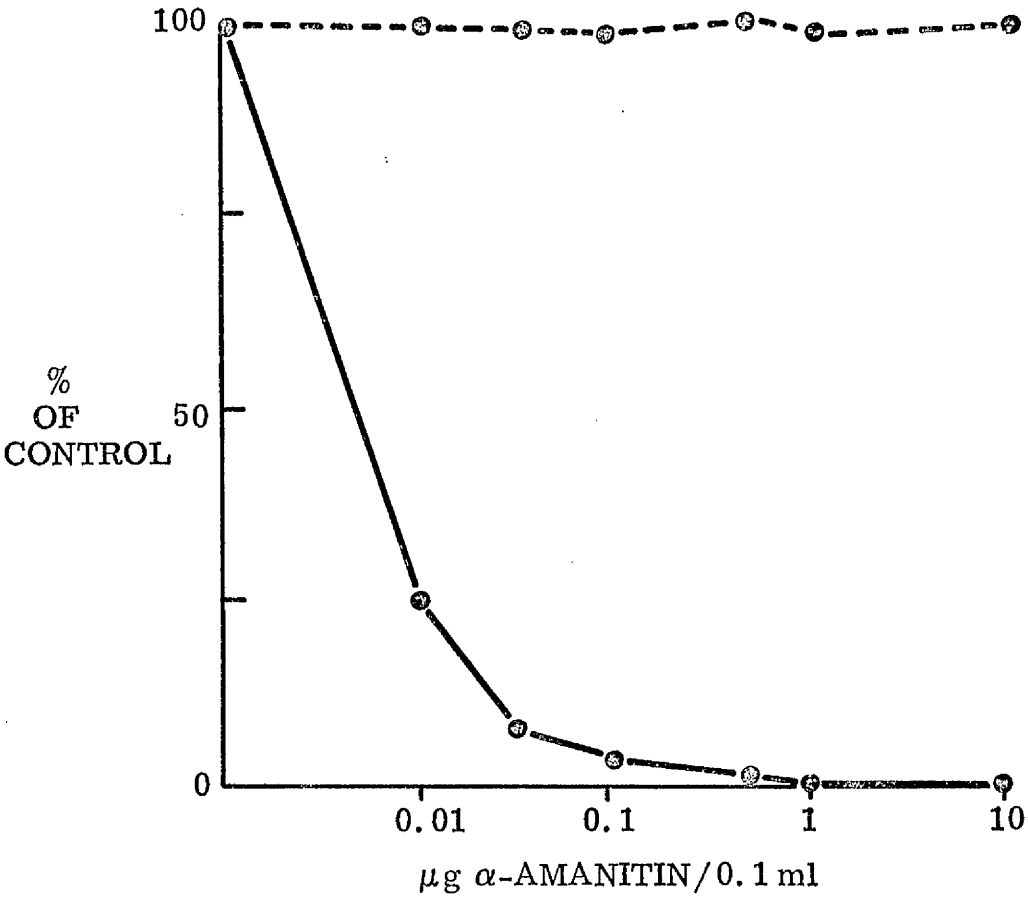
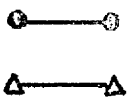

100
50
0
% OF CONTROL
0.01
0.1
1
10
μg α-AMANITIN/0.1 ml

FIG. 8: The Effect of Heat on Uterine RNA polymerases A and B.

20 units of both RNA polymerase A and B in TGED buffer were incubated for 15 min at various temperatures and then assayed under standard assay conditions as described in the Methods section. Results are expressed as a percentage of the activity measured after pre-incubation for 15 min at 37°.

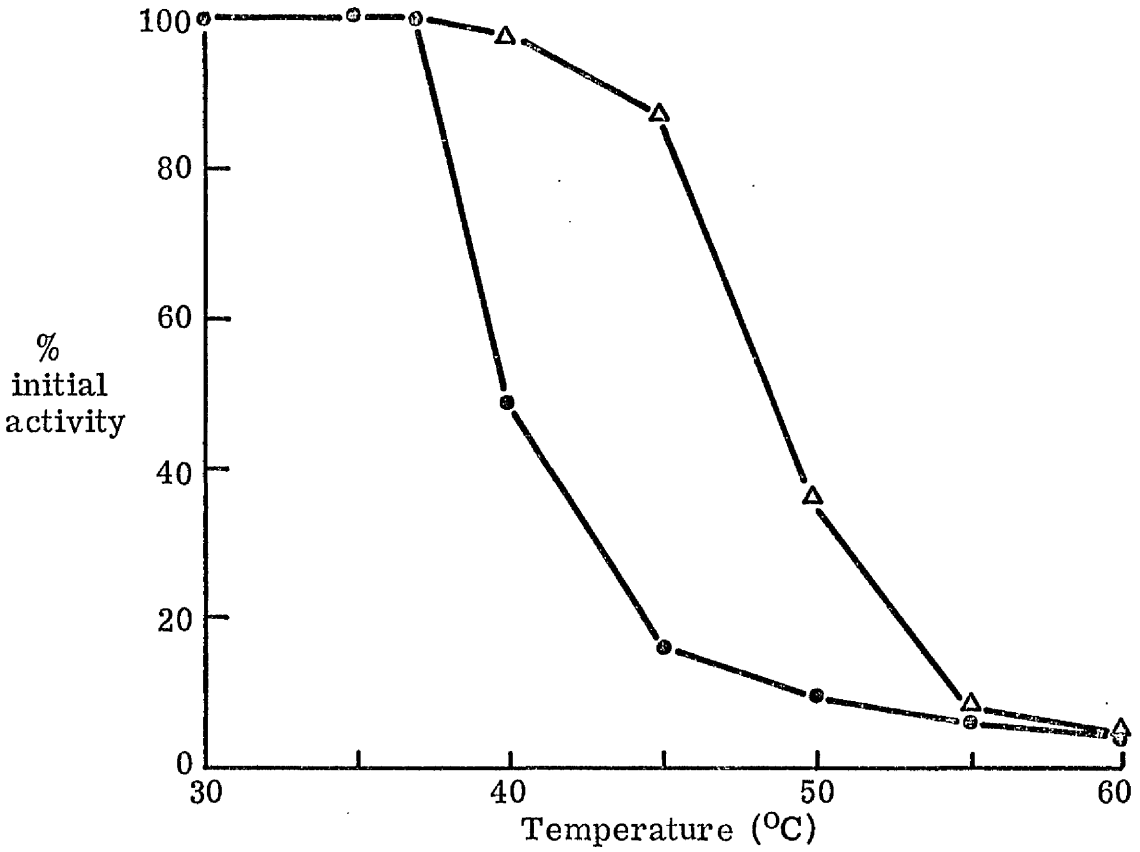
RNA polymerase A control incorporated 19.2 p mol UMP into RNA in 30 min.

RNA polymerase B control incorporated 22.4 p mol UMP into RNA in 30 min.



RNA polymerase A activity

RNA polymerase B activity.


100
80
60
40
20
0
% initial activity
30
40
50
60
Temperature (°C)

RNA polymerases are shown in Table 1. Both enzymes are dependent on the presence of all four triphosphates. If one triphosphate was omitted from the assay mixture, activity in both enzymes was decreased by about 30-35% while removal of two triphosphates from the assay mixture depressed enzymic activity to only 5-10% of control levels. Virtually no activity was measured for both enzymes when three triphosphates were absent from the system.

Another essential requirement for RNA synthesis is the presence of a reducing agent with free thiol groups such as 2-mercaptoethanol or DTT. 2-Mercaptoethanol was used in all experiments and had an optimal concentration for both RNA polymerases of 20mM. When 2-mercaptoethanol was omitted from the assay mixture only 10-15% of control activities were recorded.

2.6. Template Requirements.

There is an absolute dependence for DNA for both solubilised forms of uterine RNA polymerase as shown in Table 2. The role of DNA in the reaction was confirmed by the action of actinomycin D which completely depressed RNA synthesis.

RNA polymerase A is more active on double-stranded DNA templates such as rabbit liver DNA and calf thymus DNA than on their heat-denatured counterparts. On the other hand, RNA polymerase B catalyses RNA synthesis more efficiently on heat-denatured templates under the conditions employed. Both enzymes are capable of catalysing RNA synthesis on the synthetic polydeoxyribonucleotide poly d(AT) which is a much more efficient template for both RNA polymerase A and B than 'native' template. Rabbit uterine DNA was not used as a template because the small amount which could be extracted from uteri would necessitate using a large number of animals to obtain a sufficient amount of DNA. In the absence of DNA

TABLE 1 : Substrate Requirements for Uterine RNA Polymerases A and B.

10 units of either RNA polymerase A or B were incubated in the standard RNA polymerase assay mixture as described in the Methods section, using either ^{3}H-UTP or ^{3}H-GTP as the labelled substrate. The effect of removal of each of the substrates is expressed as a percentage of the activity with the complete system. The results are the average of at least three determinations.

Table 1.

Substrate Requirements for Uterine RNA Polymerases A and B.

	RNA Polymerases			
	A		B	
Using ^{3}H-UTP	pmol UMP incorpd	% of control	pmol UMP incorpd	% of control
Complete system :	9.70	100	10.60	100
-GTP	6.67	68.8	6.91	65.2
-ATP	6.44	66.4	7.01	66.1
-CTP	6.31	65.0	7.12	67.2
-GTP -ATP	0.83	8.6	0.71	6.7
-GTP -CTP	0.58	6.0	0.78	7.4
-CTP -ATP	0.51	5.3	0.93	8.8
-GTP -CTP -ATP	0.11	1.2	0.09	0.9
-mercaptoethanol	1.02	10.5	1.30	12.3
Using ^{3}H-GTP				
Complete system :	9.85	100	10.40	100
-UTP	6.58	66.8	7.00	67.3
-ATP	6.91	70.2	6.75	64.9
-CTP	6.74	68.4	6.82	65.6
-UTP -ATP	0.93	9.4	0.92	8.9
-UTP -CTP	0.75	7.6	0.83	8.0
-CTP -ATP	0.81	8.2	0.68	6.5
-ATP -CTP -UTP	0.18	1.8	0.11	1.1
-mercaptoethanol	1.31	13.3	1.34	12.9

TABLE 2 : Template Requirements for Uterine RNA Polymerases A and B

10 units of either RNA polymerase A or B were incubated in the standard RNA polymerase assay mixture as described in the Methods section using 10 µg of native and heat-denatured DNA as a template or 10 µg poly d(AT). In some experiments 1 µg of actinomycin D was present and when DNA was not included, 28S rRNA (10 µg) or poly A (10 µg) was used as a template. The results are the average of at least three determinations and are expressed as a percentage of the activity using double-stranded rabbit liver DNA as template.

Table 2.

Template Requirements for Uterine RNA Polymerases A and B.

Template	RNA Polymerases A pmol UMP incorpd	A % of control	B pmol UMP incorpd	B % of control
Native rabbit liver DNA	10.30	100	11.20	100
Denatured rabbit liver DNA	7.03	68.3	23.80	212.5
Native calf thymus DNA	9.74	94.6	12.20	108.9
Denatured calf thymus DNA	6.57	63.8	32.08	286.4
+actinomycin D	0.62	6.2	0.89	8.0
Poly d(AT)	22.97	223.0	20.13	179.7
-DNA	0	0	0	0
-DNA +rRNA	0.01	0.1	0	0
-DNA +poly A	0	0	0	0

neither RNA polymerase catalyses RNA synthesis on a template of either 28S rRNA or the synthetic polynucleotide poly A.

2.7. Action of Pronase and Nucleases on Enzyme Activity.

The effect of pre-incubation of both RNA polymerases with Pronase, RNase and DNase are shown in Table 3. Incubation of either enzyme with Pronase for 60 min prior to assay virtually abolishes enzyme activity. This is presumably due to the action of the proteolytic enzyme on the individual RNA polymerases showing that they are indeed proteins. Similarly no activity is observed when either of the enzymes are assayed in the presence of pancreatic RNase. This is almost certainly a result of the action of the nuclease on the product of the reaction, and in the case of both RNA polymerases about 90% of the activity is restored when heparin is added to the assay mixture to inhibit RNase.

When low concentrations of DNase are added to the RNA polymerase assay then an increase in RNA synthesis catalysed by both enzymes is observed and is due presumably to the DNase introducing single-strand 'nicks' into the DNA template. When higher concentrations of DNase are employed very little RNA polymerase activity is found and this is probably a consequence of the nuclease breaking the template into small fragments.

2.8. Contaminating Enzyme Activities.

2.8.1. DNase.

The results of DNase assays on enzyme fractions at various stages during the purification procedure are shown in Fig. 9. DNase assays

TABLE 3 : The Action of Pronase and Nucleases with Uterine RNA Polymerases A and B.

10 units of either RNA polymerase A or B were incubated in the standard RNA polymerase assay mixture as described in the Methods section.

The enzymes were pre-incubated with Pronase (50 µg) for 15 min at 37° before assaying.

In the experiments with nucleases, 10 units of either RNA polymerase A or B were incubated in the standard RNA polymerase assay mixture in the presence of either pancreatic RNase (50 µg) with and without heparin (50 µg) or DNase at concentrations of either 1 mg or 10 ng per assay.

The results which represent the average of at least three determinations are expressed as a percentage of the activity in untreated samples.

Table 3.

Action of Pronase and Nucleases with Uterine RNA Polymerases A and B.

	RNA Polymerase			
	A		B	
Preincubation with	pmol UMP incorpd	% of control	pmol UMP incorpd	% of control
-	12.60	100	14.00	100
Pronase	0	0	0	0
Pancreatic RNase	1.46	11.6	1.43	10.2
RNase + heparin	10.94	86.8	12.88	92.0
DNase (1mg)	0	0	0	0
DNase (10ng)	16.13	128.0	15.26	109.0

FIG. 9: DNase Assays on Fractions containing RNA polymerases A and B during the purification procedure.

10 units of RNA polymerases A and B from various steps during the purification of the enzymes were assayed in the standard RNA polymerase assay mixture for DNase activity as described in the Methods section.

——— RNA polymerase A

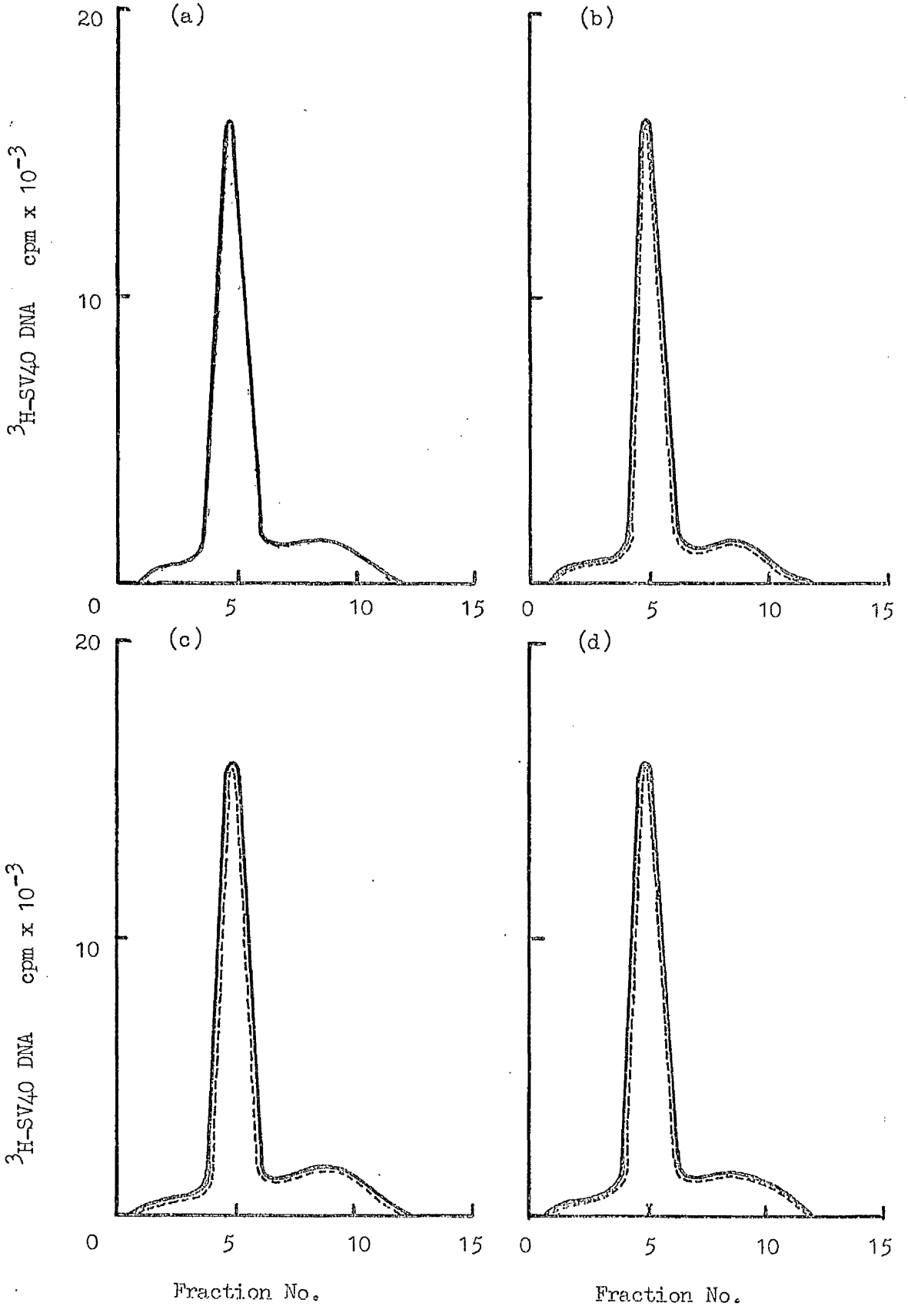
- - - - RNA polymerase B

a) Control

b) After DEAE-cellulose chromatography

c) After phosphocellulose chromatography

d) After glycerol gradient sedimentation.


(a)
(b)
(c)
(d)
20
10
0
5
10
15
^{3}H-SV40 DNA cpm x 10^{-3}
Fraction No.

were carried out as described in the Methods section. Very little DNase activity can be detected even in the first step of the purification, DEAE-cellulose chromatography. The final purified enzyme fraction does not contain any detectable DNase activity under the conditions employed.

2.8.2. RNase.

The results of RNase assays on enzyme fractions at various stages during the purification procedure are shown in Fig. 10. Slight contamination by RNase can be detected after DEAE-cellulose chromatography but this is removed by phosphocellulose chromatography. The final purified enzyme fractions do not contain any detectable RNase activity under the conditions used.

2.8.3. DNA Polymerase.

No DNA polymerase activity could be detected in any of the enzyme fractions during the purification of either of the uterine RNA polymerases.

2.9. Structural Studies on RNA Polymerases A and B.

RNA polymerases A and B and E. coli RNA polymerase were run in parallel on 15-30% glycerol gradients in the presence of 100mM ammonium sulphate (Fig. 11). Details of the procedure are given in the legend. The fractions from the gradient were assayed for RNA polymerase activity after three different treatments. Firstly, RNA polymerase activity was measured in the presence of α-amanitin (1 µg) and rifampicin (1 µg) to inhibit RNA polymerase B and E. coli RNA polymerase respectively.

FIG. 10: RNase Assays on Fractions containing RNA polymerases A and B during the purification prccedure.

10 units of RNA polymerases A and B from various steps during the purification of the enzymes were assayed for RNase activity as described in the Methods section.

——— RNA polymerase A

----- RNA polymerase B

a) Control

b) After DEAE-cellulose chromatography

c) After phosphocellulose chromatography

d) After glycerol gradient sedimentation.

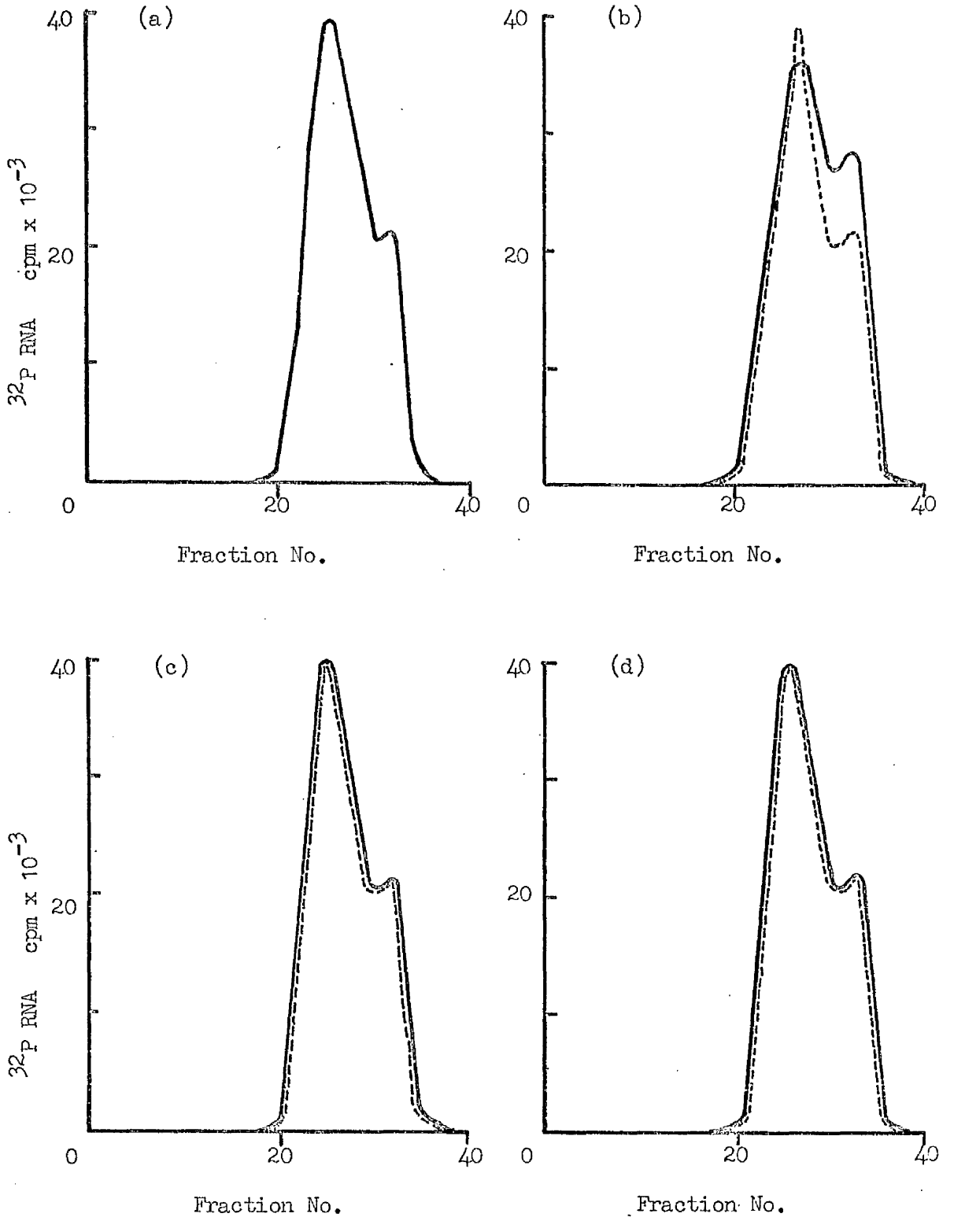
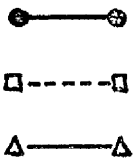

(a)
(b)
(c)
(d)
40
20
0
20
40
^{32}P RNA cpm x 10^{-3}
Fraction No.

FIG. 11: Cosedimentation of Uterine RNA polymerases A and B and E. coli RNA polymerase in glycerol gradient.

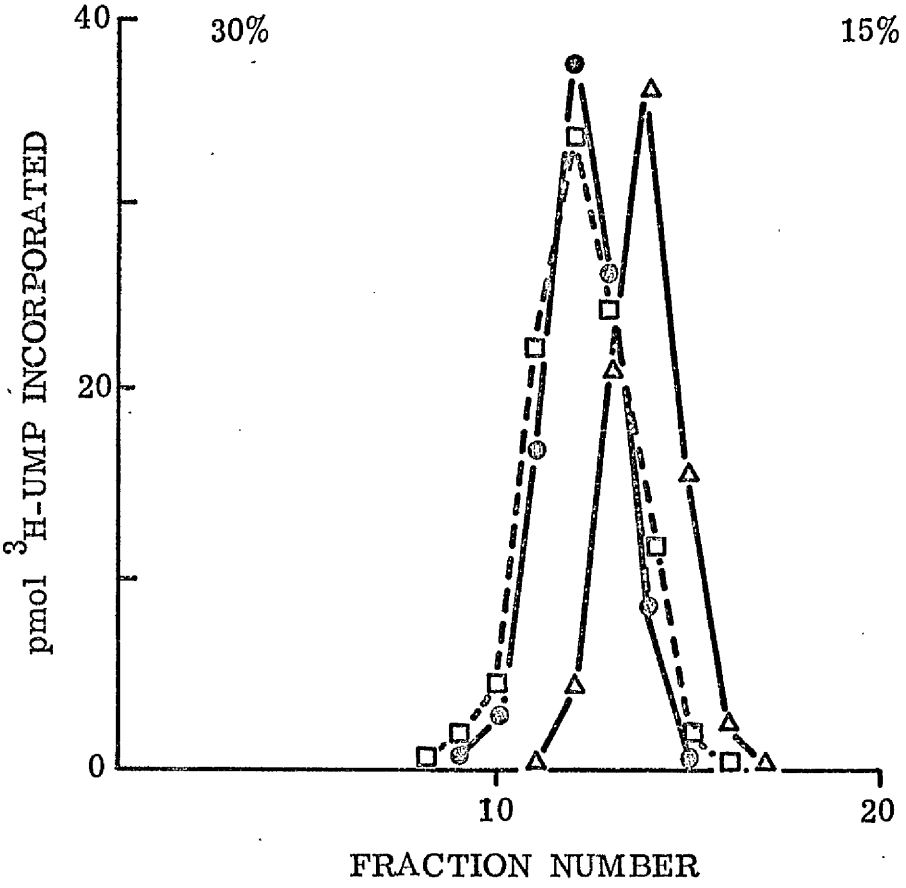
40 units of RNA polymerases A and B and 2 µg E. coli RNA polymerases were cosedimented in a 15-30% glycerol gradient and fractions assayed for RNA polymerase activity under the conditions described in the Methods section.



