THE EFFECT OF OESTRADIOL-17β ON RNA SYNTHESIS IN THE UTERUS OF THE IMMATURE RAT

by

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ABBREVIATIONS

Abbreviations are, in general, as recommended in the Instructions to Authors of the Biochemical Journal, 1972. Enzyme Commission numbers are not used since many of the enzymes referred to are incompletely characterized and are inadequately described by the numbering system. A further contradiction is that d.p.m. (disintegrations/min) are here referred to as dpm. Additional abbreviations used are:-

Bisacrylamide	NN'-methylene bisacrylamide
Cyclic AMP	Adenosine 3':5' cyclic monophosphate
DES	diethylstilboestrol
rDNA	DNA containing the genes for 28S and 18S RNA
FSH	follicle stimulating hormone
LH	luteinizing hormone
MAK	methylated albumin kieselguhr
RER	rough endoplasmic reticulum
HnRNA	heterogeneous nuclear RNA
pre-rRNA	precursors of ribosomal RNA
pre-tRNA	precursors of transfer RNA
28SA RNA	28S associated RNA
SDS	sodium dodecyl sulphate

CONTENTS

MATERIALS AND METHODS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

1. Control in Living Systems.

A central problem in molecular biology is the manner in which a cell can respond to a changing external environment by changing the rate of synthesis and the activity of specific proteins. During the last decade, extensive investigations principally with micro-organisms, have resulted in considerable achievements. The theories postulated in 1961 by Jacob and Monod to account for repression and induction of protein synthesis have found support in the isolation of repressor proteins (Gilbert & Müller-Hill, 1966; Ptashne, 1967) and more recently in the development of *in vitro* systems responding to the regulator substances (de Crombrugghe *et al.*, 1971).

Conclusive evidence, in support of one of the several theories which have been put forward to account for feed-back and related control mechanisms in bacteria and other organisms, has not yet emerged (Atkinson, 1966; Koshland and Neet, 1968; Umbarger, 1969) although it is clear that in many of the synthetic pathways of biological compounds, products formed late in a reaction chain, control the activities of enzymes catalysing earlier steps. Thus, the accumulation of end products is used by the cell to inhibit their further synthesis.

In multicellular organisms, the term "control" must involve far more than is implied above. In higher organisms considerations must be extended to the mechanism of differentiation, organ development, homeostasis, morphogenesis and maturation. The inter-relationship of cells within a population and the manner in which the

productions of one cell type might influence another must be investigated. Finally, an understanding must be reached of the co-ordination of control mechanisms throughout the whole organism.

Higher animals possess two obvious systems by which one tissue might influence the activity of another; these are the nervous system and the hormones. Nerves transmit information in the form of an electrical impulse, a process which is incompletely understood especially at the level of the brain. Here, nervous activity is co-ordinated and, in highly evolved animals, a memory has developed to allow the organism to draw on previous experience of the external environment in the control of its internal environment.

By definition, a hormone is a chemical substance which is produced in one part of the body, enters the circulation and is carried to distant organs and tissues to modify their structure and function (Bayliss & Starling, 1902). The action of hormones is not fully understood at the molecular level but, by observing the physiological actions of a spectrum of these compounds, it is immediately clear that they are intimately concerned with the control mechanisms of higher animals. Thus, the metamorphosis and post-embryonic differentiation of amphibian larvae is strongly influenced by thyroid hormone (Frieden & Just, 1970). Ecdysone, an invertebrate steroid hormone, governs insect metamorphosis (Lang, 1971) and the action of insulin and glucagon are involved in the homeostatic control of blood sugar levels (Hales, 1967).

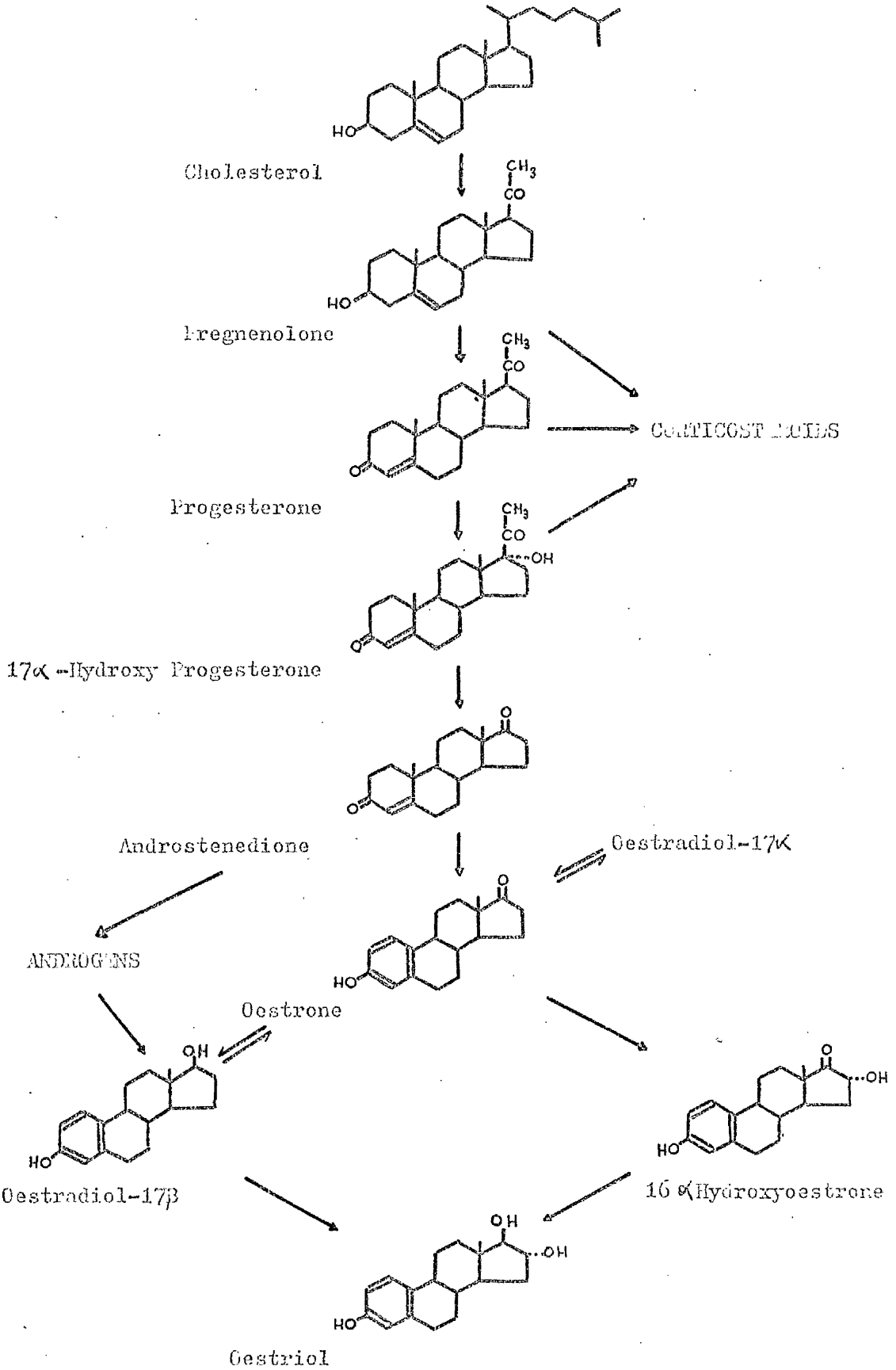
The maturation of higher animals is controlled by the sex hormones; the androgens and the oestrogens in male and female animals respectively. The mode of action of oestrogens at a molecular level is the subject of this thesis.

2. The Synthesis and Metabolism of Oestrogens.

Present knowledge of oestrogen synthesis and metabolism is the product of many laboratories and is the subject of many reviews and texts (Topják & Cornforth, 1960; Breuer, 1962; McKerns, 1967; Heftmann, 1970). The steroidal hormones, in common with many other steroid and terpenoid compounds, are synthesized from acetate. In brief, three acetate moieties, joined and enzymatically modified, form an isopentenyl unit. Repeated condensation then gives rise to many terpenoid compounds; among them squalene. This C30 isoprenoid cyclizes to produce the cyclopentanophenanthrene steroidal skeleton in the form of lanosterol and this, via several intermediates, gives rise to cholesterol. Cholesterol is the parent compound of all steroid hormones and the major conversions by which it is thought to give rise to oestrogens are shown in Fig.I.

Oestradiol-17β and oestrone are freely interconvertable in many species but the equilibrium is displaced in favour of oestrone. However, the biologically active component appears to be oestradiol-17β. Flesher et al. (1960) reported that the only radioactive substance found in the uterus following the administration of tritiated oestradiol-17β was free oestradiol-17β whereas liver contained a mixture of derivatives. Oestriol is a major end product of oestrogen metabolism in man, dog and rat and is produced mainly via 16α hydroxy oestrone. Oestradiol and oestrone, but not oestriol, are made continuously by the ovaries in small amounts but production increases considerably in the maturing follicle so that in women, excretion of 15μg/day in the first week of the menstrual cycle rises to 50μg/day at the time of ovulation. For the first three months of pregnancy the ovaries furnish most of the oestrogens but subsequently, the

FIGURE I


Cholesterol
Pregnenolone
CORTICOSTEROIDS
Progesterone
17α-Hydroxy Progesterone
Androstenedione
Oestradiol-17α
ANDROGENS
Oestrone
Oestradiol-17β
16 α Hydroxyoestrone
Oestriol

foeto-placental takes over the role. By the end of pregnancy a woman's urinary excretion is in excess of 30mg per day. The adrenals produce small amounts of oestrogens and in some animals, notably stallions, the testes produce large quantities.

The secretion of oestrogen is under the control of gonadotrophins secreted by the pituitary adenohypophysis. Follicle-stimulating hormone (FSH) brings about initiation of follicle growth and further development and increased secretion is induced by the combined effects of FSH and luteinizing hormone (LH) (Fevold, 1944). As well as being under the control of gonadotrophins, oestrogens themselves control the pituitary secretions so that high levels inhibit the release of FSH and LH. This interplay is responsible for the rhythmic uterine development in the menstrual cycle. In pregnancy, the role of the pituitary is largely replaced by the placenta which produces large amounts of chorionic gonadotrophin.

Oestrogen turnover is rapid and incompletely understood. The liver is responsible for much degradation to produce un-identified products and it is also the key site at which oestrogens are conjugated with sulphuric and glucuronic acid. If labelled oestrone or oestradiol is injected into women, about half passes into the enterohepatic circulation and is metabolised in this way, whilst the remainder goes through the kidneys and is eliminated in the urine.

3. The Physiological Response to Oestrogens.

Oestrogens are responsible for the development of the female reproductive organs and for the development of the secondary sex characteristics which, in women, include the texture of the skin,

the texture and distribution of hair, character of the voice and distribution of body fat. Ovariectomy results in an atrophy of the reproductive organs which can be reversed by oestrogen therapy.

Administration of oestrogens to an immature or ovariectomized animal, or to an animal at a preovulatory stage, induces proliferation of the epithelium of the vagina, cervix, endometrium and fallopian tubes. In the latter it also induces contraction and motility which promotes the transport of the ova. The mucosa of the endometrium grows and receives an increased blood supply. The myometrium hypertrophies and in some species undergoes rhythmic contractions. In rats, rabbits and guinea pigs water imbibition by the uterus is an early consequence of oestrogen treatment and in rodents, cornification of the vaginal epithelium forms the basis of an oestrogen bioassay. Primates accumulate glycogen and mucopolysaccharide in the vagina when they respond to oestradiol whilst in rabbits oestriol causes an increase in the size of the vagina. If given in sufficient amounts, oestrogens will induce mating in mice but simultaneous administration of oestradiol and progesterone is necessary to produce the same response in guinea pigs. In man, however, mating is mainly under nervous control. It is clear from the above that there are marked species variations in the physiological response to the female sex hormones.

4. <u>The Biochemistry of Target Tissue Response to Oestrogens.</u>

A hormone is carried in the blood system from its site of synthesis to its target tissue, where it is accumulated. Entry into the target cell must then set in motion a sequence of biochemical events which will ultimately result in the observed physiological

changes. This section sets out to review what is known of the biochemistry of oestrogen action and, where relevant, to compare and contrast its response to that of other hormones.

4. 1. Transport to the Target Organs.

Oestrogens associate spontaneously with serum proteins to form reversible complexes. Serum albumin is well known for its ability to associate with many compounds including steroid hormones (Bennhold, 1966). The binding affinity of oestrogen is low and the amount bound would be insignificant were it not for the high concentration of albumin in the blood. Conversely, Tavernetti et al. (1967) showed that there is also a protein in the β globulin fraction which, although present in small amounts, binds oestrogens with high affinity. Competition experiments by Murphy (1968) and Mercier-Bodard & Baulieu (1968) have shown that this protein is probably identical to that which binds to testosterone; it can, however, be separated from the corticosteroid binding globulin (Gueriguian & Pearlman, 1968). The oestrogen binding globulin does not appear to be present in all animals. Murphy (1968), DeMoor et al. (1969) and Diamond et al. (1969) reported its presence in man, two other species of primate, cows, pregnant guinea pigs and frogs. They also found that the level rose in man during portal cirrhosis and pregnancy. No activity was found in rat, dog, rabbit, duck and seven species of ruminant.

It has been assumed that the binding of steroid hormones in the plasma is necessary to effect their solubilization but in fact, levels at which the hormones occur in serum is far below their solubility in water. It seems likely that this reversible binding

is of importance in the control of hormone distribution. The hormone-protein complex is inactive but the fact that it readily dissociates provides a means whereby active hormone can be delivered to the target cells when it is required.

4. 2. Entry of Oestradiol-17β into Target Cells.

Little appears to be known about the passage of oestrogens into target tissues. The fact that these tissues accumulate hormone does not in itself imply an active transport mechanism. This accumulation is due to the presence, in the target cell cytoplasm, of receptor proteins. Szego & Davis (1967) have shown that injection of oestradiol results in very rapid changes in uterine cyclic AMP (adenosine 3':5' cyclic monophosphate) levels. Most work in this field indicates that these increases in cyclic AMP turnover are related to the oestrogen-induced changes in uterine permiability. However, since Szego & Davis (1967) report that there is a fall in the level of uterine cyclic AMP within 15s of oestradiol administration, it is conceivable that these changes are associated with the entry of oestradiol into the cells.

4. 3. Target Cell Oestrogen Receptor Proteins.

In the last decade, numerous studies pioneered by the work of Jensen, have revealed that there exists, in oestrogen-sensitive and other hormonal target tissues, specific receptor proteins which seem to be responsible for the accumulation of hormone in the cytoplasm and for its transport to the nucleus.

4. 3. 1. The Cytoplasmic and Nuclear Receptor Proteins.

Oestradiol is rapidly taken up by the uterus, vagina and adenohypophysis (Jensen & Jacobson, 1962), by the mammary gland (Puca & Bresciani, 1969) and by the hypothalamus (Eisenfeld & Axelrod, 1966). Autoradiographical studies by Stumpf & Roth (1966) and cell fractionations by Noteboom & Gorski (1965) revealed that although much of the accumulated hormone was to be found in the nucleus, a substantial proportion, 20-30%, was present in the cytoplasm. The binding of hormone was abolished by treatment with proteolytic enzymes though not by nuclease action (Toft & Gorski, 1966) and was destroyed by heating at 80° for 10min (Talwar et al., 1964) or by sulphydryl blocking reagents (Jensen et al., 1967). These observations, together with the binding specificity, indicated that the receptors were proteins.

The specificity of oestradiol binding in the cytoplasm of the uterus was impressive; especially when compared with the broad spectrum of ligands which bind to serum albumin, the protein chiefly responsible for transport in the blood. As well as being specific to target tissues, the receptor did not bind the non-oestrogenic corticosteroids or testosterone (Talwar et al., 1964; Toft & Gorski, 1966). The receptors also showed considerable stereospecificity in that the binding of tritiated oestradiol-17β was inhibited by an excess of non-radioactive hormone but not by its 17-epimer, oestradiol -17α (Noteboom & Gorski, 1965). The non-steroidal synthetic oestrogen, diethylstilboestrol (DES), would compete with oestradiol for binding sites (Toft & Gorski, 1966) and the anti oestrogenic compound, U-11,100 [(1-(2-(p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthylphenoxy-ethyl)pyrolidine, HCl], inhibited binding (Puca & Bresciani, 1968).

Further evidence for the protein nature of the uterine cytoplasmic receptor came from fractionation studies. Talwar et al. (1964) showed that activity was associated with the first 280nm peak eluted from Sephadex G100 and Toft & Gorski (1966) sedimented the 105,000g supernatant through sucrose gradients and obtained a sedimentation value of 9.5S for the receptor. By comparison with the sedimentation coefficient of yeast alcohol dehydrogenase, this value has since been found to be nearer 8S (Erdos, 1968; Rochefort & Baulieu, 1968) but a precise value will require the pure protein.

The nuclear receptor proved more resistant to solubilization but Jungblut & Jensen (1967) found that a receptor sedimenting at 5S could be extracted from uterine nuclei by high salt. Further characterization by Maurer & Chalkley (1967) and Puca & Bresciani (1968) established that it was proteinaceous, had a specificity similar to the cytosol receptor and was best extracted in 0.4M KCl at pH 8.5. The 5S complex was initially thought to be an artifact of the high salt concentrations used in its extraction (Korenman & Rao, 1968) but Harris (1971) found that it could be released from nuclei by deoxyribonuclease in the absence of magnesium ions and KCl. When KCl was not present, there was a strong tendency towards aggregation.

When the cytosol 8S receptor was treated with NaCl or KCl at concentrations of 0.2M or higher, it was found to break down and subunits were formed sedimenting in sucrose-KCl gradients at 4S (Erdos, 1968; Jensen et al., 1969a). The situation was confused by disagreement over precise sedimentation values of these particles as some workers (Erdos, 1968; Beziat et al., 1970) considered the dissociated cytosol receptor to have a sedimentation value of 5S. In the interests of uniformity this particle is identified by a

sedimentation value of 4S throughout this review.

The nuclear receptor and dissociated cytosol receptor were clearly different. Apart from having different sedimentation values (Jensen et al., 1969b), they differed in their recombination characteristics. Thus, the 4S receptor could reform into the 8S receptor on removal of KCl (Erdos, 1968) but the 5S receptor had no such reassociation properties. Furthermore, the 8S or 4S oestradiol-complex could be made in vitro by adding oestradiol to the uterine high speed supernatant (Toft et al., 1967). No oestradiol-5S complex was formed, however, by addition of oestradiol to uterine nuclei, or an extract of nuclei, except in the presence of the cytosol receptor (Jensen et al., 1968).

4. 3. 2. The Relationship between the Nuclear and Cytoplasmic Receptor Proteins.

Clearly there was some relationship between the different receptors and Jensen et al. (1968) and Shyamala & Gorski (1969) reported a temperature-dependent transfer of bound oestradiol from the cytosol receptor to the nuclear receptor. At 0°, most of the retained oestrogen was bound in the cytoplasm but, if the temperature was raised to 37°, bound hormone was lost from the cytosol and was irreversibly transferred to the nuclear binding site.

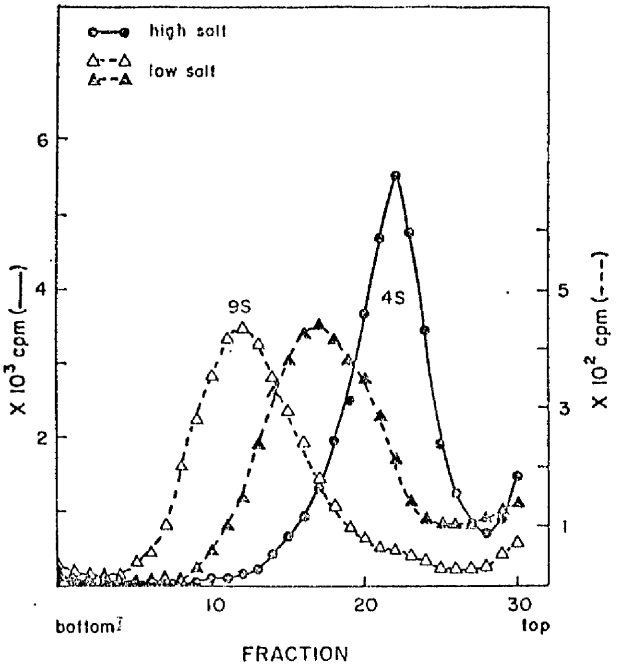
From the early studies of Erdos (1968), it appeared that the 8S cytosol receptor was an aggregate of two 4S receptors. More recent work, however, showed it to be heterogeneous. If the 8S receptor was dissociated on a KCl-sucrose gradient, the front fractions of the 4S peak would reassociate to the 8S receptor but the trailing fractions combined to a form of intermediate size (See

Fig. II). From these and similar results obtained using gel filtration, Vonderhaar et al. (1970) suggested that the cytosol receptor was composed of a 4S oestrophilic A subunit and a non-oestrogenic B subunit which would itself dissociate. The absence of part of the B subunit from the rear fractions of the 4S peak resulted in the observed incomplete reassociation.

A number of treatments modify the 4S receptor so that it can no longer reassociate to form the 8S complex. The stabilization of the 4S subunit with calcium ions has allowed DeSombre et al. (1969) to effect a 5000-fold purification and to estimate a molecular weight of 75,000 and an iso-electric point of 6.4. The fact that respective values of 200,000 and 5.8 were obtained for the 8S complex also pointed to heterogeneity. Modified 4S subunits, unable to reassociate into the 8S moiety, have also been prepared by ageing the 4S receptor in 0.3M KCl for 4h (Vonderhaar et al., 1970) and by incubating the 8S receptor in the presence of oestradiol at 37° (Vonderhaar et al., 1970; Brecher et al., 1970). It was further shown by Brecher et al. (1970) that if the temperature-dependent conversion was carried out at pH 8.0 rather than at pH 7.0, the resulting oestrophilic component sedimented at 5S. Thus, in sedimentation constant and in reassociation characteristics, the product resembled the nuclear receptor. The complex could still enter the nuclei and was only made in the presence of oestradiol.

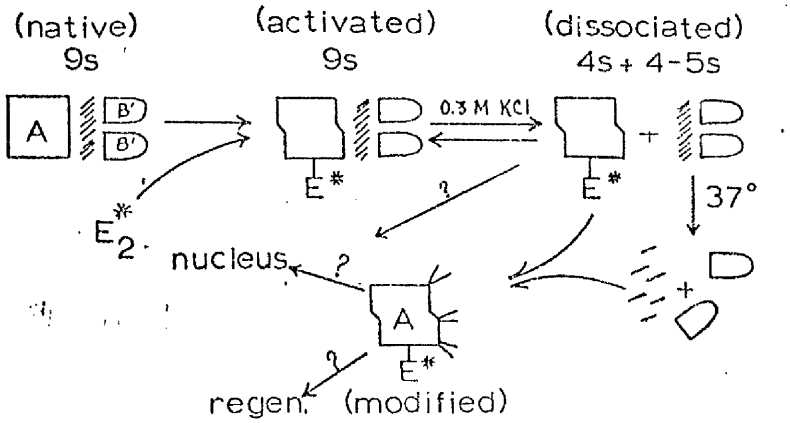
Perhaps more relevant to the in vivo situation were the results obtained using a polyvinyl (N-phenylenemaleimide) resin onto which was coupled the 17∝ propyl mercaptan derivative of oestradiol (Vonderhaar & Mueller, 1969). The 8S oestrogen receptor absorbed

Figure II



The reassociation of receptor subunits in a low ionic environment. The 105,000 × g supernatant prepared in 0·01 M Tris-0·0015 M EDTA buffer was combined with 0·3 M KCl and 10^{-3} μg ^{3}H-estradiol per ml at 0° for 15 min. 0·3 ml aliquots were layered on 4·3 ml gradients of 5–20% sucrose prepared in Tris-EDTA containing 0·3 M KCl. After centrifuging for 12 hr at 38,000 rev/min (2°) in a SW 39 rotor, the tubes were pierced and fractions collected by gravity flow. The equivalent fractions from these separate gradients were pooled and 150 μl aliquots of fractions (20 (△– –△) and 23 (▲– –▲) were diluted with 300 μl and 250 μl of Tris-EDTA buffer respectively. 0·3 ml aliquots of each were then layered on 4·3 ml gradients of 5–20% sucrose made in the Tris-EDTA buffer and re-centrifuged for 12 hr at 38,000 rev/min at 2° in a SW 39 rotor. Fractions were collected by gravity flow and analyzed for ^{3}H-estradiol. Reprinted from Vonderhaar *et al.* (1970*b*).

Figure III



A diagram of the subunit character of the cytosol receptors for estradiol. The native receptor (8–9S) becomes labellized for dissociation (activated state) on binding estradiol to the estrophilic subunit (A). Treatment with 0·3 M KCl dissociates native or activated complexes to yield the 4S estrophilic subunit (A) and a 4–5S non-estrophilic unit (B). With aging in 0·3 M KCl at 0° or incubation briefly at 37° in the absence of KCl the B units dissociate further to yield B′ and C units; the latter react with A units to modify their ability to re-associate with native B units.

Fig. III is taken from Mueller (1971)

onto this resin and treatment with 0.3M KCl resulted in the dissociation of the non-oestrogen binding subunits. If this resin extract was then added to more freshly dissociated 8S receptor, reassociation no longer occurred. The active substance bringing about this change could be dialysed and extracted with ether (Mueller, 1971).

It appears then that oestradiol, on entry into the cell, associates spontaneously with a cytoplasmic 8S receptor protein which consists of a 4S oestrogen-binding subunit and a second subunit which is composed of more than one entity. This association allows a temperature-dependent transfer of the hormone to the nucleus, where it is found associated with a 5S receptor. The diagram of the above process in Fig. III is taken from Mueller (1971). It seems likely that the 5S receptor is the modified 4S subunit but whether, in vivo, this modification occurs before entry into the nucleus is unclear. It is known that the entry of hormone into the nucleus is accompanied by depletion of cytosol receptor (Jensen et al., 1968).

The extent to which all these events are involved in the primary action of oestradiol is unclear. Association with the cytosol receptors is not in itself an early step in the uterine response to oestradiol. Their formation is not inhibited by puromycin or actinomycin D, both of which block overall oestrogenic response (Jensen, 1965). It seems likely that the cytosol receptor functions in the delivery of the hormonal regulatory message to the nucleus. Alternatively, Jensen et al. (1971) has suggested that the hormone could merely function in the transport of the protein into the nucleus and that once there, the protein functions in a manner analogous to the microbial sigma factors.

In conclusion to this section it should be mentioned that

oestradiol is far from being the only hormone which binds to specific cytoplasmic and nuclear receptors. Evidence is accumulating that a similar system to that outlined above exists for dihydrotestosterone (Mainwaring, 1969; Baulieu & Jung, 1970), progesterone (O'Malley et al., 1970), corticosteroids (Beato et al., 1970; Baxter & Tomkins, 1970) and aldosterone (Swaneck et al., 1970).

4. 4. The Binding of Oestradiol to Chromatin.

Teng & Hamilton (1968) reported that within 2min of administration of radioactive oestradiol to ovariectomized rats, the hormone had entered the uterine cell, penetrated the nucleus and bound to the chromatin. Binding, as measured by resistance to dialysis at 0^{o}, was maximal after 8h.

The function of the receptor proteins is not restricted to transport of hormone. Steggles et al. (1971a) showed that they are also involved in the binding of hormone to target cell chromatin. Thus, tritiated oestradiol, after formation of its receptor complex by incubation with uterine cytosol, was bound to uterine chromatin far more than it was to liver, spleen or lung chromatin. Similarly, dihydrotestosterone, complexed to the receptor protein from prostate gland cytoplasm, only bound extensively to the chromatin prepared from the prostate gland or the testes. Furthermore, complexes between dihydrotestosterone or oestradiol with non-target organ cytosols would not bind well to any chromatin. Similar findings and additional insights into the binding process have been provided by studies on the association of the progesterone-oviduct cytosol complex with oviduct chromatin (Spelsberg et al., 1971a,b; Steggles et al., 1971b). In this instance, the use of the target organ cytosol and the target

organ chromatin was again essential for extensive binding and it was also shown that the binding of receptor protein-hormone complex was ten times that of hormone alone. The binding characteristics of the chromatin were not brought about by the hormone but were the property of the tissue. Oviducts from young, untreated animals showed the same specificity.

Another interesting finding with this system was that the chromatin could be dissociated and reconstituted without loss of binding but, if it was reconstituted in the absence of acidic proteins, binding was greatly reduced. These observations were extended by O'Malley et al. (1972). They found that if dissociated oviduct chromatin was reassociated, replacing the native histone with non-target tissue histones, the reconstituted product bound the receptor in a manner similar to native chromatin. If, however, the acidic proteins were replaced by erythrocyte acidic proteins, the hormone-receptor complex was no longer bound. Conversely, acidic proteins from oviduct, inserted onto erythrocyte DNA, bestowed binding capacity on the hybrid chromatin. Thus, the inherent acceptor capacity of target tissue chromatin for the hormone receptor of that tissue could be transferred to a non-target DNA by transfer of the acidic protein fraction. The above workers went on to demonstrate that a specific fraction of the oviduct acidic protein contained the acceptor molecules.

On the basis of the earlier work of Steggles et al. (1971a) it seems likely that the above findings with progesterone will be relevant to the binding of oestrogen to chromatin. King et al. (1969) reported the isolation of a chromatin associated acidic protein from rat mammary tumour and rat liver, that binds tritiated

oestradiol *in vivo* and Alberga *et al.* (1971) have recently described a hon-histone chromatin protein with a very high affinity for oestrogen.

The basic entities of chromatin may also be important in the binding process. It is known that the receptor complex shows a strong tendency to interact with basic proteins and peptides, hence its precipitation by protamine, histones or polylysine (King *et al.*, 1969) and its reaction with ribonuclease (Jensen *et al.*, 1969b).

4. 5. The Effect of Oestrogen on the Chemical Composition of Chromatin.

Teng & Hamilton (1968) studied the effect of oestrogen on the chemical composition of the uterine chromatin of ovariectomized rats. They found that the RNA to DNA ratio increased 17% within 15 min of treatment and by 2-4h the protein to DNA ratio had increased 35%. During this time, however, the level of histones fell and it was interesting that after 8h of hormone treatment, when the template activity of the chromatin and the protein to DNA ratio were maximal, histone levels were reduced by 20%.

Obviously, the likeliest candidates for the increased nuclear protein levels were the acidic proteins. Teng & Hamilton (1969) investigated this possibility and found that within 8h of oestrogen treatment the incorporation of precursor into these uterine proteins was increased by 30%. Liver acidic nuclear proteins were unaffected. These workers also investigated the inhibition of uterine DNA-dependent RNA synthesis by histones and reported that all fractions, particularly the arginine rich histones, were potent inhibitors of RNA synthesis. The acidic proteins were not inhibitors

and they reversed the inhibition by histones. Teng & Hamilton (1969) concluded that a major feature of early oestrogen action, in the uterus of ovariectomized rats, was a stimulation in the synthesis of acidic proteins which reversed the effect of histones on transcription by RNA polymerase. It should be noted, however, that other results of these workers and others, indicate that RNA synthesis is stimulated in uteri much earlier than at 8h after hormone administration and it seems unlikely that the above mechanism is solely responsible for these changes. Furthermore, the inhibition of RNA synthesis by histones *in vitro* may be an artifact due to the precipitation of template (Hoare & Johns, 1971).

Other workers have also reported oestrogen-induced changes in nuclear acidic proteins. Smith *et al.* (1970) have reported increases in the synthesis in mouse uterine epithelium responding to oestradiol. Similarly, Spelsberg *et al.* (1971b) have shown that the levels of chromatin associated acidic proteins and RNA, in chick oviduct responding to DES, were dramatically increased over the first 8 days of treatment. Perhaps more relevant to early oestrogen action was the discovery by Barker (1971) of an acidic protein associated with uterine FIII histone. It was synthesized in increased amounts in ovariectomized rats which had received a 30min pulse of precursors 15min after administration of oestradiol. Concomitantly with this increase, histone levels were decreased and a histone complexing function was again postulated for the acidic protein. Teng & Hamilton (1970) have separated uterine nuclear acidic proteins on polyacrylamide gels. They also observed an oestrogen-stimulated synthesis of a specific fraction of their preparation. The work of Chytil & Spelsberg (1971) indicated that there were definite tissue dependent differences in nuclear acidic

proteins. They found that an antibody to the non-histone protein-DNA complexes of diethylstilboestrol treated chick oviduct did not cross react with similar preparations from liver, heart or spleen.

A possibility, as yet little investigated, is that hormones might induce changes in histone structure and in this way influence chromatin template activity. Cyclic AMP, but not testosterone, was found by Reddi et al. (1971) to stimulate phosphorylation of certain testes histones. Burdon & Pearce (1971), however, have indicated that certain steroid hormones, including testosterone and oestradiol, could influence the effect of adenine nucleotides on histone modification by inhibiting the conversion of cyclic AMP to AMP.

It has already been mentioned that the oestrogen-receptor complex has a high affinity for polyamines, and studies have revealed that the level of these compounds, and the activities of the enzymes orthinine decarboxylase and S-adenosyl methionine decarboxylase, are increased in oestrogen-stimulated rat uterus (Russell & Taylor, 1971; Cohen et al., 1970; Kaye et al., 1971).

4. 6. Effect of Oestrogen on the Template Activity of Chromatin.

It has been seen that oestrogen enters the nucleus and binds to chromatin, so it is pertinent to ask whether the association increases the transcription of DNA and how such increases might be brought about. Barker & Warren (1966) and Warren & Barker (1967) showed that the template activity of chromatin was increased by the administration of oestradiol to ovariectomized rats. Increases also occurred concomitantly with rising oestrogen levels throughout the oestrous cycle of golden hamsters. Similar results have been presented by Teng & Hamilton (1968) but all of these workers used bacterial DNA polymerase rather than native enzyme.

The dangers of such an approach were revealed by Dati & Maurer (1971). They used a similar system and recorded an oestrogen-induced increase in the chromatin template activity of rat uterus. In the hormone treated mouse uterus, however, the template activity was reduced.

Church & McCarthy (1970), working with chromatin isolated from rabbit endometrium and native enzyme, recorded increases in template activity at earlier times than the above workers. A stimulation of 200% of control levels was recorded 10min after oestradiol administration, rising to 500% within 2h of hormone treatment. Addition of E. coli RNA polymerase to this system affected the response of neither the controls nor the hormone treated animals. On this basis, they concluded that the stimulation was due to increased availability of sites for transcription rather than increased polymerase activity. Further studies separating these two parameters would be beneficial.

4. 7. The Effect of Oestrogen on DNA-Dependent RNA Polymerase.

As indicated above, when the chromatin and RNA polymerase are both derived from the target tissue, hormone induced increases in template activity could reflect increased availability of transcription sites or increased enzyme activity or both. That the latter is stimulated as a result of oestradiol action is well documented.

Gorski (1964) first characterized the enzyme from uterine nuclei and showed that rats, pre-treated with oestradiol 1-4h earlier, exhibited greater activity than controls. At this time Widnell & Tata, (1964, 1966a) investigating the RNA polymerase of rat liver nuclei, found an activity stimulated by magnesium ions which appeared to catalyse the synthesis of rRNA in the nucleoli. In the presence

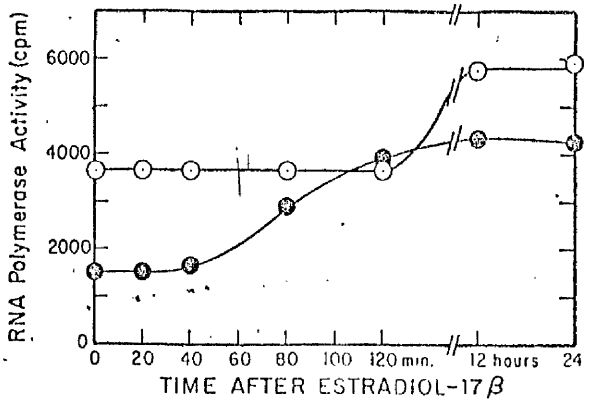
of manganese ions and 0.4M ammonium sulphate a more DNA-like RNA was made in the nucleoplasm. Hamilton et al. (1965, 1968) characterized similar activities in the uterus of ovariectomized rat.

Ovariectomy was followed by decreases in both of these enzyme activities but the administration of 10µg oestradiol to rats ovariectomized 3 weeks previously resulted in the stimulation of the magnesium ion activated reaction after 1h. This rose to 2-2.5 times the control levels 2-12h after treatment and was maintained at this level for 12-24h. No change in the manganese/high salt-activated enzyme was observed in the first 12h following hormone treatment after which 1.5-1.6-fold increases were observed (See Fig. IV).

Nicolette & Mueller (1966) showed that oestrogen-stimulated levels of RNA polymerase were maintained if the stimulated uterus was incubated in tissue culture medium but that the increased levels, though not the control levels, were highly sensitive to the presence of cycloheximide in the medium. This, and the similar observations by Noteboom & Gorski (1963) and Gorski et al. (1965) using puromycin in vivo, suggested that increased RNA polymerase activity was dependent on protein synthesis.

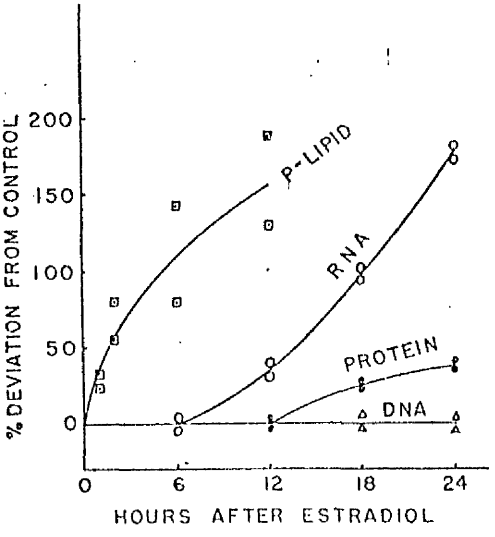
The findings of Hough et al. (1970) were somewhat at variance with these conclusions. They reported that an RNA polymerase, extracted from heifer endometrium and since shown to be associated with the nucleoli (Arnaud et al., 1971a), was stimulated in vitro by added oestradiol-4S receptor complex. (The receptor is 5S in their hands). In addition, when tritiated oestradiol was used to form the complex, the enzyme became radioactive and Arnaud et al. (1971b) postulated that the hormone-receptor complex could be functioning in a manner analogous to the microbial sigma factors. These workers

Figure IV



Time course for the effect of administration of oestradiol-17β on RNA polymerase in nuclei isolated from the uterus of the ovariectomized rat. All animals received 10 μg of the hormone at zero time. At the time indicated, the animals were killed, and the nuclei of the uteri were isolated and assayed for RNA polymerase activity in the presence of 5 mM Mg^{2+} ions or of 4 mM Mn^{2+} ions and 0·4 M ammonium sulphate, as previously described. ●——●, Activity of Mg^{2+}-activated RNA polymerase; ○——○, activity of Mn^{2+}-$(NH_4)_2SO_4$-activated RNA polymerase. The figure is taken from Hamilton, Widnell & Tata (1968).

Figure V



Alterations in the composition of rat uteri following a single dose of estradiol (10 μg) injected at zero time. DNA was measured in μmoles of thymine/uterus. RNA was measured at μmoles of uridine and calculated as the ratio of uridine to thymine. Phospholipid was measured as μmoles of ethanolamine phosphate. All data are expressed as the per cent deviation from the control during the first 24 hr after hormone treatment. Reprinted from Aizawa and Mueller (1961).

also indicated that oestradiol might serve a second function in this process. They reported that the hormone-receptor complex was phosphorylated by a myometrium kinase which was stimulated by cyclic AMP. The stimulation of RNA polymerase by the complex was increased after phosphorylation and oestradiol appeared to influence this system by stimulating adenyl cyclase.

Barry & Gorski (1971) investigated whether precursor was incorporated into the 3' end or internal positions of newly synthesized RNA of rat uterus and found that oestrogen only stimulated the latter parameter. On the basis of their findings they also concluded that the hormone increased the activity of RNA polymerase rather than the absolute amount of the enzyme. They suggested that the continued synthesis of protein was necessary for the activation because some protein factor was involved in the process.

Increases in RNA polymerase activity have also been recorded in response to thyroid hormone (Tata & Widnell, 1966), testosterone (Pegg & Korner, 1965), growth hormone (Widnell & Tata, 1966b), progesterone (McGuire & O'Malley, 1968) and corticosteroids (Yu & Feigelson, 1971).

4. 8. The Effect of Oestrogen on RNA Synthesis.

One of the most clearly established biochemical effects of oestrogen is the stimulation of RNA synthesis in its target tissues. (See Fig. V). Aizawa & Mueller (1961) found that from 6h after the administration of oestradiol to rats, the measurable RNA content of the uterus increased steadily and, if the RNA synthesis was measured in terms of precursor incorporation, much earlier increases were recorded (Hamilton, 1964). However, the efforts of the many workers

studying this response over the last decade has not elucidated the role of RNA in mediating hormone control. Frequently, they have only added to the confusion. This is largely due to the fact that mammalian RNA and protein synthesis is not well understood, but some of the systems used have been incompletely characterized. Data has frequently been presented on the incorporation of precursors into nuclear, mitochondrial, ribosomal and cell sap fractions without any attempt having been made to demonstrate the integrity or the purity of the preparations. Few workers have developed RNA purifications which ensured the extraction, without degradation, of the very-high-molecular-weight RNA species, which form the bulk of the rapidly labelled RNA. Finally, in many cases, little effort appears to have been made to reduce to a minimum the many variables inherent in the injection of radioactive precursors into whole animals. The work described in the results section of this thesis sets out to obtain a better characterized system and to use modern methodology and knowledge of mammalian nucleic acid biochemistry to derive an improved understanding of oestrogen-induced changes in RNA synthesis.

4. 8. 1. The Oestrogen-Stimulated Synthesis of RNA of Possible Messenger Function.

Since the suggestion by Karlson (1963) that hormones induce the synthesis of specific messenger RNAs, numerous attempts have been made to detect the early appearance of unique RNA species in the responding cells. Direct demonstration of particular messengers has not yet been shown in these systems and a number of indirect approaches have had to be relied upon.

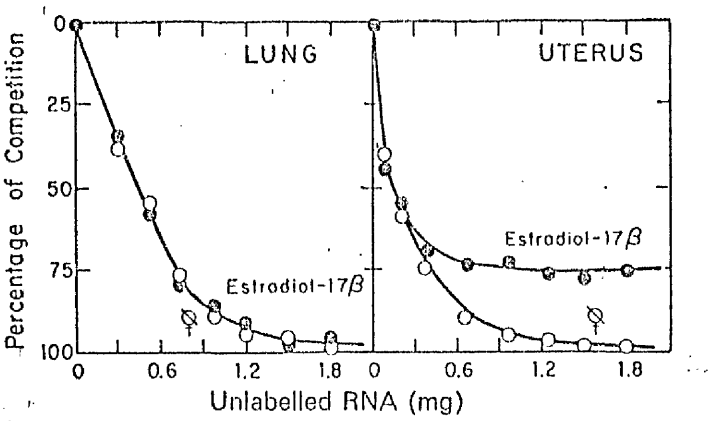
One of the most used techniques has been that of hybridization.

Church & McCarthy (1970) carried out RNA/DNA hybridization on ovariectomized rabbit tissues and demonstrated changes in the pattern of RNA transcription in the uterus and the liver, but not the lung, 1h after the administration of oestradiol. (See Fig. VI). Only 25% of the new RNA was common to both liver and uterus. O'Malley et al. (1968) measured the ability of RNA extracted from untreated chick oviduct to compete with the binding of RNA extracted from hormone treated animals. They showed that differentiation of chick oviduct subsequent to diethylstilboestrol treatment was accompanied by the production of new RNA species. Similar results have been recorded by Hahn et al. (1968) in the chick oviduct responding to oestrogen and progesterone and by Hahn et al. (1969) in domestic fowl producing hepatic yolk proteins in response to oestrogens. However, hybridization studies in mammalian systems are not totally unambiguous and the new RNA sequences need not necessarily be messengers.

Segal et al. (1965) introduced the RNA extracted from the uterus of oestrogen-stimulated rats into the uteri of unstimulated rats via an indwelling cathetre. This RNA, which was shown to be free of oestradiol, promoted a hormone-like histological response. Ribonuclease treatment of the RNA abolished the effect.

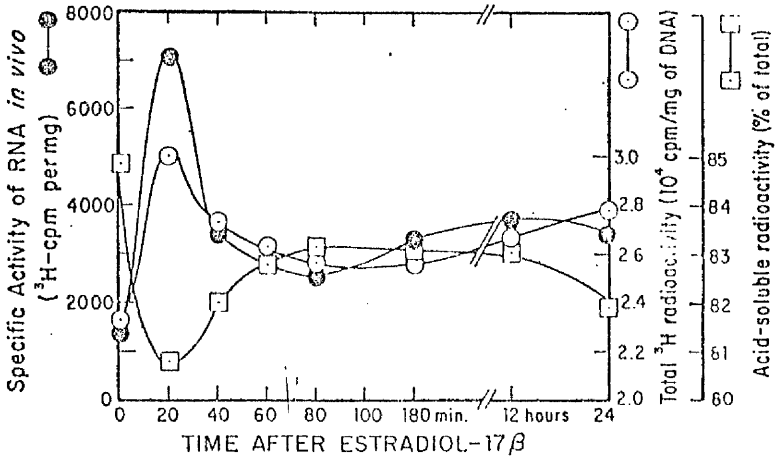
Means & Hamilton (1966b) demonstrated a 40% increase in the incorporation of precursors into the nuclear RNA of ovariectomized rats within 2min of oestradiol administration. After 20min the incorporation was increased by 500%. (See Fig. VII). This effect preceded by several hours the stimulation by oestrogen of RNA polymerase activity and ribosome formation and was significant after allowing for the increases in precursor uptake. Other workers, however, have not

Figure VI



Competition by unlabelled RNA, isolated from the lung or the uterus of ovariectomized or hormone-treated rabbit, in the reaction that hybridizes nuclear RNA with DNA. Experimental animals received 200 μg of oestradiol-17β intravenously at 1 hr before killing. Either 20 μg (lung) or 7 μg of rapidly labelled nuclear RNA was incubated with 7 μg of filter-bound DNA in the presence of increasing amounts of unlabelled lung or uterine RNA. The results are taken from Church & McCarthy (1970).

Figure VII



Early and later effects of administration of oestradiol-17β on the uptake of [^{3}H]uridine and its incorporation into nuclear RNA rapidly labelled *in vivo* in the uterus of the ovariectomized rat. All animals received intraperitoneally 100 μC of [^{3}H]uridine at 10 min before killing. Experimental animals received similarly 10 μg of the hormone at zero time, and were killed at the time indicated. Nuclei were isolated from the uteri as described by Widnell, Hamilton & Tata (1967). The figure is taken from Hamilton, Widnell & Tata (1968).

repeated these findings. Gorski & Nelson (1965) studied rapidly labelled RNA in immature rat uteri and Gorski & Nicolette (1965) followed the *in vivo* incorporation of ^{32}P into subcellular fractions of ovariectomized and immature rats. In both cases, stimulated RNA synthesis was observed 1h after oestradiol administration, but earlier effects were not recorded. Since it is known that after short pulses of radioactive precursors, virtually all labelled RNA is found in the nucleus, there is no special merit in purifying nuclei for these studies. Such purification can only increase the risk that RNA is degraded or lost by leaching during the preparative steps. In addition, it is difficult to explain the early results of Hamilton (1964) which only recorded a very slight stimulation of precursor incorporation into total uterine RNA 30min after oestradiol administration.

Further evidence that the oestrogen-induced synthesis of mRNA might precede that of other RNA species came from inhibition studies. Notides & Gorski (1966) found that the synthesis of a specific protein was induced in rat uteri within 30min of oestradiol administration, well before the stimulation of total RNA synthesis, but production of this protein was inhibited by Actinomycin D (De Angelo & Gorski, 1970).

Results to be presented in this thesis show that an early response to oestrogen is the increased synthesis in immature rat uteri of heterogeneous nuclear RNA. A messenger precursor function has been proposed for this fraction (Darnell *et al.*, 1971a).

4. 8. 2. The Oestrogen-Stimulated Synthesis of Ribosomal RNA.

The uptake of many growth promoting hormones into their

target tissues is followed by increases in the synthesis of ribosomes and polysomes and in polysome-directed polypeptide synthesis. Billing et al. (1969c) used MAK columns to separate the RNA species of immature rat uteri. After 30-60min of in vivo oestradiol treatment, they detected slight stimulation in the incorporation of precursors into pre-ribosomal and ribosomal RNA. Uptake of radioactivity continued to rise from that time, reaching 500% of control levels in the ribosomal peak 6h after hormone administration. Hamilton et al. (1968) found that increased incorporation of precursors into the microsomal fraction of ovariectomized rat uteri was stimulated from 60-90min after hormone administration. Gorski & Nicolette (1963) reported similar results with immature rats. Presumably, the slightly later stimulation in the incorporation of precursor into cytoplasmic ribosomes reflected the time taken for ribonucleoprotein to migrate out of the nucleus and into the cytoplasm.

The polyribosome concentration in the cytoplasm of the ovariectomized rat uterus increased linearly from 2-24h after hormone administration (Teng & Hamilton, 1967). It reached 400-500% of control levels after 24-36h of treatment but declined to 200-300% after 72h. Several groups of workers, including Greenman & Kenney (1964) and Teng & Hamilton (1967), have isolated cell-free ribosomal or polysomal preparations which incorporated amino acids into polypeptides in vitro. The latter workers showed that incorporation by preparations from oestrogen-treated ovariectomized rats, was increased by 30% and 100%, 2h and 8-12h respectively, after hormone administration. At later times incorporation fell off. The continual presence of oestradiol was found to be essential to maintain increased polysome levels.

The degree by which the synthesis of ribosomes was increased and the generality of this response throughout a spectrum of growth-promoting hormones, led several workers to postulate that the production of new ribosomes had an important function in the mediation of hormone control. Tata (1970a) proposed a control function based on the segregation of ribosomes into populations. The oestrogen-stimulated synthesis of ribosomes is confirmed in the results presented in this thesis, but no evidence has been found which would indicate that the newly synthesized ribosomes fulfil an important control function other than increased protein synthesis.

4. 8. 3. The Oestrogen-Stimulated Synthesis of Transfer RNA.

Billing et al. (1969c) have shown that the immature rat uterine tRNA, separated from other RNA species on MAK columns, was synthesized more rapidly when the animals had been treated with oestrogen. In fact, incorporation of precursor 1h after hormone administration was increased nearly 2-fold, a significantly greater stimulation than was found at this time in ribosomal RNA. After 6h the increase was greater than 5-fold. My own work also indicates that the oestrogen-induced stimulation of tRNA synthesis may precede that of rRNA but not hnRNA synthesis. Dingman et al. (1969) reported large increases in the incorporation of precursor into chick oviduct tRNA as a result of diethylstilboestrol and progesterone treatment. They claimed that the increased synthesis was largely in tRNA located in the nucleus and suggested that their findings were indicative of an important control function of tRNA in marmalian systems.

Several reports indicated that oestrogen-stimulation of tRNA

synthesis could be selective towards particular amino acid-carrying species or might affect the methylation or modification of these species. Thus in chicken, where oestrogen stimulated the synthesis in the liver of phosvitin, a protein containing 50% serine residues, Beck et al. (1970) reported that the rate of $tRNA^{ser}$ synthesis was increased to a greater extent than that of total tRNA. Mays & Borek (1971) have reported that, in this system, a single species of N^2-methylguanine methyl transferase activity was stimulated whereas other methyl transferase activities were reduced. Sharma & Borek (1970) observed a change in the elution pattern of $tRNA^{ser}$ and a decrease in tRNA methyl transferase activity in pig uteri following ovariectomy. The situation was reversed by oestrogen administration and it appeared that methyl transferase activities were restored because the hormone reduced the levels of specific inhibitors. Hacker (1969) demonstrated that diethylstilboestrol treatment of chicks resulted in increased oviduct tRNA methyl transferase activity.

4.9. The Effects of Oestradiol on Protein Synthesis.

The proteins synthesized in response to oestradiol fall into two groups. Firstly, there seem to be a few poorly characterized species produced early in the hormone-elicited sequence of events, which may be essential for a major part of the observed changes in RNA and phospholipid production. Secondly, there is an increase in total protein synthesis which follows the stimulation in the synthesis of RNA and the accumulation in the cytoplasm of newly-made polysomes. These latter proteins play important structural and functional roles in the hormone-differentiated target tissue.

