INVESTIGATIONS INTO THE MECHANISM OF ACTION OF OESTRADIOL - 17β

by

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CONTENTS

GENERAL INTRODUCTION

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At the present time we do not know the mode of action at the molecular level of any of the known hormones. This remains today one of the major unsolved problems in biochemistry and molecular biology. Particularly baffling is the mode of action of the growth and developmental hormones such as androgens, oestrogens, growth hormone and trophic hormones. They all increase protein synthesis and eventually cause an increase in the rate of cell division. But how do they initiate this process?

To study this problem I chose to study the mode of action of increased growth of the rat uterus under the influence of oestrogens. There are three main advantages with this system, (1) the target organ is a good one to work with because it is well defined and easily removed in a clean manner and because it is metabolically less complex than other organs such as the liver, (2) the control animal (ovariectomised or immature) is easily obtained, (3) the hormone can be obtained in a radioactive form of high specific activity which permits localisation studies. Perhaps it is for these reasons that this system seems to be one of the most popular that research workers have used to study the mode of action

of growth and developmental hormones.

A DISCUSSION OF RECENT WORK ON OESTROGEN ACTION

The animals used in all the following experiments are mainly female rats. The control animals are either 3 weeks old immature rats or adult rats which have been ovariectomised for about 3 weeks prior to the experiment. The response to oestrogens of these two groups has not been compared one to the other specifically for any differences in response between them. I think we can assume that they respond to oestrogens in the same way, but any apparent discrepancies in behaviour between the two groups will be discussed.

The study of oestrogens over the last few years has taken two main lines: (1) the study of the binding of radioactive oestrogens to uterine components (2) the study of the early effects of oestrogens on uterine biochemical parameters.

In the following pages recent work in this field is discussed. This discussion does not include all the publications in this area but is restricted to those most relevant to the work described in the later part of this thesis. Some of the papers are discussed in the light of my own observations and this does not necessarily therefore represent the only

interpretation that could be placed upon them. Nevertheless it is felt that such discussion is desirable for further progress.

1. Binding of Oestradiol in the uterus

Following this line of research Jensen and Jacobson (1962) in a classical experiment showed that oestradiol -17β interacted with and bound to some receptor in oestrogen sensitive tissues but not in other tissues. They injected ^{3}H oestradiol -17β into 23 day old immature female rats and at various time intervals measured the amount of radioactivity per mg. dry weight for several different tissues The results are shown in fig. 1. (page 3A).

This work stimulated further research into the chemical nature of this receptor and its intracellular localisation. 4 hr. after an intraperitoneal infection of 0.01 ug ^{3}H oestradiol -17β into immature female rats, differential centrifugation revealed that the heavy nuclear + myofibrillar fraction contained approximately 55%, the mitochondria 10 the microsomes 5% and the soluble fraction 30% of the total radioactivity in the uterus. By treating the rats for 2 hrs. with 1 ug. unlabelled oestradiol -17β or diethylstilboestrol (D.E.S.), before administering ^{3}H oestradiol the uptake of the ^{3}H oestradiol -17β

FIG. 1

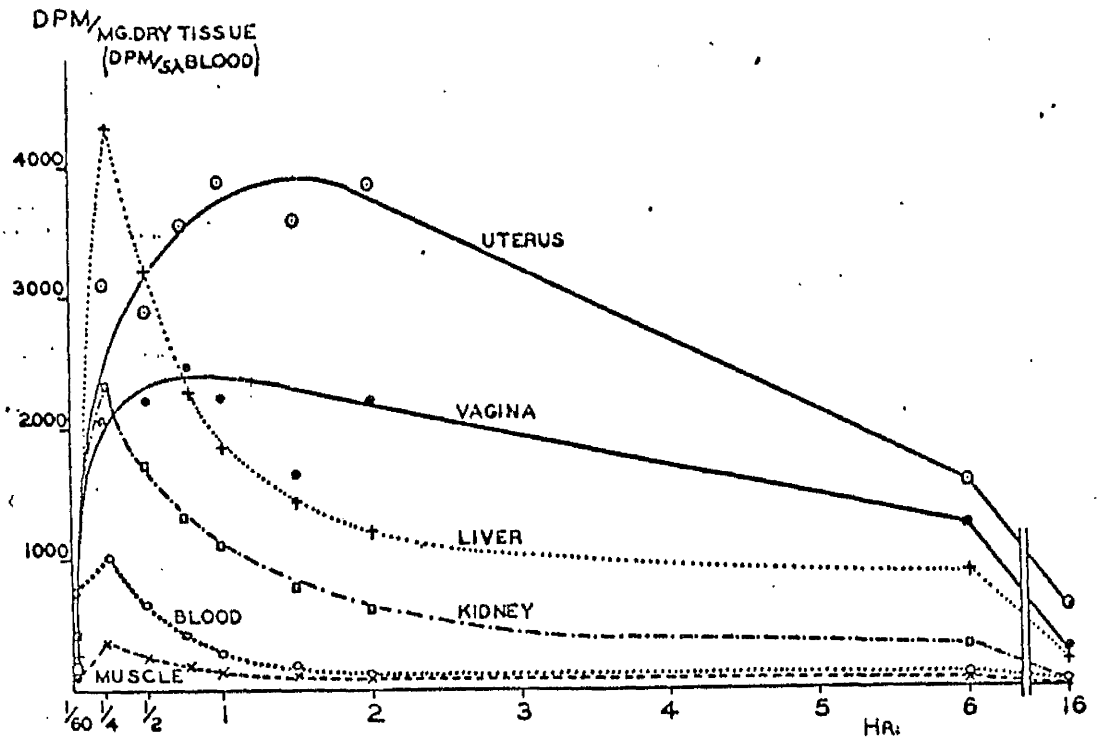


FIG. 2. Concentration of radioactivity in rat tissues after single subcutaneous injection of 0.098 μg. (11.5 μc.) of estradiol-6,7-H^3 in 0.5 ml. saline. Liver and kidney points are mean values of 3 aliquots of dried pooled tissue; other points are median values of individual samples from 6 animals. Muscle is M. *Quadriceps femoris.*

into the nuclear-myofibrillar fraction could be inhibited by 75%-80%, but no inhibition occurred when animals were pretreated with oestradiol -17α, cortisol or testosterone. The radioactivity could not be releas from the nuclear-myofibrillar fraction by incubation with DNA-ase or RNA-ase for 2 hrs., but it was released by pretreatment within 15 mins. (Noteboom and Gorski, 1965). This suggested that the binding component was a protein and was stereospecific for oestrogenic molecules. Further experiments confirmed this and showed that the "receptor was a macromolecule of sedimentation coefficient 9.5S (Toft and Gorski, 1966), and of nuclear origin (King and Gorden, 1967). It was suggeste that this 9.5S protein bound oestradiol in the cytoplasm and that the complex was transferred to the nucleus where it was cleaved into a 5S component (Jensen et al., 1968). But recently this cleavage of the 9.5 complex was shown to be an artefact caused by 0.3 M KCl being presen in the sucrose gradient (Korenman and Ramanath Rao, 1968).

Maurer and Chalkley (1967) measured the extent of binding of oestradiol with calf endometrium in vitro. They found that oestradiol was first concentrated from the culture medium into the cell and then further concentrated from the cytoplasm into the nucleus. They confirmed the observation of Noteboom and Gorski (1965) that the receptor

appeared to be stereospecific for oestrogens and that the number of binding sites per nucleus was 2,000-2,500. They also showed by caesium chloride density gradient centrifugation that the oestradiol was bound to a protein component of the chromosomal material and that the binding was reversible.

Recently Teng and Hamilton (1968) showed that the binding in vivo of oestradiol -17β to chromatin from the uterus of adult ovariectomised rats reached a maximum at 8 hrs. after injection. They also showed that the template activity of the chromatin when added to a DNA-dependent RNA polymerase system reached a maximum at 8 hrs.

Autoradiography has also been used to locate the binding sites of oestradiol in the uterus (Jensen et al., 1968) but this will be discussed in a later section. This binding of oestrogens to chromatin suggests that the genome may be the site of action

2. Early effects of oestrogens

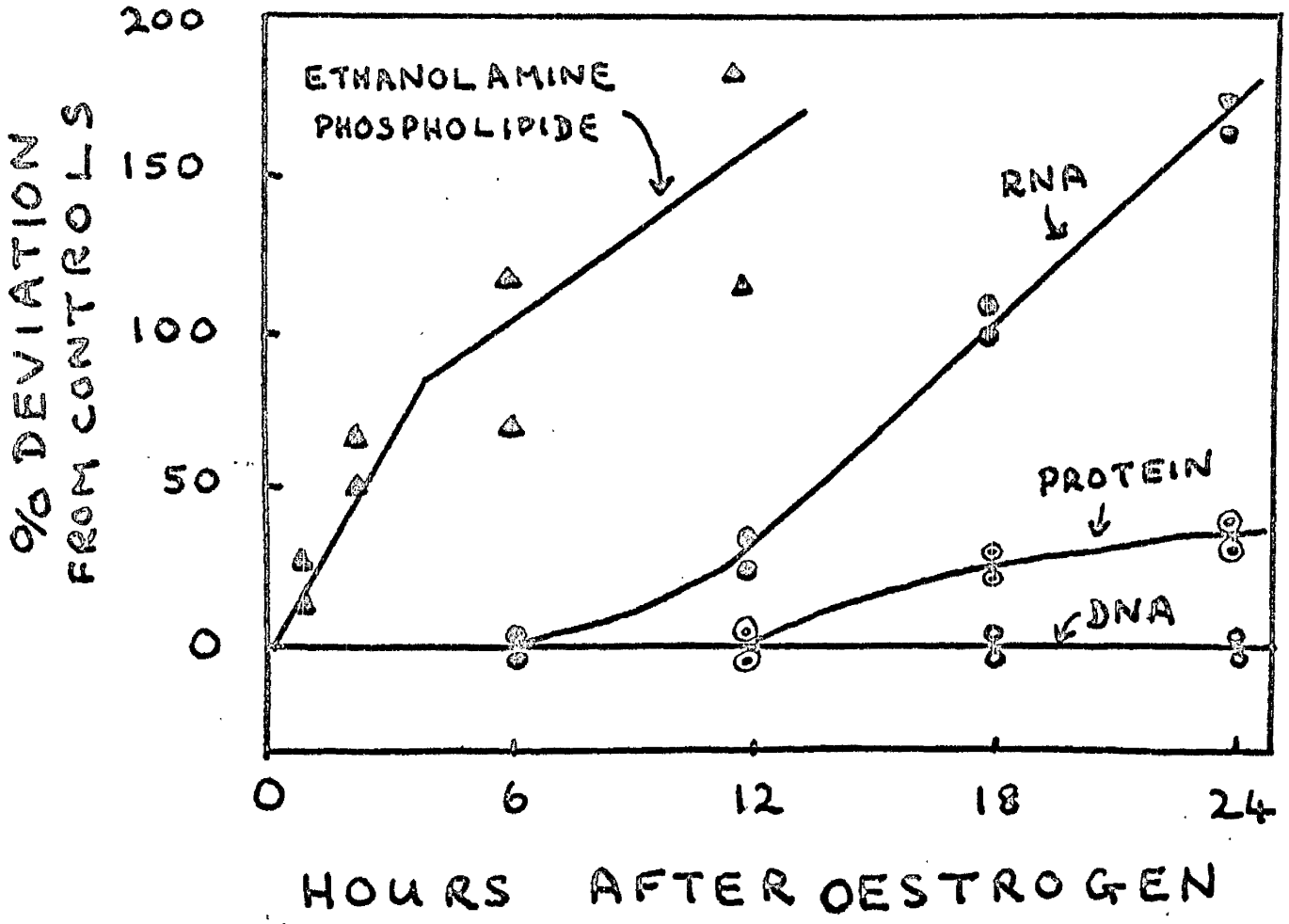
Workers following this line of research have tried to find out which biochemical parameters of the uterus undergo changes in response to hormone action, and in what order in time these changes occur. Carry this line back to its limit it was hoped to identify the earliest change tha occurs, to show how the hormone is responsible for this and to charact

ise other events which follow as a consequence of this first event. In other words we are looking for primary and secondary effects of the hormone.

(i) Chemical Parameters

The response of the uterus to oestradiol -17β has been called "a symphony in anabolism". The hormone stimulates a series of anabolic pathways which culminate in cell division. This can be seen if we measure the amounts of the main constituents in the uterus at various times after hormone action. If 10 ug of oestradiol - 17β are injected into 3 week old immature rats the pattern shown in fig. 2 (page 6A) is obtained (Aizawa and Mueller, 1961). The earliest, and one of the most striking changes concerns the level of phospholipids which rises concommitantly with the imbibition of fluid and which in tur is responsible for an increase in wet weight. The gross level of RNA increases after six hrs. of hormone action and protein only after 12 hr. The amount of DNA in the uterus does not change appreciably during th 24 hr. of this experiment, but my own observations show it to increase after about 30 hr. This is the order of events one would expect from current ideas of protein synthesis. An increase in RNA would cause a

FIG. 2.


200
150
100
50
0
% DEVIATION FROM CONTROLS
ETHANOLAMINE PHOSPHOLIPIDE
RNA
PROTEIN
DNA
0
6
12
18
24
HOURS AFTER OESTROGEN

increase in protein synthesis which would mean an increase in the enzymes and structural proteins. When the amount of protein of the cell reaches a certain level DNA synthesis seems to occur, followed by cell division. This kind of pattern is seen in the effect of other growth and developmental hormones, and in other systems such as the action of phytohaemaglutinin on lymphocytes. It is changes such as these which occur in the normal cycle of cells in culture. (This cell cycle will be described in more detail later in the thesis).

Finally in this section Teng and Hamilton (1968) measured the changes that occur in the RNA/DNA and total protein/DNA ratios of uterine chromatin after oestradiol - 17β treatment of adult ovariectom rats. The RNA component increased by 17% in amount during the initi 15 min. of hormone action. The amount of this component then remain constant for about 8 hrs. Between 12-72 hr. after treatment, the RNA DNA ratio increases again, and there is an increase in the total protein DNA ratio. There was no detectable change in the RNA/DNA and total protein/DNA ratios of liver chromatin as a result of the hormone treatment. This supports the view given by Hamilton and his co-worke from ^{3}H uridine incorporation studies that oestrogens have an early effect on RNA synthesis at the genome level.

(ii) Incorporation of radioactive precursors into macromolecules.

Much of this work was designed to show the pattern of increased RNA synthesis over the early time period of hormone stimulation up to 6 hr. after which an increase in the amount of RNA in the uterus begins to appear. Unfortunately a lot of this work is difficult to interpret in terms of a genuine net change in macromolecular synthesis. This is because the hormone changes the amount of precursor entering the uterus which will change the specific activity of the precursor pool. The rate of incorporation of precursors into macromolecules, as measured in terms of radioactivity in the macromolecules, is not only dependent on the rate of synthesis but is also dependent on the specific activity of the precursor pool. Very few workers have taken these pool changes into account and therefore the change in incorporation of precursors into macromolecules has been interpreted as being entirely due to an increased macromolecular synthesis which in most cases is not true.

Corski and Nicolette (1963) measured the amount of ^{32}P recovered as AMP -3' from the nuclear RNA of immature rats 1 hr. after the administration of ^{32}P orthophosphate. In animals which had received

oestrogen 1 hr. before killing the level of ^{32}P AMP was twice that in controls while in animals receiving the hormone 2 hr. before killing the level was five times that in the controls. They obtained similar results using adult ovariectomised rats (fig. 3i; page 9A)

Hamilton, Widnell and Tata (1965) measured the incorporation of ^{3}H uridine into uterine RNA of ovariectomised adult rats. They showed that the specific activity of nuclear RNA increases very rapidly on oestradiol treatment reaching a peak 5 times the control value at 20 min. By 40 min. this value declines again to almost the control level at which it remains for the remainder of the experiment (160 min). In later experiments Hamilton, Widnell and Tata (1968) extended this experiment and showed that the specific activity of nuclear RNA increase again at 2 hr. and continues at a new level of 2 times the control value for at least 24 hr. (fig. 3ii; page 9A).

Hamilton, Widnell and Tata (1965) also showed that the <u>total</u> tissue amount of ^{3}H uridine entering the uterus increases by 2.5 times at 20 min. and then falls to 2 times the control level at 40 min. Unfortuna in this particular experiment the authors expressed the radioactivity per mg. of tissue, but the wet weight of the uterus is itself changing over this time period. Nevertheless this experiment suggests that the hormo

FIG. 3(i)