Activity due to RNA polymerase A

Activity due to RNA polymerase B

Activity due to E. coli RNA polymerase.


40
30%
15%
20
0
pmol ^{3}H-UMP INCORPORATED
10
20
FRACTION NUMBER

Secondly, RNA polymerase activity was measured with rifampicin after 15 min pre-incubation at 45° to inhibit E. coli RNA polymerase and RNA polymerase A respectively and finally measured in the presence of α-amanitin after 15 min pre-incubation at 45° in order to inhibit RNA polymerase A and B respectively. Thus, the three enzyme activities recorded represent the activity due to RNA polymerase A, RNA polymerase B and E. coli RNA polymerase respectively.

Under the conditions employed the bacterial enzyme is in its monomeric form suggesting that the mammalian enzymes which sedimented further in the glycerol gradient both have molecular weights in excess of 500,000.

3. RNA Polymerase C.

RNA polymerase C was isolated from rabbit uterus by a modification of the method outlined in section 1.2. of Results. The excised uteri were homogenised as described in the Methods section. The filtered homogenate was centrifuged at 800xg for 15 min and the supernatant fraction was decanted and centrifuged for 1h at 105,000xg in a Spinco SW40 rotor so as to give a preparation of soluble uterine proteins. The composition of this fraction was adjusted to final concentrations of 25mM tris-HCl pH 7.9, 0.5mM EDTA, 3.5mM DTT, 5mM $MgCl_2$ and 20% (v/v) glycerol and solid ammonium sulphate was added to give 100% saturation as described in the Methods section. The pellet obtained after centrifugation was suspended in a small volume of TGMED buffer and dialysed overnight against TGMED containing 0.08M ammonium sulphate to remove excess salt. DEAE-cellulose chromatography was carried out as already described in section 1.3. of Results. RNA polymerase C activity was eluted from the column in the column wash (Fig. 1) and the fractions containing enzyme activity were pooled and dialysed against TGED buffer

containing 0.08M ammonium sulphate for 6h at 4^{o} before being applied to a phosphocellulose column. The enzyme was eluted by 0.2M ammonium sulphate (Fig. 12). No further purification of this species of RNA polymerase was attempted because of the very low levels of activity present after phosphocellulose chromatography. All the studies on the characteristics of RNA polymerase C were carried out using preparations of the enzyme which had been partially purified by DEAE- and phosphocellulose chromatography as described above.

The template and substrate requirements for RNA polymerase C are shown in Table 4. It can be seen that the enzyme is completely dependent on the presence of all four triphosphates as well as a DNA template.

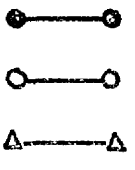
A comparison of some of the properties of RNA polymerase C with those of RNA polymerases A and B is shown in Table 5. RNA polymerase C exhibits some of the properties of both A and B enzymes. Thus, its ionic requirements are intermediate between those of RNA polymerases A and B, it is partially inhibited by α-amanitin whereas enzyme A is unaffected and enzyme B is completely inhibited. It is less sensitive to heat treatment than RNA polymerase A and more sensitive than RNA polymerase B. It resembles both the A and B enzymes in its susceptibility to actinomycin D, rifampicin and cycloheximide and the resistance of enzyme C to rifampicin suggests that it is not mitochondrial in origin since the mitochondrial RNA polymerase has been shown to be sensitive to this compound (Reid & Parsons, 1971). It is possible that this enzyme may be of cytoplasmic origin or it may appear in the soluble fraction of the homogenate because of leaching out from the nuclei during the isolation procedure.

4. Isolation of Uterine Nuclei.

Great difficulty was experienced in obtaining a satisfactory

FIG. 12: Purification of RNA polymerase C by phosphocellulose chromatography.

The pooled fractions which contained RNA polymerase C after DEAE-cellulose chromatography were passed on to a phosphocellulose column which was developed with an ammonium sulphate gradient as described in the Methods section. Each fraction was assayed in column buffer in duplicate for RNA polymerase activity and protein content.



RNA polymerase activity

Absorbance at 610nm

Salt concentration.

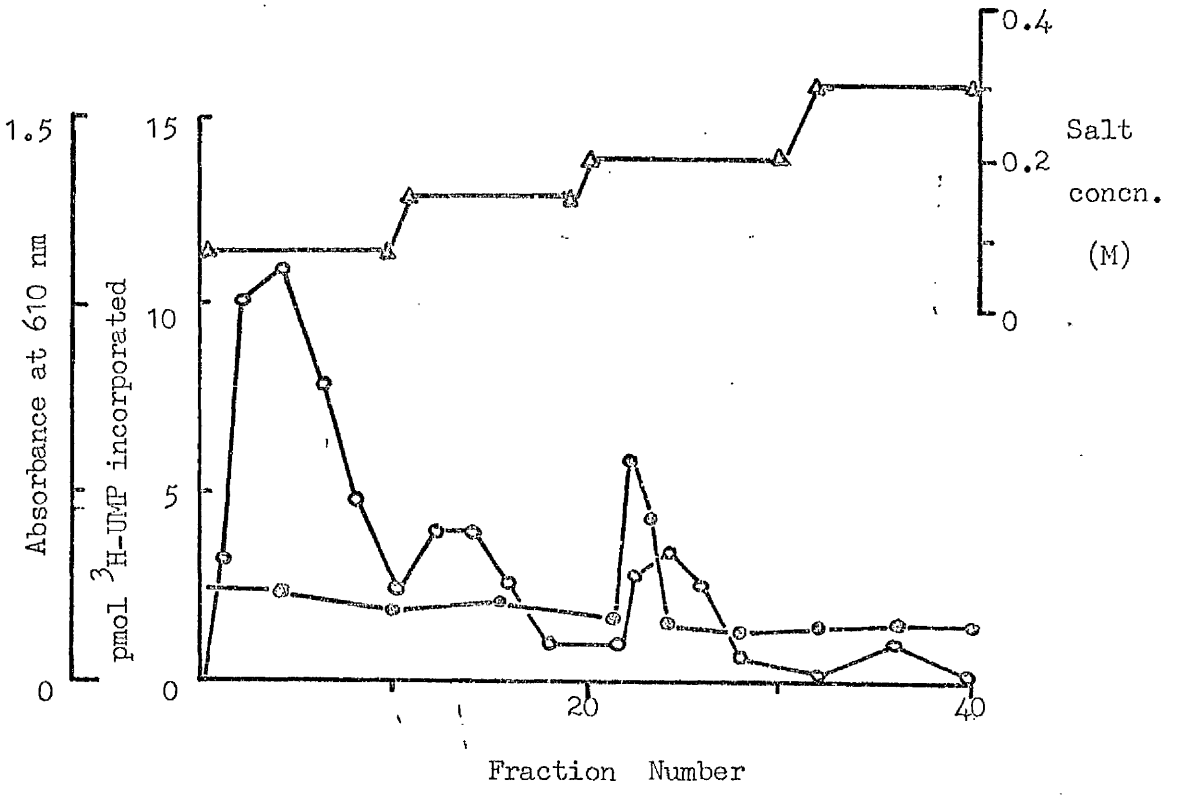

1.5
0
Absorbance at 610 nm
15
10
5
0
pmol ^{3}H-UMP incorporated
0.4
0.2
0
Salt
concn.
(M)
20
40
Fraction Number

TABLE 4 : Substrate and Template Requirements for RNA Polymerase C.

a) 5 units of RNA polymerase C were incubated in the standard RNA polymerase assay mixture as described in the Methods section. The effect of removal of each of the substrate is expressed as a percentage of the activity with the complete system. The results are the average of at least three determinations.

b) 5 units of RNA polymerase C were incubated in the standard RNA polymerase assay mixture as described in the Methods section in the presence of either 10 µg of DNA or poly d(AT). The results are the average of at least three determinations and are expressed as a percentage of the activity using double-stranded rabbit liver DNA as the template.

Table 4.

Substrate and Template Requirements for RNA Polymerase C.

a) Substrate Requirements

Substrate	pmol UMP incorpd	% of control
Complete system :	4.62	100
-ATP	2.83	61.2
-GTP	2.75	59.5
-CTP	2.99	64.7
-ATP -GTP	0.24	5.3
-CTP -GTP	0.35	7.5
-CTP -ATP	0.32	6.9
-ATP -CTP -GTP	0.04	0.8

b) Template Requirements

Template	pmol UMP incorpd	% of control
Native rabbit liver DNA	4.60	100
Denatured rabbit liver DNA	5.45	118.5
Native calf thymus DNA	4.31	93.8
Denatured calf thymus DNA	5.85	127.2
+actinomycin D	0.34	7.5
Poly d(AT)	6.35	138.0
-DNA	0	0

TABLE 5 : Characteristics of Uterine RNA Polymerases A, B and C.

a) 10 units of RNA polymerase A and B and 5 units of RNA polymerase C were assayed in various ionic and salt conditions. The results show the concentrations which gave maximal enzymic activity. RNA polymerase A is most active in 20 mM ammonium salphate in the presence of Mg^{++} and 60 mM ammonium sulphate in the presence of Mn^{++}.

The effect of heat on the enzymes is expressed as a percentage of the activity in samples incubated for 15 min at 37^{o}.

b) 10 units of RNA polymerase A and B and 5 units of RNA polymerase C were assayed in the standard RNA polymerase assay mixture as described in the Methods section in the presence of either α-amanitin (1 µg), actinomycin D (1 µg), rifampicin (1 µg) or cycloheximide (1 µg).

The results are expressed as a percentage of the activity in samples which did not contain any inhibitor.

Table 5.

Characteristics of Uterine RNA Polymerases A, B and C.

a)

Salt and ionic requirements	A	B	C
Optimal Mg^{++} concn.	8mM	6mM	5mM
Optimal Mn^{++} concn.	2-3mM	3mM	3mM
Optimal ammonium sulphate concn.	20mM 60mM	110mM	80mM
Thermal treatment : 45° for 15 min Loss of activity	90	20	30
b) Action of inhibitors	% age of control activity		
α-amanitin	100	0	80
Actinomycin D	6	6	5
Rifampicin	100	100	100
Cycloheximide	100	100	100

method for the isolation of enzymically active uterine nuclei in a sufficiently high yield without too much cytoplasmic contamination. The most successful method of isolation gauged in terms of recovery of nuclei based on ratio of the amount of DNA in the nuclear and homogenate fractions was a modification of the method of Blobel & Potter (1966) as described in the Methods section in which the tissue homogenate was sedimented through concentrated sucrose solutions. The isolated nuclei had a RNA/DNA ratio of 0.23-0.27 and a protein/DNA ratio of 2.6-2.7. The average recovery of nuclei was between 35-40% using the homogenisation conditions described in the Methods section. This was the best average recovery obtained due to the muscular nature of the uterus and the difficulty encountered in homogenisation. Fig. 13 is an electronmicrograph of a preparation of uterine nuclei magnified 12,500 times. Although many nuclei have disrupted membranes, very little cytoplasmic contamination can be detected.

5. RNA Polymerase Activities in Intact Nuclei.

The measurement of endogenous RNA polymerase activities in isolated intact nuclei depends on the presence of several factors in the assay mixture. It is well known that nuclei are capable of synthesising RNA in the presence of either Mg^{++} ions or Mn^{++} ions and ammonium sulphate. Synthesis of RNA under conditions of low ionic strength and in the presence of Mg^{++} has been equated with RNA polymerase A activity, while RNA synthesis occurring in high salt conditions has been attributed mainly to RNA polymerase B.

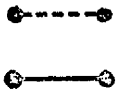
In the experiments with isolated nuclei reported here it was observed that addition of α-amanitin to the incubation mixture caused a loss of 80% of the activity measured under high salt conditions and only about 20% of the activity measured under low salt conditions (Fig. 14).

FIG. 13: Nuclei from the Uteri of Immature Rabbits.

Nuclei were prepared from the uteri of immature rabbits by method described in the Methods section and prepared for examination in the electron microscope also as described in the Methods section. The electron micrograph is a 12500 x magnification.

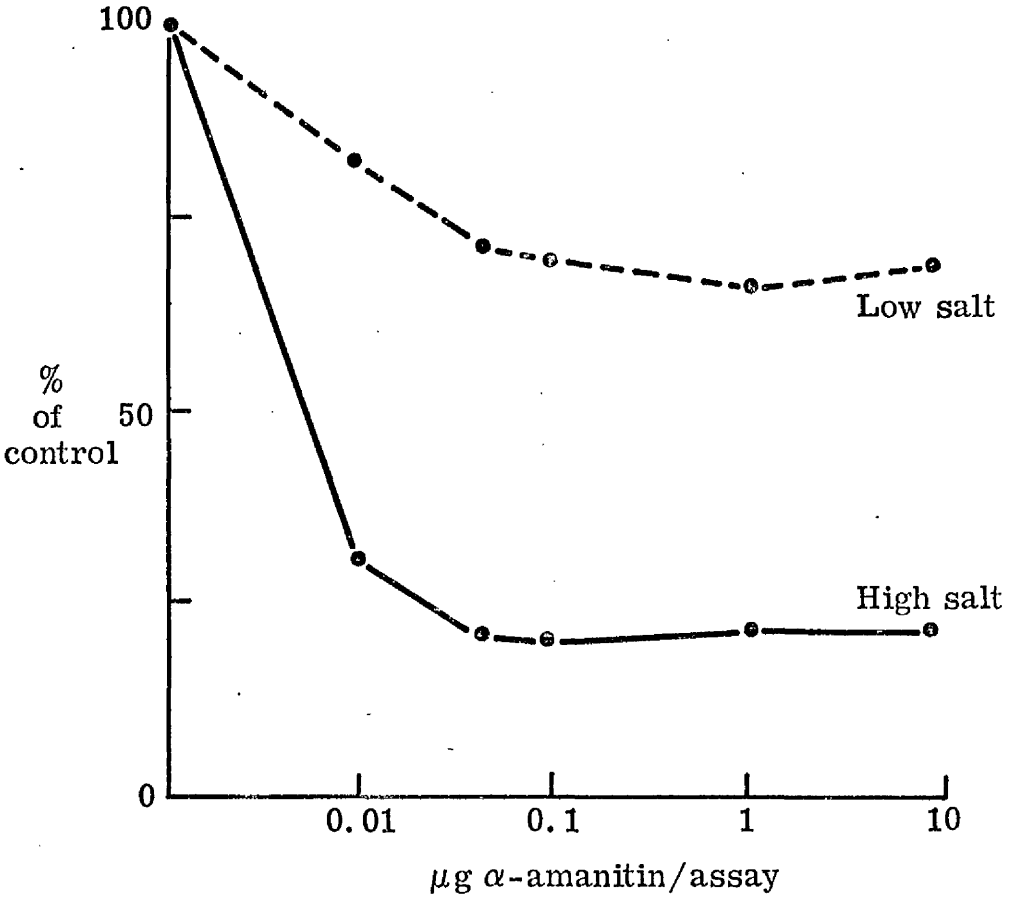
FIG. 14: The Effect of α-amanitin in vitro on RNA synthesis in Uterine Nuclei.

Uterine nuclei, prepared as described in the Methods section, containing between 25-30 µg DNA were assayed in either low salt or high salt conditions in the presence of increasing amounts of α-amanitin. Results are expressed as a percentage of the activity in controls which did not contain any α-amanitin.



Low salt RNA polymerase activity

High salt RNA polymerase activity.


100
% of control
50
0
Low salt
High salt
0.01
0.1
1
10
μg α-amanitin/assay

It has already been demonstrated that α-amanitin specifically inhibits RNA polymerase B *in vitro* and the above result is interpreted as indicating that the residual activity in each case is due to RNA polymerase A which is insensitive to the action of the toxin. In subsequent experiments on the RNA polymerases in isolated nuclei, it is assumed that the activity recorded when the nuclei are incubated in low salt in the presence of 1 μg of α-amanitin corresponds to RNA polymerase A while the activity measured under high salt conditions less the activity measured under high salt conditions in the presence of 1 μg of α-amanitin is taken to represent RNA polymerase B.

5. The Effect of Oestradiol on RNA Synthesis.

5.1. Effect of Hormone Concentration.

The effect on RNA synthesis of 2h treatment of increasing amounts of oestradiol-17β to immature rabbits is shown in Fig. 15. The animals were injected with a mixture of [^{3}H]-guanosine and [^{3}H]-uridine 30 min prior to sacrifice. The results are expressed as a percentage of control values. It can be seen that 10 μg oestradiol per kg body weight caused a considerable increase in the incorporation of precursor into acid-insoluble material while the increase in labelling of the acid-soluble fraction was quite small. Larger doses of oestradiol did not increase significantly the incorporation of labelled precursors into either the acid-soluble or acid-insoluble material.

5.2. Effect of Oestradiol Administered *In Vitro* on RNA Synthesis in Isolated Nuclei.

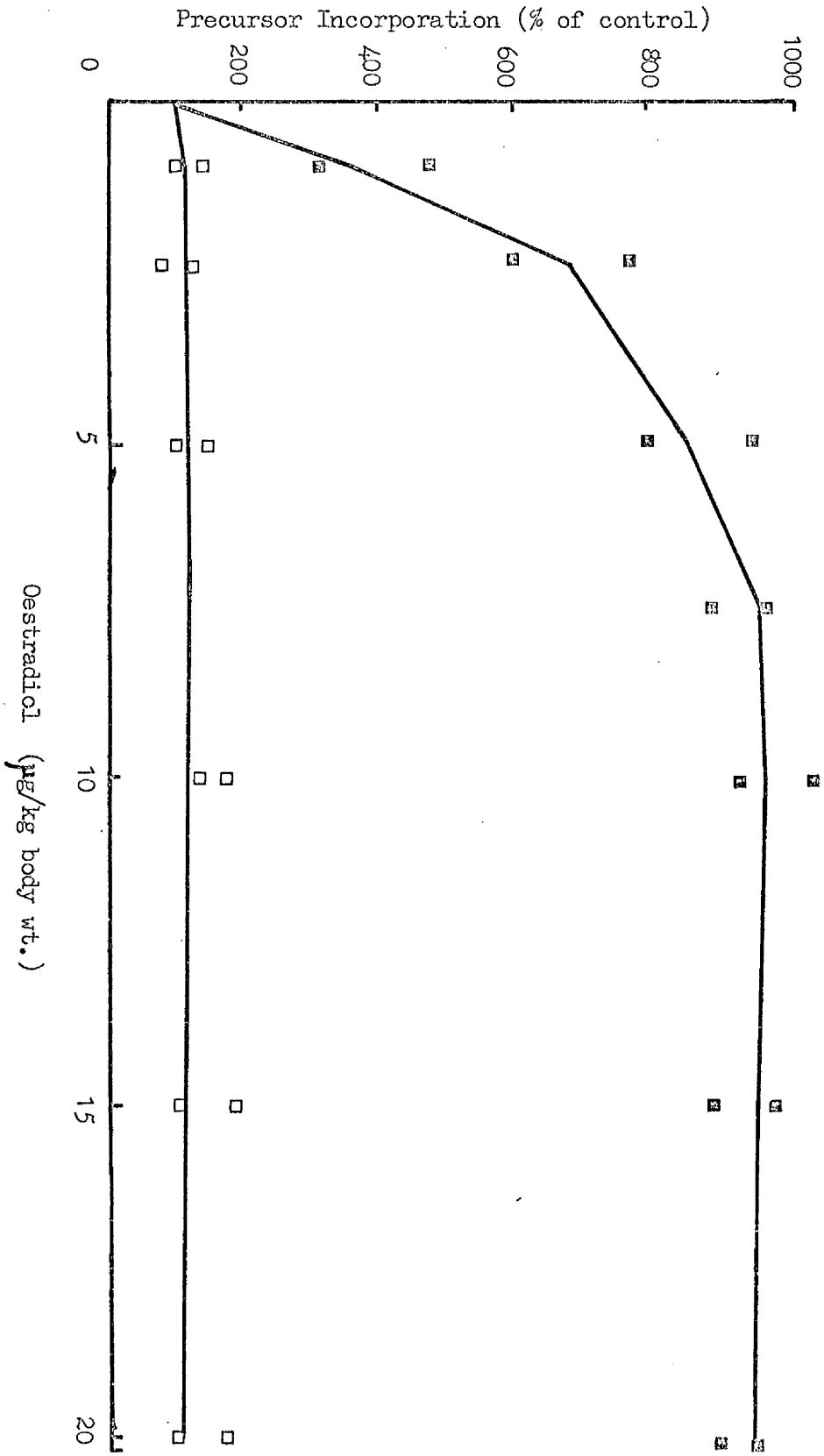
Uteri from immature animals which had not been treated with oestradiol were incubated for varying periods of time in Eagle's medium

FIG. 15: The effect of the level of oestradiol-17β on the stimulation of RNA synthesis in immature uteri.

Hormone was given intravenously 2h before death and 50μC$_i$ each of [5-^{3}H]uridine and [8-^{3}H]guanosine were administered intraperitoneally 30 min before death. Acid-soluble and acid-insoluble fractions were prepared from the excised uteri as described in the Methods section. Results were calculated as dpm/μg DNA and expressed as a percentage of control incorporation. Each point represents a mean of at least three animals.

■——■ Acid-insoluble fraction

□——□ Acid-soluble fraction


Precursor Incorporation (% of control)
0
200
400
600
800
1000
5
10
15
20
Oestradiol (µg/kg body wt.)

containing 1nM oestradiol as described in the Methods section. Nuclei were prepared after this incubation and the results of the assays for RNA polymerase activity in the nuclei from uteri treated *in vitro* with oestradiol are shown in Fig. 16.

These results show that incubation of the excised tissue with oestradiol *in vitro* does not cause a significant stimulation of either RNA polymerase A or B. The RNA polymerase A and B activities show hardly any stimulation and both show a definite decrease in activity when incubated *in vitro* for more than 4h.

In subsequent experiments on the action of oestradiol on RNA synthesis in nuclei, the animals were treated with oestradiol *in vivo*.

6. Effect of Oestradiol Administered In Vivo on RNA Synthesis in Isolated Nuclei.

6.1. The Effect of Time of Oestradiol Treatment.

Oestradiol was administered to animals for various periods of time including intervals of less than 1h before death which have not usually been included in previous investigations of the RNA polymerase activity in nuclei from responding uterine tissue (Hamilton *et al.*, 1965). The RNA polymerase activities were measured in low salt and high salt conditions using nuclei isolated from the uteri of animals treated with oestradiol over periods of up to 3h before death, and the results of these experiments are shown in Fig. 17.

It can be seen that the RNA polymerase A activity remains at control levels for the first hour then increases to reach a maximum at 2h and remains constant up to 3h. On the other hand, RNA polymerase B activity increases rapidly to a peak of about 6 times the control values after 30-45 min of hormone treatment. Thereafter this activity declines reaching control levels by 2h before beginning to rise again.

FIG. 16: The Effect of Treatment of Excised Uteri with Oestradiol-17β in vitro on RNA polymerase activities in Nuclei.

Excised uteri from immature rabbits were incubated for up to 4h in Eagles medium containing 1nM oestradiol as described in the Methods section. After this incubation, nuclei were prepared and assayed for RNA polymerase activities also as described in the Methods section.

■——■ RNA polymerase B activity

▲——▲ RNA polymerase A activity.