4. 9. 1. Early Proteins.

The oestrogen-induced increases in the synthesis of rRNA are dependent on protein synthesis (Nicolette & Gorski, 1964b; Noteboom & Gorski, 1963; Gorski & Axman, 1964), but as yet the nature of these proteins is largely unknown. It should be emphasized, however, that rRNA synthesis appears to be dependent on protein synthesis in many, if not all, systems. Thus, in conditions of curtailed protein synthesis, ribosome processing beyond the 45S precursor stage is inhibited, probably due to a deficiency in ribosomal proteins (Pederson & Kumar, 1971). There is evidence that 18S RNA is degraded at an increased rate when protein synthesis is reduced in resting cells or by treatment with cycloheximide (Cooper & Gibson, 1971). Therefore, the fact that hormone-induced synthesis of rRNA is dependent on protein synthesis does not necessarily indicate the previous synthesis of oestrogen specified proteins.

An important protein, apparently activated as an early result of oestrogen action, is the magnesium ion-stimulated RNA polymerase which appears to be bound to nucleolus chromatin and to be functional in the synthesis of rRNA. As already discussed, the synthetic activity of this enzyme was maximally increased after 2h of oestradiol treatment (Hamilton et al., 1968) and seemed to be stimulated after as little as 40min (See Fig. IV). The stimulated RNA polymerase activity was inhibited by puromycin (Noteboom & Gorski, 1963; Gorski et al., 1965) and by cycloheximide (Nicolette & Mueller, 1966), indicating its dependence on new protein synthesis.

Early changes in the synthesis of uterine proteins were also detected by Notides & Gorski (1966) when they followed the incorporation of radioactive amino acids into immature rat protein. They

separated the soluble uterine proteins on starch gels and found an oestrogen-stimulated peak of precursor incorporation within 30min of hormone treatment. Synthesis of the protein or proteins in this peak was not stimulated in similarly treated rat ileum but continued in the uterus until the increases in total protein synthesis masked the effect. Barnea & Gorski (1970) demonstrated that this protein was synthesized de novo and Mayol & Thayer (1970) indicated that a group of acidic proteins, also synthesized early on in the oestrogenic response, might be the same protein. Barker (1971) studied rats receiving similar lengths of hormone treatment, and found an acidic protein synthesized in response to the hormone. This protein was associated with the FIII histone fraction and its relationship to the starch gel-detected protein is unknown. These findings are interesting in view of the already discussed possibility that acidic proteins are important in the effect of oestrogen on chromatin.

DeAngelo & Gorski (1970) showed that the synthesis of the specific protein of Notides & Gorski (1966) was dependent on RNA synthesis, as was the group of acidic proteins reported by Mayol & Thayer (1970). Barker (1971), however, claimed that the histone-associated acidic protein was still produced when RNA synthesis was 90% inhibited. It may be that 90% inhibition of RNA synthesis is insufficient to block messenger RNA production. DeAngelo & Gorski (1970) found that a more complete inhibition of RNA synthesis was necessary to totally inhibit the synthesis of the starch gel-detected protein.

4. 9. 2. Later Proteins.

2h after the administration of oestrogen to rats, there is

a rise in the synthesis of total uterine protein which, after 12h, is manifested in measurable increases in the total protein content (Means & Hamilton, 1966a; Aizawa & Mueller, 1961) (See Fig. V). The uterus has not proved as amenable to the study of later protein synthesis as other oestrogen-responsive tissues. Thus, although the structural protein involved in hormone-induced uterine differentiation will obviously be synthesized and despite the fact that increases in many enzyme activities have been demonstrated, there does not appear to be a readily detected single protein, the synthesis of which has been followed. However, 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 (Brant & Halbandor, 1956; Kohler et al., 1968). O'Malley et al. (1967) observed that in chicks, treated with 5mg of diethylstilboestrol (DES) daily, the synthesis of ovalbumin and lysozyme is initiated and by 15 days production of these proteins is increased to as much as 300 times the basal levels. Synthesis is dependent on continued hormone administration. If, after 12-18 days of treatment, progesterone is given instead of DES, the synthesis of avidin begins within 6h. Production of this protein is much lower if progesterone administration is not preceded by DES treatment. It appears, therefore, that oestrogen can induce the synthesis of some oviduct proteins and prime the tissue for the synthesis of others. The production of avidin can also be promoted by progesterone in vitro in oviduct derived from DES treated chicks (O'Malley, 1967). Similarly, monolayers of oviduct cells, treated with DES for 14 days and then with progesterone, will produce avidin within 12h (O'Malley &

Kohler, 1967). Avidin synthesis is inhibited by cycloheximide added at any time and by Actinomycin D if added with progesterone but not if it is administered at later times (O'Malley, 1967).

Another readily detected product of oestrogen action is phosvitin, a protein containing many phosphorylated serine residues. This yolk protein is normally produced in the livers of laying fowl (Heald & McLachlan, 1963), but within 24h of the administration of oestrogen to cockrels, they also initiate its synthesis (Greengard et al., 1965). Actinomycin D, given simultaneously with the hormone, inhibits the synthesis but when given 6-8h after the hormone, it has no effect. (Greengard et al., 1964). In the African Clawed Toad Xenopus laevis, oestradiol stimulates the synthesis of another phosphorylated protein - vitellogenin (Dolphin et al., 1971).

It should be noted that these proteins are merely examples, the synthesis of which is readily followed; they are not, however, made in isolation. By this point in the response to hormone, the previously quiescent target tissue is mobilising its total protein synthetic machinery and making many proteins necessary for differentiation into a fully functional form.

4.10. Oestrogen-Stimulated Glucose Metabolism.

2h after hormone administration, the activities of many enzymes of glucose metabolism are stimulated. Few, if any, of the increases have been shown to be definitely due to enzyme synthesis, though in many cases, protein synthesis is necessary before the change occurs. Thus, the rate of glucose catabolism and of glucose incorporation into protein, RNA, lipid and glycogen in rats is

increased by oestradiol administration (Gilmour & McKerns, 1966; Macleod & Hollander, 1961; Williams & Provine, 1966; Nicolette & Gorski, 1964b). The latter workers showed that inhibition of protein or RNA synthesis blocks these oestrogen-induced increases in glucose metabolism. The mobilization of biosynthetic pathways necessitates increased supplies of NADPH. For this reason, much has been made in the past of the stimulation by oestrogen of the key enzymes of the pentose phosphate pathway, particularly glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Scott & Lisi, 1960). These increases occurred after more than 10h of oestrogen treatment and probably represent protein synthesis. However, McKerns (1967) has suggested that the primary effect of oestrogen action might be an earlier activation of pre-existing molecules of these enzymes. No direct evidence is available to support this concept.

4.11. The Effect of Oestrogen on Phospholipid and Membrane Synthesis.

The effect of oestrogen on the synthesis of lipids and the manufacture of membranes has not been studied as fully as those concerning RNA and protein synthesis. Aizawa & Mueller (1961) showed that in the first 6h following the administration of oestradiol to rats, the levels of uterine ethanolamine, choline and inositol phospholipids increased rapidly (See Fig. V), and uteri from hormone-treated rats, incubated *in vitro*, incorporated ^{32}Pi into phospholipid at increased rates. Gorski & Nicolette, (1963) made similar observations but most work in this field has been with other hormones. Under the influence of testosterone, growth hormone, tri-iodothyronine and thyrotrophic hormone, phospholipid is synthesized

at increased rates in the respective target tissues of rats and amphibians. All subcellular fractions are affected but the stimulation is most noticeable in the membranous elements of the microsome fraction. The increase is co-ordinated with stimulated RNA synthesis and with the accumulation in the cytoplasm of rough endoplasmic reticulum (Tata, 1967a,b & c; Kerkof & Tata, 1967).

4.12. Effects of Oestrogen on DNA Synthesis and Cell Division.

Eventually, the growth promoting effects of oestradiol leads not only to the hypertrophy, measurable as an early response, but also to hyperplastic growth. Brody (1958) found that during pregnancy, a 5-fold increase in size of the human uterus was caused by cell multiplication and a concomitant 6-fold enlargement was accounted for by increase in cell size. However, DNA synthesis, mitosis and cell division are late responses to oestradiol when compared with the other metabolic parameters covered in this review. Billing et al. (1969b) found that the measurable DNA content of the immature rat uterus did not increase until 32h after hormone treatment. Brody & Wiqvist (1961) found no change in the uterine DNA content of ovariectomized rats 12h after oestradiol treatment, but a definite increase after 36h. Spaziani (1963) investigated the effect of oestradiol on rat uterine cell division. 24h after intraluminal administration of hormone, division of epithelial cells was increased 112% over control levels. Some mitosis was evident in the epithelial lining of the uterine glands but was not evident elsewhere at that time.

4.13. The Effect of Oestrogen on Uterine Water Imbibition and Uptake of Small Molecules.

The effects of oestrogen so far discussed appear to be interrelated and to some extent sequential. In the rat uterus, however, there is one hormone-induced response which seems to be distinct from the others; namely the increased uptake of water. Oestradiol causes an imbibition of water by the rat uterus which is detectable as increased tissue wet weight 2h after hormone treatment and continues to rise for at least 36h (Billing et al., 1969b). The effect may be distinguished from the oestrogen-induced changes in uterine metabolism.

Cortisol inhibits oestrogen-induced water imbibition, but has no effect on the stimulated protein, RNA and phospholipid synthesis by the hormone (Spaziani & Szego, 1958; Nicolette & Gorski, 1964a & b). Furthermore, Spaziani & Szego (1958) have shown that uterine histamine levels decrease after oestrogen injection and they suggest that increased capillary permeability, due to histamine release, causes the water and electrolyte accumulation. In support of these ideas, Spaziani & Szego (1959) have shown that histamine and its analogues mimic the oestrogenic effects on rat uterine water uptake and that antihistamines inhibit the response. Neither histamines nor antihistamines have any effect on oestradiol-stimulated RNA synthesis (Hamilton et al., 1968).

The oestrogen-induced increases in the uptake of sugars, amino acids and nucleosides are more difficult to analyse. Increased uptake of these metabolites could reflect the increased requirement in polymer synthesis. Conversely, the stimulated synthesis of protein and RNA could merely be due to increased precursor availability.

The latter argument was supported by Billing et al. (1969a), who concluded that the increased transport of RNA precursor into the uterus accounted for most of the stimulated RNA synthesis in the first 5h of hormone action. However, results to be presented in this thesis show that the stimulation of uterine RNA synthesis can occur at much higher levels than can be explained by increased precursor uptake. Moreover, Oliver (1971) has shown that nucleosides are not actively transported into the uterus but enter by facilitated diffusion. Amino acids are actively transported into the uterus and accumulation is stimulated by oestradiol (Riggs et al., 1968). Actinomycin D and cycloheximide inhibit oestradiol-stimulated uptake of ∝aminoisobutyric acid, but do not inhibit similar transport stimulation by insulin acting on muscle tissue (Roskoski & Steiner, 1967). Thus, it appears that oestrogen-induced transport of amino acids is dependent on, and secondary to, nucleic acid and protein synthesis.

The alternative argument that oestrogen stimulation of precursor uptake is due to increased utilization in synthetic pathways, is also not supported by initial evidence. It was found that accumulation also occurs with the non-metabolizable amino acid, ∝ amino-isobutyric acid (Noall et al., 1957), the non-utilizable sugar, D-xylose (Walkerston et al., 1960) and the non-utilizable nucleoside, cytosine arabinoside (Oliver, 1971). However, the increase in the uptake of cytosine arabinoside was wholly explained by oestrogen-induced water imbibition and was considerably less than the stimulation of uridine uptake. As a result, Oliver (1971) concluded that the elevation in uridine uptake was due either to more rapid delivery of nucleoside to the uterus or to a more rapid removal of nucleoside for

the synthesis of uterine nucleotides and nucleic acids.

5. The Synthesis of Mammalian RNA.

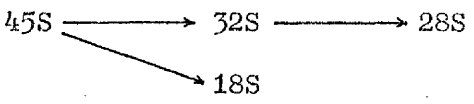
The above review has shown that following oestradiol binding to target tissue there is stimulation of the cellular synthetic processes. Many theories exist as to the nature of the activation of these events, the majority of which envisage either a transcriptional or a translational control of protein synthesis. Since the production of all major RNA species is stimulated by oestradiol, it follows that if control is transcriptional, it could be at the level of messenger, ribosomal or transfer RNA. The further elucidation of these possibilities requires a more refined investigation of the nature of the RNA species synthesized in response to hormone. Before describing the results obtained in attempting to achieve this, it is desirable to briefly review what is known of RNA synthesis in mammalian cells.

5. 1. The Synthesis of Ribosomal RNA.

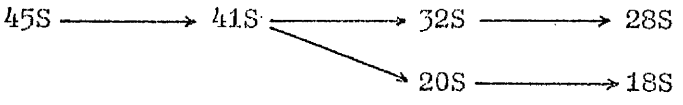
In the eukaryotic cell, ribosomal RNA is synthesized in the form of a giant precursor molecule containing the sequence of both 28S and 18S RNA together with long stretches of non-ribosomal sequences. This synthesis occurs in the nucleolus (Penman et al., 1966) where several hundred copies of the genes for rRNA are concentrated (Steele, 1968). These apparently form a linear entity, known as rDNA, within which the genes for 28S and 18S RNA alternate (Brown & Weber, 1968b; Quagliarotti & Ritossa, 1968). Transcription may well be directed by a specific RNA polymerase, (RNA polymerase I) which is located in the nucleolus, is magnesium ion dependent and is

sensitive to both cordycepin (3' deoxyadenosine) and high ionic strength but relatively insensitive to ∝ amanitin (Widnell & Tata, 1966a; Siev et al., 1969; Roeder & Rutter, 1969; Jacob et al., 1970).

The giant precursor molecule, which appears in nucleolar RNA after a short pulse of radioactive precursors, sediments at 45S. Actinomycin D chase and kinetic experiments revealed that this molecule was converted to a smaller species, migrating at 32S, and that simultaneously 18S rRNA appeared and immediately migrated to the cytoplasm. With longer chases, the 32S RNA disappeared and was replaced by the 28S ribosomal species (Scherrer et al., 1963; Soeiro et al., 1966). On the basis of these findings, a sequence of events was postulated as follows:



However, polyacrylamide gel separations revealed further short lived intermediate species and the fact that certain of these, sedimenting at 41S and 20S, accumulated during polio virus infection of HeLa cells (Weinberg et al., 1967), allowed them to be fitted into the scheme as follows:



Ribosomal RNA is methylated and in eukaryotes the substitution is largely at the 2'O position of the ribose moieties rather than methylation of the bases. With the exception of one methyl group, which is probably added during maturation (Zimmerman, 1968),

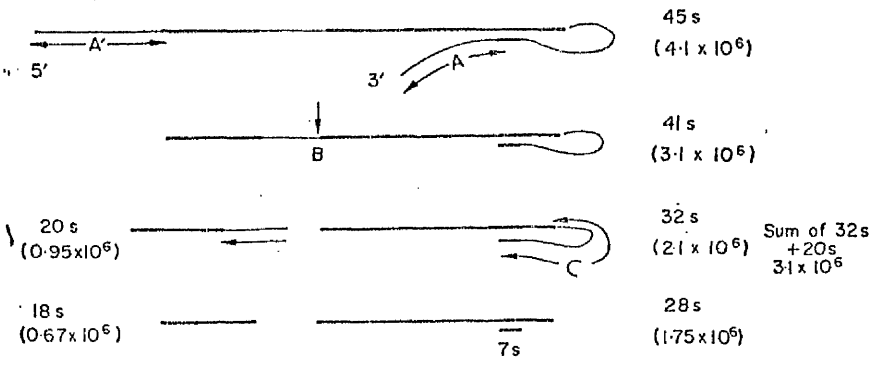
the methylation appears to occur during the synthesis of 45S RNA (Greenberg & Penman, 1966). Methyl labelled methionine has been used by Salim et al. (1970) to label the methyl groups of rRNA and its precursors. Enzyme digests of the various species were then separated electrophoretically and the resulting separation patterns, when compared, confirmed the maturation processes outlined above.

Little is known of the non-ribosomal sequences of 45S RNA except that comparison with mature rRNA indicates that they have a high G+C content and are unmethylated (Amaldi & Attardi, 1968; Willems et al., 1968). The presence or absence of the ribosomal species in the various precursors has been used to postulate an arrangement of ribosomal and non-ribosomal sequences within 45S RNA (Fig. VIII). The 18S sequence has been placed nearest the 5' end of the molecule since only 18S sequences are found in RNA corresponding to 45S RNA when transcription is prematurely terminated by cordycepin (Siev et al., 1969). It is seen from Fig. VIII that the so called 7S RNA, which in fact sediments at 5.7S and is strongly hydrogen bonded to 28S RNA, also has its origins in the 45S precursor.

The synthesis of 5S ribosomal RNA is little understood but, since anucleolate toads, which are unable to synthesize rRNA, still make 5S RNA (Brown & Weber, 1968a), it can be assumed that it is made outside the nucleolus. There appear to be many more genes for 5S RNA than there are for rRNA (Brown & Weber, 1968a).

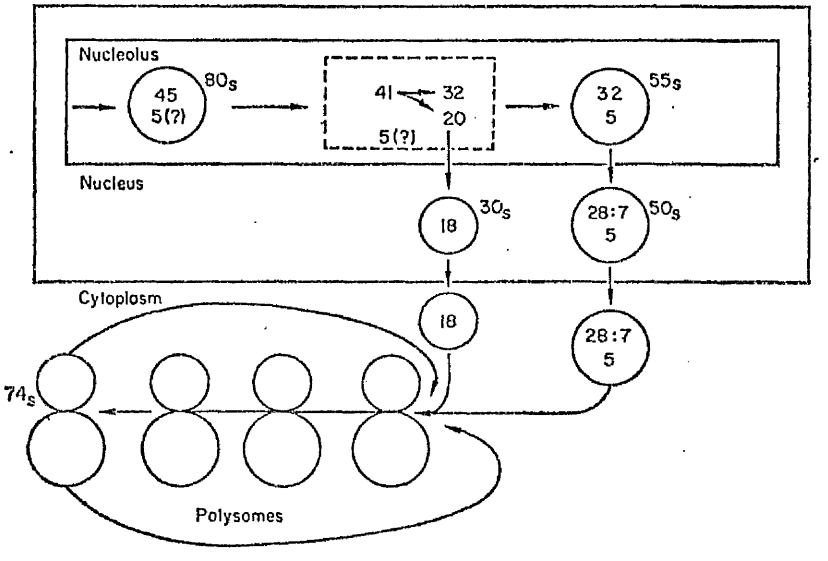
The ribosomal 28S and 18S RNA species occur in the 60S and 40-45S ribosomal subunits respectively, and Girard et al. (1965) have shown that they are found in ribonucleoprotein complexes when they first enter the cytoplasm. Further work has revealed that nuclear pre-ribosomal RNA also occurs as ribonucleoprotein particles.

Figure VIII



A possible arrangement for the ribosomal and non-ribosomal sequences in Hela cell 45s RNA, based on the clues mentioned in the text. The ribosomal sequences are indicated by heavy lines. Removal of the non-ribosomal sequences A and A' would generate 41s molecules containing both 28s and 18s sequences. Cleavage at B would then generate 32s and 20s RNA. Cleavage and removal of the non-ribosomal sequence C would generate 28s RNA with its attached 7s fragment. Cleavage at B before removal of A and A' would generate 36s and 24s molecules. The latter might still be capable of giving rise to mature rRNA. Since non-ribosomal fragments are not found unattached to rpre RNA it is possible that they are removed by exonuclease action, following preliminary cleavage of rpre RNA where necessary by endonuclease. Slightly different models are discussed by Weinberg and Penman (1970), the main difference being that in their models there is no "hairpin loop" of non-ribosomal material separating 28s and 7s sequences in 32s RNA. The molecular weight values are also those of Weinberg and Penman.

Figure IX



Schematic representation of ribosome formation in Hela cells. The numbers outside the particles represent the sedimentation constants of the particles themselves. The numbers inside the particles represent the sedimentation constants of the RNA species which they contain. Note that the 30s particle is found in the nucleoplasm only in vanishingly small amounts. Note also that the *exact* relationship between the protein components of the various nucleolar particles and cytoplasmic ribosomes is not yet clear.

Figs. VIII and IX are taken from Maden (1971)

They were first detected by electron microscopy (Bernhard & Granboulan, 1963) and were later extracted from nucleoli by Warner & Soeiro (1967) as particles sedimenting at 55S and 80S in EDTA-containing buffer. Under these conditions ribosome subunits sediment at 50S and 30S. Analysis of the protein and RNA within these particles, together with pulse label studies, have shown that the 55S particles were precursors to the 50S ribosomal subunit and contained 32S RNA, 5S RNA and most of the 50S ribosomal proteins. The 80S particle contained 45S RNA and some 35S and 41S intermediates (Soeiro, 1968). Figure IX shows a schematic representation of ribosome formation.

5. 2. The Synthesis of Transfer RNA.

Hybridization experiments by Ritossa et al. (1966), autoradiography by Woods & Zubay (1968) and studies with anucleolate mutants of Xenopus laevis by Brown & Gurdon (1964) have established that tRNA is synthesized on non-nucleolar chromosomes. The product of transcription, which rapidly leaves the nucleus, has been found to be a precursor molecule known as pre-tRNA. Pulse-chase experiments revealed that it matures in the cytoplasm (Burdon et al., 1967). The precursor is found to be deficient in methyl groups and, on Sephadex G100 or polyacrylamide gels, it migrates to a position between 5S RNA and tRNA (Burdon et al., 1967; Burdon & Clason, 1969). This migration characteristic is not the result of differences in secondary structure (Burdon & Clason, 1969) and appears to be due to the presence in the precursor of approximately 30 extra nucleotides. Smillie (1970) showed that these extra sequences were rich in pyrimidines and largely located at the 5' end of the molecule. Both

the 3' and 5' end of the precursor possess different terminal sequences from mature tRNA. Cell-free preparations which convert pre-tRNA to a tRNA-like species have been described by Smillie & Burdon (1970) and by Nowshowitz (1970). The product, although apparently trimmed to the right size, is deficient in methylated nucleosides and pseudouridine. It appears that the primary gene product first undergoes some base modifications, as the pre-tRNA does contain some pseudouridine (Nowshowitz, 1970). It is then trimmed to the right size, probably by an exonuclease (Smillie & Burdon, 1970) and then undergoes further base alterations by cytoplasmic enzymes.

5. 3. The Synthesis of Messenger RNA.

The manner in which higher animals synthesize messenger RNA and the manner in which it is protected during its passage out of the nucleus are matters of considerable controversy. Indeed, it is only recently that RNA molecules, which appear to be messengers for the synthesis of specific eukaryotic proteins, have been identified (Mathews et al., 1971; Lazarides & Lukens, 1971; Williamson et al., 1971).

Evidence has also been accumulating which suggests that a rapidly-labelled, high-molecular-weight RNA, known as heterogeneous nuclear RNA (HnRNA), is a precursor to cytoplasmic messengers. Heterogeneous nuclear RNA has been found in vertebrates, insects and plants (Warner et al., 1966; Attardi et al., 1966; Brown & Gurdon, 1966; Edström & Daneholt, 1967; Retèl & Planta, 1967) and is comprised of a wide spectrum of polymers with sedimentation values ranging from 20S to 80S (Warner et al., 1966). It is synthesized

on non-nucleolar chromosomes, (Penman et al., 1968) possibly by a distinct RNA polymerase (RNA polymerase II) which can be separated from the nucleolar enzyme by its nucleoplasmic location and its sensitivity to the fungal toxin ∝amanitin (Roeder & Rutter, 1970; Jacob et al., 1970).

At first sight, HnRNA is a poor candidate for a messenger precursor; at least 90% of it turns over without leaving the nucleus and its average half life may be as little as 3min (Shearer & McCarthy, 1967; Soeiro et al., 1968). However, it has a DNA-like base composition (Attardi et al., 1966; Soeiro et al., 1966) and comparisons by DNA-RNA hybridization indicate some sequence homology between HeLa cell HnRNA and a "messenger" fraction isolated by EDTA treatment of HeLa cell polysomes (Soeiro & Darnell, 1970). Thus, a small proportion of the nuclear species might consist of messenger sequences which could survive the breakdown of the remainder. In further hybridization experiments with HnRNA and mRNA derived from cells transformed by the oncogenic virus SV40, Lindberg & Darnell (1970) obtained more evidence in support of this hypothesis. Such cells are known to contain at least 20 copies of the viral genome incorporated into their DNA. The above workers found that RNA which specifically hybridizes to viral DNA is present in both the HnRNA and the cytoplasmic mRNA of the transformed cells.

Further evidence that the heterogeneous nuclear species is a messenger precursor came with the finding that both species contain polyadenylate (poly A) sequences about 150-200 nucleotides long (Darnell et al., 1971b; Lee et al., 1971; Edmonds et al., 1971). Poly (A) is also found in both the nuclear and polysomal fractions of viral specific RNA from adenovirus infected HeLa cells

(Philipson et al., 1971), yet the viral DNA does not contain sequences complementary to poly (A). It appears that the poly (A) is added in the nucleus by a host cell post-transcriptional mechanism. Such addition provides a possible explanation for the finding by Penman et al. (1970) that cordycepin inhibits the appearance of labelled RNA in the polysome derived mRNA but has little effect on the synthesis of HnRNA. Since the antibiotic is an analogue of adenosine, it seemed likely that it might inhibit poly (A) synthesis. This was found to be the case by Darnell et al. (1971a) who suggest the following scheme for the synthesis of mRNA.

By some unknown mechanism, HnRNA molecules are selected for conversion to mRNA and a poly (A) segment is attached, probably at one terminus. Nucleases then recognise and remove the mRNA plus poly (A) after which it is transported to the cytoplasm while the remainder of the HnRNA is destroyed. The further elucidation of these possibilities is of utmost importance in unravelling the manner in which higher animals control their synthetic processes.

Bacterial messenger RNAs are susceptible to nuclease attack and have very short half lives. Eukaryotic messenger species would be expected to be equally vulnerable but, despite the fact that they must cross a nuclear membrane and much greater cytoplasmic barriers, they appear to have relatively long half lives. It may be that poly (A) serves a protective function (Lee et al., 1971) but there is evidence that not all messengers carry poly (A) (Edmonds et al., 1971). There are at least two other postulated transport mechanisms. The first is that messengers leave the nucleus attached to the small ribosomal subunit and the second that they are transferred as ribonucleoprotein particles known as informosomes.

The former argument is supported by the work of Henshaw et al. (1965) and McConkey & Hopkins (1965) who have shown that in rat liver, rapidly labelled RNA is first found in the cytoplasm in association with a 40-45S ribonucleoprotein aggregate. The particle contains messenger-like RNA and ribosomal RNA and appears to be a newly synthesized ribosomal subunit. Similar evidence has been published by Joklik & Becker (1965), Latham & Darnell (1965) and Köhler & Arends (1968). The informosome theory has been proposed largely as a result of the finding that fish and sea urchin embryos contain DNA-like RNA in ribonucleoprotein particles with sedimentation values ranging from 15-110S (Spirin & Nemer, 1965; Infante & Nemer, 1968; Spirin, 1969). These particles are formed at a time when the embryos are not producing ribosomes.

Evidence obtained to date does not conclusively support either of these theories and interpretation of results is confused by the finding that ribosome particles may themselves undergo modification and maturation in the cytoplasm. A number of forms with varying buoyant densities have been reported by Sugano et al. (1971) and Chen et al. (1971). It seems very likely, however, that messengers are transported as ribonucleoprotein particles and in this respect it is interesting that HnRNA has been reported to occur as ribonucleoprotein aggregates with sedimentation values as high as 5000S (Penman et al., 1968).

Although there is still much to learn, this short review has shown that some understanding of RNA transcription and maturation has been achieved in recent years. These advances have depended, in large measure, on the development of improved methods of RNA fractionation. The experimental section of this thesis describes the

results obtained when some of this methodology was used in an attempt to better understand the effects of oestrogen on uterine RNA synthesis.

MATERIALS AND METHODS

1. Materials.

1. 1. Hormones, Enzymes and Inhibitors.

Oestradiol-17β, oestrone, diethylstilboestrol, dibutyryl adenosine 3' 5'-cyclic monophosphate, epinephrine, cycloheximide, ribonuclease and pancreatic deoxyribonuclease (ribonuclease free) were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6., U.K. Actinomycin D and Pronase were purchased from Calbiochem Ltd., London W1H 1AS, U.K. Oestrone sulphate was the generous gift of Professor G.S. Boyd, Department of Biochemistry, Edinburgh University.

1. 2. Nucleic Acids.

Salmon sperm DNA (grade A) was the product of Calbiochem Ltd., London W1H 1AS, U.K. and yeast RNA and "Soluble" *E. coli* RNA were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6., U.K.

1. 3. Reagents for the Purification of RNA.

Bentonite powder and sodium dodecyl sulphate (specially pure) was supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. and the bentonite was purified essentially by the method of Fraenkel-Conrat *et al.* (1961). 20g of bentonite was suspended in 400ml of distilled water and centrifuged at 800 x g for 15min. The supernatant fraction was recentrifuged at 8000 x g for 20min and the resulting sediment was resuspended in 0.1M disodium EDTA (pH 7.0)

for 48h at 25°. The suspension in EDTA was recentrifuged differentially and the 8000 x g sediment suspended in the buffer in which it was to be used (either 0.05M sodium acetate buffer, pH 5.2, or 0.1M tris-HCl, pH 7.5, containing 1mM $MgCl_2$) and was again centrifuged at 8000 x g for 20min. The sediment was suspended in either of the above buffers at a concentration of 20mg/ml, as determined by dry weight estimation, and the preparations were sterilized by autoclaving at 15 p.s.i. for 30min. Phenol was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. and was redistilled before use.

1. 4. Reagents for the separation of RNA.

Acrylamide, agarose and NNN'N'-tetramethylethylenediamine were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. The acrylamide was purified as described by Loening (1967). Acrylamide was dissolved at a concentration of 70g/l in chloroform at 50°, filtered hot without suction and recrystallized at -20°. The crystals were collected by filtration in a chilled filter funnel. NN'-Methylene bisacrylamide was supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. and purified according to the method of Loening (1967). The solid was dissolved at 10g/l in acetone at 50°, filtered hot and recrystallized by slowly cooling to -20°. The crystals were recovered by filtration and washed with cold acetone. Ethylene diacrylate was purchased from Kodak Ltd., Kirkby, Lancs., U.K.

1. 5. Reagents for Histology.

DePeX mounting medium and eosin (yellowish:water and

alcohol soluble) were obtained from George T. Gurr Ltd., London S.W.6., U.K. and eosin was made up as a 1% (w/v) aqueous solution. Haemalum (Mayer's) was prepared from 3g of Haematein, provided by BDH Chemicals Ltd., Poole, Dorset, U.K., which was dissolved in a little glycerol and added to 1l of 5% potassium alum ($KAl(OH)_4$) in tap water. Bouin's fixative was prepared from a mixture of 750ml of saturated picric acid and 250ml of 40% aqueous formaldehyde, neutralized with $MgCO_3$ and 50ml of glacial acetic acid. Paraffin wax (congealing point 54.5°C) was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

1. 6. Reagents for In Vitro Incubations.

Eagle's medium (Glasgow modification) (Busby et al., 1964) containing 100µg/ml of streptomycin, 100 units/ml of penicillin and 0.002% w/v phenol red was the usual medium in which excised uteri were incubated in vitro. In some experiments, calf serum and foetal calf serum, purchased from Flow Laboratories Ltd., Victoria Park, Heatherhouse Rd., Irvine, Ayrshire, U.K., was added to Eagle's medium and in others a complex medium NCTC 109, purchased from Microbiological Associates Inc., Bethesda, USA., was used instead.

1. 7. Radioisotopes and Materials for Liquid Scintillation Counting.

[5-^{3}H]uridine (5Ci/mmol), [2-^{14}C]uridine (60 mCi/mmol), [8-^{3}H]guanosine (500mCi/mmol), [methyl-^{14}C]methionine (53.6 mCi/mmol), [4,5-^{3}H]lysine (360 mCi/mmol) and [^{32}P]-orthophosphate (78 Ci/mgP) were purchased from The Radiochemical Centre, Amersham, Bucks, U.K. Cellulose acetate filters, 2.5cm diameter and 0.45µ pore size, were

obtained from Sartorius Membranfilter GHBM,34, Gottingen, West Germany. Kieselguhr (Hyflo-supercell) was purchased from Koch-Light Ltd., Colnbrook, Bucks, U.K. Toluene scintillator was 0.5% 2,5-diphenyl-oxazole in toluene and in counting radioactivity in gel slices this was mixed with 2-methoxyethanol (3:2v/v). Dioxan scintillator was 0.7% 2,5-diphenyloxazole and 10% naphthalene in scintillation grade dioxan. Koch-Light Laboratories, Colnbrook, Bucks., U.K. supplied 2,5-diphenyl oxazole and dioxan, BDH Chemicals Ltd., Poole, Dorset, U.K. provided 2-methoxyethanol and Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh. supplied naphthalene and 1M hyamine hydroxide in methanol. Planchettes were the product of McGreggor and Alves Ltd., Glasgow.

1. 8. Miscellaneous.

Cellulose nitrate and polyallomer centrifuge tubes were a product of Beckman Spinco Ltd., Palo Alto, California, USA. All other chemicals were Analar grade where possible and were usually obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

2. Biological Methods.

2. 1. Experimental Animals.

The rats, which were derived from the Wistar strain and bred at Glasgow University, were fed *ad libitum* on diet 41B (Bruce & Parkes, 1956). They were used when 18-21 days old and in all later experiments were limited to a weight of 25-30g. The rats were killed by cervical dislocation and, in most experiments, the excised uteri were carefully dissected free of connective tissue

before further treatment. An exception was the attempt to demonstrate oestrogen-stimulated RNA synthesis in an artificial medium. Here, damage to the uterine cells was minimized by removing the connective tissue after the in vitro incubation.

2. 2. Administration of Hormone, Inhibitors and Radioactive Precursors to Experimental Animals.

a) Hormone: Oestradiol-17β was solubilized by the method of Roberts & Szego (1947) using methyl red as an indicator. Except where stated it was injected intraperitoneally in 0.1ml of 0.9% NaCl (saline) containing 0.5% (v/v) ethanol. Control animals received saline-ethanol only.

b) Inhibitors: Actinomycin D and cycloheximide were injected intraperitoneally in 0.2ml of saline.

c) Radioactive Precursors: Unless otherwise noted, radioactive precursors were given intravenously via the lateral tail vein in 0.2 or 0.25ml of saline. To facilitate easier handling, animals were kept under light ether anaesthesia during the injection and their tails were pre-warmed in water at 48° for approximately 20s. Tritiated ribonucleosides were administered as an equal mixture of [5-^{3}H]uridine and [8-^{3}H]guanosine. This increased the total uptake of precursor but for any given experimental technique the validity of the results was checked by the administration of [5-^{3}H]uridine alone. When uteri were used for the preparation of total uterine acid-soluble and acid-insoluble fractions, the rats received 20μCi of the ribonucleoside mixture. When they were to be used for the purification of uterine RNA or the preparation of subcellular particles, they received 250μCi of the precursors, although in acid-insoluble preparations of uterine nuclei, only three out of a group

of nine rats were so treated. In one experiment, where protein synthesis was investigated, each rat received 20 μCi of [4,5-^{3}H] lysine.

2. 3. In Vitro Incubations.

The uteri of 1-12 animals were incubated in 5ml conical flasks under an atmosphere of 95% O_2/5% CO_2 at 37° in a shaking water bath. Incubation was usually in 2-4ml of Eagle's medium but precise conditions varied with the experiments as follows:

a) Oestrogen-Stimulation of RNA Synthesis In Vitro: In most experiments, uteri were incubated individually in 5ml conical flasks in 2ml of a medium containing 0.1 μCi/ml of each of [5-^{3}H] uridine and [8-^{3}H] guanosine. Where uterine horns were floated on lens tissue rafts, the incubation was in 5cm diameter petri dishes and a border of silicon grease was applied to the 2cm square rafts to keep them afloat. The many modifications to the artificial medium are described in the Results section. Incubations were for 30min after which the uteri were washed twice in cold saline, blotted dry and dissected free of connective tissue before being frozen. Acid-soluble and acid-insoluble fractions were then prepared as described in section 3. 1.

b) Methylation of rRNA: Four uteri were dissected free of connective tissue and incubated for 30 or 60min in a 5ml conical flask, in 2ml of methionine-free Eagle's medium, containing 5 μCi of [methyl-^{14}C] methionine and 20mM sodium formate. The latter addition inhibits incorporation of the methonine into the purine ring structure. After washing and drying, the uteri were frozen and RNA prepared from them as described in section 5. 1.

c) Decay of RNA: Four uteri, which had been freed of connective tissue, were incubated for 15min in 2ml of Eagle's medium containing 3 μCi/ml of each of [5-^{3}H]uridine and [8-^{3}H] guanosine. After washing in 2 x 20ml of pre-warmed Eagle's medium, they were then transferred to a further 2ml of Eagle's media which contained 100μg/ml of each of uridine, guanosine and actinomycin D. The incubation was continued for varying lengths of time, after which the uteri were washed, dried and frozen prior to the preparation of RNA.

d) Base-Ratio Determination: Four uteri were incubated for 1h in Eagle's medium in which the phosphate content was reduced to 10% of the normal level but [^{32}P]-orthophosphate was present at 1 mCi/ml. The washed, dried and frozen uteri were used to prepare ^{32}P labelled RNA for base composition analysis.

e) Nuclei Preparations: Uteri from 12 rats were incubated for 30min in 2ml of Eagle's media which, in early preparations, contained 3 μCi/ml of each of [5-^{3}H]uridine and [8-^{3}H]guanosine. In later experiments, the level of each precursor was raised to 9 μCi/ml. The uteri were washed, dried and frozen before being used to prepare nuclear RNA.

2. 4. HeLa Cells.

S3 HeLa cells were the generous gift of Dr B.E.V. Haden. They were grown in spinner culture to a concentration of 7×10^{5} cells/ml.

3. Preparation of Acid-Soluble and Acid-Insoluble Fractions.

3. 1. Acid-soluble and Acid-Insoluble Fractions of Whole Uteri.

Acid-soluble and acid-insoluble fractions were prepared by a modification of the method of Billing et al. (1969a).

Uteri, removed from treated animals or from in vitro incubations, were washed three times with cold saline, placed individually in universal containers and rapidly frozen in a solid-CO_2/methanol bath. They were then either stored for up to 3 days at-60° or used immediately.

The uteri were thawed, finely chopped with scissors and homogenized in 2.5ml of ice-cold distilled water using a glass homogenizer with a motor driven teflon pestle (Jencons, Hemel Hempstead, Herts.). All subsequent steps were performed at 0-4°. The homogenate plus a further 2ml of water, used to wash the homogenizer, were added to 0.5ml of 50% (w/v) trichloroacetic acid and mixed. After standing for 15min, one quarter of the homogenate was separated from the remainder and both aliquots were sedimented at 800 x g for 5min. The supernatant from the smaller fraction was discarded and the pellet put to one side for DNA estimation. The supernatant fluid from the larger fraction was retained and the pellet washed with a further 2ml 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 2ml of 2% (w/v) kieselguhr in 5% (w/v) trichloroacetic acid. An additional 2ml of kieselguhr suspension was added to a millipore filtration unit and the kieselguhr collected as a pad on a 2.5cm Whatman No. 1 filter paper disc. The acid-insoluble pellet

bound to kieselguhr was collected as a second layer on this pad and washed with 3 x 15ml portions of 5% (w/v) trichloroacetic acid, 1 x 15ml portion of absolute alcohol and 2 x 5ml portions of diethyl ether. The pad was extracted with 0.5ml of 1M hyamine hydroxide for 10min at 60° and the radioactivity measured by scintillation counting in 10ml of toluene scintillator with tritium counting efficiencies of 16-18%. The radioactivity in 0.4ml aliquots of the acid-soluble fraction was counted in 10ml of dioxan scintillator with tritium counting efficiencies of 18-21%.

3. 2. Preparation of Acid-Insoluble Fractions from Sub-cellular Preparations.

The above method was modified slightly for nuclei. Uterine nuclear pellets were suspended in 1.0ml of 1mM $MgCl_2$ and divided into unequal fractions of 0.3ml and 0.7ml. The larger fraction was precipitated at 0-4° with 0.2M perchloric acid, washed three times in cold 0.2M perchloric acid and used to estimate RNA and DNA. The smaller fraction was precipitated with ice cold 5% (w/v) trichloroacetic acid, washed twice in 5% (w/v) trichloroacetic acid and the acid-insoluble radioactivity estimated as above.

Microsomal and submicrosomal fractions were treated similarly to whole uteri. The pelleted fractions were suspended in 5% (w/v) trichloroacetic acid in the presence of 200µg of bovine serum albumin and divided in two. After standing at 0-4° for 15min, the precipitates were collected by sedimentation at 800 x g and washed twice with 5% (w/v) trichloroacetic acid. Half of the preparation was then collected on a kieselguhr pad so that the acid-insoluble radioactivity could be estimated as described above. The other

half of the preparation was used to estimate RNA content.

3. 3. Preparation of Acid-Insoluble Fractions from Less than 100µg of Material.

Small amounts of material, such as the RNA in fractions from sucrose density gradients, were trapped on membrane filters. The fractions were collected into an equal volume of ice cold 10% (w/v) trichloroacetic acid and a further 5ml of 5% (w/v) trichloroacetic acid together with one drop of 2% (w/v) bovine serum albumin was added. After mixing and standing at 0-4° for at least 15min, the precipitates were collected on 2.5cm diameter membranes with a 0.45µ pore size. The membranes were washed with a further 5ml of 5% (w/v) trichloroacetic acid, dried in scintillation vials at 50° for 1h and the radioactivity measured by scintillation counting in 10ml of toluene scintillator with efficiencies of 27-30% for ^{3}H and 80-82% for ^{14}C.

4. Preparation of Subcellular Fractions.

4. 1. Nuclei Preparation (A).

Twelve uteri, removed from treated animals or from in vitro incubations, were rapidly frozen in a solid CO_2/methanol bath and broken up with a footed-glass rod. They were then homogenized in 2ml of RSB (0.01M tris-HCl, pH 7.4, 0.01M NaCl, 0.0015M $MgCl_2$) containing 1% (w/v) Triton X100 in an Ultra Turrax homogenizer run at 60V for 20s. This, and all subsequent steps were carried out at 0-4°. The homogenate was filtered through two thicknesses of muslin and to it was added a further 2ml of the homogenization medium, which

was used to wash the homogenizer and muslin. A nuclear pellet was obtained by sedimenting the filtrate at 300 x g for 5min and it was suspended in 2ml of the homogenization buffer. A further 2ml of homogenization buffer, containing 0.5M sucrose, was mixed with the suspension and 2ml of 0.32M sucrose in homogenization buffer was layered beneath it. The nuclei were collected by sedimentation at 800 x g for 5min.

4. 2. Nuclei Preparation (B).

Twelve uteri, collected, rapidly frozen and broken up as in method (A), were homogenized in 2ml of 1mM $MgCl_2$ in an Ultra Turrax homogenizer run at 40V for 20s. The homogenization and all subsequent steps were carried out at 0-4°. After filtration of the homogenate through two thicknesses of muslin, the filtrate was mixed with 2ml of 0.1M citric acid in 1mM $MgCl_2$, this having first been used to wash the homogenizer and muslin. The nuclei were collected by sedimentation at 300 x g for 5min and the pellet resuspended in 1mM $MgCl_2$ containing 0.05M citric acid and 1% (w/v) Triton X100. The mixture was made 0.25M with respect to sucrose as in method (A) and underlaid with 2ml of 1mM $MgCl_2$ containing 0.32M sucrose, 0.05M citric acid and 1% (w/v) Triton X100. The nuclei were collected by sedimentation at 800 x g for 5min.

4. 3. Preparation of Microsomal and Submicrosomal Fractions (A).

The method used was based on that described by Tata (1970b) and differed mainly in modifications to the constituents and volumes of the buffers used. All steps were carried out at 0-4°. The uteri of 24 rats were collected into a homogenization medium which

consisted of TKMC buffer (0.05M tris-HCl, pH 7.5, 0.025M KCl, 0.003M $MgCl_2$, 0.002M $CaCl_2$) containing 0.32M sucrose. After draining and finely chopping with scissors, they were homogenized in 5ml of homogenization media in a motor driven, teflon-glass, Potter-Elvehjem-type homogenizer. The homogenizer was loose fitting and was run at the lowest speed at which it could operate. A further 3ml of homogenizing medium, used to wash the homogenizer, was added to the homogenate which was then sedimented for 7 and 10 min at 8000 x g to obtain a mitochondria-free supernatant. This was then layered over 4ml of 1.3M sucrose in TKMC buffer in a $^5/_8$in diameter x 3in cellulose nitrate tube and centrifuged at 105,000 x g for 2.5h in a Beckman 50 Ti angle head rotor. Two fractions were collected after centrifugation; an opaque layer which collected at the interface and a pellet which was resuspended in homogenization medium by careful hand homogenization in an all glass homogenizer. Each fraction was carefully layered onto a discontinuous sucrose density gradient consisting of 1.25ml of each of 2M sucrose, 1.5M sucrose and 1.15M sucrose, all in TKMC buffer. The gradients, in $\frac{1}{2}$in diameter x 2in cellulose nitrate tubes, were centrifuged at 50,000 x g for 16h in a Beckman SW40 rotor.

Material collected at each of the three interfaces of the gradient and as a pellet. In the original method of Tata (1970b) these fractions were equivalent to smooth membrane at the first interface, light and heavy rough membrane at the second and third interfaces and polysomes forming the pellet. Here they are referred to as fractions 1, 2, 3 and 4 respectively. The interface aggregate of the first discontinuous gradient partitioned between fractions 1 and 2 while the pellet of the first fractionation

partitioned between fractions 2, 3 and 4. These fractions, together with the phase above them, were carefully collected and the two second interface fractions combined. Each preparation was diluted with TMKC buffer or homogenizing medium and pelleted by sedimentation at 105,000 x g. One half of each pellet was used in the preparation of an acid-insoluble fraction for the assay of RNA and the other half was used to measure acid-insoluble radioactivity or to assay phospholipid. In one experiment, RNA was prepared from the preparations as described in section 5. 2.

4. 4. Preparation of Microsomal and Submicrosomal Fractions (B).

The method followed that of Andrews & Tata (1971) for the preparation of free and membrane-bound ribosomes of skeletal muscle. It was scaled down and adapted to suit the small amount of tissue available and all steps were carried out at 0-4°. The uteri of 24 rats were collected into a homogenization medium which consisted of 0.05M tris-HCl, pH 7.6, containing 0.1M KCl and 0.01M $MgCl_2$. After collection, the tissue was drained, finely chopped and homogenized in 5ml of homogenization medium in an Ultra Turrax homogenizer run at 80V for 2 x 15s. The homogenate was sedimented for 30min at 40,000 x g in a Griffin Christ SW40 rotor and the pellet was discarded. The supernatant was layered onto 1.5 volumes of 2M sucrose in homogenization medium and sedimented at 60,000 x g in a Griffin Christ SW40 rotor for 16h. Three fractions were collected from this gradient. These were referred to as fraction A which accumulated at the interface, B occurring as a diffuse band just below the interface and C forming a pellet. The isolated fractions were pelleted by sedimentation at 105,000 x g. Aliquots were

prepared for the assay of acid-insoluble radioactivity, RNA and phospholipid or the whole pellet was used to purify RNA as described in method A.

4. 5. Preparation of HeLa Cell Nucleoli.

Nucleoli were prepared from 1.5 x 10^8 Hela cells which had been grown for 2.5h in the presence of 0.1 μCi/ml of [2-^{14}C]uridine. They were isolated by a slight modification of the method of Penman et al. (1966) and, except where stated, all steps were carried out at 0-4°. The HeLa cells were collected by sedimentation at 800 x g for 5min and washed twice in Buffered Saline Solution (BSS) (0.116M NaCl, 5.4mM KCl, 1mM $MgSO_4$, 1mM NaH_2PO_4, 1.8mM $CaCl_2$, 0.002% w/v phenol red adjusted to pH 7.0 with 8.4% w/v $NaHCO_3$). They were then resuspended in 4.5ml of reticulocyte saline buffer (RSB) (0.01M tris-HCl, pH 7.4, containing 0.01M NaCl and 0.0015M $MgCl_2$) and, after standing for 10min, were homogenized by 25 strokes of a Dounce homogenizer with a clearance of 0.003mm. A crude nuclear pellet was collected by sedimentation at 800 x g for 10min and this was resuspended in a further 2ml of RSB and again collected by sedimentation at 800 x g. The pellet was resuspended in 3ml of RSB and to it was added 0.6ml of a mixture consisting of two thirds 10% (w/v) Tween 40 and one third 10% (w/v) sodium deoxycholate. After mixing, the clean nuclei were collected by sedimentation at 800 x g for 5min. The nuclei were suspended in 2ml of high salt buffer (HSB) (0.01M tris-HCl, pH 7.4, 0.5M NaCl, 0.05M $MgCl_2$), containing 100μg of ribonuclease-free deoxyribonuclease and the mixture was warmed to 37° until it became clear. The digest was layered onto 20% sucrose in HSB and sedimented at 40,000 x g for 15min at 0° in order to pellet the nucleoli.

5. Preparation of RNA.

5. 1. Preparation of High-Molecular-Weight RNA.

With minor modifications, high-molecular-weight RNA was purified by the method of Joel & Haggerman (1969). Uteri removed from treated animals or from *in vitro* incubations were rapidly frozen in a solid-CO_2/methanol bath. Four to eight frozen uteri, broken up with a footed glass rod, were homogenized in a conical, ground glass, Potter-Elvehjem type homogenizer to which had been added 2ml of 0.05M sodium acetate buffer, pH 5.2, containing 1mg bentonite/ml and 2ml of 88% (v/v) phenol in the same buffer. Homogenization was carried out by hand in a water bath at 5-10°. A further 1.6ml of 0.05M sodium acetate buffer containing 1mg bentonite/ml, 2ml of 88% (v/v) phenol and 0.4ml of 10% (w/v) SDS in acetate buffer was added to the homogenate. The mixture was then blended for 30s in an Ultra Turrax homogenizer operated at 60V at room temperature.

Phenol extraction was carried out by rapidly stirring the homogenate for 3min at 52°. After cooling in ice for 2min, the mixture was centrifuged at 20,000 x g for 6min at 4°. The phenol layer was discarded and the aqueous layer and interface were made 1mg/ml with respect to bentonite and re-extracted with a further 3.2ml of 88% (v/v) phenol. After re-centrifugation, the aqueous phase of this second extraction was removed, 4mg of bentonite was added and it was set aside at 0-4°. The phenol layer and interface were re-extracted for a third time with a further 2ml of 0.05M acetate buffer, pH 5.2, containing 2mg of bentonite. The aqueous layer from this extraction, combined with that from the second

extraction, was centrifuged at 20,000 x g for 15min to remove most of the bentonite. RNA and DNA was then precipitated from the resulting supernatant in the presence of 0.15M NaCl and 2 volumes of ethanol at -20° overnight.

The precipitate was collected by centrifugation, washed twice with cold 95% (v/v ethanol and dried in a stream of nitrogen. The pellet was digested at 0° for 5min and at 25° for 10min with 0.15mg of ribonuclease-free deoxyribonuclease, dissolved in 1.0ml of 0.01M tris-HCl (pH 7.5) containing 1mM $MgCl_2$. This was the only step in which glassware was sterilized since, in the remainder of the preparation, the high levels of uterine nucleases (unpublished observations of Greenman & Kenney, 1964) necessitated the use of ribonuclease inhibitors.

After cooling in ice, the digest was made 1mg/ml with respect to bentonite and to 1% with SDS before being extracted with 0.5 volumes of 88% (v/v) phenol for 10min at 10°. The extract was centrifuged as before and 1mg bentonite was added to the aqueous phase which was retained at $0-4^{\circ}$. The phenol and inter-phase were re-extracted with 0.5ml of 0.01M tris-HCl (pH 7.5) containing 1mM $MgCl_2$ and the combined aqueous phases centrifuged, as before, to remove bentonite. RNA was precipitated from the supernatant at -20° for $1\frac{1}{2}$h in the presence of 0.15M NaCl and 2 volumes of ethanol. The precipitate, collected by centrifugation, was dissolved in 0.5ml of 1mM $MgCl_2$ and re-precipitated by treatment with 2M potassium acetate and 25% ethanol at -20° for $1\frac{1}{2}$h. This re-precipitation was performed three times and the final precipitate washed twice with cold 95% (v/v) ethanol.