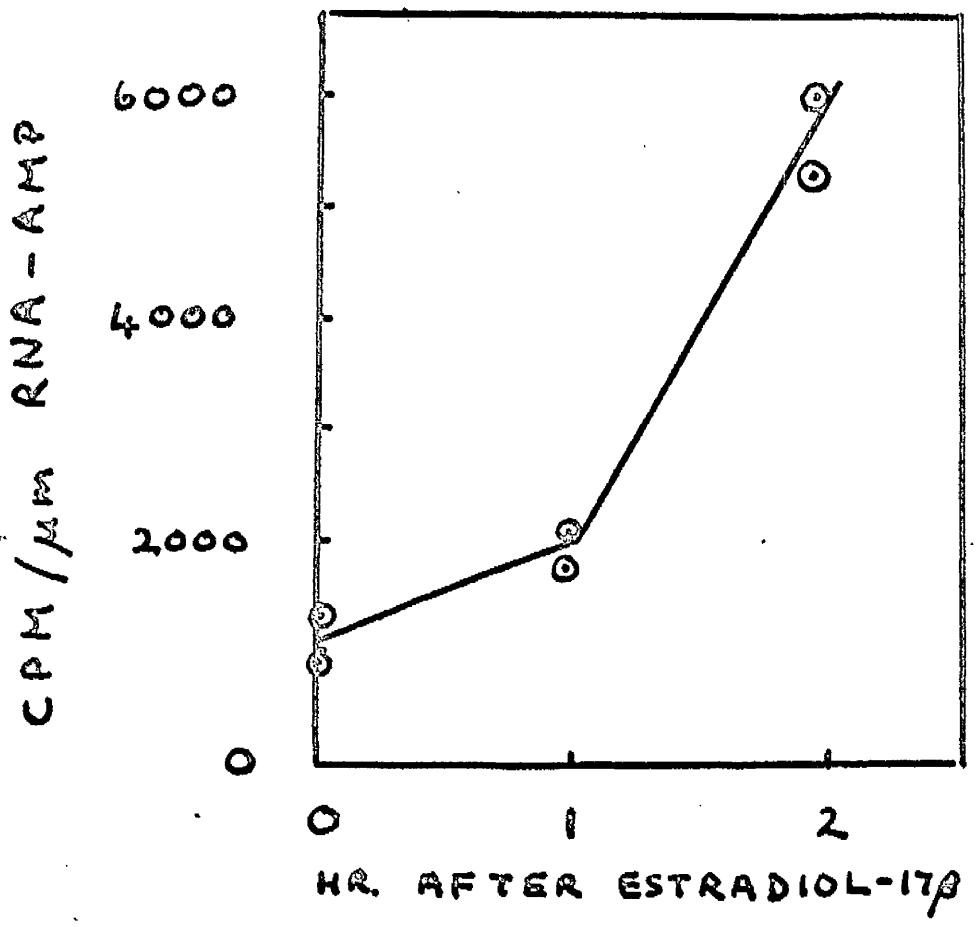
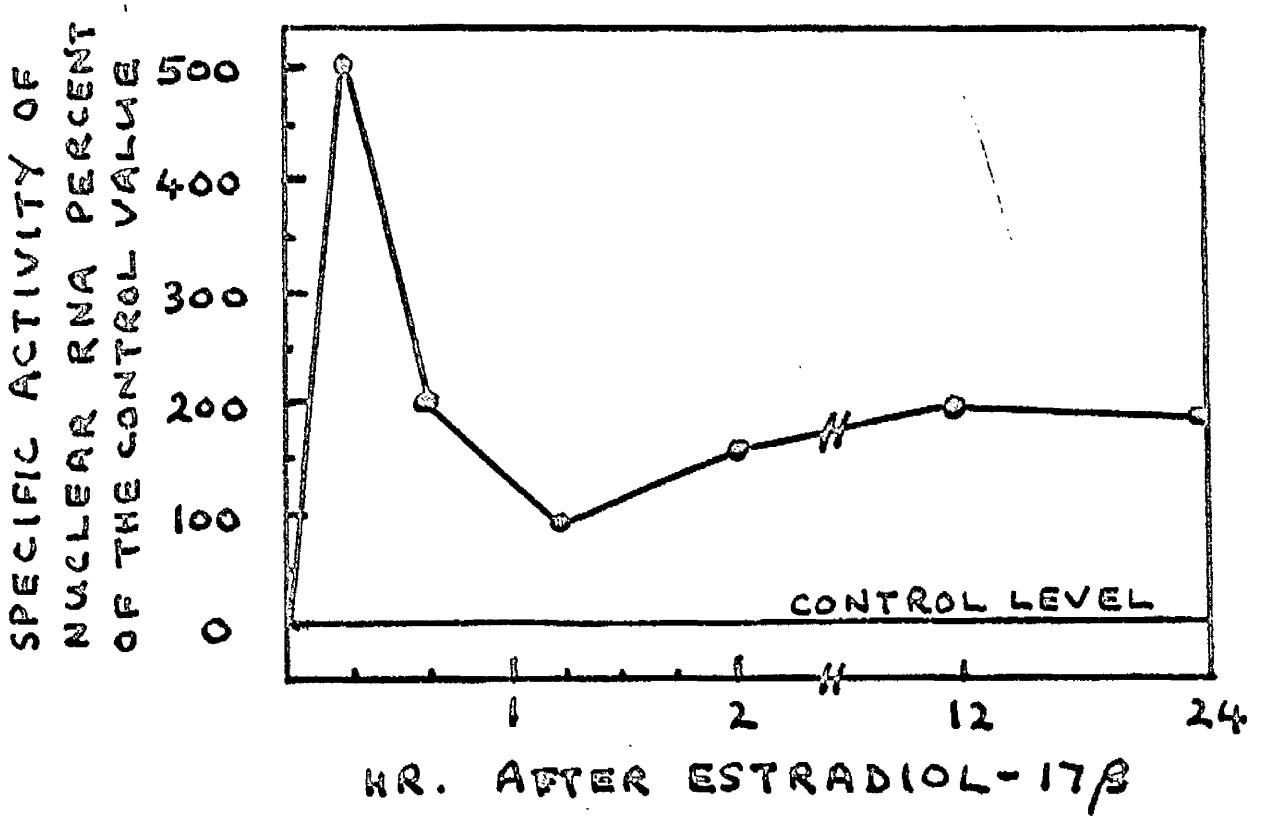


FIG. 3(ii)



in causing an increase in the amount of ^{3}H uridine in the uterus which itself would contribute to an increased incorporation into uterine RNA. Means and Hamilton (1966a) confirmed this increase in specific activity of nuclear RNA, but interpreted the results as meaning an increase in RNA synthesis of several hundred per cent. Clovati and Nicolette (1963) also interpreted their increase in incorporation of ^{32}P into uterine nuclear RNA as representing an increase in RNA synthesis of this order. No account was taken of any changes in the specific activity of the precursor ^{32}P orthophosphate pool which may have arisen from oestrogen causing an increased transport of precursor from the blood stream into the uterus. Until the effect of oestrogens on permeability is separated from the effect on RNA synthesis it is dangerous to draw firm conclusions about the effect on RNA synthesis alone from this type of incorporation experiment.

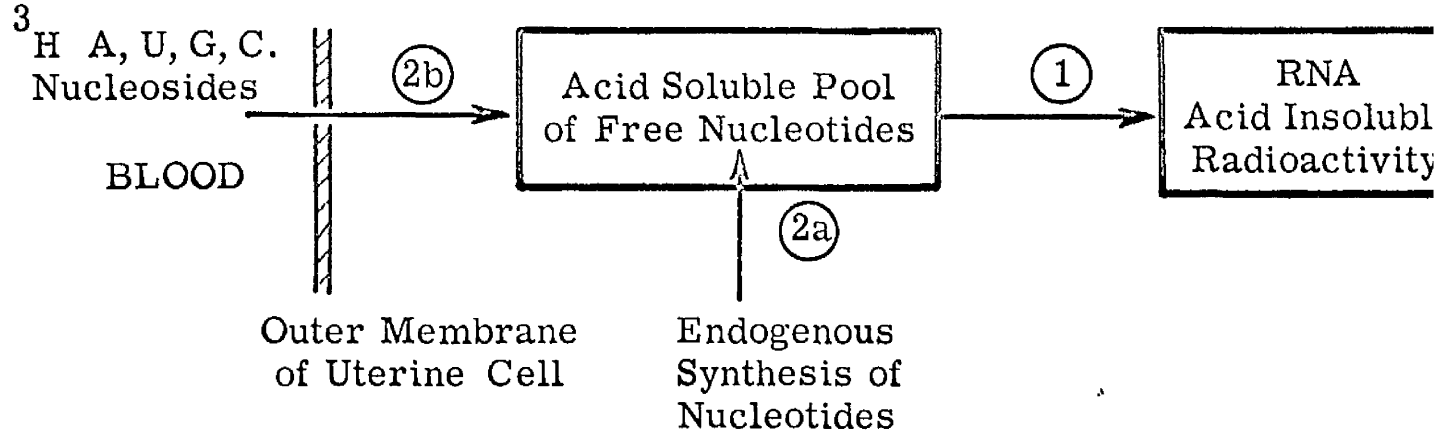
To achieve an accurate measurement of the changes in RNA synthesis from this type of experiment, using the incorporation of radioactive precursors such as ^{3}H uridine, the changes in the specific activity of the UTP pool must be known. In the case of UTP this is very difficult to measure because of the rapid turnover of the pool and

because of its small size. A very rough approximation would be to measure the changes in the acid soluble material which Hamilton has done in certain cases. This would include ^{3}H uridine which had entered the cell but also the amount which was still left in the blood vessels, and it would not show how changes in the pool occur with time. I have tried to overcome some of these problems by using ^{3}H adenosine which enters a larger pool with a proportionally smaller turnover, and by measuring changes in the specific activity of the total adenine nucleotide pool. If we assume that any of the injected ^{3}H nucleoside which is found as nucleotide is inside the cell then this may lead to a closer approximation of the true changes in RNA synthesis (fig. 4 page 11A).

Means and Hamilton (1966 b) have confirmed again their earlier observation of a rapid increase in the specific activity of nuclear RNA and have also shown increases in total uptake of precursor by the uterus. They also show that both these increases are detectable as early as 2 min. after the administration of oestrogen (fig. 5 page 11B) It should be noted that increases in acid insoluble radioactivity (RNA) always correspond with increases in total uptake of ^{3}H uridine into the tissue. While a 5 fold increase in the specific activity of nuclear

FIG. 4

UTERINE CELL



Increased radioactivity in RNA can be due to:-

1. Increased synthesis of RNA.

2. Increased specific activity of the free nucleotide pool.
   a. Decrease in endogenous pool.
   b. Increase in uptake of ^{3}H nucleoside from external sources e. g. blood stream.

FIG. 5

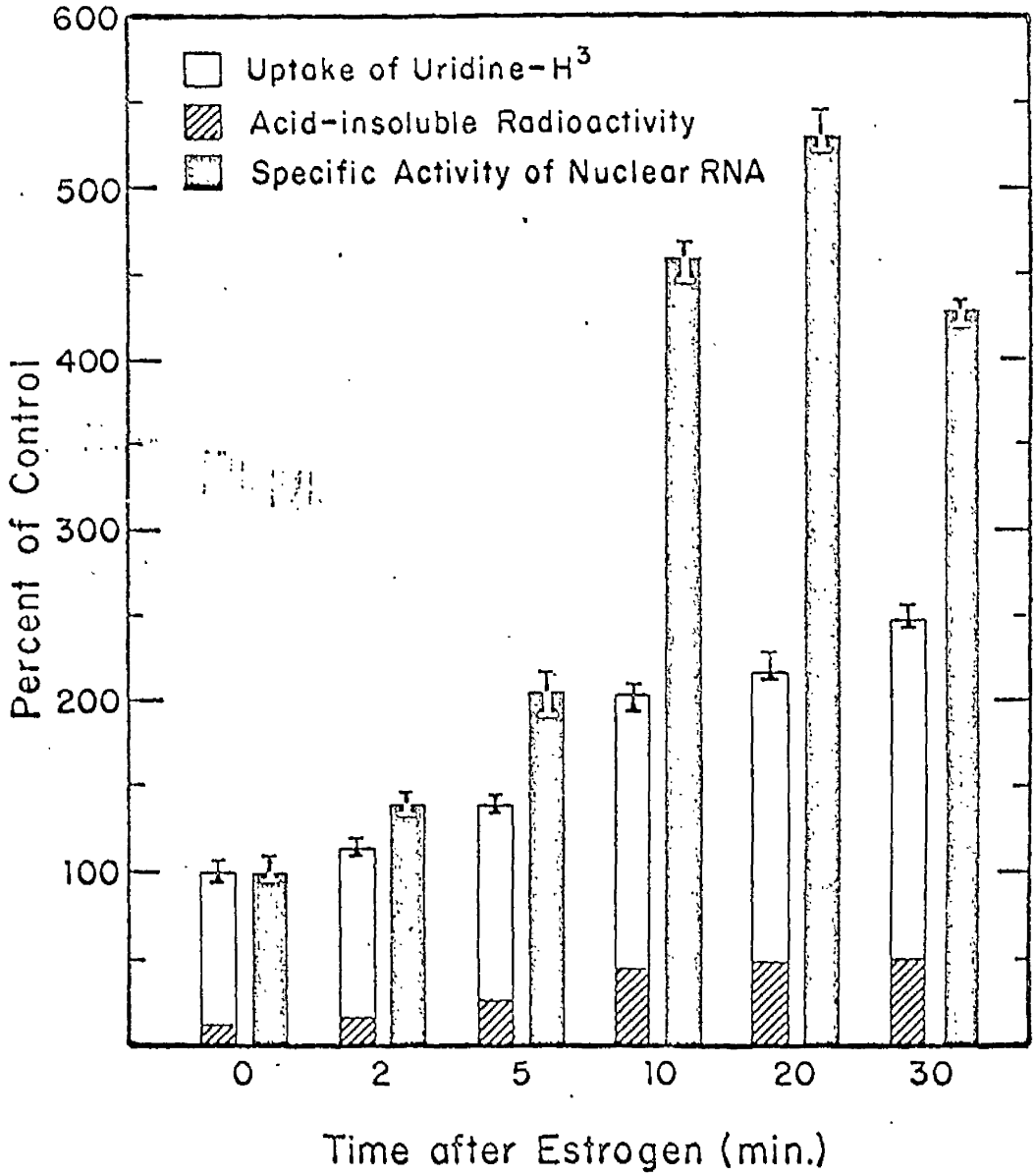


FIG. 1.—Effect of estrogen on uptake of uridine-H^3 by the uterus and its incorporation into acid-insoluble material and nuclear RNA. The data are expressed as per cent of control value. Brackets show the ranges of triplicate experiments. Five uteri were pooled for each experimental or control group. The ranges of variation for the acid-insoluble data are given in Table 1. The control values are: 6,920 cpm/mg homogenate DNA; 11.0% acid-insoluble radioactivity; and 1,204 cpm/mg nuclear RNA (see Table 1). For each period of time tested for estrogen action, separate control experiments were performed (see *Materials and Methods*). Thus the control values given here at time zero for tissue radioactivity and nuclear RNA specific activity, and for acid-insoluble radioactivity given in Table 1, represent respectively the mean for 15 control groups (i.e., three for each time period of estrogen action tested).

RNA was observed at 20 mins. after oestradiol treatment, the increase in the total amount of radioactivity in the tissue was only two-fold. Although this is just a very rough approximation to changes in the UTP pool it may indicate that there is a genuine increase in the rate of RNA synthesis at this time. Hamilton has suggested that the increased uptake of precursor into the tissue may be a response to a fall in the ribonucleotide pool caused by utilisation of nucleotides for the synthesis of RNA. This would therefore be a secondary effect. There is however no evidence to suggest that a fall in the ribonucleotide pool occurs so quickly after the onset of increased RNA synthesis. Indeed there is a lot of evidence which points to the fact that oestrogens can increase the transport of precursors into the uterus irrespective of macro molecular synthesis.

Kalman and Daniels (1961), Kalman, Lombrozo and Lewis (1961), Daniels and Kalman (1961) have measured the transport into the uterus of the amino acid α amino-iso butyric acid which is not utilised for portein synthesis. They show that the uptake of this amino acid is increased 2 hr. after oestrogen administration and reaches a maximum at 12 hr. Szego and Davies (1967) show that changes in the level of 3' 5' cyclic AMP occur as early as 15 seconds after hormone treatment.

Bargo (1969) shows that release of histamine in the uterus is another of the earliest effects of oestrogens and that this probably has effects on permeability of the organs and the blood supply. The transport of water into the uterus is another of the early effects of the hormone which is unlikely to be a consequence of macromolecular synthesis. As water is taken into the uterus other small precursor molecules may be taken in with it. In my own experiments with immature rats the rate of inhibition of water and increased blood supply are very apparent at about 2 hr. after hormone administration. It is at this time that a 5 fold increase in uptake of RNA precursors is observed in both acid insoluble and acid soluble material. In every occasion when increases were observed in the incorporation of nucleosides into acid-insoluble material increases were also found in the incorporation of nucleosides into the acid-soluble pool. This close correlation suggests that the observed changes in incorporation of labelled precursors in RNA may, to a large extent, be accounted for in terms of changes in the specific activity of the precursor pool, and not to increased RNA synthesis which is the interpretation given by other workers in the field.

I now want to compare the pattern of incorporation of precursors into RNA obtained by Clever and Nicolette (1969) (fig. 31 page 94) with

the pattern obtained by Hamilton and his co-workers (fig 3ii page 9A). Briefly Hamilton shows a peak of incorporation at 20 min. 5 times the control value after hormone administration which falls quickly and levels off at about 2 times the control value. Gorski and Nicolette do not show this early peak but show a 5 fold increase from 1 to 2 hr. after oestrogen administration: they find the same pattern using immature rats or adult ovariectomised rats. Our results with immature rats give the same pattern as Gorski and Nicolette and also show that there is in fact a peak of incorporation at 2 hr. Hamilton usually shows his peak at 20 mins. using adult ovariectomised rats, but Hamilton, Widnell and Tata (1968) also observe a 2 fold increase over the control after 20 min. of hormone action using 3 week old immature rats.

What are the reasons for these differences in incorporation pattern There may be a difference between animals (immature against ovariectomised), but there may also be differences due to the different techniques used by workers. I myself using immature rats have not been able to observe any increase after 20 mins. of hormone treatment using a 15 min. labelling time. It is possible that Gorski and Nicolette (1963) did not detect this rapid increase in incorporation into nuclear RNA at 20 min. because of the longer labelling period (1 hr.) they used.

This could happen if the rapidly labelled nuclear RNA observed by Hamilton and his co-worker is rapidly broken down again. But there may be other differences in the techniques used necessary to explain this difference in pattern. Some of the difficulties emerging from the use of these techniques are discussed in the following paragraph but it may be that these do not of themselves explain the discrepancies mentioned above.

Subcellular fractions such as nuclei are very difficult to isolate from uterus owing to its fibrous nature. This is evident in the amount of whole tissue DNA recovered in from isolated nuclei from uterus which varies from 15-20% (Means and Hamilton, 1966 a and b) to 45-65% (Hamilton, Widnell and Tata, 1968). This raises two points (i) is it possible to determine reproducibly the RNA from these recovered nuclei (ii) are there variations in the type of nuclei recovered bearing in mind the differences in RNA synthesis and response to oestrogens that may occur in myometrial and endometrial cells? In experiments of very short duration (10 min.) used by Hamilton and his co-workers it is know that nearly all the ^{3}H uridine incorporated into RNA is localised with the cell nuclei. In such circumstances it is doubtful whether anything is to be gained from experiments in which nuclei are isolated prior to

analysis of the RNA.

Could discrepancies in incorporation patterns arise from the mode of injection? Miller and Emmens (1967) have shown that ^{3}H uridine injected intraperitoneally leads to a higher but less reproducible levels of radioactivity in uterine RNA than is the case when the precursor is administered subcutaneously or intravenously. They suggest that from an intraperitoneal injection some radioactivity enters the uterus directly from the peritoneal cavity besides entering by way of the circulation. Bearing this in mind it is not difficult to understand why I obtained an even higher incorporation of ^{3}H uridine into the uterus from an intraperitoneal injection if the same amount of radioactivity was injected with a large injection volume. Thus if the control animal is not injected with an equal volume of saline exactly at the same time as the hormone then discrepancies in radioactivity due to volume could occur.

I have compared in detail these patterns showing early rapid increase in uptake of ^{3}H uridine into uterine RNA at 20 mins. and at 2 hr. after oestrogen administration because it is a very important point If there is an early burst of RNA synthesis at 20 mins. as Hamilton suggests then it occurs before a more general increase in RNA

synthesis and therefore it may well have an important role to play in the initiation of the response of the uterus to oestradiol. If it is just an artefact then this too should be cleared up.

Finally in this section on uptake of radioactive precursors Means and Hamilton (1966a) working with adult ovariectomised rats have shown that uptake of tritiated methionine into uterine protein is depressed in all cellular fractions 30 mins. after oestradiol administratio but reaches the control level again at about 2 hrs. and thereafter increase rapidly. The least depression and most rapid increase occurs in the nuclear fraction while the greatest depression and slowest increase occur in the soluble protein fraction. The authors find this result difficult to explain, but they suggest that the depression in incorporation at 30 min. could be due to a lowering of the uterine ATP pool or a slow mobility of this pool which they say would be depleted owing to an increase in RNA synthesis.