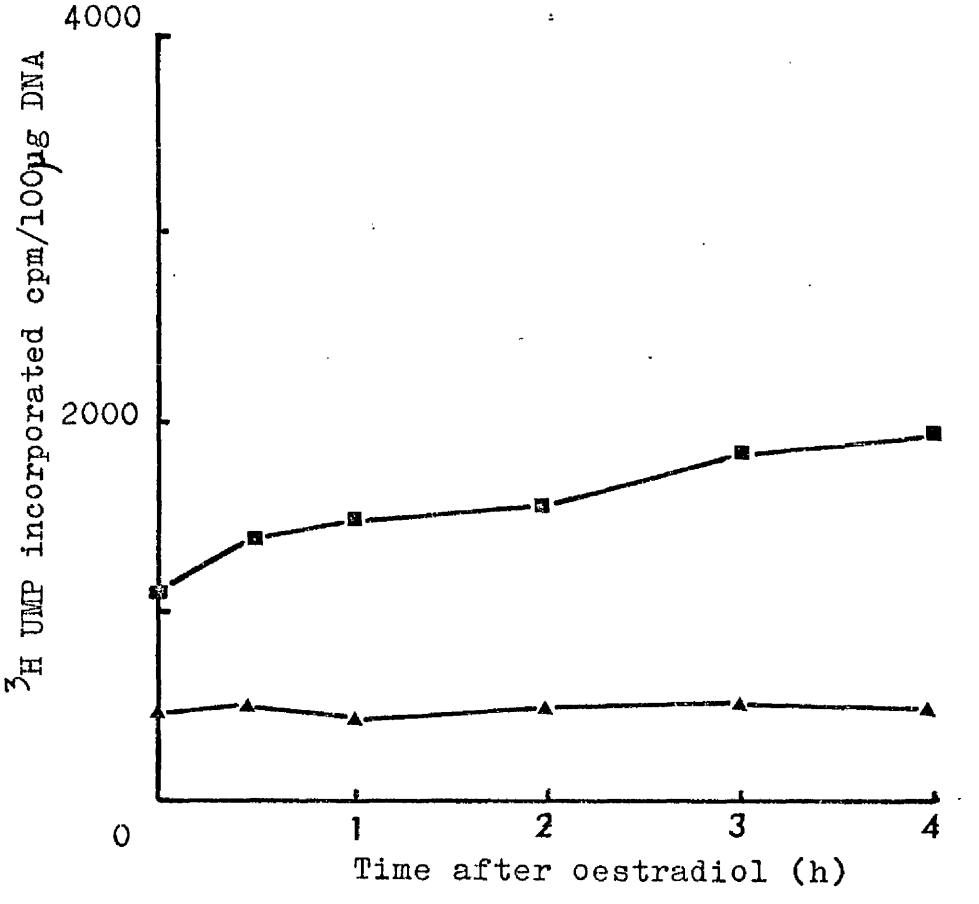
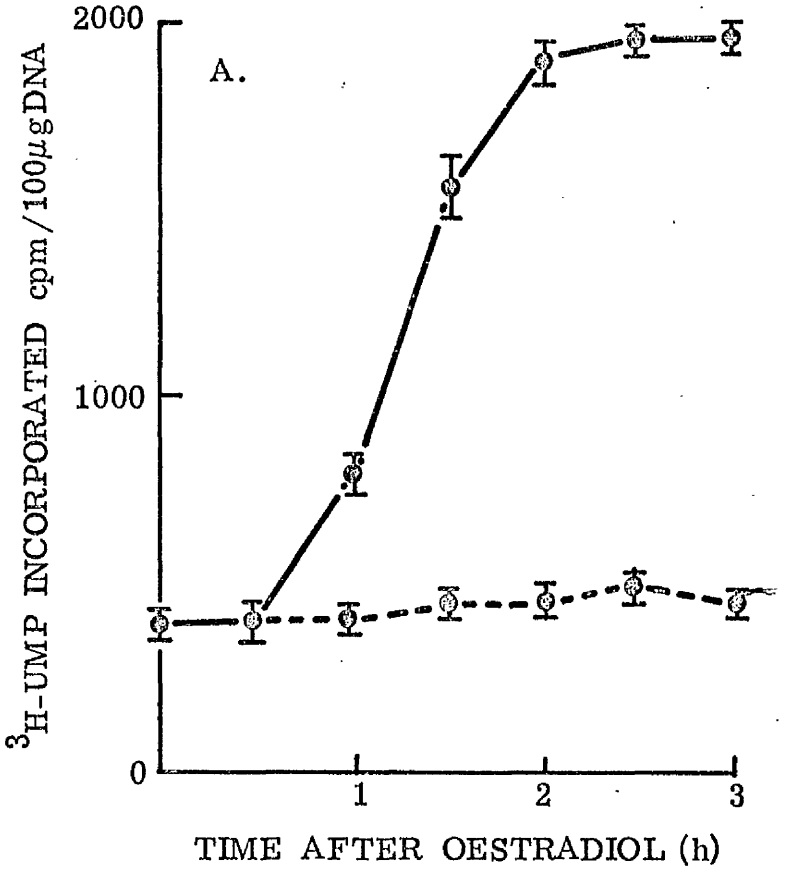

4000
2000
0
^{3}H UMP incorporated cpm/100µg DNA
1
2
3
4
Time after oestradiol (h)

FIG. 17: Effect of Time of Treatment with Oestradiol-17β on the RNA polymerase activities in Uterine Nuclei.

Rabbits were treated with hormone for periods up to 3h and the nuclei prepared from uteri and assayed for RNA polymerase activity as described in the Methods section. Results are expressed in cpm/100 μg DNA and each point shows the range of values obtained from at least three determinations.

a) RNA polymerase A activity in oestrogen-treated (⊗——⊗) and control uteri (⊗----⊗)

b) RNA polymerase B activity in oestrogen-treated (⊙——⊙) and control uteri (⊙----⊙).


A.
2000
1000
0
1
2
3
^{3}H-UMP INCORPORATED cpm/100μg DNA
TIME AFTER OESTRADIOL (h)

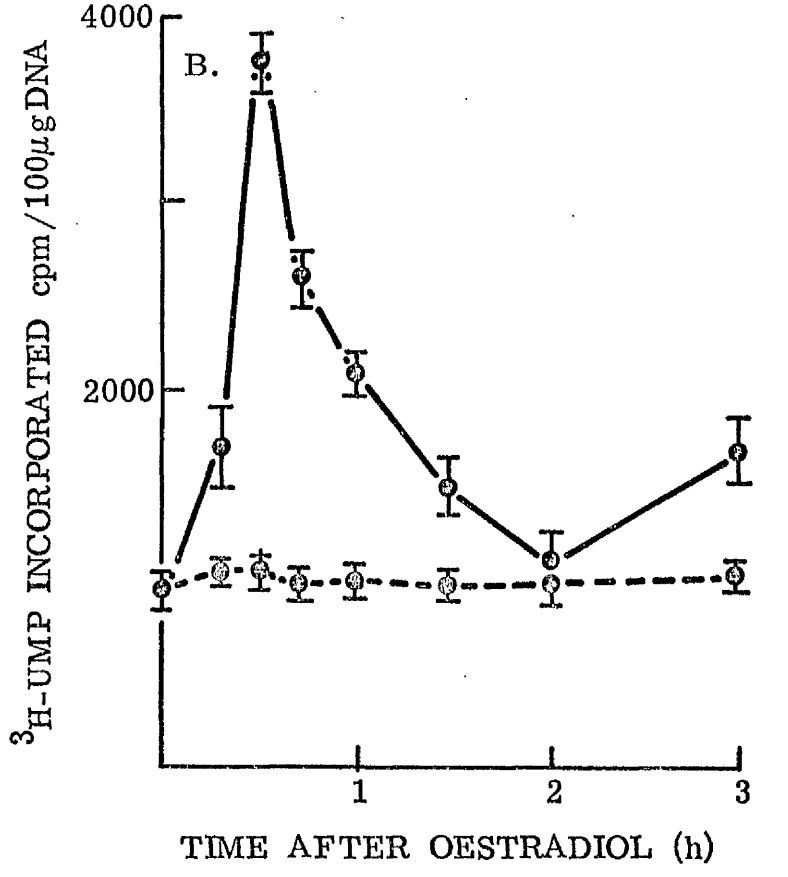

B.
4000
2000
1
2
3
^{3}H-UMP INCORPORATED cpm/100μg DNA
TIME AFTER OESTRADIOL (h)

Fig. 18 shows the results of experiments carried out over a period of treatment with oestradiol up to 12h. After its initial increase at 1-2h, RNA polymerase A remains on a plateau at about 5 times the control level for up to 12h. On the other hand, RNA polymerase B shows a biphasic response to the hormone, with the previously observed peak at about 30-45 min followed by a second rise over the period 2-5h to a maximum and remains fairly constant up to 12h.

In order to determine whether the stimulation of the RNA polymerase A activity was dependent on the synthesis of some product from the early high salt activity stimulation, animals were treated *in vivo* with various inhibitors of RNA and protein synthesis.

6.2. The Effect of Inhibitors on Uterine RNA and Protein Synthesis.

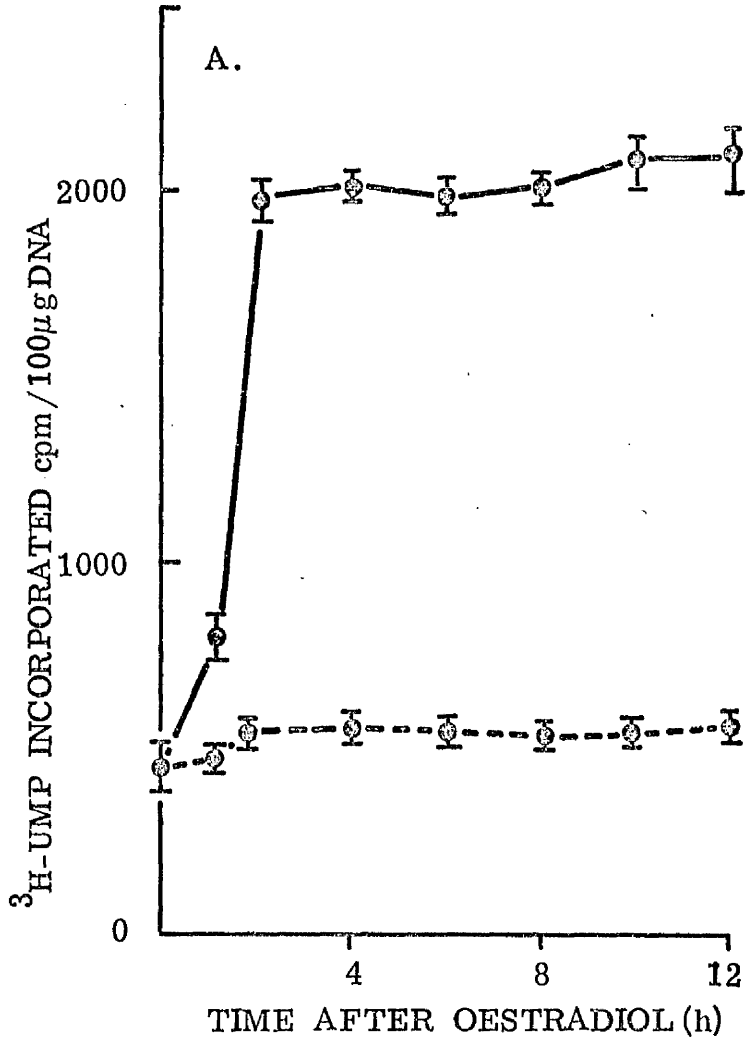
Fig. 19 shows the effect of treatment of animals *in vivo* with actinomycin D, α-amanitin and cycloheximide on RNA and protein synthesis in the immature rabbit uterus. The animals were injected intraperitoneally with the inhibitor 2h before death. In the case of actinomycin D and α-amanitin the animals received a mixture of [^{3}H]-guanosine and [^{3}H]-uridine 30 min before sacrifice, while the animals treated with cycloheximide received a mixture of [^{3}H]-leucine and [^{3}H]-lysine. Acid-insoluble fractions were prepared as described in the Methods section and the results are expressed as percentage inhibition of an untreated control. It can be seen that the inhibitors greatly affect RNA and protein synthesis in the rabbit uterus. The levels at which the inhibitors are used in subsequent experiments are shown by the arrows. These concentrations are 0.5 mg/kg body weight for actinomycin D, 0.05 mg/kg body weight for α-amanitin and 0.4 mg/kg body weight for cycloheximide and are sufficient to inhibit RNA synthesis by 98% in the case of actinomycin D and α-amanitin and protein synthesis by 98% in the case of cycloheximide.

FIG. 18: Effect of Extended Time of Treatment with Oestradiol-17β on the RNA polymerase activities in Uterine Nuclei.

Rabbits were treated with hormone for periods up to 12h and the uterine nuclei prepared and assayed for RNA polymerase activity as described in the Methods section. Results are expressed as cpm/100 μg DNA and each point shows the range of values obtained from at least three determinations.

a) RNA polymerase A activity in oestrogen-treated (●——●) and control uteri (●- - -○)

b) RNA polymerase B activity in oestrogen-treated (●——●) and control uteri (●- - -●).


A.
2000
1000
0
^{3}H-UMP INCORPORATED cpm/100μg DNA
4
8
12
TIME AFTER OESTRADIOL (h)

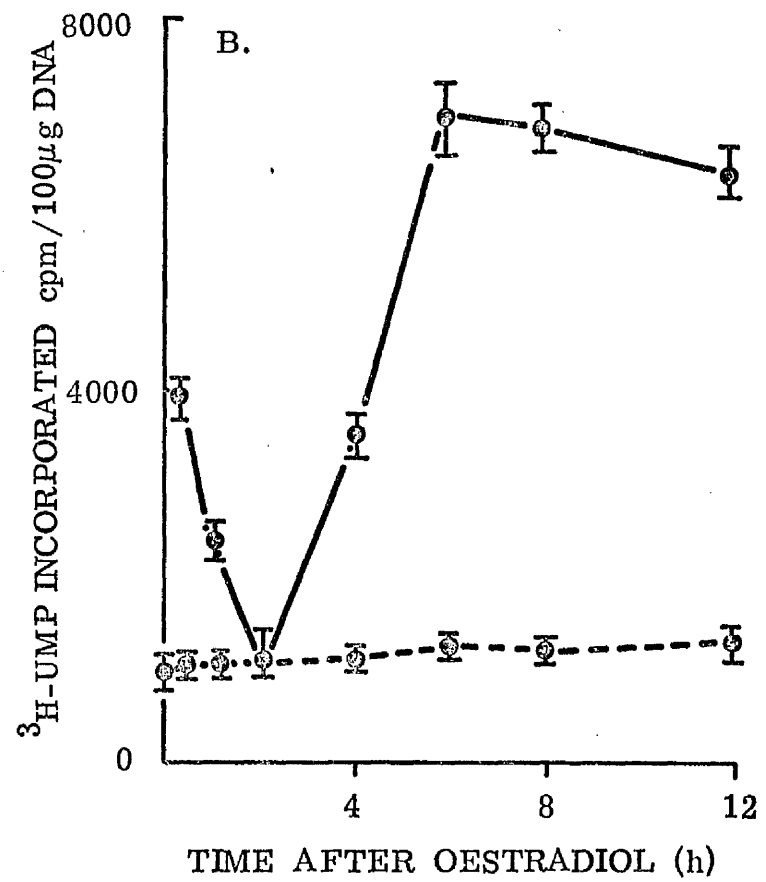

B.
8000
4000
0
^{3}H-UMP INCORPORATED cpm/100μg DNA
4
8
12
TIME AFTER OESTRADIOL (h)

FIG. 19: Effect of Inhibitors on Uterine RNA and Protein Synthesis

Immature rabbits weighing 1.2 - 1.4 kg received 10 μg of oestradiol/kg body wt 4h before death.

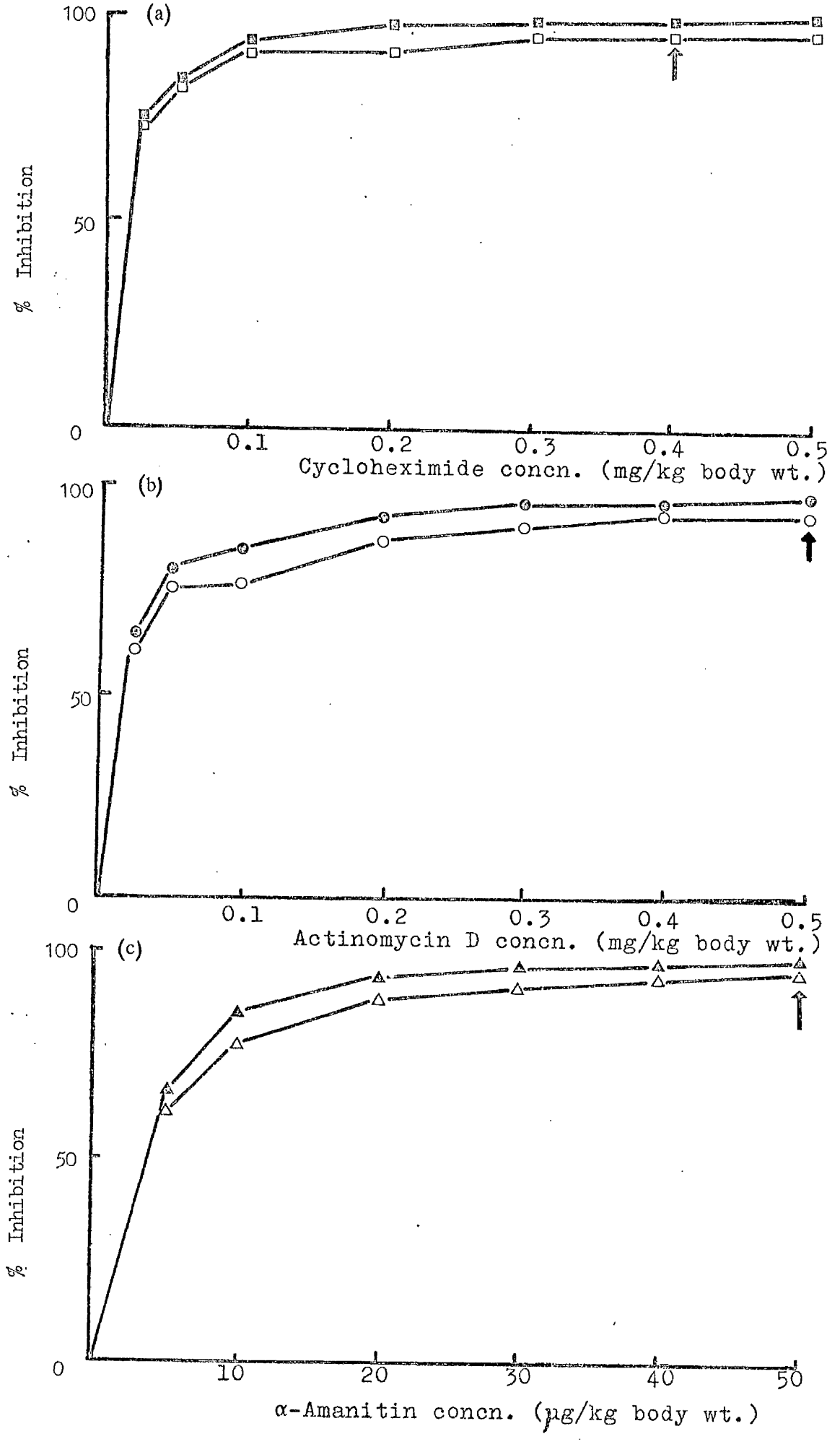
a) Animals treated with cycloheximide 1h before death and 50 μC_i each of $[4,5\text{-}^3H]$ lysine and leucine 30 min before death. Incorporation of radioactivity was calculated as acid-insoluble dpm/μg DNA and expressed as a percentage of uninhibited controls. Each point represents a mean of two determinations.

b) Animals treated with actinomycin D 1h before death and 50 μC_i each of a mixture of $[5\text{-}^3H]$ uridine and $[8\text{-}^3H]$ guanosine 30 min before death. Results expressed as above.

c) Animals treated with α-amanitin 1h before death and 50 μC_i each of a mixture of $[5\text{-}^3H]$ uridine and $[8\text{-}^3H]$ guanosine 30 min before death. Results expressed as above.

■—■ ●—● ▲—▲ Hormone-treated animals

□—□ ○—○ △—△ Control animals


(a)
100
50
0
% Inhibition
0.1
0.2
0.3
0.4
0.5
Cycloheximide concn. (mg/kg body wt.)
(b)
100
50
0
% Inhibition
0.1
0.2
0.3
0.4
0.5
Actinomycin D concn. (mg/kg body wt.)
(c)
100
50
0
% Inhibition
10
20
30
40
50
α-Amanitin concn. (μg/kg body wt.)

6.2.1. Effect of Time of Treatment with Inhibitors.

Fig. 20 shows the effect of the time of administration of the inhibitors on the synthesis of RNA and protein. The amount of inhibitor used was sufficient to inhibit the reaction by 98% after 2h. It can be seen from the results that treatment with actinomycin D and α-amanitin for 30 min causes more than 95% inhibition of RNA synthesis and that administration of cycloheximide 30 min before death inhibits protein synthesis by more than 90%.

6.3. The Effect of the Administration of Actinomycin D *In Vivo* on RNA Polymerase Activities in Isolated Nuclei.

Actinomycin D, 0.5 mg per kg body weight, was administered to experimental animals 30 min before the injection of oestradiol. The animals were killed at various times thereafter and uterine nuclei were prepared as before. The RNA polymerase activities in these nuclei are shown in Fig. 21. Actinomycin D completely abolishes the oestradiol-induced stimulation of both RNA polymerases A and B under the conditions employed. The endogenous RNA polymerase activities in nuclei extracted from control animals are also inhibited. This is compatible with the mechanism of action of actinomycin D which binds to the DNA template preventing further transcription.

6.4. The Effect of the Administration of α-Amanitin *In Vivo* on RNA Polymerase Activities in Isolated Nuclei.

α-Amanitin, 0.05 mg per kg body weight, was administered to animals 30 min prior to the administration of oestradiol. Uterine nuclei were prepared and assayed for RNA polymerase activities as before, and the results of these experiments are shown in Fig. 22. Treatment with

FIG. 20: Effect of Time of Treatment with Inhibitors on Uterine RNA and Protein Synthesis.

Immature rabbits received 10 μg oestradiol or saline with the appropriate concentration of inhibitor as indicated by the arrows in Fig. 19.

a) 400 μg cycloheximide injected with oestradiol. Animals received 50 μC_i each of $[4,5\text{-}^3H]$ lysine and $[4,5\text{-}^3H]$ leucine 30 min before death. Incorporation of radioactivity was calculated as acid-insoluble dpm/ μg DNA and expressed as a percentage of uninhibited controls. Each point represents the mean of two determinations.

b) Animals treated with 500 μg actinomycin D with oestradiol and 50 μC_i each of $[5\text{-}^3H]$ uridine and $[8\text{-}^3H]$ guanosine 30 min before death. Results expressed as above.

c) Animals treated with 50 μg α-amanitin with oestradiol/saline and 50 μC_i each of $[5\text{-}^3H]$ uridine and $[8\text{-}^3H]$ guanosine 30 min before death. Results expressed as above.

■—■ ●—● ▲—▲ Hormone-treated animals

□—□ ○—○ Δ—Δ Control animals

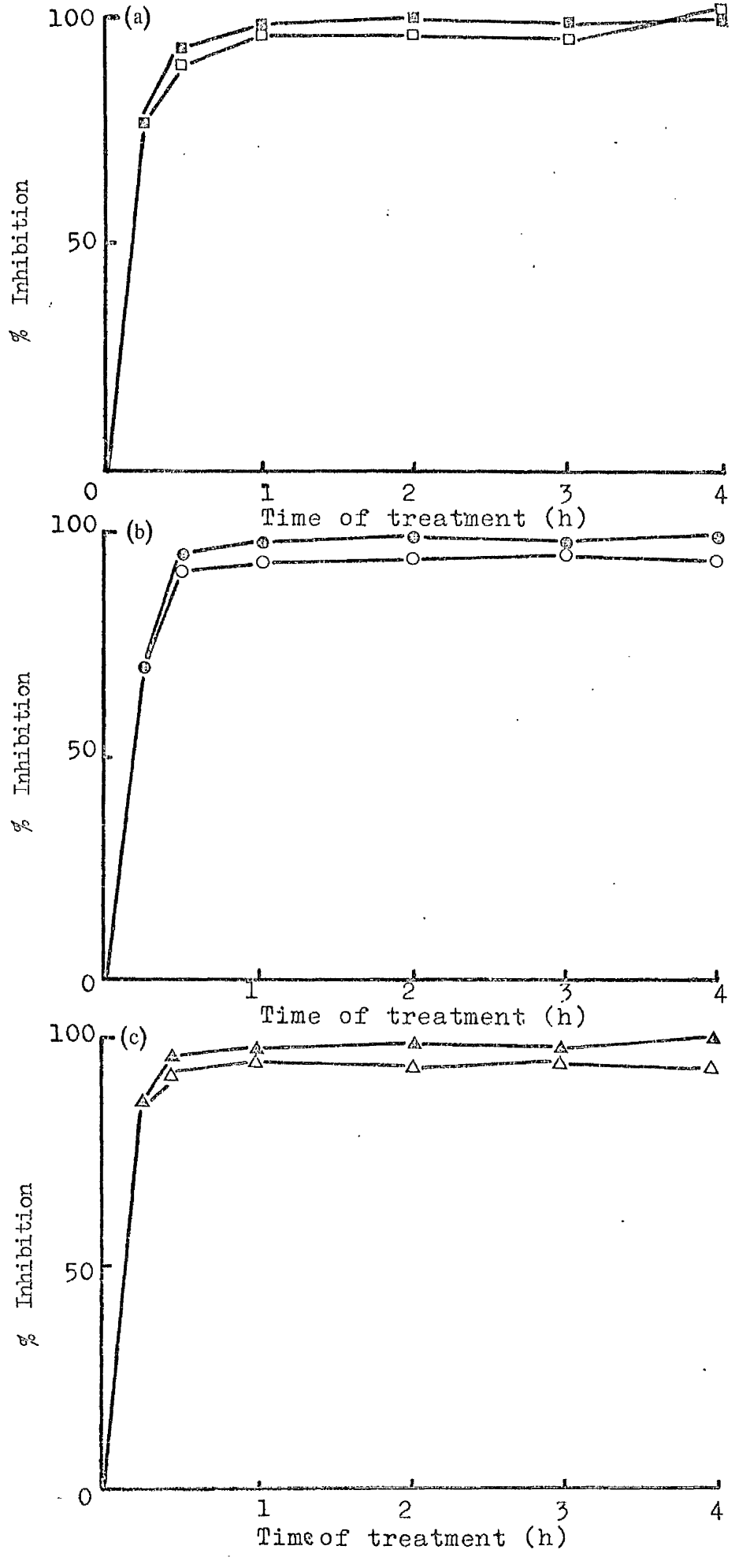

(a)
100
50
0
% Inhibition
1
2
3
4
Time of treatment (h)
(b)
100
50
0
% Inhibition
1
2
3
4
Time of treatment (h)
(c)
100
50
0
% Inhibition
1
2
3
4
Time of treatment (h)

FIG. 21: The Effect of Treatment with Actinomycin D in vivo on the RNA polymerase activities of Isolated Uterine Nuclei.

Animals were treated with 500 µg actinomycin D/kg body wt 30 min before the administration of 10 µg oestradiol or saline. Nuclei were isolated and assayed for RNA polymerase activities as described in the Methods section. Each point represents the range of at least two determinations.

a) RNA polymerase A activity in OE_2-treated animals (●—●)

RNA polymerase A activity in control animals (▲—▲)

RNA polymerase A activity in OE_2 + actinomycin D animals (■—■)

RNA polymerase A activity in control + actinomycin D animals (□—□)

The curves for both oestrogen-treated and control animals treated with actinomycin D are virtually identical and have been slightly displaced in the figure for the sake of clarity.