5. 2. Preparation of RNA from Subcellular Particles.

The above method of RNA preparation was also used to prepare nuclear RNA except that it was only necessary to perform one potassium acetate/ethanol precipitation in order to remove the DNA oligonucleotides remaining after the deoxyribonuclease digestion.

RNA from other subcellular fractions, such as microsomal preparations and nucleoli, was prepared by following the above method of Joel & Haggerman (1969) as far as the overnight ethanol precipitation. The virtual absence of DNA in these preparations precluded the need for the subsequent steps. In extracting these small amounts of RNA, all reagent volumes were reduced by half and it was sometimes necessary to add unlabelled uterine RNA to effect precipitation.

5. 3. Preparation of Low-Molecular-Weight RNA.

In preparing RNA for the study of the low-molecular-weight species, the method of Joel & Haggerman (1969) was followed as far as the first ethanol precipitation but the deoxyribonuclease step and subsequent extraction and purification was omitted. Instead, the ethanol precipitate was dissolved in 1mM $MgCl_2$ and the high-molecular-weight nucleic acids were precipitated with 1M NaCl in the presence of 1mg bentonite/ml at 0° for 4-6h. The precipitate was removed by sedimentation at 800 x g and the RNA remaining in the supernatant was recovered by precipitation with 2 volumes of ethanol at -20° overnight.

6. RNA Fractionation.

6. 1. Fractionation of RNA on Polyacrylamide Gels.

Gels were prepared as described by Loening (1967) except that in all later experiments, NN'-methylene bisacrylamide was replaced by ethylene diacrylate. The electrophoresis buffer (36mM tris, 30mM NaH_2PO_4 and 1mM EDTA adjusted to pH 7.7-7.8 with glacial acetic acid) was that described by Loening (1969). For the preparation of 2.7% acrylamide/bisacrylamide gels, a stock solution was made containing 15% (w/v) recrystallized acrylamide and 0.75% w/v) recrystallized bisacrylamide in water while, for 2.7% acrylamide/ethylene diacrylate gels, the stock solution contained 27% (w/v) recrystallized acrylamide and 2.5% (v/v) ethylene diacrylate. 7.5% acrylamide/ethylene diacrylate gels were prepared from stock solutions containing 25% (w/v) recrystallized acrylamide and 0.416% (v/v) ethylene diacrylate. These stock solutions were unaffected by storage at 4^{o} for at least 2 months. When used, they were diluted such that the required concentrations were present in electrophoresis buffer in the presence of 0.33ml of 10% w/v) ammonium persulphate and 33µl of NNN'N' tetramethylethylenediamine per gram of acrylamide.

The prepared solutions were carefully mixed to avoid aeration and 3ml aliquots were rapidly pipetted into vertical $\frac{1}{4}$ x 5in plexiglass tubes. Water was then carefully layered over the solution using a Hamilton syringe. The 2.7% gels set in approximately 15min and the 7.5% gels in about 2min. All gels were pre-electrophoresed at 5mA/gel for 30min before RNA (40-120µg), dissolved in 0.05ml of electrophoresis buffer containing 10% sucrose, was layered on the top. Electrophoresis was for 2-6h at 5mA/gel. After separation, the gels were scanned at 260nm in the linear transport attachment for the Gilford 240 recording spectrophotometer,

but prior to this it was found advantageous to allow them to stand in water for 30min. This procedure almost completely removed the humps of 260nm absorbing material which collected at either end of all gels, including blanks. Gels containing radioactive RNA were frozen in powdered solid CO_2 and sliced into 1mm sections by using a Mickle gel slicer. Slices of acrylamide/ethylene diacrylate gels containing ^{3}H or ^{14}C labelled RNA were digested individually in vials with 0.5ml of aqueous 2M NH_3 solution at 50°. After evaporation to dryness, the digests were taken up in 0.3ml of water and left for 30-60min to allow the gel residue to swell and the RNA hydrolysate to dissolve. Radioactivity was counted by scintillation counting in 10ml of toluene/2-methoxyethanol-based scintillator with efficiencies of 16-18% for ^{3}H and 71-74% for ^{14}C. Where acrylamide/bisacrylamide gels were used, the individual slices were dried in scintillation vials by incubation at 60° for approximately 2h. The slices were then totally digested by further incubation at 60° for 18h in the presence of 0.3ml of 30% (v/v) hydrogen peroxide. Radioactivity was then assayed as above in 10ml of toluene/2-methoxyethanol scintillator with ^{3}H counting efficiencies of 16-19%.

scintillator with efficiencies of 16-18% for ^{3}H and 71-74% for ^{14}C.

When RNA was labelled with ^{32}P, the gel slices were dried on 2.5cm diameter Whatman No.1 filter paper discs which were glued to planchettes. The radioactivity was measured in a Nuclear Chicago, low background, gas flow counter.

6. 2. Fractionation of RNA on Agarose Gels.

Agarose was dissolved to 1% in electrophoresis buffer (36mM tris, 30mM NaH_2PO_4, 1mM EDTA, brought to pH 7.7-7.8 with glacial

acetic acid) in a boiling water bath and 3ml aliquots were rapidly pipetted into $\frac{1}{4}$ x 5in plexiglass tubes. After setting, the top of the gels were cut level with a scalpel blade. The gels were pre-electrophoresed for 30min at 5mA/gel before approximately 40µg of RNA was carefully layered on top in 0.05ml of 10% sucrose in electrophoresis buffer. Electrophoresis was carried out at 5mA/gel for $2\frac{1}{2}$h after which the gels were scanned at 260nm and sliced as in section 6. 1. Gel slices were extracted overnight in 4ml of 2-methoxyethanol and 6ml of toluene scintillator was added to the extract for scintillation counting. ^{3}H and ^{14}C were counted with efficiencies of 21-23% and 72-76% respectively.

6. 3. RNA Analysis on Sucrose Density Gradients.

The method used was a modification of that described by Girard et al. (1965). 80µg of RNA in approximately 0.1ml of LETS buffer (0.01M tris-HCl, pH 7.4, 0.1M LiCl, 0.001M EDTA and 0.2% SDS) was layered onto a 13ml, 15-30% linear sucrose density gradient in LETS buffer in a $^{9}/16$in diameter x $3\frac{3}{4}$in cellulose nitrate tube. Centrifugation was for 16h at 31,800 x g and at 20^{o} in the SW40 rotor of a Beckman model L2 65B ultracentrifuge. Gradients were eluted, by the use of a peristaltic pump, through the flow cell of a Gilford 240 recording spectrophotometer and the extinction at 260nm was continuously monitored. 10s fractions were collected (about 0.35ml) into an equal volume of 10% w/v) trichloroacetic acid and the acid insoluble material was collected on membranes as described in section 3. 5.

7. Band Sedimentation Studies.

Total uterine DNA was dissolved at 100µg/ml in a buffer containing 0.01M tris-HCl, pH 7.0, 0.1M NaCl and 0.004M EDTA. A 20µl sample of the solution was layered onto 10ml of 0.01M tris-HCl, pH 7.0, containing 1.3M NaCl and 0.004M EDTA and its band sedimentation characteristics were kindly analysed by Dr. R. Eason of this department. Sedimentation rate was measured at 20° and 30,000rpm in the AnD rotor of a Beckman Model E analytical ultracentrifuge, fitted with UV scanner-multiplex accessories and R.T.I.C. temperature control. Traces of the rate of sedimentation were recorded every 8min.

8. Digestion and Denaturation of DNA.

8. 1. Ribonuclease Digestion.

Approximately 100µg of DNA was purified as far as the deoxyribonuclease treatment of section 5. 1. It was then incubated for 15min at 37° or 25° with 100µg ribonuclease in 1ml of 0.1M tris-HCl, pH 7.5, containing 1mM $MgCl_2$. The digest was then subjected to the second phenol extraction and subsequent purification as in section 5. 1.

8. 2. Pronase Digestion.

100µg of DNA, purified as above to the deoxyribonuclease stage of section 5. 1., was incubated with 100µg Pronase in 1ml of 0.01M tris-HCl, pH 7.5, containing 1mM $MgCl_2$. Treatment was for 1h at 37°, in the absence of any ribonuclease inhibitors, and for 24h at 37° in the presence of SDS. The purification of the products of the incubation was then continued

as in section 5. 1.

8. 3. Denaturation.

The method was a slight modification of that described by Katz & Penman (1966). Purified RNA was dissolved at 50µg/ml in 0.01M tris-HCl, pH 7.4, containing 0.1M NaCl, 0.001M EDTA and 0.5% SDS. Six volumes of dimethyl sulphoxide was mixed with the solution which was then incubated at 37° for 18min. A similar incubation without dimethyl sulphoxide formed a control. The RNA was precipitated with two volumes of ethanol at -20° for 1h and the precipitate was collected and washed once with 95% ethanol before being loaded onto 2.7% polyacrylamide gels as described in section 6. 1.

9. Base Composition Analysis.

9. 1. Recovery and Hydrolysis of RNA.

Selected gel slices containing ^{32}P labelled RNA were cut from the filter paper discs on which they had been counted (see section 6. 1.) and were extracted overnight at 65° in 1ml of 4 x SSC (0.52M NaCl, 0.052M trisodium citrate adjusted to pH 7.0 with HCl). This was essentially the method described by Marcaud et al. (1971). The extracts were diluted ten fold with saline and water such that the NaCl concentration was reduced to 0.15M. RNA was precipitated, in the presence of 1mg of yeast RNA, by two volumes of ethanol at -20° overnight. The hydrolysis and subsequent separation of the hydrolysate was a modification of the method of Sebring & Salzman (1964). Precipitated RNA was washed twice with ethanol/ether

(3:1 v/v) and dried in a $CaCl_2$ desiccator under vacuum. The dry solid was then dissolved in 0.25ml of 0.3M KOH and digested for 18h at 37°. The digest was carefully adjusted to pH 3.5 with perchloric acid and the precipitated $KClO_4$ was removed by centrifugation. It was found beneficial to freeze and thaw the samples and recentrifuge them in order to completely remove the $KClO_4$.

9. 2. Electrophoretic Separation of RNA Digests.

The digests were spotted onto 2cm long origins on 46 x 57cm sheets of Whatman 4MM paper together with 10µl of a mixture containing 1mg/ml of each of the RNA monophosphates. After a preliminary 20min pre-electrophoresis at 300V to remove salts, the monophosphates were separated in pH 3.5 acetate/pyridine buffer (1ml pyridine, 10ml glacial acetic acid, water to 300ml) containing 0.01M EDTA at 3000V for 2½h on a 10kV electrophoresis unit (Miles Hivolt Ltd., Shoreham, Sussex.). The separated spots were cut out, placed in scintillation vials and the radioactivity determined by scintillation counting in 10ml of toluene scintillator.

10. Histology of the Uterus.

10. 1. Fixation and Embedding for Light Microscopy.

Uteri from treated animals or from in vitro incubations were loosely attached to a square of filter paper to keep the horns straight and then fixed for 2h in Bouin's fixative. They were left overnight in 70% ethanol before preparation for embedding as follows:

The tissue was immersed in -

70% ethanol for - - - - - - - - - 1h

96% ethanol for - - - - - - - - - 1h

absolute ethanol for- - - - - - 1h

absolute ethanol/chloroform for 1h

chloroform for - - - - - - - - - 1h

xylol for - - - - - - - - - - - - 1h

The fixed and dehydrated tissue was then immersed in two changes of paraffin wax at 57° for 3h before being embedded in paraffin wax.

10. 2. Staining and Mounting for Light Microscopy.

Using a rocking arm microtome, 0.005mm sections of the embedded samples were cut, floated on water at approximately 40° and deposited onto alcohol-cleansed slides. After drying overnight at 37°, the preparations were stained according to the following schedule:

Slides were immersed in -

xylol for - - - - - - - - - - - - 2min

absolute ethanol for - - - - - - 2min

methylated spirits for - - - - - 2min

tap water for - - - - - - - - - - 5min

filtered haemalum for - - - - - - 6min

tap water for - - - - - - - - - - 5min

1% eosin for - - - - - - - - - - $1\frac{1}{2}$min

They were then rinsed successively in tap water, methylated spirits and absolute alcohol, immersed in xylol for 2min and mounted in DePeX resin.

10. 3. Light Microscopy.

Phase contrast micrographs of nuclei were made on a Gilette & Sibert Conference Microscope fitted with an Ilford Sportsman camera.

10. 4. Electron Microscopy.

The quality of subcellular preparations was kindly investigated by the personnel, under the direction of Mr. H. Elder, of the Electron Microscopy Unit of the Physiology Department of this University. Briefly, samples supplied as sedimented pellets in polyallomer centrifuge tubes were fixed in gluteraldehyde-osmium tetroxide, embedded in plastic and cut sections were supported on grids. These were stained with lead and uranium for visualization in the electron beam. The electron microscope was an A.E.I. EM6B.

11. Assay Procedures.

11. 1. Assay of DNA.

Samples for the assay of DNA were hydrolysed in 0.5M perchloric acid at 70° for 1h and were then assayed by the method of Burton (1956).

11. 2. Assay of RNA.

Samples for analysis were hydrolysed in 0.5M perchloric acid for 1h at 70°. RNA was then determined by the method of Kerr & Seraidarian (1945).

11. 3. Assay of Phospholipid.

Material for assay was precipitated and washed twice in ice cold 5% (w/v) trichloroacetic acid and phospholipid was extracted by a slight modification of the method of Tata (1967b). The washed precipitate was homogenized in a conical, all glass, Potter-Elvehjem-type homogenizer with chloroform/methanol (2:1 v/v) and then extracted by shaking in the same solvent for 1 min at 20° and 10min at 45°. The organic solvent extract was washed once with one fifth of its volume of 0.1M HCl and twice with similar volumes of water. The residue was evaporated to dryness under a stream of air in a water bath at 40°. Phospholipid in the residue was measured by the method of Ames & Dubin (1960) by ashing and measuring inorganic phosphate. The residue was dissolved in 0.05ml of water and 0.05 ml of 10% $Mg(NO_3)_2.6H_2O$ (w/v) in ethanol. This solution was then carefully evaporated to dryness over a strong flame and heating was continued until no more brown fumes evolved. The ash was taken up in 0.3ml of 1M HCl and heated in a boiling water bath for 15min. 0.7ml of a mixture of 1 part of 10% (w/v) ascorbic acid in water to 6 parts of 0.42% (w/v) ammonium molybdate in 1M H_2SO_4 was added to the digest. The assay was developed in a water bath at 45° for 20min and read at 825mm against a standard curve of 0.01-0.06μmol of phosphate. Phospholipid was taken as 25 times the inorganic phosphate (Ansell & Hawthorne, 1964).

RESULTS

1. Factors Influencing Oestrogen-Stimulated RNA Synthesis in Immature Rat Uterus.

1. 1. Effects of Injection Route.

In studying oestradiol-induced stimulation of RNA and protein synthesis, most workers have administered both hormone and radioactive precursor intraperitoneally and many have reported large variations in the responses of replicate animals (Hamilton, 1964; Greenman, 1970). Fig. 1 compares the responses of animals receiving radioactive ribonucleosides and hormone by various injection routes. It is apparent that the mode of administration of labelled precursors exerts a profound effect on the observed stimulation of RNA synthesis, while the route of administration of hormone makes little difference to the response.

Intraperitoneal injection of labelled ribonucleosides gives a comparatively low and very variable stimulation of incorporation into the acid-insoluble fraction. The results shown for this method of precursor administration are better than average in that the variation between duplicate animals is often greater than shown in Fig. 1 and occasionally so great as to invalidate the experiment. Furthermore, increased incorporation into RNA is frequently less than the 3.5-fold shown in Fig. 1 and is often as low as 2-fold.

It can also be seen from Fig. 1 that incorporation into the acid-soluble fraction is stimulated 2-2.5-fold by oestradiol and, if uptake into the acid-insoluble fraction is only increased to the same extent, it becomes possible that the whole effect is merely a

Figure 1.

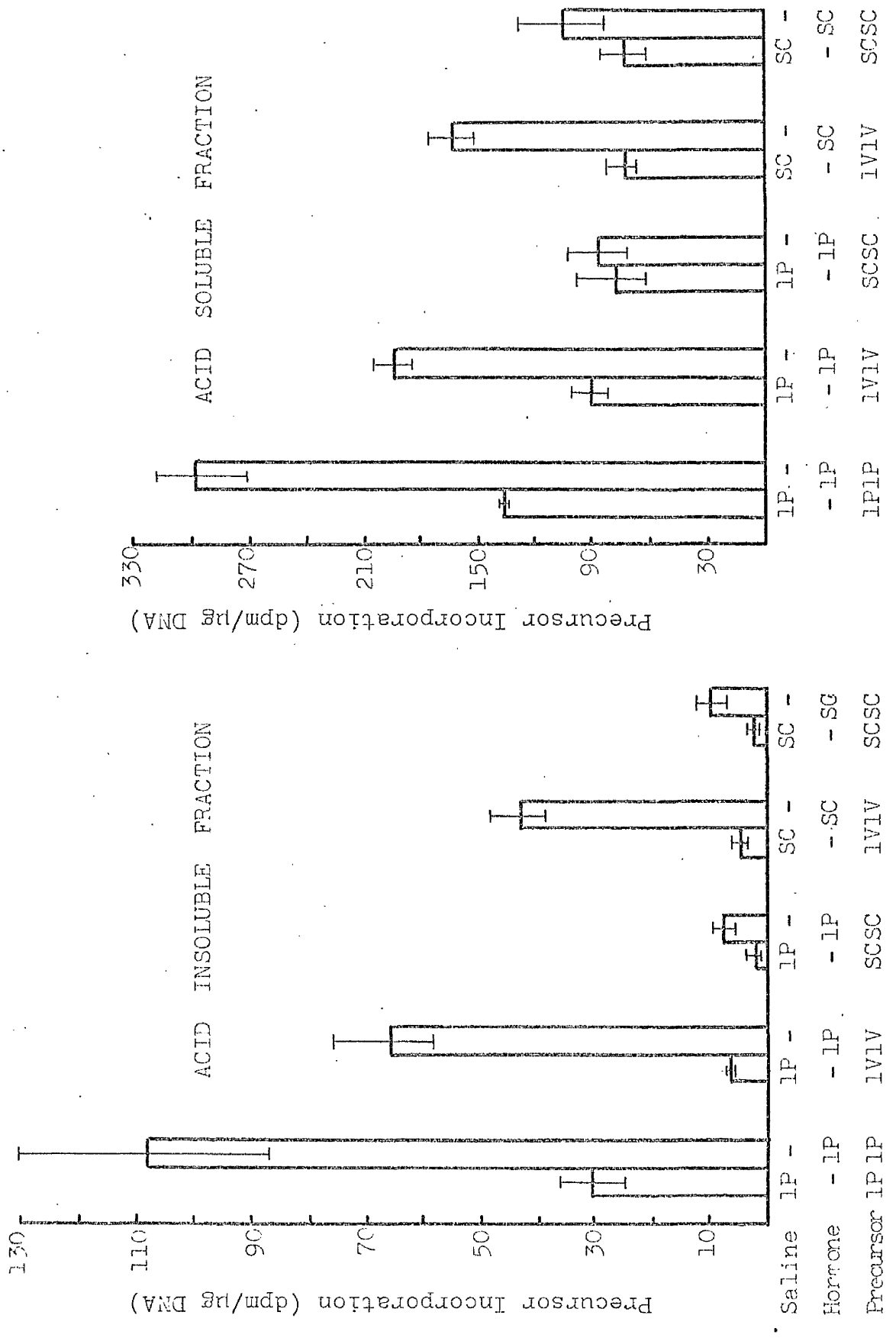
Comparison between different injection routes for the administration of oestradiol-17β and RNA precursors to 18-21 day old rats.

Oestradiol (5μg/rat) in 0.1ml of 0.5% ethanol in saline, or 0.5% ethanol in saline alone, was administered 2h before death. 10μCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine were given 30min before death. Acid-soluble and acid-insoluble fractions were prepared from the excised uteri as described in the Materials and Methods section. The histogram shows the mean and range of three determinations.

I.P. = Intraperitoneal injection.

I.V. = Intravenous injection.

S.C. = Subcutaneous injection.


ACID SOLUBLE FRACTION
Precursor Incorporation (dpm/µg DNA)
330
270
210
150
90
30
ACID INSOLUBLE FRACTION
Precursor Incorporation (dpm/µg DNA)
130
110
90
70
50
30
10
Saline 1P - 1P - 1P - SC - SC -
Hormone - 1P - 1P - 1P - SC - SC
Precursor 1P 1P 1V1V SCSC 1V1V SCSC
1P - 1P - 1P - SC - SC -
- 1P - 1P - 1P - SC - SC
1P1P 1V1V SCSC 1V1V SCSC
Injection Route

consequence of increased labelling of the precursor pools.

Intravenous injection of radioactive precursor, while giving somewhat lower values for total radioactivity in both acid-soluble and acid-insoluble fractions, consistently gives rise to an 8-11 fold stimulation of uptake into RNA. Incorporation into the acid-soluble fraction is still only stimulated 2-2.5 fold and variation between duplicate animals is greatly reduced.

When the labelled precursors are given by subcutaneous injection, the total uptake into RNA is low although the response to oestradiol is greater than that obtained using intraperitoneal injections.

1. 2. Effect of Oestradiol-17β Concentration.

Fig. 2 shows the effect of varying doses of oestradiol on the incorporation of labelled ribonucleosides into the acid-soluble and acid-insoluble fractions of immature rat uterus. With concentrations as low as 0.01μg oestradiol-17β/rat, the incorporation of labelled nucleosides into the acid-insoluble fraction is still 4 times higher than in control animals. Maximum responses are obtained at doses of approximately 0.3μg/rat and at higher levels the curve forms a plateau.

1. 3. Effect of Rat Weight on Response to Oestradiol.

It can be seen from Fig. 2 that at the higher doses of oestradiol, there is still considerable variation between different sets of results. This is caused, at least in part, by variations in the weights of the rats since, depending partly on litter size, 18-21 day old rats weigh anything from 20-40g. Fig. 3 shows the

Figure 2.

The effect of the level of oestradiol-17β on the stimulation of RNA synthesis in 18-21 day old rat uteri.

Hormone was given intraperitoneally 2h before death and 10μCi each of [5-^{3}H]uridine and [8-^{3}H]guanosine were given intravenously 30min before death. Acid-soluble and acid-insoluble fractions were prepared from the excised uteri as described in the Materials and 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.

—o— = Acid-insoluble fraction

—o— = Acid-soluble fraction.

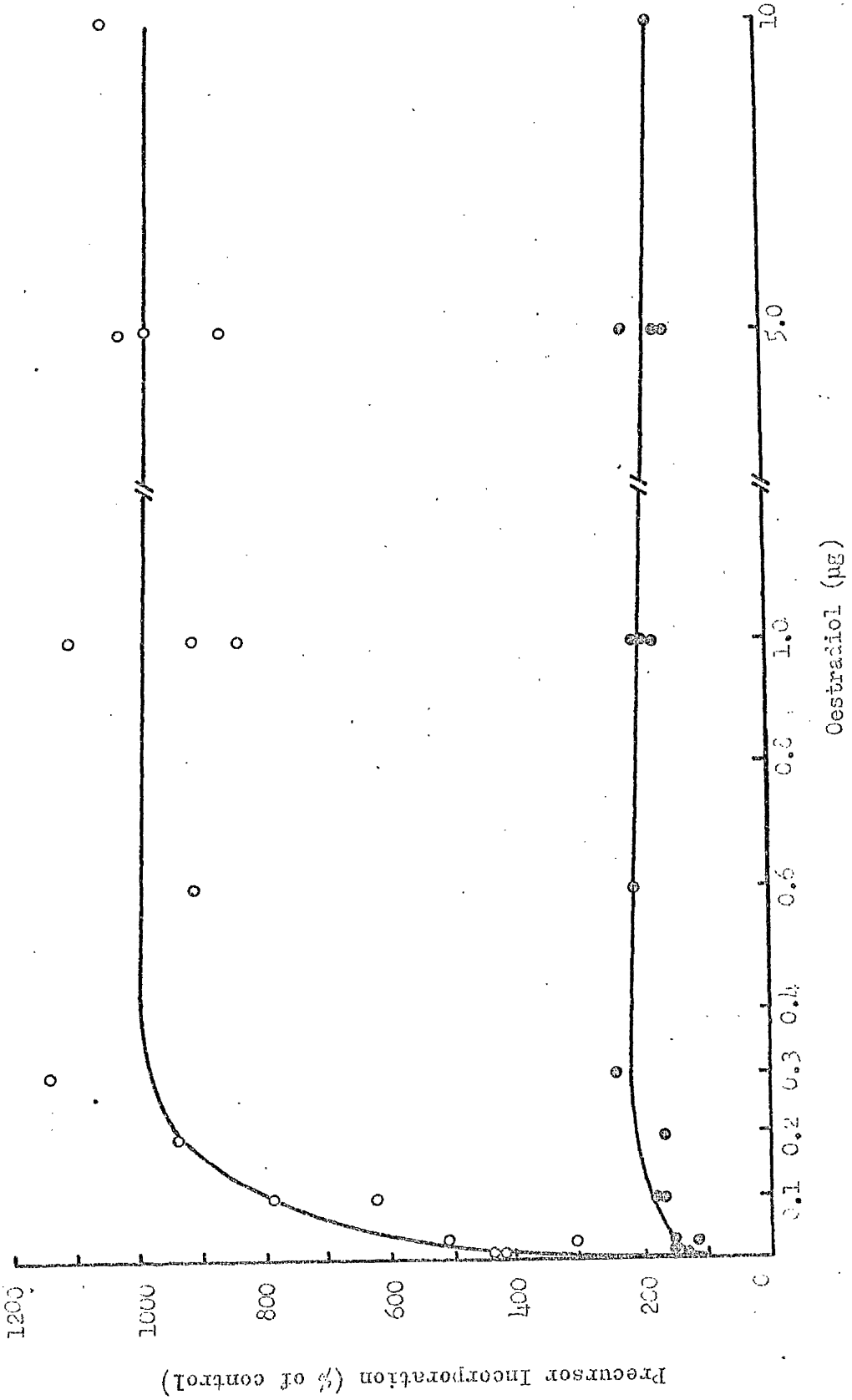

Precursor Incorporation (% of control)
1200
1000
800
600
400
200
0
0.1
0.2
0.3
0.4
0.6
0.8
1.0
5.0
10
Oestradiol (µg)

Figure 3.

Time course for the effect of oestradiol-17β (1μg/rat) on the incorporation of DNA precursor into 18-21 day old rat uteri.

Hormone was administered intraperitoneally and 10μCi each of [5-^{3}H] uridine and [8-^{3}H] guanosine were given intravenously 30min before death. Results were calculated as dpm/μg DNA and expressed as a percentage of the uptake in non-hormone treated controls. Each point represents a mean of at least three animals.

△ = 20-25g rats.

○ = 25-30g rats.

● = 30-35g rats.

——— = Acid-insoluble fraction.

- - - = Acid-soluble fraction.

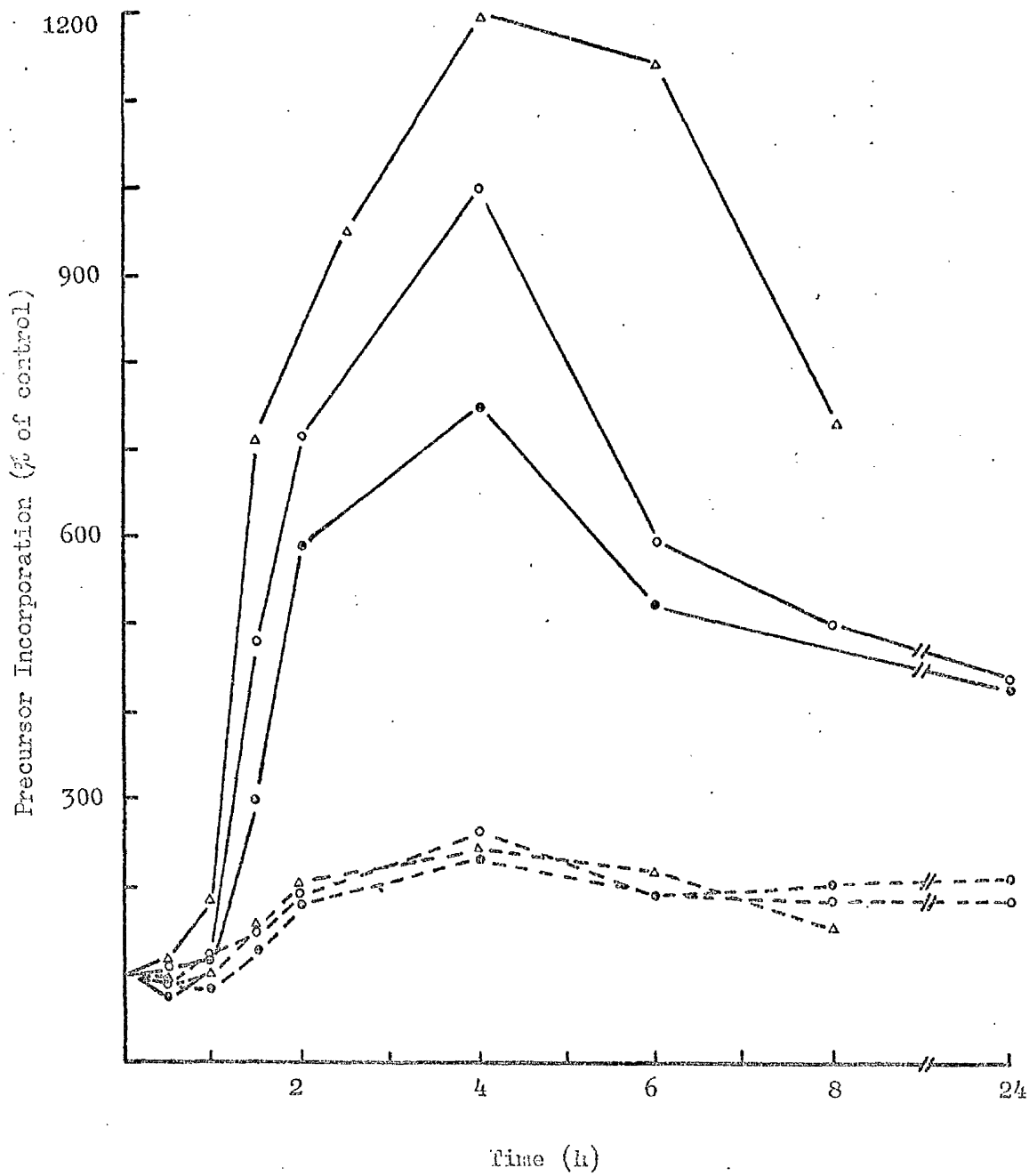

1200
900
600
300
Precursor Incorporation (% of control)
2
4
6
8
24
Time (h)

Figure 4.

Time course for the effect of oestradiol-17β (0.1 μg/rat) on the incorporation of DNA precursor into 18-21 day old rat uteri.

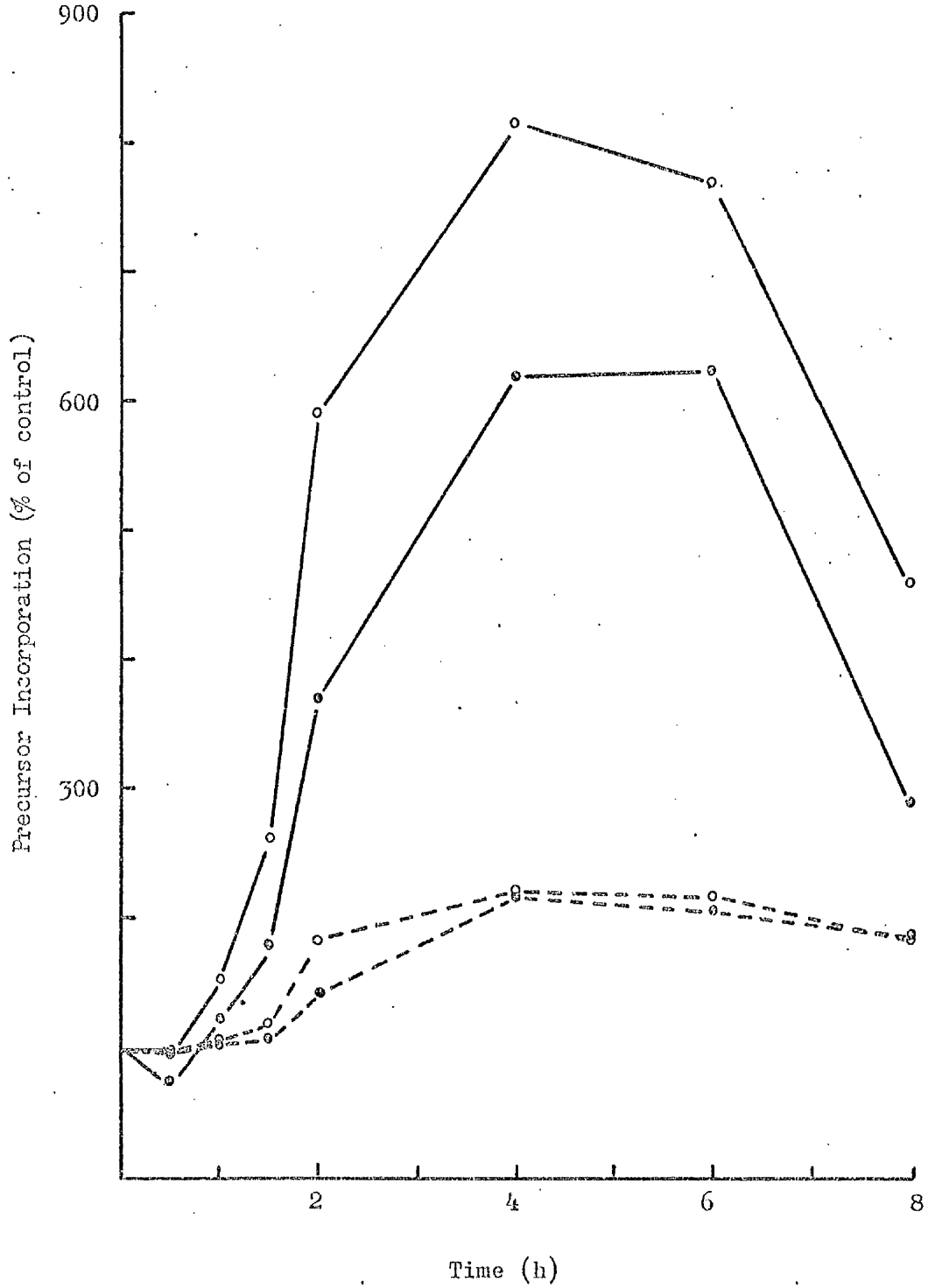
Hormone was administered intraperitoneally and 10μCi each of [5-^{3}H]uridine and [8-^{3}H]guanosine were given intravenously 30min before death. Results were calculated as dpm/μg DNA and expressed as a percentage of the uptake in non-hormone treated controls. Each point represents a mean of at least three animals.

o = 20-25g rats.

• = 25-30g rats.

——— = Acid-insoluble fraction.

- - - = Acid-soluble fraction.


900
600
300
Precursor Incorporation (% of control)
2
4
6
8
Time (h)

time course of incorporation of precursors into the acid-soluble and insoluble fractions of uteri from 18-21 day old rats selected in 5g weight ranges. The greatest response is observed in 20-25g rats where the peak of incorporation into the acid-insoluble fraction is 12 times the control level at 4h after the administration of oestradiol-17β (1μg/rat). 25-30g rats, which made up the bulk of the numbers, peak at 10 times control incorporation, whilst in 30-35g rats the maximal response is reduced to 7.5 fold. Sufficient rats in the age range and weighing 35-40g were not available for a complete time course, but individual experiments indicated that the peak response is reduced to less than 6 times the control value. Incorporation of radioactivity into the acid-soluble fraction follows a similar pattern in all the weight ranges, peaking at 2-2.6 times the control level, 4h after hormone administration.

Fig. 4 shows the results of an identical experiment to that of Fig. 3 except that the rats received 0.1μg of oestradiol. This level of hormone produces slightly less than the maximal response (Fig. 2), and the time courses are very similar to those in Fig. 3 with the peak for 25-30g rats and 30-35g rats being reduced to 8 and 6 times the control values respectively.

2. Oestrogen-Stimulated RNA Synthesis in Immature Rat Uterus in Vitro.

2. 1. Effects of Modifications to the Incubation Media.

In the early studies on the biochemical effects of oestradiol-17β, extensive use was made of a system whereby uteri of hormone-treated rats were placed in an incubation medium in order to

Figure 5.

Time course for the effect of oestradiol-17β on the incorporation of DNA precursor into 18-20 day old rat uteri in vivo and in vitro.

(a) Oestradiol-17β (1μg/rat) was administered intraperitoneally at various times and 10μCi of each of [5-^{3}H]uridine and [8-^{3}H] guanosine were given intravenously 30min before death. Each point is the mean of at least three animals.

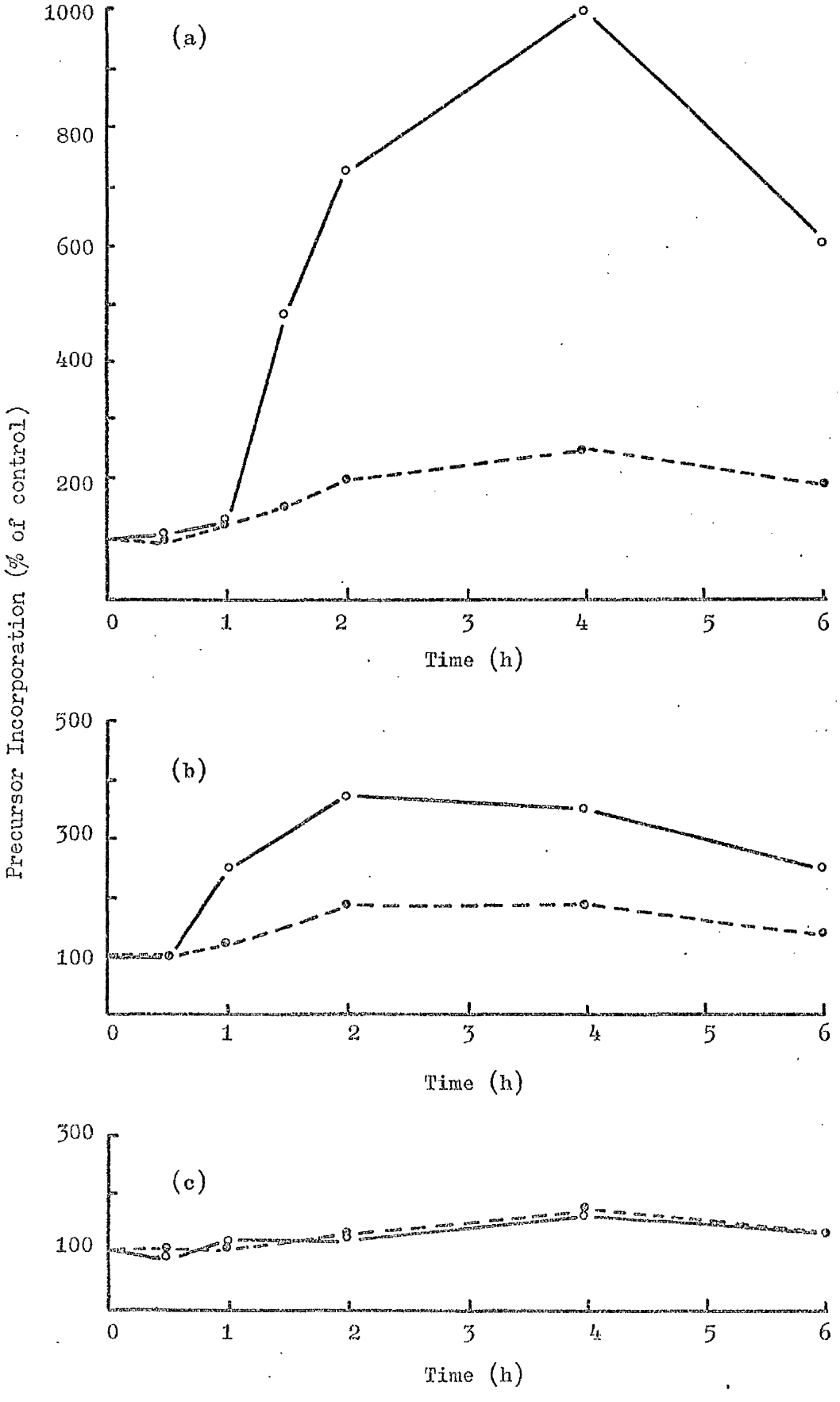
(b) Oestradiol-17β (5μg/rat) given at varying times and 10μCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine given 30min before death, were both administered intraperitoneally. Each point is a mean of three animals.

(c) 5μg of oestradiol was given intraperitoneally at various times before death. The excised uteri were incubated in vitro individually, as described in the Materials and Methods section, in 2ml of Eagle's medium containing 0.1μCi/ml of each of [5-^{3}H] uridine and [8-^{3}H]guanosine. The incubation was for 30min. Each point is a mean of three animals.

All results were calculated as dpm/μg DNA and expressed as a percentage of the uptake in non-hormone treated controls.

——o—— = Acid-insoluble fraction.

- -o- - = Acid-soluble fraction.


(a)
1000
800
600
400
200
0
1
2
3
4
5
6
Time (h)
Precursor Incorporation (% of control)
(b)
500
300
100
0
1
2
3
4
5
6
Time (h)
(c)
300
100
0
1
2
3
4
5
6
Time (h)

follow any stimulation in the uptake of precursors. Using these techniques, Mueller (1953) demonstrated increased incorporation of amino acids into proteins, Aizawa and Mueller (1961) observed the increased uptake of acetate and phosphate into lipids and Mueller and Herranen (1956) recorded increased incorporation of one carbon precursors into metabolic components. However, Gorski and Nicolette (1963), using a similar system, found only a marginal increase in the incorporation of precursors into RNA. My findings are in agreement with this observation and Fig. 5 shows a comparison between the oestrogen-stimulated incorporation of radioactivity into RNA in vivo with the uptake of precursor in vitro. The in vivo incorporation, Fig. 5(a) and 5(b), again emphasizes the effect of injection route on the uptake of precursor and reveals that when radioactivity is administered intraperitoneally, there are differences not only in the degree of stimulation but also in the time at which response is maximal.

Fig. 5(c) illustrates the results of an experiment in which immature rats were first treated with oestradiol-17β, and then the excised uteri were incubated in Eagle's medium in the presence of tritiated ribonucleosides. The effects of the hormone pretreatment on the in vitro RNA synthesis were slight. When rats received oestradiol 4h before death, the incorporation of precursor was increased 50% over that of control animals but at this, and all other times, the uptake into the acid-insoluble fraction was mirrored by increases in acid-soluble radioactivity.

It seemed possible that this poor response was caused by deficiencies in the incubation system or by components active in hormone action leaching out of the uteri. Accordingly, the effect

Table 1.

The rats received 1μg oestradiol-17β 2h before death. The excised uteri were incubated *in vitro* in 2ml of Eagle's medium, as described in the Materials and Methods section, in the presence of 0.1μCi/ml of each of [5-^{3}H]uridine and [8-^{3}H] guanosine. Steroids were added to the incubation medium in 0.1ml of saline containing 0.5% ethanol and the remaining additions were in saline only, though the solution was made slightly alkaline to dissolve epinephrine. The incubations were continued for 30min and the acid-soluble and acid-insoluble radioactivity was calculated as dpm/μg DNA and expressed as a percentage of controls which had no additions to the *in vitro* system.

TABLE I (A) The Effect of Various Hormones on the Incorporation of Nucleosides, In Vitro, into Uteri from Oestrogen-treated rats.

ADDITIONS	NUCLEOSIDE INCORPORATION (% of non-hormone treated controls)	
	ACID SOLUBLE FRACTION	ACID INSOLUBLE FRACTION
None	123	157
10^{-6}M Oestradiol 17B	138	169
10^{-6}M Oestrone	152	194
10^{-6}M Oestrone Sulphate	233	261
10^{-6}M Diethylstilbestrol	107	116
10^{-6}M Progesterone	121	141
10^{-6}M Oestradiol-17B plus 10^{-6}M Progesterone	125	156
10^{-4}M Dibutyryl Cyclic AMP	111	127
10^{-6}M Oestradiol-17B plus 10^{-4}M Dibutyryl Cyclic AMP	106	123
10^{-6}M Epinephrine	101	108
10^{-3}M Histamine	140	121

(B) The Effect of Increased Aeration on the Incorporation of Nucleosides In Vitro by Uteri from Oestrogen Treated Rats.

CONDITIONS	NUCLEOSIDE INCORPORATION (% of non-hormone treated controls)	
	ACID SOLUBLE FRACTION	ACID INSOLUBLE FRACTION
Oxygen/CO_2 (95:5) continuously aerating the medium	131	137
Whole uterine horns supported on lens tissue rafts	127	139
Shredded uterine horns supported on lens tissue rafts	127	48
Finely chopped uterine horns supported on lens tissue rafts	77	69

of various additions to the incubation medium was investigated. The results of some of these are shown in Table 1(A). Only the three oestrogenic steroids give rise to increased levels of precursor incorporation and in each case the increases they bring about occur in both the acid-soluble and acid-insoluble fractions. It is likely, therefore, that the effects of these hormones are on the rate of precursor entry into the tissue.

A number of modifications to the basic incubation medium were also investigated. These included the addition of various amounts of bovine serum, or foetal bovine serum, to the medium and the substitution of Eagle's medium with the complex medium, NCTC 109. These alterations did not stimulate the in vitro synthesis of DNA.

Fig. 6 shows histological preparations comparing the uterus immediately after removal from the rat with uteri which have been incubated in vitro for 6h. It is clear that after incubation for this length of time, the endometrium was unhealthy and the epithelium lining the lumen sloughing. Similar results were obtained whether the uteri were incubated in vitro with or without oestrogen.

Electron microscopy has recently been used to investigate the morphology of the mouse uterus after incubation in a synthetic medium, (Ljungkvist & Terenius, 1970). This technique enabled these workers to demonstrate that after incubation for periods as short as 30min, pronounced mitochondrial and nuclear changes were apparent in all cell layers, particularly the epithelium. Mitochondria were swollen with the inner chistae lost and the chromatin of nuclei was often condensed to a few spots. Some cells were totally disintegrated. It was also found that even without incubation, the time taken to fix fresh tissue was sufficient for some

Figure 6.

The effect of *in vitro* incubations on the morphology of the immature rat uterus.

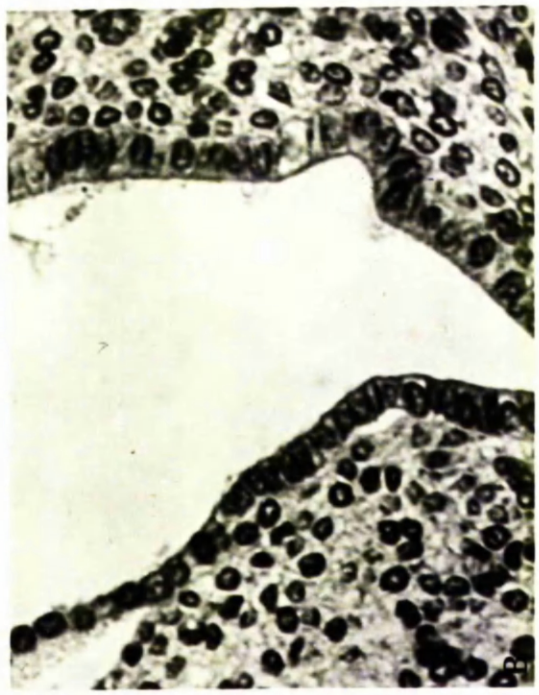
18-21 day old rats were administered oestradiol-17β at 1μg/rat and, after varying lengths of treatment, the uteri were gently excised, fixed, sectioned and stained. The uteri of other non-treated rats were incubated *in vitro*, with or without oestrogen, for up to 6h before they too were used for histological preparations.

(A) = Oestrogen treatment *in vivo* for 6h, magnification x 450.

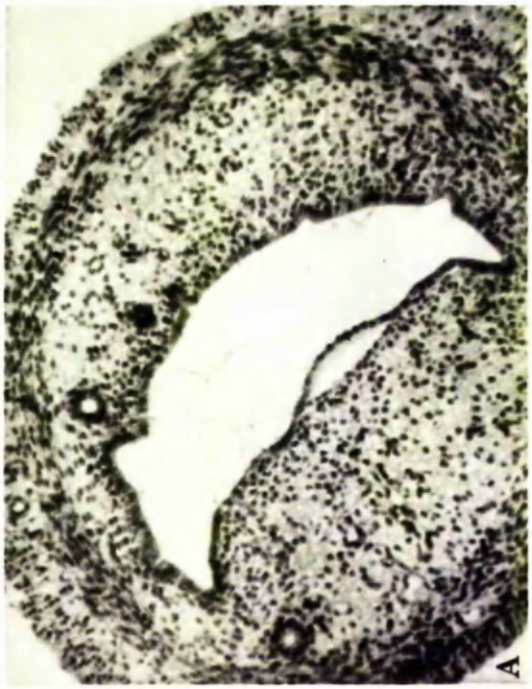
(B) = Oestrogen treatment *in vivo* for 6h, magnification x 1800.

(C) = Oestrogen treatment *in vitro* for 6h, magnification x 450.

(D) = Oestrogen treatment *in vitro* for 6h, magnification x 1800.


B


D


A


C

mitochondrial damage to occur.

A possible explanation of this cellular breakdown is that the uterine cells are very sensitive to anoxia. Accordingly, various means of increased aeration were tried. These included continuous bubbling of 95% O_2/5% CO_2 through the incubation medium while the tissue itself was variously sliced, shredded or supported on lens tissue rafts. Table 1(B) shows that none of these alterations resulted in significant increases in the incorporation of RNA precursors into the tissue but the damage caused by excessive maceration reduced uptake.

3. Effects of Oestrogen on the Synthesis of High-Molecular-Weight Uterine RNA.

3. 1. Purification of High-Molecular-Weight Uterine RNA.

In all studies on purified uterine RNA, a standard system was used in which 18-21 day old rats, weighing 25-30g, were treated with 1µg of oestradiol-17β administered intraperitoneally. The effects of this treatment upon RNA synthesis were determined by following the incorporation of intravenously administered radioactive ribonucleoside precursors.

The investigation of RNA synthesis in a differentiating system, such as a target tissue responding to hormone, demands an efficient and reliable method of RNA purification which extracts all species of RNA and avoids concomitant degradation. The earliest changes in the synthesis of uterine RNA in response to oestrogen might be expected to take place in the nucleus but the purification of uterine nuclei is difficult and liable to result in RNA

degradation. Because of this, it was decided to prepare total RNA from uteri and to ensure that in so doing the nuclear species were extracted without degradation. These difficulties have not always been recognized in previous studies of oestrogen action although Joel & Haggerman (1969) have compared many methods of RNA extraction from immature rat uteri and have described one which fulfilled the above criteria. Only this method successfully preserved the rat uterus RNA species with sedimentation coefficients greater than 28S.

The recovery of total uterine RNA by the method of Joel & Haggerman (1969) is shown in Table 2. It can be seen that, after the first phenol extractions and precipitation in the presence of NaCl and ethanol (the modified extraction procedure of Warner et al., 1966), there is considerably more DNA than RNA in the preparation. Subsequent purification, essentially by the method of DiGirolamo et al. (1964), completely removes the DNA, as detected by the diphenylamine reaction, but in my hands also results in losses of RNA. Later results will show that the losses of 4S RNA during this purification are substantial but losses of high-molecular-weight RNA does not result in differences in the relative abundance of the various species.

The nature of the RNA species purified from immature rat uteri was investigated by analytical centrifugation. Band sedimentation values of two major peaks were found to be 29.6S and 18.7S, while a third minor peak had a value of 3.6S. Despite the slightly high values, which may have been caused by traces of SDS or alcohol in the preparation, these results confirm that the bulk of the RNA isolated is derived from the large and small ribosomal subunits.

Table 2.

The uterine RNA of eight 18-21 day old rats, weighing 25-30g, was purified by the method of Joel & Haggerman (1969) (see Materials and Methods). At each precipitation step an aliquot was removed, precipitated and washed with 5% trichloroacetic acid at 0-4°, digested in 0.5M perchloric acid at 70° for 1h and used to assay RNA and DNA. The results are expressed as a percentage of total uterine content (110µg RNA and 225µg DNA) after correction had been made for sampling losses.