Finally I will summarise the main points under this heading of incorporation of radioactive precursors into uterine macromolecules.

(1) Many experiments of this type have been performed over recent years but very few have taken into account the changes in the specific activity of the precursor pool. It is dangerous to draw conclusions

about changes in the synthesis of macromolecules from these experiment when the extent of pool changes are not known. (ii) Where changes in the uptake of precursors have been noted it is not known whether or not they are secondary effects to changes in the synthesis of macromolecules. (iii) There are differences between workers in the pattern of incorporation of RNA precursors into the uterus after oestrogen administration. Hamilton and his co-workers on one hand observed a peak of incorporation at 20 mins. after hormone administration while Gorski and Nicolette on the other hand observe no peak at 20 mins. but a peak at 2 hr.

(iii) Enzyme activities.

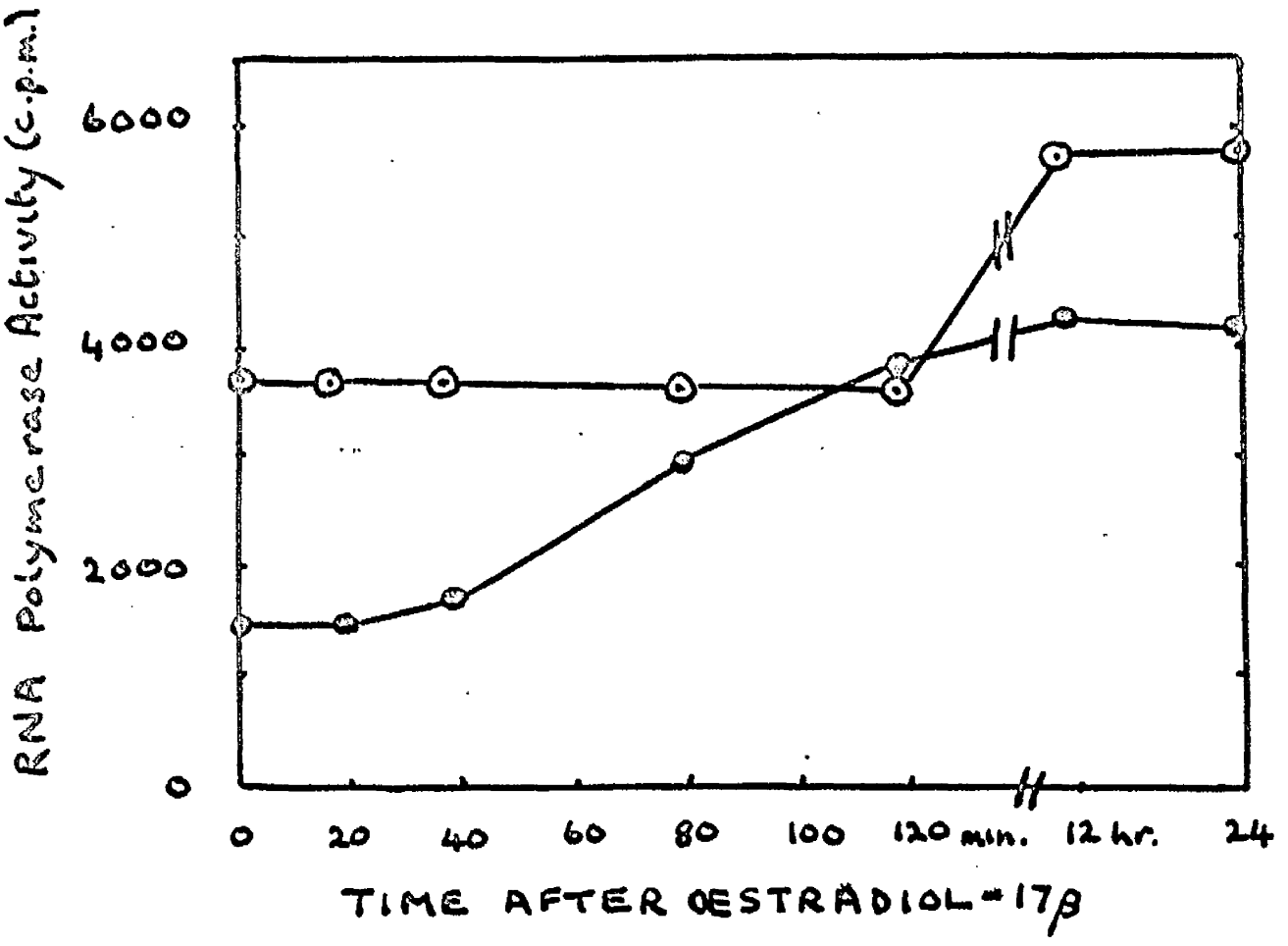
(a) DNA-dependent RNA polymerase

Gorski (1964) showed that DNA-dependent RNA polymerase activi from immature rat uterus increased two-fold 1 hr. after oestrogen treatment in vivo. There was no DNA primer or ammonium sulphate added to this assay system. When ammonium sulphate was added this increase in activity disappeared. Hamilton, Widnell and Tata (1965) clarified this point by showing that there were two polymerase assays

one with Mg^{++} ions but no salt, and the other with Mn^{++} ions plus ammonium sulphate. The assay of the polymerase in the presence of Mg^{++} showed a similar increase after 1 hr. of oestrogen treatment to that which Gurdon (1964) obtained. Hamilton, Widnell and Yoko (1969) extended this work (fig. 6 page 19A). They showed that the peak of the Mg^{++} activated polymerase activity occurred at about 12 hrs. after hormonal stimulation when it levelled off at about 2½ times the control level. The Mn^{++} plus ammonium sulphate activated polymerase did not increase in activity until about 12 hrs of hormone action when it showed only a 50% - 60% stimulation over the control level. These experiments were performed using ovariectomised adult rats, but the authors say they obtained similar results using immature rats. In the same paper it was shown that the base composition of the product of the Mg^{++} activated polymerase similar to ribosomal RNA, whereas the product of the reaction in the presence of Mn^{++} ions and ammonium sulphate may be stripping more protein from the DNA making more sites available for template function. Hamilton, Teng & Means (1968) continued this work.

In all the above mentioned experiments the template for RNA synthesis was endogenous DNA which was part of the chromatin. Therefore in the Mg^{++} dependent polymerase activity we are presumably left

FIG. 6



o—o Mn^{++} $(NH_4)_2SO_4$ activated RNA polymerase

●—● Mg^{++} activated polymerase

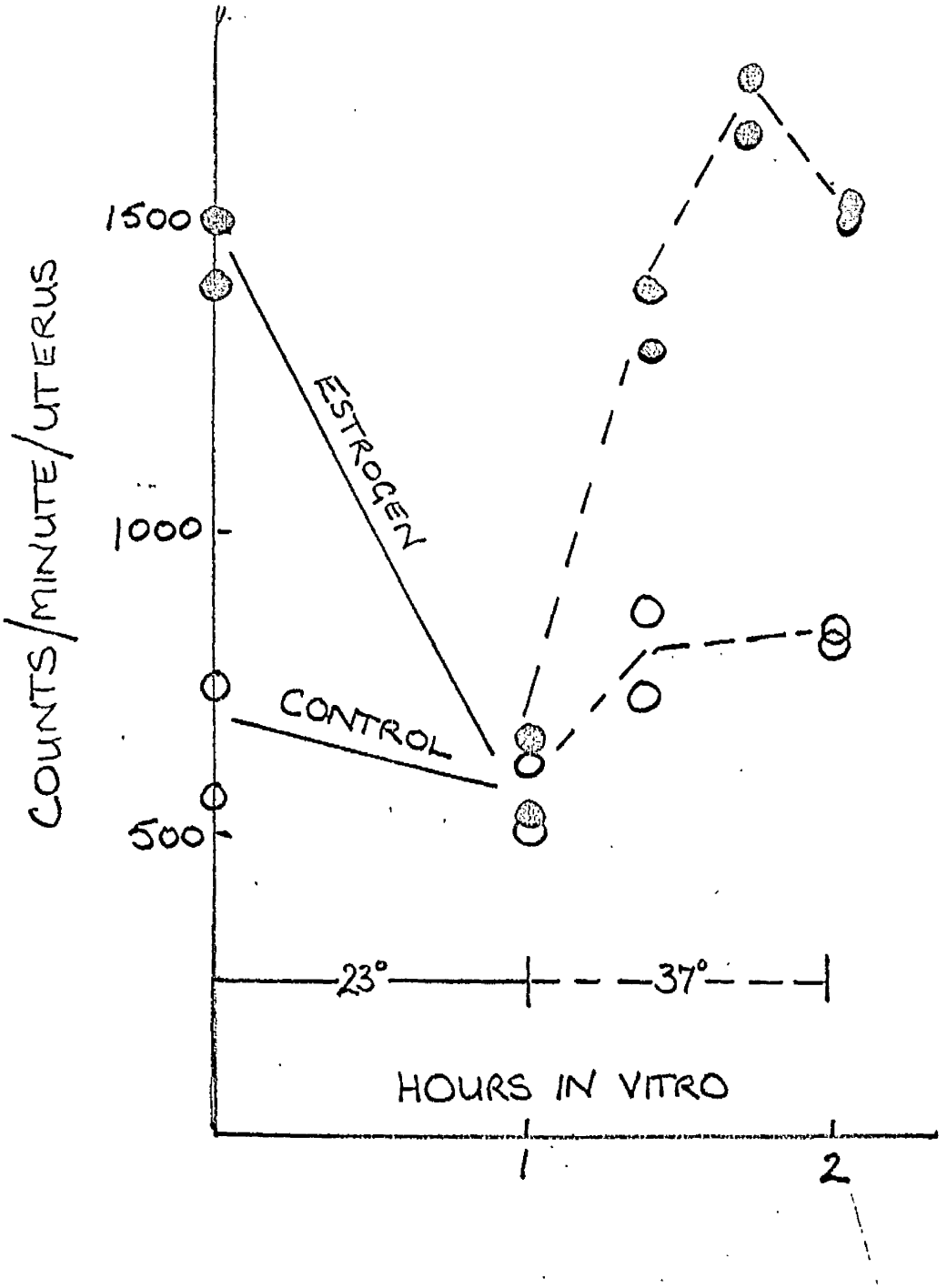
complete availability and polymerase activity. The Mn^{++} concentration inhibits polymerase assay may markedly stimulate the template penetration and show changes only in the amount or activity of the actual enzyme. Therefore it seems that oestradiol may increase the template activity of the chromatin from 1 hr. to 12 hrs., but the amount or activity of DNA polymerase enzyme does not increase until later. This was confirmed by Tsuji and Hamilton (1966), where they add exogenous bacterial DNA polymerase and in a low salt medium observe only the change in template activity of the added bacteria chromatin.

Some elegant experiments on DNA - dependent RNA polymerase from immature rat uterus were performed by Nicodeto and Mueller (1966) showing the effect of temperature and inhibitors on the increased activity under increase stimulation. They killed the rats 4 hrs. after a 5 mg injection of oestradiol -17β and the uteri were incubated in Eagle culture medium which was regulated to either 37° or 23°. At various times after being placed in culture medium the uteri were removed and assayed for RNA polymerase activity by the method of Gerard (1964). The RNA polymerase activity rose 2-fold on *in vivo* treatment with oestradiol as shown by Gerard (1964) but after half an hour incubated at

23° the polymerase activity fall back to the control level which was itself only slightly affected by the fall in temperature. However the lost polymerase activity was quickly restored by raising the temperature of the incubation medium to 37° again (fig. 7 page 21A). If an amount of cycloheximide which caused 95% inhibition of protein synthesis in the uterus was added to the incubation medium, it reduced within 1 hr. the increased polymerase activity of the hormone treated uteri to that of the control level. The amount of cycloheximide used had no effect on the level of polymerase activity of the control uteri. This paper demonstrates the striking dependence of the oestrogen - induced increase in polymerase activity on continued synthesis of new protein and reveals that a highly temperature - sensitive process is involved.

(b) Ribonuclease.

To summarise events concerning RNA metabolism it can be said that after 6 hrs. of oestradiol action there is an increase in the amount of RNA in the uterus. This could be due to increased synthesis of, or to decreased breakdown of RNA. As we have seen the activity of DNA - dependent RNA polymerase is increased after 1 hr. of oestrogen activity. This alone may account for the increased amount of RNA, but little or no attempt seems to have been made to measure changes in nuclease activity


1500
1000
500
COUNTS/MINUTE/UTERUS
ESTROGEN
CONTROL
23°
37°
HOURS IN VITRO
1
2

in response to hormone stimulation.

It is possible that ribonuclease activity is associated with the DNA-dependent RNA polymerase systems that have been employed in these studies. It should be noted that bentonite, which is an inhibitor of ribonuclease, will appreciably enhance RNA synthesis when added to the polymerase assay system (Teng and Hamilton, 1968). If there were inhibition of ribonuclease by oestradiol it would throw a different interpretation on the results of the RNA polymerase work.

(iv) Actinomycin D and early effects of oestrogens

It is known that actinomycin D will inhibit RNA synthesis probably by binding to the DNA template. Thus the following experiments seek to show whether or not RNA synthesis is required for subsequent response to oestrogens. However, it should be remembered that actinomycin D is very toxic to cells in general and may have effects on cellular metabolism other than on RNA synthesis.

Talwar and Segal (1963) showed that the effect of oestrogen on proliferation and cornification of the vaginal epithelium could be blocked by a local application of actinomycin D.

It was shown by Ui and Mueller (1963) that earlier effects of oestradiol on rat uterus were also inhibited by actinomycin D. The

incorporation of ^{3}H uridine into RNA and the incorporation of ^{14}C glycine into lipid and protein during the final 2 hr. of a 4 hr. oestrogen treatment were measured, (fig. 8 page 23A). It was found that the actinomycin D was equally effective in blocking RNA synthesis in the uteri of both control and oestradiol treated rats, but leaving in both cases a small amount of incorporation which was insensitive to the action of actinomycin D. As seen in fig. 8 page 23A, the incorporation of ^{14}C- glycine into protein was inhibited to the same extent in both hormone treated and untreated uteri showing that the increased incorporation due to hormone stimulation was lost. But again this increas may have been due to an increased uptake of ^{14}C - glycine from the blood stream, and thus the authors might have been measuring the inhibition of this process and not protein synthesis by actinomycin D. Most, but not all, the increased stimulation of ^{14}C - glycine incorporation into lipid was inhibited by actinomycin, but this again may have been an effect on uptake of ^{14}C - glycine from the blood. The most genuine effect of actinomycin was the inhibition of the increase in wet weight which represents the water inhibition. Does this process require new RNA synthesis or is the inhibition a direct effect of actinomycin D? Mueller, Gorski & Aizawa (1961), showed that

FIG. 8

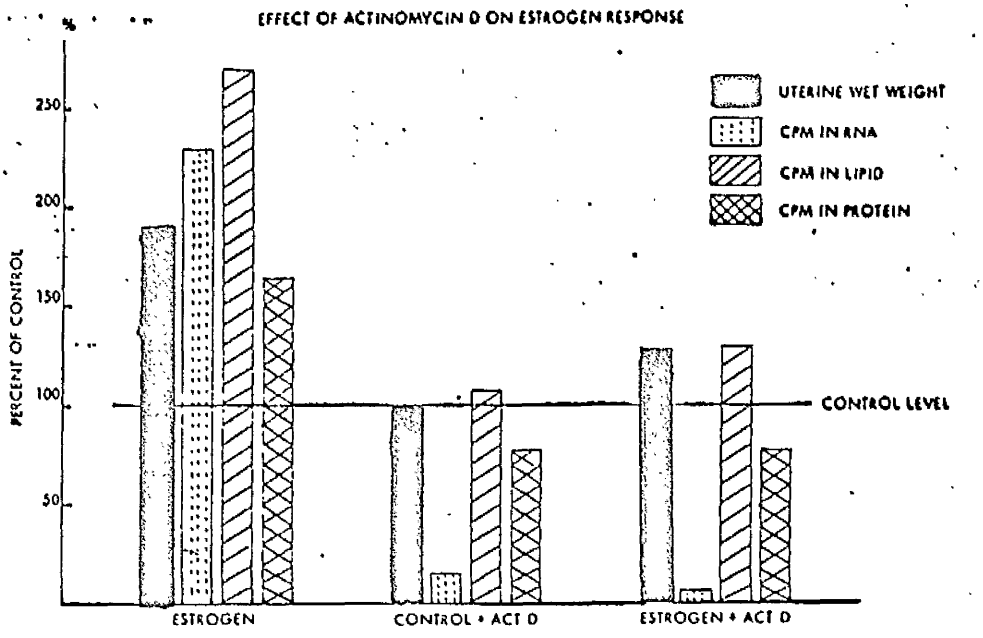


FIG. 1.—Effects of actinomycin D on the early estrogenic response. Groups of 3 rats were injected intraperitoneally with 375 μg of actinomycin D or control solution 30 min prior to the administration of 10 μg of estradiol or control solutions. At 2 hr and again 3 hr after the hormone treatment 25 μc of uridine-H^3 and 6 μc of glycine-$2C^{14}$ were injected intraperitoneally. Four hr after the hormone treatment, the rats were killed and the uteri removed and analyzed for the wet weight, CPM of uridine-H^3 incorporated into RNA, and CPM of glycine-$2C^{14}$ incorporated into protein and mixed lipid fractions. Data are expressed as per cent of values obtained for control uteri which were: wet weight = 71 mg; CPM in RNA per uterus = 986; CPM per mg protein = 81; CPM in lipid fraction per uterus = 235.

puromycin, an inhibitor of protein biosynthesis, also blocks the increase in water inhibition. Therefore this effect of oestrogens may require both new RNA synthesis and new protein synthesis.

(4) Puromycin and Cycloheximide and early Effects of Oestrogens

Both these drugs efficiently inhibit protein biosynthesis. Puromycin acts as an analogue of aminoacyl - sRNA (Nathans, 1964). Mueller, Gerard & Alonso (1961) showed that the increased incorporation of orthophosphate ^{32}P into RNA and phospholipid in ovariectomized adult rats after 4 hrs. of oestrogen action was abolished when puromycin was present. They interpreted this to mean that the synthesis of RNA and phospholipid was increased by oestradiol and that the process required new protein synthesis. They also showed that the increased water inhibition was abolished with puromycin.

Again these experiments showing increased incorporation of precursors never show to what extent the uptake of the precursor into uterine cells has increased under hormone action. In the absence of this information we do not know how much of the inhibition of increased incorporation is due to an inhibition of increased synthesis or simply due to an inhibition of increased uptake. Mueller, Gerard & Alonso (1961) were aware of this point, but came to the conclusion from a paper

by Halkerston, Eichhorn, Feinstein, Scully and Hechter (1960) that there was no increased uterine permeability in their experiment. Hechter and Lester (1960) come to the conclusion that there were no striking changes in cellular permeability in the uterus over the first 6 hr. of oestrogen action, but they noted that there was an increased inhibition of water over this period. Kalman, Lombrozo and Lavis (1961) disagreed with the conclusions of Halkerston et al. (1960), and they showed that oestrogens do increase uterine permeability which is detectable after 1 hr. of hormone action. This point needs more clarification before the action of inhibitors on incorporation studies such as those of Mueller, Gorski and Aizawa (1961) can be assessed accurately.