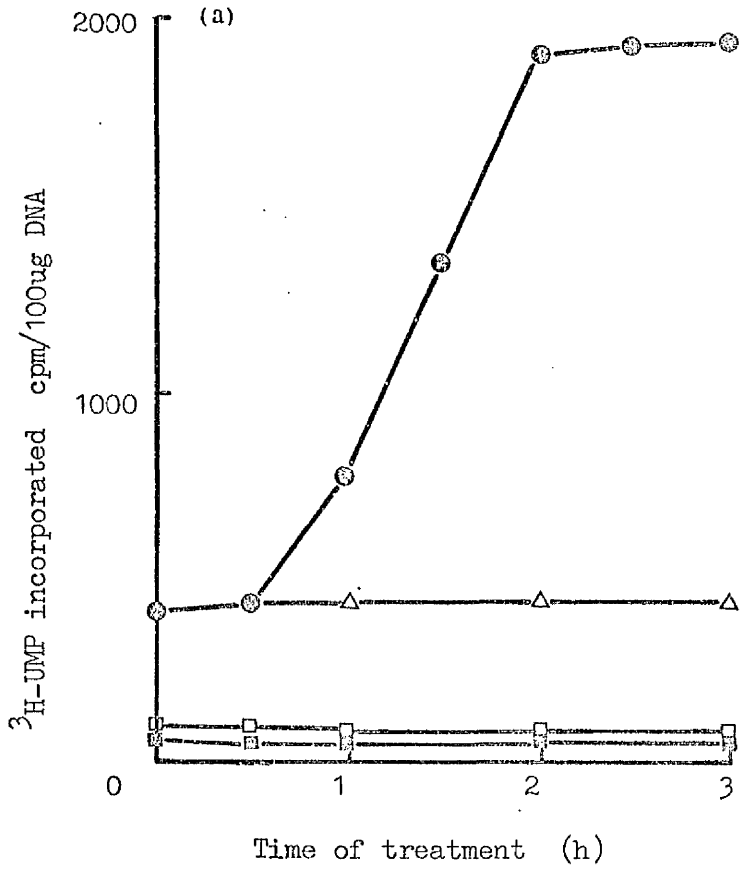
b) RNA polymerase B activity in OE_2-treated animals (●—●)

RNA polymerase B activity in control animals (▲—▲)

RNA polymerase B activity in OE_2 + actinomycin D animals (■—■)

RNA polymerase B activity in control + actinomycin D animals (□—□)

The curves for both oestrogen-treated and control animals treated with actinomycin D are virtually identical and have been slightly displaced in the figure for the sake of clarity.


(a)
2000
1000
0
1
2
3
^{3}H-UMP incorporated cpm/100ug DNA
Time of treatment (h)

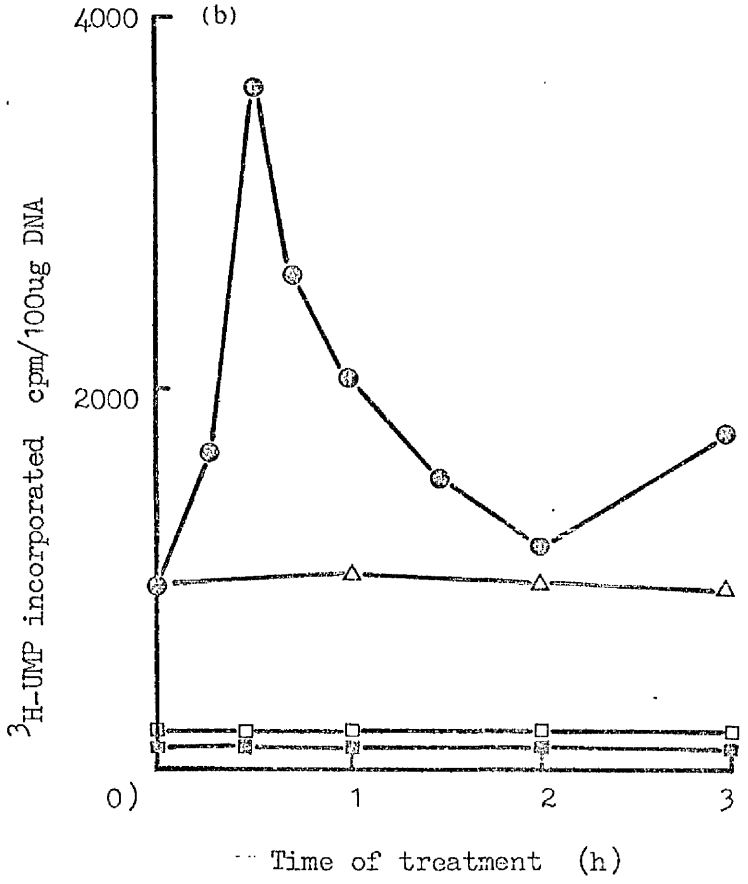

(b)
4000
2000
0)
1
2
3
^{3}H-UMP incorporated cpm/100ug DNA
Time of treatment (h)

FIG. 22: The Effect of α-amanitin on the RNA polymerase activities in Isolated Uterine Nuclei from Control Animals and Oestradiol-treated Animals.

Animals were treated with 50 μg α-amanitin/kg body wt 30 min before treatment with 10 μg oestradiol or saline. Nuclei were isolated and assayed for RNA polymerase activities as described in the Methods section. Each point represents the range of at least three determinations.

a) RNA polymerase A activity in OE_2-treated animals (●——●)

RNA polymerase A activity in control animals (▲——▲)

RNA polymerase A activity in OE_2 + α-amanitin treated animals (■——■)

RNA polymerase A activity in control + α-amanitin treated animals (□- - -□)

The curves for both oestrogen-treated and control animals treated with α-amanitin have been slightly displaced for the sake of clarity.

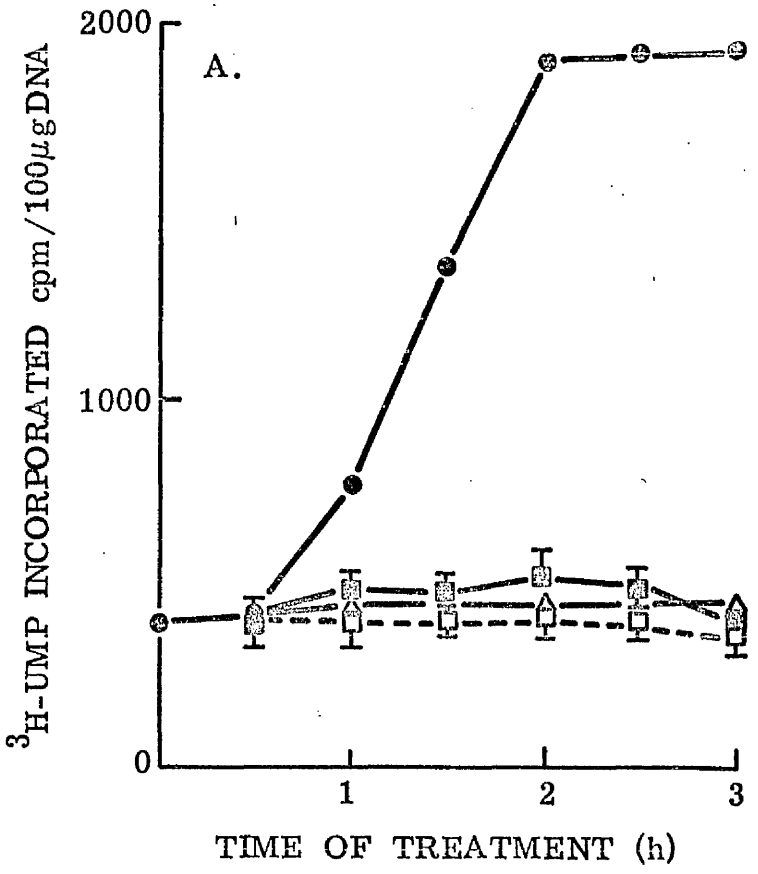
b) RNA polymerase B activity in OE_2-treated animals (●——●)

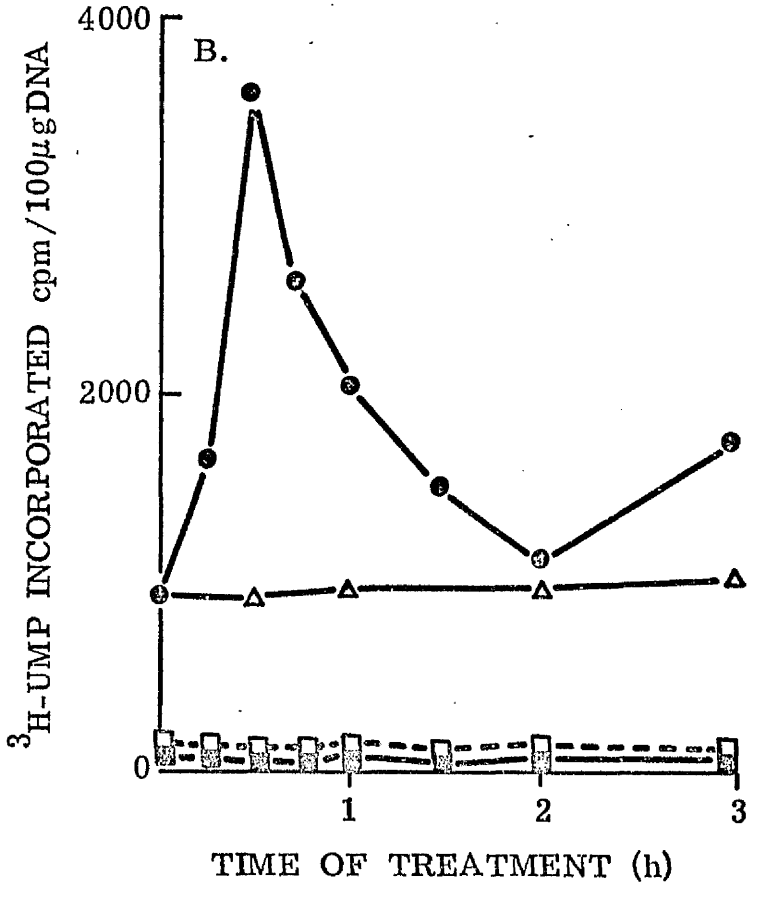
RNA polymerase B activity in control animals (▲——▲)

RNA polymerase B activity in OE_2 + α-amanitin treated animals (■——■)

RNA polymerase B activity in control + α-amanitin treated animals (□- - -□)

The curves for both oestrogen-treated and control animals treated with α-amanitin have been slightly displaced for the sake of clarity.


A.
2000
1000
0
1
2
3
^{3}H-UMP INCORPORATED cpm/100μg DNA
TIME OF TREATMENT (h)


B.
4000
2000
0
1
2
3
^{3}H-UMP INCORPORATED cpm/100μg DNA
TIME OF TREATMENT (h)

∝-amanitin causes the total inhibition of RNA polymerase B in nuclei from both control and hormone treated animals. This result is as expected since ∝-amanitin has already been shown to be a potent inhibitor of RNA polymerase B *in vitro* .

However, RNA polymerase A activity is also affected by prior administration of the toxin to the rabbits (Fig. 22). In these experiments only the increase in RNA polymerase A activity caused by treatment of the animals with oestradiol is prevented by ∝-amanitin.

These results suggest that some product formed during the early phase of increased RNA polymerase B activity may be required in order to produce the subsequent observed increase in RNA polymerase A activity.

In an attempt to confirm this conclusion, animals were treated with oestradiol as before followed by ∝-amanitin 30 min later. The object of this experiment was to delay the administration of ∝-amanitin until after the increase in RNA polymerase B activity had occurred so as to determine whether in these circumstances the increase in RNA polymerase A activity could still be inhibited by ∝-amanitin. Fig. 23 shows the results obtained from such an experiment. The initial enhancement of RNA polymerase B activity is still observed although following the administration of ∝-amanitin at 30 min this activity is almost totally inhibited. However, the increase in RNA polymerase A activity is unaffected by the administration of ∝-amanitin *in vivo* 30 min after the hormone, the values obtained being identical to those observed in nuclei from animals treated with oestradiol alone.

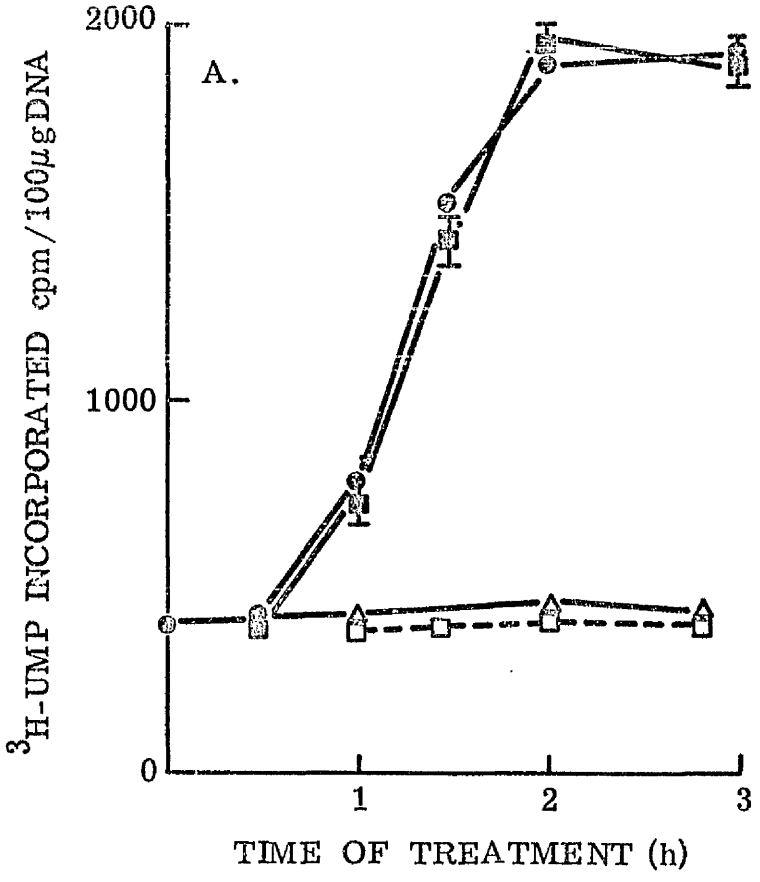
Thus, while administration of ∝-amanitin before oestradiol totally inhibits RNA polymerase B activity and prevents the later oestradiol-induced rise in RNA polymerase A activity, administration of the toxin after the oestradiol-induced increase in RNA polymerase B activity has occurred permits the oestradiol-induced rise in RNA polymerase A activity. It seems probable that the product of the elevated RNA polymerase B is

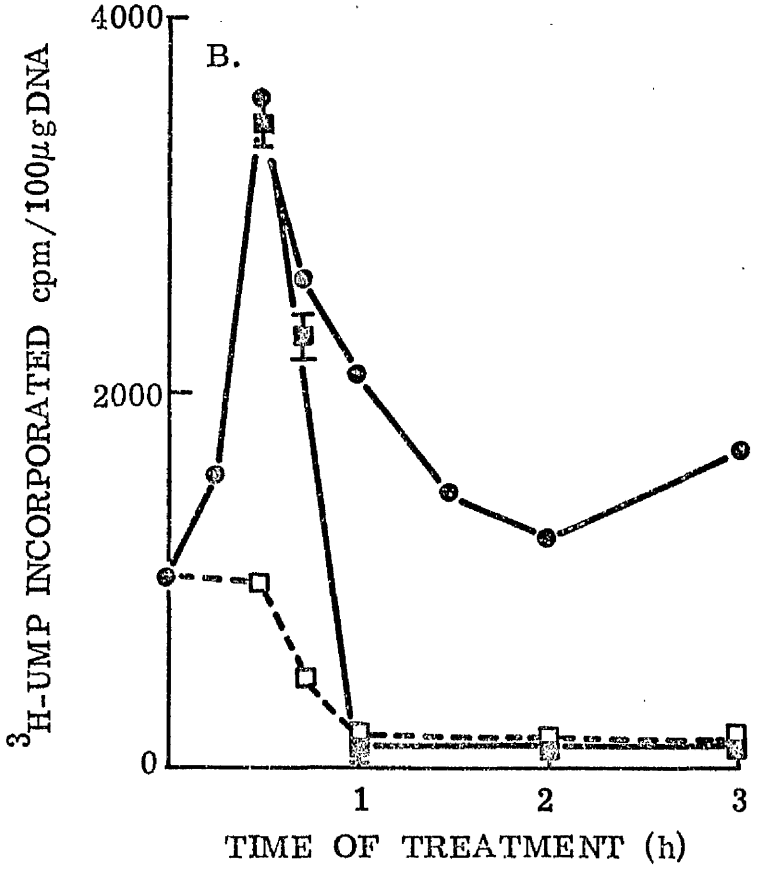
FIG. 23: The Effect of α-amanitin administered 30 min after Oestradiol on the RNA polymerase activities in Isolated Uterine Nuclei.

Animals were treated with 50 µg α-amanitin/kg body wt 30 min after 10 µg oestradiol/kg body wt. Nuclei were isolated and assayed for RNA polymerase activities as described in the Methods section. Each point shows the range of at least three determinations

a) RNA polymerase A activity in OE_2-treated animals (●——●)
RNA polymerase A activity in saline-treated animals (▲——▲)
RNA polymerase A activity in OE_2 + α-amanitin treated animals (■——■)
RNA polymerase A activity in saline + α-amanitin treated animals (□- - -□)

b) RNA polymerase B activity in OE_2-treated animals (●——●)
RNA polymerase B activity in saline-treated animals (▲——▲)
RNA polymerase B activity in OE_2 + α-amanitin treated animals (■——■)
RNA polymerase B activity in saline + α-amanitin treated animals (□- - -□).


A.
2000
1000
0
1
2
3
^{3}H-UMP INCORPORATED cpm/100μg DNA
TIME OF TREATMENT (h)


B.
4000
2000
0
1
2
3
^{3}H-UMP INCORPORATED cpm/100μg DNA
TIME OF TREATMENT (h)

required for the stimulation of RNA polymerase A.

This product could be either RNA or protein and attempts were made to distinguish between these possibilities in experiments in which animals were treated with an inhibitor of protein synthesis in order to determine the effect of this compound on the levels of RNA polymerase A and B activity in isolated nuclei.

6.5. The Effect of the Administration of Cycloheximide *In Vivo* on the RNA Polymerase Activities in Isolated Nuclei.

The effect of cycloheximide administered 30 min prior to oestradiol on the RNA polymerase activities is shown in Fig. 24. Under the conditions employed, 98% of the total measurable protein synthesis was inhibited. However, *in vivo* treatment with cycloheximide does not inhibit the early stimulation of RNA polymerase B activity and there is even a slight increase in activity in animals treated with cycloheximide and oestradiol compared with animals treated with oestradiol alone. The increase in RNA polymerase A activity usually produced by oestradiol is prevented by treatment of the rabbits with cycloheximide 30 min before oestradiol.

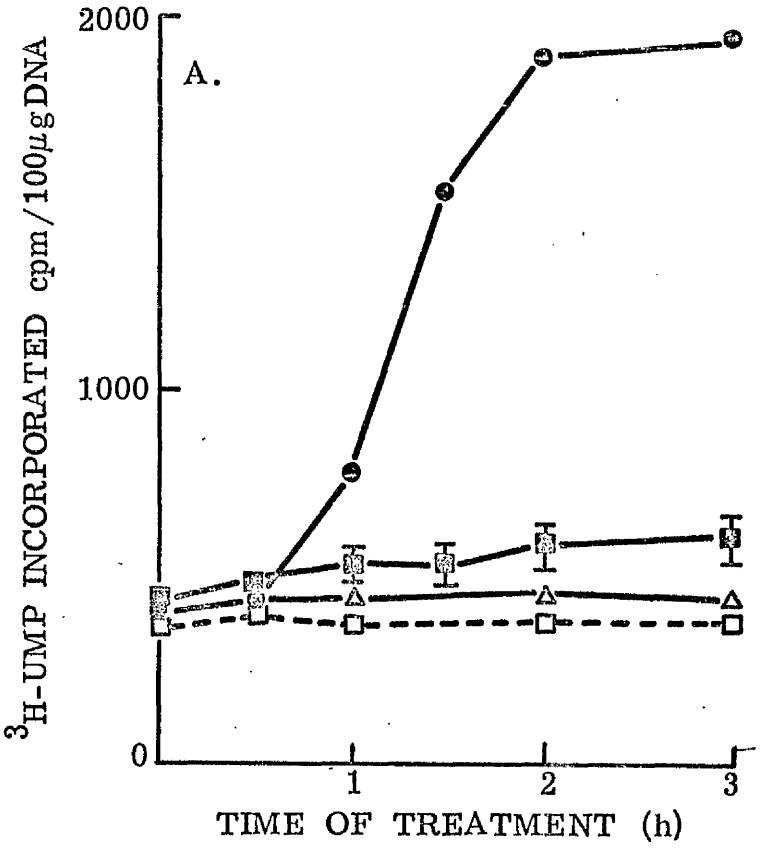
When cycloheximide was administered to rabbits 30 min after treatment with oestradiol, the early stimulation of RNA polymerase B activity is observed as before (Fig. 25). However, the enhancement of the RNA polymerase A activity is no longer prevented. It seems probable from these observations that treatment with cycloheximide prior to hormone administration causes the inhibition of the synthesis of a protein or proteins which are necessary in order that the RNA polymerase A activity can increase in response to oestradiol treatment. Such a protein or proteins must be synthesised during the first 30 min after oestrogen treatment.

FIG. 24: The Effect of Cycloheximide administered prior to Oestradiol on the RNA polymerase activities in Isolated Uterine Nuclei.

Animals were treated with 400 µg cycloheximide/kg body wt 30 min before oestradiol administration. Nuclei were isolated and assayed for RNA polymerase activities as described in the Methods section. Each point shows the range of at least three determinations.

a) RNA polymerase A activity in OE_2-treated uteri (●——●)
RNA polymerase A activity in saline-treated uteri (▲——▲)
RNA polymerase A activity in OE_2 + CH (■——■)
RNA polymerase A activity in saline + CH (□- - -□)

b) RNA polymerase B activity in OE_2-treated uteri (●——●)
RNA polymerase B activity in saline-treated uteri (▲——▲)
RNA polymerase B activity in OE_2 + CH (■——■)
RNA polymerase B activity in saline + CH (□- - -□)


A.
2000
1000
0
1
2
3
^{3}H-UMP INCORPORATED cpm/100μg DNA
TIME OF TREATMENT (h)

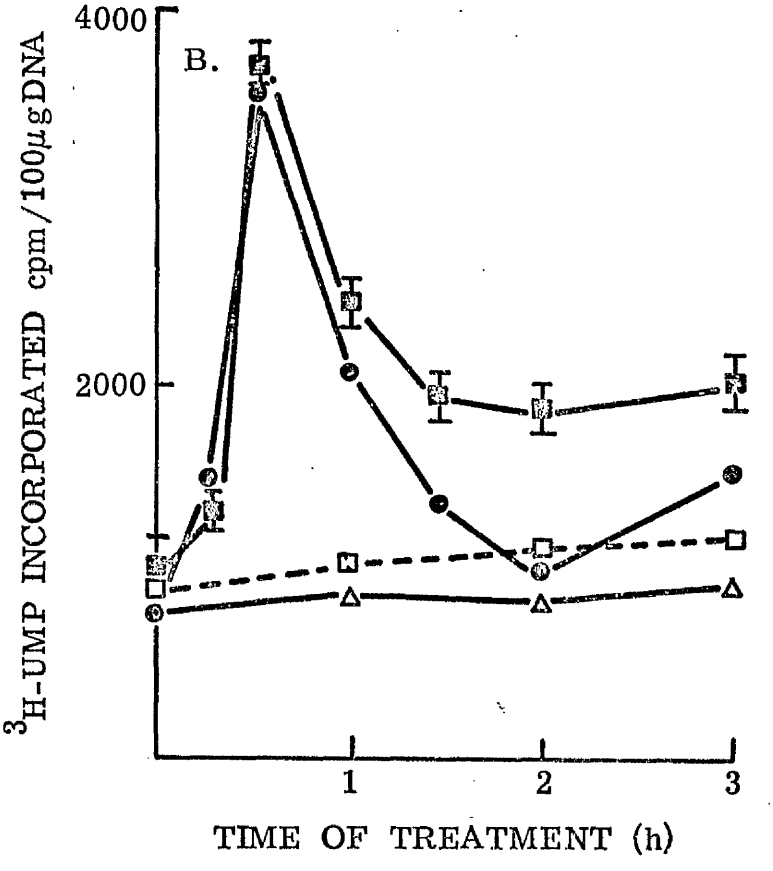
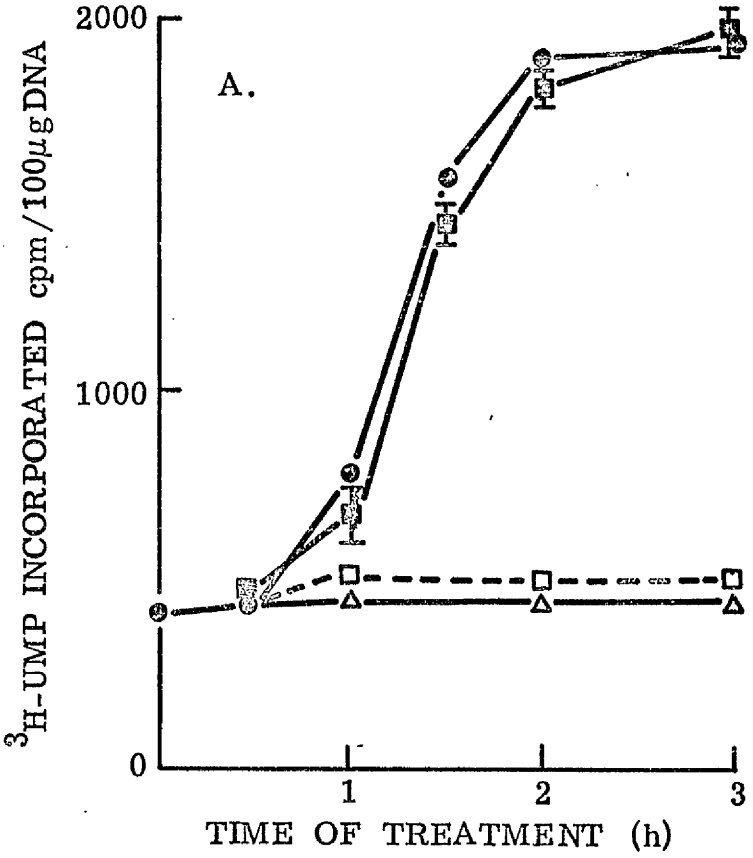

B.
4000
2000
1
2
3
^{3}H-UMP INCORPORATED cpm/100μg DNA
TIME OF TREATMENT (h)

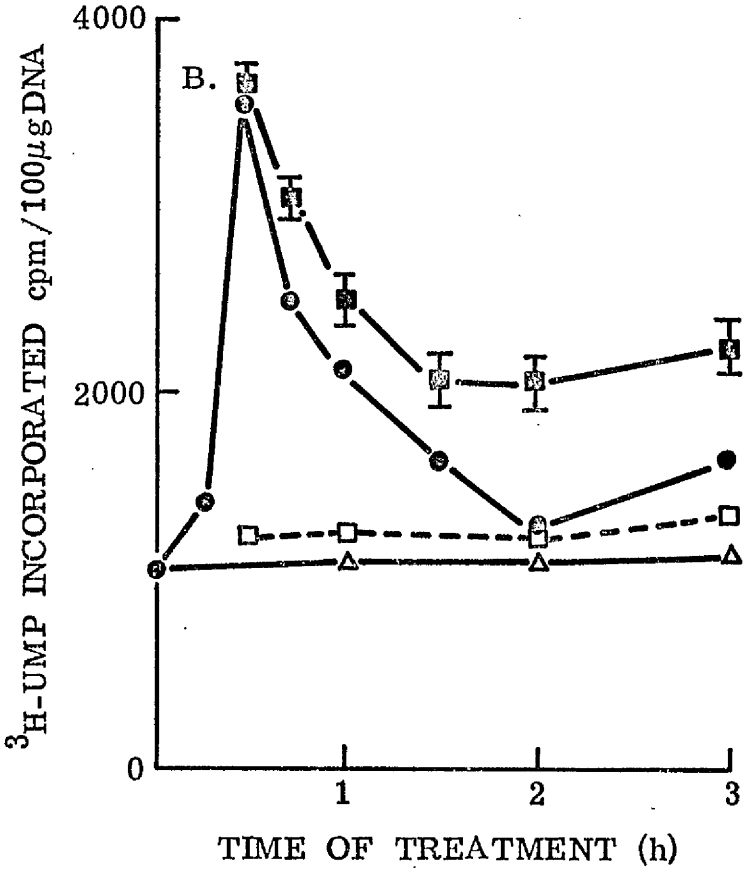
FIG. 25: The Effect of Treatment with Cycloheximide 30 min after Oestradiol administration on the RNA polymerase activities in Isolated Uterine Nuclei.