TABLE 2

The Purification of RNA from the Uterus of the Immature Rat.

PRECIPITATE	RNA		DNA	
	µg/rat	% recovery	µg/rat	% recovery
1st NaCl/EtOH precipitate	92.5	84.0	139.0	61.7
2nd NaCl/EtOH precipitate	49.6	45.0	44.0	19.4
1st K acetate/EtOH precipitate	49.3	44.7	4.8	2.1
3rd K acetate/EtOH precipitate	48.8	44.4	0	0

3. 2. Separation of High-Molecular-Weight RNA on Polyacrylamide Gels.

Initially, RNA purified by the method of Joel & Haggerman (1969) was separated on 2.0-2.7% polyacrylamide gels as described by Loening (1967) but using the electrophoresis buffer of Loening (1969). This method gave adequate separations when RNA was electrophoresed for 2-2½h, a length of time suitable for separating 28S, 18S and 4S RNA on one gel (Fig. 7a). However, during longer electrophoretic separations, suitable for investigating RNA with sedimentation values greater than 28S, the peaks of RNA became very diffuse (Fig. 7b). This problem did not occur if the bisacrylamide crosslinker used by Loening (1967) was replaced by ethylene diacrylate. Figs. 7(c) and 7(d) show the separation of RNA on gels containing 2.7% polyacrylamide and 0.25% ethylene diacrylate. Using these modified gels, the peaks corresponding to rRNA remain sharp whether the gels were run for 2¼h or 5h. Ethylene diacrylate was, therefore, used as the crosslinker in all subsequent experiments.

Two further points are noteworthy regarding these early gel separations. Firstly, Figs. 7(a) and 7(c) illustrate the low yield of 4S RNA in these preparations. Secondly, the optical density traces of these gels tend to be "U" shaped with a hump at either end. This always occurred, even with blank gels containing no RNA, but it was found that if the gels were soaked in water for 30min before scanning at 260nm, the humps largely disappeared. Presumably they are due to some diffusable substance which absorbs at 260nm and which accumulates at the two ends of the gel in contact with the electrophoresis buffer.

Figure 7.

Comparison between bisacrylamide and ethylene diacrylate as crosslinkers in polyacrylamide gels.

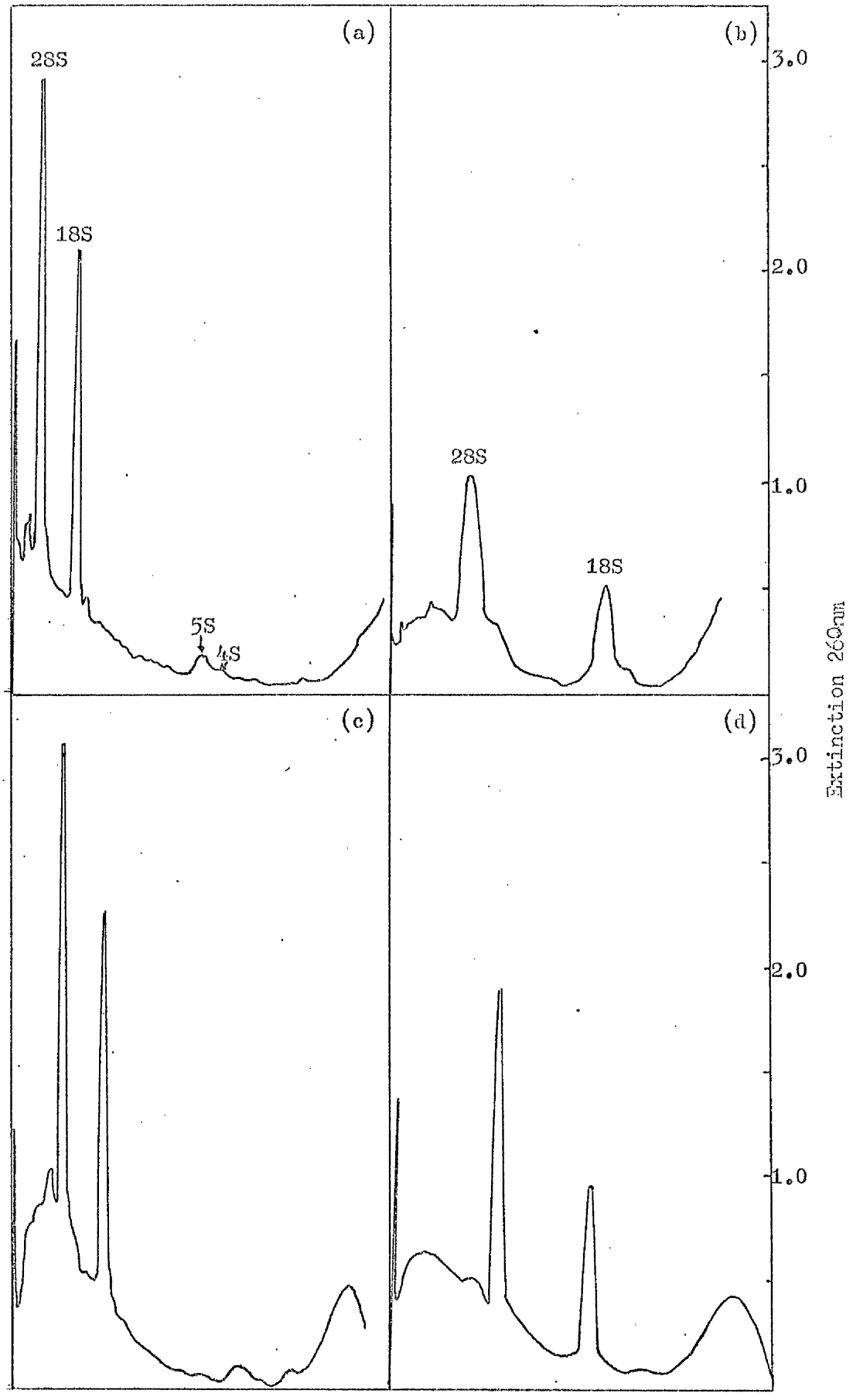
RNA purified from the uteri of 18-21 day old rats, weighing 25-30g, was separated on 2.7% acrylamide gels at 5mA/gel, as described in the Materials and Methods section. The extinction at 260nm was recorded with the aid of a gel scanning attachment for the Gilford 240 spectrophotometer.

(a) = 2½h electrophoresis in a polyacrylamide/ bisacrylamide gel.

(b) = 5h electrophoresis in a polyacrylamide/ bisacrylamide gel.

(c) = 2½h electrophoresis in a polyacrylamide/ ethylene diacrylate gel.

(d) = 5h electrophoresis in a polyacrylamide/ ethylene diacrylate gel.


(a)
28S
18S
5S
4S
(b)
28S
18S
(c)
(d)
3.0
2.0
1.0
3.0
2.0
1.0
Extinction 260nm

3. 3. Effect of Oestrogen on Uterine High-Molecular-Weight RNA Synthesis.

3. 3. 1. 15min Pulse of Precursors.

RNA from the uteri of 18-21 day old rats, weighing 25-30g, was separated for 5h on 2.7% polyacrylamide gels. Fig. 8 shows the effect of oestradiol on the incorporation of tritiated ribonucleosides into RNA at various times after oestradiol treatment and 15min after administration of the radioactive precursor. Incorporation into control animals is low and confined to RNA species of very-high-molecular-weight. 15min after oestradiol administration (not shown in Fig. 8), incorporation of precursor is identical to the control but after 30min treatment there is a marked increase in the incorporation into RNA species that remained in the first two or three slices of the gel. This increase is similarly apparent 45min and 1h after oestradiol administration. It is interesting that after 30min and 45min treatments, the synthesis of RNA migrating in the position of the ribosomal precursors appears slightly reduced. This decrease, although reproducible, is small and may not be significant. It seems possible, however, that a temporary reduction of rRNA synthesis, during initial oestrogen action, could be the result of precursors being used for the synthesis of very-high-molecular-weight RNA. Furthermore, such a shift in emphasis could explain why this early response to the hormone is not detected in uterine acid-insoluble preparations (Fig. 3). By 1h after hormone treatment, the synthesis of RNA migrating in the position of ribosomal precursors is restored to control levels and continues to rise from that time. After 2h and 4h of hormone treatment, there is a strongly labelled

Figure 8.

Incorporation into uterine RNA of radioactive precursor administered 15min before death.

18-21 day old rats, weighing 25-30g, received 1µg of oestradiol or vehicle by intraperitoneal injection and 125µCi each of [5-^{3}H]uridine and [8-^{3}H]guanosine by intravenous injection. Purified uterine RNA was separated for 5h in 2.7% polyacrylamide gels at 5mA/gel.

(a) = Control.

(b) = 30min after oestradiol-17β administration.

(c) = 45min after oestradiol-17β administration.

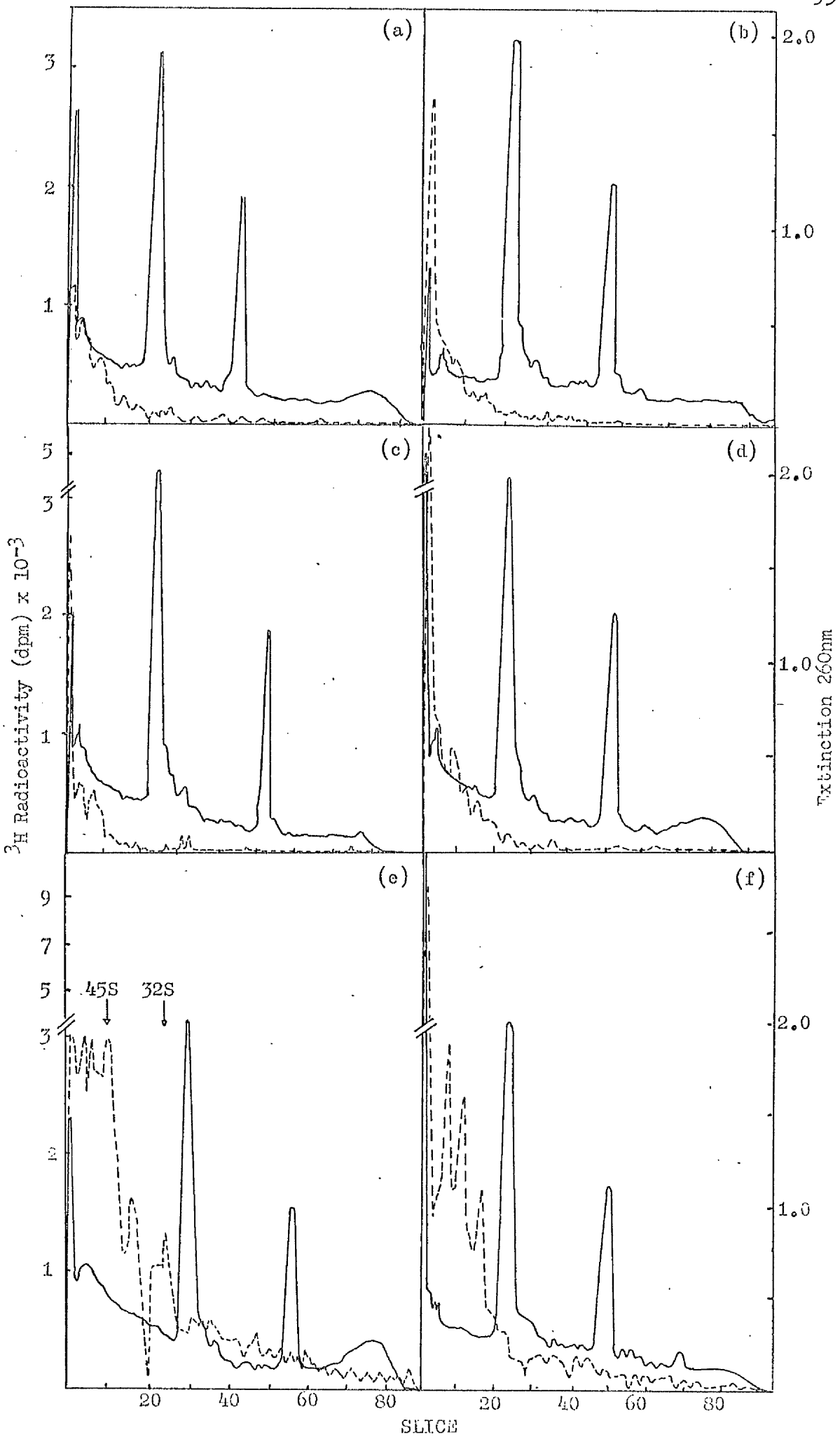
(d) = 1h after oestradiol-17β administration.

(e) = 2h after oestradiol-17β administration.

(f) = 4h after oestradiol-17β administration.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


(a)
(b)
(c)
(d)
(e)
(f)
45S
32S
^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm
SLICE
20
40
60
80
1.0
2.0

peak corresponding to 45S rRNA and a pronounced peak corresponding to the 32S precursor. The former of these two peaks was not always clearly separated from the radioactivity corresponding to higher-molecular-weight species and in general the ribosomal precursor RNA peaks tended to be superimposed on a background of heterogeneous radioactivity.

3. 3. 2. 30min Pulse of Precursor.

Fig. 9 shows the results of similar experiments in which the radioactive ribonucleosides were given 30min before the death of the rat. Here again, the earliest response is in the increased incorporation of precursor into RNA species remaining in the first few slices of the gel. With the longer pulse length this early stimulation is not detectable quite so soon. Thus, 30min after oestradiol treatment, where hormone and precursor are given simultaneously, there is only a marginal stimulation in the synthesis of the very-high-molecular-weight RNA. Presumably this is because much of the labelled precursor is utilised before the hormone begins to act. By 1h the increased synthesis of RNA species remaining in the first few slices of the gels is clearly discernible and, at 2 and 4h after hormone administration, incorporation into ribosomal precursor RNA is again strongly stimulated. With the longer pulse length, radioactivity moves out of the 45S rRNA precursor into the 32S species and incorporation is also apparent in the RNA of the ribosomal subunits. Again due to differences in the degree of hormonal response at the time of precursor administration, the longer pulse length gives rise to more obvious differences between the results obtained 2 and 4h after hormone treatment than

Figure 9.

Incorporation into uterine DNA of radioactive precursor administered 30min before death.

18-21 day old rats, weighing 25-30g, received 1µg of oestradiol or vehicle by intraperitoneal injection and 125µCi each of [5-^{3}H]uridine and [8-^{3}H]guanosine by intravenous injection. Purified uterine DNA was separated for 5h in 2.7% polyacrylamide gels at 5mA/gel.

(a) = Control.

(b) = 30min after oestradiol-17β administration.

(c) = 1h after oestradiol-17β administration.

(d) = 2h after oestradiol-17β administration.

(e) = 4h after oestradiol-17β administration.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.

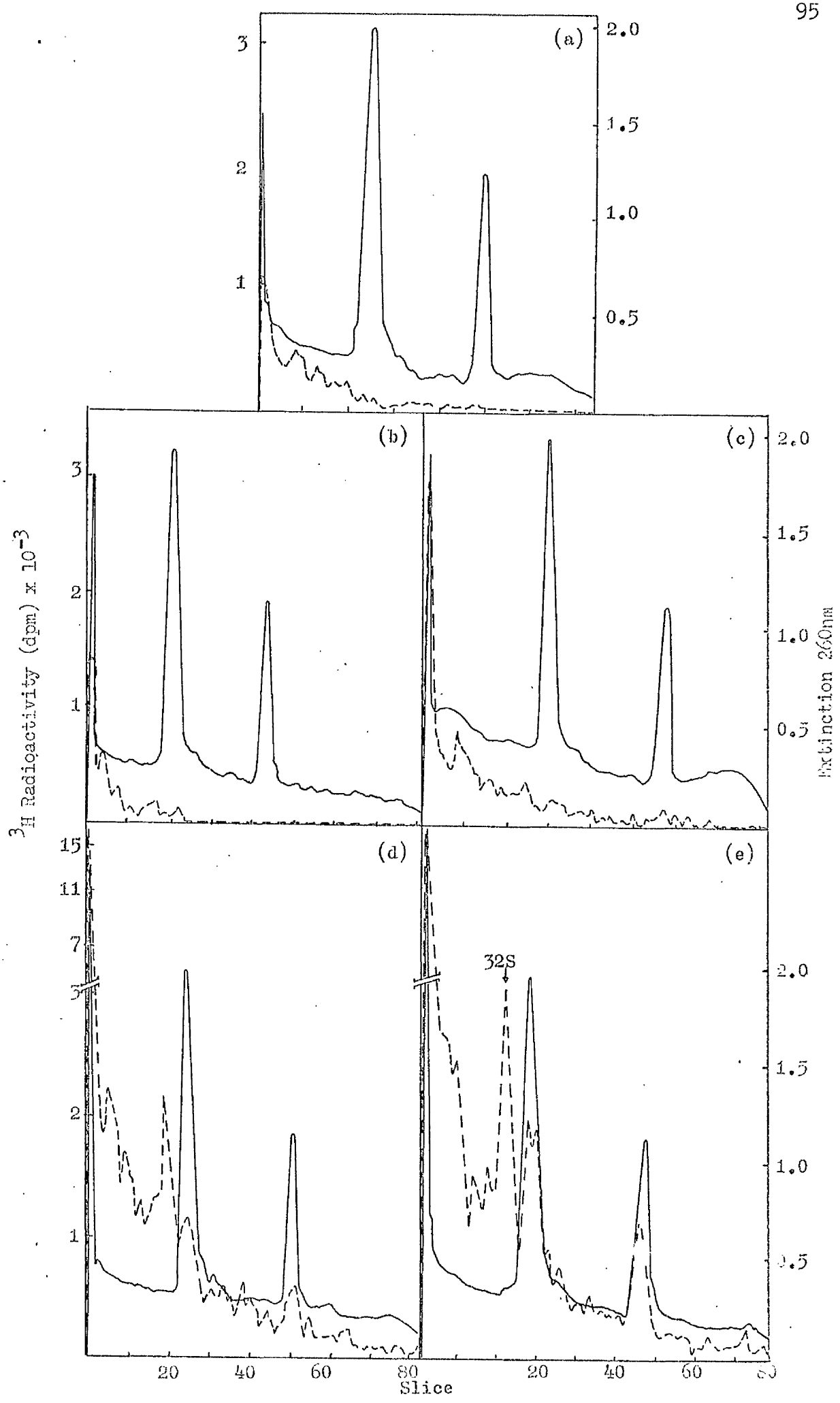

(a)
(b)
(c)
(d)
(e)
^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm
Slice
32S
20
40
60
80

Figure 10.

Incorporation into uterine RNA of radioactive precursor administered 1h before death.

18-21 day old rats, weighing 25-30g, received 1µg of oestradiol or vehicle by intraperitoneal injection and 125µCi each of [5-^{3}H]uridine and [8-^{3}H]guanosine by intravenous injection. Purified uterine RNA was separated for 5h in 2.7% polyacrylamide gels at 5mA/gel.

(a) = Control.

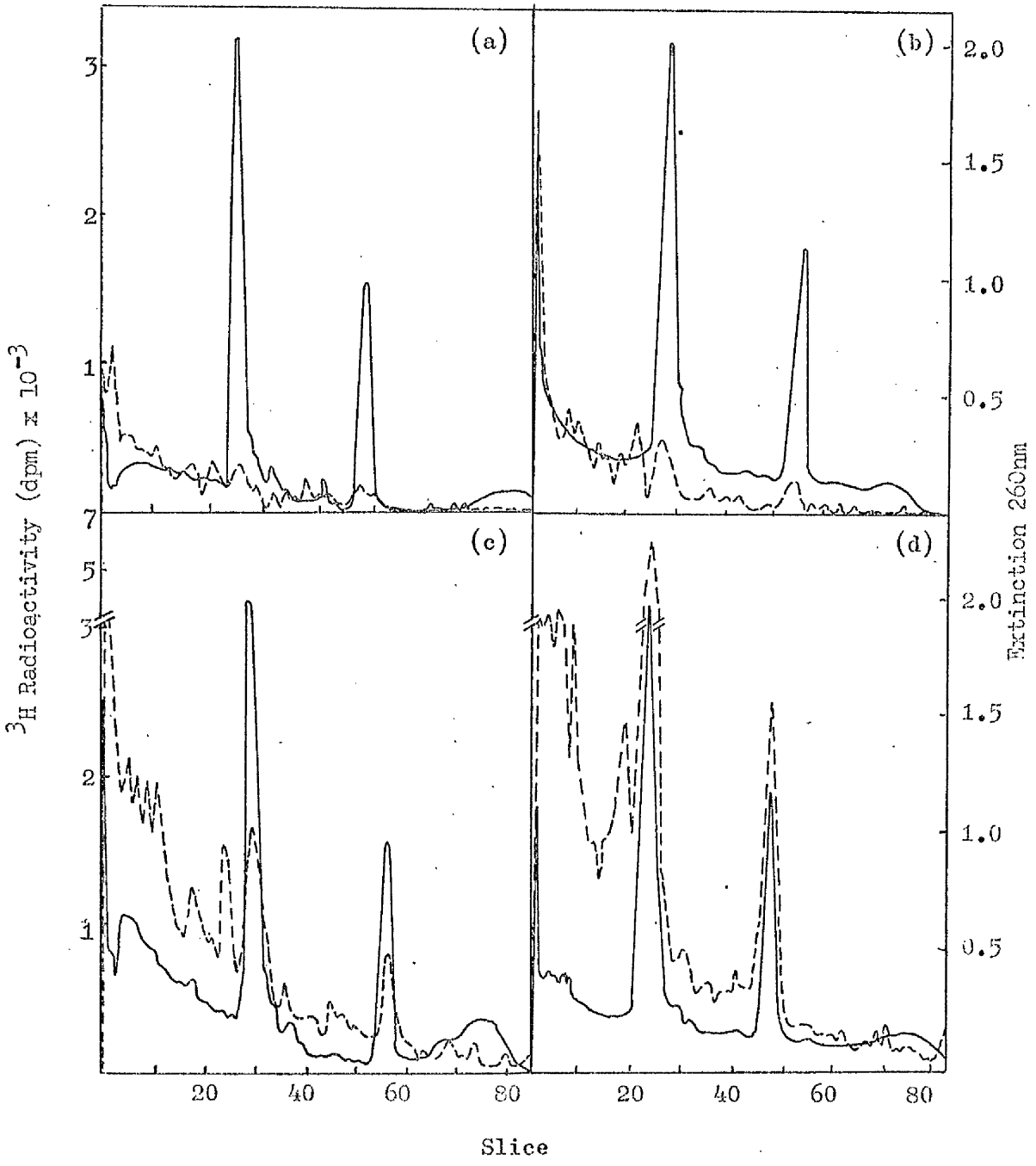
(b) = 1h after oestradiol-17β administration.

(c) = 2h after oestradiol-17β administration.

(d) = 4h after oestradiol-17β administration.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


(a)
(b)
(c)
(d)
^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm
Slice
20
40
60
80
0.5
1.0
1.5
2.0
1
2
3
5
7

was observed when 15min incorporation periods were used. As in the previous experiment (Fig. 8), the synthesis of ribosomal precursor RNA appears slightly reduced after 30min of hormone treatment.

3. 3. 3. 60min Pulse of Precursor.

Fig. 10 shows the distribution of radioactivity in RNA species of rat uterus 1h after administration of precursors. The increased incorporation into RNA species remaining in the first few slices of the gel is again the earliest observable change and is clearly apparent 1h after hormone treatment of the rats. At later times, 2 and 4h after oestradiol administration, incorporation into the RNA of the ribosomal subunits is strongly stimulated with incorporation into the pre-ribosomal species still apparent.

3. 4. Methylation of Uterine High-Molecular-Weight RNA.

From Figs. 8, 9 and 10 it can be seen that with increasing time after precursor administration the radioactivity in the RNA appears to move from a peak, assumed to be 45S RNA, through a second peak, assumed to be 32S RNA, and finally into the rRNA species. The identities of the 45S and 32S RNA peaks have been confirmed by methylation experiments.

Attempts to methylate uterine RNA *in vivo* showed that incorporation was much too low for the experiment to yield meaningful results. Therefore, in these experiments, one sample of ^{3}H-labelled RNA was prepared in the usual way from rats receiving 4h oestradiol treatment and 30min after administration of radioactive precursors. A second sample of RNA was prepared after labelling the uteri with [methyl-^{14}C] methionine *in vitro* as described in the Methods section,

Figure 11.

Methylation of uterine RNA.

18-21 day old rats, weighing 25-30g, received intraperitoneal injections of 1µg of oestradiol-17β 4h before death. The uteri were either labelled *in vivo* with a 30min pulse of 125µCi of each of [5-^{3}H]uridine and [8-^{3}H] guanosine or were incubated *in vitro* with 5µCi/ml of [methyl-^{14}C] methionine. The purified RNAs were mixed and separated on 2.7% acrylamide gels for 5h at 5ml/gel.

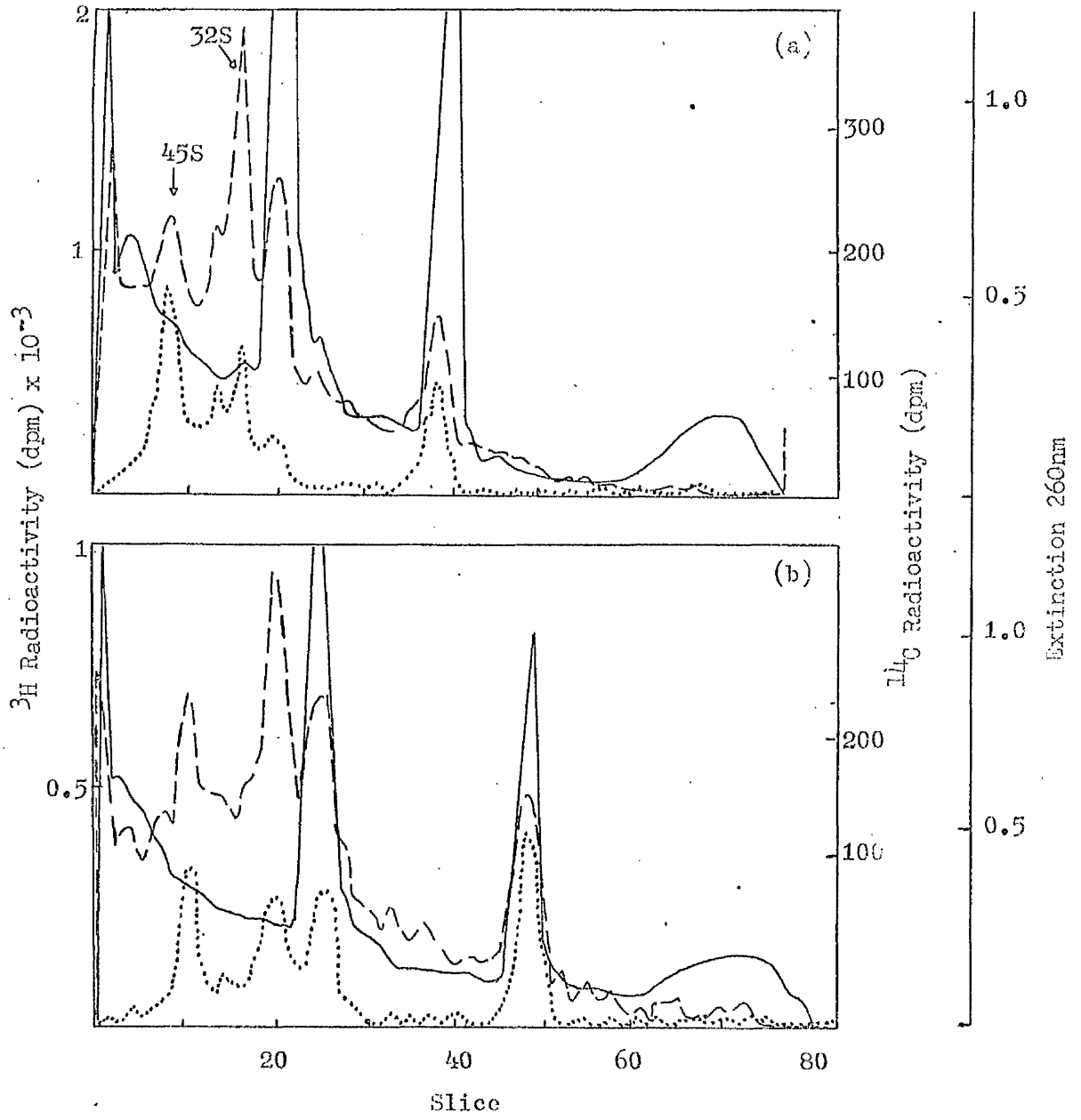
(a) = *In vitro* labelled for 30min.

(b) = *In vitro* labelled for 60min.

_____ = Extinction at 260nm.

\- - - = Radioactivity due to ^{3}H.

..... = Radioactivity due to ^{14}C.


32S
45S
(a)
(b)
2
1
0.5
300
200
100
1.0
0.5
20
40
60
80
Slice
^{3}H Radioactivity (dpm) x 10^{-3}
^{14}C Radioactivity (dpm)
Extinction 260nm

4h after oestradiol treatment of the rats. The ^{3}H and ^{14}C labelled RNA samples were mixed and fractionated on 2.7% polyacrylamide gels for 5h. Fig.11(a) shows the distribution of ^{3}H and ^{14}C in the various RNA species when the labelling with [methyl-^{14}C] methionine in vitro had been carried out for 30min and Fig. 11(b) shows the results obtained after 1h of in vitro methylation.

In Fig. 11(a), the RNA in the 45S, 32S and 18S peaks are methylated, whereas the 28S peak contains little ^{14}C. Fig. 11(b) shows that after 1h, a substantial amount of methylation of 28S RNA has taken place and that the 18S peak is still more heavily labelled. In both experiments, there is some evidence of other doubly labelled components migrating between 45S and 32S. They may represent short lived intermediates or degradation products as the existence of such short lived species is well documented (Weinberg et al., 1967) and they have been assigned sedimentation values of 41S and 36S. The sequence of methylation observed in Fig. 11 fits in with the rate of rRNA synthesis observed in Figs. 8, 9 and 10 and also with the now accepted sequence in the formation of rRNA (Scherrer et al., 1963; Soeiro et al., 1966; Greenberg & Penman, 1966). It is noteworthy that the high-molecular-weight RNA close to the gel origin does not appear to be methylated, either after 30min or 1h of precursor incorporation.

3. 5. Cofractionation of High-Molecular-Weight Uterine RNA with HeLa Cell Nucleolar RNA.

The identities of the uterine 45S and 32S RNA peaks have also been confirmed by cofractionation with known samples of ^{14}C labelled 45S and 32S RNA prepared from HeLa cell nucleoli. Uterine

Figure 12.

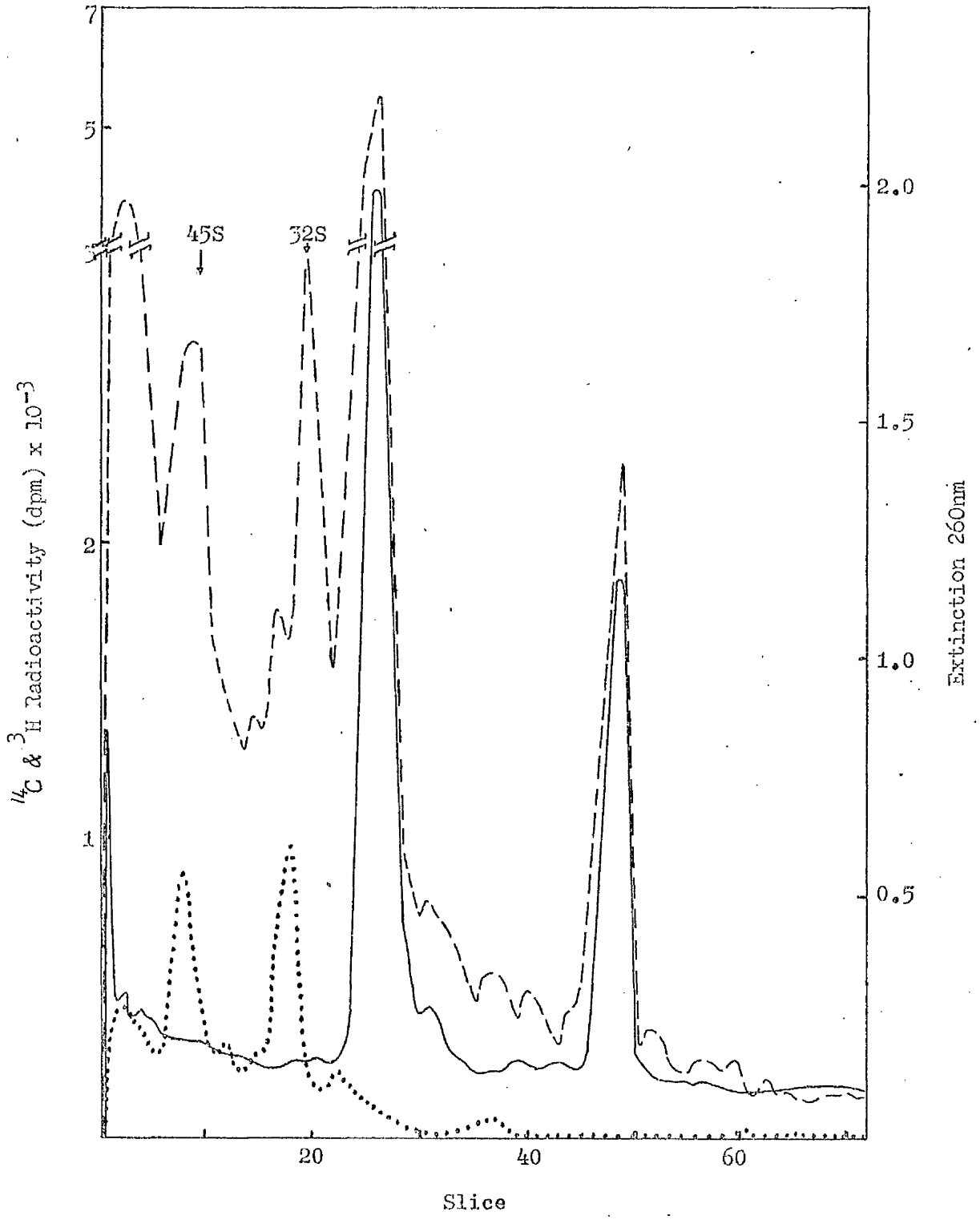
Comparison between uterine RNA and HeLa nucleolar RNA.

18-21 day old rats, weighing 25-30g, received 1µg oestradiol intraperitoneally 4h before death and 125µCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine by intravenous injection 1h before death. The purified RNA was co-electrophoresed with ^{14}C labelled HeLa nucleolar RNA, prepared as described in the Materials and Methods section. Electrophoresis was for 5h in a 2.7% polyacrylamide gel.

——— = Extinction at 260nm.

- - - = Radioactivity due to ^{3}H.

..... = Radioactivity due to ^{14}C.


45S
32S
7
5
3
2
1
20
40
60
Slice
2.0
1.5
1.0
0.5
Extinction 260nm
^{14}C & ^{3}H Radioactivity (dpm) x 10^{-3}

^{3}H labelled RNA was prepared from immature rats 4h after oestradiol treatment and 1h after administration of radioactive precursors. This was mixed with RNA prepared from the nucleoli of HeLa cells which had been grown in the presence of [2-^{14}C] uridine. HeLa cell nucleoli have been shown to contain predominantly 45S and 32S RNA (Perman et al., 1966). The combined RNAs were separated on 2.7% polyacrylamide gels for 5h and the results obtained are shown in Fig. 12.

The ^{14}C labelled HeLa nucleolar 45S and 32S RNA species essentially correspond to the ^{3}H labelled peaks of uterine RNA. They do, however, migrate slightly slower than the uterine preparation. It is possible that this reflects differences between rat uterus and HeLa cells or that it is an artifact of the mixing of the two different RNA preparations.

3. 6. The Inhibition of High-Molecular-Weight RNA Synthesis by Actinomycin D.

It has been established in tissue culture cells that actinomycin D inhibits rRNA synthesis to a greater extent than that of other RNA species (Perry, 1962). Experiments were therefore performed to determine whether this observation applied to the synthesis of uterine RNA. This was important for two reasons.

(1) If the synthesis of rRNA in the uterus was inhibited by actinomycin, without markedly affecting the higher-molecular-weight species, this would have provided a useful tool in investigating the nature of the latter species.

(2) DeAngelo & Gorski (1970) have demonstrated that the synthesis of a new protein, at short time intervals after

treatment of rat uteri with oestrogens, was only totally inhibited by high concentrations of actinomycin D. It was of interest, therefore, to determine whether some RNA was still made at relatively high levels of the inhibitor.

Fig.13 shows the effect of treating immature rats with various levels of actinomycin D. The inhibitor was given simultaneously with oestradiol 4h before the death of the animals and radioactive precursors were given 1h before death. Purified RNA was then separated on 2.7% polyacrylamide gels for 2¾h. The short electrophoresis time was designed to reveal the effects of the inhibitor on all RNA species simultaneously. However, the low yield of 4S RNA affected the validity of the results with respect to the low-molecular-weight RNA species and the effects of actinomycin D on these will be presented later.

As can be seen from Fig.13, the increased synthesis of rRNA in response to oestradiol was more susceptible to actinomycin D inhibition than the synthesis of other RNA species. This differential effect was not, however, as clear cut as in tissue culture systems (Perry, 1962). At doses of 15µg actinomycin D/rat, incorporation of radioactivity into rRNA was partially inhibited but incorporation into the RNA remaining in the first few slices of the gel was affected to a much smaller extent. When the dose of inhibitor was increased to 30µg/rat, synthesis of rRNA was almost totally eliminated whereas the synthesis of the very-high-molecular-weight RNA was still only partially inhibited and even when the dose of actinomycin D was increased to 120µg/rat, there was still some incorporation into this fraction.

Figure 13.

Effect of actinomycin D on oestradiol-17β-stimulated uterine DNA synthesis.

18-21 day old rats, weighing 25-30g, received 1μg of oestradiol-17β and actinomycin D simultaneously 4h before death. 125μCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine were administered 1h before death. Purified DNA was separated on 2.7% polyacrylamide gels for 2¼h.

(a) = No actinomycin D.

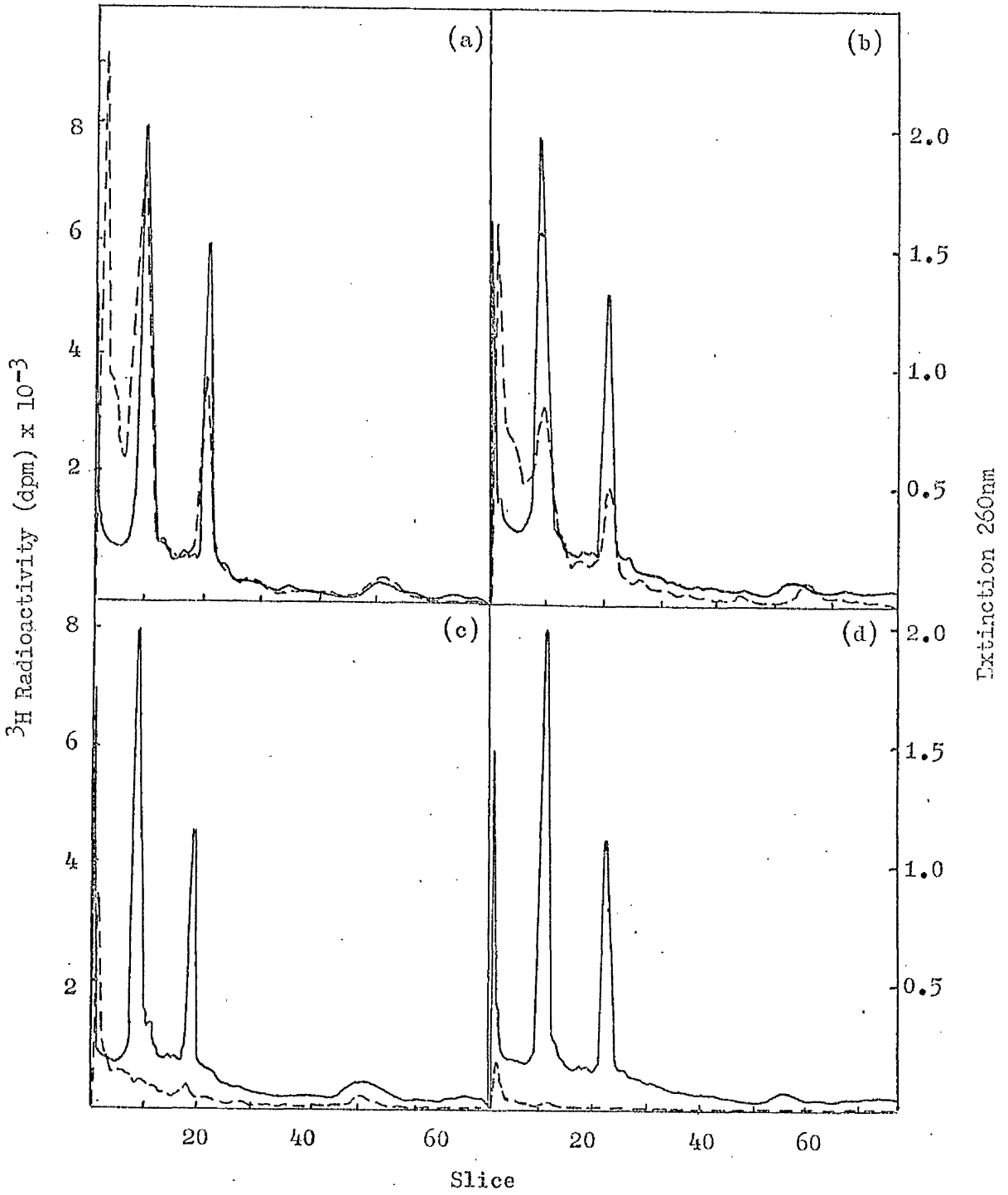
(b) = 15μg/rat of actinomycin D.

(c) = 30μg/rat of actinomycin D.

(d) = 120μg/rat of actinomycin D.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


(a)
(b)
(c)
(d)
^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm
Slice
8
6
4
2
2.0
1.5
1.0
0.5
20
40
60

3. 7. The Effect of Cycloheximide on Uterine Protein Synthesis.

The oestrogen-dependent increases in the synthesis of rRNA and in the activity of RNA polymerase are eliminated by inhibitors of protein synthesis (Noteboom & Gorski, 1963; Gorski & Axman, 1964). However, this finding does not necessarily mean that the increased production of ribosomes depends on the hormone-induced synthesis of protein. It is established that inhibitors of protein synthesis markedly affect RNA synthesis in situations not involving hormones (Pederson & Kumar, 1971; Cooper & Gibson, 1971) and it is likely that the same alterations occur in the uterus, even in the absence of any involvement of oestradiol. In view of this, it is important to establish the effects of inhibitors of protein synthesis on the synthesis of RNA in uterine tissue.

Fig. 14 shows the effect of cycloheximide inhibition on protein synthesis in immature rat uterus. The animals either received no hormone or injections of oestradiol 4h before death. Cycloheximide was administered at various doses 1h before death and 20μCi of [4,5-^{3}H] lysine was given intravenously 30min before death. Acid-insoluble fractions were prepared from the uteri and the radioactivity determined as described in the Materials and Methods section. Inhibition of [4,5-^{3}H] lysine uptake increased rapidly with rising dosage of cycloheximide and at 75μg/rat, reached 92.7% and 94% in control and hormone-treated animals respectively. At higher doses, the curve tended to form a plateau and more complete inhibition required high levels of inhibitor. Thus, 600μg cycloheximide/rat was required to obtain a 96-98% inhibition of protein synthesis.

Figure 14.

Effect of cycloheximide on uterine protein synthesis.

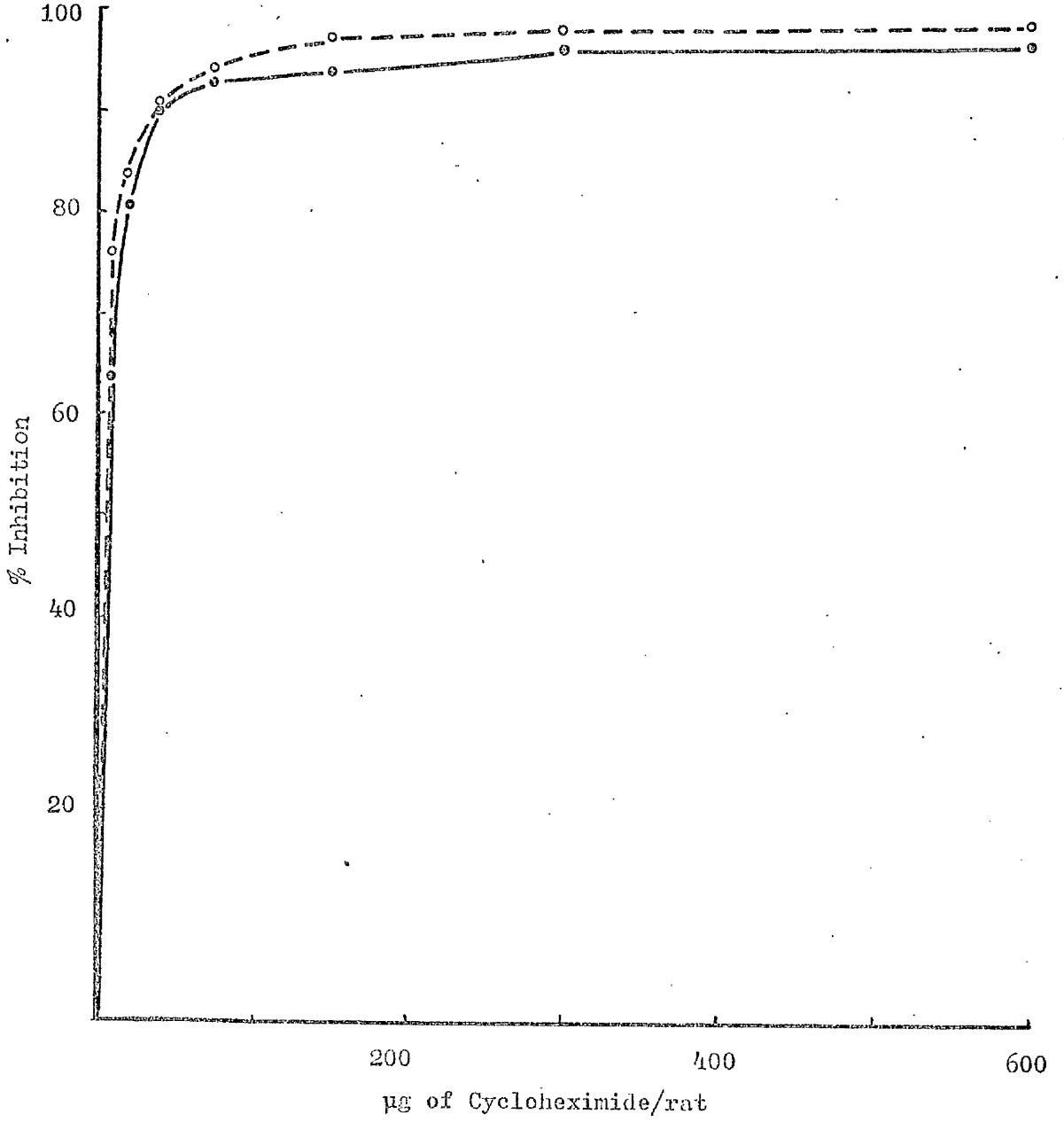
18-21 day old rats, weighing 25-30g, received 1µg of oestradiol-17β or vehicle 4h before death, cycloheximide 1h before death and 20µCi [4,5-^{3}H]lysine 30min before death. Incorporation of radioactivity was calculated as acid-insoluble dpm/µg DNA and expressed as a percentage of uninhibited controls. Each point represents a mean of two determinations.

—●— = Control rats.

---o--- = Hormone-treated rats.

Uninhibited control rats incorporated 44.8 dpm/µg DNA.

Uninhibited hormone-treated rats incorporated 77.5 dpm/µg DNA.


100
80
60
40
20
% Inhibition
200
400
600
µg of Cycloheximide/rat

The results show that the effects of cycloheximide were rapid since the inhibitor was injected only 30min before the administration of radioactive precursor. At the higher levels of inhibitor, the effects were also long-lasting; 600µg cycloheximide caused 96-98% inhibition of [4,5-^{3}H] lysine incorporation whether the inhibitor was given ½, 1½ or 3½h before the administration of the precursor. When lower doses of cycloheximide were used, however, the inhibition was less persistant. In hormone-treated rats receiving 60µg/rat of cycloheximide ½, 1½ and 3½h before the administration of [4,5-^{3}H] lysine, inhibition of precursor incorporation was 93%, 94% and 86% respectively. Furthermore, where animals were not treated with hormone, the effect of 60µg cycloheximide/rat was even more short lived and the inhibition of 92%, when there was 30min between treatment with inhibitor and injection of radioactive precursor, was reduced to 82% if the time interval was 1½h and to 64% when it was 3½h. It was interesting that the slightly increased susceptibility to cycloheximide, shown by the hormone-treated animals in Fig. 14, was exaggerated with the longer exposure to the inhibitor. Two other points arise from the experiments described above.

(1) In experiments without cycloheximide, the incorporation of [4,5-^{3}H] lysine into uterine protein in rats treated with oestradiol 4h before death was significantly higher (77.5 dpm/µg DNA) than in untreated animals (44.8 dpm/µg DNA).

(2) At higher doses of cycloheximide there was some evidence of toxic effects on the animals. They became slow in their movements and were more susceptible

to ether anaesthetisation.

On the basis of the above results, it was decided to investigate the effect of cycloheximide at 600µg/rat and 60µg/rat on the synthesis of RNA.

3. 8. Effects of Cycloheximide at 600µg/rat on the Synthesis of High-Molecular-Weight RNA.

Fig. 15 shows the effect of cycloheximide at 600µg/rat on the incorporation of radioactive precursors into uterine RNA. The immature rats were given oestradiol 4h before death, cycloheximide at various times before death and labelled ribonucleosides were administered 30min before death. When given 1h before oestradiol, cycloheximide severely curtailed the incorporation of radioactivity into all RNA species. Administration of cycloheximide simultaneously with oestradiol strongly inhibited incorporation into rRNA and the 45S and 32S pre-ribosomal peaks but the synthesis of very-high-molecular-weight RNA at the gel origin was affected much less. When the inhibitor was given 1h after oestradiol, the principle effect appeared to be the inhibition of rRNA maturation. Thus, after a 30min pulse of radioactivity, when the 32S RNA should have been most strongly labelled and the 28S and 18S RNA peaks should also have been labelled, only the 45S RNA peak showed marked incorporation.

3. 9. Effects of Cycloheximide at 60µg/rat on the Synthesis of High-Molecular-Weight RNA.

Fig. 16 shows the results of an identical experiment to that shown in Fig. 15 except that the rats received 60µg cyclo-

Figure 15.

Effect of cycloheximide at 600µg/rat on oestradiol-17β-stimulated uterine RNA synthesis.

18-21 day old rats, weighing 25-30g, received 1µg of oestradiol 4h before death and cycloheximide at various times. Both were given by intraperitoneal injection but 125µCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine was administered intravenously 30min before death. The purified RNA was separated on 2.7% polyacrylamide gels for 5h.

(a) = No cycloheximide treatment.

(b) = Cycloheximide given 1h before hormone.

(c) = Cycloheximide given simultaneously with hormone.

(d) = Cycloheximide given 1h after hormone.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.