It was reported in the section on DNA-dependent RNA polymerase (iii, a) that Nicolette and Mueller (1966) had shown that cycloheximide blocked the oestradiol induced increase in DNA-dependent RNA polymerase activity. Therefore it seems that this effect of oestrogen requires new synthesis of protein. They also showed that cycloheximide had this blocking effect when added some time after the initiation of the increase in polymerase activity which seems to indicate a necessity for continued synthesis of protein to produce the increase in enzyme activity.

Finally if cycloheximide is used to inhibit protein synthesis in control rats the amount of incorporation of ^{3}H uridine into nuclear RNA increases between two and seven-fold (Means and Hamilton 1966a; Hamilton, Widnell and Tata; 1968). This means that cycloheximide mimics to a certain extent the effect of oestradiol in the control animal. One possible explanation of these observations is that oestradiol could inhibit the synthesis of a protein which itself blocks the accumulation of new RNA. This protein could be a ribonuclease. The blocking of a molecule which is inhibiting growth in the resting (oestrogen-less) uterus is a possible mechanism for the mode of action of oestrogens. The blocking of a ribonuclease would be one possibility.

3. Other aspects of oestrogen action.

(i) Oestrogen-induced synthesis of a specific uterine protein.

Notides and Gorski (1966) ran the soluble fraction of proteins from oestrogen treated and untreated immature rat uteri on starch-gel electrophoresis. After various times of in vivo oestrogen treatment the uteri were removed and incubated in vitro for 1 hr. with ^{3}H - leucine. The radioactivity of all protein fractions increased. The pattern of labelling was essentially the same in both treated and untreate animals except for one particular zone (A) which contained a relatively

small proportion of the uterine proteins. This zone was more negatively charged than the main protein components, and it exhibited a large increase in radioactivity even after ½ hr. of in vivo oestrogen treatment. The liver and the ileum soluble proteins did not show this change in pattern with oestrogen treatment. If the rats received 200 ug of actinomycin D ½ hr. before oestrogen treatment the increase in radioactivity of zone A was still observed. The authors suggest that oestrogen is causing an increase in the synthesis of a specific uterine protein which may be under translational control, and which occurs before an over-all stimulation of protein synthesis.

(ii) Adenosine 3' - 5' cyclic monophosphate and histamine involvement in oestrogen action

Szego and Davis (1967) showed that within 15 seconds of oestrogen treatment the level of 3' - 5' cyclic AMP increased by over 100%. This effect is specific for oestrogens, 17α - oestradiol would not give this response. This increased level of 3' - 5' cyclic AMP is not maintained but gradually falls to the control level after 1 hr. The authors say that this does not represent the release of a bound form of the molecule because they would have detected this form in the untreated control. The enzyme adenyl cyclase which synthesises 3' - 5' cyclic AMP is membrane bound Davoren and Sutherland (1963), and it may have come

connection with increased permeability. Szego (1965) shows that oestrogens cause a release of histamine from the uterus at this early time which continues for 2 or 3 hours. She suggests that there may be some connection between the histamine release and the 3' - 5' cyclic AMP level. The fact that the increased level of cyclic AMP persists only for a short time suggests it may have some kind of a "triggering" function directed at histamine release.

Szego (1965) shows, by using india-ink perfusion, some very good photographs of the increased blood supply to the uterus after 4 hours of oestrogen action. She suggests that the release of histamine may have an important role to play in this increase in blood supply or in hyperemia which is well established by 15 min.

It seems however from the following experiments that histamine has very little effect on changes in RNA synthesis. Hamilton, Widnell and Tata(1968) using immature rats observed a twofold increase in the specific activity of nuclear RNA 20 min. after oestradiol treatment.

Neither histamine or the antihistamine compound mepyramine had any significant effect on the specific activity of nuclear RNA even after 2 hours of exposure and the specific activity of the nuclear RNA obtained after treatment with histamine or antihistamine plus oestradiol was not

significantly different from the value obtained with oestradiol alone. The dose of histamine employed did however cause an increase of about 70% in the wet weight of the uteri at 2 hours which is comparable to the change produced by treatment with oestradiol. These authors have concluded that histamine, antihistamine and histamine liberator do not affect the uptake of ^{3}H uridine into nuclear RNA. While these experiments may show that histamine is not involved in the increased synthesis of nuclear RNA they do not exclude the possibility that it may be responsible for imbibition of water, and (as suggested earlier) there may well be a relationship between the rate of uptake of water and the rate of transport of substrates into the uterus, both of which could be mediated by histamine.

In the same paper the authors confirm their suggestion that histamine has no mediating role in oestrogen induced increases in RNA synthesis. They show that histamine does not cause the increase in DNA-dependent RNA polymerase activity which is caused by the hormone.

Kalman and Daniels (1961), Kalman, Lombrozo and Lavis (1961) Daniels and Kalman (1961) have shown that there is an increased uptake of amino acids from the blood stream in response to oestrogens and my own results show that there is an increased uptake of nucleosides. As

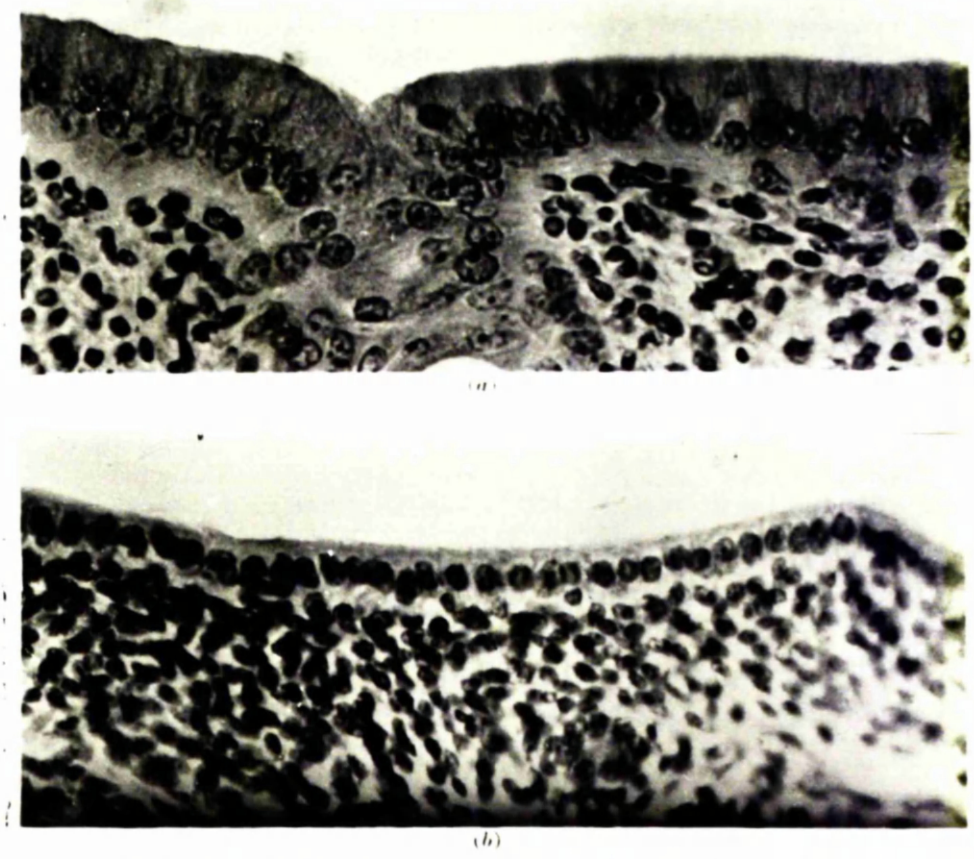
has been mentioned already many of the experiments with radioactive precursors which are interpreted as representing increased macromolecular synthesis are also open to the interpretation that there is an increased uptake of precursors from the blood stream into the uterus.

From the evidence that has been discussed it appears most likely that there exist at least two sites of action of oestrogens in the uterus: one site which is situated at the periphery causing an increased uterine blood supply and permeability, and which may be mediated by histamine; and the other site which is in the nucleus causing an increased RNA synthesis at the genome level, and which is not mediated by histamine.

(iii) Effect of oestrogens on the rat endometrium mediated by RNA

Segal, Davidson, and Wada (1965) showed that the histological changes in the rat endometrium caused by oestrogen could also be caused by uterine RNA which had been extracted from oestradiol -17B treated ovariectomised adult rats. Fig. 9(a) (page 30A) shows a cross-section of endometrium of an ovariectomised rat which had received intrauterine applications of oestradiol 17B. Fig. 9(b) (page 30A) shows the opposite horn of the same animal which had only received saline. This figure is taken from the above paper by Segal et al. (1965). In this paper he

FIG. 9



(*a*) Cross-section of endometrium of ovariectomized rat which received intrauterine application of estradiol-17β. A total dose of 6×10^{-1} μg was administered in divided doses every 4 hr for 48 hr and the animal sacrificed 4 hr after the twelfth intraluminal application. ×900. (*b*) Opposite uterine horn of animal shown in (*a*). This horn treated with saline on same schedule. ×900.

shows photographs similar to fig. 9(a) produced by uterine RNA which had been extracted from oestradiol-17β treated ovariectomised rats. The opposite horn of the same uterus showed no effect and looked like fig. 9(b). This horn had been treated in the same way with the same RNA extract incubated with pancreatic RNase for 60 min. at 37°C. The authors checked the RNA preparation for any contamination with oestradi which may have been giving the effect. The rat from which the RNA was extracted was labelled with ^{3}H - oestradiol-17β, but no radioactivity was found in the final extract of RNA. There was no effect on uteri treated with liver RNA extracts. Actinomycin D which had been shown to inhibit the uterine response to oestradiol-17β itself, did not block the response of the uterus to the RNA fraction.

From this remarkable experiment it seems as if the response of the uterus to oestradiol-17β is mediated via RNA.

(iv) Effect of Oestrogens on DNA

Goldberg and Atchley (1966) showed that oestrogens changed the melting profile of placental DNA by weakening the strength of the intrastrand bonds. 17α - oestradiol (the biologically inactive isomer) was ineffective. DNA isolated from B subtilis and synthetic DNA's, poly d (A. T.) and poly D (G. C.) were unaffected by the hormones. A

deoxyribonucleoprotein complex isolated from the nuclei of placental cells was affected by oestrogens in the same way as pure DNA. The authors reach the conclusion that one action of hormones is to activate genes by promoting the separation of complementary strands of specific segments of the DNA double helix prior to transcription. Supporting this idea is a paper by Frenster (1965) who showed that inactive chromat gave rise to a higher degree of hyperchromicity on heating than did activ chromatin. It is believed that in active chromatin the strands of DNA are already partially separated due to transcription by RNA polymerase.

(v) Transhydrogenase Activity and Oestrogen Action.

This theory of oestrogen action was postulated by Talalay and Williams-Ashman (1958) and (1960). It originated from work on the placenta but it could not be confirmed in the uterus because a specific uterine transhydrogenase could not be found, and there was no oxido reduction action at the C-17 position of 17B- oestradiol in the uterus (Jensen and Jacobson, 1962). See review No. 3: The Hormones ed. G. Pincus, K. V. Thimann and E. B. Astwood, pages 786-808 for further discussion on transhydrogenase activity.

Summary of Important Early Effects of Oestrogens

1. Oestrogens cause an increased accumulation of RNA in the rat uterus from 6 hours after hormone administration.

2. Oestrogens bind to proteins in the cytoplasm and nucleus of uterine tissue.

3. Oestrogens increase the activity of the Mg^{++} activated DNA dependent RNA polymerase after about 1 hour of hormone action.

4. Oestrogens increase the uptake of radioactive precursors from the blood stream into the uterus, and increase its blood supply.

5. Oestrogens increase the amount of water taken into the uterus.

6. Effects 4 and 5 may be connected in some way to present a site of action of oestrogens at the periphery and effects 1 and 3 at a site of action at the genome.

Reviews on the Effects of Oestrogens

1. Mechanisms of Hormone Action (1965) ed. Karlson: chapter on oestrogen action by G. C. Mueller.

2. Journal of Cellular and Comparative Physiology Vol. 66 No. 2 Supplement 1 (1966) Symposium on hormonal control of protein synthesis Chapter on oestrogens by Gorski, Noteboom and Nicolette page 91.

3. The Hormones V (1964) ed. G. Pincus, K. V. Thimann, and E. B. Astwood, pages 786-808.

4. Cancer Research (1965) 25, 1096. Williams-Ashman.

5. Progress in Nucleic Acid Research and Molecular Biology 5. (1966) ed. J. N. Davidson and W. E. Cohn: Chapter by J. R. Tata on

Hormones and RNA synthesis.

PART I

I THE STUDY OF THE ACTION OF OESTRADIOL ON R RAT UTERUS USING TISSUE CULTURE TECHNIQUES

INTRODUCTION

The main advantage of studying the mode of action of hormones in tissue or organ culture is that the environment of the target tissue is controlled. The system is independent of blood and nerve supply, and from effect of other hormones and metabolites which are carried by the blood stream. Examples of hormones acting in tissue or organ culture are given by Willmer (1965). These are not very numerous, and the results have not yielded very much positive information about hormone action. Steroid hormones seem especially difficult in this respect. However hormone-dependent differentiation of mammary gland epithelial cells studied in organ culture, has been shown. This requires the presence of insulin, hydrocortisone and prolactin (Lockwoo Stockdale and Topper, 1967). Synthesis of specific milk proteins such as casein are induced but there are very few other cases of specific effects of steriod hormones in vitro.

Maurer, Rounds and Raiborn (1967) showed that oestradiol caused an increase in cell division in calf endometrial tissue in vitro. They were only able to study the binding of ^{3}H-oestradiol -17B on this system because the levels of endogenous oestradiol were too high to permit

studies involving changes in metabolism (Maurer and Chalkley, 1967).

For the purpose of studying the effect of oestrogens in vitro I have tried to grow primary explants of rat uterus in culture. The tissue is cut up into small pieces, and when these are placed in culture medium cells proliferate out from the explants. (Willmer, 1965; Paul, 1966). The main difficulties in growing cells in vitro by these methods are (1) the cells in the centre of the explants tend to die through lack of oxygen (2) the conditions for cellular proliferation are often varied and complex (3) the numbers of cells obtained are small (4) the cells are probably dedifferentiated from the type in the original tissue (5) the life span of the proliferating cells can be short.

Another method which is frequently used to obtain cell cultures is to treat the original tissue with trypsin. This enzyme does not enter the cells, and therefore it does not harm them, but it breaks down the inter-cellular protein. This frees single cells or groups of cells from the rest of the tissue, and these will grow and divide in culture (Willmer, 1965; Paul, 1966). Similar difficulties to those listed above are encountered.

The cell cycle

The cell cycle; the sequence of events affecting the cell emerging from a

division until the end of the following mitosis. The duration of the cell cycle is called the generation time, and it can be subdivided into 4 stages.

G1 (first gap) is a period of protein synthesis, and is thought to represent the physiological resting state of cells. DNA is stable. The duration of this period is variable, but in HeLa cells it is about 14 hr. S (synthesis) and is the period when the amount of DNA in the cell doubles. The synthesis of DNA can be shown by the incorporation of labelled thymidine into the cell nucleus. The duration of this period in HeLa cells is 5-6 hr.

G2 (second gap) is the next period. DNA is stable (but it is double the amount in G1); the cell prepares for division. The duration of G2 in HeLa cells is 2-8 hr. M (mitosis) is the period when DNA is quantitatively and qualitatively halved and distributed between daughter cells.

Intracellular localisation of oestrogens by autoradiography

Intracellular localisation of oestrogens by autoradiography have met with many difficulties, but some attempts have been made (Ullberg & Bengtsson 1963; Inman, Banfield and King, 1965). Ullberg and Bengtsson (1963) showed autoradiographs which suggested a nuclear

site of action for oestrogens, in the uterus, but low levels of labelling made the evidence inconclusive. Inman, Banfield and King (1965) tried various methods to overcome the fixation difficulties which are encountered owing to the high solubility of oestradiol in alcohol. They obtained quite high grain densities by injecting large amounts of oestradiol -17B of very high specific activity (51.6 c/m-mole but they were unable to obtain results with uterus; they were only able to obtain results with anterior pituitary, liver and erythrocytes. The liver showed grains only in the cytoplasm, but the pituitary showed many cases of nuclear grains. These authors performed autoradiograph on sections from whole organs, and encountered embedding difficulties besides the others which will be mentioned later in the thesis.

Aims of the study of oestrogen action on rat uterus in vitro

At the beginning of this work it was hoped to grow a cell line in culture from rat uterus which would respond to the addition of oestrogen to the culture medium. This did not prove possible. Cells did proliferate out from uterine explants. These cells were viable and synthesised DNA which was shown by autoradiography of cells labelled with ^{3}H thymidine. But so few cells were obtained that only experiments involving qualitative autoradiography were possible. Experiments were performed to determine the intracellular localisation

of oestradiol -17β by autoradiography. Alcohol containing fixatives could not be used because they would extract oestradiol from the tissue. Although oestradiol of very high specific activity was used long exposure times were necessary because of the low levels of hormone present in the cells.

METHODS AND RESULTS

1. Cell proliferation from uterine tissue explants

Methods

The uterus was removed aseptically from an immature rat. It was placed into NCTC 109 medium and was cut up into very small fragments. These were placed onto glass slides which were in large petri dishes containing just enough NCTC 109 medium to cover the slides (fig. 10 page 40A). The petri dishes containing the uterine tissue explants were then placed in a 37^{o} incubator which was supplied with CO_2: air in the ratio of 5:95 or CO_2: O_2 in the ratio 5:95. The amount of CO_2 was regulated to give the medium a final pH of 7.4. Each day the explants were viewed under the microscope in order to see whether or not any cells were proliferating out from the explants. The medium in the petri dishes was changed every 1 or 2 days.

Another method of growing cells from uterine tissue explants was to place the explants in bottles (fig. 11 page 40A). The experimental details were the same as those used for the growth in petri dishes except that the bottles were gassed with the correct mixture and then stoppered.