Animals were treated with 400 µg cycloheximide per kg body wt 30 min after oestradiol administration. Nuclei were isolated and assayed for RNA polymerase activities as described in the Methods section. Each point shows the range of at least three determinations.

a) RNA polymerase A activity in OE_2-treated uteri (●——●)
RNA polymerase A activity in saline-treated uteri (▲——▲)
RNA polymerase A activity in OE_2 + CH (■——■)
RNA polymerase A activity in saline + CH (□----□)

b) RNA polymerase B activity in OE_2-treated uteri (●——●)
RNA polymerase B activity in saline-treated uteri (▲——▲)
RNA polymerase B activity in OE_2 + CH (■——■)
RNA polymerase B activity in saline + CH (□----□)


A.
2000
1000
0
1
2
3
^{3}H-UMP INCORPORATED cpm/100μg DNA
TIME OF TREATMENT (h)


B.
4000
2000
0
1
2
3
^{3}H-UMP INCORPORATED cpm/100μg DNA
TIME OF TREATMENT (h)

6.6. The Effect of Cytoplasm from the Uteri of Oestrogen-treated Animals on the RNA Polymerase Activities in Isolated Nuclei.

The experiments reported above suggested that a specific protein or proteins may be required for the activation of RNA polymerase A. This protein would obviously need to be synthesised after oestradiol treatment but in advance of the observed increase in RNA polymerase A activity which occurs from 1-2h after hormone administration. In an attempt to demonstrate the existence of such a protein or proteins, nuclei prepared from the uteri of rabbits which had not been exposed to oestradiol were incubated with cytoplasm from the uteri of rabbits which had been treated with the hormone for up to 2h (Fig. 26). Treatment of these nuclei with cytoplasm from oestradiol-treated rabbit uteri gave rise to an increase in RNA polymerase A activity. In these experiments the nuclei were incubated for 15 min at 37° to ensure disruption of the nuclear membranes and so permit access of any protein factors to the nuclear contents. However, the stimulation of RNA polymerase A activity is also observed when untreated uterine nuclei are incubated with cytoplasm prepared from animals which had not been exposed to oestradiol. It is probable that this enhancement of RNA polymerase activity is due to the action of nucleases in the cytosols particularly DNase which is introducing single-strand breaks into the DNA of the chromatin template where non-specific initiation of RNA synthesis can proceed.

The contamination of treated and untreated cytoplasm with RNase (Fig. 27) and DNase (Fig. 28) are shown. It can be seen that there is considerable contamination with both RNase and DNase in all cytosol preparations.

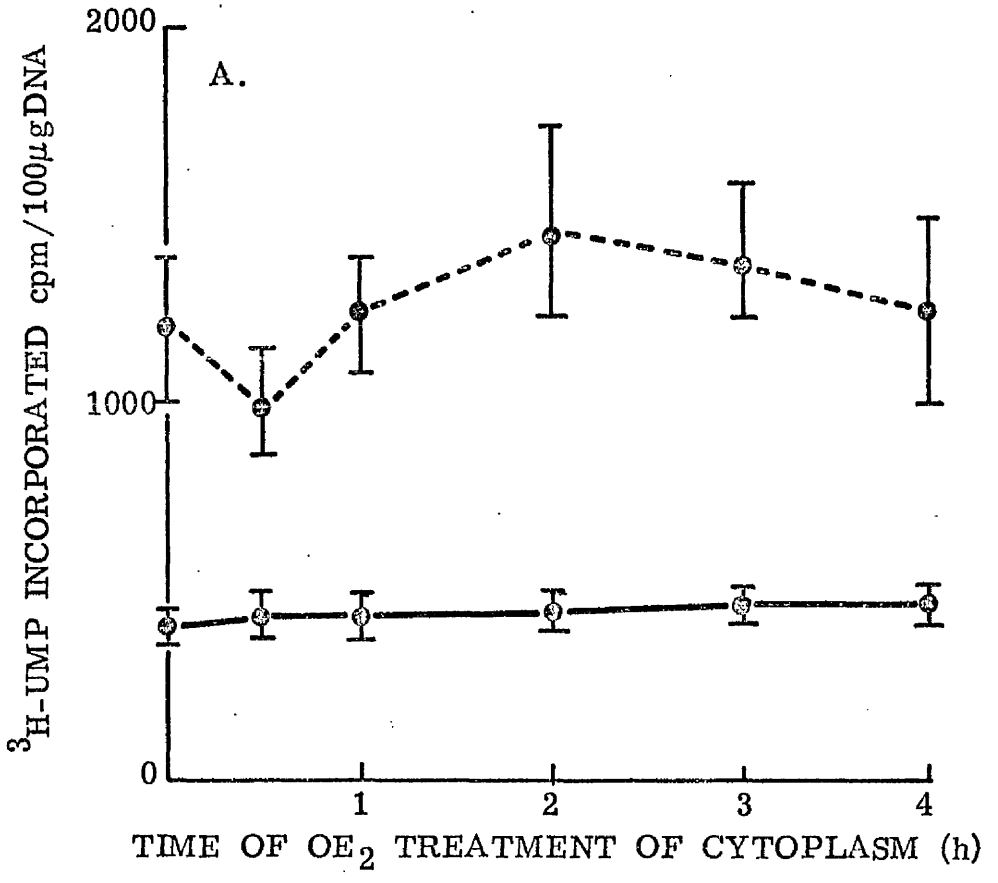
In an attempt to inhibit the RNase activity, control nuclei were incubated with a mixture of cytoplasm and the RNase inhibitor heparin (Rhoads et al., 1973). The effect of heparin alone on the RNA polymerase activities in intact isolated nuclei is shown in Fig. 29. It is evident

FIG. 26: The Effect of the addition of Cytoplasm from Oestradiol-treated Uteri on the RNA polymerase activities in Isolated Uterine Nuclei.

Cytoplasm was prepared from rabbit uteri which had been treated with oestradiol as described in the Methods section and added to nuclei isolated from untreated uteri. RNA polymerase activities were assayed as described in the Methods section. Each point represents the range of three determinations.

a) RNA polymerase A activity with cytoplasm from OE_2-treated uteri (⊙----⊙) and control uteri (⊙——⊙)

b) RNA polymerase B activity with cytoplasm from OE_2-treated uteri (⊙- - -⊙) and control uteri (⊙——⊙)


A.
2000
1000
0
^{3}H-UMP INCORPORATED cpm/100μgDNA
1
2
3
4
TIME OF OE_2 TREATMENT OF CYTOPLASM (h)

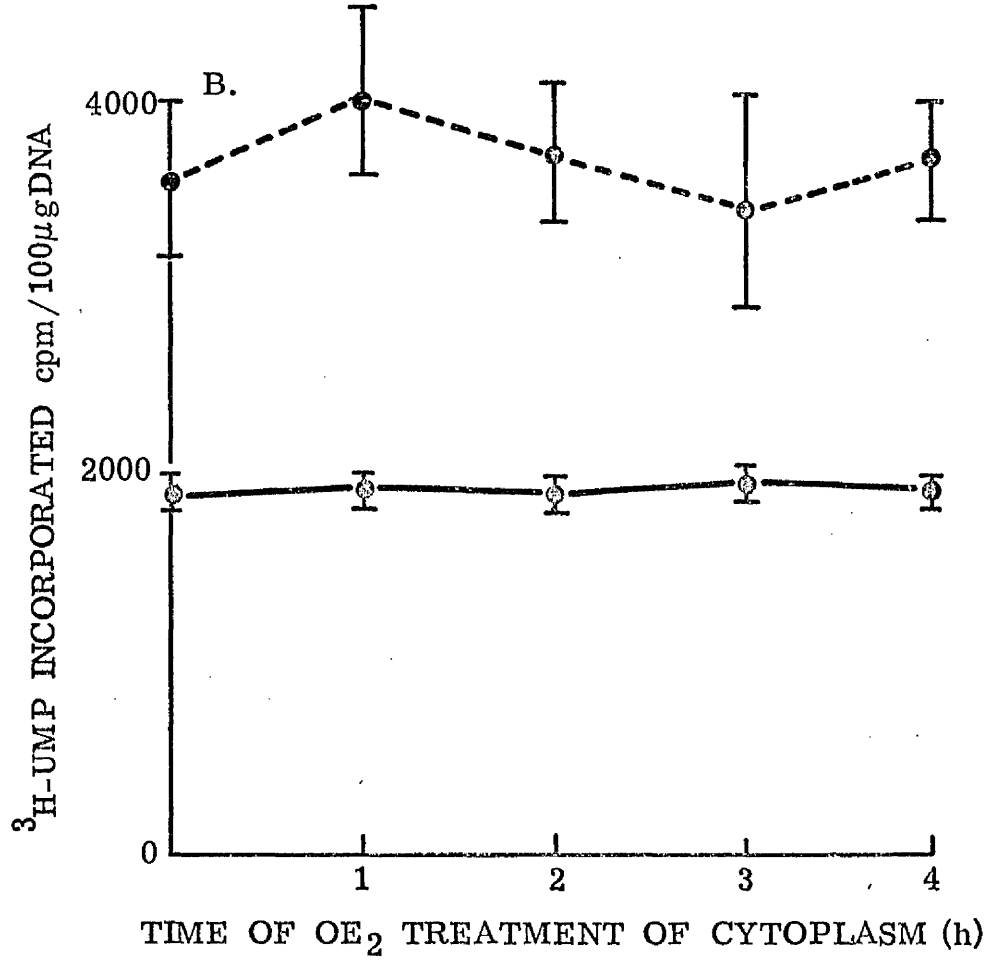

B.
4000
2000
0
^{3}H-UMP INCORPORATED cpm/100μgDNA
1
2
3
4
TIME OF OE_2 TREATMENT OF CYTOPLASM (h)

FIG. 27: RNase activity in Cytoplasm from Oestrogen-treated and Control Rabbit Uteri.

Uterine cytoplasm was prepared from oestrogen-treated and control animals and assayed for RNase activity as described in the Methods section by following the degradation of ^{32}P ribosomal RNA on sucrose gradients.

a) Control

b) Cytoplasm from control uteri

c) Cytoplasm from uteri treated for 30 min with oestradiol

d) Cytoplasm from uteri treated for 60 min with oestradiol

Similar profiles were obtained with cytoplasm treated for 2, 3 and 4h with oestradiol.

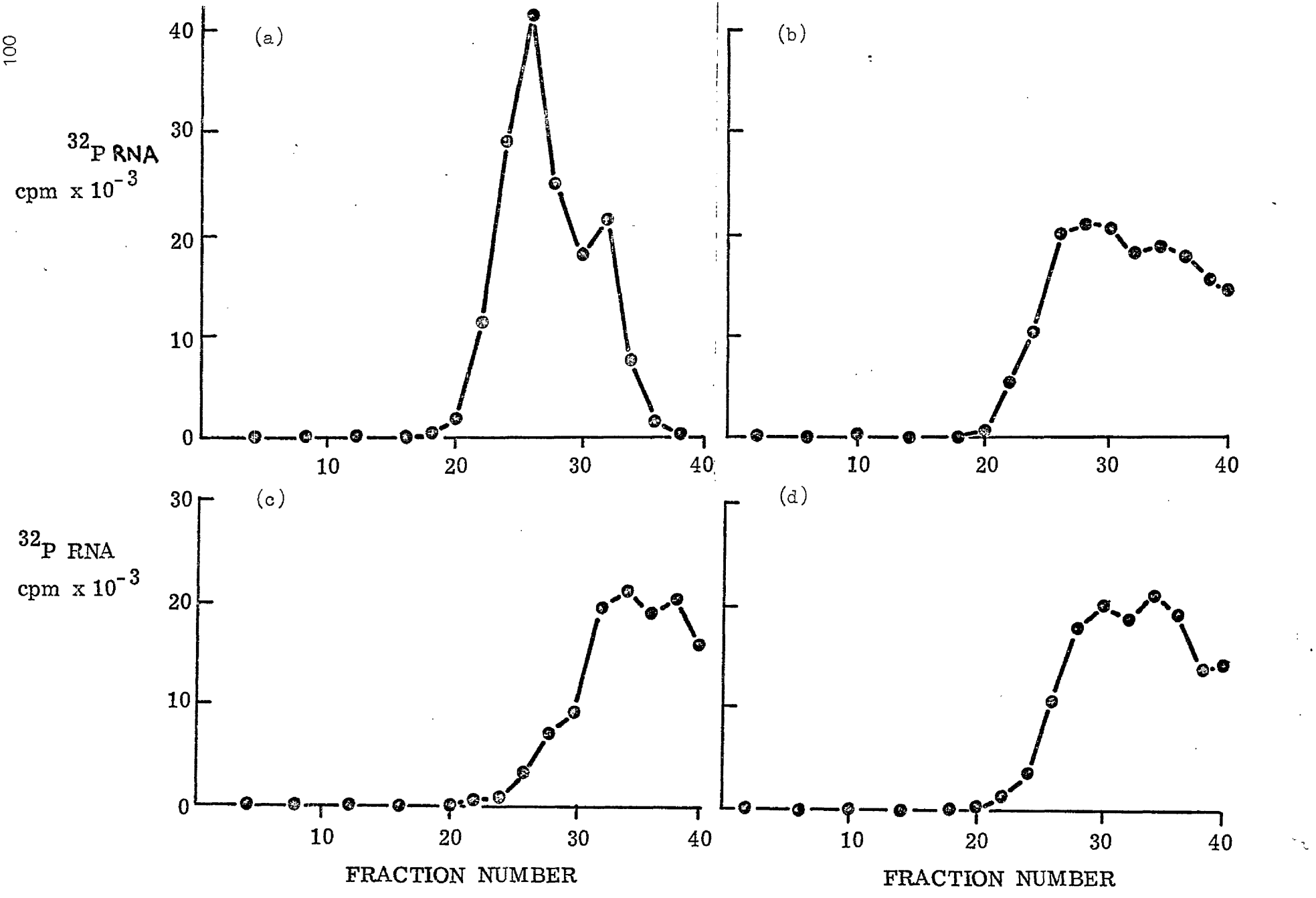

(a)
(b)
(c)
(d)
^{32}P RNA
cpm x 10^{-3}
^{32}P RNA
cpm x 10^{-3}
0
10
20
30
40
FRACTION NUMBER
FRACTION NUMBER

FIG. 28: DNase activity in Cytoplasm from Oestrogen-treated and Control Rabbit Uteri.

Uterine cytoplasm was prepared from oestrogen-treated and control animals and assayed for DNase activity as described in the Methods section by following the degradation of ^{3}H labelled SV40 DNA on CsCl gradients.

a) (○——○) Control

(●——●) Control cytoplasm

b) (○——○) Control

(●——●) Cytoplasm from 30 min OE_2-treated uteri

c) (○——○) Control

(●——●) Cytoplasm from 1h OE_2-treated uteri

d) (○——○) Control

(●——●) Cytoplasm from 2h OE_2-treated uteri.

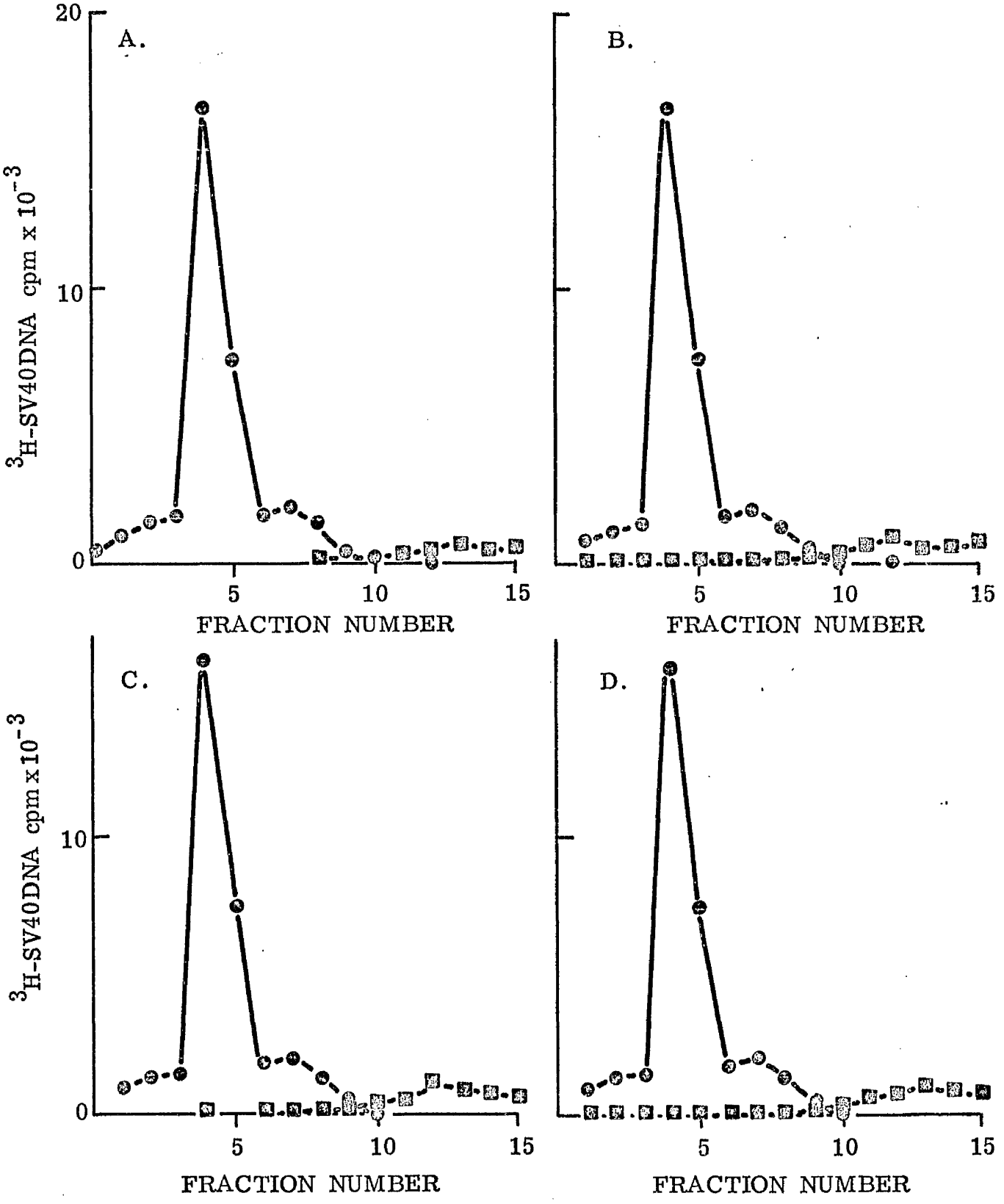
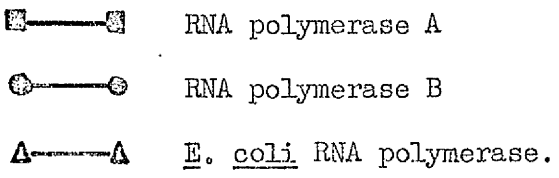

A.
B.
C.
D.
20
10
0
5
10
15
FRACTION NUMBER
^{3}H-SV40DNA cpm x 10^{-3}

FIG. 29: The Effect of Heparin on the RNA polymerase activities in Isolated Nuclei.

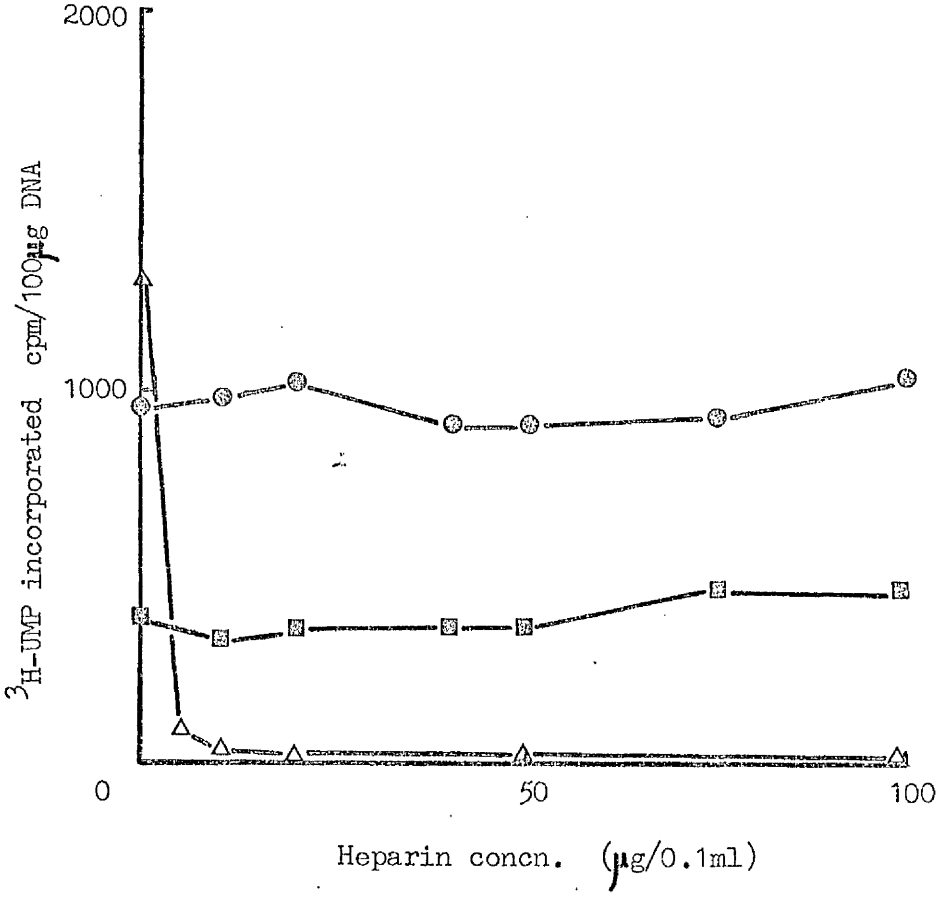
Uterine nuclei isolated from untreated animals were assayed for RNA polymerase activities in the presence of increasing amounts of heparin as described in the Methods section. Also shown is the effect of heparin on exogenous E. coli RNA polymerase (1 μg). Each point shows the range of two determinations.



RNA polymerase A

RNA polymerase B

E. coli RNA polymerase.


2000
1000
0
50
100
^{3}H-UMP incorporated cpm/100µg DNA
Heparin concn. (µg/0.1ml)

that heparin has very little effect on RNA synthesis catalysed by the endogenous enzymes while exogenous E. coli RNA polymerase is completely inhibited.

When nuclei isolated from animals treated with saline alone are incubated for 15 min with oestradiol-treated cytoplasm and heparin, very little significant variation is observed in the RNA polymerase activity (Fig. 30). It is probable that the slight increase is due to the action of DNase on the template.