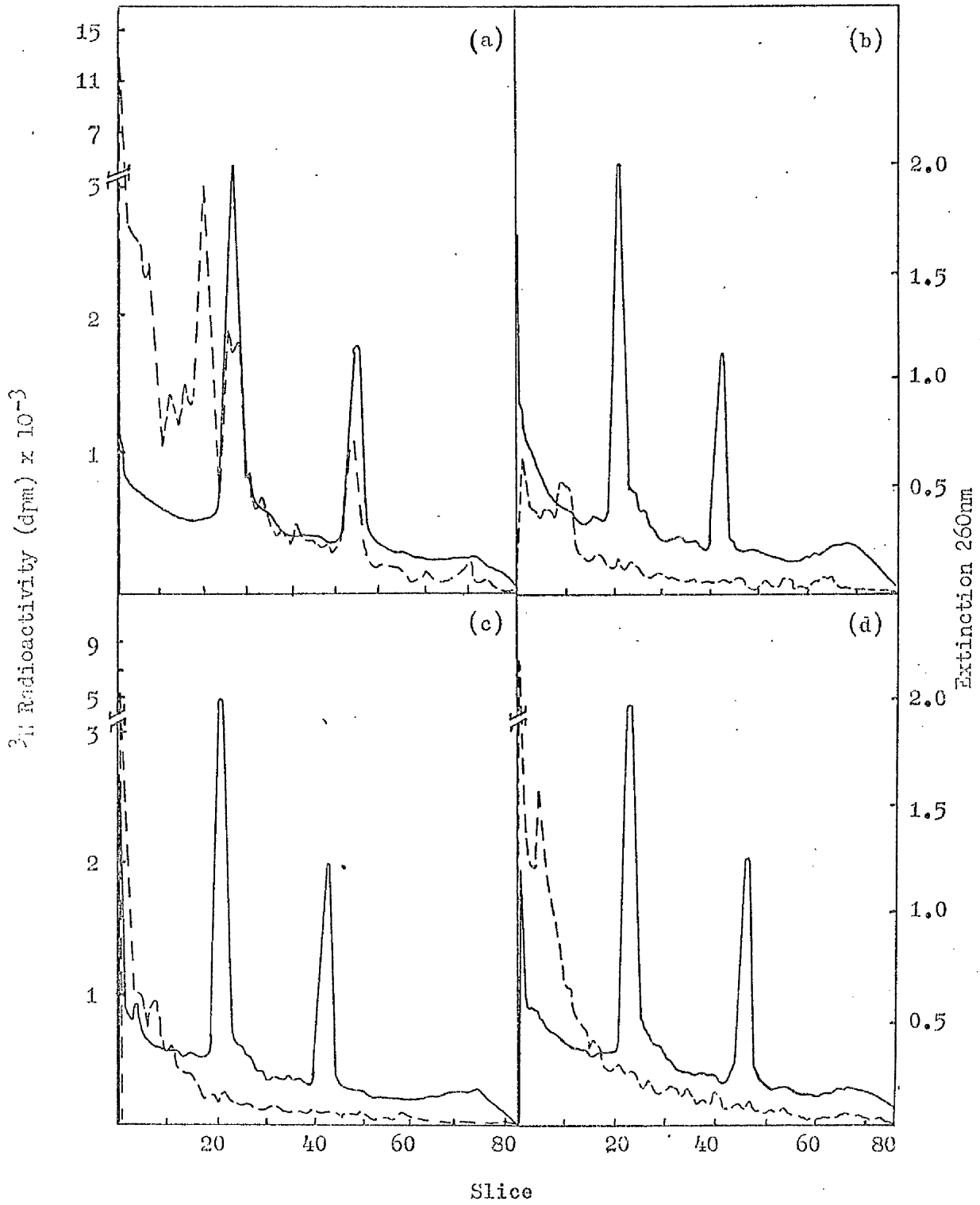

(a)
(b)
(c)
(d)
15
11
7
3
2
1
9
5
3
2
1
2.0
1.5
1.0
0.5
20
40
60
80
Slice
Extinction 260nm
^{3}H Radioactivity (dpm) x 10^{-3}

Figure 16.

Effect of cycloheximide at 60μg/rat on oestradiol-17β-stimulated uterine RNA synthesis.

18-21 day old rats, weighing 25-30g, received 1μg of oestradiol 4h. before death and 60 μg of cycloheximide at various times. Both were given by intraperitoneal injection but 125μCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine was administered intravenously 30min before death. The purified RNA was separated on 2.7% polyacrylamide gels for 5h.

(a) = No cycloheximide treatment.

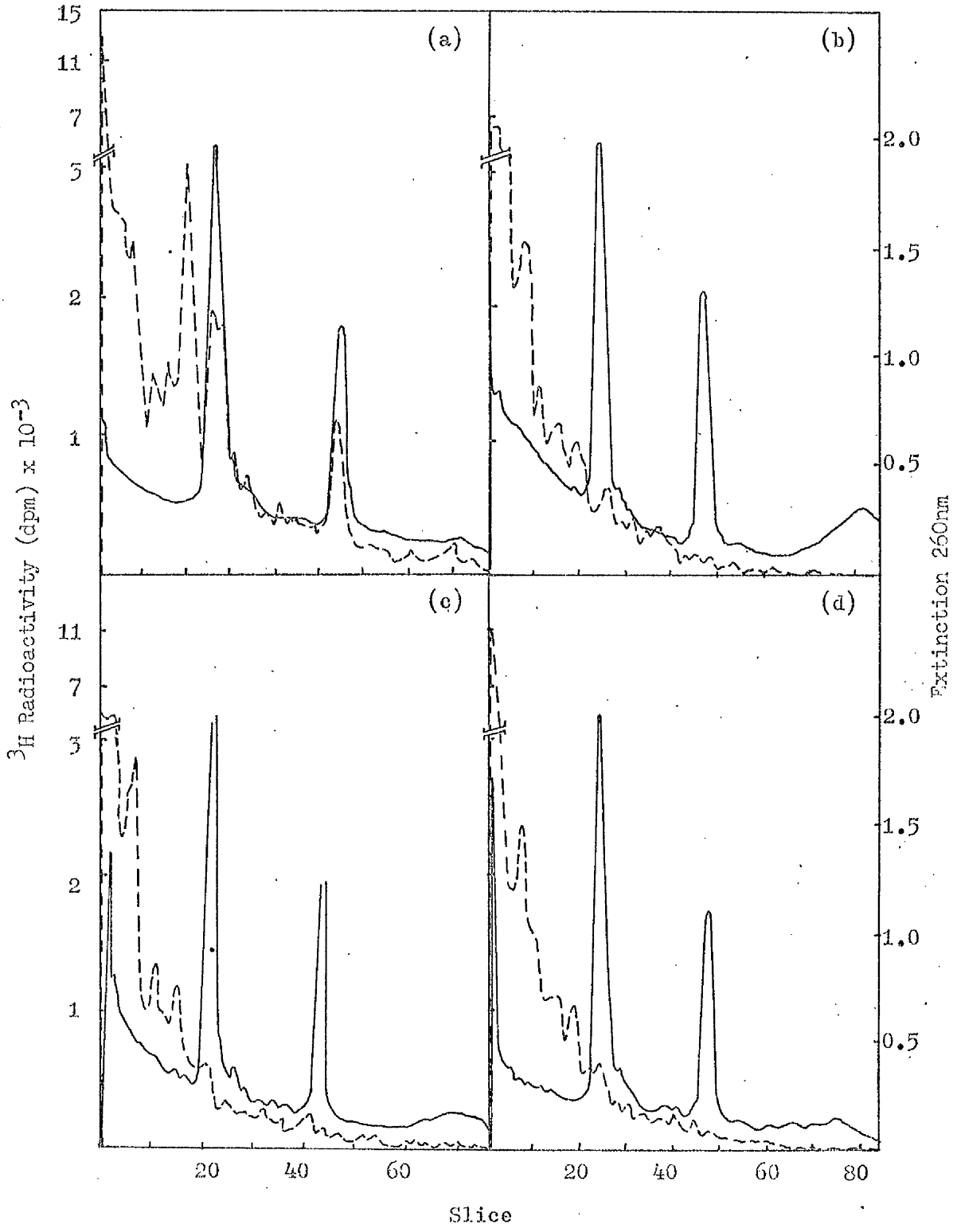
(b) = Cycloheximide given 1h before hormone.

(c) = Cycloheximide given simultaneously with hormone.

(d) = Cycloheximide given 1h after hormone.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


(a)
(b)
(c)
(d)
15
11
7
3
2
1
2.0
1.5
1.0
0.5
20
40
60
80
Slice
^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm

hoximide/rat. The uninhibited control result of Fig. 15 is repeated in Fig. 16 to facilitate comparison. In this case, the effect on ribosome maturation was observed whether the inhibitor was given before, with or after the hormone. The inhibition by cycloheximide of the maturation of ribosomes has been recorded by Willems et al. (1969) in HeLa cells and by Craig & Perry (1970) in L cells.

3.10 Characterization of Uterine RNA: (a) Synthesis In Vitro, (b) The Effects of the Later Purification Steps on the RNA Recovered.

A number of questions can be asked about the nature of what appears to be RNA remaining in the first few slices of polyacrylamide gels after the separation of total uterine RNA. It is possible that the radioactivity could represent higher-molecular-weight heterogeneous RNA species. It is also possible, however, that it represents aggregation products formed during the purification of the RNA, or that it is RNA complexed to protein. It is even possible that it is not RNA at all. If it is a high-molecular-weight RNA, the experiments in Fig. 12 show that it is not methylated in vitro. Nevertheless, it is possible that this RNA is not made under the conditions prevailing in vitro . The experiments shown in Figs. 17, 18 and 19 investigated these possibilities.

Fig. 17(a) shows the incorporation of radioactivity into the RNA of uteri incubated in vitro for 30min in the presence of 3μCi/ml of each of [5-^{3}H]uridine and [8-^{3}H]guanosine. The purified RNA was separated on 2.7% polyacrylamide gels for 5h. It was evident that the ratio of radioactivity in the high-molecular-weight

Figure 17.

(a) Synthesis of uterine RNA in vitro.

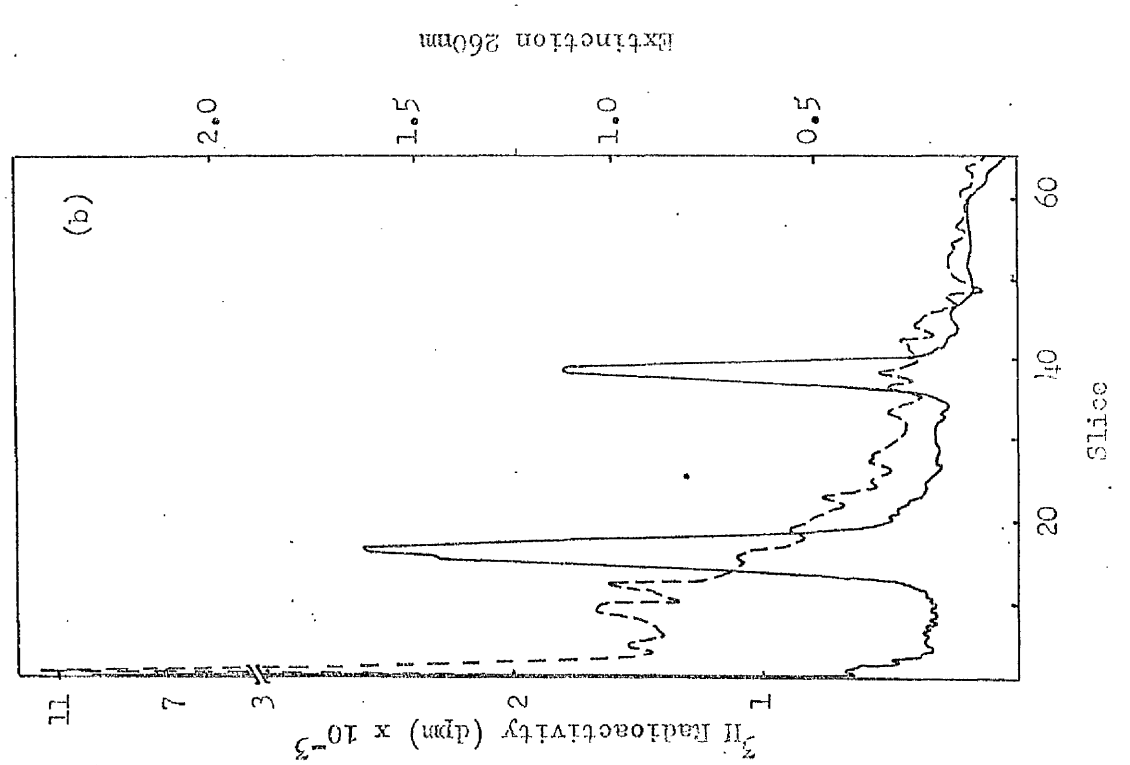
The uteri of 18-21 day old rats, weighing 25-30g, were incubated in 2ml of Eagle's medium containing 5μCi/ml of each of [5-^{3}H]uridine and [8-^{3}H]guanosine for 30min. RNA was purified and separated for 5h on 2.7% polyacrylamide gels.

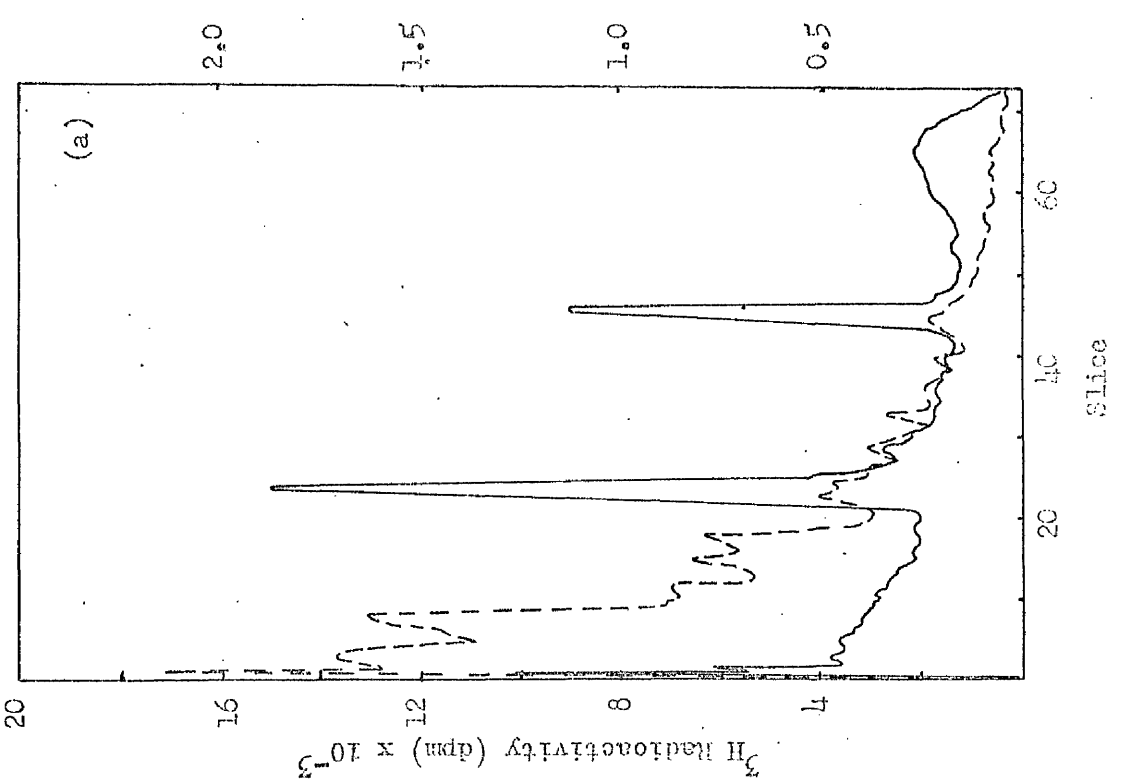
(b) Electrophoresis of uterine RNA which had not been purified after deoxyribonuclease digestion.

18-21 day old rats, weighing 25-30g, received 1μg of oestradiol-17β by intraperitoneal injection 2h before death and 125μCi of each of [5-^{3}H]uridine and [8-^{3}H] guanosine by intravenous injection 15min before death. Uterine RNA was purified as described in the Materials and Methods section as far as the deoxyribonuclease digestion and, without further purification, was separated electrophoretically on a 2.7% polyacrylamide gel for 5h.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


Extinction 260nm
2.0
1.5
1.0
0.5
(b)
^{3}H Radioactivity (dpm) x 10^{-3}
11
7
3
2
1
20
40
60
Slice


Extinction 260nm
2.0
1.5
1.0
0.5
(a)
^{3}H Radioactivity (dpm) x 10^{-3}
20
16
12
8
4
20
40
60
Slice

RNA to that in 32S, 28S and 18S RNA was higher than would have been expected after a 30min pulse *in vivo*. However, this almost certainly reflected the relatively limitless availability of labelled precursor in the *in vitro* incubations which permitted the continuous synthesis of the high-molecular-weight species. Notwithstanding these differences, it was clear that the uterine RNA, synthesized at short time intervals *in vitro*, was not grossly different from that made *in vivo*.

Fig. 17(b) shows the separation, on a polyacrylamide gel, of RNA purified from rat uteri 2h after oestradiol treatment and 15min after administration of labelled ribonucleosides. This RNA was not, however, purified free of DNA and was applied to the gel immediately after DNase treatment. The sample thus contained DNA oligonucleotides and protein and was an unpurified equivalent of the RNA used in Fig. 8(e). The profile of radioactivity in this separation was essentially the same as that of purified RNA, though the migration through the gel appeared to be slightly slower. The preparation contained at least as much very-high-molecular-weight RNA as DNA-free preparations and at first it was thought to contain rather more. This was probably not the case, as it was later found that high-molecular-weight RNA from the uteri of rats, which had received oestradiol 2h before death, incorporated radioactive precursor to a very variable extent (compare Figs. 8e and 28d). Such findings would be expected because, after 2h exposure to the hormone, the rate of RNA synthesis was changing rapidly (Figs. 3 and 4). It was concluded from Fig. 17(b) that the radioactivity remaining in the first few slices of the gel could only be the product of aggregation, if the condensation

occurred before the final purification of the RNA. It was also concluded that the losses incurred during the purification of the RNA did not markedly alter the distribution or relative amounts of the high-molecular-weight RNA species.

3.11. Effects of Ribonuclease and Pronase on High-Molecular-Weight RNA.

The effects of Pronase and ribonuclease on the profile of RNA separated on polyacrylamide gels were examined to determine the nature of the very-high-molecular-weight RNA. Immature rats were treated for 2h with oestradiol and received radioactive precursors 30min before death. The RNA was then purified as far as the deoxyribonuclease digestion step and thereafter it was treated with self-digested Pronase or ribonuclease as described in the Materials and Methods section. Purification of the RNA then continued in the usual way.

Some difficulty was experienced in the Pronase digestion in that the enzyme is rather slow acting. Ideally, the RNA should be treated for several hours. However, despite the use of self-digested enzyme and sterilized glassware, it was found that when digestion was carried out for extended periods, some effects due to ribonuclease contamination were found in all the RNA species. It was possible to continue the Pronase digestion for 1h without marked RNA degradation, but longer treatments necessitated protection with a ribonuclease inhibitor. SDS would protect the RNA for at least 24h but the detergent probably also affected the Pronase activity.

Fig. 18(a) shows the results of treatment of approximately 100µg uterine RNA with 100µg Pronase for 1h at 37° and Fig. 18(b)

Figure 18.

Effect of Pronase and ribonuclease on uterine DNA.

18-21 day old rats, weighing 25-30g, were given 1µg of oestradiol-17β intraperitoneally 2h before death and 125µCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine intravenously 30min before death. The DNA was purified from the excised uteri as far as the deoxyribonuclease digestion after which aliquots were further digested with Pronase or ribonuclease. The purification was then continued as described in the Materials and Methods section and the DNA was separated on 2.7% polyacrylamide gels for 5h.

(a) = Digestion with 100µg of Pronase at 37° for 1h.

(b) = Digestion with 100µg of Pronase at 37° for 24h in the presence of 1% SDS.

(c) = Digestion with 100µg ribonuclease for 15min at 37°.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.

^{3}H Radioactivity (dpm) x 10^{-3}
7
5
3
2
1
(a)
(b)
(c)
20
40
60
Slice
Extinction 260nm
2.0
1.5
1.0
0.5

illustrates a similar experiment in which the Pronase digestion was continued for 24h in the presence of 1% SDS. Under these conditions, protein digestion had absolutely no effect on the profile of radioactivity due to high-molecular-weight RNA. The only observable difference between these separations and those of untreated preparations (See Figs. 9d and 11) was that the 260nm trace indicated some loss of the 18S RNA species.

Fig. 18(c) shows the optical density and radioactivity profiles obtained after a 15min treatment of 100µg of uterine RNA with 100µg ribonuclease at 37°. Virtually all radioactivity and all extinction at 260nm due to the 28S and 18S ribosomal RNA peaks had disappeared. Identical results were obtained when the digestion was carried out at 25°.

In view of the fact that the radioactivity in the first few slices of the polyacrylamide gels was highly susceptible to attack by ribonuclease and was unaffected by Pronase, it was concluded that RNA was present in this segment of the gels.

3.12. Effects of Dimethylsulphoxide on High-Molecular-Weight RNA.

When radioactive uterine RNA is separated on 2.7% polyacrylamide gels for 5h, a peak of radioactivity is found in the first few slices. The counts have been shown to be associated with RNA not complexed to protein (Fig. 18). The RNA must, however, be of very-high-molecular-weight (>45S) since it has been established that migration of RNA in polyacrylamide gels is a function of molecular weight (Loening, 1969). Two possibilities therefore remain; either the RNA is a very-high-molecular-weight species, such as

heterogeneous nuclear RNA, or it is a product of aggregation. At the temperatures used and the RNA concentrations obtained (never more than 0.1mg/ml), aggregation should not occur during the extraction procedure (Wagner et al., 1967). Furthermore, if it occurs, it does so before the final purification steps (Fig. 17b).

Assuming that it is composed entirely of RNA, an aggregate should have regions of double strandedness. Katz & Penman (1966) have described a method which achieves strand separation of double stranded RNA without degrading the single strands released. This method, involving treatment of RNA with 85% dimethylsulphoxide (see Materials and Methods), was used on half a preparation of uterine RNA purified from immature rats 2h after oestradiol treatment and 15min after administration of radioactive ribonucleosides. The other half of the RNA preparation was incubated under identical conditions without dimethylsulphoxide. One deviation from the method of Katz and Penman (1966) was found to be necessary. Dextran sulphate, which was added as a ribonuclease inhibitor, had to be omitted as it markedly affected the polyacrylamide gels, causing them to warp badly and producing unsatisfactory separations.

Fig. 19 shows the results of incubations of uterine RNA with or without dimethylsulphoxide. The treated RNA was in fact more normal than the untreated preparation, the latter showing some signs of degradation of the species migrating in the 45S-18S size range. Ribonuclease may have been the cause of this difference, the dimethylsulphoxide perhaps having some inhibitory effect on ribonuclease. However, the SDS present in the incubation medium should have afforded sufficient protection.

Notwithstanding these differences, it was clear that the

Figure 19.

Effects of dimethyl sulphoxide on uterine RNA.

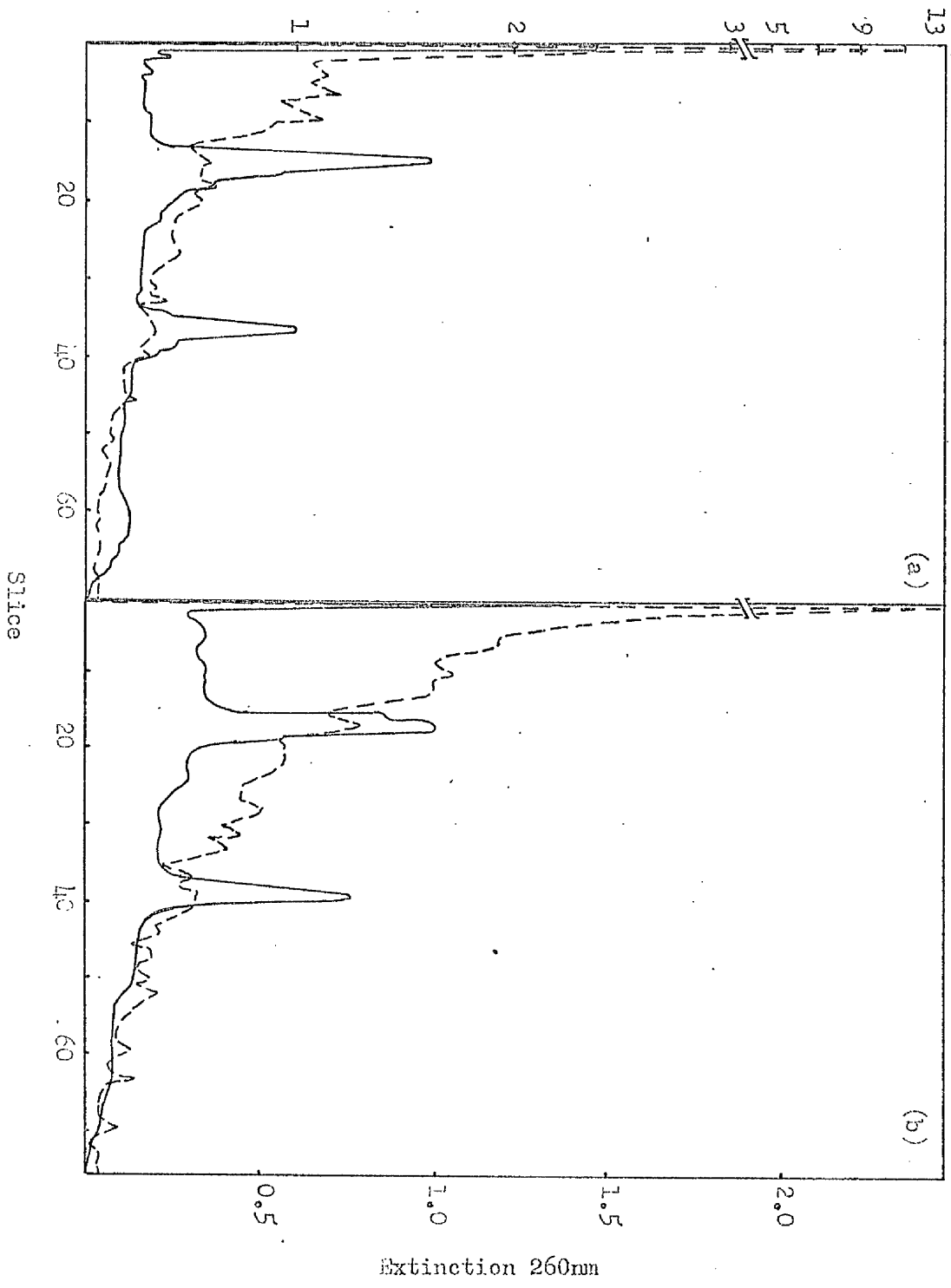
RNA was purified from the uteri of 18-21 day old rats, weighing 25-30g, these having received an intraperitoneal injection of 1µg of oestradiol-17β, 2h before death and an intravenous injection of 125µCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine, 15min before death. Half of the preparation (about 60µg) was incubated in 88% dimethyl sulphoxide at 37° for 18 min while the remainder was similarly incubated without the denaturant. The precipitated RNA was separated for 5h on 2.7% polyacrylamide gels.

(a) = Without dimethyl sulphoxide.

(b) = With dimethyl sulphoxide.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


^{3}H Radioactivity (dpm) x 10^{-3}
1
2
3
5
9
13
20
40
60
(a)
20
40
60
(b)
Slice
0.5
1.0
1.5
2.0
Extinction 260nm

denaturation of the secondary structure of RNA has no effect on the amount of radioactivity present in the first few slices of the gel and it was concluded that these RNAs were discrete species of very-high-molecular-weight.

As further confirmation of the above experiment, denaturation of uterine RNA was attempted by incubation and electrophoresis in 8-12M urea as described by Dobos & Faulkner (1970). Even with very extended electrophoresis periods (27h), however, RNA did not enter the gels under these conditions. It should be mentioned at this point that, although ethylene diacrylate as a crosslinker has pronounced advantages when electrophoresis is continued for relatively long periods (Fig. 7), it is not without disadvantages. The method of Dobos & Faulkner (1970) apparently worked adequately when bisacrylamide was the crosslinker. Furthermore, the staining with toluidine blue of RNA separated on acrylamide/bisacrylamide gels (McIndoe & Munro, 1967) was unsuccessful in my hands when ethylene diacrylate was used as a crosslinker.

3.13. The Decay of Very-High-Molecular-Weight Uterine RNA.

From the results obtained so far, it seems that very-high-molecular-weight RNA in the first few slices of the gel is in fact heterogeneous nuclear RNA (HnRNA). Two of the characteristics of this species have already been demonstrated in the uterine material, namely the high-molecular-weight and the absence of methylation (Fig. 11). HnRNA has also been shown to have a very rapid turnover, a low G+C content and a nuclear location, so it remained to see whether these properties were also shared by the uterine RNA.

Many attempts were made to demonstrate a short half life

for the high-molecular-weight RNA *in vivo*. These largely involved labelling these species of RNA in isolation by using a short pulse of radioactive precursor in conjunction with a low dose of actinomycin D. Incorporation of radioactivity was then stopped by the administration of high levels of actinomycin D and decay of the RNA was followed. In practice, the variability and slow response of the *in vivo* system marred the results. The time taken for the inhibitor to get to its site of action, and exert its full effect, was uncertain and variable. Moreover, it proved impossible to completely stop RNA synthesis quickly enough. Although there seemed to be a rapid decay of the high-molecular-weight RNA, results were not clear cut and the *in vivo* approach was abandoned.

Since it had been shown that the very-high-molecular-weight RNA was made *in vitro*, it seemed reasonable to study its synthesis and decay in an artificial medium. Even here, it was found that drastic conditions were required to inhibit RNA synthesis fast enough. To stop incorporation of radioactivity into RNA it was not sufficient to transfer uteri from an incorporation medium into a system not containing radioactive precursor. Even if an excess of unlabelled precursor was added, intracellular pools were not diluted fast enough to inhibit further incorporation of radioactivity. The *in vitro* incubations were, therefore, carried out as described in the Materials and Methods section. Radioactive uridine and guanosine were incorporated into rat uteri for 15min. The tissue was then rapidly washed and either used to make RNA immediately or transferred to a second incubation medium containing 100µg/ml of each of unlabelled uridine, guanosine and actinomycin D. In this system RNA synthesis appeared to stop almost immediately and the decay of

Figure 20.

Decay of uterine high-molecular-weight RNA.

The uteri from 18-21 day old rats, weighing 25-30g, were incubated in groups of four, *in vitro*, in 2ml of Eagle's medium containing 5μCi of each of [5-^{3}H]uridine and [8-^{3}H] guanosine. The incubation was continued for 10min, and the uteri washed twice in Eagle's medium at 37°. The newly-synthesized RNA was then allowed to decay for various lengths of time by incubating the uteri in a further 2ml of Eagle's medium containing 100μg/ml of each of uridine, guanosine and actinomycin D. The purified RNA was separated on a 2.7% polyacrylamide gel for 5h.

(a) = No decay.

(b) = 10min decay.

(c) = 20 min decay.

(d) = 30min decay.

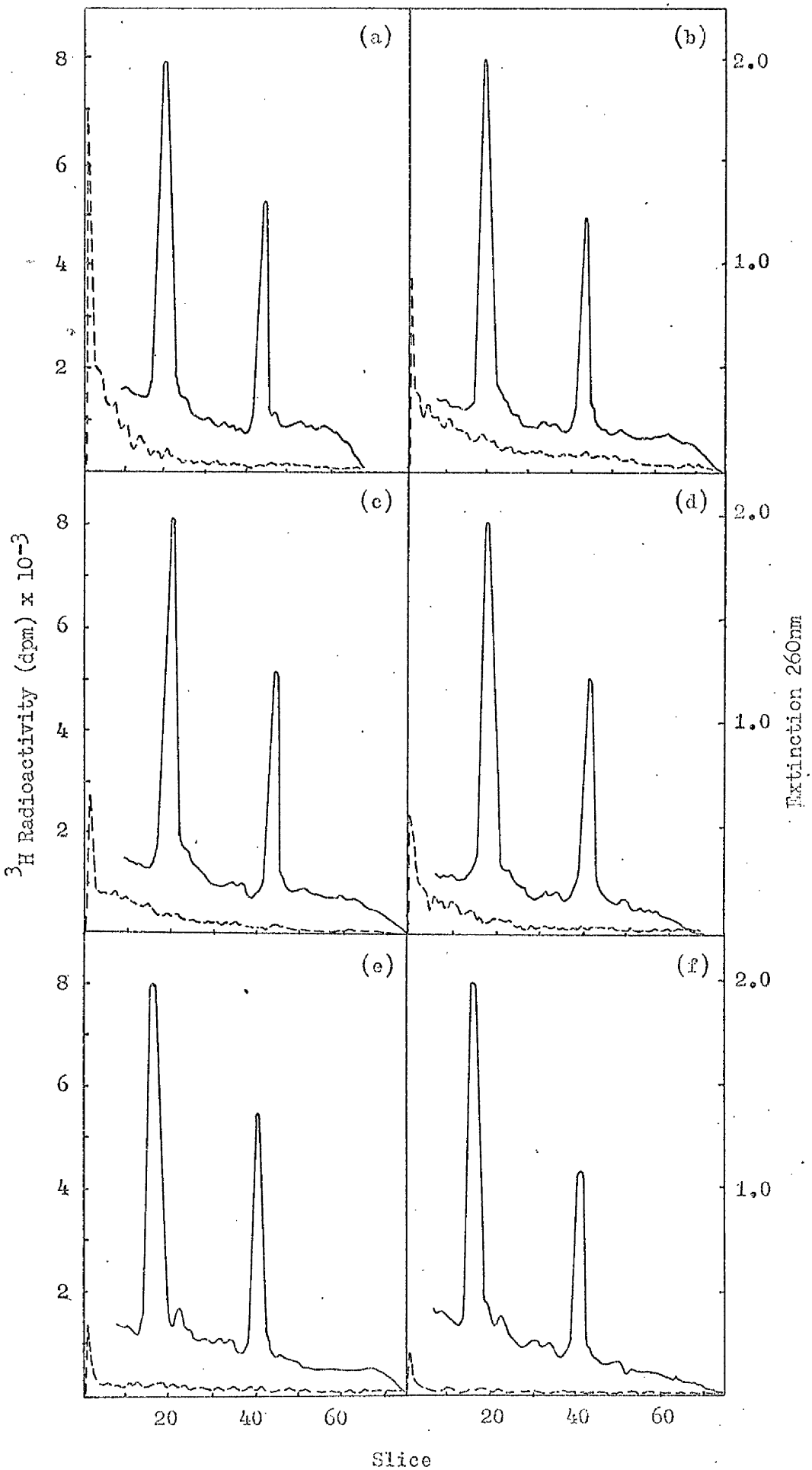
(e) = 45min decay.

(f) = 60min decay.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.

For reasons of clarity the E260nm trace is omitted from the first few slices of the gels.


(a)
(b)
(c)
(d)
(e)
(f)
8
6
4
2
2.0
1.0
20
40
60
Slice
^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm

previously synthesized RNA was followed in uteri incubated for varying lengths of time. The purified RNA was separated on 2.7% polyacrylamide gels for 5h.

Fig. 20 compares the profile of radioactivity in RNA which had been allowed to decay for 10, 20, 30, 45 and 60min, with a control. Breakdown was rapid, especially during the first 10min, when the radioactivity in the first four slices of the gel were reduced by 43%. After 1h of decay, the radioactivity in these first four slices was only 11% of the controls. No attempt has been made to estimate a half life since the in vitro conditions may not accurately reflect those in vivo and it is possible that the high levels of actinomycin D speeded up the decay.

3.14. Decay of Uterine Ribosomal RNA.

In the following experiment attempts were made to determine whether the decay observed in the very-high-molecular-weight species also occurred with other species of RNA. The results shown in Fig. 21 compare the profile of radioactivity in RNA labelled in vitro for 1h with that from a similar preparation that had been allowed to decay for a further hour. Again, after 1h decay the radioactivity in the first four slices of the gel was reduced to 10%. Furthermore, the radioactivity in all RNA of molecular weight greater than 28S was drastically reduced, presumably through both decay and ribosome maturation. When the decay of heterogeneous RNA associated with the ribosomal peaks had been allowed for, it was clear that the 28S and 18S RNA had not undergone marked degradation. It was concluded that the very-high-molecular-weight RNA had a much faster turnover than ribosomal RNA.

Figure 21.

The decay of uterine ribosomal RNA.

The uteri from 18-21 day old rats, weighing 25-30g, were incubated in groups of four, in vitro, in 2ml of Eagle's medium containing 5μCi/ml of each of [5-^{3}H]uridine and [8-^{3}H] guanosine. The incubation was continued for 1h after which the uteri were washed and the RNA from one group was purified. The newly-synthesised RNA in a second group was allowed to decay for 1h by incubating the uteri in a further 2ml of Eagle's medium, containing 100μg/ml of each of uridine, guanosine and actinomycin D. The RNA of these uteri was also purified and the two preparations were separated on 2.7% polyacrylamide gels for 5h.

(a) = No decay.

(b) = 1h decay.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.

^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm
Slice
(a)
(b)

3.15. Base Composition of Uterine RNA.

Heterogeneous nuclear RNA is characterized by a base composition high in U and low in G+C (Attardi et al., 1966; Soeiro, et al., 1966). Experiments were, therefore, performed to determine whether the uterine species which exhibit many of the properties of HnRNA also shared this characteristic.

To determine the base ratios of uterine RNA it was again necessary to label the tissue in vitro. Incorporation of ^{32}P into immature rat uterus RNA in vivo was low, presumably because the isotope was being utilized in many ways and at many sites. Incorporation also posed problems of isotope equilibration. Uterine RNAwas, therefore, labelled in vitro in the presence of high levels of ^{32}P and in a medium of low phosphate content (See Materials and Methods section). The purified RNA was separated on 2.7% polyacrylamide gels which were then sliced and the radioactivity in the separate slices determined. RNA was re-extracted from selected slices, hydrolyzed, and the hydrolysate separated chromatographically. The percentage of the radioactivity in each monophosphate was then determined.

Fig. 22 shows the radioactivity profile of ^{32}P labelled uterine RNA and includes the base composition of the gel slices corresponding to the major peaks of precursor incorporation. Slices 1 and 2 of the polyacrylamide gel were selected as an area containing possible HnRNA. Slice 5 was chosen as an area containing HnRNA contaminated with 45S RNA whilst slice 9 was chosen as an area of 45S RNA contaminated with HnRNA (45S RNA was very poorly differentiated). Slices 15, 20 and 21 contained 32S, 28S and 18S RNA respectively and slice 12 was taken to contain a possible intermediate

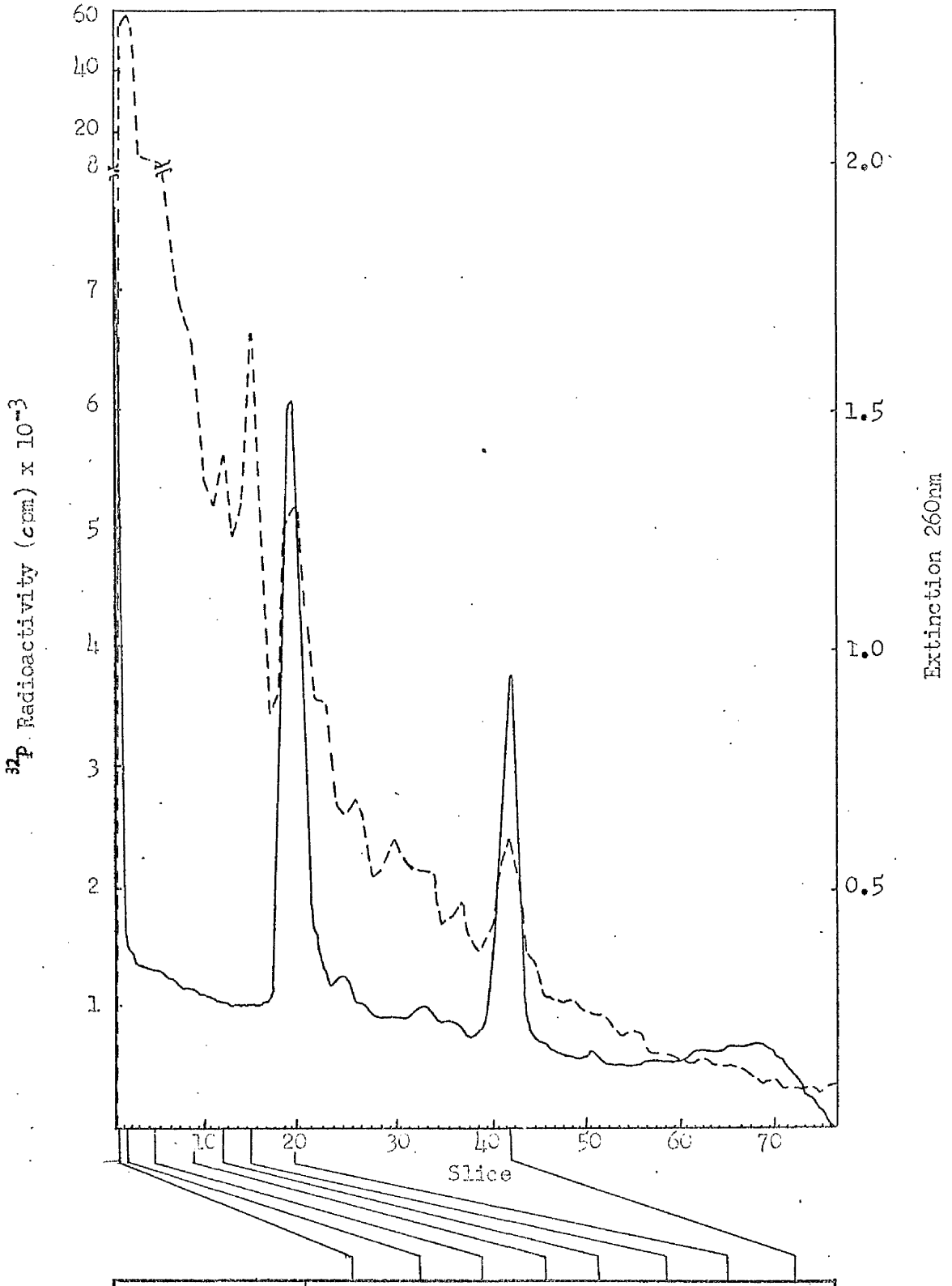
Figure 22.

Analysis of the base composition of uterine RNA.

The uteri of four 18-21 day old rats, weighing 25-30g, were incubated in 2ml of Eagle's medium which had its phosphate content reduced to 10% of the normal concentration but contained 2mCi $[^{32}P]$orthophosphate. After washing the incubated uteri thoroughly, the total RNA was purified and separated on a 2.7% polyacrylamide gel for 5h. The extinction at 260nm and the radioactivity were determined throughout the gel and the RNA was extracted from selected slices. This was precipitated in the presence of unlabelled RNA, hydrolysed and its base composition analysed.

_____ = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.



Base Ratio (%)								
Slice / Base	1	2	5	9	12	15	20	42
C	23.2	19.3	20.7	30.3	24.1	20.2	27.3	25.2
A	26.1	28.4	21.9	17.7	22.2	20.2	20.3	22.7
G	19.8	22.6	24.1	25.8	27.0	28.4	29.2	27.0
U	30.9	29.7	27.3	26.3	26.7	23.2	23.2	25.1
G + C	43.0	41.9	50.8	56.1	51.1	56.6	56.5	52.2

between 45S and 32S RNA. It must be pointed out that the radioactivity peaks of these rRNA and pre-rRNA species were superimposed on a background of heterogeneous radioactivity and extracts would therefore contain a mixed RNA population. The base compositions shown in Fig. 22 correlate well with the expected results. The composition of the RNA in slices 1 and 2 can be compared with HnRNA from nuclear supernatant fractions of HeLa cells (Soeiro, et al., 1966) and with rapidly labelled RNA of duck erythrocytes (Attardi et al., 1966).

Base composition (%)	C	A	G	U	G+C	Reference
Slice 1 from Fig. 22	23	26	20	31	43	
HeLa RNA>65s	22	26	20	32	42	Soeiro et al., 1966
Erythrocyte RNA>75s	22	25	24	30	46	Attardi et al., 1966
Slice 2 from Fig. 22	19	28	23	30	42	
HeLa RNA of ≈ 65s	21	27	20	32	41	Soeiro et al., 1966

The base composition of the ribosomal RNA peaks also gave a result which might have been predicted from their known base ratio, bearing in mind the fact that they were contaminated with labelled heterogeneous RNA. Thus, Willems et al. (1968) have reported G+C compositions for 45S, 32S, 28S and 18S RNA of 69.7%, 70.1%, 66.8% and 55.7% respectively. The lower values of 56.1%, 56.6%, 56.5% and 52.2% obtained in Fig. 22 seem reasonable assuming a dilution with an RNA of low G+C content. If, for example, one postulates that in slice 9 radioactivity is roughly divided between 32S RNA

and a DNA-like RNA with a G+C content of 44%, then one would expect a G+C value of approximately 57% in the mixture. In a similar situation, where total HeLa cell RNA was separated on sucrose density gradients, Soeiro et al. (1966) obtained G+C values of 60%, 56% and 48% for 45S, 28S and 18S RNA respectively. From purified ribosomes, however, they obtained G+C values of 67.3% and 57.1% for the 28S and 18S species.

It is concluded that the base ratios in Fig. 22 accurately reflect the compositions of the radioactive RNAs in the respective slices. Furthermore, the RNA remaining in the first few slices of the polyacrylamide gels after separations of uterine RNA preparations, has been shown to have an HnRNA-like base composition. That is, it has a low G+C and a high U content.

3.16. Isolation and Characterization of Uterine Nuclei.

Great difficulty was experienced in obtaining satisfactory preparations of immature rat uterine nuclei. Many methods of isolation were tried. They included:-

(1) Homogenization in sucrose containing buffer and differential sedimentation through varying sucrose concentrations (Blobel & Potter, 1966; Hamilton et al., 1965; Seshadri & Warren, 1969).

(2) Non-aqueous nuclear isolations (Kay et al., 1956; Dounce et al., 1950).

(3) Preparations in buffers of low ionic strength (Penman et al., 1966; AB & Malt, 1970).

(4) Isolations in buffers containing citric acid (Smellie et al., 1955).

The nuclei recovered from all of these methods were in low yield and were invariably damaged to a greater or lesser extent. In addition, many of the methods did not yield clean preparations and took longer than was desirable for preservation of nuclear RNA. The best results were obtained after a mild, and of necessity incomplete, homogenization in low ionic strength buffer followed by a rapid purification.

Method (A.) in the Materials and Methods section is an example of such a preparation. The uteri were gently homogenized in the hypotonic buffer, RSB and the nuclei were purified by two low speed centrifugations. The second of these was through a discontinuous sucrose density gradient and all steps were in the presence of the non-ionic detergent Triton X 100 to remove the outer nuclear membrane. The nuclei took 30min to prepare in a yield of 16-20% and appeared clean by phase contrast microscopy. However, when the nuclear RNA was separated on polyacrylamide gels the very-high-molecular-weight RNA was not present. Fig. 23(a) shows an example of a separation of nuclear RNA prepared after a 30min incubation of the uteri, in vitro, in the presence of radioactive ribonucleosides. The profile of radioactivity indicated an absence of counts in the initial slices of the gel, but showed distinct peaks corresponding to 45S, 32S, 28S and 18S RNA. The 45S peak was not always so prominent. The ribosomal and pre-ribosomal peaks appeared to be superimposed on a background of heterogeneous radioactivity and it seemed likely that the very-high-molecular-weight RNA was being degraded to smaller entities during isolation of nuclei. Nevertheless, the inclusion of ribonuclease inhibitors such as polyvinyl sulphate, diethyl pyrocarbamate, and Macaloid during the isolation

Figure 23.

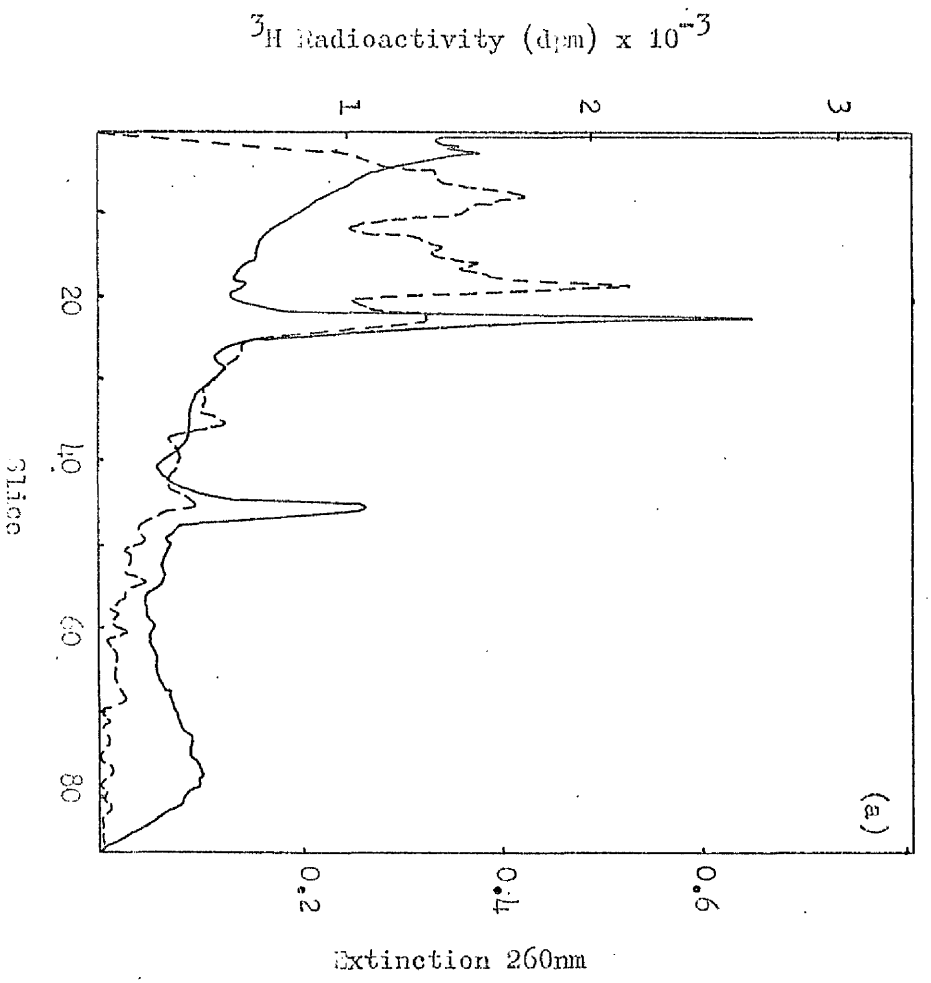
RNA synthesized in the nuclei of immature rat uteri.

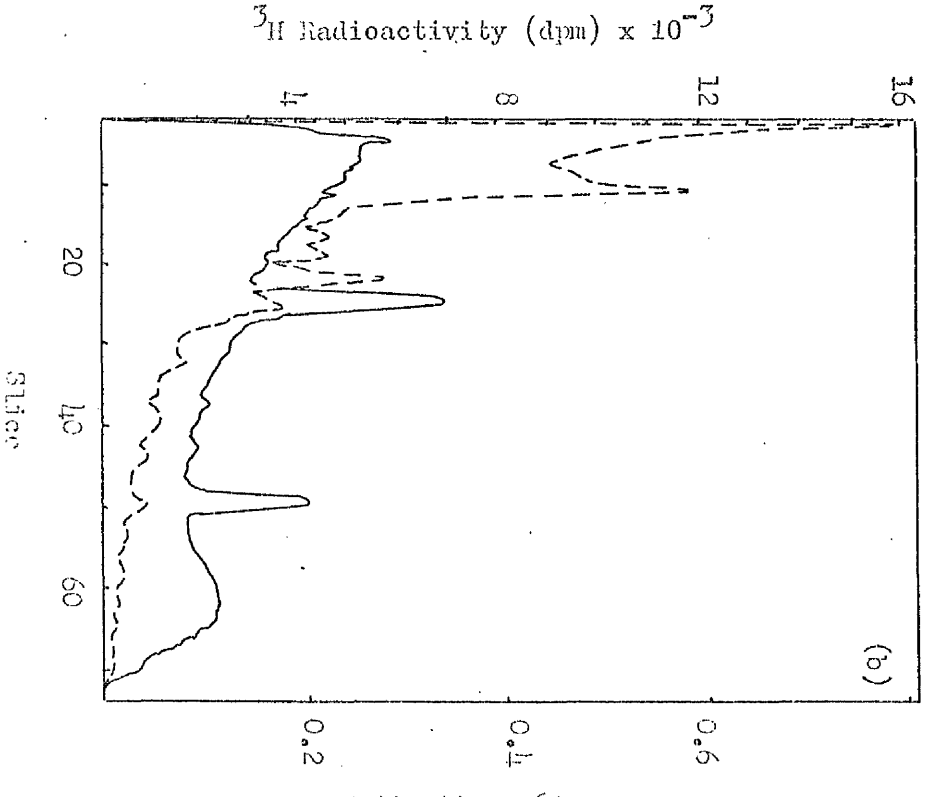
(a) The uteri of twelve 18-21 day old rats, weighing 25-30g, were incubated in 4ml of Eagle's medium which contained 3μCi/ml of each of [5-^{3}H]uridine and [8-^{3}H]guanosine. The nuclei were then prepared by method A in the Materials and Methods section and the RNA was extracted from the pellet, purified and separated on a 2.7% polyacrylamide gel for 5h.