FIG. 10

GROWTH OF CELLS FROM PRIMARY EXPLANTS IN PETRI DISHES (SIDE VIEW)

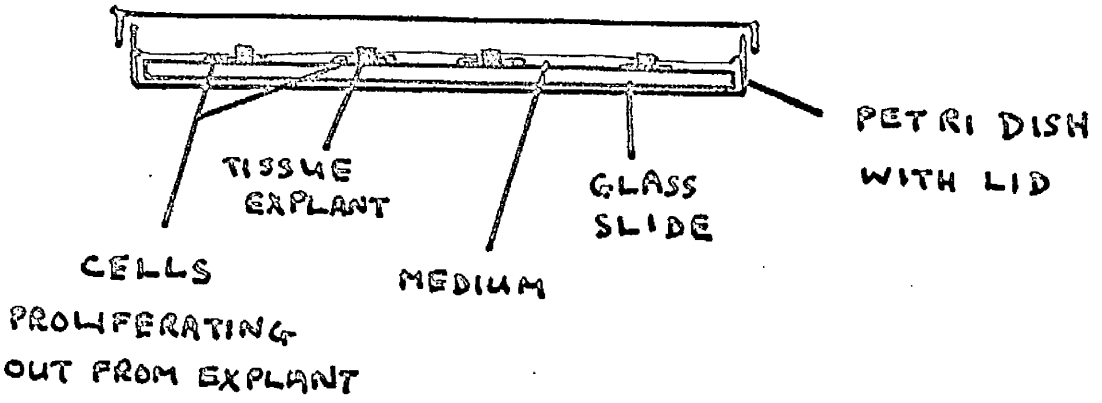
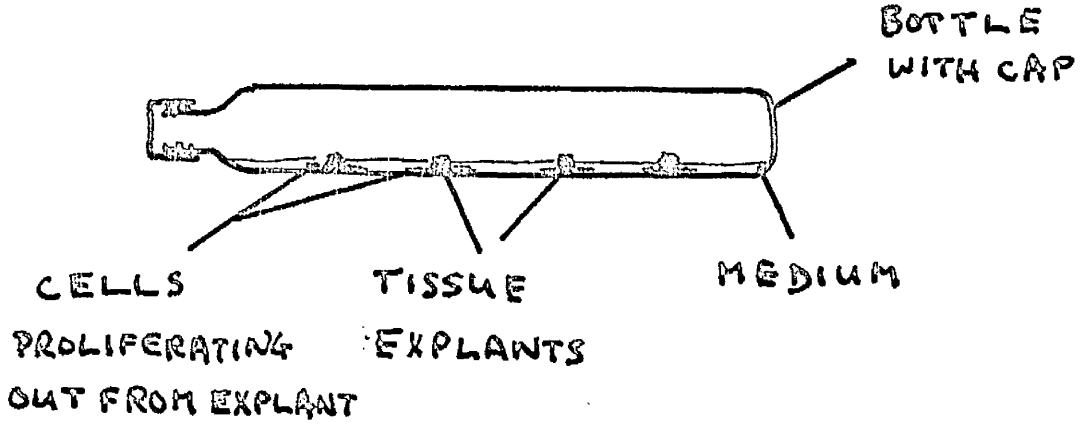


FIG. 11.

GROWTH OF CELLS FROM PRIMARY EXPLANTS IN BOTTLES (SIDE VIEW)



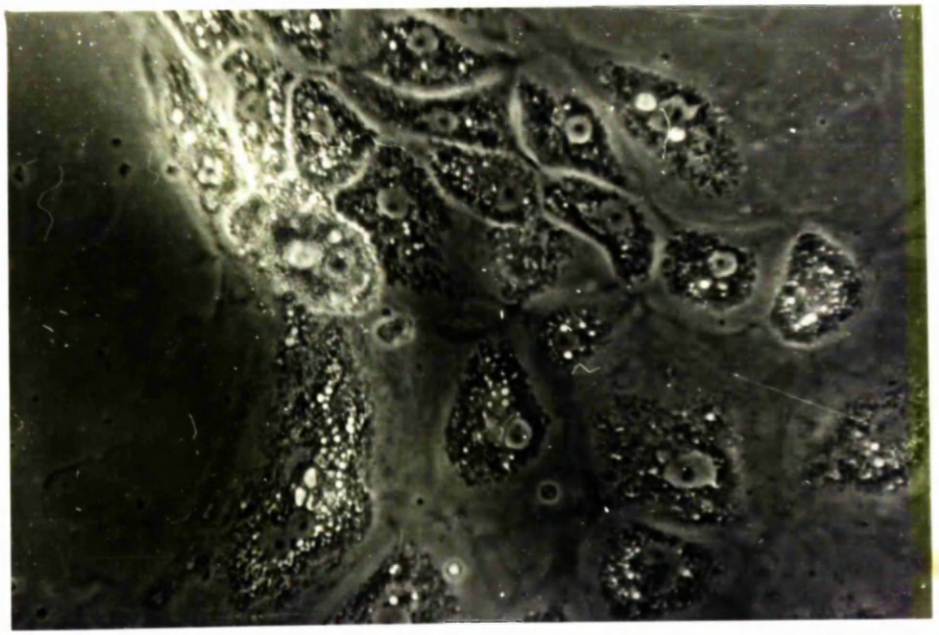
Results

Uterine tissue from 3 week old immature rats showed very little proliferation of cells from explants whether they were grown in bottles or in petri dishes. If the medium was supplemented with 15% foetal calf serum and with 1-2 ug/ml of insulin then some proliferation did occur. This proliferation was not noticeably increased by adding oestradiol to the medium. There was more proliferation if the animal received oestradiol in vivo before the uterus was removed and placed in culture. The maximum amount of proliferation did not exceed a distance of a cm. from the explant and the cells did not live for more than 8 or 9 days after which time they died and became detached from the glass.

Two main types of cell proliferated from the explants (fig. 12 page 41A)- (1) epithelial type-large, round cells forming sheets (2) fibroblastic type - long, thin cells migrating separately and which formed the major species. Attempts were made to increase the numbers of proliferating cells by using large round rotating bottles, but the explants would not stay attached to the glass walls of the bottle. The trypsinisation technique of forming primary cells for culture also proved unsuccessful. Therefore because of the small numbers of cells available qualitative

FIG. 12
Magnification 100x

Epithelial type



Fibroblastic type

autoradiography was the only best technique to use to study these cells.

2. Autoradiography

Methods

i) Incorporation of ^{3}H Thymidine into DNA

Cells were allowed to proliferate out from uterine explants in petri dishes as described above. The pieces of tissue explants were removed and fresh medium was added to the remaining cells. 5 uc of ^{3}H thymidine (4 c/mMole) were added to each dish. After 6 hr. incubation the medium was taken off, and the cells were washed with fresh medium and then 0.9% NaCl. 1-2 ml of NaCl were left in the dish and the cells were fixed by adding dropwise down the side of the petri dish absolute alcohol/glacial acetic acid (v/v 3/1). This was carried out until 100% fixative was present, and then the cells were left with fixative for 15-30 min. They were washed repeatedly with 5% TCA, then with fixative again and finally they were air dried.

The area of the slide containing the fixed cells was dipped into NTB3 liquid photographic emulsion (diluted 1 to 3 with H_2O) which was then allowed to dry. The cells were exposed to the emulsion for one week before they were developed. Finally they were stained with

ACKNOWLEDGEMENTS

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Most of all I would like to thank my wife Angela for her patience and understanding over the past three years.

I would finally like to thank Mr. D. Forrester for his technical assistance, and to acknowledge the receipt of a Science Research Council grant.

ABBREVIATIONS

These were as laid down in the Biochemical Journal instructions to Authors (revised, 1969) with the following additions:-

t RNA	transfer RNA
r RNA	ribosomal RNA
m RNA	messenger RNA
D-RNA	DNA-like RNA
TD-RNA	tenaciously bound D-RNA
SDS	sodium dodecyl sulphate
DES	Diethyl stilboestrol
MAK	Methylated albumin kieselguhr

haematoxylin, dehydrated and mounted in DPX or Canada Balsam.

ii) ^{3}H - Oestradiol -17B localisation in rat uterine cells

a) Purification of ^{3}H oestradiol -17B by thin layer chromatography (TLC)

High specific activity oestradiol -17B (58.7 c/m-mole) must be periodically purified from breakdown products. This was done by chromatography on TLC with a solvent system of 1 volume of ethyl acetate with 1 volume of cyclohexane. The standard oestradiol -17B spots were detected by viewing the plate under ultra violet light. The silica-gel (Kieselgel H F_{254}) fluoresces showing the oestradiol standards as black spots. The ^{3}H oestradiol was extracted from the gel with ethyl alcohol.

b) Treatment of cells

Cells were prepared in a similar way to those used in the ^{3}H thymidine autoradiography. They were incubated with 0.2 ug (15 uc) of purified 6,7 ^{3}H oestradiol 17B for 2 hr. They were then washed with fresh medium and 0.9% NaCl.

c) Fixation

i) Osmium Tetroxide

The cells were treated with 1% OsO_4 in 0.028 M. acetate-veronal buffer pH 7.4 for 30 min.

ii) Formalin

The cells were fixed with 10% (w/v)

formalin in 1.0 M phosphate buffer pH 7.4 for 5-10 min.

iii) Freeze drying

The cells were plunged into liquid propane at -180°C which was achieved by passing propane gas through liquid nitrogen. Lowering the temperature of the cells very quickly avoids the formation of large ice crystals which would damage them. Liquid propane is better than liquid nitrogen because the latter which boils at -196°C forms a layer of gas around the slide which insulates the cells from the most rapid cooling. Liquid propane at -180° does not form a gaseous layer because it does not become gaseous until a much higher temperature (-42°C) is reached.

The frozen cells were then placed under high vacuum overnight which causes dehydration and fixation due to the sublimation of the ice.

iv) Air drying fixation

Some cells were fixed by allowing them to dry in air.

d) Autoradiography

i) Wet method

This has already been described under the section on autoradiography with ^{3}H thymidine.

ii) Dry method

Loops of wire 6 cm in diameter were dipped into liquid photographic emulsion, and a thin film of emulsion formed within the loop (Prescott, 1964). When the film had dried (20 min.) it was stretched over the fixed ^{3}H oestradiol treated cells and exposed for periods up to 2 months. They were finally developed and stained as previously described.

3. Smears of immature rat uterine cells

This was another technique used to obtain uterine cells for autoradiography. The ^{3}H oestradiol was applied either locally to the uterus or injected intraperitoneally into the animal some hours before death. The uterus was removed and thoroughly washed by passing several ml. of 0.9% NaCl, by means of a syringe, through the lumen. The endometrium was exposed by inserting a long syringe needle into the lumen and prizing the organ open. The uterus was then laid on a glass slide with the endometrium uppermost. The surface cells were scraped off using a scalpel blade, and smeared over the rest of the slide. The cells were then air dried and dry autoradiography performed on them.

Results and General Discussion on the Experiments in Tissue Culture

The result of the thymidine autoradiographs of cells proliferating

from the uterine explants are shown in fig. 13 page 46A. There are grains over quite a large proportion of the nuclei showing that the cells are synthesising DNA. Not all the cells would be expected to show incorporation of thymidine into DNA; only cells in S phase of the cell cycle during the incubation would show grains.

Fig. 14 i & ii page 46A shows sheets of cells prepared by making a smear of endometrium. These are actually autoradiographs of uterine cells from a 3 week old immature rat which had received an intraperitoneal injection of ^{3}H oestradiol 1.25 hr. before killing. There are very few visible grains although the cells were exposed to photographic emulsion for 6 weeks. This may have been because only a small amount of oestrogen reached the uterus by this intraperitoneal route and some may also have been extracted with the fixing process. The cells were fixed with 10% (w/v) formalin which contained methanol (1% v/v). Fig. 15 i, ii & iii page 47A shows autoradiographs of smeared uterine cells from rats which received an intra-luminal application of ^{3}H oestradiol for a short period. The cells were air dried, and dry emulsion in the form of a film was applied. They were exposed for 6 weeks. There are many more grains present by using these methods. The nucleus is very large in these cells, but

Magnification 500x

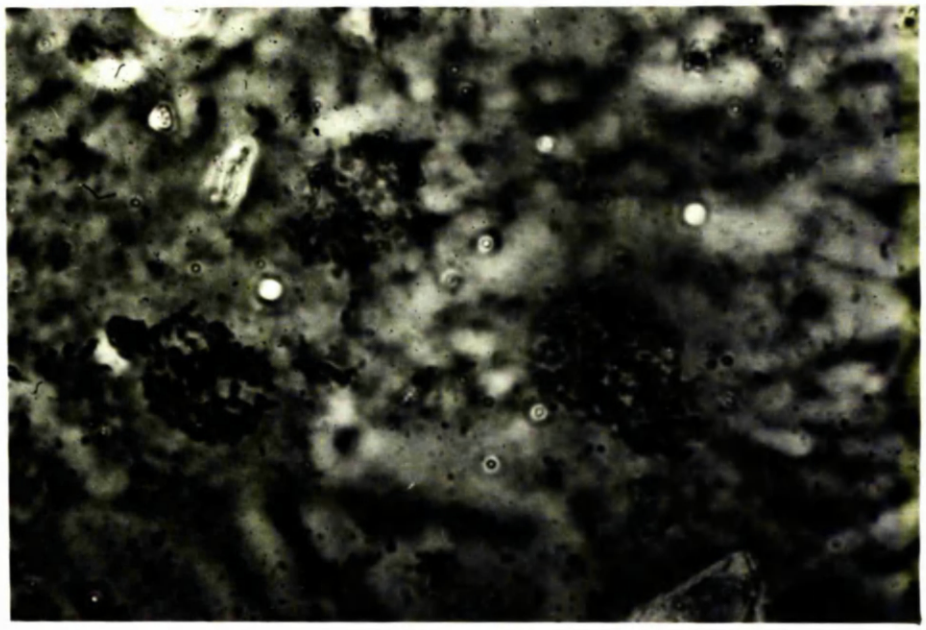


FIG. 13

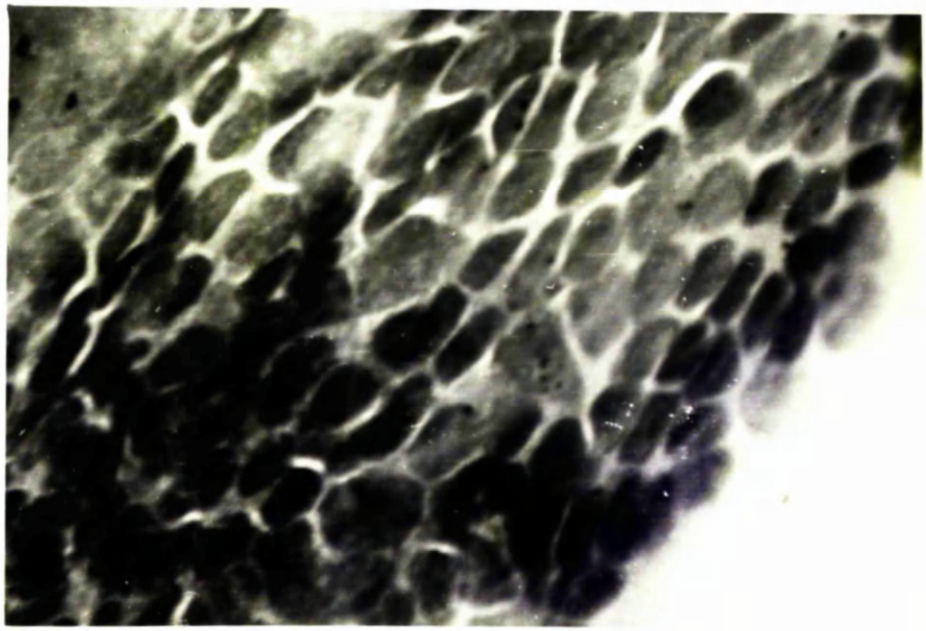


FIG. 14. I.

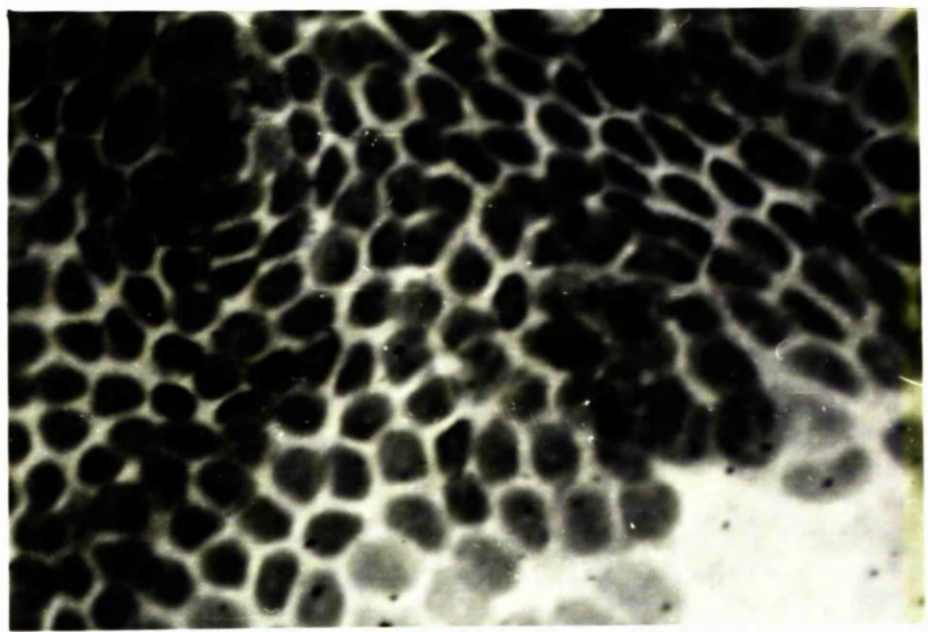


FIG. 14. II.

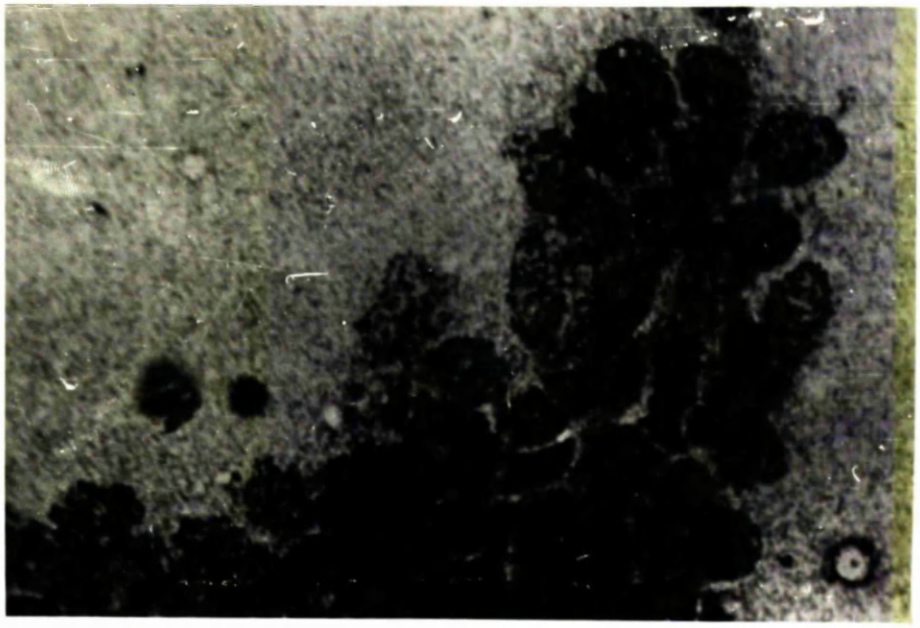
this is also the case with the cells in fig. 14. This is probably a feature of the cell type (columnar epithelium) and the smeared preparation of the cells. It seems from fig. 15 that most of the grains are in the nucleus. This would be in agreement with the binding studies of other workers in the field which were mentioned in the introduction. It should be mentioned however, that some results showed a general distribution of grains throughout the cell, but this could be due to dislocation due to washing.