Since some indications were obtained of an effect of cytoplasm from oestradiol-treated rabbit uteri on the RNA polymerase activity in nuclei, attempts were made to concentrate any such components. The cytoplasm from uteri of oestradiol-treated animals was fractionated on a CM-cellulose column and fractions were assayed with nuclei extracted from saline-treated animals in order to determine the RNA polymerase activities (Fig. 31). There appears to be a fraction present in the cytoplasm of uteri treated for 30 min with oestradiol which will stimulate RNA polymerase A activity without enhancing RNA polymerase B activity

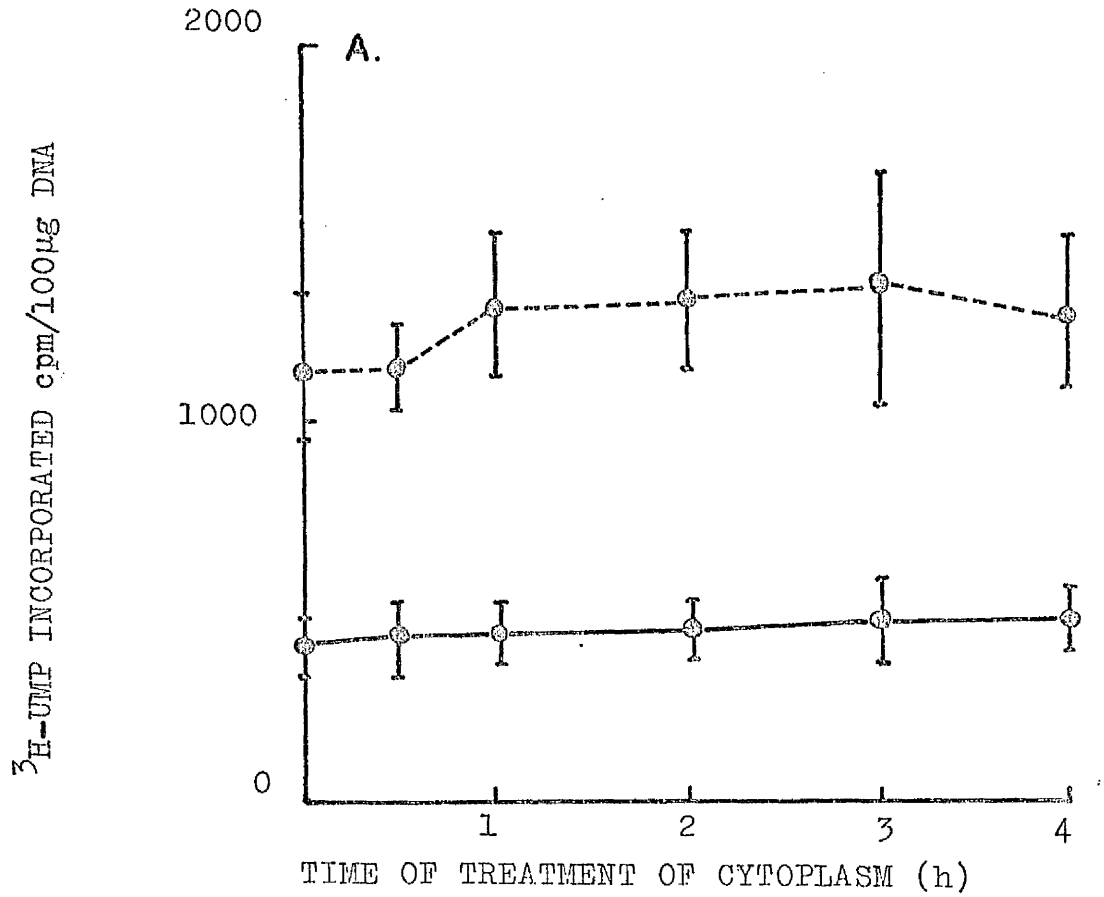
Thus, there is evidence for the presence in cytoplasm of uteri from rabbits responding to oestradiol of a protein component which can enhance the RNA polymerase A activity in isolated uterine nuclei. Such a fraction, which appears to be transient, could lead to modification and activation of RNA polymerase A or could be involved in modifying the template function of the nuclear chromatin. In an attempt to distinguish between these possibilities, experiments will now be described on the action of oestradiol on the solubilised RNA polymerases from immature rabbit uterus.

FIG. 30: The Effect of the presence of Heparin on the addition of Cytoplasm from Oestradiol-treated Uteri on the RNA polymerase activities in Isolated Uterine Nuclei.

Cytoplasm was prepared from rabbit uteri which had been treated with oestradiol as described in the Methods section and added to nuclei isolated from untreated uteri in the presence of 50 µg heparin which was added after the cytoplasm. RNA polymerase activities were assayed as described in the Methods section. Each point represents the range of three determinations.

a) RNA polymerase A activity with cytoplasm from OE_2-treated uteri (●----●) and control uteri (●——●)

b) RNA polymerase B activity with cytoplasm from OE_2-treated uteri (●----●) and control uteri (●——●)


A.
2000
1000
0
1
2
3
4
^{3}H-UMP INCORPORATED cpm/100µg DNA
TIME OF TREATMENT OF CYTOPLASM (h)

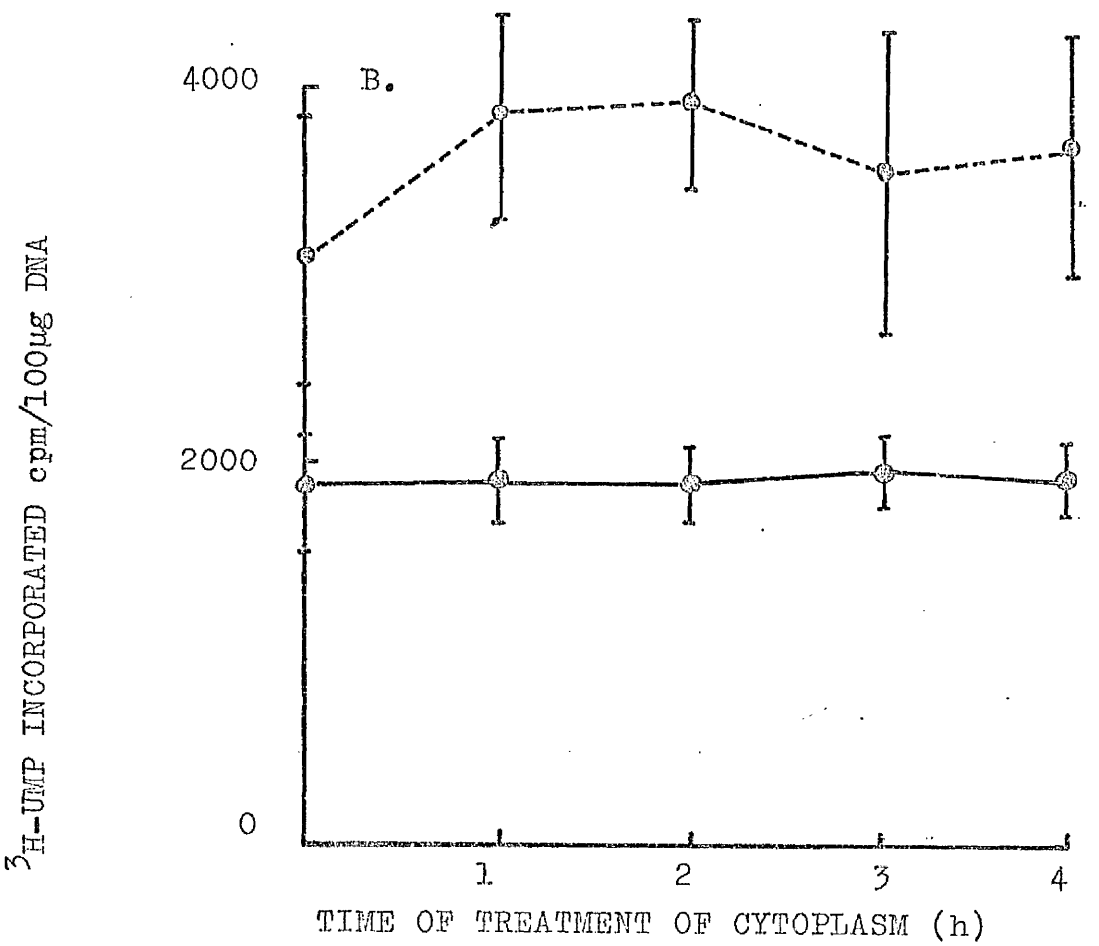
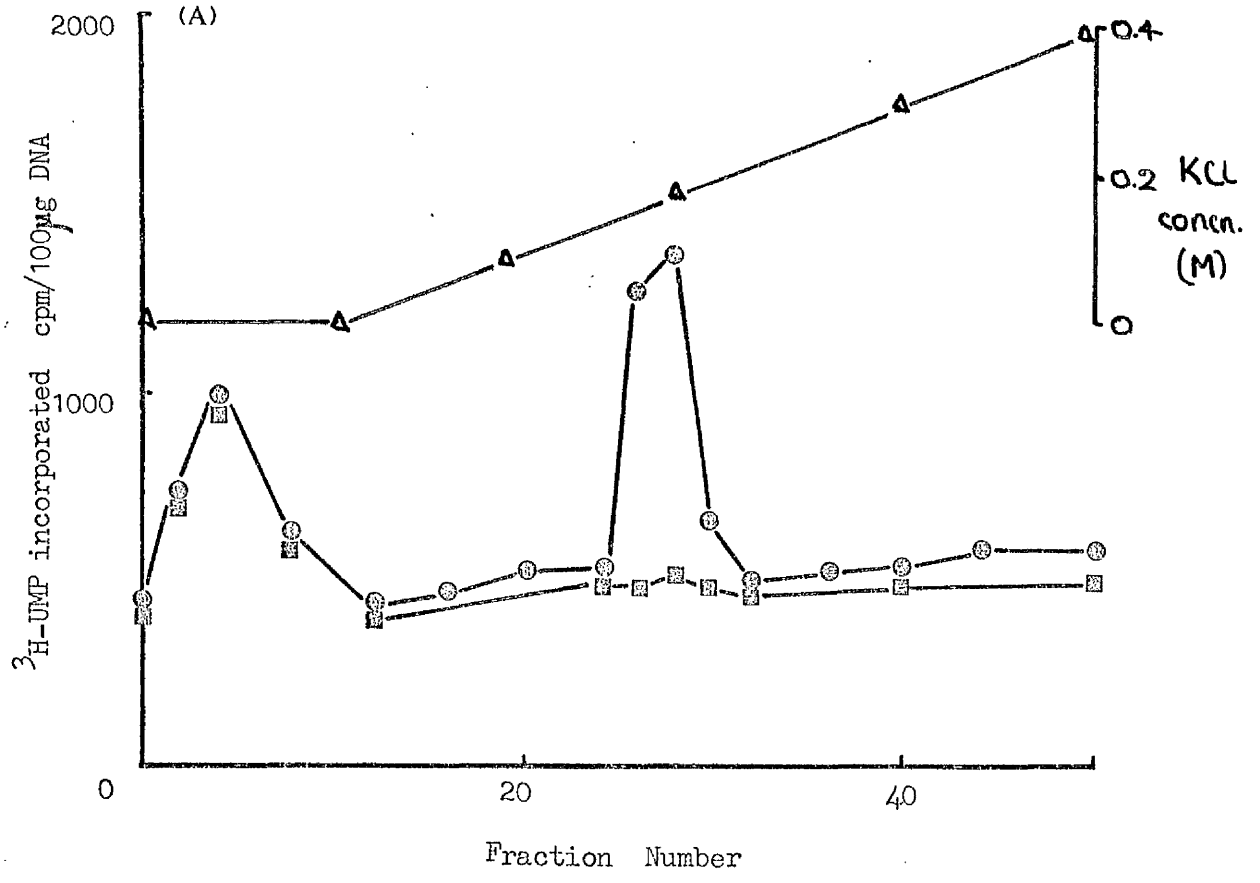

B.
4000
2000
0
1
2
3
4
^{3}H-UMP INCORPORATED cpm/100µg DNA
TIME OF TREATMENT OF CYTOPLASM (h)

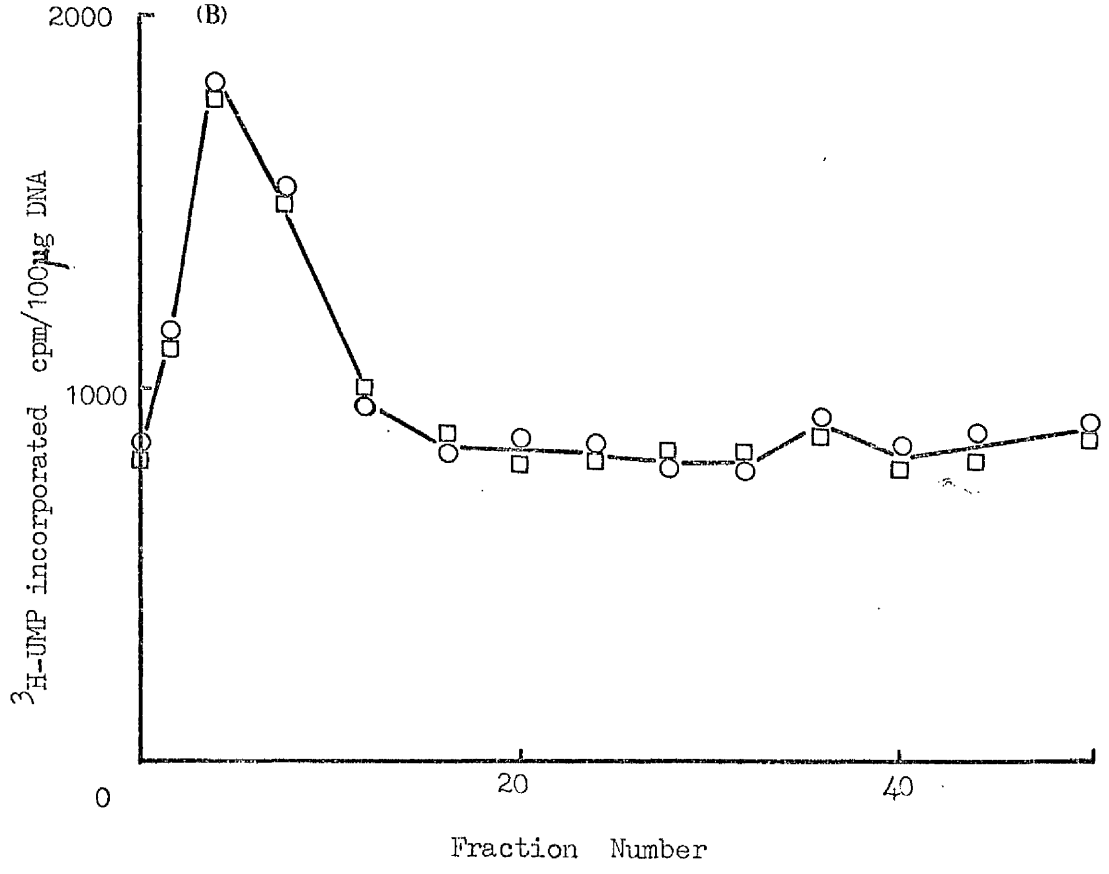
FIG. 31: Fractionation of Cytoplasm from the Uteri of Rabbits treated with Oestradiol.

Cytoplasm prepared from immature rabbit uteri at various times after treatment with oestradiol as described in the Methods section was fractionated on columns of CM-cellulose. Nuclei prepared from uteri of untreated animals were assayed for RNA polymerase activities in a total volume of 0.1 ml in the presence of samples of each fraction obtained by CM-cellulose chromatography of the cytoplasms.

a) RNA polymerase A activity in nuclei assayed with cytoplasm from uteri treated with oestradiol for 30 min (⊙——⊙) and 60 min (▣——▣)

b) RNA polymerase B activity in nuclei assayed with cytoplasm from uteri treated with oestradiol for 30 min (○——○) and 60 min (□——□)


(A)
2000
1000
0
20
40
0.4
0.2 KCL
concn.
(M)
0
^{3}H-UMP incorporated cpm/100µg DNA
Fraction Number


(B)
2000
1000
0
20
40
^{3}H-UMP incorporated cpm/100µg DNA
Fraction Number

8. The Effect of Oestradiol Administered to Rabbits *In Vivo* on the Solubilised Uterine RNA Polymerases.

Several forms of DNA-dependent RNA polymerases were detected in preparations from the uteri of immature rabbits which had previously been treated with oestradiol *in vivo* for varying periods of time. A series of DEAE-cellulose columns of identical size were prepared and identical amounts of protein fractions prepared as described in the Methods section from uteri at varying times after oestradiol treatment were applied to the columns. After elution of the columns, the fractions were assayed for RNA polymerase activities using double-stranded rabbit liver DNA as the template. Fig. 32 shows the effect of treating the animals for 15 min, 30 min and 45 min with oestradiol *in vivo*. It is apparent that under the conditions employed for the assay of RNA polymerase activity *in vitro* there is no detectable change in the activity of the solubilised enzymes. Similarly, when the time of treatment with oestradiol *in vivo* is increased up to 4h (Fig. 33) no detectable change in the activities of the solubilised enzymes is observed.

Experiments were also performed to determine whether oestradiol itself was associated with either of the uterine RNA polymerases. Tritiated oestradiol was administered to rabbits, the enzymes isolated as before and measurements of the radioactivity in each fraction performed (Fig. 34). Within the limits of detection for oestradiol, it seems that oestradiol is not associated in any way with either of the RNA polymerases. Over 70% of the radioactivity which was originally administered to the animals is recovered as a discreet peak in the material which flows through the DEAE-cellulose column.

It is possible that some factor, which is required for the activation of the RNA polymerases *in vivo* in response to the hormone, is lost during the isolation procedure. In an attempt to demonstrate

FIG. 32: Isolation of Multiple Forms of RNA polymerase in Uteri from Animals treated with Oestradiol.

RNA polymerases were solubilised from immature rabbit uteri as described in the Methods section. 40 mg samples of uterine protein prepared at each time interval after oestradiol treatment were applied to identical 10 x 0.9 cm columns of DEAE-cellulose and column chromatography carried out as described in the Methods section.

a) RNA polymerases from uteri treated for 15 min with OE_2 (——) and control uteri (------)

b) RNA polymerases from uteri treated for 30 min with OE_2 (——) and control uteri (------)

c) RNA polymerases from uteri treated for 45 min with OE_2 (——) and control uteri (------).

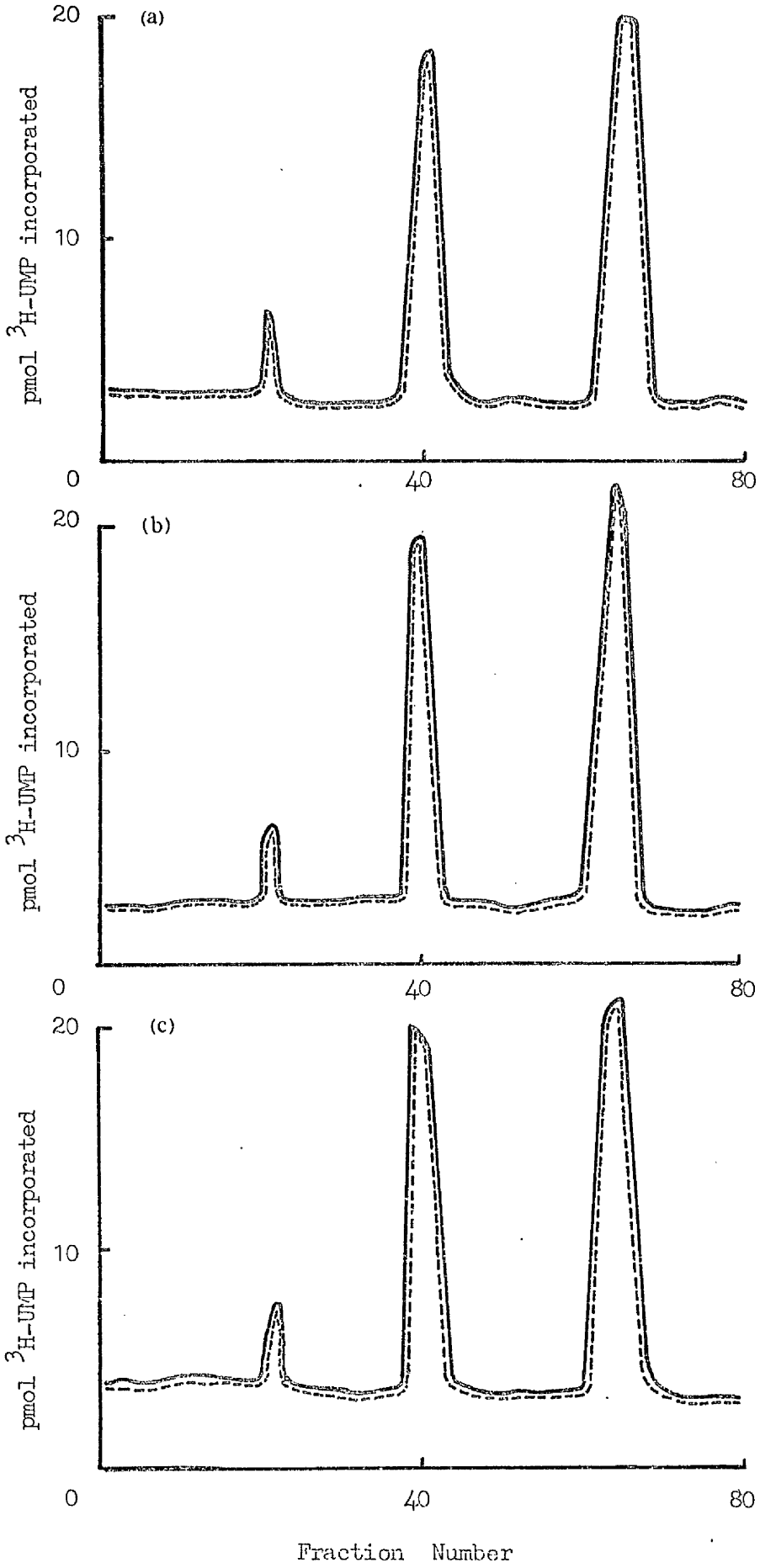

(a)
(b)
(c)
20
10
0
40
80
pmol ^{3}H-UMP incorporated
Fraction Number

FIG. 33: Isolation of Multiple Forms of RNA polymerase in Uteri from Animals treated with Oestradiol.

RNA polymerases were solubilised from immature rabbit uteri as described in the Methods section. 40 mg samples of uterine protein prepared at each time interval after oestradiol treatment were applied to identical 10 x 0.9 cm columns of DEAE-cellulose and column chromatography carried out as described in the Methods section.

a) RNA polymerases from uteri treated for 1h with OE_2 (——) and control uteri (------)

b) RNA polymerases from uteri treated for 2h with OE_2 (——) and control uteri (------)

c) RNA polymerases from uteri treated for 4h with OE_2 (——) and control uteri (------).

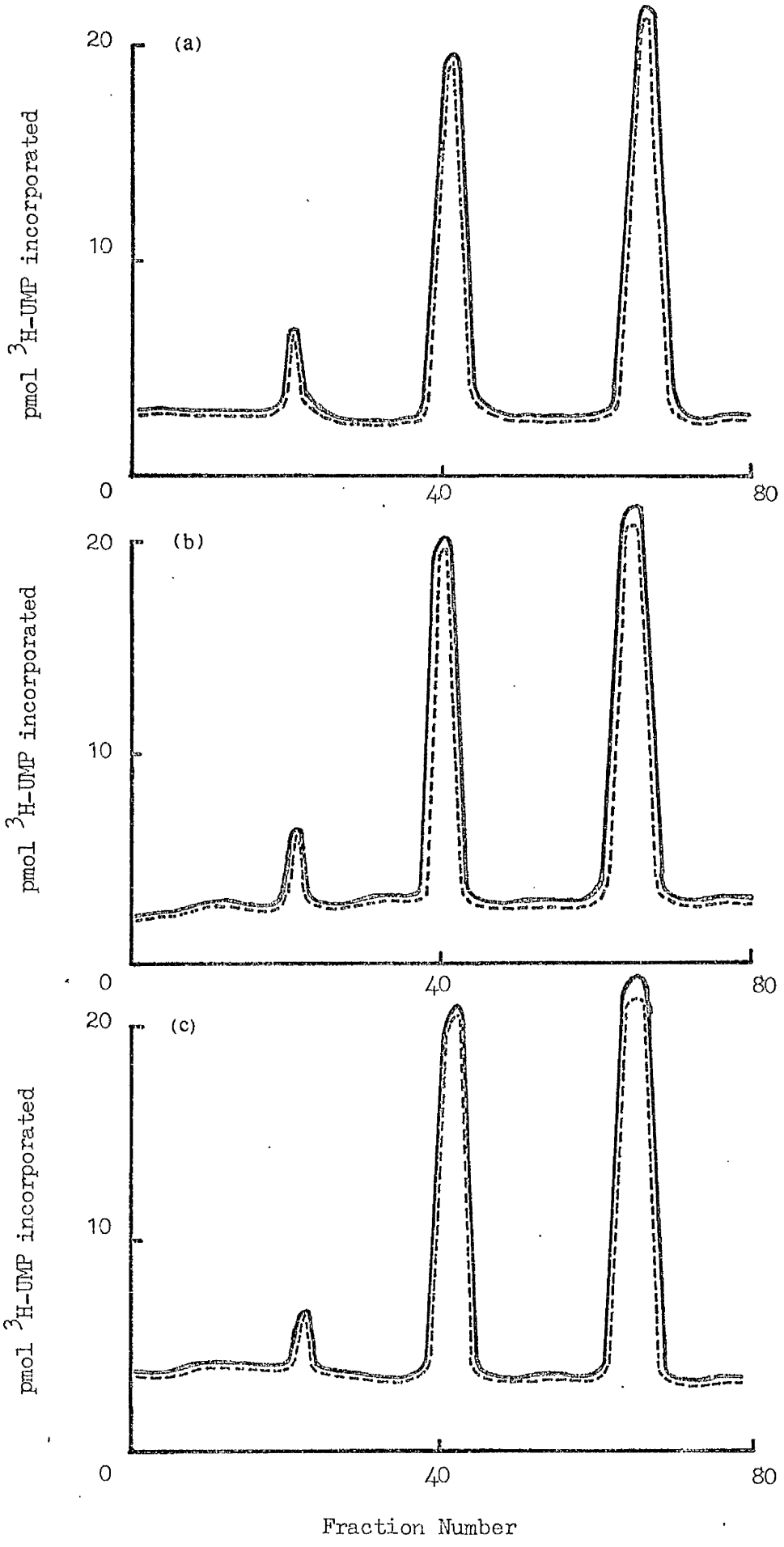
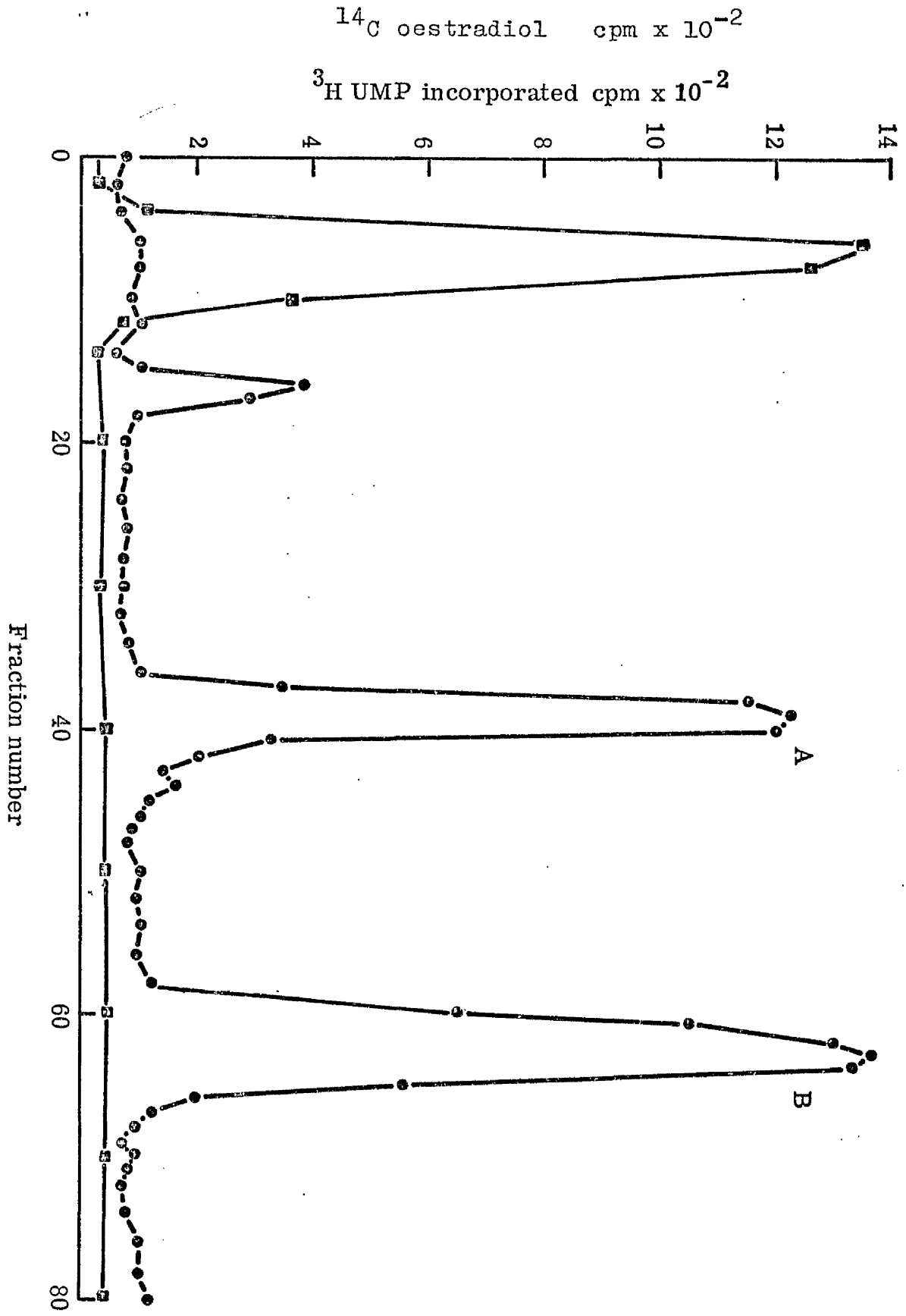

(a)
(b)
(c)
20
10
0
40
80
pmol ^{3}H-UMP incorporated
Fraction Number

FIG. 34: The Association of Oestradiol with Uterine RNA polymerases.