(b) The experimental details were as in Fig. 23(a) except that the Eagle's medium contained 9μCi/ml of each of [5-^{3}H] uridine and [8-^{3}H]guanosine, and the nuclei were prepared by method B in the Materials and Methods section.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


^{3}H Radioactivity (dpm) x 10^{-3}
1
2
3
20
40
60
80
Slice
(a)
0.2
0.4
0.6
Extinction 260nm


^{3}H Radioactivity (dpm) x 10^{-3}
4
8
12
16
20
40
60
Slice
(b)
0.2
0.4
0.6
Extinction 260nm

of the nuclei, did not alter the profile of the RNA recovered.

When the nuclei were examined by electron microscopy, it was found that almost all of them were damaged and had lost some extranucleolar contents. Almost certainly the heterogeneous nuclear RNA had leached out into the isolation medium. In order to avoid this loss, the preparation was modified to that described in method (B) in the Materials and Methods section. Here, the nuclei were liberated into a homogenization medium of even lower ionic strength (1mM $MgCl_2$) but were then immediately protected by the addition of citric acid. All subsequent purification steps were in the presence of 0.05M citric acid which, by lowering the pH, reduced nuclear fragility (Dounce, 1955). Higashi et al. (1966) reported that, in Walker carcinoma tissue, citric acid permitted the isolation of nuclei which retained high-molecular-weight RNA. They found considerable changes in the ultra structure of the nuclei but the nucleolus was intact and the RNA/DNA ratio was unchanged. Inclusion of citric acid in my preparations also seemed to cause more structural damage and, although the nuclei appeared intact when observed by phase contrast microscopy, they were again found to be badly damaged when examined in the electron microscope (Fig. 24). However, when the RNA from citric acid-protected nuclei was separated on polyacrylamide gels (Fig. 23b), the very-high-molecular-weight RNA was found to be present and the radioactivity profile was similar to that observed in total RNA.

In nuclear RNA preparations from HeLa cells, 45S, 32S and 28S RNA are present but there is an almost total absence of 18S RNA (Penman, 1966). This was not the case with uterine nuclear RNA where most

Figure 24.

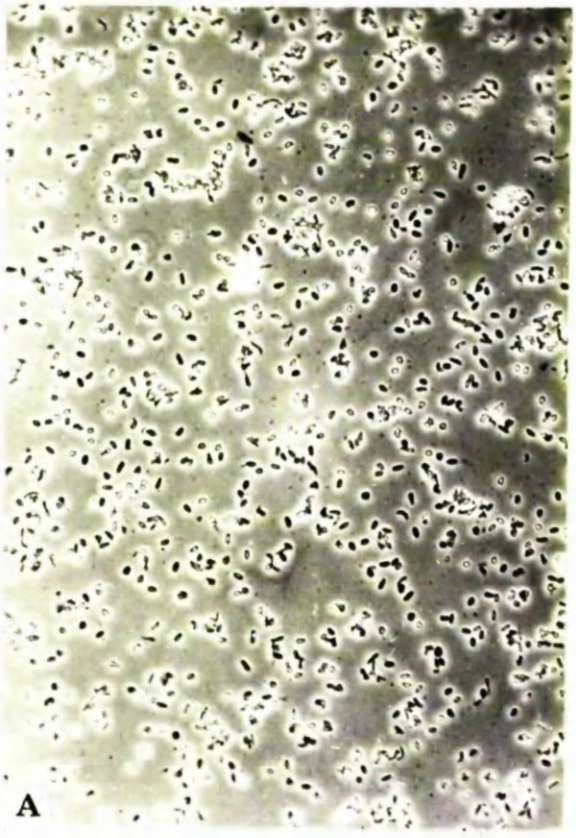
Nuclei from the uteri of immature rats.

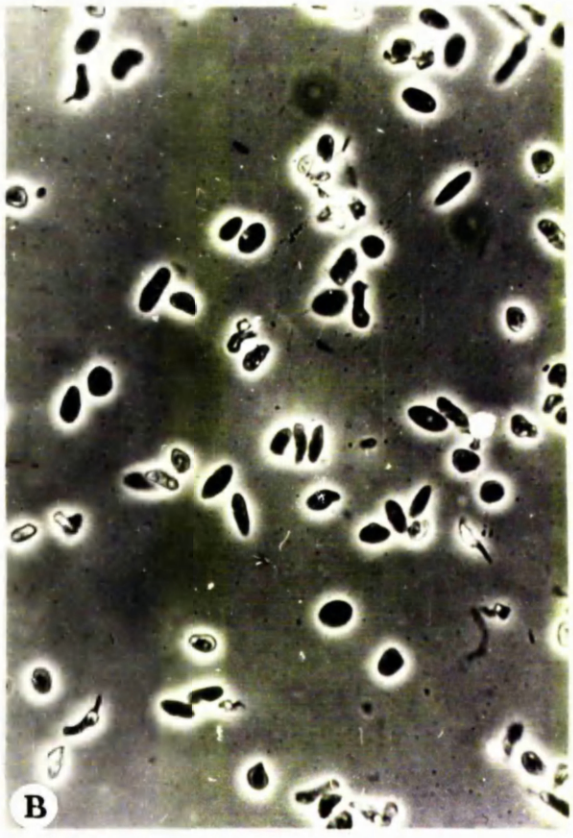
Nuclei were prepared from the uteri of 18-21 day old rats by method B in the Materials and Methods section in which they were protected by citric acid.

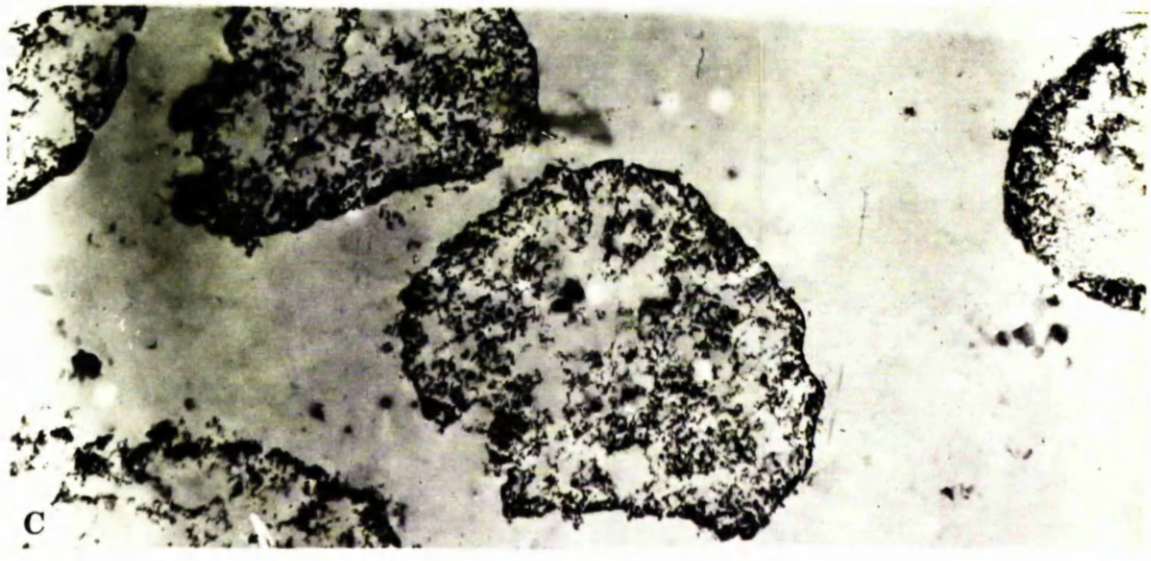
(A) = Phase contrast light micrograph, magnification x 450.

(B) = Phase contrast light micrograph, magnification x 1800.

(C) = Electron micrograph, magnification x 12500.


A


B


C

of extinction at 260nm was associated with 28S and 18S RNA. This difference may reflect the relative impurity of the uterine nuclei but it should be mentioned that nuclear RNA from other mammalian sources also differs from HeLa cells in containing readily detectable levels of the 18S species (AB & Malt, 1970; Higashi et al., 1966; Steele et al., 1965). Whether the difference is due to cell type or to contamination is unclear.

The finding that uterine nuclei contain very-high-molecular weight RNA, together with the already established rapid synthesis and decay, the DNA-like base ratios and other characters of this species, permit the conclusion that it is heterogeneous nuclear RNA.

3.17. Effect of Oestradiol-17β on Uterine Nuclear RNA Synthesis.

When HeLa cells are exposed to short pulses of labelled RNA precursors, almost all the incorporated radioactivity is found in the nucleus (Penman et al., 1966; Scherrer et al., 1963). After similar short exposures to radioactive precursors, it would be expected that the incorporation into uterine RNA in response to oestradiol would be the same in both isolated nuclear RNA and total RNA. Nevertheless, Means & Hamilton (1966) have reported a rapid increase in the incorporation of radioactivity into nuclear acid-insoluble material following administration of oestrogen to ovariectomized rats. Figs. 3 and 4 showed no comparable increase in the stimulation of total RNA synthesis in the immature rat uterus. The 30min pulse length that was employed in this work may have been too long to detect this change, and in consequence it was worth while investigating the isolated nuclei.

Uterine nuclei were prepared by method (B) in the Materials and Methods section from immature rats which had received oestradiol at various times before death and radioactive ribonucleosides 10min before death. Fig. 25 shows the incorporation into the acid-insoluble fraction expressed as a function of control values. The stimulation of nuclear RNA synthesis approximated to that of total RNA in Fig. 3, except that the maximal stimulation was lower. This difference would be expected if, as Hamilton et al. (1968) have suggested, oestrogen accelerates the entry of newly formed RNA into the cytoplasm as well as stimulating the rate of synthesis. Under these conditions, the observed increase in the incorporation of precursors into nuclear RNA would be less than that of total RNA whilst the increased accumulation of label in the cytoplasm would exceed that of total RNA. Some evidence in support of this will be presented later.

The RNA content of the uterine nuclei increased with oestrogen treatment. Thus, the RNA/DNA ratios of 0.24-0.29, recorded during the first 2h of oestrogen treatment, rose to 0.3 after 4h and to 0.33 after 6h of treatment. When incorporation of precursor was expressed as specific activity (dpm/µg RNA), the increasing RNA concentration of the nuclei resulted in an apparent early fall in the rate of stimulated RNA synthesis. There was no indication of the rapid rise and fall in the specific activity of nuclear RNA which was described by Means & Hamilton (1966) during the first 30min of oestrogen action in ovariectomized rats.

3.18. Separation of RNA on Sucrose Density Gradients and Agarose Gels.

The HnRNA which was synthesized in increased amounts as an

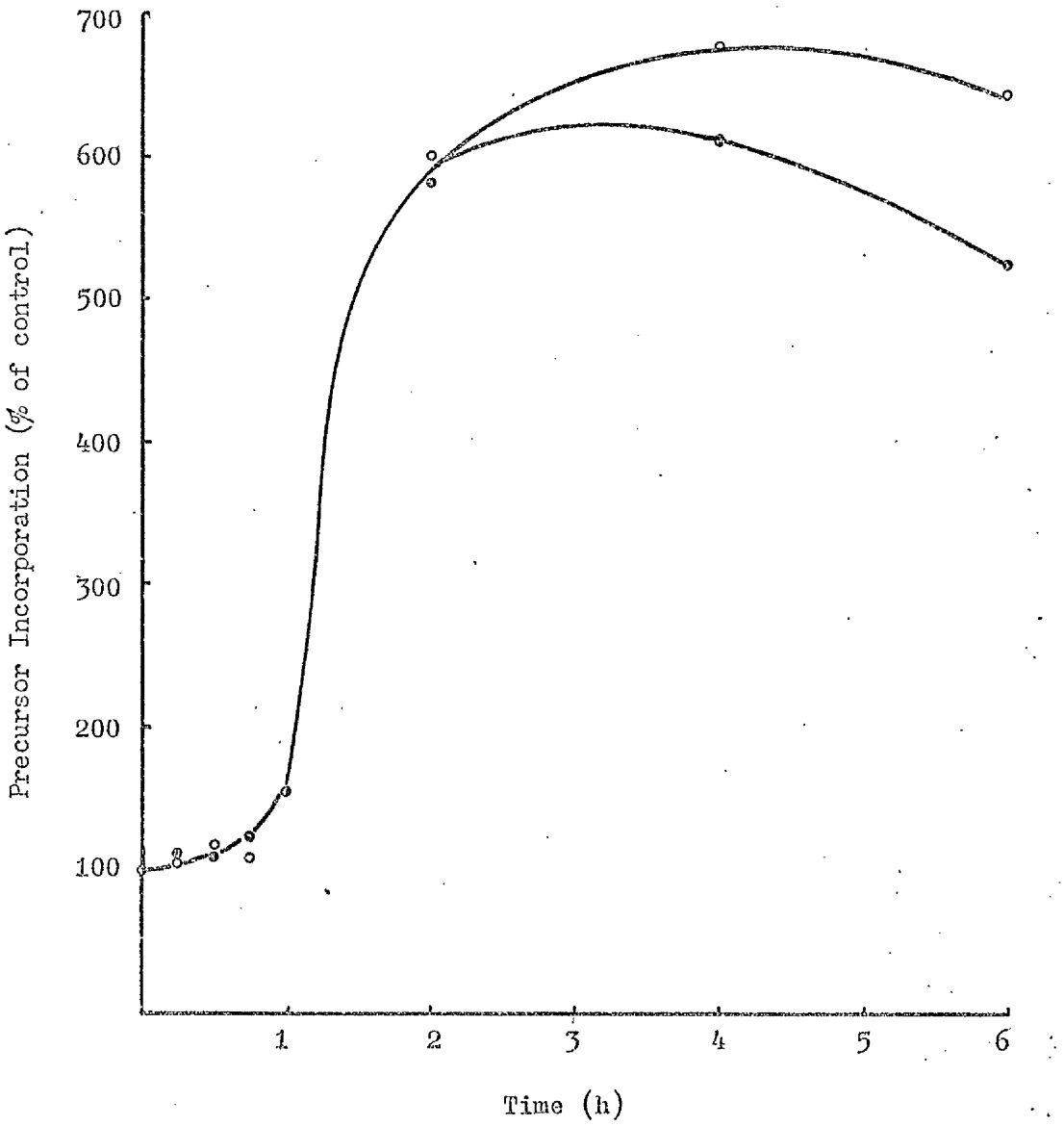
Figure 25.

Effect of oestradiol on the synthesis of RNA in uterine nuclei.

Groups of nine 18-21 day old rats, weighing 25-30g, received 1µg oestradiol-17β intraperitoneally at various times before death. Three of each group were also given 125µCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine 10min before death. The nuclei of the combined uteri were prepared by method B in the Materials and Methods section and were used to assay RNA, DNA and the incorporation of radioactivity into the acid-insoluble fraction. The synthesis of RNA was measured as dpm/µg RNA and dpm/µg DNA and was expressed as a percentage of the uptake in rats not receiving hormone. Control values were 21.7 dpm/µg DNA and 80.7 dpm/µg RNA. Over the first 2h of the time course, the RNA/DNA ratio of the nuclei ranged from 0.24-0.29, after 4h it was 0.3 and at 6h it was 0.33.

—o— = Incorporation measured as dpm/µg DNA.

—•— = Incorporation measured as dpm/µg RNA.


700
600
500
400
300
200
100
Precursor Incorporation (% of control)
1
2
3
4
5
6
Time (h)

early result of oestrogen action was located in the first few slices of polyacrylamide gels. This was not an entirely desirable situation but it was little changed by prolonged electrophoresis. Other separation procedures were tested in order to confirm and extend the polyacrylamide gel results and to investigate the degree of heterogeneity of these very-high-molecular-weight RNA species.

The most commonly used systems in the study of HnRNAs have been sucrose density gradient separations based on the method of Girard et al. (1965). RNA was separated on 15-30% sucrose density gradients in the presence of SDS and under conditions in which rRNA underwent little migration. In these experiments, the heavier HnRNA species occupied most of the gradient (Soeiro et al., 1966). An alternative separation procedure was indicated by my own observations with discontinuous gels. When a step of agarose was put on top of a polyacrylamide gel, HnRNA tended to accumulate at the step interface rather than at the origin. It was clear that migration of very large RNA molecules was retarded much less in agar. A third possibility, namely the separation of RNA on MAK columns as described by Roberts & Quinlivan (1969), was rejected because the HnRNA was eluted as defined peaks and was not, therefore, in a form in which its heterogeneity could be investigated. It was decided to look at uterine HnRNA separated in 15-30% sucrose density gradients and in 1% agarose gels.

Both systems were initially characterized with regard to the migration of rRNA and pre-rRNA. Fig. 26(a) shows the separation of a mixture of total uterine RNA and HeLa cell nucleolar RNA on a 15-30% sucrose gradient for 16h at 31,800 x g and at 20°. Uterine RNA was prepared from rats which had been treated for 4h

Figure 26.

The separation of RNA on sucrose density gradients and agarose gels.

(a) Sucrose density gradients.

Uterine RNA was purified from 18-21 day old rats, weighing 25-30g, which had received 1µg oestradiol-17β 4h before death and 125µCi of each of [5-^{3}H]uridine and [8-^{3}H] guanosine 15min before death. To the preparation was added ^{14}C labelled HeLa cell nucleolar RNA and the mixture was layered onto a 15-30% sucrose density gradient in LETS buffer and sedimented for 16h at 31,800 x g and at 20°. The fractions were numbered from the bottom to the top of the gradient.

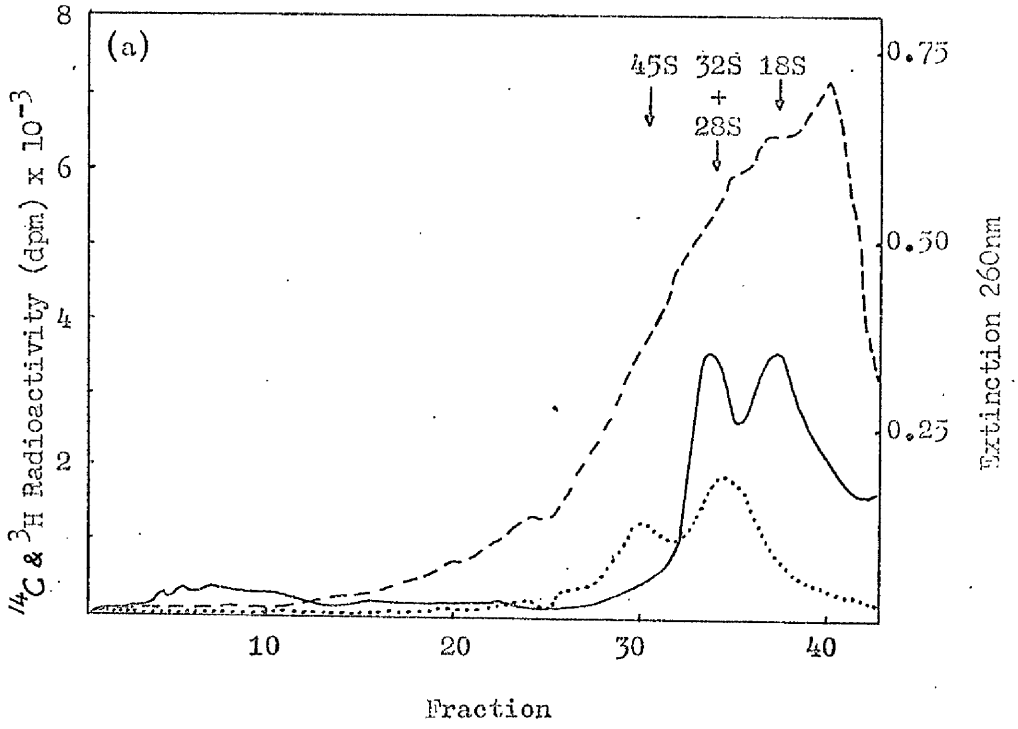
(b) Agarose gels.

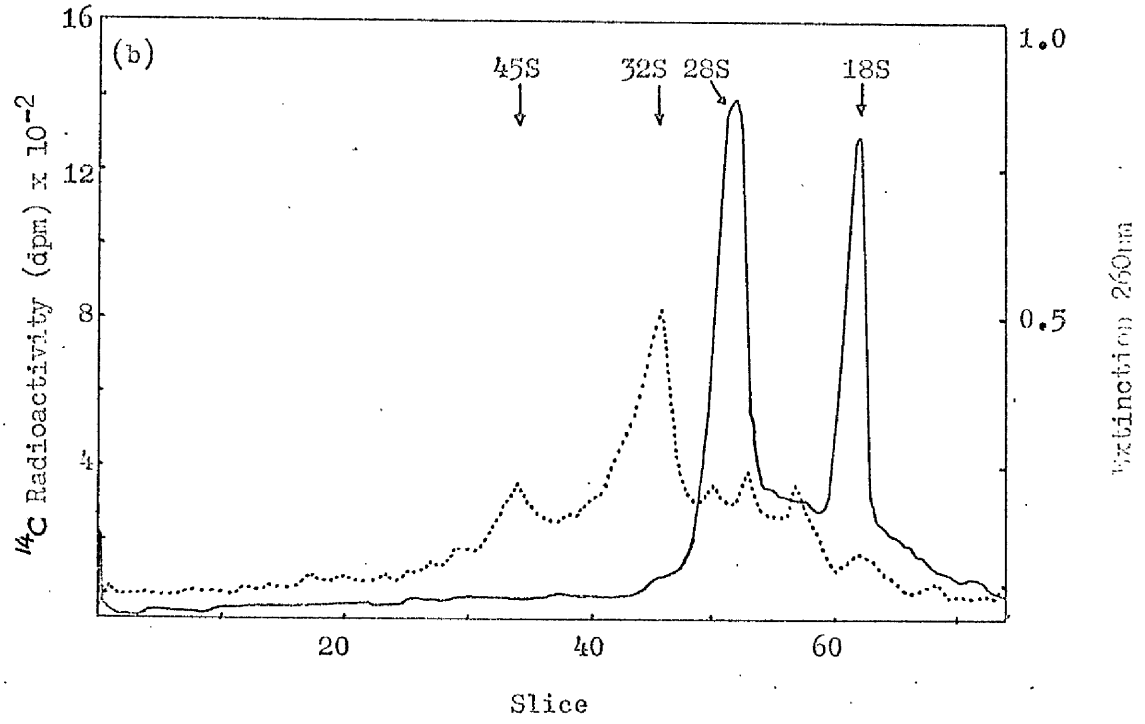
Unlabelled uterine RNA was mixed with ^{14}C labelled HeLa cell nucleolar RNA and the mixture was co-electrophoresed on a 1% agarose gel for 2½h.

——— = Extinction at 260nm.

- - - = Radioactivity due to ^{3}H.

..... = Radioactivity due to ^{14}C.


(a)
45S
32S + 28S
18S
^{14}C & ^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm
Fraction


(b)
45S
32S
28S
18S
^{14}C Radioactivity (dpm) x 10^{-2}
Extinction 260nm
Slice

with oestradiol and given a 15min pulse of tritiated ribonucleosides. HeLa nucleolar RNA was prepared from cells grown in the presence of [2-^{14}C]uridine. The conditions of sedimentation resulted in the bulk of the RNA, the ribosomal subunit species, remaining at the top of the gradient with the 28S and 18S peaks only partially separated. HeLa cell pre-rRNA was closely associated with rRNA; the 32S peak being indistinguishable from 28S RNA whilst the 45S species had migrated only slightly further. More than half of the gradient contained RNA species with sedimentation values in excess of 45S. The profile of ^{3}H counts revealed that most of the labelled RNA was located at the top of the gradient. Presumably this represented newly synthesized ribosomal and pre-ribosomal species, low-molecular-weight cytoplasmic RNA and the smaller species of HnRNA but these were totally undifferentiated. Tritium was also incorporated into the uterine RNA heavier than 45S, some of it so heavy that it almost sedimented to the bottom of the gradient.

Fig. 26(b) shows the separation, on a 1% agarose gel, of unlabelled uterine RNA mixed with ^{14}C labelled HeLa cell nucleolar RNA. The ribosomal species were clearly separated from each other and from the pre-ribosomal HeLa RNA. Some radioactivity was present in the first 30 slices of the gel. This may have been caused by impurities in the nucleolar preparation or may have been due to diffusion of the pre-rRNA species. The sharpness of the 28S and 18S peaks suggested that diffusion should not have been a problem with this system.

3.19. Comparison between Polyacrylamide Gels and Sucrose Density Gradients.

It was difficult to equate the separation of tritiated

Figure 27.

The distribution of uterine RNA on sucrose density gradients.

The RNA, which formed part of the preparation used in Fig. 25(a), was purified from rats which had received 1µg of oestradiol-17β 4h before death and 125µCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine 15min before death. It was layered onto a 15-30% sucrose density gradient in LETS buffer and sedimented for 16h at 31,800 x g and at 20°. The gradient fractions 1-15, 15-30 and 30-45 were pooled and the constituent RNA precipitated by two volumes of ethanol in the presence of approximately 40µg of unlabelled uterine RNA. Recovered RNA was separated on 2.7% polyacrylamide gels for 5h.

(a) = The sucrose density gradient indicating the positions of the three composite fractions.

(b) = Polyacrylamide gel separation of the RNA in the gradient fractions 1-15.

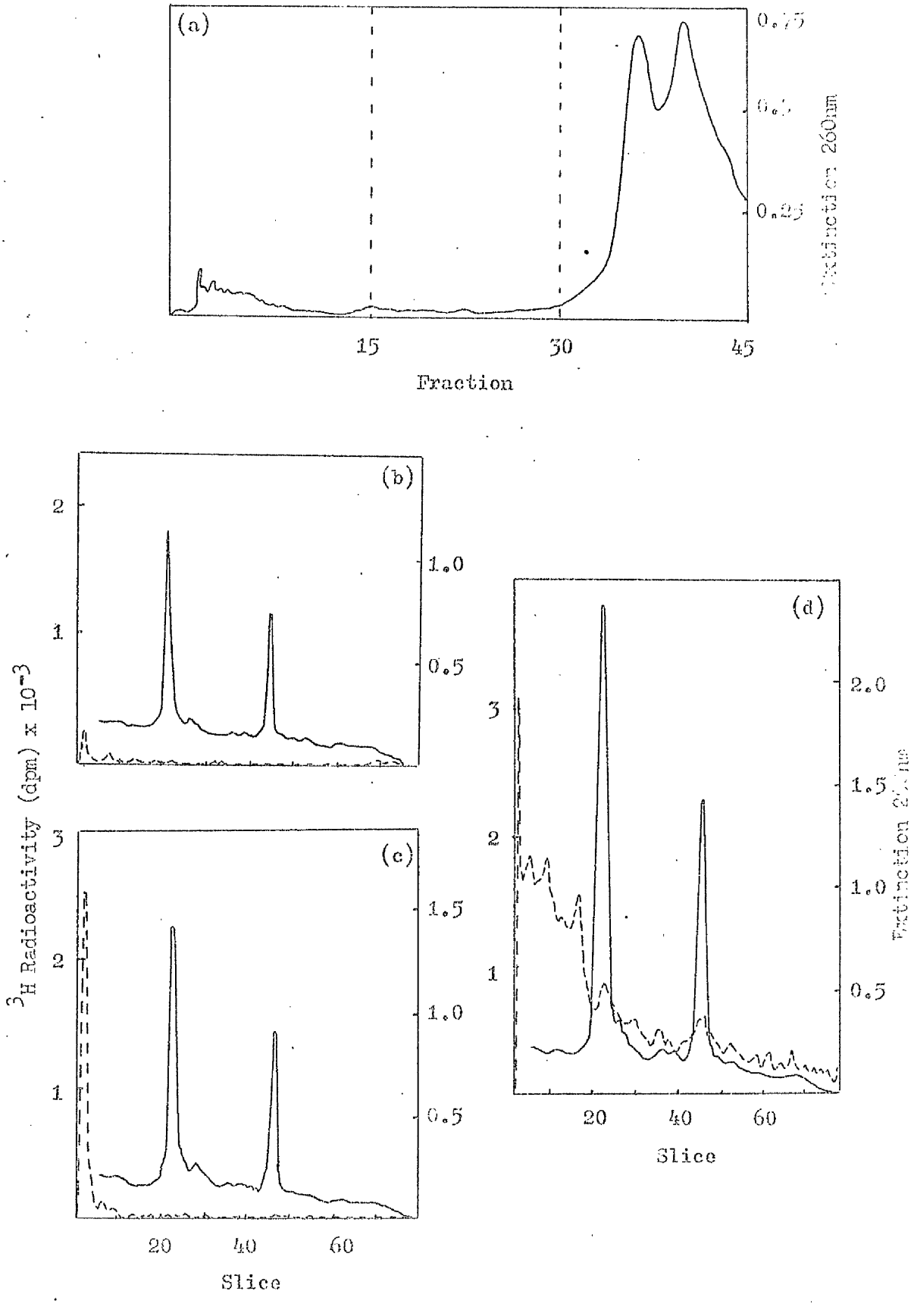
(c) = Polyacrylamide gel separation of the RNA in the gradient fractions 15-30.

(d) = Polyacrylamide gel separation of the RNA in the gradient fractions 30-45.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.

For reasons of clarity the E 260nm trace is omitted for the first few slices of the gels.


(a)
0.75
0.5
0.25
Extinction 260nm
15
30
45
Fraction
(b)
2
1
1.0
0.5
(c)
3
2
1
1.5
1.0
0.5
20
40
60
Slice
^{3}H Radioactivity (dpm) x 10^{-3}
(d)
3
2
1
2.0
1.5
1.0
0.5
20
40
60
Slice

uterine RNA on a sucrose density gradient (Fig. 26a) with that on polyacrylamide gels. The reason was obvious; the latter system concentrated high-molecular-weight RNA at one end while the former did the same with low-molecular-weight species. Fig. 27 shows an experiment which attempted to compare the two systems. Uterine RNA, from rats treated with oestradiol for 4h and receiving a 15min pulse of radioactivity, was separated on a 15-30% sucrose density gradient. The RNA formed part of the preparation from which the sample in Fig. 26(a) was derived and the gradient would have had a similar profile of tritium counts. The radioactivity in each fraction was not, however, determined. Instead, the 45 fractions were combined in three lots of 15, as indicated in Fig. 27(a). The RNA in each composite fraction was precipitated by two volumes of ethanol at -20° in the presence of approximately 40µg of unlabelled uterine RNA and the three preparations were separated on 2.7% polyacrylamide gels. Fig. 27(b) shows the distribution of labelled RNA from the bottom 15 fractions of the gradient. Relatively little RNA was heavy enough to have sedimented this far but there was a very definite peak of radioactivity at the gel origin. The middle fifteen fractions of the gradient also contained only very-high-molecular-weight RNA, but in increased amounts (Fig. 27c). The top fractions contained the ribosomal RNA species as well as a high percentage of the heterogeneous species (Fig. 27d).

It appeared that sucrose density gradients were suitable for looking at the heaviest of the HnRNA species. These molecules were spread over two thirds of the gradient in the absence of other types. The method used was less suitable for investigating the

only slightly heavier than 45S RNA but the character of the agarose separations suggested that they were ideal for resolving these molecules.

3.20. Separation of Uterine RNA on Sucrose Density Gradients, Polyacrylamide Gels and Agarose Gels.

In this experiment RNA was isolated in fairly large batches so that each preparation could be analysed on both of the gel types and on sucrose density gradients. In this way, it was hoped to gain the maximum information about the effect of oestrogen on the synthesis of uterine HnRNA. Control rats, or rats that had been treated with oestradiol 30min, 60min or 120min before death, received radioactive ribonucleosides 15min before death.

3.20. 1. Separation on Polyacrylamide Gels.

Fig. 28 shows the separation of the above RNA on polyacrylamide gels. The profile of precursor incorporation was essentially comparable with Fig. 8 (a, b, d and e). The first observable effects were again in the synthesis of RNA species remaining at the gel origin and now known to be HnRNA. There were, however, some differences. The results in Fig. 8 indicate very little, if any, stimulation in the synthesis of pre-rRNA after 1h of oestrogen treatment but in Fig. 28(c) there was a definite stimulation at this time. After 2h of oestrogen treatment, there was considerably more label in 28S RNA in Fig. 28(d) than there was in Fig. 8(e). These results were reproducible but the only known difference between the two experiments was that those in Fig. 8 and associated figures were done in the Winter whilst those in Fig. 28

Figure 28.

Comparison of polyacrylamide gels, sucrose density gradients and agarose gels in the analysis of uterine RNA synthesized in response to oestrogen.

(A) Separation on polyacrylamide gels:

18-21 day old rats, weighing 25-30g, received 1µg oestradiol-17β, or carrier, by intraperitoneal injection at varying times before death and 125µCi of each of [5-^{3}H] uridine and [8-^{3}H] guanosine by intravenous injection 15min before death. Purified RNA was separated for 5h on 2.7% polyacrylamide gels.

(a) = Control.

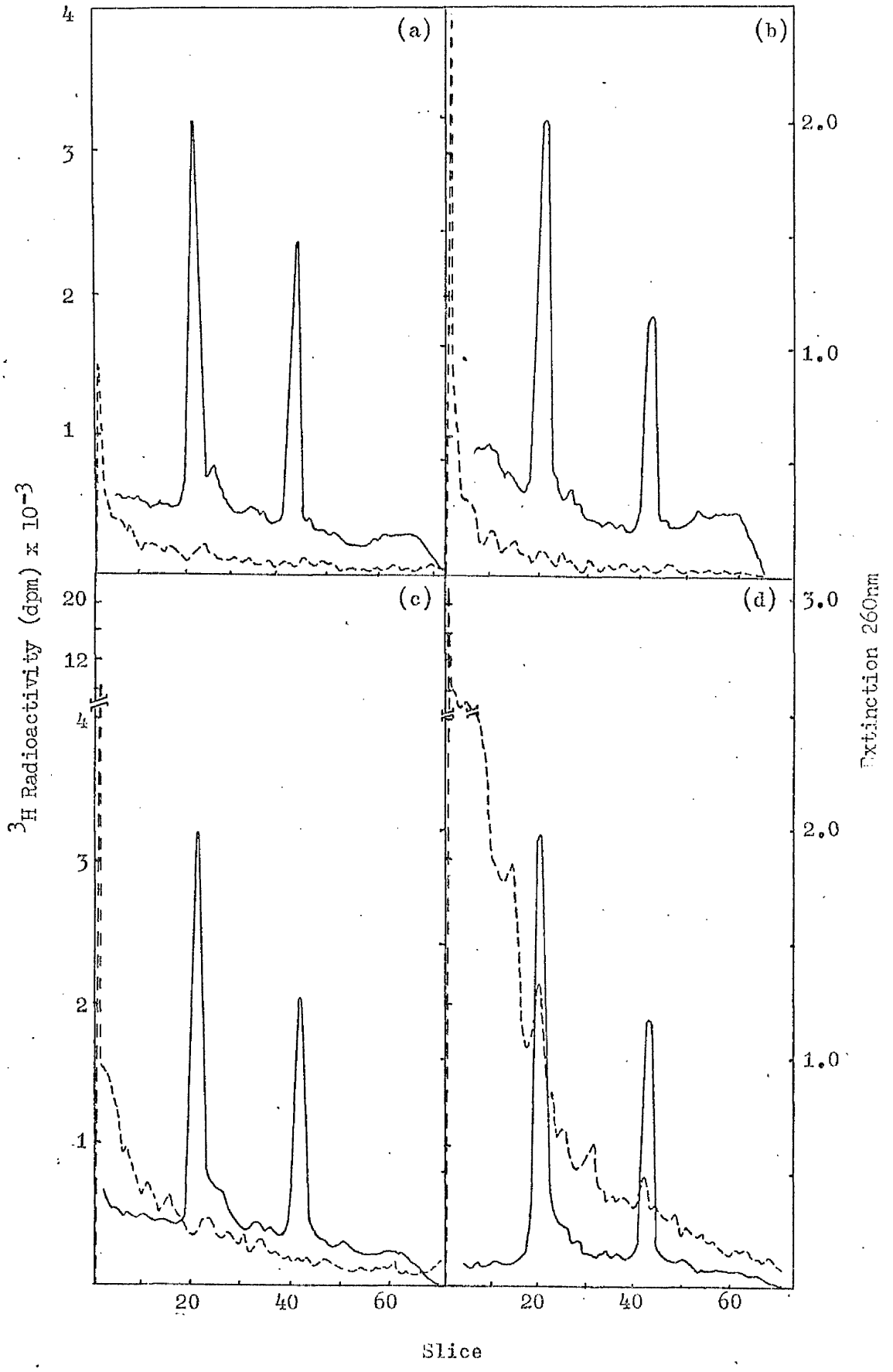
(b) = 30min treatment with oestradiol-17β.

(c) = 1h treatment with oestradiol-17β.

(d) = 2h treatment with oestradiol-17β.

——— = Extinction at 260nm.

– – – = Radioactivity per slice in dpm.


(a)
(b)
(c)
(d)
^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm
Slice
20
40
60
1
2
3
4
12
1.0
2.0
3.0

were done during the Summer. It is tempting to speculate that some factor, such as day length or air temperature, may sufficiently affect the metabolism of immature rats to influence the speed of their response to hormone and the rate of ribosome maturation. Further experimentation would be required to confirm these possibilities.

Notwithstanding the above differences, the polyacrylamide gels repeat the established pattern of response to oestrogen; namely the initial stimulation of HnRNA synthesis followed by increased production of ribosomes.

3.20. 2. Separation on Sucrose Density Gradients.

The early stimulation of HnRNA synthesis, seen when Fig. 28(a) was compared to Fig. 28(b), was less obvious when the same preparations were separated on 15-30% sucrose density gradients. This was expected, since it had been shown in Fig. 27 that the RNA in the first few slices of polyacrylamide gels was spread over two thirds of such gradients. Nevertheless, Figs. 29(a) and 29(b) revealed that, when RNA from control animals was compared with that isolated from rats treated with oestradiol for 30min, there was a definite increase in radioactivity levels throughout the bottom thirty fractions of the gradient. These increases were more obvious in the radioactivity profiles of rats which had been exposed to hormone for longer periods and Figs. 29(c) and 29(d) show that, 1h and 2h after treatment, the incorporation of precursor into HnRNA and lower molecular weight RNAs was strongly stimulated. As already shown, the slower sedimenting species were totally undifferentiated in this system. There was no indication of a

Figure 29.

Comparison of polyacrylamide gels, sucrose density gradients and agarose gels in the analysis of uterine RNA synthesized in response to oestrogen.

(B) Separation on sucrose density gradients:

The RNA preparations were aliquots of those described in Fig. 28. They came from 18-21 day old rats, weighing 25-30g, which had received 1µg oestradiol-17β, or carrier, intraperitoneally at various times before death and 125µCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine intravenously 15min before death. Purified RNA was layered onto 15-30% sucrose density gradients and sedimented for 16h at 31,800 x g and at 20°. The fractions are numbered from the bottom of the gradients.

(a) = Control.

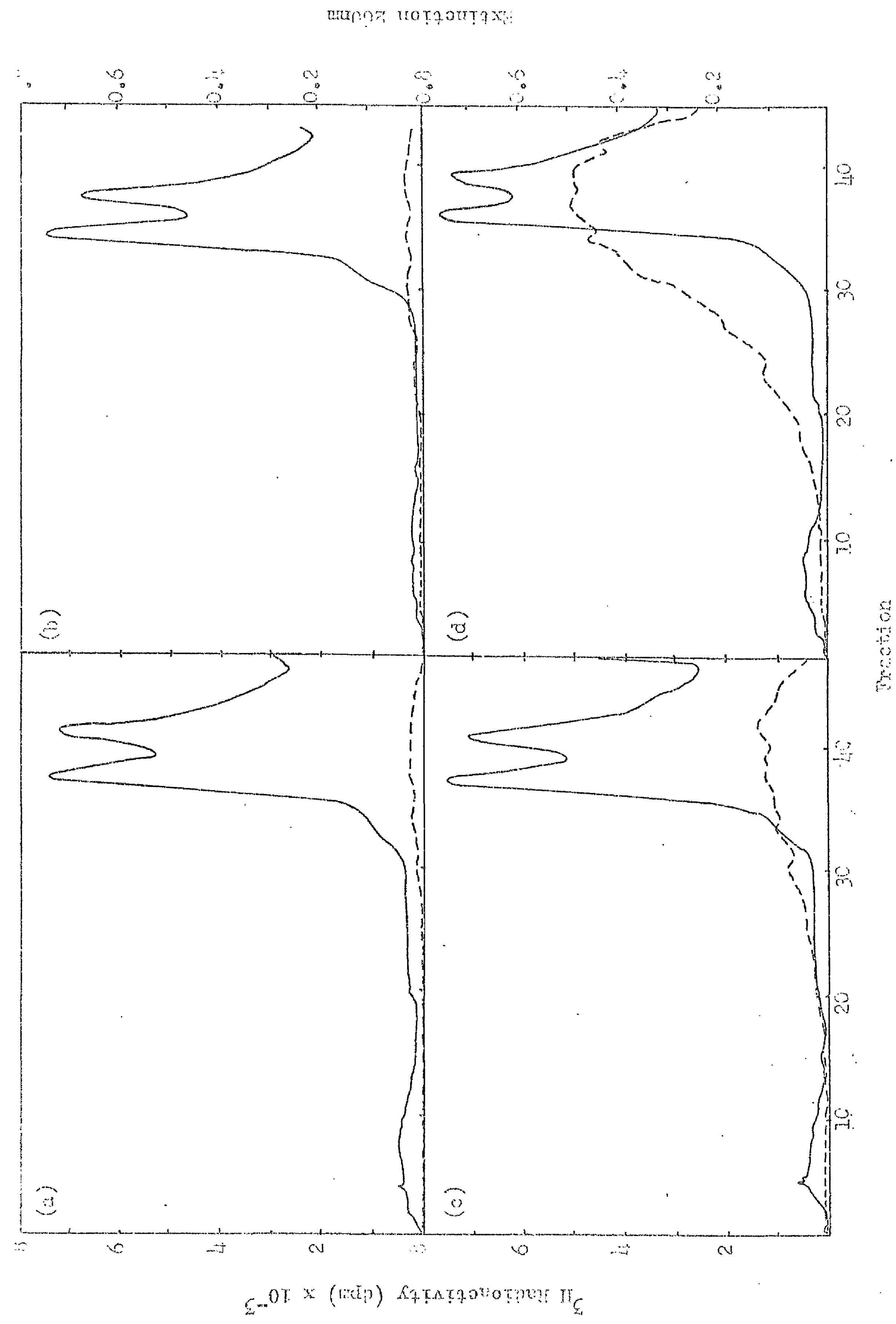
(b) = 30min treatment with oestradiol-17β.

(c) = 1h treatment with oestradiol-17β.

(d) = 2h treatment with oestradiol-17β.

——— = Extinction at 260nm.

- - - = Radioactivity per fraction in dpm.


Extinction 260nm
(a)
(b)
(c)
(d)
Fraction
³H Radioactivity (dpm) x 10⁻³

selective stimulation of discrete HnRNA species at earlier times or to a greater extent than the remainder.

3.20. 3. Separations on Agarose Gels.

The preparations of uterine RNA, examined in Figs. 28 and 29 on polyacrylamide gels and sucrose density gradients, were further investigated by separating them on 1% agarose gels. Fig. 30 shows that the earliest effects of the hormone, seen in HnRNA from rats exposed to oestrogen for 30min, were detected as increased levels of radioactivity in the first 30 slices of the gel. These slices would contain RNA larger than the ribosomal precursor species. Longer hormone treatments resulted, as expected, in the stimulated synthesis of pre-rRNA as well as large increases in the heterogeneous background radioactivity throughout the length of the gel. Again, there was no detectable individual species of HnRNA, the synthesis of which was stimulated to a greater extent than the rest.

The separation of immature rat RNA on sucrose density gradients and agarose gels was undertaken because it was felt that these systems would clarify the oestrogen-induced synthesis of HnRNA. The early stimulation of the synthesis of these species was indeed confirmed by the results obtained with both systems, but it must be said that the increased incorporation of radioactivity was much more apparent where the HnRNA was collected into a few slices of a polyacrylamide gel. The greater resolution of the gradient and agarose techniques served to illustrate the heterogeneous nature of the very-high-molecular-weight HnRNA and it revealed that the increased synthesis was not restricted to a few species but was of a general nature.

Figure 30.

Comparison of polyacrylamide gels, sucrose density gradients and agarose gels in the analysis of uterine RNA synthesized in response to oestrogen.

(C) Separation on agarose gels:

The RNA preparations were aliquots of those described in Figs. 28 and 29. They came from 18-21 day old rats, weighing 25-30g, which had received 1µg of oestradiol-17β, or carrier, intraperitoneally at various times before death. and 125 µCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine intravenously 15min before death. Purified RNA was separated for 2½h on 1% agarose gels.

(a) = Control.

(b) = 30min treatment with oestradiol-17β.

(c) = 1h treatment with oestradiol-17β.

(d) = 2h treatment with oestradiol-17β.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.

(a)
(b)
(c)
(d)
16
12
8
4
1.0
0.5
20
40
60
Slice
^{3}H Radioactivity (dpm) x 10^{-2}
Extinction 260nm

4. Low-Molecular-Weight Uterine RNA.

4. 1. Purification of Low-Molecular-Weight RNA.

It has already been mentioned that the high-molecular-weight RNA, purified by the method of Joel & Haggerman (1969), was unsuitable for studies on the low-molecular-weight species. In my hands, the precipitation procedures designed to remove contaminating oligonucleotides, also gave rise to substantial losses of 4S RNA. The extent of these losses is shown in Fig. 31(a), where RNA prepared by the above method was separated on a 2.7% polyacrylamide gel for 2¼h. The extinction at 260nm by 4S RNA was insignificant compared to the 28S and 18S peaks and was slightly smaller than the 5S peak. Fig. 31(c) shows the separation, on a 7.5% polyacrylamide gel, of a similar uterine RNA preparation in which the rats were given radioactive ribonucleosides and were stimulated by oestradiol to promote 4S RNA synthesis. The extinction at 260nm showed that the 4S RNA peak was considerably smaller than that of the 5S RNA. It also shows a reproducible pattern of minor peaks, some of which were rapidly labelled in rats exposed to oestradiol for several hours. The incorporation of precursor into 4S RNA produced an interesting artifact, in that the peak of radioactivity was always displaced to the right of the peak of extinction at 260nm. Whether this finding, and the narrowness of the 4S peak, reflect a preferential loss during the purification of those tRNA species which normally comprise the trailing edge of the 4S peak, is unknown.

The losses of 4S RNA could not be overcome by omitting the deoxyribonuclease digestion and subsequent purification steps because

Figure 31.

Characterization of uterine low-molecular-weight-RNA and its purification.

(a) Uterine RNA, prepared by the method of Joel & Haggerman (1969) from 18-21 day old rats, weighing 25-30g, was separated for 2¼h on a polyacrylamide gel.

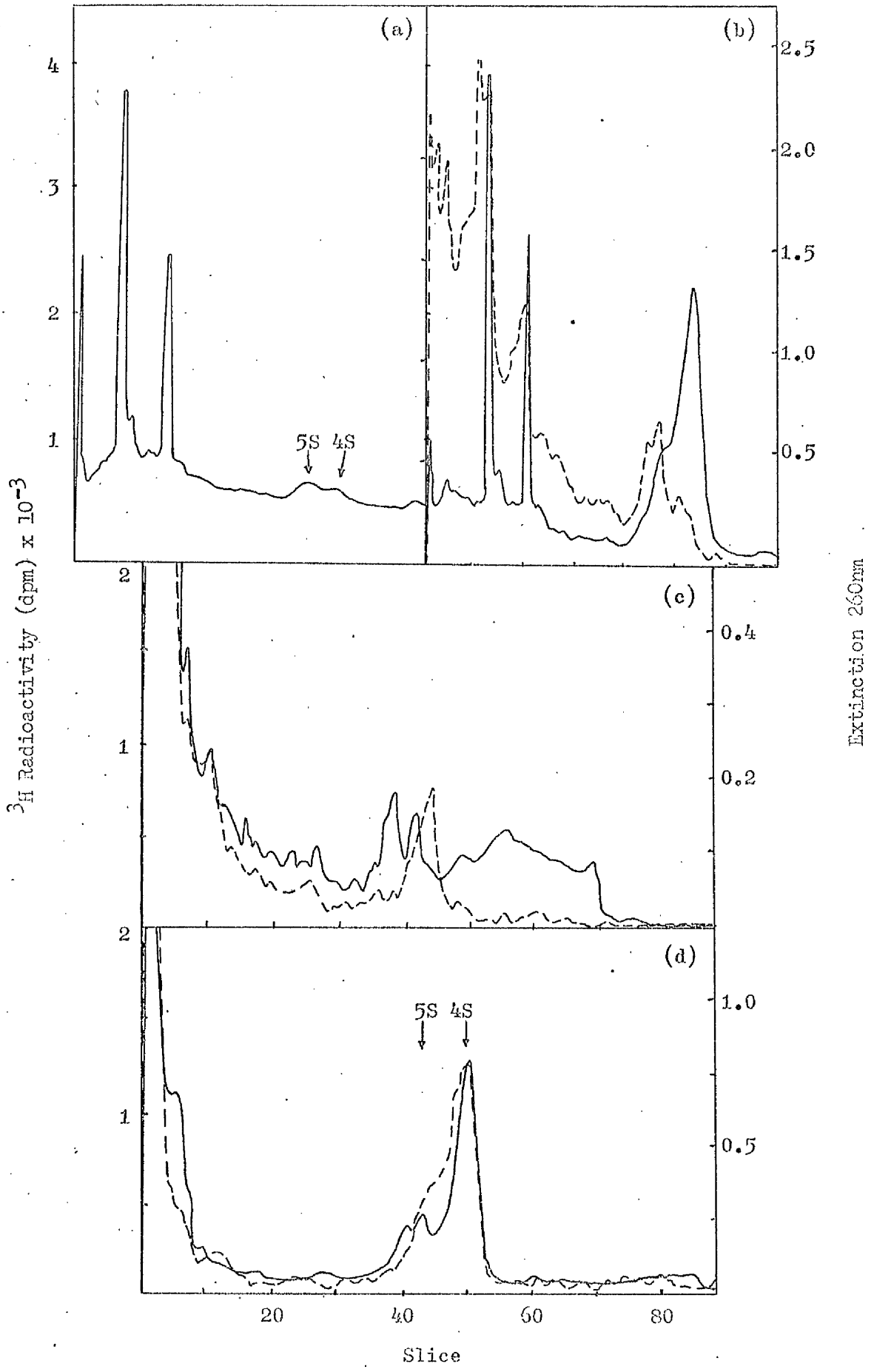
(b) 18-21 day old rats, weighing 25-30g, received 1µg oestradiol-17β intraperitoneally 4h before death and 125µCi of each of [5-^{3}H] uridine and [8-^{3}H] guanosine intravenously 30min before death. Uterine RNA was prepared by the method of Joel & Haggerman (1969) as far as, and including, the deoxyribonuclease digestion and then separated on a 2.7% polyacrylamide gel for 2¼h.

(c) RNA was prepared by the method of Joel & Haggerman (1969) from the uteri of 18-21 day old rats, weighing 25-30g. They had received 1µg oestradiol-17β intraperitoneally 4h before death and 125µCi of each of [5-^{3}H]uridine and [8-^{3}H] guanosine intravenously 30min before death. The purified RNA was separated on 7.5% polyacrylamide gels for 4h.

(d) Uterine RNA was prepared from 18-21 day old rats, weighing 25-30g, by the method for low molecular weight RNA described in the Materials and Methods section. The rats received 1µg oestradiol intraperitoneally 4h before death and 125µCi of each of [5-^{3}H]uridine and [8-^{3}H] guanosine intravenously 30min before death. The purified RNA was separated on 7.5% polyacrylamide gels for 4h. Fig. 31(d) is reproduced from figure 33(d).

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


(a)
(b)
(c)
(d)
5S 4S
5S 4S
^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm
Slice
20
40
60
80

the DNA in the preparation then overloaded the polyacrylamide gels. Nor was the problem solved by not removing the DNA oligonucleotides. Fig. 31(b) shows a separation of uterine RNA which had not been purified after digestion of the DNA. The oligonucleotides completely masked the extinction at 260nm due to 4S and 5S RNA.