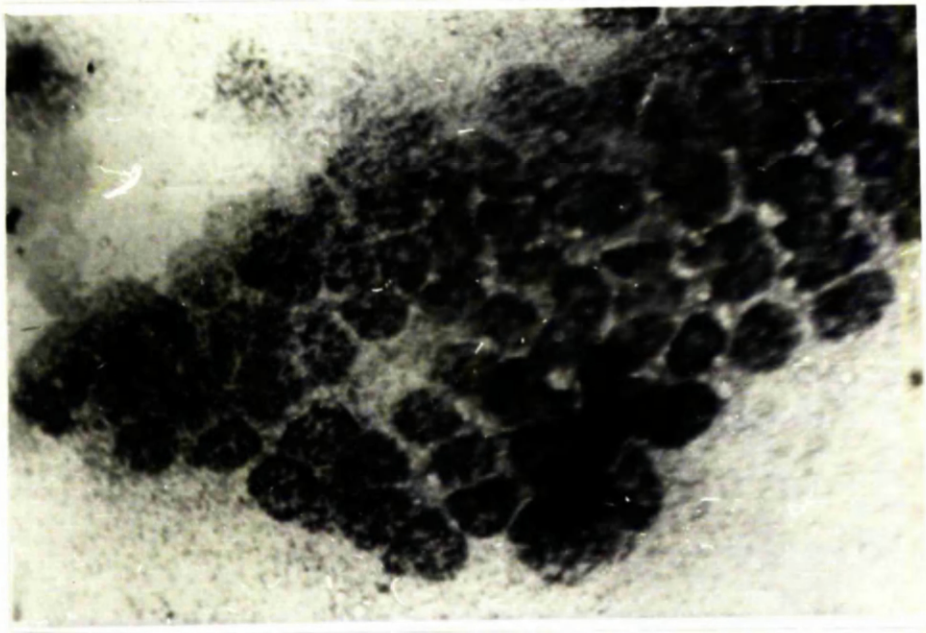
Fig. 16 page 47B shows the usual pattern of grain distribution obtained with autoradiographs of cells proliferating from explants of uterus in culture. The grains are widely distributed throughout the cell suggesting that the hormone may not be localised in any particular place within these cells. The explanation of this pattern could be the lack of a particular receptor in these cells.

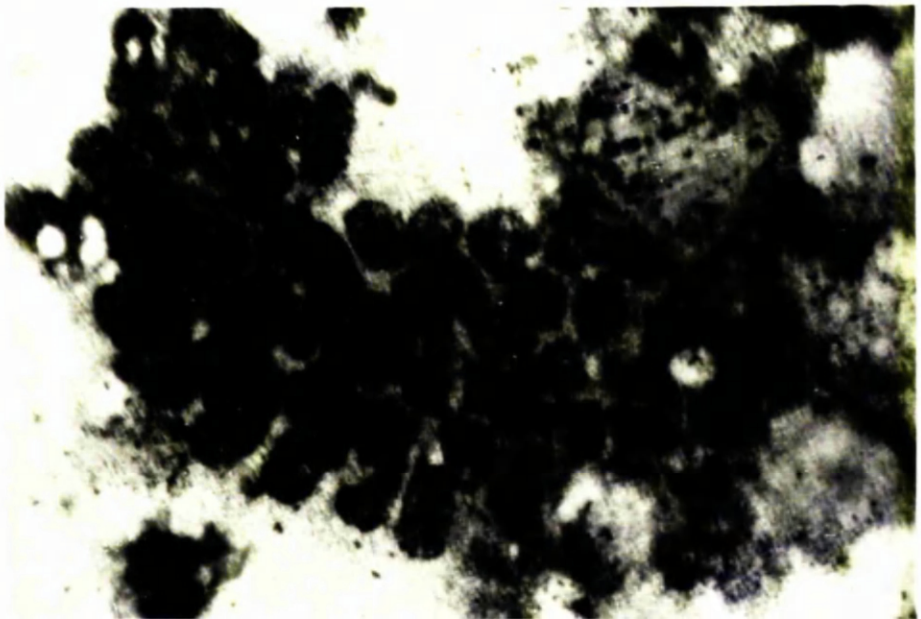
Many of the attempts to study the localisation of oestradiol by autoradiography failed to give results. The difficulties with localisation of oestradiol by autoradiography are great. Some of these difficulties arise in fixation and from the long exposure times which are necessary (Inman, Banfield and King, 1965). Other difficulties arise from the interpretation of the grain distribution. For example if there are grains over the cytoplasmic area of the cell it may not

FIG. 13

Magnification 500x





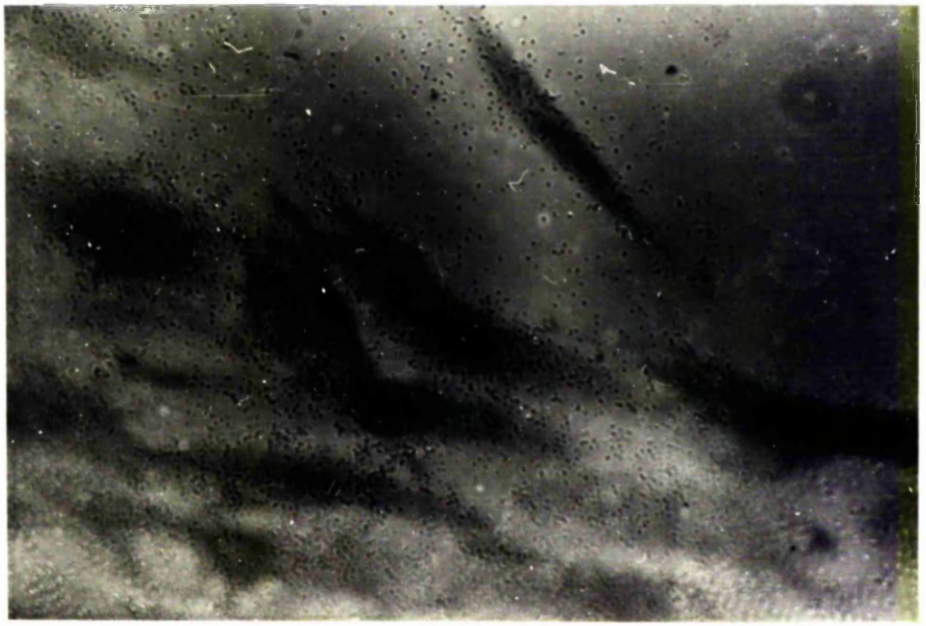


necessarily mean that this is a site of action of the hormone. These grains may be due to hormone in transit to the real site of action in the nucleus, or else they may be due to hormone which has been dissociated from the real receptor. It is difficult to avoid these artefacts completely. If the cells are washed too little then there are many "background" grains and if the cells are washed too much then the hormone may become dissociated from its receptor site. For these reasons good, reliable results for the localisation of oestradiol by autoradiography are very difficult to obtain. The best and probably most accurate studies on intracellular localisation of oestradiol have been obtained from fractionation of cells from uterus which has been treated with ^{3}H oestradiol. These results have been discussed in the introduction.

In summary it can be said that the study of the action of oestradiol on uterus in tissue or cell culture presents very many difficulties. The main problem, which is perhaps insurmountable, is the fact that the hormone does not seem to affect uterus in vitro. It may well be that the uterine cells have dedifferentiated and lost their response to oestrogens. When these cells are placed in a culture medium they are changing as they try to adapt to the new environmental conditions.

FIG. 16

Magnification 500x



Under these circumstances of continual change it is difficult to obtain consistant control cells with which to compare treated cells. For these reasons the study of oestradiol (and probably hormones in general) using presently available systems of primary cell culture of the target organ can only be of limited value.

PART II

THE EARLY EFFECTS OF OESTRADIOL ON RNA SYNTHESIS IN RAT UTERUS USING IN VIVO STUDIES ON WHOLE ANIMALS

INTRODUCTION

A review of recent work done in this field has been considered in some detail in the general introduction. In the following studies I have analysed the type of RNA synthesised under early hormone action, and I have looked again at the uptake of precursors into uterine RNA taking into account changes in the specific activity of the cellular precursor pool before assessing the extent of the changes in RNA synthesis.

1. The changes in the patterns of synthesis of RNA species in response to oestradiol 17β

The type of the RNA synthesised under early hormone action has been observed using sucrose density centrifugation (Mueller, 1965; Gorski and Nelson, 1965). It was found that apart from the increased amount of labelling in RNA from the hormone treated animals, the general pattern shown by this method does not change very much from the control pattern. One drawback of this method of RNA fractionation is that it is difficult to account for all the labelled RNA; some of it is dispersed throughout the sucrose gradient. In experiments described in this section the RNA synthesised under oestrogen treatment has been

fractionated on columns of kieselguhr coated with methylated bovine serum albumin (MAK columns). This method will fractionate all species of nucleic acid including the species of RNA called DNA like RNA (D-RNA) which is dispersed on sucrose density gradients.

D-RNA is so called because it has base composition corresponding very closely to that of DNA (40% G+C). MAK column chromatography has proved a useful method of separating D-RNA from other species of RNA (Ellem 1966; Ellem 1967). The D-RNA fractionated on MAK columns can be subdivided into two groups: one which elutes towards the end of a NaCl concentration gradient (called Q2 RNA) and the other which is tenaciously bound to the column after salt elution (called TD-RNA). The latter can be eluted from the column with (1) a solution of sodium dodecyl sulphate (SDS) at 35° and then at 70°, or (2) a solution of guanidine thio cyanate (Ellem and Rhodes, 1969). The function and the relationship to each other of these species of D-RNA is unknown. Some of their properties are known. Q2 RNA has a sedimentation value of 50 S (Yoshikawa - Fukada et al., 1965) which is much higher than that of TD-RNA (16-18 S) (Ellem, 1966). Both of these species are rapidly labelled and exhibit a rapid turnover. It has been suggested that one of them could be m-RNA. Recently it has been shown that TD RNA is confined to the nucleus of normal liver cells

(Billing, Inglis and Smellie, 1969). This suggests that Q2 RNA is more likely to contain m RNA than is the TD-RNA fraction.

Q1 RNA which is eluted from MAK columns with NaCl solution after the ribosomal peak but before the Q2 peak represents the 45 S ribosomal precursors (Muramatsu, Hodnett and Busch, 1966).

The method of extraction of RNA is important in that all the labelled RNA should be extracted and that it should not be degraded. The hot phenol - SDS method of Warner et al., (1966) with the use of freshly redistilled phenol and bentonite which prevents RNA degradation meets these requirements.

By using this method of RNA extraction and by using MAK chromatography which allows a recovery of over 96% of the RNA almost all the RNA synthesised under hormone action has been accounted for. If it is assumed that there is a common pool of precursors for the synthesis of all the species of RNA, then by using the above methods, one species can be compared to another with respect to changes in rate of synthesis. It was found that after oestradiol treatment there is increased labelling in t-RNA and r-RNA species compared to D-RNA species.

2. The transport of RNA precursors into the uterus and the synthesis of uterine RNA in response to oestrogen treatment

Experiments showing the increase in incorporation of labelled precursors into uterine RNA in response to oestrogen treatment have been discussed in some detail in the general introduction to this thesis. The main criticism of this work was that the results were taken to represent increases in RNA synthesis of this order without due consideration being taken of changes in the specific activity of the precursor pool. The purpose of the experiments I have done under this heading was (1) to measure the extent of the changes in specific activity of the ribonucleotide precursor pool (2) to see if these changes were a consequence of changes in RNA synthesis (3) to measure the real changes in RNA synthesis by taking into account changes in specific activity of the pool.

Preliminary experiments showed that oestradiol caused an early increase in the transport of labelled RNA precursors into the uterus which occurred before any large changes in RNA synthesis. To observe increases in incorporation of labelled precursors into RNA which were directly proportional to the rate of RNA synthesis, the specific activity of the precursor pool was stabilised by eliminating the effect of the hormone on the transport of labelled precursors into the uterus. The labelled precursor was injected into the animal 3 hr.

before the administration of the hormone. This resulted in the precursor being removed from the blood stream before the hormone was administered, and therefore its effect on the transport was eliminated. ^{3}H adenosine was chosen as the precursor because the endogenous adenine nucleotide pool was large and stable over the initial period of hormone action. This resulted in a constant specific activity of precursor during the length of the experiment.

The result of these experiments show that there is no increase in RNA synthesis in the rat uterus until after 5 hr. of hormone action. This is in agreement with the timing of increases in uterine RNA content as measured by chemical methods.

METHODS

1. Materials

5 - ^{3}H Uridine (24 c/m - mole), 2 - ^{14}C uridine (53 mc/m-mole), 8 - ^{3}H Guanosine (4.76 c/m - mole) G (n) - ^{3}H Cytidine (4.51 c/m-mole) and G (n) - ^{3}H Adenosine (2.34 c/m-mole) were purchased from the Radiochemical Centre, Amersham.

Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

Kieselguhr (Hyflo-super cell)

Highly polymerised yeast RNA for use as carrier was purchased from British Drug Houses Ltd., Poole, Dorset.

Dowex -1- Chloride AGl - X8 200-400 mesh anion exchange resin was obtained from Bio-Rad Laboratories.

Micro analysis Filter Holders were obtained from Millipore (U.K.) Ltd., Millipore membranes 0.45 μ and 0.22 μ (pore size) white, plain, 25 mm.

Actinomycin D was a gift from Merck, Sharp & Dohme Inc., New Jersey.

2. Biological Methods

Three-week-old immature female albino rats of the Wistar strain weighing 35-40g. were used in all experiments. The rats were killed by cervical dislocation: the uteri were removed cleanly without any mesentery and immediately placed into cold 0.9% NaCl. They were

washed with fresh cold 0.9% NaCl, dried with absorbent tissue and placed into clean universal containers for homogenising. In some experiments the uteri were weighed at this stage.

In all experiments 1 µg of oestradiol -17β was injected intraperitoneal in 0.1 ml 0.9% NaCl into the test rats in the form of the sodium salt as prepared by Roberts and Szego, 1947 using aqueous phenol red as the indicator. Control animals received 0.1 ml of 0.9% NaCl alone.

Radioactive precursors were administered in various volumes of 0.9% NaCl either intraperitoneally or intravenously via the external jugular vein. In most experiments radioactive precursors were injected 30 min. before death. In one particular group of experiments using ^{3}H adenosine alone the precursor was injected 3 hr. before the hormone.

3. Measurement of changes in the content of RNA, acid-soluble ribose, DNA and protein in immature rat uterus following oestrogen treatment

(a) Treatment of animals and uteri

At various times after the administration of oestrogen the animals were killed and the uteri removed and washed as described above. Individual uteri were homogenised in 4 ml. of cold distilled water at full speed for 1 min. in a Silverson Vortex homogeniser. 0.45 ml. 2M $HClO_4$ was added to the homogenate giving a final concentration of 0.2M and after standing in ice for 15 min. the mixture

was centrifuged at 600 xg for 5 min. The supernatant fluid was decanted into a clean tube and labelled the acid-soluble fraction. The sediment was suspended in 2.0 ml. of H_2O and 1.0 ml. of the suspension was taken for the measurement of protein. 1.0 ml. of 1M H Cl O_4 was added to the remaining 1 ml. of suspension and the mixture was heated to 70° in a water bath for 1 hr. After centrifuging at 600g for 5 min. the supernatant fluid was taken for the estimation of DNA and RNA.

(b) The determination of RNA and acid-soluble ribose

This was essentially the method of Kerr and Seraidarian (1945).

Reagents:

20% (W/v) orcinol in 95% ethanol (freshly prepared)

0.03% (W/v) Fe Cl_3 in conc. H Cl.

Standard RNA solution. 50 µg/ml. in 0.05N NaOH

0.2 ml. aliquot of the "hot H Cl O_4 extracts" or aliquots from the acid soluble fraction in 6 x $\frac{5}{8}$" test tubes and the volume made up to 3 ml. with water. 3.0 ml. of 0.03% Fe Cl_3 and 0.2 ml. 20% orcinol were added. They were mixed thoroughly and placed in a vigorously boiling water bath for 30 min. A blank and a set of standards were also prepared. Glass "dew-drops" were also used to prevent a loss

of volume. The tubes were cooled in an ice-water slurry, and then read on a spectrophotometer at 665 mμ. The calibration curve was also constructed and the RNA content of the extract determined.

(c) The determination of DNA (Burton; 1956)

Reagents:

Burton Diphenylamine Reagent. To 150 ml. glacial acetic acid (redistilled if necessary) add 1.5 ml. conc. H_2SO_4 and 1.5g of diphenylamine and store in the dark. On the day of use add 0.10 ml. of 1.6% ($^w/_v$) aqueous acetaldehyde to 20 ml. of reagent required.

1 ml. of the "hot H Cl O_4 extract" containing DNA was added to 2 ml. of Burton reagent. After mixing the tubes were covered and left to stand at 30° for 16-20 hr. A blank and a set of standards were similarly treated. The solutions were finally read on a spectrophotomet at 600 mμ.

(d) Determination of protein (Lowry, Rosenbrough, Farr and Randall, 1951)

Reagents:

13% ($^w/_v$) Na_2CO_3

2% ($^w/_v$) $CuSO_4 \cdot 5H_2O$

4% ($^w/_v$) Sodium Potassium Tartrate $4H_2O$

0.66N and 2N Na OH

Solution A (freshly prepared) 100 ml. 13% Na_2CO_3 3 ml. 4% Sodium Potassium Tartrate and 3 ml. 2% Cu SO_4. $5H_2O$ are added together in this order and mixed quickly.

Folin and Ciocalteau's Reagent (2N stored at 4°C).

Bovine serum albumin standard 150µg/ml.

The protein containing extract was made 0.66N with respect to NaOH and incubated at 30°C for 16-24 hr. with occassional shaking. A blank and a set of standards were similarly incubated. 0.5 and 1.5 ml aliquots of the protein digests were made up to 1.5 ml. with 0.66 NaOH. 1.5 ml. of Solution A was added and the solution mixed immediately. After exactly 10 min. 0.5 ml. of Folin - Ciocalteau reagent was added and mixed thoroughly and immediately. The mixture stood at room temperature for 30 min. and the extinction at 625 mµ then read.

4. Analysis of the type of uterine RNA synthesised under early hormone action as fractionated by MAK column chromatography

(a) Treatment of animals

Test animals were killed at ½, 1, 2 and 6 hr. after administration of 1 µg of oestradiol. They were each given an intraperitoneal injection of 25 µc ^3H uridine and 25 µc ^3H guanosine in 0.2 ml. 0.9% NaCl 30 min. before death.