Animals were treated for 1h with 200 μC_i of tritiated oestradiol *in vivo*. Total uterine protein was prepared and the RNA polymerases separated on DEAE-cellulose columns as described in the Methods section. Each fraction was assayed for RNA polymerase activity using ^{14}C-UTP as the labelled substrate. 0.2 ml samples were counted in toluene-methoxyethanol scintillator to determine the oestradiol content.

RNA polymerase activity

Oestradiol.

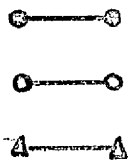

^{14}C oestradiol cpm x 10^{-2}
^{3}H UMP incorporated cpm x 10^{-2}
0
2
4
6
8
10
12
14
20
40
60
80
Fraction number
A
B

the existence of such a stimulatory factor or factors, the protein which was not bound to the DEAE-cellulose and appeared in the column wash was fractionated on a CM-cellulose column as described in the Methods section. Each fraction was treated with respect to its effect on the activity of both RNA polymerase A and B using double-stranded rabbit liver DNA as the template (Fig. 35). None of the fractions obtained exerted any stimulatory effect on either RNA polymerase A or B.

Baulieu et al. (1972) proposed that a 'key intermediary protein' may act to modify gene expression following the administration of oestradiol to immature uteri. This protein would have to be synthesised early in the oestrogenic response probably after the early observed synthesis of HnRNA (Knowler & Smellie, 1973). In order to show whether or not there is such a protein fraction present in the rabbit uterus after treatment with oestradiol, soluble cytoplasmic preparations from uteri treated with oestradiol for varying periods of time up to 90 min were assayed with respect to their effects on the activity of both RNA polymerase A and B (Fig. 36). There is great variation in the results from one experiment to another although there is no significant increase in the activity of either RNA polymerase A or B. It has already been demonstrated that cytoplasmic preparations from rabbit uteri are contaminated with both RNase and DNase (Figs. 27 & 28), and it seems probable that any increases in RNA polymerase activity are the consequence of the action of DNase introducing 'nicks' into the template where transcription can be non-specifically initiated. This effect may be balanced by the action of RNase on the product of the RNA polymerases. Heparin is known to inhibit RNase activity (Rhoads et al., 1973) although it does not affect the endogenous RNA polymerase activities in isolated nuclei (Fig. 29). When heparin is added to the incubation mixture after the addition of cytoplasm to inhibit RNase, then this treatment has little effect on the RNA polymerase activities (Fig. 37).

FIG. 35: Fractionation of Uterine Protein on Columns of CM-cellulose.

The protein which was eluted from the DEAE-cellulose column by 0.08M ammonium sulphate in the column wash was fractionated on a CM-cellulose column. Each fraction was assayed with soluble forms of RNA polymerase A or B in a total volume of 0.1 ml as described in the Methods section.



RNA polymerase A

RNA polymerase B

salt concentration.

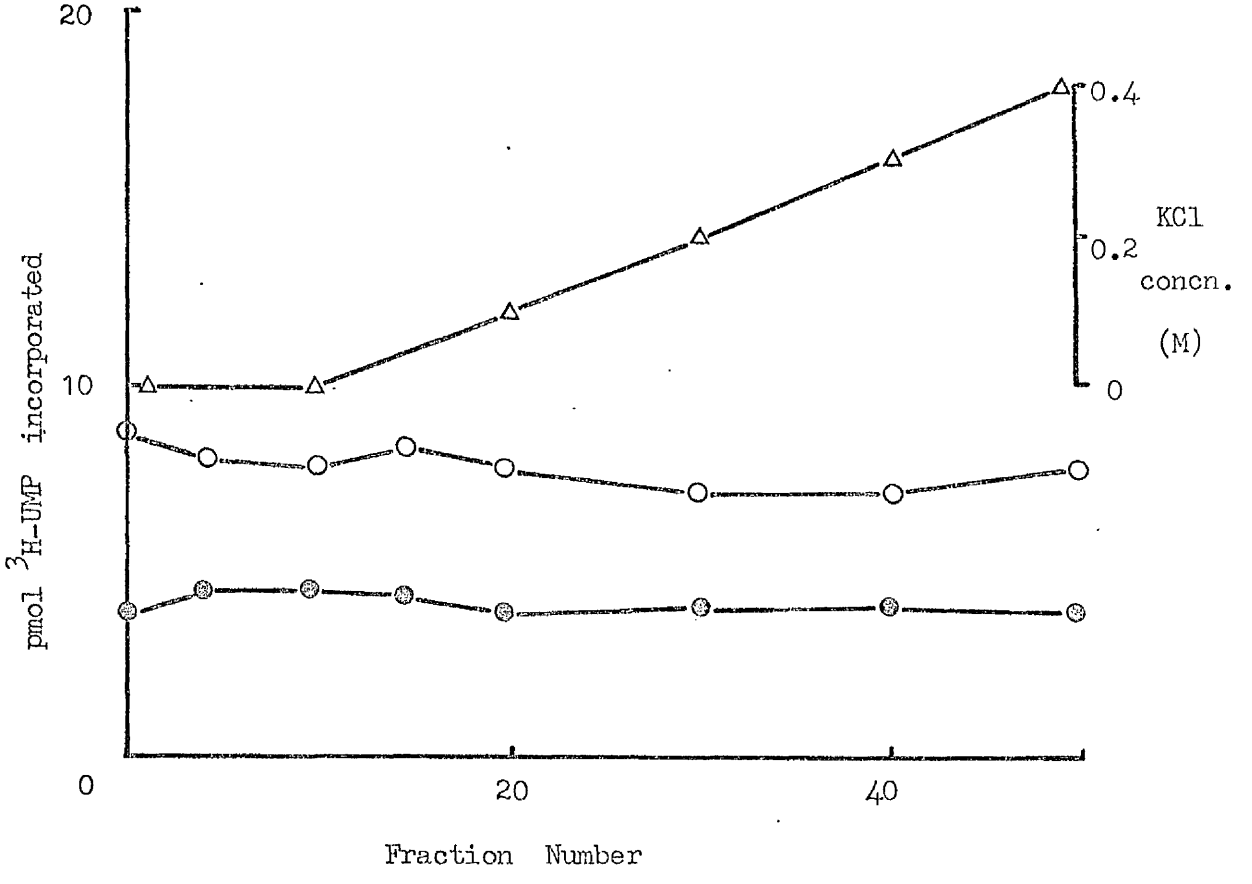
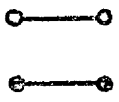

20
10
0
20
40
Fraction Number
pmol ^{3}H-UMP incorporated
0.4
0.2
0
KCl
concn.
(M)

FIG. 36: Effect of Cytoplasms from Oestrogen-treated Uteri on Soluble Uterine RNA polymerases.

Cytoplasms were prepared from rabbit uteri after various times of treatment with oestradiol as described in the Methods section. 0.05 ml samples were incubated with RNA polymerase A or B in a total volume of 0.1 ml for 30 min at 37°.



RNA polymerase B

RNA polymerase A

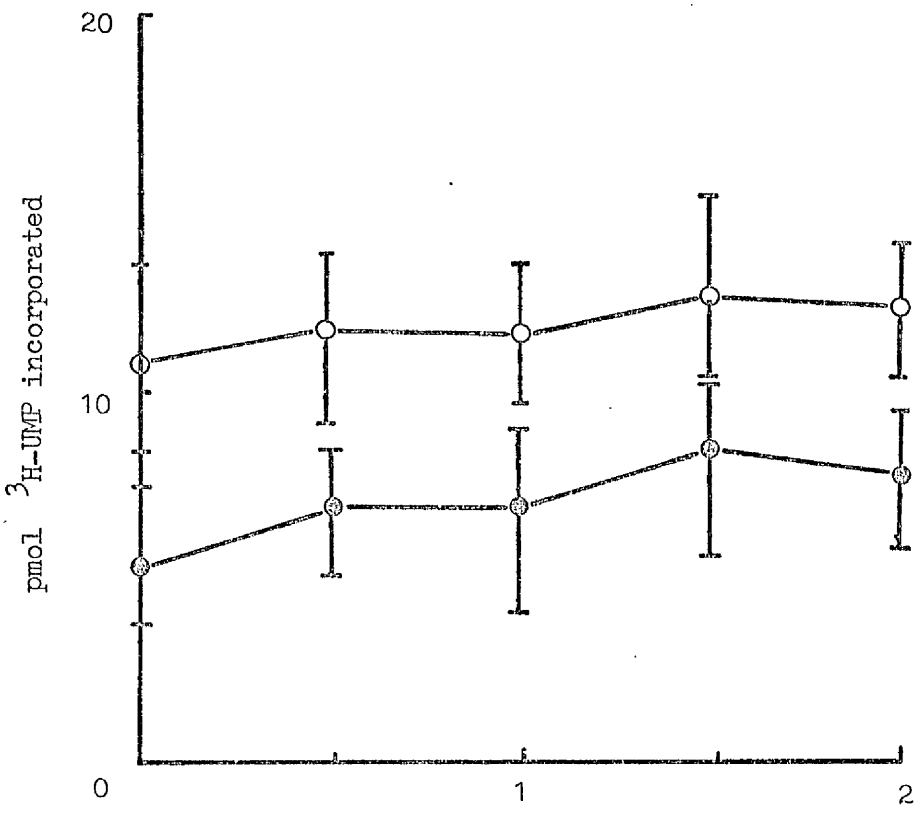
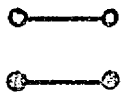

20
10
0
1
2
pmol ^{3}H-UMP incorporated
Time of oestradiol-treated uterine cytoplasm (h)

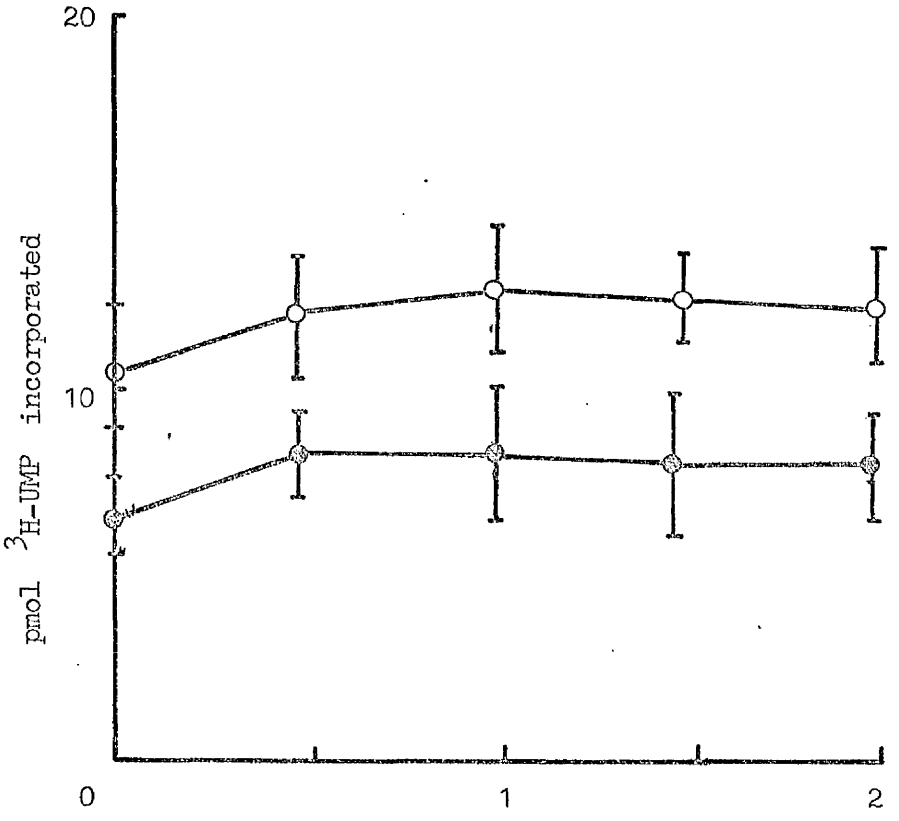
FIG. 37: Effect of Cytoplasm from Oestrogen-treated Uteri on Soluble Uterine RNA polymerases in the presence of Heparin.

Cytoplasm was prepared from rabbit uteri after various times of treatment with oestradiol as described in the Methods section, and 0.05 ml assayed with either RNA polymerase A or B in a total volume of 0.1 ml in the presence of 50 µg heparin. Results are expressed as p mol ^{3}H-UMP incorporated per 30 min and show the range of values obtained in at least three determinations.



RNA polymerase B

RNA polymerase A


20
10
0
1
2
pmol ^{3}H-UMP incorporated
Time of oestradiol-treated uterine cytoplasm (h)

If some protein synthesised early in response to the hormone is required for the initiation of transcription, then it may be possible to demonstrate its existence in the cytoplasm of oestrogen-treated uteri. Consequently, cytoplasms from rabbit uteri which had previously been exposed to oestradiol were fractionated on CM-cellulose and assayed for their capacity to stimulate RNA polymerase activity (Fig. 38). None of the fractions examined produced any significant stimulation of either RNA polymerase A or B. However, it is possible that the fractions may have been too dilute or inactivated because experiments conducted with fractions which had been concentrated by dialysis against 30% (v/v) polyethylene glycol produced a similar result. Thus, it appears that other components of the chromatin are required for an authentic transcription by the RNA polymerases.

One of the most serious problems encountered in the experiments with purified RNA polymerase preparations is the choice of template, the most commonly used being synthetic templates such as poly d(AT), viral DNAs of known structure and poorly preserved natural templates. In most of the experiments already described, double-stranded rabbit liver DNA has been used as the template for the RNA polymerases. Studies on the molecular weight of the DNA prepared by the method described in the Methods section were carried out following the method of Studier (1965). Samples were layered on either 1M NaCl for analysis of double-stranded DNA or 0.9M NaCl containing 0.1M NaOH for analysis of denatured DNA. The molecular weight for double-stranded rabbit liver DNA was 1.8×10^7 while single-stranded DNA had a molecular weight of 0.6×10^6. Thus, it is obvious that there are several 'nicks' in this DNA preparation where RNA synthesis could be non-specifically initiated. Most commercial preparations of eukaryotic DNA have similar or slightly lower molecular weights and it is only recently that more success has been achieved in the isolation of high-molecular weight DNA (Gross-Bellard et al., 1973).

FIG. 38: Chromatography of Uterine Cytoplasm on CM-cellulose.

Uterine cytoplasm was prepared from rabbits after various times of treatment with oestradiol and CM-cellulose chromatography carried out as described in the Methods section. 0.05 ml of each fraction was assayed with respect to its effect on either RNA polymerase A or B in a total volume of 0.1 ml. Only the results for cytoplasm from uteri treated for 30 min and 60 min are shown. Control values were identical to these.

a) RNA polymerase A

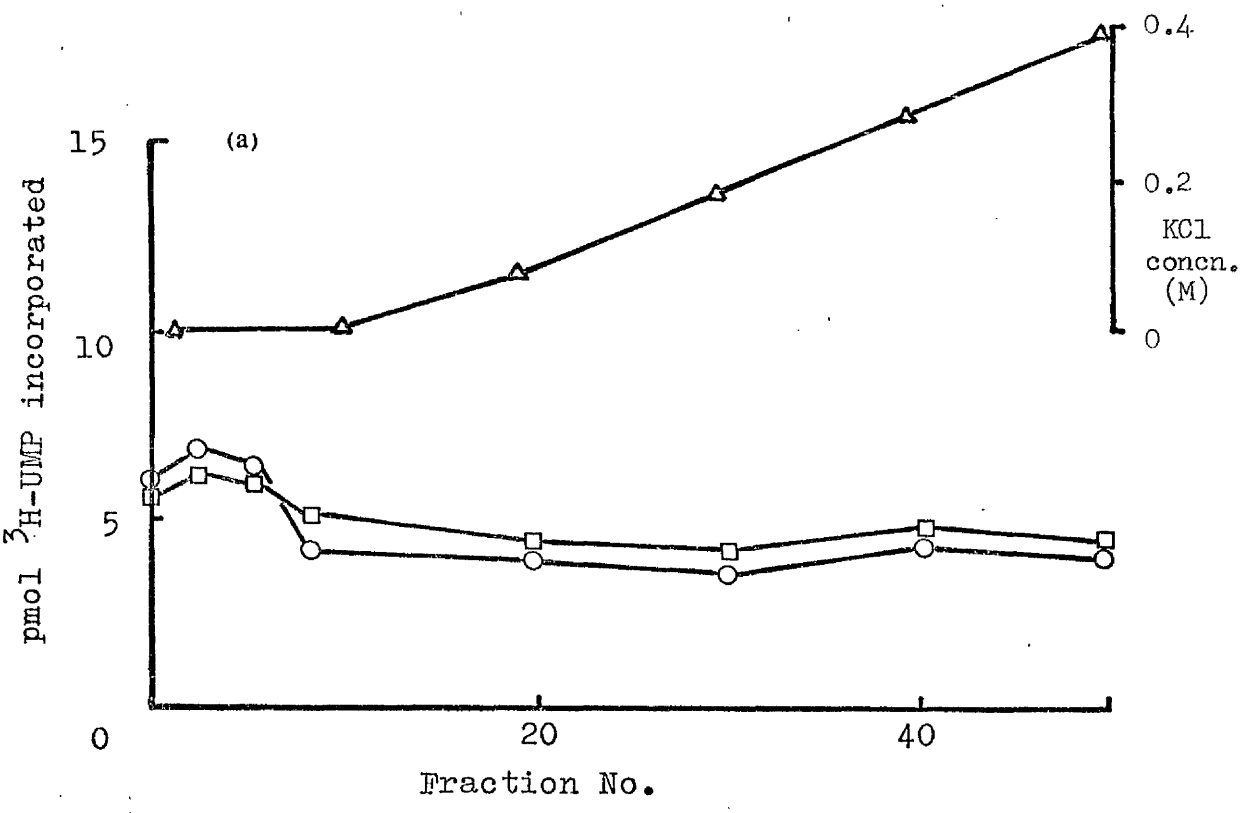
(o—o) 30 min oestradiol

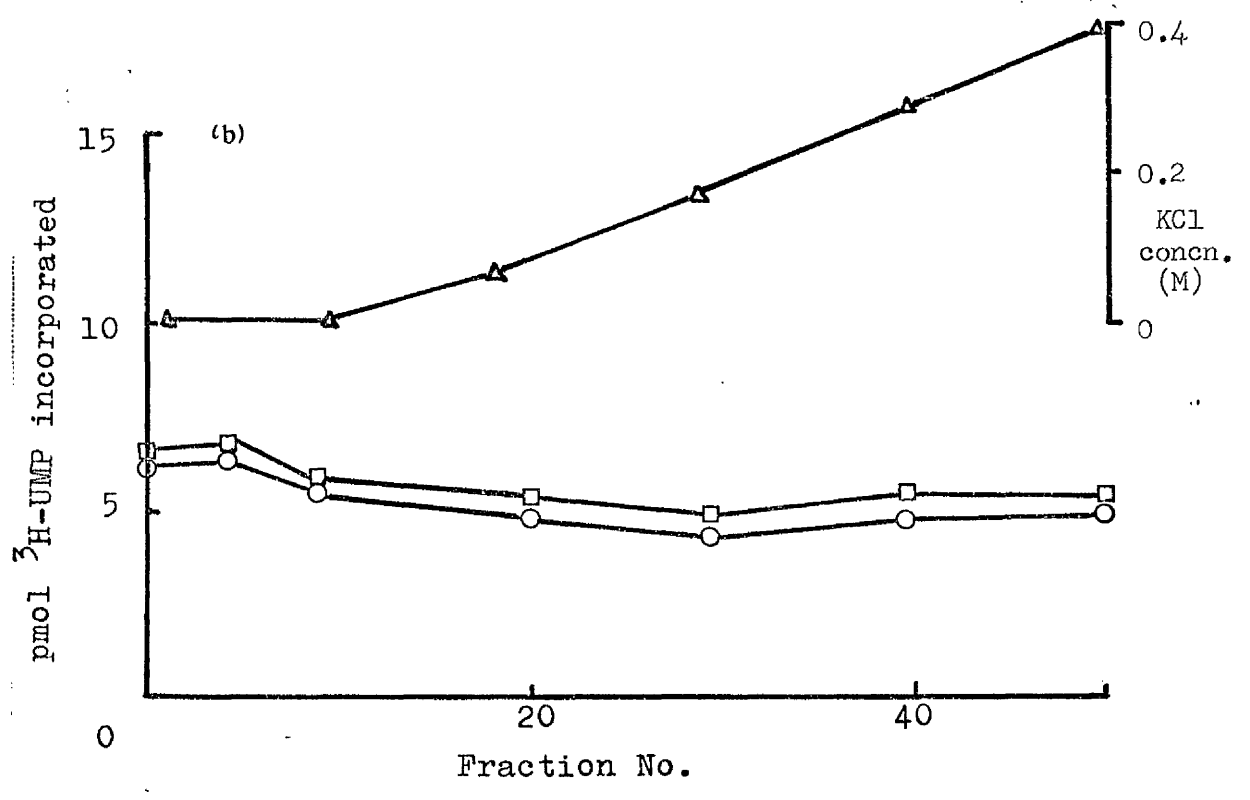
(□—□) 60 min oestradiol

b) RNA polymerase B

(o—o) 30 min oestradiol

(□—□) 60 min oestradiol


(a)
15
10
5
0
pmol ^{3}H-UMP incorporated
0.4
0.2
KCl
concn.
(M)
0
20
40
Fraction No.


(b)
15
10
5
0
pmol ^{3}H-UMP incorporated
0.4
0.2
KCl
concn.
(M)
0
20
40
Fraction No.

DISCUSSION

Most tissues in higher animals are in some way dependent on hormones for their growth and development. It is well known that a wide spectrum of steroid and polypeptide hormones stimulate protein synthesis in their respective target tissues (Korner, 1965; Manchester, 1968; Tata, 1970). Corticosteroids, which normally inhibit protein synthesis, stimulate the production of certain key enzymes of gluconeogenesis and amino acid metabolism while hormones such as insulin and ACTH also stimulate protein synthesis (Manchester, 1968). In virtually every case, the stimulation of protein synthesis is dependent upon and preceded by RNA synthesis (Tata, 1966).