In preparing RNA for separation on 7.5% polyacrylamide gels, the deoxyribonuclease and purification steps were substituted by a single precipitation with 1M NaCl. The removal of higher-molecular-weight RNA by this method was not very efficient but was sufficient to avoid overloading the gels. Fig. 31(d) shows a typical separation of uterine RNA from rats in which the incorporation of tritiated ribonucleosides into RNA had been stimulated by oestradiol (The figure is reproduced from Fig. 32 for convenient comparison). The 5S and 4S RNA were in the accepted relative proportions to each other (Burdon & Clason, 1969) and, after 4h electrophoresis on a 7.5% gel, were almost completely separated from each other. There was no sign of the minor peaks found in the previous preparations but the 5S RNA had a double peak which became more noticeable after longer separations (see Fig. 34). There were two possible explanations for this phenomenon. It seemed most likely that the component with the slower migration characteristics was the 28S associated RNA discovered by Pene et al. (1968) and widely known as 7S RNA. They found that this species was normally strongly bound to 28S RNA but was released by hot phenol with a maximum yield at 50°. Originally the species was assigned a sedimentation constant of 7S, but Weinberg & Penman (1968) found it had an electrophoretic mobility corresponding to a value of approximately 5.5S and they renamed it 28S associated RNA (28SA RNA). Its

conditions of extraction and electrophoretic mobility were consistent with the slower migrating component in the 5S peak of Fig. 31(d).

A less attractive possibility was that the bimodal 5S peak represented the two forms of 5S RNA reported by Weinberg & Penman (1968) and Weinberg & Penman (1969). One of these forms was a denaturation product of hot phenol extraction but it usually only formed a shoulder on the 5S peak and would probably not have been detectable where the separation of 4S and 5S RNA was as incomplete as in Fig. 31(d). The apparent labelling of one of the components of the 5S peak to a greater extent than the other in Fig. 31(d) also argued against the two entities being forms of the same species. Conversely, the finding that the slower migrating component was not markedly labelled after a 30min pulse of precursors but was after a 1h pulse (compare Fig. 31d and 34d), was consistent with its identification as 28SA RNA since, after a 30min pulse, most 28SA RNA would be a covalently bound part of 32S RNA (Fig. 9; Pene et al., 1968).

4. 2. Characterization of Uterine 4S and 5S RNA.

To confirm the identity of the 4S and 5S RNA peaks, uterine low-molecular-weight RNA, from rats which had received oestradiol 2h before death and radioactive ribonucleosides 30min before death, was coelectrophoresed with commercial E. coli "Soluble RNA". Fig. 32 shows the separation of the mixture on a 7.5% polyacrylamide gel for 4h. The radioactivity profile corresponded to the extinction at 260nm of the combined uterine and bacterial 4S and 5S RNA species without any apparent overlap. It was concluded that the three peaks detectable after separation of low-molecular-weight uterine

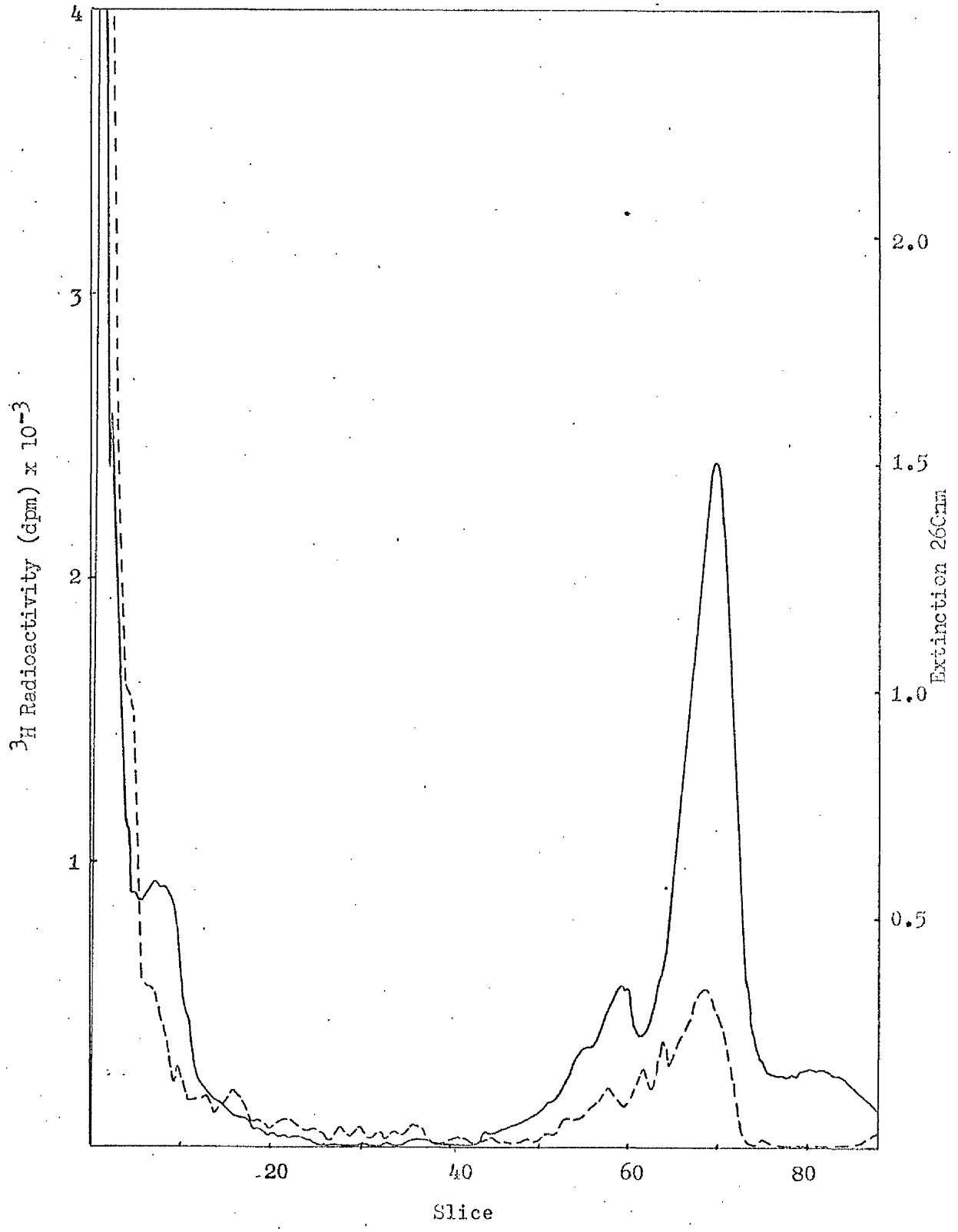
Figure 32.

Coelectrophoresis of uterine and E. coli low-molecular-weight RNA.

18-21 day old rats, weighing 25-30g, received 1μg of oestradiol-17β intraperitoneally 2h before death and 125μCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine intravenously 30min before death. Uterine low-molecular-weight RNA was prepared and mixed with 50μg of E. coli "soluble" RNA. The mixture was cofractionated by electrophoresis on a 7.5% polyacrylamide gel for 4h.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


4
3
2
1
^{3}H Radioactivity (dpm) x 10^{-3}
2.0
1.5
1.0
0.5
Extinction 26Cnm
20
40
60
80
Slice

RNA on 7.5% polyacrylamide gels were 4S, 5S and 28SA RNA.

4. 3. The Effect of Oestradiol on the Synthesis of Low-Molecular-Weight RNA.

Fig. 33 shows the effect of oestradiol-17β treatment of rats on the incorporation of tritiated ribonucleosides into 4S and 5S uterine RNA when the precursor had been administered 30min before the death of the animals. It was found that, compared with the controls, incorporation was slightly enhanced after 1h and strongly stimulated after 2 and 4h of oestradiol treatment. It was interesting that the increased synthesis appeared to precede slightly the stimulated production of rRNA.

4. 4. Immature Rat Uterus Pre-tRNA.

The incorporation of radioactive ribonucleosides into 4S and 5S RNA in the above experiment indicated that there was some newly-synthesized RNA of an intermediate size. Fig. 33(b) in particular, had three definite tritium peaks; a double peak associated with 5S and 28SA RNA, a 4S peak and a third peak migrating at approximately 4.5S. Pre-tRNA has been shown to migrate between 4S and 5S on polyacrylamide gels (Burdon & Clason, 1969) and in Fig. 34 an attempt is made to demonstrate this species in immature rat uteri. The animals received oestradiol 1½h before they were given a 5, 10, 20 or 60min pulse of tritiated ribonucleosides. The purified RNA was separated for 6h on polyacrylamide gels, the longer electrophoretic period giving a slightly improved separation of 4S and 5S RNA. After a 5min pulse of radioactivity, the biggest peak of precursor uptake was into RNA migrating to the left of 4S RNA

Figure 33.

Effect of oestrogen on the synthesis of uterine low-molecular weight RNA.

Low-molecular-weight uterine RNA was isolated from 18-21 day old rats, weighing 25-30g. They had received 1µg of oestradiol-17β by intraperitoneal injection at various times before death and 125µCi of each of [5-^{3}H]uridine and [8-^{3}H] guanosine intravenously 30min before death. Purified RNA was separated on 7.5% polyacrylamide gels for 4h.

(a) = Control.

(b) = 1h after oestradiol-17β administration.

(c) = 2h after oestradiol-17β administration.

(d) = 4h after oestradiol-17β administration.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.

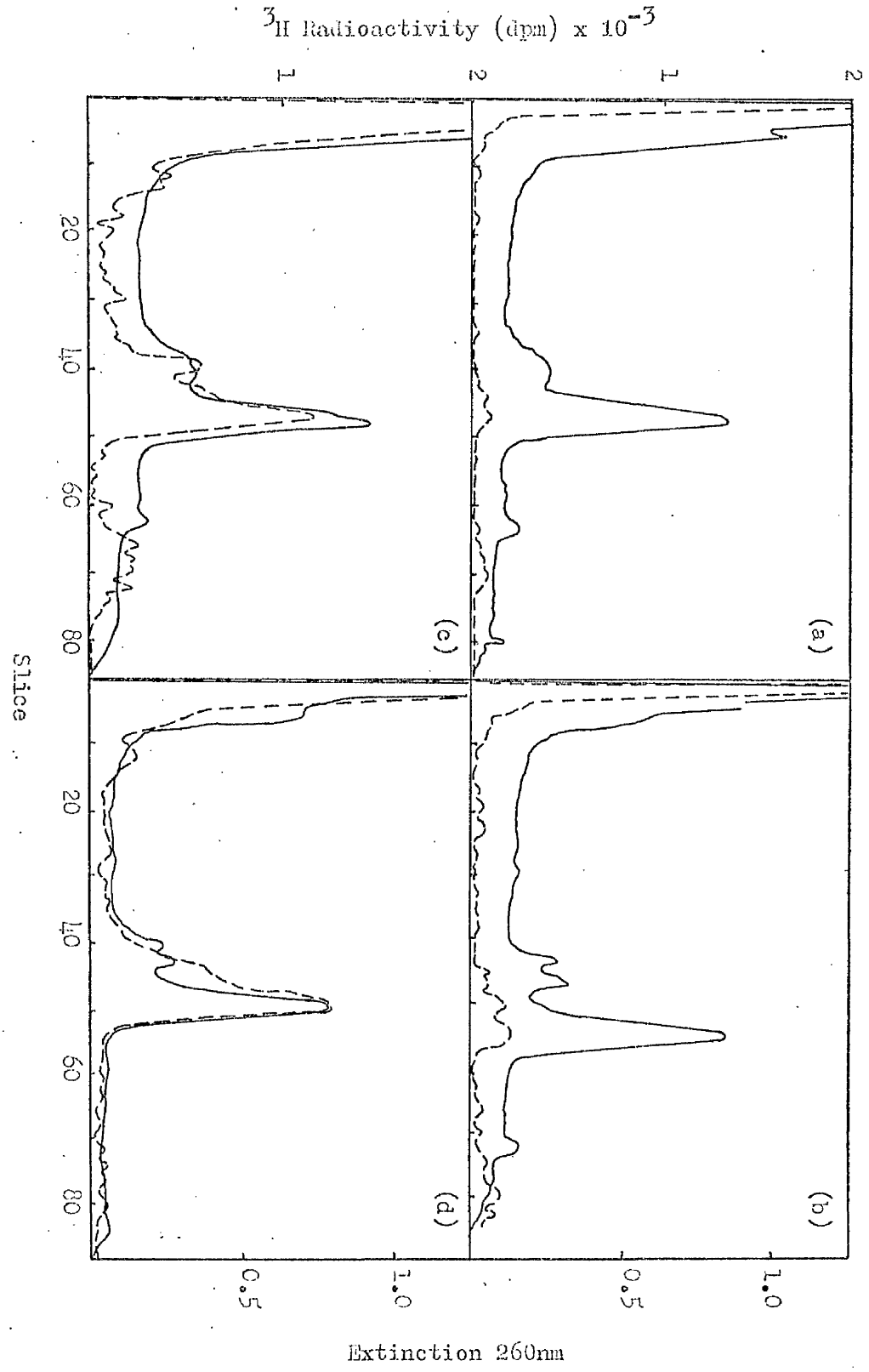

^{3}H Radioactivity (dpm) x 10^{-3}
1
2
1
2
20
40
60
80
(c)
(a)
(d)
(b)
20
40
60
80
0.5
1.0
0.5
1.0
Slice
Extinction 260nm

Figure 34.

Attempts to detect uterine pre-tRNA.

18-21 day old rats, weighing 25-30g received 1μg of oestradiol-17β, intraperitoneally, 1½h before the intravenous administration of 125μCi of each of [5-^{3}H]uridine and [8-^{3}H] guanosine. Purified low-molecular-weight uterine RNA was separated on 7.5% polyacrylamide gels for 6h.

(a) = Radioactive precursors given 5min before death.

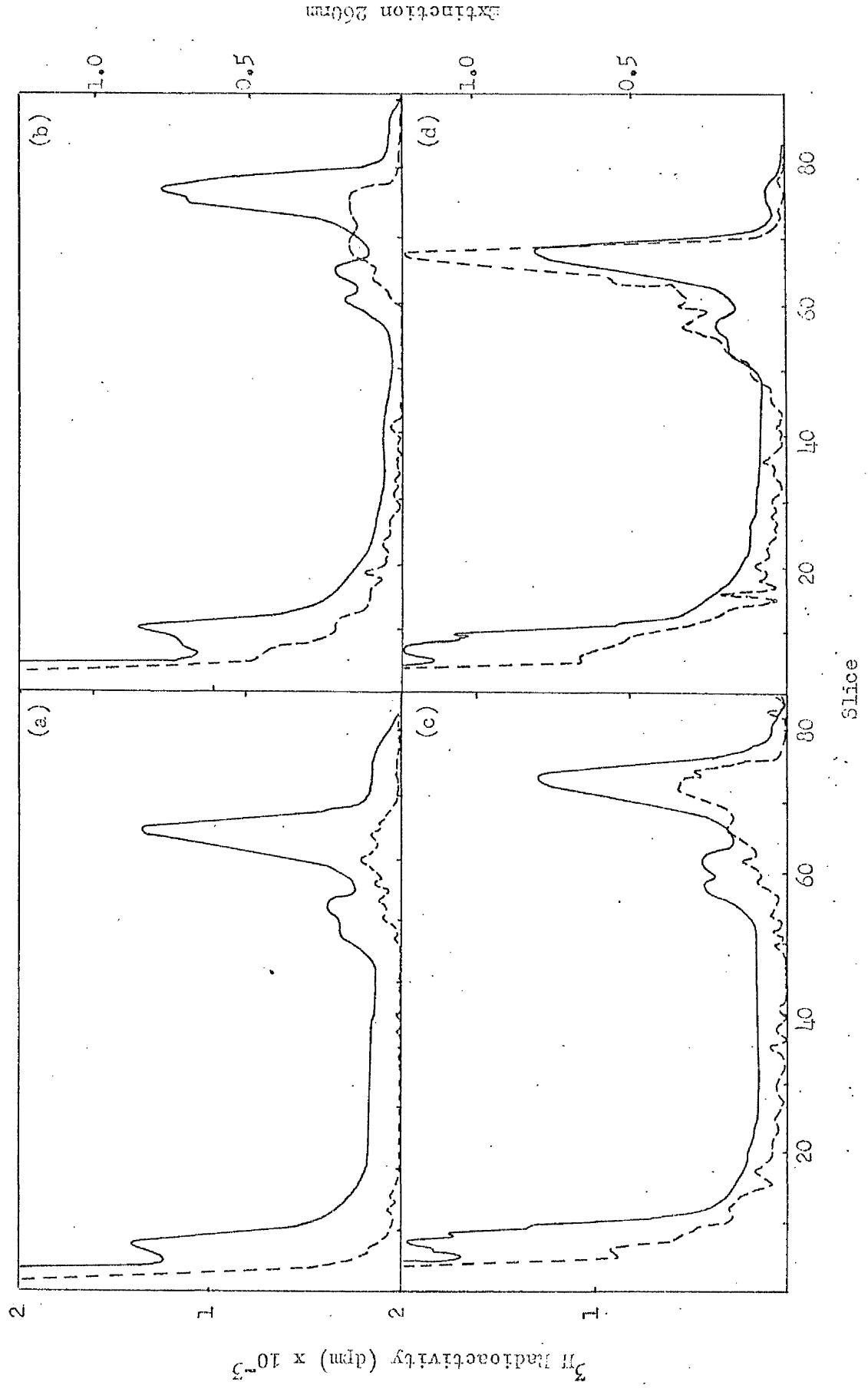
(b) = Radioactive precursors given 10min before death.

(c) = Radioactive precursors given 20min before death.

(d) = Radioactive precursors given 1h before death.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


(a)
(b)
(c)
(d)
2
1
20
40
60
80
1.0
0.5
Slice
^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm

and, after 10min incorporation, the peak migrating at approximately 4.5S was still slightly larger than that of the 4S species. A 20min pulse length resulted in the incorporation into 4S RNA overtaking that of the intermediate species, whilst after a pulse of 1h, precursor uptake into the 4S and 5S peaks was sufficient to obscure the intermediate peak.

The precursor-product relationship demonstrated above is insufficient to establish the identity of pre-tRNA. Indeed, in the absence of amino acid-acceptance determinations, the 4S RNA cannot be categorically called tRNA. Nevertheless, the results shown in Fig. 34 can be taken as strong evidence that the synthesis of tRNA, via a precursor species migrating at approximately 4.5S, occurs in rat uterus as well as in the tissue culture systems in which it is usually studied.

4. 5. Effects of Actinomycin D on the Synthesis of Low-Molecular-Weight RNA.

Fig. 35 shows the effects of actinomycin D at 30µg, 60µg and 120µg/rat on the hormone-stimulated synthesis of 4S and 5S RNA in immature rat uteri. The inhibitor was given simultaneously with oestradiol 4h before rat death and radioactive ribonucleosides were given 30min before death. The synthesis of 4S and 5S RNA was considerably more resistant to inhibition by actinomycin D than was the synthesis of rRNA (compare Fig. 35 with Fig. 13). At a dose of 30µg/rat of actinomycin D, which totally suppressed rRNA synthesis, incorporation of radioactivity into 4S and 5S RNA was only partially inhibited. As with HnRNA synthesis, some low-

Figure 33.

The effect of actinomycin D on the oestradiol-17β-stimulated synthesis of uterine low-molecular-weight RNA.

18-21 day old rats, weighing 25-30g, received intraperitoneal injections of 1μg of oestradiol-17β and varying amounts of actinomycin D simultaneously 4h before death. They also received 125μCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine intravenously 30min before death. Purified low-molecular-weight RNA was separated on 7.5% polyacrylamide gels for 4h.

(a) = No actinomycin D. (reproduced from figure 33d).

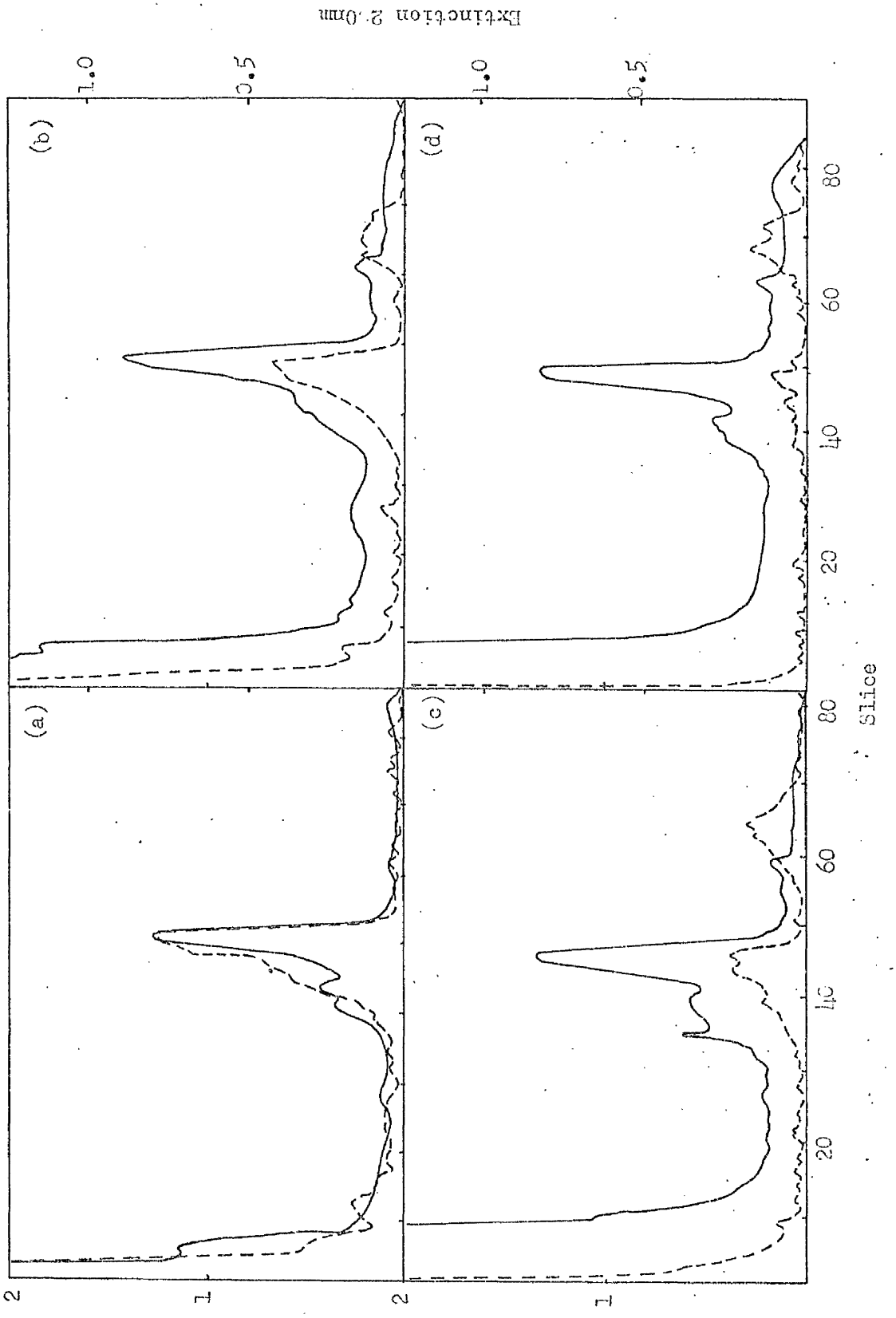
(b) = 30μg/rat of actinomycin D.

(c) = 60μg/rat of actinomycin D.

(d) = 120μg/rat of actinomycin D.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


Extinction 2.0mm
1.0
0.5
(b)
(a)
(d)
(c)
2
1
20
40
60
80
Slice
^{3}H Radioactivity (dpm) x 10^{-3}

molecular-weight RNA was made at inhibitor concentrations of 120µg/rat. The untreated control in Fig. 35 is a repeat of Fig. 33(d), included to facilitate comparison.

In all of the actinomycin D treatments, there was an accumulation of radioactivity migrating further than 4S RNA in polyacrylamide gels. The number of counts in this peak was fairly constant at all inhibitor levels and was equally pronounced at 15µg of actinomycin D/rat. For this reason it was felt that the peak was probably degradation products of ribosomal or pre-ribosomal RNA. Reich & Goldberg (1964) have suggested that actinomycin D may cause some RNA degradation.

4. 6. Effects of Cycloheximide on the Synthesis of Low-Molecular-Weight RNA.

Fig. 36 compares the effect of cycloheximide at 600µg/rat, on the hormone-stimulated synthesis of rat uterine low-molecular-weight RNA, with the uninhibited result shown in Fig. 33(d). The rats were given 4h oestradiol treatment, a 30min pulse of precursors and the inhibitor was administered 1h before, 1h after or simultaneously with the hormone. Cycloheximide strongly inhibited tRNA synthesis, the suppression being most severe in animals receiving the inhibitor before the hormone but only slightly less marked after the shorter treatments.

5. The Ribosomes of the Oestrogen-Treated Immature Rat Uterus.

Tata (1967a) reported that many hormones stimulated the production of ribosomes and membranes and the accumulation, in

Figure 36.

Effect of cycloheximide at 600µg/rat on oestradiol-stimulated low-molecular-weight RNA synthesis in immature rat uterus.

18-21 day old rats, weighing 25-30g, received 1µg oestradiol-17β 4h before death and cycloheximide at various times. Both were given by intraperitoneal injection but 125µCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine were administered intravenously 30min before death. The purified uterine low-molecular-weight RNA was separated on 7.5% polyacrylamide gels for 4h.

(a) = No cycloheximide treatment (figure taken from Fig. 33d).

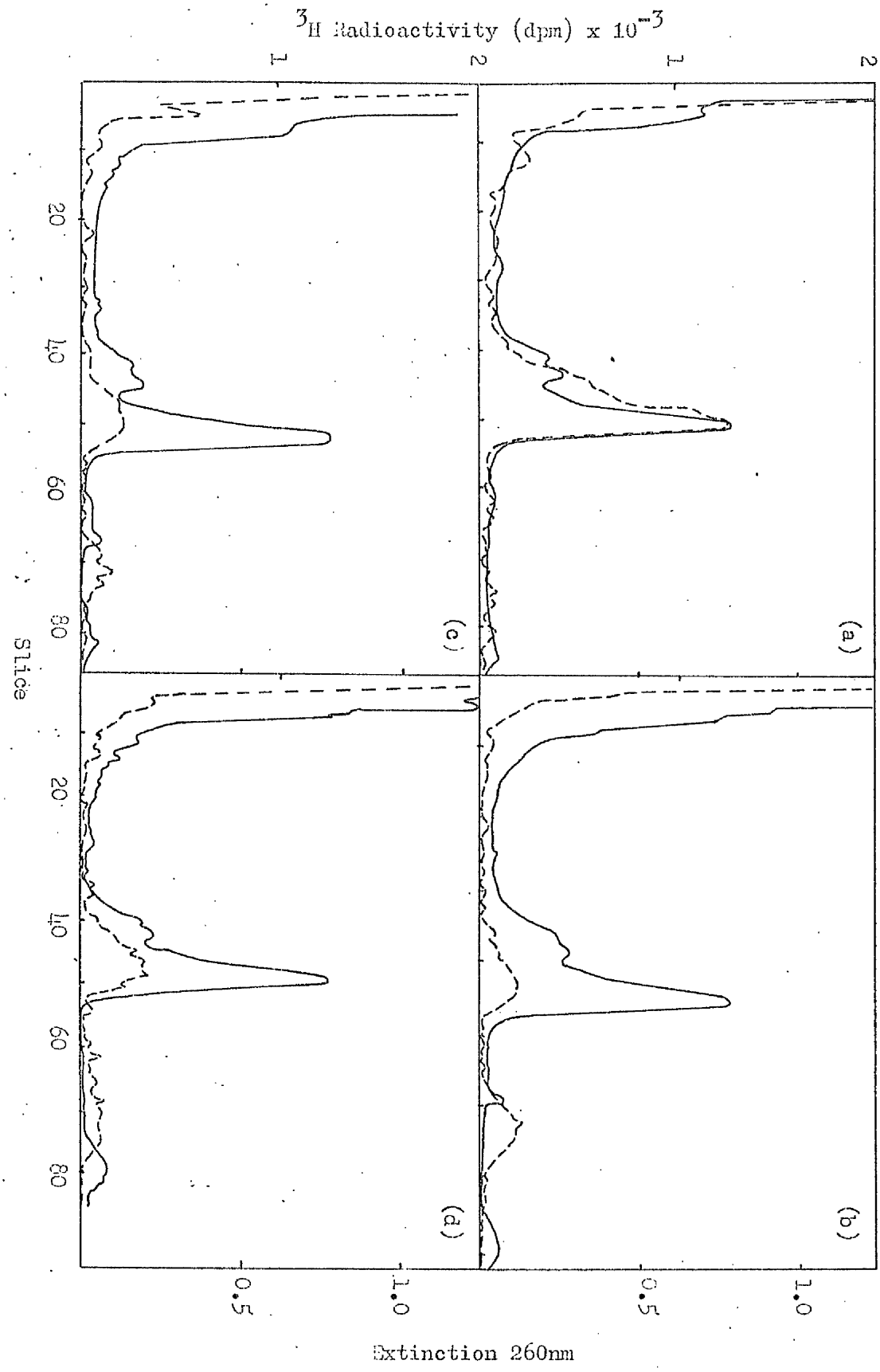
(b) = Cycloheximide given 1h before hormone.

(c) = Cycloheximide given simultaneously with hormone.

(d) = Cycloheximide given 1h after hormone.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


^{3}H Radioactivity (dpm) x 10^{-3}
1
2
1
2
20
40
60
80
Slice
(c)
(a)
20
40
60
80
(d)
(b)
0.5
1.0
0.5
1.0
Extinction 260nm

responsive cells, of rough endoplasmic reticulum. As a result of his studies, he postulated that the hormone-induced response promoted the formation of a separate population of ribosomes which controlled the subsequent growth and development of target cells. Little work of this kind has been done with the uterus responding to oestrogen. Although it was known that phospholipid and ribosome synthesis were stimulated during oestrogen action (Aizawa & Mueller, 1961; Hamilton et al., 1968), Davies (1967) showed only slightly more rough endoplasmic reticulum in the endometrium of the pregnant rabbit than in a nonpregnant animal. In both cases, the amount of rough endoplasmic reticulum was negligible compared to that in the liver of Rana catesbeaina responding to tri-iodothyronine (Tata, 1967a,b). Conversely, Nemetschek-Gansler (1967) has reported that the granular reticulum and free ribosomes were greatly increased under the influence of oestrogen in the epithelial, muscle and connective tissue cells of the uterus. The importance of the implications in Tata's (1970a) hypothesis warranted further investigations of his ideas and it was decided to attempt to purify uterine submicrosomal fractions, to look at their structure, the RNA they contained and to investigate whether the ribosomes produced in response to oestrogen entered one fraction to a greater extent than the remainder.

5. 1. Preparation and Characterization of Microsomal Fractions.

Of several methods tried, the best results were obtained with a modified version of that described by Tata (1970b). The excised uteri were very gently homogenized and a mitochondria-free

supernatant produced from the homogenate. Microsomal fractions were then isolated, as described in the Materials and Methods section, by sedimentation through two discontinuous sucrose density gradients. The method gave rise to four preparations, called here 1, 2, 3 and 4 and equivalent in sedimentation characteristics to the smooth membrane, light rough membrane, heavy rough membrane and polysome fractions respectively, of Tata's preparations (1970b).

Initial analysis of the microsomal preparations were encouraging. Fractions 1, 2, 3, and 4 had RNA/phospholipid ratios of 0.123, 0.823, 2.0 and 3.96 respectively and thus appeared to consist of the expected submicrosomal entities. When the fractions were investigated by electron microscopy, it became clear that they did not equate with the liver preparations (Tata, 1970b). Fig. 37 shows that fraction 1 was composed very largely of smooth membrane in the form of tightly packed vesicles and contained some particulate matter which was probably ribonucleoprotein but appeared smaller than ribosomes. Fraction 2 could only be described as debris. It contained some membranous elements and some particulate material but the bulk of the preparation was essentially structureless. Fraction 3 consisted of purified ribosomes containing polysomes but with very little evidence of membranous elements. Fraction 4 was again largely debris but contained ribosomes, some of which appeared to be membrane-bound. The preparations shown in Fig. 37 were from rats which had received 24h oestradiol treatment; the fractions from control animals were similar except that the smooth membrane appeared somewhat less abundant and the ribosomes were in much smaller numbers. Again, there was little evidence of membrane-bound ribosomes.

Figure 37.

Submicrosomal fractions of immature rat uterus.

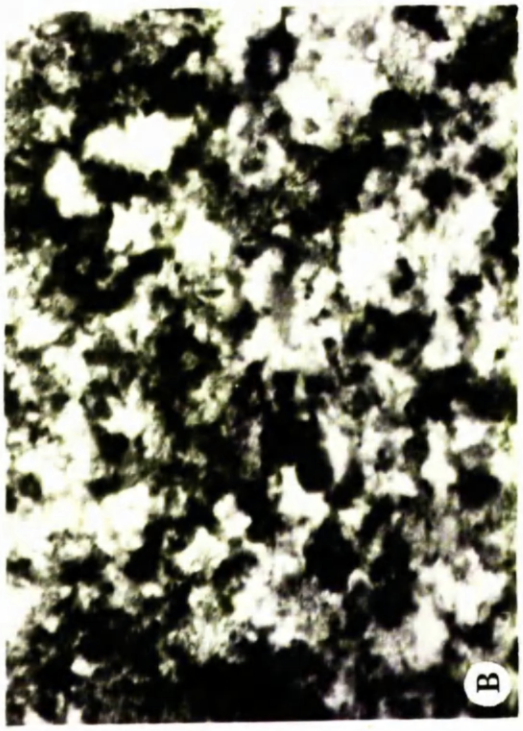
Submicrosomal fractions were prepared from the uteri of 18-21 day old rats by method A in the Materials and Methods section. All rats received 1µg of oestradiol intraperitoneally 24h before death. The pelleted fractions were prepared for visualization in an electron beam as described in the Materials and Methods section and all preparations are at a magnification of 50,000 (20,000 mag. x 2.5 enlargement).

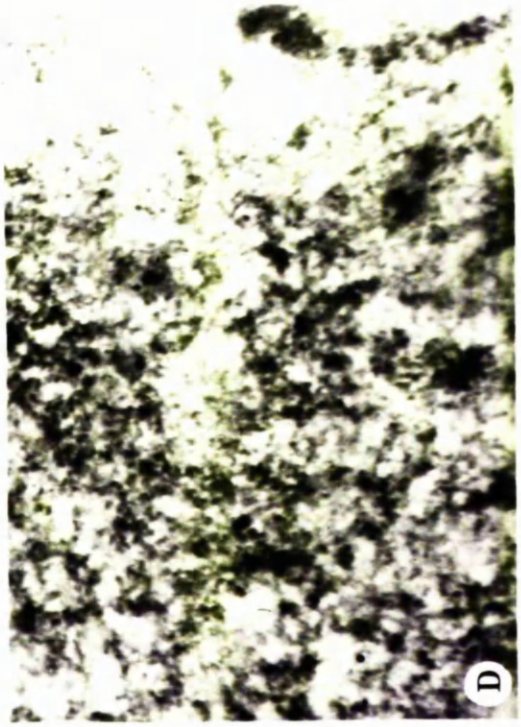
(A) = Fraction 1.

(B) = Fraction 2.

(C) = Fraction 3.

(D) = Fraction 4.


B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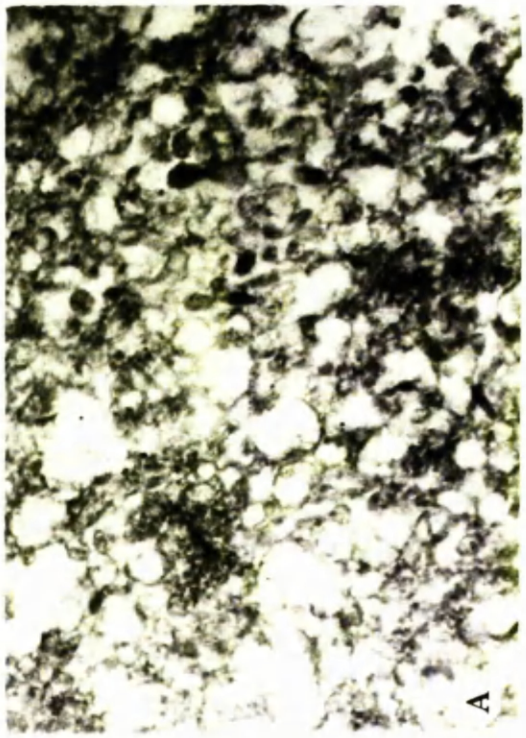
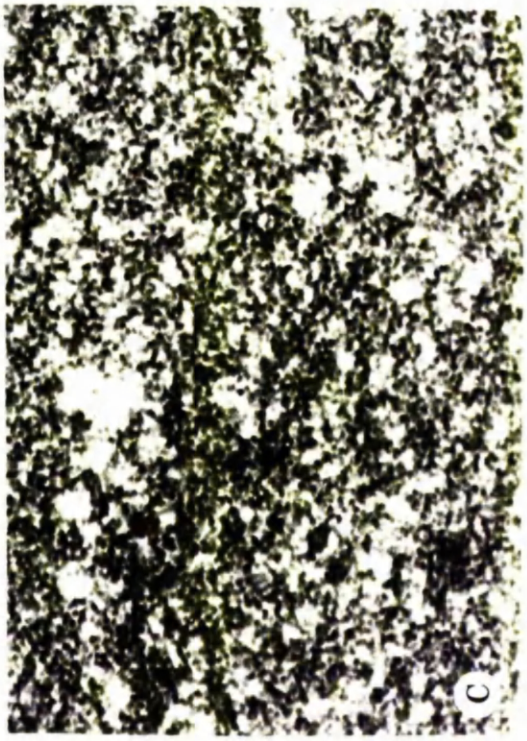

A


C

5. 2. RNA in the Microsome Fractions.

The 24 rats all received 4h oestradiol treatment and five were given radioactive ribonucleosides 2h before death whilst another five were given the precursors 1h before death. The reason for the dual pulse length was largely convenience. The time taken to administer intravenous injections of one pulse length to ten rats would have prolonged the period of killing the animals, with the result that some uteri would be excised but unhomogenized for an unnecessary length of time. The isolated microsome fractions were extracted with hot phenol and the RNA precipitated as described in the Materials and Methods section. Unlabelled total uterine RNA was added to fractions 1 and 2 to effect precipitation.

Fig. 38 shows $2\frac{3}{4}$h electrophoretic separations of the microsomal RNA on 2.7% polyacrylamide gels. Fraction 1, the smooth membrane fraction, appeared to contain free 40S ribosomal subunits, as radioactivity in 18S RNA greatly exceeded that in 28S RNA. The existence of free subunits explained the small size of the particulate material in this preparation. Fraction 2 contained the ribosomal subunit species in the expected ratio, as did fractions 3 and 4. The latter preparations incorporated more radioactivity than the former. Extinction at 260nm in Figs. 38(a) and 38(b) was due to the added carrier RNA rather than fractions 1 and 2 but Figs. 38(c) and 38(d) show that fractions 3 and 4 contained strongly labelled peaks which migrated between 28S and 18S RNA. These species, which were present to a much smaller extent in all uterine RNA preparations, have been reported by many workers (Peacock & Dingman, 1967; McIndoe & Munro, 1967; Dingman et al., 1970). A recent report by Aaij et al. (1971) suggested that they were degradation

Figure 23.

RNA in the submicrosomal fractions of immature rat uterus.

24 rats, 18-21 days old and weighing 25-30g, were given 1µg oestradiol 17β intraperitoneally 4h before death. 5 of the rats were administered 125µCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine by intravenous injection 2h before death and another 5 animals received the same treatment 1h before death. Uterine submicrosomal fractions were isolated as described in method A of the Materials and Methods section and RNA was purified from each fraction. Unlabelled uterine RNA was added to the first two fractions to effect their recovery. The purified RNA was separated on 2.7% polyacrylamide gels for 2½h.

(a) = Fraction 1.

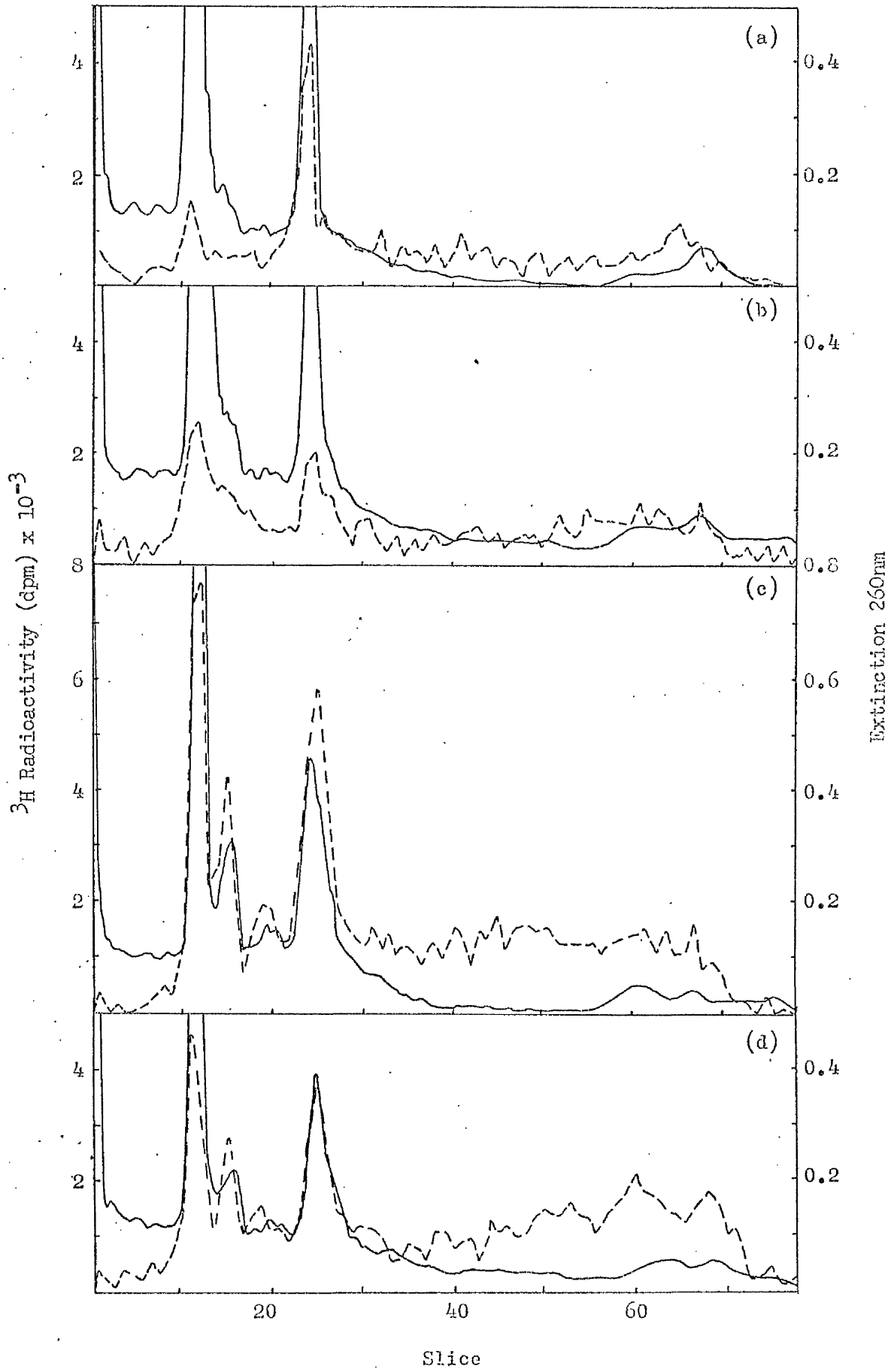
(b) = Fraction 2.

(c) = Fraction 3.

(d) = Fraction 4.

——— = Extinction at 260nm.

- - - = Radioactivity per slice in dpm.


(a)
(b)
(c)
(d)
^{3}H Radioactivity (dpm) x 10^{-3}
Extinction 260nm
Slice
20
40
60

products of ribosomal RNA and in this context preparations 3 and 4 showed other signs of degradation, both in the size and shape of the 18S peak at E260nm and in the amount of radioactivity associated with RNA smaller than 18S. Some of the latter would, however, have been due to ribosome-associated species such as 4S and 5S RNA. All four fractions showed evidence of slight contamination with nuclear material, since there was always some radioactivity in the top slices of the gel, presumably due to the presence of high-molecular-weight nuclear RNA.

5. 3. Effects of Oestrogen on Ribosome Synthesis.

Oestrogen was administered at varying times to groups of 12 rats, 6 of which received radioactive ribonucleosides 1h before death. The uterine microsomal fractions were prepared and the acid-insoluble fractions used to measure precursor incorporation and RNA content. The stimulation of RNA synthesis, compared with that of non-hormone treated animals, was then recorded as dpm/μg RNA. This was not an entirely satisfactory parameter to measure, since the total RNA of these preparations increased with time. However, all other measurable quantities such as lipid, protein and enzyme activities would also be expected to increase after hormone treatment and, since bovine serum albumin was added to ensure trichloroacetic acid precipitation, dry weight estimation was not an alternative.

Fig. 39(a) shows the total RNA content of the four microsomal preparations. Fractions 3 and 4 contained more than four times the RNA of fractions 1 and 2 and, after 24h of oestrogen treatment, the level of RNA in fraction 3, the ribosome preparation, had risen dramatically. This observation confirmed the electron

Figure 39.

The effects of oestradiol-17β on the incorporation of RNA precursor into uterine submicrosomal fractions.

Groups of 24 rats, 18-21 days old and weighing 25-30g, received 1μg of oestradiol-17β by intraperitoneal injection at various times before death. 6 of the animals in each group also received 125μCi of each of [5-^{3}H]uridine and [8-^{3}H]guanosine intravenously 1h before death. Uterine submicrosomal preparations were isolated as described in method A of the Materials and Methods section and were used to measure RNA content and the incorporation of RNA precursor into the acid-insoluble fractions. Results were calculated as dpm/μg RNA and expressed as a percentage of non-hormone treated controls.

(a) = RNA content of the fractions.

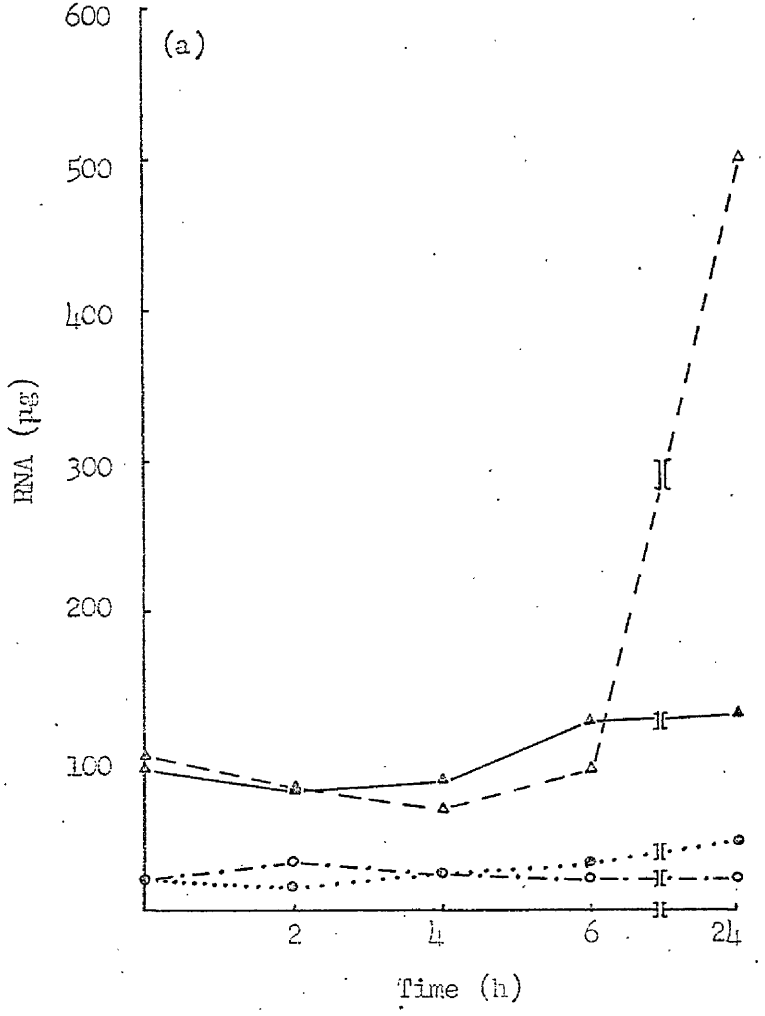
(b) = RNA synthesis in the fractions.

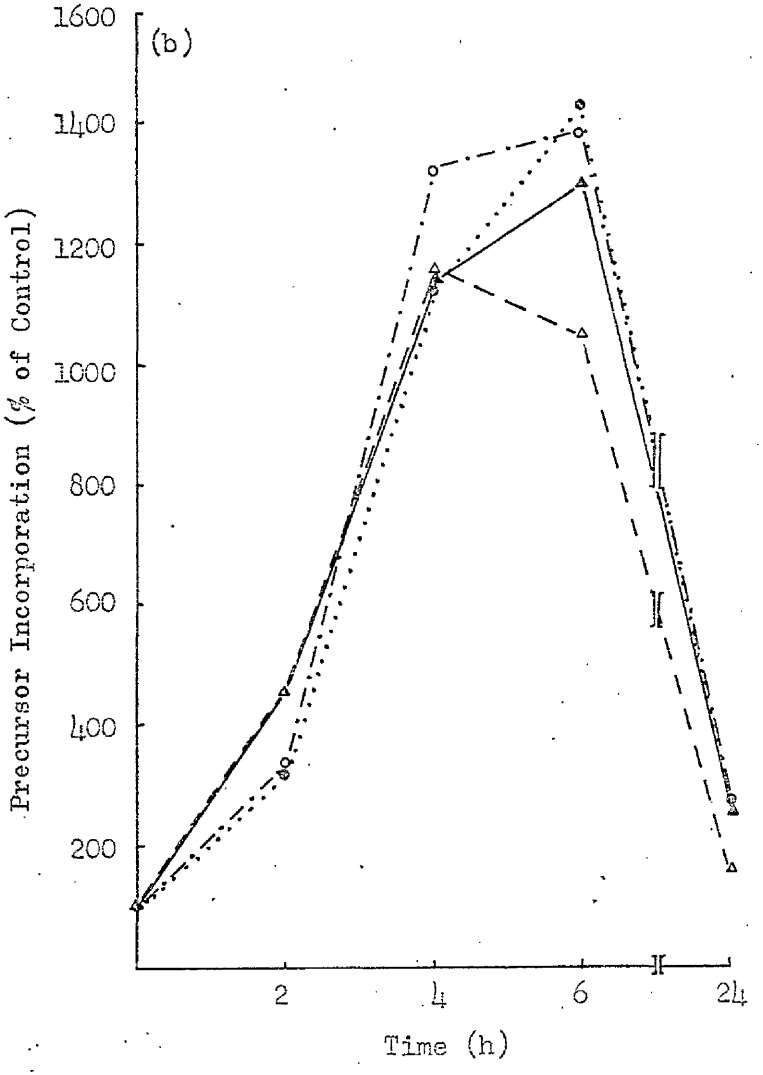
-·-·-·o·-·-·- = Fraction 1.

.....e..... = Fraction 2.

-------Δ------- = Fraction 3.

_____Δ_____ = Fraction 4.


(a)
600
500
400
300
200
100
RNA (µg)
2
4
6
24
Time (h)


(b)
1600
1400
1200
1000
800
600
400
200
Precursor Incorporation (% of Control)
2
4
6
24
Time (h)

microscope findings. The RNA content of other fractions changed marginally or not at all. When the incorporation of precursor into RNA was followed, fractions 3 and 4 again contained approximately four times the radioactivity of fractions 1 and 2. The maintenance of these ratios throughout the time course of oestrogen action was such that, when uptake of precursor was measured as specific activity (dpm/µg RNA), the curves of stimulated ribosome synthesis were almost identical in all four preparations (Fig. 39b). With the exception of the 24h oestrogen treatments, the levels of RNA did not alter much throughout the time course. Thus, the amount of new RNA entering the four fractions was directly correlated with the ribosome content of that fraction. Except in the case of fraction 1, the factors influencing the partition of ribosomes between the four preparations is unknown but it is clear that most of them eventually enter fraction 3, the main ribosomal pool. The low levels of membrane-bound ribosomes, the labelling of all microsome fractions to equal specific activities and the fact that most ribosomes eventually enter the main population, is not consistent with the proposal that oestrogen initiates the synthesis of a topographically separate population of these particles.