(b) Extraction of RNA

The method of RNA extraction was essentially that of

Warner, Sociro, Birnboim, Girand and Darnell (1966) using hot phenol and SDS. The uteri from 14 rats were pooled and homogenised in 6 ml. of 0.05M ice-cold sodium acetate buffer pH 5.1 containing 0.01M EDTA and 0.66% w/v Bentonite with a Silverson Vortex homogeni The final volume of the uterine homogenate was 20 ml. which contained 0.2% (w/v) Bentonite and 1% (w/v) SDS. This was deproteinised with 20 ml. of 90% (v/v) redistilled phenol by shaking at 60° in a water bath for 5 min. (the final temperature of the emulsion was approximately 58°) The emulsion was broken into 3 layers by centrifuging at 600g for 3 min. The bottom (phenol) layer was discarded. 16 ml. of fresh 90% phenol was added to the remaining two layers and the mixture shaken again for 5 min. in a 60° water bath. The emulsion was again broken by centrifugation at 600g for 3 min. This time the upper (aqueous) layer was retained. This contained most of the RNA. 20 ml. of fresh buffer was added to the remaining interphase and phenol layer, and the process of shaking in a 60° water bath for 5 min. and centrifuging to break the emulsion were repeated. The upper (aqueous) layer was added to the other aqueous layer. The RNA was precipitated by (1) making the combined aqueous layers 2% with respect to sodium acetate (2) adding 2 volumes of cold ethanol (3) allowing the solution to stand for at least 30 min. at -20° (4) centrifuging at 1,360g for 30 min. at -5°.

The precipitate was dissolved in a small volume of buffer and extracted three times with ether to remove residual phenol. The recovery of RNA was always the same (40 E_{260}) to within 5%.

Notes on extraction of RNA

(i) The interphase material left at the end of the extraction which contained bentonite, was found to contain quite large amounts of acid-insoluble radioactivity (RNA). When bentonite was not present, there was not very much residual acid-insoluble radioactivity. An experiment was performed in which two similar groups of uteri labelled with RNA precursors were extracted for RNA in the same way except that one group had bentonite present from the beginning of the extraction. The amount of radioactivity extracted as RNA was similar for each group, but the group which were extracted in the presence of bentonite had much more acid-insoluble radioactivity in the final interphase material. This led to the conclusion that the bentonite was binding acid-soluble radioactivity which was precipitated with acid in the interphase. Most of the radioactivity in RNA in the uterus had been extracted by this method because very little acid-insoluble radioactivity remained at the end of the extraction either in the phenol layer or the interphase layer. Bentonite was added to all the preparations of RNA analysed on MAK columns because it is an inhibitor of ribonuclease and there is evidence

that it gives a better quality of RNA preparation.

(ii) In preparation of liver RNA by this method some of the labelled RNA enters into the phenol layer. This can be overcome by making the lower layer more dense by adding chloroform-isoamyl alcohol to the emulsion (Penman, 1968)

(iii) Preparation of Bentonite

(1) 20g of Bentonite were suspended in 400 mls. of distilled water and centrifuged at 600g for 15 min. (2) The supernatant was then centrifuged at 8,500g for 20 min. (3) The sediment from this centrifugation was resuspended in 0.1M EDTA pH 7.0 and left for 40 hr. (4) This was centrifuged as in (1) and (2). (5) The sediment from the 8,500g centrifugation was suspended in 0.1M sodium acetate buffer pH 6.0 and centrifuged at 8,500g for 20 min. (6) This final sediment was suspended to a known concentration (1.5 - 6.0%) in the sodium acetate buffer. The concentration was measured by evaporating to dryness a known volume and measuring the weight of the residue.

(c) Chromatography on MAK columns

(i) Preparation of the column

This was essentially the method of Mandell and Hershey (1960).

(a) Preparation of the methylated albumin.

5g of bovine serum albumin (fraction V from bovine plasma - Armour Pharmaceuticals) were suspended in 500 ml. of absolute methanol (Analar) and 4.2 ml. of 12N HCl was added. The albumin dissolves, but eventually reprecipitates. The solution was allowed to stand in the dark for 3-5 days (longer the time the more basic the product) with intermittent shaking. The precipitate was collected by centrifugation at 5,000g. The residual acid was removed by washing twice with absolute methanol, then with absolute methanol containing a slight amount of ammonia (S. G. 0.880) and then with absolute methanol again. The precipitate was finally washed twice with ether to remove any water. The residual ether was removed by evaporation in air and then in vacuo over KOH. The methylated albumin was ground into a powder and stored at -20°C.

(b) Preparation of MAK stock solution for the middle layer of the column

10 ml. of a 1% solution of methylated albumin in distilled water was made. 40g of kieselguhr was boiled in 200 ml. of 0.1M NaCl in 0.2M phosphate buffer pH 6.7 to remove the air. The 10 ml. of 1% solution of methylated albumin were added dropwise with stirring to the cooled boiled suspension of kieselguhr. 10-20 ml. portions of

this MAK suspension were applied to a glass column 2-3 cm in diameter and they were washed with 200-300 ml. of .4M NaCl under 3 lb. per sq. in. pressure. The washed MAK was suspended and could be stored for weeks at 0° in 200 ml. 0.4M NaCl in 0.2M phosphate buffer pH 6.7.

(c) Preparation of the 3 layer MAK column

The following suspensions were boiled and cooled in three separate beakers.

(1) 8g kieselguhr in 40 ml. of 0.1M NaCl

(2) 6g kieselguhr in 40 ml. of 0.4M NaCl

(3) 1g kieselguhr in 10 ml. of 0.4M NaCl

All NaCl solutions are made in 0.2M phosphate buffer pH 6.7.

2 ml. of 1% solution of methylated albumin was added dropwise with stirring to 8g of kieselguhr in 40 ml. of 0.1M NaCl in the first beaker. This MAK was applied to the column (2-3 cm diameter) which had a small layer of acid washed pure sand to prevent clogging of the sinter. This first layer was allowed to settle and it was washed with 100 ml. of 0.1M NaCl solution. The 2nd layer which consisted of pure kieselguhr mixed with MAK was made by adding dropwise with stirring 10 ml. of MAK stock solution to the suspension of 6g of kieselguhr in the second beaker. This layer was not prepared in advance because

transfer of the methylated albumin may occur. This layer was added to the column by means of a large pipette and it was allowed to settle. The suspension of 1g of kieselguhr in the third beaker was then added to the column to make the third layer. The complete column of three layers was washed with 150 ml. of 0.4M NaCl. This column has a capacity of 70 E 260 units of RNA.

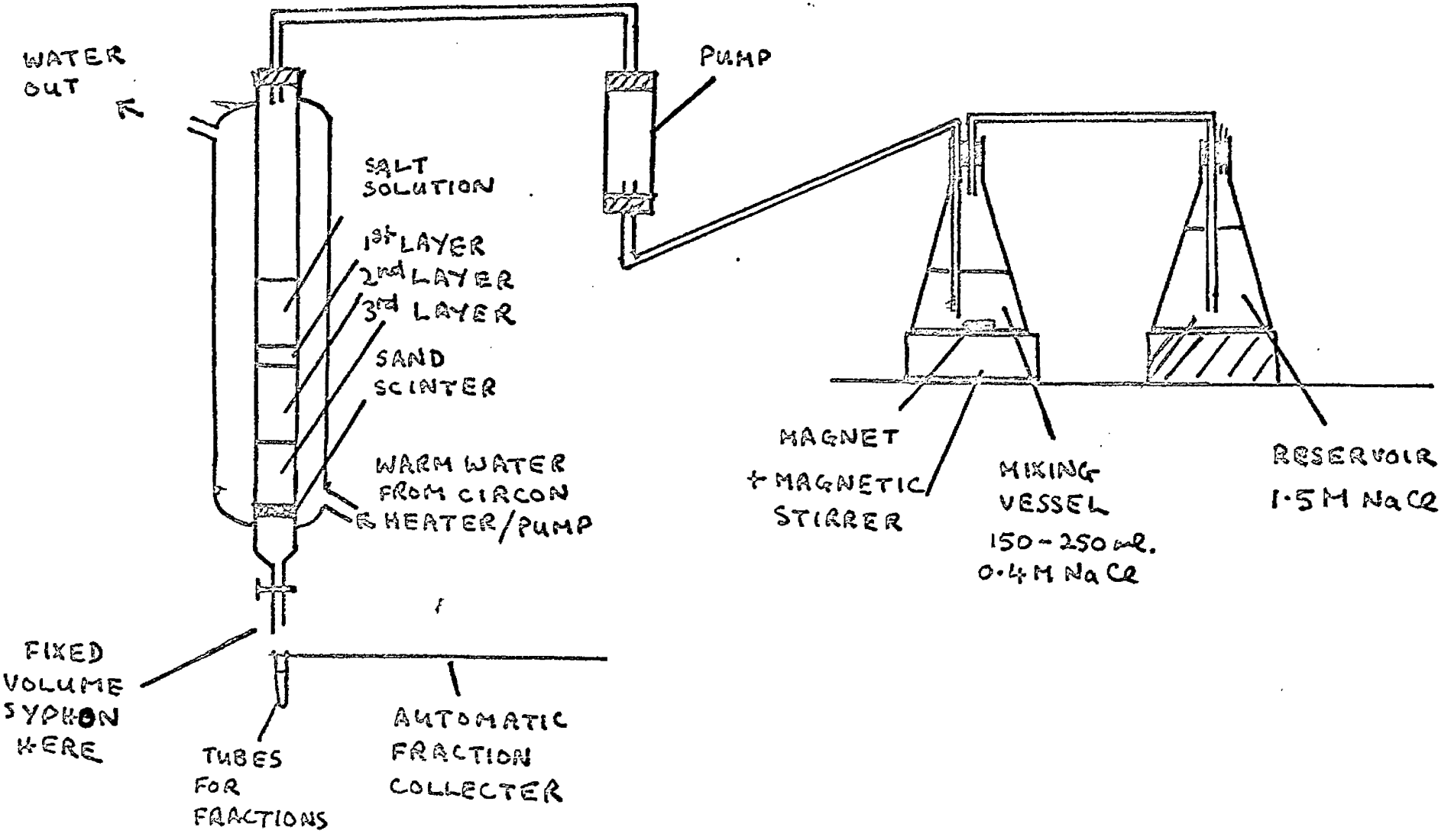
A column of internal diameter of 1.5 cm was used for most separations of uterine RNA. It consisted of 5g of kieselguhr and 1.2 ml. of 1% methylated albumin solution for the first layer and 3.4g of kieselguhr with 5.5 ml of MAK stock for the second layer. 40 E 260 units were applied.

(ii) Application and elution of the RNA

The RNA from 14 uteri which usually amounted to 40 E 260 units was applied (under pressure) to the column in 100 ml. of 0.4M NaCl (10-20 µg RNA per ml.) is recommended. The column plus RNA was washed with 0.4M NaCl until no further $OD_{260\ m\mu}$ was present in the washings (70 ml.).

Fig. 17 page 65A shows the apparatus for elution from the column. The RNA was eluted with a linear salt gradient from 0.4M NaCl to 1.5M NaCl at 35° with a flow rate of 60 ml/hr. 1 ml. fractions were collected and placed in ice. The E 260 was read immediately.

FIG. 17 APPARATUS FOR MAK COLUMN CHROMATOGRAPHY



Adjacent fractions were pooled in pairs, 50 µg of RNA carrier was added and the solution was made 10% with respect to trichloracetic acid. The RNA was collected on Millipore filter membranes (0.22µ pore size). The membranes were placed into scintillation vials and dried at 50° in an oven. 10 ml. of toluene based scintillator were added and the radioactivity was counted in a liquid scintillation counter.

After the salt elution was completed the tenaciously bound RNA was eluted with a solution of 0.2% SDS in 0.4 M NaCl first at 35° and then at 80°. 10 ml. fractions were collected and the radioactivity of the RNA was measured in a similar way to that employed for the salt eluted fractions. The method of elution of the column was taken from Yoshikawa - Fukada, Fukada and Kawadi (1965) Muramatsu, Hodnett and Busch (1966) and Ellem (1966).

5. <u>Dual labelling experiments to show the change in pattern of RNA synthesis in uteri from control and hormone treated animals.</u>

One group of six rats received 1 µg of oestradiol in 0.1 ml. of 0.9% NaCl intraperitoneally 90 min. before they were killed; the controls received 0.1 ml. of 0.9% NaCl. At the same time (again 90 min. before death) the control group were each given intraperitoneally 100 µc of ^{3}H uridine (212 mc/m-mole) and the hormone-treated animals received 25 µc of ^{14}C uridine (53 mc/m-mole).

The uteri from all 12 animals were pooled for the extraction of RNA

by the same method as described above and the RNA was fractionated by MAK column chromatography.

This method eliminated any differences in preparation between the control and the hormone-treated rat uterine RNA. RNA from the controls were distinguished from the RNA of the hormone treated rats by their different isotope labels.

6. Dual labelling technique to estimate the base ratios of the various RNA species fractionated by MAK column chromatography

Five 3 week old female rats were each given 1 μg of oestradiol -17β intraperitoneally $3\frac{1}{4}$ hr. before they were killed. After killing the uteri were removed and washed in NCTC medium 109, and placed in a 25 ml. conical flask containing 2 ml. of the same medium + 0.2 ml. (10 μc) ^{14}C uridine + 5 μl (5 μc) ^{3}H guanosine. The flask was gassed with a mixture O_2: CO_2, 95:5, sealed and shaken in a 37° water bath for 45 min. The uteri were removed, placed in ice cold saline, washed several times with fresh saline and finally blotted dry with absorbent tissue. RNA was extracted and fractionated by MAK chromatography by the methods described above. The ratio of $^{3}H/^{14}C$ is proportional to the ratio of guanosine/uridine. This method gives an indication of the base ratios of the various RNA species fractionated by MAK chromatography.

7. Experiments to show the changes in uptake of tritiated nucleosides into acid-soluble and acid-insoluble fractions of rat uterus following oestradiol administration.

(a) Biological methods

Test rats were killed at various times after administration of 1 μg of oestradiol. Control rats received the vehicle only, 0.1 ml. of 0.9% NaCl. Each animal received an intravenous injection of 12.5 μc of each of the tritiated ribonucleosides adenosine, guanosine, uridine and cytosine 30 min. before death.

In another series of experiments each animal received an intraperitoneal injection of 25 μc each of ^{3}H uridine and ^{3}H guanosin These two RNA precursors are not directly involved in terminal turnover of t-RNA.

In some experiments changes in the transport of RNA precursors into the uterus were investigated when RNA synthesis was inhibited by actinomycin D. 150 μg of actinomycin D were injected intraperitoneally 30 min. before the administration of hormone (or vehicle dose) and 2.5 hr. before death.

(b) Determination of radioactivity in acid-soluble and acid-insoluble fractions

Individual uteri were homogenised in 5 ml. of cold distilled water at full speed for 1 min. in a Silverson Vortex homogeniser. The homogeniser was washed with a further 2 ml. of water and the washings

added to the initial homogenate. Cold 50% (w/v) trichloroacetic acid was added to the homogenate plus washings to give a final concentration of 5%. After standing in ice for 10 min. the mixture was centrifuged at 1000g for 5 min. The resulting precipitate was washed with 2 ml. of 5% (w/v) trichloroacetic acid to give the acid-insoluble fraction. The supernatant plus washings were combined to give the acid-soluble fraction. The radioactivity in these fractions was measured as described below.

8. Ion-exchange chromatography of the acid-soluble fraction

Uteri from 12 to 14 rats were pooled and homogenised in 6 ml. of cold water as described above. 12M H Cl O_4 was added to the homogenate to give a final concentration of 0.4N. After standing in ice for 15 min. the mixture was centrifuged at 1,000g for 15 min. and the supernatant fluid was decanted and kept. The sediment was washed with 4 ml. of 0.3M H Cl O_4 and the washings added to the first supernatar to give the acid-soluble fraction. The radioactivity in the acid-insoluble residue was determined as described below.

The acid-soluble fraction was neutralised with cold 10N KOH and the precipitate of K Cl O_4 was separated by centrifugation.

The E_{260} of the final acid-soluble fraction was measured and samples were taken for measurement of radioactivity. It was then

applied to a column of anion-exchange resin (10 cm x 1 cm) (Cohn, 1955) of Dowex 1 x 8, chloride form, 200-400 mesh. The column was washed with water to remove the nucleosides and other unabsorbed material. The total free nucleotides were eluted with 1N HCl as one complete fraction. The radioactivity and the E_{260} of the washing and the eluant were measured.

9. Measurement of the extent of changes in RNA synthesis in uterus under the action of oestrogen.

RNA synthesis was measured by the amount of incorporation of radioactive precursors into RNA, but the effect of the hormone on the transport of labelled precursors into the uterus was abolished by administering the ^{3}H adenosine 3 hr. before the hormone.

50 μc of ^{3}H adenosine was injected intraperitoneally into each rat. Three hours later the test rats received 1 μg of oestradiol in 0.1 ml. 0.9% NaCl and the control rats received 0.1 ml. 0.9% NaCl only. The rats were killed at 1 hr. intervals after the initial injection. The acid-soluble and acid-insoluble fractions of each uterus were obtained, and the amount of radioactivity in each fraction was measured as described below.

10. Measurement of radioactivity

(1) Acid-insoluble radioactivity

(a) Amounts less than 100 μg.

Small amounts of acid-insoluble material such as

that in fractions from MAK chromatography were collected on millipore membrane filters of either 0.22 μ or 0.45 μ pore size. The material was usually added to the membrane in 5% trichloroacetic acid and then washed with a further 5 ml. of 5% trichloroacetic acid. The membranes were placed into scintillation vials and dried in a 50° oven for 30 min. Toluene-based scintillator was added and the radioactivity was counted on a liquid scintillation analyser.

(b) Amounts of acid-insoluble material greater than 100 μg

Although measuring acid-insoluble radioactivity on millipore membranes was found to be the quickest and most efficient method large amounts of material will block these membranes. The acid-insoluble material from whole uteri came into this category, and in this case the method using filter-aid was employed (Lieberman, Abrams and Ove, 1963). 2.0 ml. of a suspension of 2% (W/v) kieselguhr (hyflo-supercel) in 5% (W/v) trichloroacetic acid were (1) added to the acid-insoluble material and (2) added to a millipore filter unit containing a 2.5 cm diameter Whatman No. 1 filter paper disc. A thin layer of kieselguhr was formed on the filter paper which made a pad onto which the acid-insoluble material could be layered. The acid-insoluble precipitate was washed on the pad with (1) 3 x 15 ml. of 5% (W/v) trichloroacetic acid (2) 15 ml. of absolute alcohol and (3) 2 x 5 ml. of

ether. The pad of kieselguhr with the acid-insoluble material was scraped into a scintillation vial. 0.5 ml. of 1.0M hyamine hydroxide was added, and the vial incubated at 60° for 10 min. or 37° for 30 min. This solublised the radioactivity. 10 ml. of toluene-based scintillator was added and after allowing the toluene-hyamine to dark adapt for at least 3 hr. the radioactivity was counted in a liquid scintillation analyser.