The precise role of RNA synthesis in the hormone-induced response has been the subject of much intensive research and many theories have been proposed to accommodate this observation. The majority of these theories envisage either a transcriptional or a translation control mechanism. Transcriptional control could involve either the unmasking of genes by removal of proteins from the chromatin or could involve the activation of key proteins such as DNA-dependent RNA polymerase. As the hormone-stimulated production of RNA involves all species of RNA, control could be effected at the level of rRNA, mRNA or tRNA.

The most obvious change in the early stages of response of target cells to many growth and developmental hormones is a stimulation in the rate of rRNA synthesis. This effect has been demonstrated in uteri responding to oestradiol (Hamilton, 1968), in rat prostate responding to testosterone (Liao et al., 1966) and rat liver in response to triiodothyronine (Tata, 1966). The larger increases in rRNA synthesis after the administration of oestradiol to ovariectomised rats led Hamilton et al. (1968a) to conclude that the regulation of rRNA synthesis was a major and possibly rate limiting step in the growth response of the uterus to oestradiol.

The concept that in higher animals hormones could control the synthesis of new species of mRNA was a logical extension of the findings that certain small molecules were responsible for the control of transcription in bacteria (Jacob & Monod, 1961). This concept was expressed first by Karlson (1963) after finding that the insect hormone ecdysone induced the unfolding and priming of specific regions of the insect chromosomes followed by the appearance of enzymes associated with insect moulting. It has been suggested that the stimulation of chromatin template activity in hormone-treated animals could reflect the control of mRNA production by the hormone. Dahmus & Bonner (1965) reported that the template activity of liver chromatin was enhanced when hydrocortisone was injected into adrenalectomised rats and that this increase was associated with the proteins complexed to the DNA since removal of these proteins yielded only DNA whose template activity was not affected by hormone treatment.

Further support for this idea came from the finding that the stimulation of nuclear RNA synthesis occurred in the nuclei of uterine and liver cells as an early response to the administration of oestrogen and triiodothyronine respectively (Tata & Widnell, 1966 ; Hamilton et al., 1968a). Hybridisation studies revealed new nuclear RNA species in the rabbit uterus shortly after oestradiol treatment (Church & McCarthy, 1970), in chick oviduct in response to DES and progesterone (O'Malley et al., 1968) and in rat liver responding to cortisol (Yu & Feigelson, 1969).

The mechanism by which oestradiol effects an increase in the rate of RNA synthesis in the uterine nucleus has been the subject of extensive interest and investigation. This stimulation could be brought about by one of several means: the hormone may affect directly the chromatin template activity, activate the RNA polymerases, influence

the transport of RNA from the nucleus to the cytoplasm;or the response may be a consequence of a combination of these effects. Some evidence exists in support of each of these possibilities. Increased chromatin template activity in rat uteri treated with oestrogen was observed when assayed using exogenous E.coli RNA polymerase (Barker & Warren, 1966) while similar findings were reported when rabbit endometrial chromatin was assayed using endogenous RNA polymerase (Church & McCarthy, 1970). It was suggested by Teng & Hamilton (1969) that activation of chromatin may involve the synthesis of new acidic proteins which overcome the inhibitory effect of uterine histones on RNA synthesis. It is well documented that DNA-dependent RNA synthesis is inhibited by the addition of histone to cell-free systems containing RNA polymerase and either DNA or chromatin (Hnilica, 1967).

Barry & Gorski (1971) found that the increased incorporation of precursor into RNA did not involve the synthesis of more RNA chains as would be expected if new template sites were made available. These workers found that oestradiol stimulated the production of longer RNA chains suggesting an effect on the RNA polymerase itself.

Very little is known about the effects of hormones on the selective transfer of RNA from the nucleus to the cytoplasm. It is possible that the hormone initiates a change in the synthesis of RNA but does not control the eventual selection and transfer of the various types of RNA synthesised. Growth hormone and cortisol have been shown to increase the rate of appearance of 40-50S particles in rat liver (Finkel et al., 1966 ; Sells & Takahashi, 1967). These particles are believed to be precursors to polysomes and contain mRNA attached to the small ribosomal subunit. It is also possible that they contain 'informosomes' or messenger ribonucleoprotein particles (Liao et al., 1973).

In an attempt to differentiate between these various possible mechanisms for the action of oestradiol in the uterus the present study has involved the isolation and characterisation of multiple forms of DNA-dependent RNA polymerase from immature rabbit uterus and the effects of oestradiol on these enzymes both in isolated nuclei and in purified preparations.

DNA-dependent RNA polymerases.

In recent years multiple forms of RNA polymerases have been extracted from a wide variety of animal tissues. Most of the methods employed for the isolation of the enzymes involve the sonication of nuclei from the tissue in high ionic strength conditions (greater than 0.3M ammonium sulphate) in order to remove the enzymes which are tightly bound to the chromatin. In the present studies, it did not prove feasible to prepare large amounts of uterine nuclei and, as a consequence of this, uterine RNA polymerases were solubilised by the method of Sugden & Keller (1973) which consisted of starting with a whole tissue homogenate thus eliminating the necessity to prepare nuclei as a first step.

This procedure enabled the isolation of three different RNA polymerases from rabbit uterus (Fig. 1). The two principal species are designated A and B and both of these enzymes can be further purified by phosphocellulose chromatography and glycerol density gradient sedimentation (Figs. 3 & 4). These procedures have made it possible to achieve a purification of approximately 300 fold, although it is not possible to obtain a precise value because the actual amount of each enzyme protein present after the last step of the purification scheme is very low and consequently difficult to determine accurately. One consequence of the low protein concentration has been the necessity to add stabilising protein to the RNA polymerases if they were to be stored

for any time. At low protein concentrations the activity of both uterine RNA polymerases was lost in a few days.

In addition to the RNA polymerases A and B which can be extracted from rabbit uterus, there is a third peak of RNA polymerase activity which is eluted with the bulk of the cellular protein from DEAE-cellulose. This enzyme can be further purified by phosphocellulose chromatography (Fig. 12) although this preparation still contains a considerable amount of protein contamination. It has been shown to represent a species of RNA polymerase as it is completely dependent on the presence of DNA template and all four nucleoside triphosphates and is suppressed by the inclusion of actinomycin D in the incubation mixture. When the uterine RNA polymerases were extracted from preparations of nuclei low amounts of enzymes A and B could be solubilised, but RNA polymerase C did not appear to be present (Fig. 2). Thus, it is possible that this species does not originate from the nucleus and may be located in some other cellular fraction. Seifart et al. (1973) have described the presence of a possible cytoplasmic RNA polymerase in rat liver. Alternatively, RNA polymerase C may well be nuclear in origin but is extracted from the nucleus during the process of nuclear isolation. Consequently this species has been designated 'soluble' RNA polymerase. It is unlikely that this enzyme is from the mitochondria since it has been found to be resistant to inhibition by rifampicin, while the mitochondrial RNA polymerase of rat liver has been shown to be sensitive to this antibiotic (Reid & Parsons, 1971). As yet it has not been possible to determine the origin or function of this species of RNA polymerase in uteri.

Properties of RNA Polymerases.

Using buffer conditions in which E. coli RNA polymerase sediments

as a monomer (Berg & Chamberlin, 1970) with a sedimentation coefficient of about 15S, the mammalian RNA polymerases sedimented slightly on the heavier side in the glycerol gradient (Fig. 11), suggesting that the molecular weight of these enzymes was slightly greater than that of E. coli RNA polymerase. Without a more detailed study of the subunit structure of the uterine RNA polymerases, which was not possible due to the very small amounts of purified enzymes solubilised, the molecular weight can only be estimated to be in the range of 500,000 - 600,000. This value is similar to those obtained for highly purified preparations of calf thymus RNA polymerases AI (Gissinger & Chambon, 1972), B (Kedinger & Chambon, 1972) and rat liver RNA polymerase B (Weaver et al., 1971).

The ionic and salt requirements of RNA polymerases A and B have already been described for the rat liver enzymes (Roeder & Rutter, 1970b) and those from calf thymus (Chambon et al., 1970). From the results presented here (Figs. 5 & 6) it is apparent that the optimal ionic concentrations for RNA polymerase A are 8mM Mg^{++} and 3mM Mn^{++} while the equivalent Mn^{++} concentration for RNA polymerase B was 2-3mM. The optimal ionic strengths were 60mM and 100mM ammonium sulphate for enzymes A and B respectively. These values are similar to those obtained from calf thymus. RNA polymerase C shows optima of 10mM Mg^{++}, 3mM Mn^{++} and 70mM ammonium sulphate which are intermediate between enzymes A and B. The purified enzymes require considerably lower salt concentrations than the 0.4M ammonium sulphate usually used for the analysis of enzyme activities in nuclei. Maitra & Barash (1969) have shown that salt will detach newly formed RNA from E. coli RNA polymerase and consequently allow the reinitiation of RNA chains.

The action of α-amanitin on the uterine RNA polymerases (Fig. 7) shows that enzyme A is completely unaffected by the action of the toxin while RNA polymerase B is completely inhibited at similar concentrations.

This is compatible with the finding of Chambon et al. (1970) who showed that α-amanitin was bound to one subunit of the B enzyme unlike other inhibitors of RNA synthesis which bind to the template. It is interesting to note that RNA polymerase C is inhibited by about 20% at similar concentrations. This value is comparable to that obtained by Seifart et al. (1973) for their 'cytoplasmic' RNA polymerase.

The thermal sensitivities of the enzymes are similar (Fig. 8) to the reported values obtained for the rat liver RNA polymerases (Shields & Tata, 1973). This differential sensitivity appears to reflect the subunit structure of the enzymes. From the published structures of RNA polymerases A and B (Table 1) it is clear that these are different, and it seems likely that one of the subunits of enzyme A is very heat labile and treatment at 45° causes a rapid loss of enzymic activity.

Throughout the course of the purification RNA polymerase B utilises heat-denatured DNA more efficiently than 'native' double-stranded DNA as a template while RNA polymerase A utilises double-stranded DNA preferentially. It is possible that this observation on the solubilised RNA polymerases is an artefact due to a modification of the enzymes during the purification procedure rather than an intrinsic property of the enzymes. Other important points to be remembered when dealing with RNA polymerases in vitro are that free DNA does not adequately reflect physiological conditions and that other proteins present in the chromatin are probably required for a meaningful transcription to take place. Equally important is the fact that most preparations of DNA contain a significant amount of single-stranded 'nicks' where RNA synthesis can be non-specifically initiated. Using rabbit liver DNA with a molecular weight of about 2×10^7, the enzymes can be shown to be completely dependent on DNA and all four triphosphates as well as being suppressed by actinomycin D (Table 2).

Treatment of the RNA polymerases with proteases and nucleases results in a total loss of activity from each type of treatment (Table 3). However, these effects arise in different ways. Pronase treatment results in a complete loss of activity as expected due to the protein nature of the enzymes. Incubation with RNase causes an apparent loss of activity but, as this can be restored by including an RNase inhibitor such as heparin in the incubation mixture, it is apparent that the RNase is acting on the product of the reaction and not on the enzymes themselves. Similarly, DNase treatment results in an apparent loss of RNA polymerase activity but this can be turned into an apparent stimulation of RNA synthesis by using smaller concentrations of DNase. Obviously the higher concentrations of DNase is breaking the DNA template into fragments so small that RNA synthesis cannot take place. Flint et al. (1974) have recently shown that there may be a certain minimal size of template that can support RNA synthesis. When lower concentrations of DNase are employed, this introduces 'nicks' into the template where RNA synthesis can be initiated non-specifically.

Measurement of RNA Synthesis in Nuclei.

Widnell & Tata (1966) first demonstrated the existence of two different RNA polymerases in rat liver nuclei, one of which was active in the presence of Mg^{++} and low salt while the other was more active in the presence of Mn^{++} and high salt conditions. The measurement of RNA synthesis in intact nuclei or isolated chromatin in vitro in the presence of high salt is a compromise between two opposing effects (Yu & Feigelson, 1972a). The high ionic strength of 0.4M ammonium sulphate causes dissociation of the nucleoprotein complex enabling transcription of previously repressed stretches of the template to proceed. This effect is counter-balanced by the salt mediated dissociation of the template and the RNA polymerase.

In the experiments presented in this thesis on the measurements of RNA synthesis in isolated uterine nuclei, two different incubation conditions were employed *in vitro*. The first type involved assaying for RNA synthesis under conditions of low salt and in the presence of α-amanitin which has already been shown to be a specific inhibitor of RNA polymerase B. These conditions are optimal for RNA polymerase A and the activity in low salt has been equated with this enzymic activity. The second type of condition used was to assay for RNA synthesis in high salt buffer in the presence of Mn^{++}; these conditions are optimal for RNA polymerase B activity. The contribution of RNA polymerase A to the total high salt activity was determined by estimating the amount of residual activity when α-amanitin was incubated with the high salt buffer. This value could be subtracted from the total high salt activity to give a value for the activity due to RNA polymerase B.

Another important point to note is that high salt causes lysis of the nuclei immediately on addition while this effect does not take place when low salt is added to nuclei. In addition, 0.4M ammonium sulphate has a considerably higher ionic strength than physiological conditions.

The Effect of Oestradiol on RNA Synthesis in Nuclei.

Gorski (1964) first demonstrated the oestradiol-stimulated increase in DNA-dependent RNA polymerase activity in uterine nuclei. This oestrogen-induced increase in enzyme activity was inhibited by treatment with puromycin and cycloheximide. Gorski *et al.* (1965) showed that delayed treatment with puromycin administered after oestradiol caused the rapid loss of the oestrogen-induced RNA polymerase activity. Nicolette & Mueller (1966) also demonstrated a similar decrease in RNA polymerase activity *in vitro* when inhibitors of protein synthesis were included in the incubation medium. The simplest conclusion to these findings is that the oestrogen-induced increase in RNA polymerase activity

is dependent on the availability of some protein or proteins which can be made limiting by blocking new protein synthesis. Perhaps this protein could be a specific factor for the RNA polymerase acting in a similar manner to a bacterial sigma factor by causing initiation of transcription at certain specific sites on the chromatin template. Alternatively, the protein might assist in the processing or the transport of newly synthesised RNA from the RNA polymerase site to the cytoplasm.

An early stimulation of RNA polymerase B activity has been shown in nuclei isolated from immature rabbit uteri responding to oestradiol. The initial stimulation of RNA polymerase B occurred 30-45 min after hormone administration (Fig. 17). This result correlates exactly with the previous observations of the stimulation of uterine HnRNA synthesis in response to oestradiol (Knowler & Smellie, 1973). The level of RNA polymerase B activity then falls back to near control levels at about 2h before rising again to a maximum by 4h and remaining constant to 12h (Fig. 18). From 1h after oestradiol treatment, RNA polymerase A activity in the nuclei also increases and reaches plateau levels by 4h. Both of these peaks of activity can be suppressed by actinomycin D, injected 30 min prior to oestradiol (Fig. 21). Similar observations have been reported for RNA polymerase B activity in rat uterine nuclei isolated 15-30 min after oestradiol treatment in vivo (Glasser et al., 1972). This increased activity occurred before any detectable change in template activity or RNA polymerase A activity neither of which was stimulated until about 1h after oestradiol administration. This would appear to confirm the earlier findings with inhibitors that an early increase in RNA polymerase B activity is essential for the subsequent biochemical events in the action of oestradiol in the uterus.

In vivo administration of α-amanitin to immature rats inhibits the oestradiol-induced increase in RNA polymerase A activity. Since this enzyme is insensitive to the action of the toxin (Raynaud-Jammet *et al.*, 1972), this result suggests that the oestradiol enhancement of RNA polymerase A is dependent on the earlier synthesis of an RNA which is produced by the action of RNA polymerase B which is sensitive to α-amanitin.

When α-amanitin was administered to immature rabbits *in vivo* at concentrations which caused more than 95% inhibition of RNA synthesis, then the early oestradiol-induced increase in RNA polymerase B was completely abolished. This result is very much as expected due to the known mechanism of action of α-amanitin in binding to one subunit of the RNA polymerase B molecule (Chambon *et al.*, 1970). However, the oestradiol-induced stimulation of RNA polymerase A is also abolished by treatment with the inhibitor (Fig. 22). In this case the levels of enzyme activity fall to control values in animals which have not been exposed to oestradiol. This finding is compatible with the observations of Tata *et al.* (1972) who observed that all synthesis of nuclear RNA was inhibited in rat liver following *in vivo* treatment with α-amanitin.

If α-amanitin is administered *in vivo* after 30 min of oestradiol treatment, or after the early oestrogen-induced stimulation of RNA polymerase B has taken place, then the enhancement of RNA polymerase A activity is identical to that in uninhibited hormone-treated animals (Fig. 23). This provides further evidence that a product of the early RNA polymerase B reaction could be controlling the activity of RNA polymerase A and hence rRNA synthesis. This product could be either an RNA species or a protein translated from this RNA. If it is the latter possibility, then the increase in RNA polymerase A activity in response to oestradiol should be abolished by treatment with an inhibitor of protein synthesis such as cycloheximide.

When cycloheximiade was administered *in vivo* 30 min before oestradiol, the stimulation of RNA polymerase B activity was unaffected but that of RNA polymerase A was almost completely eliminated (Fig. 24). However, administration of cycloheximide to rabbits 30 min after oestradiol gave rise to a stimulation of RNA polymerase A which could not be distinguished from that in hormone-stimulated animals which had not been treated with cycloheximide. This suggested that during the first 30 min after oestradiol treatment the uterus was synthesising some protein or proteins which control the activity of RNA polymerase A and through this the synthesis of rRNA. Schmid & Sekeris (1973) have suggested that cycloheximide inhibited rRNA synthesis in rat liver by affecting the formation of rapidly turning over proteins which are involved in the transcription of rDNA genes. It is important to note that *in vivo* administration of inhibitors may affect other cellular functions such as permeability.

In order to test the possibility that an oestrogen-induced protein or proteins may act to stimulate rRNA synthesis, nuclei extracted from the uteri of rabbits not exposed to oestradiol were treated with cytoplasm from oestrogen-treated animals. If such a protein fraction is synthesised it should appear in the soluble fraction of the cytoplasm. Unfortunately, uterine cytoplasm is heavily contaminated with both RNase and DNase. These enzymes will have opposite effects on RNA synthesis; RNase will degrade newly synthesised RNA while DNase introduces 'nicks' into the DNA template allowing non-specific initiation of RNA synthesis. The results show a slight stimulation but this is manifested even when nuclei are treated with cytoplasm from uteri not exposed to hormone even in the presence of heparin which is known to inhibit RNase action (Rhoads *et al.*, 1973). In an attempt to overcome this difficulty, uterine cytoplasm was fractionated on CM-cellulose

columns and each fraction incubated with nuclei from uteri not treated with oestradiol. Each cytoplasm contains a fraction capable of stimulating RNA polymerase A but these fractions have been shown to contain DNase which would account for the observed stimulation. However, only cytoplasm isolated from uteri treated for 30-45 min with oestradiol *in vivo* possessed another fraction, eluted by about 0.15M KCl, which stimulated RNA polymerase A activity in nuclei. None of the fractions from any cytoplasmic preparations have any stimulatory effect on RNA polymerase B in nuclei.

Mueller (1970) has also shown that a fraction of the soluble uterine cytoplasmic proteins was capable of enhancing RNA polymerase activity in nuclei isolated from untreated uteri. It is possible that this fraction contains the same protein as described in the experiments presented here.

The Action of Hormones on Solubilised RNA Polymerases.

There have been several demonstrations that administration of hormones *in vivo* to experimental animals causes a change in the activity of the RNA polymerases solubilised from the tissue which is responding to one particular hormone. Sajdel & Jacob (1971) showed that a single injection of hydrocortisone caused an increase in the activities of both nucleolar and nucleoplasmic RNA polymerases. They concluded that rRNA synthesis was stimulated in rat liver by regulating the level or the activity of the nucleolar RNA polymerase rather than by increasing the availability of template. In particular, they proposed that hydrocortisone may induce an allosteric change in the nucleolar RNA polymerase resulting in increased activity of the enzyme and an enhancement of rRNA synthesis. Rat liver RNA polymerase A activity measured *in vitro* has also been shown to be stimulated after *in vivo* treatment with human growth hormone and triiodothyronine (Smuckler & Tata, 1971).

Nucleoli isolated from rat liver showed an enhanced ability to incorporate labelled precursors into RNA *in vitro* 4h after administration of cortisone (Yu & Feigelson, 1971). By blocking the natural template with actinomycin D in the presence of exogenous poly d.C as a template, it was found that the increased RNA synthesis was a consequence of elevated levels of RNA polymerase. The same workers then demonstrated that *in vivo* treatment with cycloheximide and actinomycin D caused a rapid loss of nucleolar RNA polymerase activity (Yu & Feigelson, 1972b). This was explained on the grounds that the synthesis of some species of mRNA was inhibited, and the protein product of this mRNA might be a catalytically essential polypeptide component of the RNA polymerase. They further claimed that RNA polymerase A has a rapid turnover time so that control of rRNA synthesis could be achieved by a variation in the amount of enzyme synthesised. Benecke *et al.* (1973) found the complete reverse of this situation. In their experiments on rat liver, the RNA polymerases were entirely unaffected by treatment with cycloheximide administered *in vivo* at concentrations which severely curtailed protein synthesis. These workers claimed that the RNA polymerase molecules have extended half life periods and represent a fairly stable population of enzymes. In addition, they found that treatment with cortisol *in vivo* for 3h did not affect the amount or the activity of the RNA polymerases which could be solubilised from the tissue.

Similar observations to this have been shown in this thesis (Figs. 32 & 33) with the effect of oestradiol on the solubilised RNA polymerases from immature rabbit uterus. It is evident that treatment of the tissue with the hormone for increasing periods up to 4h has very little effect on the activity of the solubilised enzymes. It is not possible to determine whether the absolute amount of enzyme present in the tissue is increased after oestradiol treatment because of the low

levels present. That fact that no variation was found in the total activities of any of the uterine RNA polymerases after hormone treatment in complete contrast to the stimulation of both RNA polymerase A and B in nuclei (Fig. 17) could be due to the fact that the enzymes are not altered themselves per se in any manner by the hormone or alternatively that some specific factor was lost from the RNA polymerases during the isolation procedure.

When the rabbits were injected with tritiated oestradiol and the RNA polymerases solubilised as before (Fig. 34) the hormone was not found to be associated with any of the RNA polymerases. This finding is similar to that of Arnaud et al. (1971) in that the hormone is eluted in the column wash. Addition of hormone to an incubation mixture of either RNA polymerase A or B did not enhance the rate of RNA synthesis. Thus, it seems improbable that the hormone itself has any direct effect on the RNA polymerases.

If a factor which is required for accurate transcription to take place was lost during the isolation procedure, then it should be present in the material which washed straight through the DEAE-cellulose column. Consequently, this material was re-chromatographed on a CM-cellulose column and assayed for stimulatory activity. Again no fraction was found which will stimulate the activity of either RNA polymerase A or B in vitro using double-stranded rabbit liver DNA as a template (Fig. 35).

In the earlier experiments with nuclei it was observed that uterine cytoplasm from animals receiving oestrogen 30 min before death could be fractionated on columns of CM-cellulose to produce a fraction capable of stimulating RNA polymerase A activity (Fig. 31). When this experiment is repeated using soluble uterine RNA polymerases and double-stranded DNA in the assay mixture, no comparable effect could be detected for either RNA polymerase. There are some reports

of specific mammalian protein factors which are able to enhance RNA polymerase B activity *in vitro* (Seifart *et al.*, 1973) but so far there have been no reports of any factors which are specific for RNA polymerase A. Thus, it seems probable that some of the other components of the chromatin are involved in the stimulation of rRNA synthesis in response to oestradiol in the uterus. These additional components could be either proteins or nucleic acids and presumably are present in the isolated nuclei but absent from the *in vitro* system.

An alternative possibility is that the template employed in all the studies on solubilised RNA polymerases, namely 'native' double-stranded rabbit liver DNA, bears little relation to the structure of DNA as it exists in uterine chromatin *in vivo*. There is some evidence that mammalian RNA polymerases exhibit template specificity. Meilhac & Chambon (1973) have demonstrated the presence of different sites on calf thymus DNA where RNA polymerases A, B and *E. coli* RNA polymerases will bind. Similarly, Butterworth *et al.* (1971) suggested that rat liver RNA polymerase B binds to different sites on rat liver chromatin than does *Micrococcus lysodeikticus* RNA polymerase.

Most of the studies performed on solubilised RNA polymerases have utilised either synthetic templates, viral DNAs of known structure or poorly preserved 'natural' templates. Owing to the complexity of the eukaryotic genome, it is very difficult to isolate preparations of DNA with a molecular weight of greater than 2×10^7 on account of the shearing of these large molecules. In addition, most isolation procedures also introduce single-strand breaks or 'nicks' into the template where RNA synthesis can be initiated non-specifically (Dausse *et al.*, 1972).

More recently, Flint *et al.* (1974) have studied the effect of DNA structure and template integrity on the specificity of RNA polymerases. Treatment of the DNA with pancreatic DNase gives rise

to single-stranded 'nicks' where RNA synthesis can be initiated by both AI and B forms of rat liver RNA polymerases. However, this effect is balanced by an inhibition of RNA synthesis because of the production of low molecular weight DNA by double-strand scissions which may be correlated with the formation of non-productive complexes between the RNA polymerases and the ends of DNA molecules. DNA with a molecular weight of less than 5×10^6 may be inhibitory as a template.

From the results presented here, oestradiol has been shown to be capable of stimulating both RNA polymerase A and B in nuclei isolated from immature rabbit uteri but has no stimulating effect on the enzymes once they have been solubilised from the tissue. Thus, it appears that oestradiol does not exert any effect on the uterine RNA polymerases *per se*. This possibility cannot be entirely eliminated until extensive work has been carried out on the subunit structure of the enzymes as well as ensuring that the template employed *in vitro* will allow a meaningful transcription by permitting each enzyme to initiate RNA synthesis only at the sites specific for each. However, it seems more probable that the observed changes in RNA synthesis in the uterus responding to oestradiol reflects subtle changes in the structure and composition of the chromatin itself.

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