It was found that the maximal stimulation of ribosome synthesis in Fig. 39(b) exceeded the 10-fold stimulation of total RNA synthesis in 25-30g rats (Fig. 3), whereas the stimulation of nuclear RNA synthesis has been shown to be less than 10-fold (Fig. 25). These results suggest that oestrogen might accelerate the rate at which newly synthesized RNA leaves the nucleus of uterine cells.

One other experiment on isolated microsomal fractions

should be mentioned. Andrews & Tata (1971) described a method of preparing submicrosomal preparations from skeletal muscle. This differed from the above method principally in the preparation of the fractions from a 40,000 x g supernatant of the crude homogenate (see Materials and Methods section). When this was used with rat uteri, three fractions were obtained. Fraction A was very similar in RNA content (including the predominance of the 18S species) and RNA/phospholipid ratio to fraction 1 of the previous preparation, but contained very few membranous structures. Fraction B was similar to fraction 3 and fraction C approximated to fraction 4 of the previous method. The oestrogen stimulated incorporation of precursor into RNA was not significantly different to the above experiment. Thus, the method of Andrews & Tata (1971) largely confirmed the findings with the method of Tata (1970), the only major difference being the absence of smooth membranes where the homogenate had been centrifuged at 40,000 x g. The second method of microsome preparation provided no additional information on uterine cell ribosome populations.

5. 4. Examination of Whole Uteri by Electron Microscopy.

The experiments with sub-microsomal fractions of immature rat uterus indicated that uterine cells contained only one major population of ribosomes and that rough endoplasmic reticulum was not a conspicuous feature of the quiescent or oestrogen-stimulated tissue. To investigate further these indications, the uteri of untreated animals, and of rats receiving 6h and 24h oestrogen treatment, were examined by electron microscopy. Fig. 40 illustrates the columnar epithelium cells which were typical of the entire

Figure 40.

The epithelium of the immature rat uterus.

18-21 day old rats were either untreated or received 1µg oestradiol 6h or 24h before death. Their uteri were carefully excised and chopped into 1mm sections. These were then fixed, embedded and prepared for visualization in an electron microscope as described in the Materials and Methods section. All preparations are at a magnification of 12,500 (5,000 mag. x 2.5 enlargement).

(A) = Columnar epithelium from an untreated rat.

(B) = Columnar epithelium from a rat receiving oestradiol 6h before death.

(C) = Columnar epithelium from a rat receiving oestradiol 24h before death.

av = Autophagic vacuole.

fr = Free ribosomes.

g = Golgi stack.

m = Mitochondria.

mv = Microvilli.

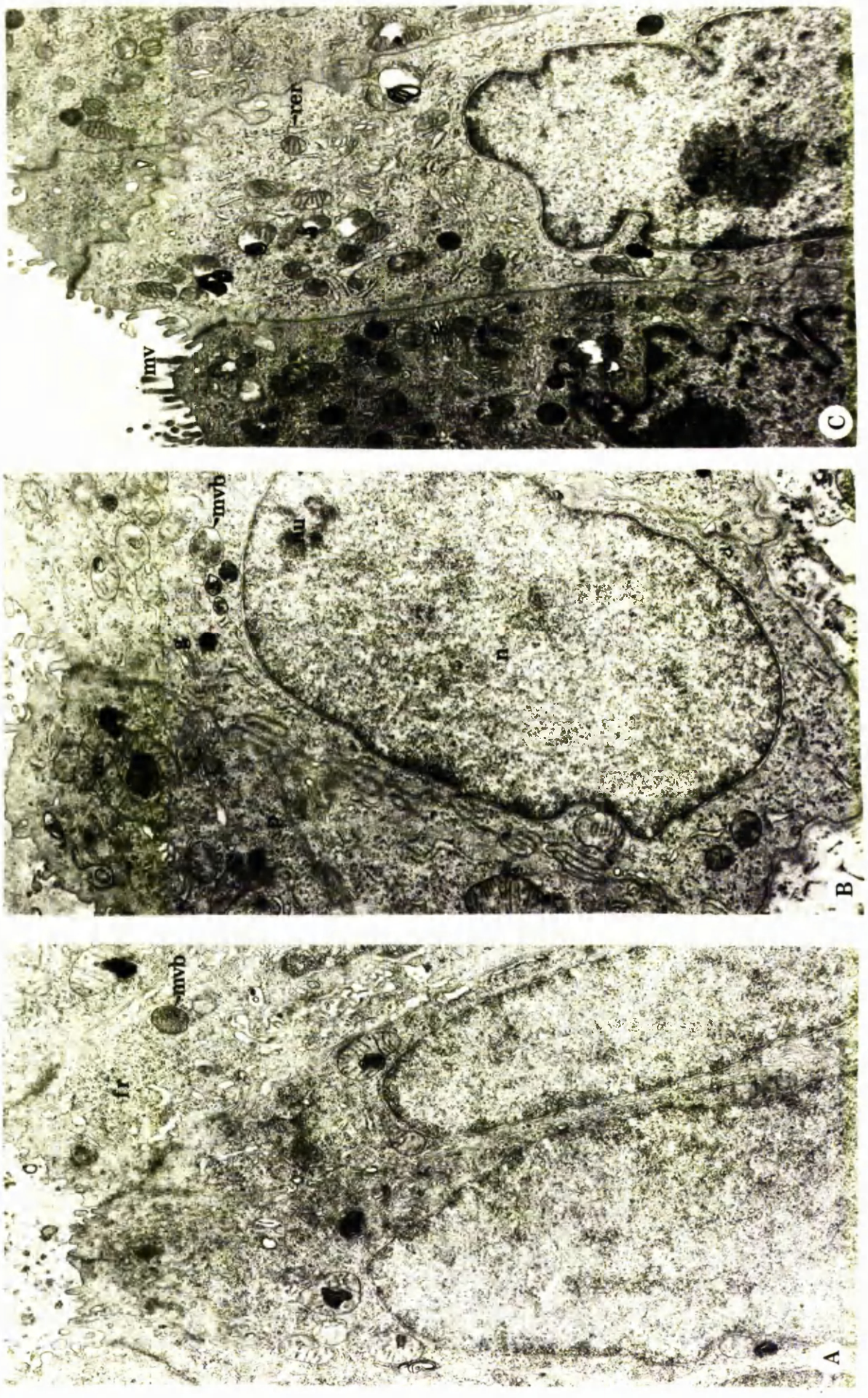
mvb = Multivesicular body.

n = Nucleus.

nu = Nucleolus.

p = Polysomes.

rer = Rough endoplasmic reticulum.


mv
ret
C
mvb
g
n
B
mvb
fr
A

epithelium except in their shape and possession of microvilli. Fig. 41 shows fibrocytes from the connective tissue and Fig. 42 shows smooth muscle. The earliest obvious change brought about by oestrogen was an aggregation of ribosomes. Thus, the untreated tissue in all cell types contained ribosomes largely in the form of monomers. In the epithelium, these were present in large numbers throughout the cell together with some polysomes. In the fibrocytes they were also present in large numbers and in smooth muscle they formed scattered pockets. By 6h of oestradiol treatment, almost all ribosomes in each of the three cell types were congregated into the typical rosette clusters of polyribosomes. They were still in this form 24h after oestradiol administration.

Care must be taken in the identification of free monoribosomes since they can be confused with glycogen granules. Glycogen is often present in uterine smooth muscle (Nemetschek-Gansler, 1967) and in certain circumstances, such as delayed implantation (Enders, 1967), can be found in large amounts in the columnar epithelium. However, although glycogen granules are in the small beta-form in smooth muscle, in the uterus they do not appear to be less than 250Å in diameter (Nemetschek-Gansler, 1967), whereas a ribosome is approximately 150Å . Measurements at high magnification show the observed particles to be of ribosomal size and comparison between the free particles and membrane-bound ribosomes, reveals no size difference. A number of other observations indicate that the free particles are ribosomes. They do not have the very dark appearance of glycogen stained with lead nor the aggregate appearance of alpha particles; the expected form of glycogen in epithelial cells. Even the small beta particle of smooth muscle would be

Figure 41.

The connective tissue of the immature rat uterus.

18-21 day old rats were either untreated or received 1µg of oestradiol 6h or 24h before death. Their uteri were carefully excised and chopped into 1mm sections. These were then fixed, embedded and prepared for visualization in an electron microscope as described in the Materials and Methods section. All preparations are at a magnification of 12,500 (5,000 mag. x 2.5 enlargement).

(A) = Fibrocyte from an untreated rat.

(B) = Fibrocyte from a rat receiving oestradiol 6h before death.

(C) = Fibrocyte from a rat receiving oestradiol 24h before death.

c = Collagen fibres

fr = Free ribosomes.

g = Golgi stack.

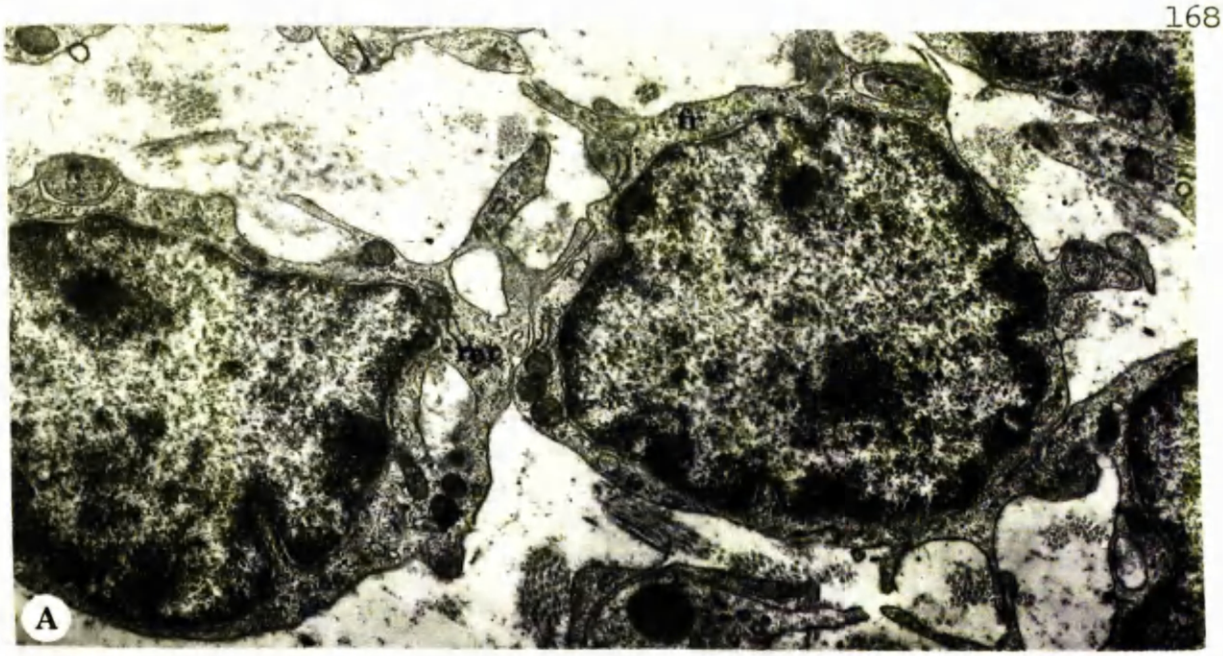
m = Mitochondria.

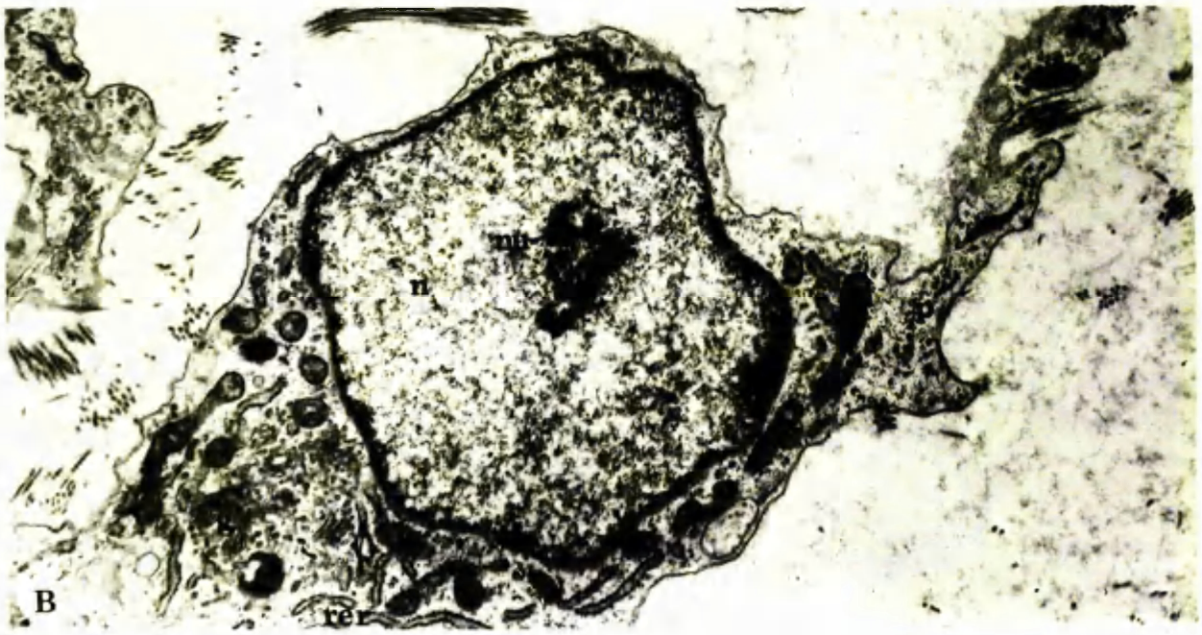
n = Nucleus.

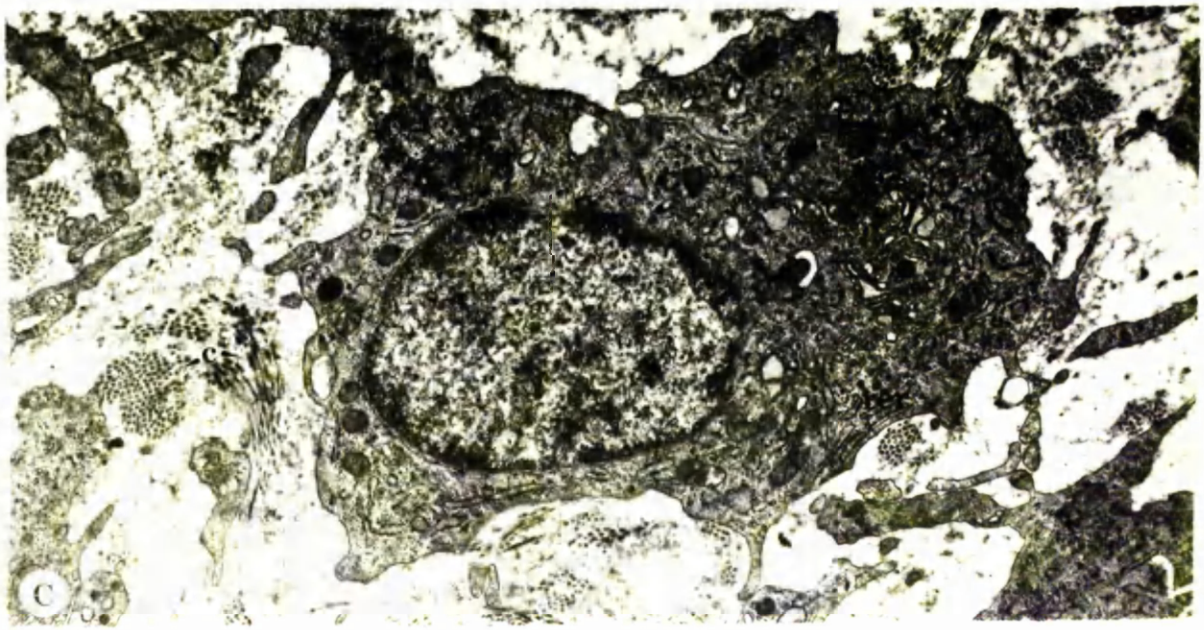
nu = Nucleoli.

p = Polysomes.

rer = Rough endoplasmic reticulum.


A


B
nu
n
re


C
c
ner

expected to show a spectrum of sizes with only the smallest nearing ribosome dimensions.

24h after the administration of oestrogen, further changes were seen in the treated tissues. The epithelium probably contained slightly more rough endoplasmic reticulum but the effect was not as marked as indicated in Fig. 40. Areas containing as much RER as the 24h treatment in Fig. 40(c) could also be found in the untreated epithelium and vice versa. The smooth muscle may also have contained more RER and the fibrocytes definitely contained more, together with increased amounts of Golgi apparatus. From the electron micrographs obtained, they also appeared to be secreting more collagen. The most pronounced change after 24h was in the smooth muscle. Here, hypertrophy and fluid imbibition was manifested in increases in the size of both the cells and the intracellular spaces, and the cells contained many more ribosomes. These were again in the form of polysomes and tended to be clustered at the apices of the nuclei and around mitochondria. The responding smooth muscle cells also contained increased amounts of Golgi apparatus which was often in the form of smooth-surfaced cisternae along the length of the nucleus.

It was concluded that many of the ribosomes in an immature rat uterus were monomers and that, by 6h after administration of oestradiol, these had aggregated into polyribosomes. Newly synthesized ribosomes were most abundant 24h after hormone treatment of the rats in the uterine smooth muscle and also formed polyribosomes. Rough endoplasmic reticulum was not a conspicuous feature of the untreated or oestradiol stimulated uterus but was fairly abundant in the collagen-secreting fibrocytes.

Figure 42.

The smooth muscle of the immature rat uterus.

18-21 day old rats were either untreated or received 1µg of oestradiol-17β 6h or 24h before death. Their uteri were carefully excised and chopped into 1mm sections. These were then fixed, embedded and prepared for visualization in an electron microscope as described in the Materials and Methods section. All preparations are at a magnification of 12,500 (5,000 mag. x 2.5 enlargement).

(A) = Smooth muscle from an untreated rat.

(B) = Smooth muscle from a rat receiving oestradiol 6h before death.

(C) = Smooth muscle from a rat receiving oestradiol 24h before death.

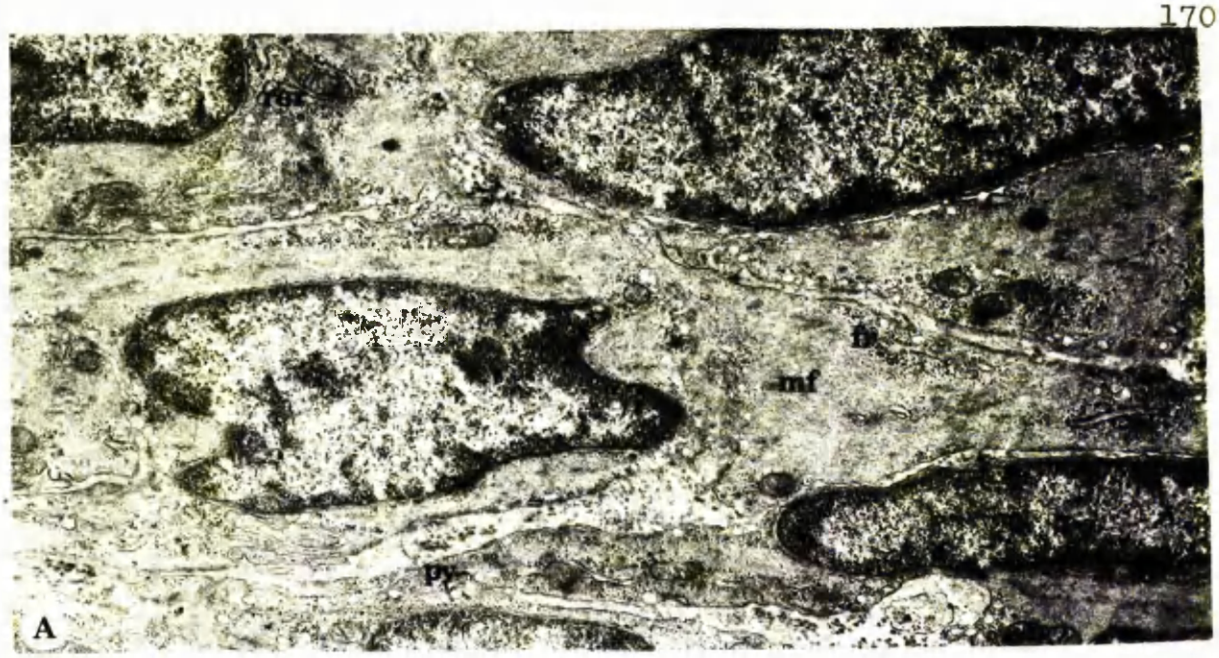
fr = Free ribosomes.

m = Mitochondria.

mf = Myofilaments.

p = Polysomes.

rer = Rough endoplasmic reticulum.


rer
mf
py
A


n
B


mf
rer
C

D I S C U S S I O N.

A wide spectrum of steroid and polypeptide hormones stimulate protein synthesis in their target tissues (Korner, 1965; Manchester, 1968; Tata, 1970a). The growth and developmental hormones initiate the synthesis of the total cellular protein complement (Tata, 1970a). Corticosteroids, which traditionally restrain protein synthesis, stimulate the production of certain key enzymes of gluconeogenisis and amino acid metabolism (Lin & Knox, 1957; Civen & Knox, 1959; Weber, 1968) while hormones such as insulin and adrenocorticotrophic hormone, not primarily thought of as growth promoting compounds, have also been shown to stimulate incorporation of amino acid into protein (Manchester, 1968; Branisome & Reddy, 1963). In almost all cases, the enhanced protein synthesis is dependent on, and preceded by, RNA synthesis although Wool et al. (1968) has presented evidence that insulin-stimulated protein synthesis need not involve RNA synthesis.

The precise role of RNA synthesis in the hormonal response has been the subject of a great deal of research and many theories have been proposed. Most of these envisage a transcriptional or a translational control mechanism. If the control is transcriptional, it could involve gene unmasking or could be the result of synthesis or activation of key proteins such as DNA-dependent RNA polymerase. Since hormone-stimulated production of RNA involves all species, it follows that control could be at the level of rRNA, mRNA or tRNA. Each of these possibilities have been proposed and it is intended to discuss them here in relation to the present studies.

Stimulation of Rat Uterine RNA Synthesis In Vivo.

In recent years, established tissue culture cell lines have been developed which respond to hormones (Thompson et al., 1966). In other cases organ explants and primary cultures have been shown to retain their in vivo properties (Turkington et al., 1965; O'Malley & Kohler, 1967). These systems are few in number and, despite the advantages of working with them, it is not certain that the hormonal response has not been modified by dedifferentiation. Most work on hormone action continues to be with whole animals.

The study of a hormonal response in vivo is hindered by the many variables inherent in such a system and it is of utmost importance to reduce these to a minimum. The common practice of giving radioactive precursors by intraperitoneal injection can result in large variations in the uterine uptake of radioactivity in replicate animals and it has been found that differences can be so great as to invalidate the experiment. Similar poor duplication has been recorded by Hamilton (1964) and Greenman (1970). The reasons are obvious. Small differences in the angle of the needle during the injection or in the physical arrangement and amount of movement of the viscera or even in the distribution of fat could mean the difference between the uterus being flooded with precursor or most of it being taken up by other tissue.

Variation between replicate animals was greatly reduced when the radioactive compounds were injected intravenously and, surprisingly, this mode of administration also resulted in a striking increase in the observed stimulation of RNA synthesis in response to oestradiol. This finding was important since the 2-3.5-fold stimulation of RNA synthesis, observed after intraperitoneal

injections of precursor, was barely significant when the effects of the hormone on the labelling of the ribonucleoside pools had been taken into account. Conversely, after intravenous administration of radioactive precursors, the 8-12-fold stimulation of RNA synthesis was much higher than the 2-2.6-fold stimulation in precursor uptake. Miller and Emmens (1967) presented similar evidence on the effect of injection route on the observed response of mouse uterus to oestradiol and, since the completion of the work described in the results section, Greenman (1970) has compared intraperitoneal and subcutaneous administration of RNA precursors to rats. While his results for total uptake were similar to these in Fig. 1, he did not appear to have exposed the animals to oestradiol for long enough to record any increase in the incorporation into RNA. Greenman (1970) suggested that intraperitoneally-injected labelled ribonucleosides entered the uterus by direct diffusion into the serosal layers and that much acid-soluble label was then extracellular. These concepts may explain the poorer stimulation of RNA synthesis following intraperitoneal injection since much of the precursor may become localized in unresponsive or less responsive cells. Attempts to confirm these possibilities by autoradiography were hampered by the difficulties encountered in obtaining sufficiently high levels of radioactivity in the uterus.

The variation between replicate animals has also been reduced by standardising the rats by weight as well as age. This followed the finding that the response of an 18-21 day old rat varied markedly with increasing weight. The heavier rats responded less dramatically to the hormone. The reason for this was unclear but there may have been higher levels of circulating hormones in the

more developed animals. Price (1947) has shown that, in a strain of rats experiencing puberty at an age of 50-60 days, some secretion of oestrogen was demonstrable as early as the second postnatal week. If this was the cause of the weight-dependent difference, it would be expected that the uterus of the heavier non-hormone-treated rats would incorporate more RNA precursor per µg of DNA than the lighter controls. However, this effect would tend to be negated by the distribution of radioactive precursor throughout a larger body mass. Thus, the low response in the heavier animals might be manifested in part by increased RNA synthesis in control animals and partly by reduced uptake in stimulated animals. The observed incorporation of radioactivity was in accordance with these ideas.

Stimulation of RNA Synthesis In Vitro.

One alternative to studying a hormonal response entirely in the whole animal is to carry out the hormone treatment in vivo and then follow the biochemical response in vitro. This method was used by Mueller (1953) and Aizawa & Mueller (1961) to follow oestrogen-directed protein and phospholipid synthesis. However, Gorski & Nicolette (1963) found that, when uteri from oestrogen-treated animals were incubated in an artificial medium, RNA synthesis was only slightly stimulated over that of non-hormone-treated animals. The results of a similar experiment, presented in Fig. 5, recorded a maximum stimulation of 50% in both RNA synthesis and precursor uptake. In the event of the incubation system being deficient in some requirement of the stimulated tissue, a range of additions and modifications to the medium were investigated. Of these, only the oestrogenic hormones had a stimulatory effect on RNA synthesis and,

in every case, the increased incorporation into the acid-insoluble fraction was mirrored by similar increases in the acid-soluble counts.

Recently, Munns & Katzman (1971) have looked at the effects of oestradiol pre-treatment of immature rats on subsequent uterine RNA synthesis in an artificial medium. They found that the *in vitro* stimulation of ^{32}P incorporation was negligible, thus confirming the findings of Gorski & Nicolette (1963). When they used tritiated uridine as their precursor, their results were similar to those presented in Fig. 5 and Table 1, except that stimulation was increased by reducing the incubation time to 15min. Finally, when they used [methyl-^{3}H]methionine as the precursor, stimulation of *in vitro* RNA synthesis, in uteri from animals treated with oestrogen for 6h, was more than 3 times that of controls. In all of these experiments, the stimulation of precursor uptake was comparable in both the acid-soluble and acid-insoluble fractions.

Oliver (1971) has concluded that the only effect oestradiol treatment has on nucleoside uptake by the uterus *in vitro* is due to the size and water content of the tissue. It seems likely that, in the experiments shown in Fig. 5 and Table 1, it was precisely this that was observed. Prior treatment of the rats with oestradiol caused water imbibition and the resulting expansion of uterine volume was manifested *in vitro* by an increased nucleoside uptake. Since, during the first few hours of the response to oestrogen, the size of the uridine pool of the uterus remains constant and that of guanosine decreases (Oliver & Kellie, 1970), increased uptake of these RNA precursors would be associated with increased RNA synthesis. When oestrogens were added to the *in vitro* incubation, they further stimulated

precursor uptake, presumably by the same mechanism. It also seems likely that the results of Munns and Katzman (1971) merely reflected the differing degrees by which oestrogen stimulated the uptake of phosphate, uridine and methionine into the uterine cells.

The large increases in RNA synthesis, observed when precursors were incorporated into the oestrogen-stimulated uterus, did not occur when the excised tissue was incubated *in vitro*. The optical microscope observations in Fig. 6 and the electron microscope studies of Ljungkvist & Terenius (1970) showed that anoxia at least contributed to the poor response *in vitro* but a method of overcoming the oxygen-starvation was not found.

The Stimulated Synthesis of Ribosomal RNA.

The most striking change in the early stages of the response of target cells to many growth and developmental hormones is a stimulation in the rate of ribosomal RNA synthesis. This effect has been clearly established for oestrogen (Hamilton, 1968), as well as for testosterone (Liao *et al.*, 1966), triiodothyronine (Tata, 1966) and corticosteroids (Greenman *et al.*, 1965). The present study has confirmed the oestrogen augmented synthesis of ribosomal RNA in the uterus of the immature rat and has taken advantage of the resolution of polyacrylamide gels to follow its formation from the 45S precursor.

The increased synthesis of rRNA, even at the level of the 45S and 32S precursor species, was not striking until a relatively late stage (2-4h) after treatment, although in some experiments, conducted in the Summer rather than the Winter, stimulation was apparent at 1h. When uterine RNA synthesis was examined in rats receiving less than 1h of oestrogen treatment, there was some

evidence that it was slightly decreased to allow the utilisation of precursor in HnRNA synthesis.

Luck & Hamilton (1972) have recently claimed that oestradiol speeds up the maturation of rRNA. In their study, using sucrose density gradients, the high-molecular-weight RNA was, however, poorly differentiated compared with the resolution which can be obtained with polyacrylamide gels. In the present study, although the 45S RNA was not always separated from HnRNA, the individual precursors could be clearly located and their identity confirmed by methylation and cofractionation with samples of HeLa cell ribosomal precursor species. When rRNA synthesis was followed with 15, 30 and 60min precursor incorporations, radioactivity was seen to move through the precursors and into the mature rRNA subunit species. Comparison of the maturation process in control and hormone-treated animals (Figs. 8, 9 and 10) revealed that, after a 30min pulse, the control animal 32S RNA peak was smaller than the 45S RNA peak whereas, in hormone-treated preparations, the 32S peak was the larger (Fig. 9). Similar arguments could be made regarding the relative sizes of the 28S and 32S peaks where the precursor had been incorporated for 1h (Fig. 10). However, these indications were not clear-cut since all radioactivity in pre-ribosomal RNA was superimposed on a background of heterogeneous counts and the peaks in the control animals were very small. Thus, the hormone may stimulate the maturation of ribosomal RNA but the effect is small compared with the increases in transcription.

The large increases in rRNA synthesis, after the administration of oestrogen to ovariectomized rats, led Hamilton et al. (1968) to conclude that the regulation of rRNA synthesis was a major,

and possibly rate-limiting step, in the growth response of the uterus to oestrogen. Hamilton (1968) further proposed that the first step in the response to the hormone was the synthesis of rRNA, followed by the formation of ribonucleoprotein particles. These then collected mRNA from its site of synthesis and transported the messenger out of the nucleus. This suggestion followed the proposal of Henshaw et al. (1965) and McConkey & Hopkins (1965) that mRNA entered the cytoplasm attached to the small ribosomal subunit. Associated with these postulates, Hamilton et al. (1968) concluded that oestradiol stimulated not only the synthesis of rRNA but also its transport from the nucleus to the cytoplasm. A clear demonstration of such an effect would be difficult to achieve. It would necessitate differentiation between the increased labelling of cytoplasmic ribosomes due to stimulated transcription, from that due to an accelerated migration from the nucleus. Some indication was obtained in the results section that a true acceleration of transport might occur. The maximal stimulation of total RNA synthesis in 25-30g rats receiving 1µg of oestradiol, was 10 times that of control animals (Fig. 3). The maximal stimulation of precursor incorporation into nuclear RNA was 7-fold (Fig. 25) while that of the combined cytoplasmic ribosome fractions was approximately 13-fold (Fig. 39). These results might indicate a stimulation in the transport of ribosomes from the nucleus to the cytoplasm. However, they could also be explained if, at the time of maximum response, the synthesis of other nuclear RNA species was stimulated to a smaller extent than rRNA synthesis.

It was concluded that a major response of the immature rat uterus to oestrogen treatment was the synthesis of rRNA. The

maturation of the product of transcription and the rate of transport of ribosomes from the nucleus may have been independently stimulated but further research would be necessary to confirm these possibilities.

The synthesis of rRNA was sensitive to actinomycin D inhibition (Fig. 13) and at 30µg inhibitor/rat, was almost totally eliminated. The synthesis of other RNA species was less susceptible. Cycloheximide also inhibited rRNA formation and 60µg/rat, a dose giving an incomplete inhibition of protein synthesis, inhibited ribosome maturation. The higher dose of 600µg/rat also inhibited ribosome maturation if given 1h after oestradiol administration. This effect of the inhibitor has been established in tissue culture cells (Willems et al., 1969; Craig & Perry, 1970) and underlines the difficulty in interpreting the results obtained with these compounds. It becomes obvious that the inhibition of oestrogen-induced RNA synthesis by cycloheximide does not necessarily show a dependence on the previous production of oestrogen-specified proteins, as indicated by Gorski & Axman (1964). Rather, it shows that in this system, as in many others, ribosome synthesis is dependent on protein synthesis. 600µg cycloheximide/rat, if given with or before oestradiol, eliminates ribosome synthesis even at the precursor level. However, the toxic effect of the drug at this dosage makes analysis of the result difficult.

Somewhat at variance with popular opinion, Tomkins et al. (1969) have suggested that the increased production of ribosomes and other RNA species may have little to do with hormone-induced production of specific proteins. During the initiation of tyrosine aminotransferase in the liver of rats treated with corticosteroids, there is an increased synthesis of DNA-like, ribosomal and transfer

RNA (Greenman et al., 1965; Wicks et al., 1965). However, when the synthetic corticosteroid, dexamethasone, induces the enzyme in hepatoma cells in tissue culture, there is no detectable increase in the synthesis of any RNA species (Gelehrter & Tomkins, 1967), although some RNA synthesis was necessary as actinomycin D inhibited the induction (Tomkins et al., 1966). This suggests that the increased synthesis of rRNA and tRNA in vivo could be a result of general hypertrophy, having nothing to do with the induced synthesis of new protein species. Tomkins et al. (1969) postulate that the synthesis of tyrosine aminotransferase is under translational control. It may, however, be unwise to study hormonal control systems in established cell lines, most of which can be shown to have undergone at least some dedifferentiation (Pitot et al., 1964). Such cell lines may have lost control over rRNA transcription with the result that they continuously produce ribosomes at a maximal rate.

Olmo et al. (1971) have reached similar conclusions to those of Tomkins et al. (1969). They studied testosterone control of enzyme induction in the kidney of normal mice and in mice carrying the X linked testicular feminization (Tfm) mutation. From their findings they concluded that two intracellular metabolites of testosterone controlled the response. 5α-androstan-3α-17β-diol stayed in the cytoplasm and induced the synthesis of alcohol dehydrogenase and β glucuronidase by translational control. A second metabolite, 5α-dihydrotestosterone, was carried to the nucleus by the 3.5S receptor protein (Fang et al., 1969) where it mediated hypertrophy.

When oestrogen acts on the uterus, it initiates first

hypertrophy and later hyperplasia. It can be concluded, therefore, that the synthesis of rRNA is very relevant to oestrogen action, even if it is unnecessary to the induced synthesis of new protein species.

The Fate of Newly-Synthesized Ribosomes.

The opposite view of the possible role of ribosomes in translational control mechanisms is that of Tata (1970a). He postulates that an important function of growth promoting hormones could be the stimulated production of ribosomes and phospholipid and their association into rough endoplasmic reticulum (RER). He further claims that this response could provide a means whereby a topographically segregated population of ribosomes would be responsible for producing the proteins bringing about hormone-induced differentiation of the target tissue. The advantages of such a system, in facilitating a rapid response to external stimuli without upsetting the synthesis of constitutive proteins, are obvious. However, this hypothesis envisages a different function for membrane-bound ribosomes to the normally accepted role in the production of secretory proteins (Palade, 1966). In a non-secretory tissue, such as muscle or brain, membrane-bound ribosomes may synthesize protein for intracellular destinations (Andrews & Tata, 1971) but Tata's most convincing results, demonstrating the development of RER in response to triiodothyronine, are with a protein-secreting tissue, the bullfrog liver (Tata, 1967a,b).

The possibility that the uterine ribosomes, produced in response to oestrogen, were membrane-bound was investigated in the immature rat. Four fractions were prepared from a mitochondria-free

supernatant. One of these was the expected smooth membrane fraction and another consisted of very pure ribosomes but no fraction contained large quantities of membrane-bound ribosomes. Strangely, most of the membrane-bound ribosomes that were seen were in fraction 4, the preparation containing the heaviest material and the smallest quantity of phospholipid. Precursor incorporation studies showed that newly-synthesized RNA was partitioned between the submicrosomal fractions with stoicheiometrical relationship to their existing RNA content, such that the four preparations were always labelled to approximately the same specific activity. Thus, for the first six hours of the time course, fraction 3 contained roughly four times as much RNA as fraction 1 but also incorporated four times as much radioactivity. Despite this initial segregation of newly-synthesized ribosomes, it was clear that most particles eventually became a part of fraction 3, the main ribosome pool, so that after 24h, the total RNA content of this fraction was greatly increased. The factors influencing the distribution of rRNA containing entities throughout all of the four fractions was unknown. There were indications that the smooth membrane fraction contained a predominance of 40S subunits and, when these were labelled, it was possible that they had just entered the cytoplasm and had not yet associated into mature ribosomes. Perhaps the ribosomes in fraction 2 were also incomplete. Such a concept would explain why the amount of RNA in fractions 1 and 2 did not increase when, after 24h, the content of fraction 3 was greatly increased and that of fraction 4 was also enlarged. It appeared fairly certain that the ribosomes in fractions 1, 3 and 4 were not separate populations in the intact cell; rather they were particles which, for some reason, differed in their sedimentation characteristics

from the main pool. In summary, fraction 3 was the main ribosome population in control and hormone-stimulated animals. Newly-synthesized ribosomes occurred free and as polysomes but few were membrane-bound. No evidence was found that they were in any way topographically segregated from pre-existing ribosomes.

As a result of the above experiments, it was desirable to determine the extent to which membrane-bound ribosomes were found in the intact uterus. It was possible that a relatively small percentage of the cells responded to oestrogen in the production of RER. The uterus has been the subject of a number of electron microscope studies. Davies (1967) examined the columnar cells of the endometrium and showed that they contained large numbers of free ribosomes and occasional areas of RER in non-pregnant rabbits and that this did not change greatly during pregnancy. Laguens (1964) and Nemetschek-Gansler (1967) have shown that the free ribosome content of uterine smooth muscle increased greatly after oestrogen treatment. The latter worker also showed that some cells in the connective tissue could have a predominance of membrane-bound. ribosomes and also stated that granular epithelium was increased in muscle and epithelial cells under the influence of oestrogen. It was decided to investigate the immature rat uterus for the presence of RER.

The epithelium, connective tissue and smooth muscle were examined in the uteri from untreated rats and from rats which had received oestradiol 6h and 24h before death. The first observed change was an aggregation of ribosomes into polysomes and was clearly visible in all cell types after 6h exposure to the hormone. Because virtually the entire ribosome population of the cell was involved in

this change, at a time when the total RNA of the tissue was not greatly changed, it was assumed that the aggregation involved pre-existing, as well as newly-made, ribosomes. This indicated that the old ribosomes were as important as new ones in the oestrogen-directed protein synthesis. The electron micrographs of Davies (1967) showed that ribosomes were largely in the form of polysomes in the epithelium of both pregnant and non-pregnant rabbits, but the non-pregnant animal was mature and, therefore, exposed to oestrogen.

After 24h of hormone treatment, the ribosomes were still in the form of polysomes and there were large amounts of new ribosomes, again occurring largely as polysomes, in the hypertrophied smooth muscle. In untreated and treated animals there were small amounts of RER in the epithelium and smooth muscle and rather more in the collagen-secreting fibrocytes. The amount of RER was increased in the fibrocytes after exposure to the hormone.

Tata (1970a) has made a good case for the hormone-induced accumulation of RER and the existence of a mechanism in the target cell for coupling the formation of membrane-bound ribosomes to an increased demand for protein. Many growth stimulating hormones, including oestrogen, stimulate the coordinated synthesis of phospholipid and RNA (Aizawa & Mueller, 1961). When growth hormone and triiodothyronine are given simultaneously to hypophysectomized rats, the hepatic synthesis of these components is additive, with two peaks of precursor incorporation corresponding to the lag periods of the two hormones (Tata, 1968). Furthermore, in the bullfrog liver, the newly-synthesized phospholipid and RNA is involved in the reorganisation of the cytoplasm and formation of RER which becomes

the most active component in protein synthesis. Accumulation of membrane-bound ribosomes is followed, in these animals, by induced synthesis of urea-cycle enzymes and serum albumin (Tata, 1967b).

In an extension of his findings, Tata (1970a) suggested that coordinated synthesis of RNA and phospholipid during oestrogen action may also result in membrane-bound ribosome accumulation. Nicolls et al. (1968) has shown that, when oestrogen stimulates Xenopus liver to produce phospholipoprotein, this is the case. The present study reveals, however, that in the immature rat uterus there is little RER before or after hormone treatment. The only exceptions were the fibrocytes which, as secretory cells, might be expected to contain RER.

The difference between these results and those of the other cited workers may lie in the fact that oestrogen, when acting on the uterus, is not noted for the initiated synthesis of specific enzymes or other proteins but rather results in general hypertrophy followed by hyperplasia. There is no known equivalent in the uterus to the induced synthesis of phosvitin in the liver responding to oestrogen (Heald & McLachlan, 1963) or of thyroglobulin induction in the thyroid gland responding to thyrotrophic hormone (Kerkof & Tata, 1967) or urea-cycle enzyme production in metamorphosing amphibia (Tata, 1967b). It therefore appears that RER may be formed when a hormone dictates the induced synthesis of new protein species. Oka & Topper (1971) have recently shown that the induction of casein synthesis, in mammary cells responding in vitro to insulin, corticosteroid and prolactin, is accompanied by the formation of RER.

Associated with these findings, a further point should be made; the Introduction and Discussion in this thesis has drawn attention

to the similarities in the biochemical responses initiated by the various growth-promoting hormones whether polypeptide or steroid. Despite this, it should be remembered that the physiological actions of these compounds are very different and, at a biochemical level, it may be the differences rather than the similarities which will prove to be the key to their mode of action.

The Stimulated-Synthesis of tRNA.

The degeneracy of the genetic code and the tRNA species has led many workers to speculate that tRNA may be involved in the translational control of protein synthesis (Sueoka & Kano-Sueoka, 1970). Numerous examples have been reported where the tRNA content of a system undergoing change has been shown to be altered, either by the synthesis of new tRNA species, the modification of existing ones or an alteration in the relative abundance of isoaccepting species. Sueoka & Kano-Sueoka (1964) reported changes in the relative abundance of two leucyl-tRNA species in E. coli infected with the bacteriophage, T2 and Kaneko & Doi (1966) have shown that the valyl-tRNA differs in the spore and vegetative forms of B. subtilis. Similar changes have been found in methionyl-tRNA in erythrocytes during chick development (Lee & Ingram, 1967) and many changes have been found between normal and tumour-derived cells (Taylor et al., 1968; Srinivasan et al., 1971). Some of the reported changes can be associated with requirement and it has already been mentioned that in the liver synthesizing the serine-rich protein, phosvitin, in response to oestrogen, there is an elevated level of seryl-tRNA (Beck et al., 1970). Several other changes in tRNAs, or their methylating enzymes, in systems sensitive

to oestrogens and progestins have also been mentioned in the Introduction to this thesis. It is clear that the possible control of protein synthesis by tRNA is relevant to oestrogen action.

The findings in the present work confirm those of Billing et al. (1969c), also with immature rats, and those of Dingman et al. (1969) working with chick oviduct, that the synthesis of tRNA is greatly increased in response to oestrogen. In the oviduct system, the tRNA synthesis was the most noticeable of all the observed changes in RNA production and in both sets of findings with the immature rat, there was evidence that the stimulated production of tRNA preceded slightly that of rRNA. It would be interesting to compare the newly synthesized tRNA with that of untreated rats.

The synthesis of tRNA in the immature rat uterus appears to be via a precursor, is less sensitive to actinomycin D inhibition than rRNA but is sensitive to levels of cycloheximide which almost totally inhibit protein synthesis. Conceivably, this effect could be correlated with the synthesis of specific tRNA modifying enzymes but in view of the observed toxicity of the levels of cycloheximide used, there is little value in such speculation.

The Stimulated Synthesis of DNA-Like RNA.

The concept that, in higher animals, hormones could promote new messenger RNA synthesis was a logical extension of the findings that similar low-information molecules are responsible for controlling transcription during bacterial induction and repression (Jacob & Monod, 1961). The idea was first expressed by Karlson (1963) after his finding that ecdysone induced the unfolding and priming of specific regions of insect chromosomes, followed by the appearance of enzymes associated with insect moulting. The concept received support from

a number of quarters. A 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 (Hamilton et al., 1968; Tata & Widnel, 1966). Hybridization studies revealed new nuclear RNA species produced in the rabbit uterus shortly after oestrogen treatment (Church & McCarthy, 1970), in oviduct responding to DES and progesterone (O'Malley et al., 1968; O'Malley & McGuire, 1969), and in liver treated with cortisol, but not when it was treated with growth hormone (Drews & Brawerman, 1967a,b). New DNA-like RNA was also reported following treatment of rat kidney and toad bladder with aldosterone (Forte & Landon, 1968; Rousseau & Crabbé, 1968) and in the rat liver responding to cortisol (Yu & Feigelson, 1969).

The above findings do not, however, add up to conclusive evidence either for synthesis of mRNA or for a decisive role for new messenger species in hormonal control. The difficulties in demonstrating mRNA in the eukaryotic cell, particularly where any new production would be totally masked by the increases in rRNA and tRNA synthesis, have led to a reappraisal of the concepts of control in higher animals. Some of the postulated alternatives to the bacterial-type control by induction and repression have been presented above, and it is clear that they deserve careful investigation. The eukaryote differs from the prokaryote in having a membrane-bounded nucleus with DNA organised into chromosomes, associated specific nuclear proteins and a specialized area synthesizing rRNA. All this indicates that higher organisms differ in their control mechanisms but at the same time the concept that hormones induce the synthesis of specific mRNA species should not be abandoned.

The earliest response to oestrogen, detected in immature rats in the present work, was the incorporation of precursor into an RNA of very-high-molecular-weight. Studies on the behaviour of this material showed that it was rapidly synthesized and had a short half life. It was unmethylated, located in the nucleus and had a base composition low in G+C and high in U. These findings supported the conclusion that the material was heterogeneous nuclear RNA and studies were undertaken to investigate whether the increased synthesis involved discrete species of this RNA type. The HnRNA was separated on two systems designed to fractionate the species but the stimulated incorporation of radioactive precursor was heterogeneous.

Simultaneously with these studies, evidence has been accumulating that HnRNA is a precursor to mRNA species (Darnell et al., 1971a). If this proves to be the case, then the study of HnRNA synthesis, in systems in which it can be separated from RNA species of lower molecular weight, may provide a way in which the synthesis of mRNA can be studied in tissues undergoing differentiation.

There is no reason why steroid hormone regulation in the target cell could not involve several of the proposals that have been put forward. The primary response could be the synthesis of specific mRNA species, the translation of which might result in the synthesis of a small number of specific proteins. These in turn could promote the synthesis of new ribosomes and tRNA which could contribute to the control of subsequent differentiation in the responding tissue. Studies on oestrogen action have produced a considerable body of evidence which can be marshalled in support of such an argument. Firstly, oestrogen is found bound to the chromatin of target organs within 2min of its administration (Teng & Hamilton,

1968) and results in increases in chromatin template activity (Church & McCarthy, 1970). This is followed by the early synthesis of new RNA species (Church & McCarthy, 1970) and the production of a small number of new protein species (Notides & Gorski, 1966; Barker, 1971) which appear to be dependent on RNA synthesis for their elaboration (DeAngelo & Gorski, 1970) and probably include an activator of magnesium ion-stimulated RNA polymerase (Barry & Gorski, 1971). Increased activity of this enzyme is associated with stimulated production of rRNA (Hamilton et al., 1968) and at the same time, or possibly earlier, synthesis of tRNA is also increased. In the uterus, the new RNA would be involved primarily in hypertrophy and later hyperplasia but in other tissues, such as the liver producing phosvitin, the ribosomes could be associated with membranes and themselves perform a regulatory role in the induction process. In such a system, a modification in the relative abundance of various tRNA species could also be important.

It is tempting to speculate that the early synthesis of HnRNA in the uterus of immature rat could reflect the synthesis of new species of mRNA and that the translation of these into protein could be a prerequisite of increases in rRNA synthesis.

SUMMARY

1) The action of oestradiol at a molecular level, together with the current concepts of RNA synthesis, maturation and transport are reviewed. RNA synthesis is investigated and characterized in the uterus of 18-21 day old rats responding to oestradiol-17β.

2) The observed stimulation of RNA synthesis, in the uterus of rats treated with oestradiol-17β, is markedly dependent on the route of injection of precursor. After intraperitoneal administration of radioactive precursors, response is low and variable. Conversely, intravenous injection of precursor gives rise to a marked stimulation of RNA synthesis which is far in excess of the stimulated uptake into the acid-soluble pools and indicates that the increased uptake into RNA represents a real stimulation in RNA synthesis. Subcutaneous injection of precursor gives an intermediate response.

3) 18-21 day old rats respond maximally to a dose of 0.3μg/rat or more of oestradiol-17β, the degree of response being dependent on the weight of the animals within the age range.

4) *In vitro* synthesis of RNA, in uteri excised from oestrogen-treated rats, is only slightly stimulated and the increase can be accounted for by hormone-activated uptake of precursor.

5) The purification and separation of uterine RNA on polyacrylamide gels, agarose gels and sucrose density gradients is described.

6) The earliest detected effect of oestradiol is the stimulated synthesis of a very-high-molecular-weight RNA from approximately 30min after the administration of oestradiol-17β. The rapid synthesis and decay of this species, together with its nuclear location, absence of

methylation and its base composition, permits its identification as heterogeneous nuclear RNA. The HnRNA made in response to oestradiol varies considerably in size.

7) Since evidence is accumulating that HnRNA contains polynucleotide sequences which ultimately become messengers, it is suggested that the stimulated production of this species in the uterus of oestrogen-treated rats may reflect hormone-induced mRNA synthesis and the translation of the messengers into protein may be a necessary prerequisite for stimulated rRNA synthesis and subsequent hormone augmented differentiation.

8) A striking change in the uteri of rats exposed to oestradiol is the stimulated synthesis of ribosomal RNA. When purified RNA is separated on 2.7% polyacrylamide gels, synthesis can be followed from the 45S precursor, through the 32S precursor and into the ribosomal subunit species. Synthesis is first stimulated at, or shortly after, 1h of oestrogen treatment and is greatly increased 2 and 4h after hormone administration. Some evidence is presented that, in addition to increasing the rate of transcription of rRNA, oestradiol may also stimulate the rate of rRNA maturation together with its transport into the cytoplasm.

9) The fate of newly-synthesized ribosomes in oestrogen-treated uterine cells is investigated. As a consequence of hormone administration, pre-existing and newly-synthesized ribosomes appear to aggregate into polysomes but there are few membrane-bound ribosomes either before or after hormone treatment. The features of the induced production of ribosomes in immature rat uteri are discussed in relation to the current concepts of their involvement in hormone action.

10) Oestrogen-induced synthesis of tRNA may precede slightly the increase in rRNA synthesis, since the labelling of 4S RNA is clearly elevated 1h after hormone administration. Synthesis of both 4S and 5S RNA is strongly stimulated after 2 and 4h of oestrogen treatment.

11) The increased synthesis of rRNA in response to oestradiol-17β is more strongly inhibited by actinomycin D than the synthesis of other RNA species. Cycloheximide, depending on the time of administration and dosage, inhibits either RNA synthesis or the maturation of rRNA.

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