(ii) Acid-soluble radioactivity

If the solution was neutral and it did not contain very much salt then up to 1 ml. could be counted in 10 ml. of dioxane-based scintillator. If the solution was acid less than 0.5 ml. was added to 10 ml. of dioxane-based scintillator. In some cases up to 0.3 ml. of solution can be counted in 8 ml. of toluene-based scintillator if 2.0 ml. of absolute alcohol are also added. (Usually toluene scintillator can only be used with non-aqueous samples).

(iii) Scintillation Fluids

(a) Toluene-based

100 ml. of toluene (Analar)

0.5g 2,5 diphenyl-oxazole (PPO)

0.03g 1,4 bis (5-phenyloxazole-2-yl) benzene (POPOP)

(b) Dioxane-based

This scintillator is used with aqueous samples. It does not give as high efficiencies of counting as toluene-based scintillator. Dioxane must be gassed with nitrogen in order to keep it free from oxygen with which it forms quenching compounds (peroxides).

100 ml. Dioxane

0.70g PPO

0.03g POPOP

10.00g Napthalene (scintillation grade)

(c) Bray's solution (Bray, 1960)

This scintillation fluid is a mixture of PPO, POPOP and naphthalene in a dioxane-methanol-ethylene glycol mixture. It has some advantages over Dioxane-based scintillator in that it does not freeze at low temperatures and it hold considerable quantities of water and salt.

RESULTS

1. Changes in the composition of the major constituents of immature rat uterus following oestrogen treatment

Fig. 18 page 74A shows the changes that occur in the RNA, DNA and protein content of immature rat uterus following an intraperitoneal injection of 1 μg. of oestradiol-17β. There is no detectable change in any of these parameters over the first 7 hr. after administration of the hormone. The RNA content is the first to change. It begins to rise rapidly 7 hr. after administration of oestradiol. The protein content does not begin to increase until 14 hr. and the DNA until 28 hr. after hormone administration, but thereafter they both increase rapidly for the duration of the experiment. (32 hr.)

Fig. 19 page 74B shows the changes that occur in the RNA, acid-soluble ribose, wet weight, DNA and protein of immature rat uterus following treatment with 5 μg. of diethylstiboestrol. The results are similar to those shown in fig. 18. It should be noted that the first time taken for all the parameters in fig. 18 is 7 hr. after hormone administration. Only the RNA content and wet weight show significant increases at this time. They both continue to increase for 40 hr. then decline, although the overall change in RNA content is relatively greater than the change in wet weight. Acid-soluble ribose begins to increase

Fig. 18. Changes in the RNA, DNA and protein content of immature rat uterus following oestradiol-17β (1 μg) administration. Data are expressed as percentage of control values and represent the mean of at least four experiments.

FIG. 18

CHANGES IN THE AMOUNT OF RNA, DNA AND PROTEIN IN IMMAT
RAT UTERUS FOLLOWING OESTRADIOL-17β ADMINISTRATION

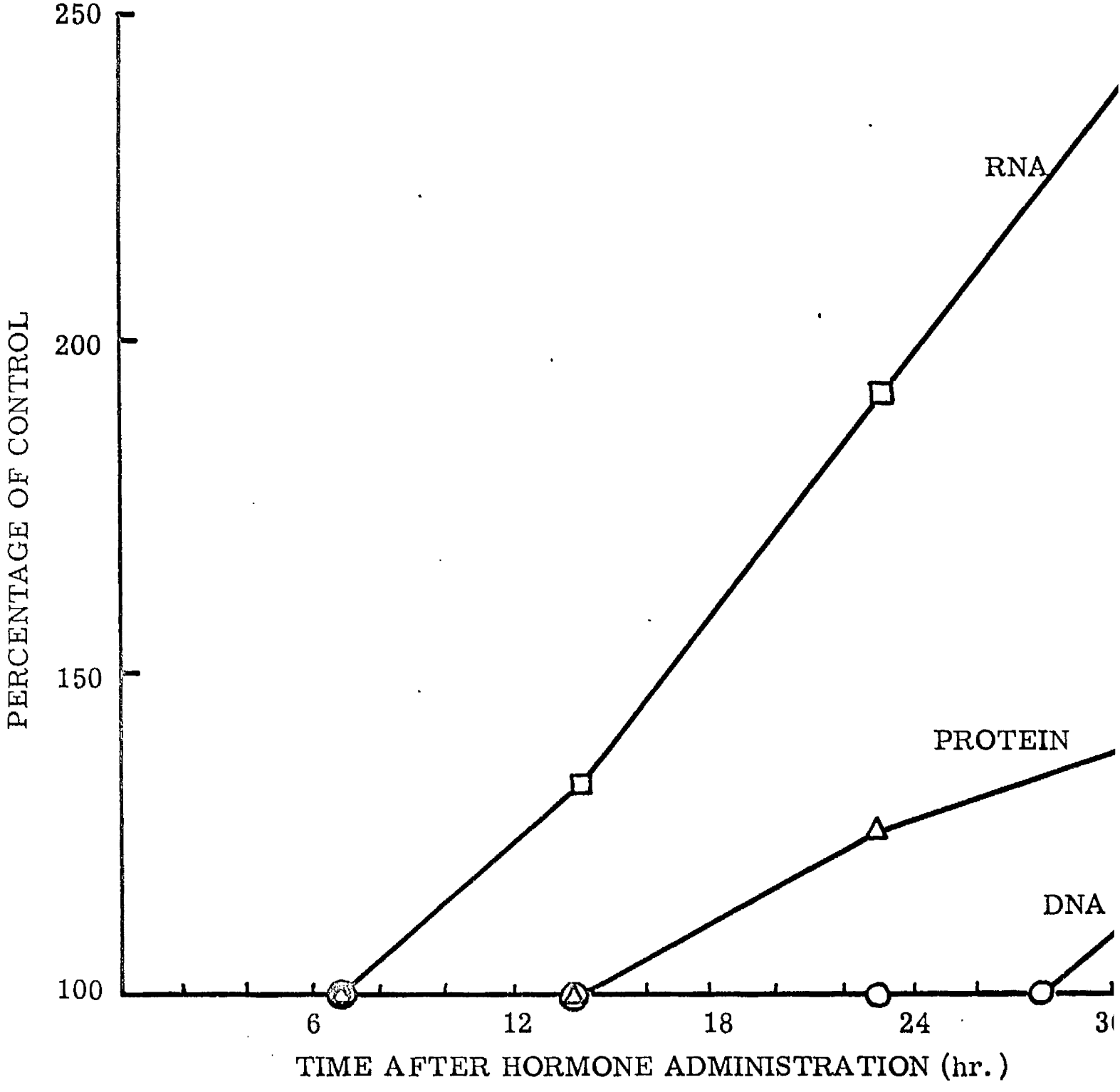


Fig. 19. Changes in the composition of immature rat uterus following oestrogen treatment. 5 μg of diethyl-stilboestrol were injected intraperitoneally at various times before killing. Data are expressed as percentage of control values.

———— RNA

—— —— Wet Weight

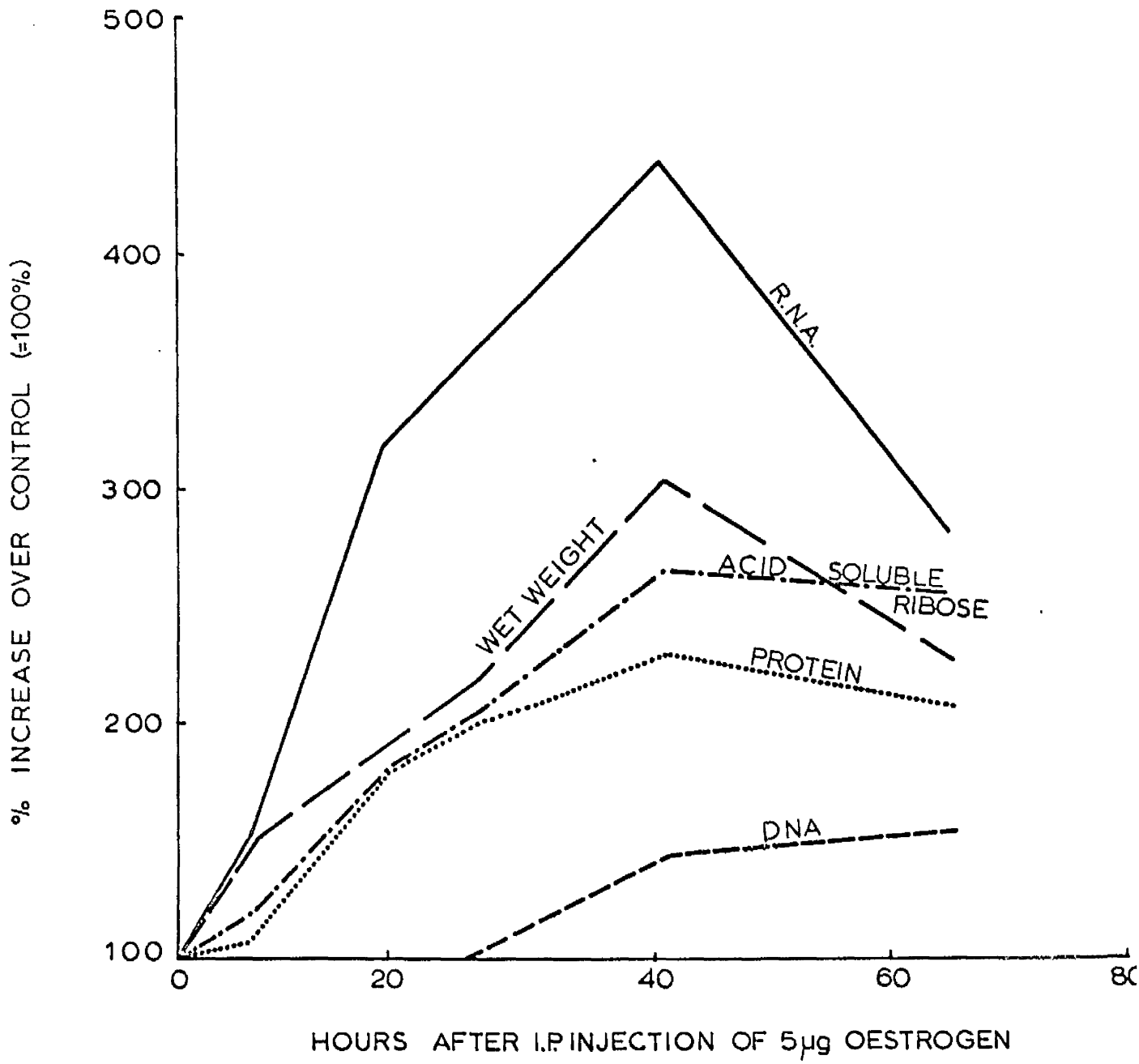
— . — Acid-soluble Ribose

. . . . . Protein

- - - DNA

FIG. 19

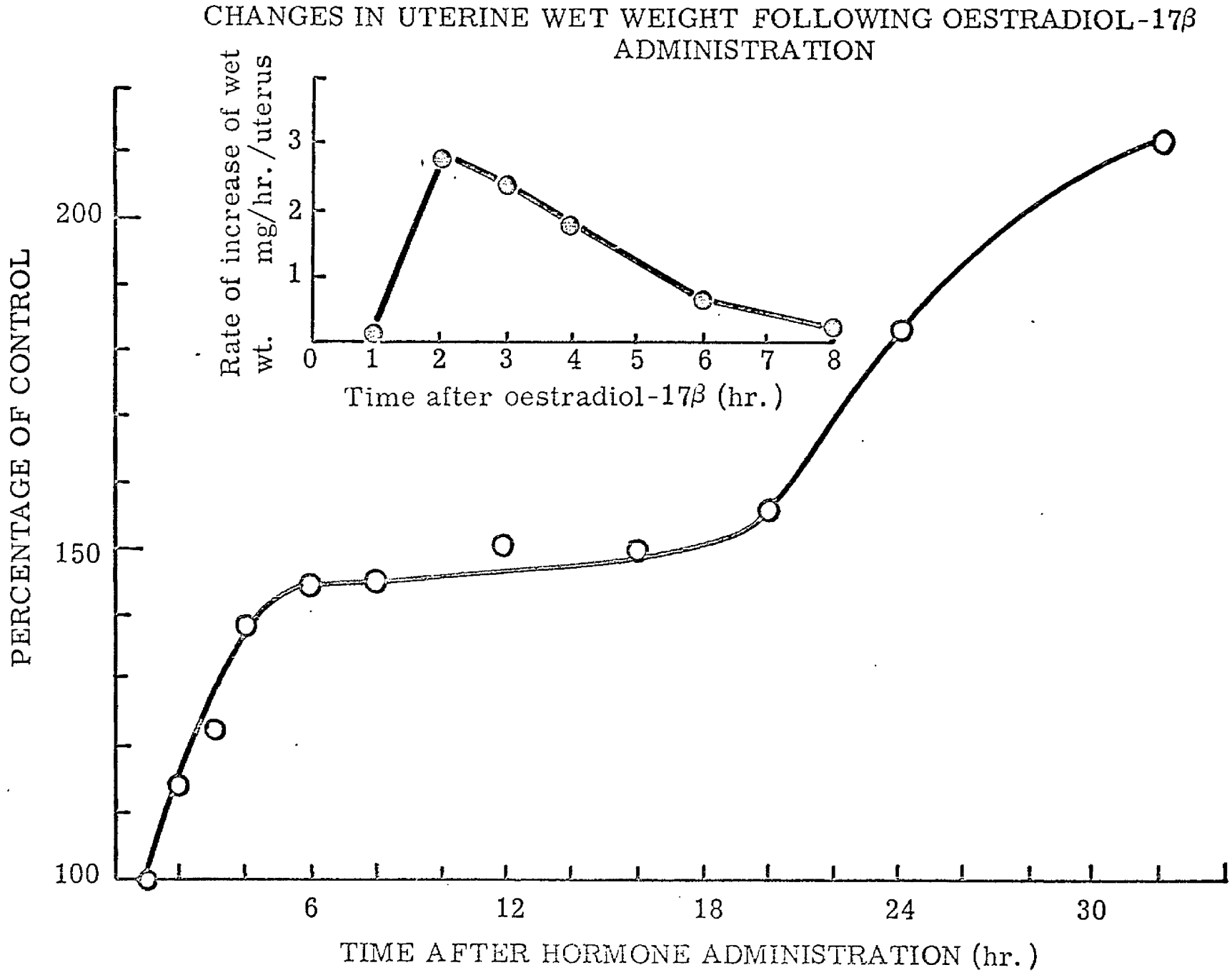
CHANGES IN THE COMPOSITION OF IMMATURE RAT UTERUS FOLLOWING OESTROGEN TREATMENT



rather more slowly than RNA and wet weight and reaches a plateau at about 40 hr. The protein content shows no significant increase at 7 hr. but it has risen to 180% of the control value at 20 hr. and continues to increase until it reaches a maximum at about 40 hr. DNA content shows no increase at 24 hr. but has increased to a level of 150% of the control at 40 hr. and 72 hr.

Fig. 20 page 75A shows in detail the changes in uterine wet weight following the administration of 1 μg of oestradiol-17β. The wet weight begins to rise 2 hr. after oestradiol administration reaching a level of 45% over the control value at 6 hr. It remains at this level for several hours, but begins to rise again at 20 hr., and continues to rise for the remainder of the experiment (32 hr.). The inset of fig. 20 shows the rate of increase in wet weight over the early period of hormone action. It is known that all of this early increase in wet weight is caused by increases in water content of the uterus (80% of the uterus is water) (Astwood, 1938). The pattern for the rate of increase in wet weight (or water uptake) is very similar to the rate of uptake of ribonucleosides into the uterus which will be described later. There is a rapid increase between $1\frac{1}{2}$ and 2 hr. which reaches a maximum at 2 hr. and then declines reaching a minimum at about 8 hr.

Fig. 20. Changes in wet weight of immature rat uterus following oestradiol-17β administration. Data are expressed as percentages of control values and represent the average of at least five experiments. Average wet weight of control uteri was 18.5 mg. The inset shows the rate of change in wet weight during the first 8 hours after oestradiol administration.


CHANGES IN UTERINE WET WEIGHT FOLLOWING OESTRADIOL-17β ADMINISTRATION
PERCENTAGE OF CONTROL
100
150
200
6
12
18
24
30
TIME AFTER HORMONE ADMINISTRATION (hr.)
Rate of increase of wet wt. mg/hr./uterus
1
2
3
0
1
2
3
4
5
6
7
8
Time after oestradiol-17β (hr.)

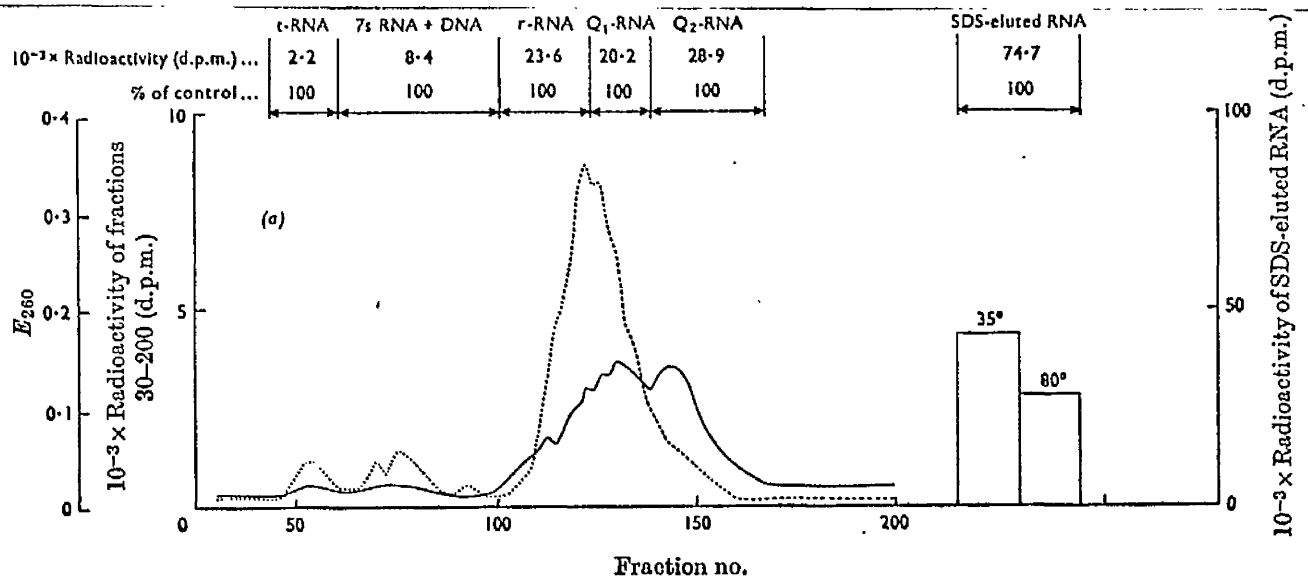
2. Changes in the patterns of synthesis of RNA in immature rat uterus in response to oestradiol-17β

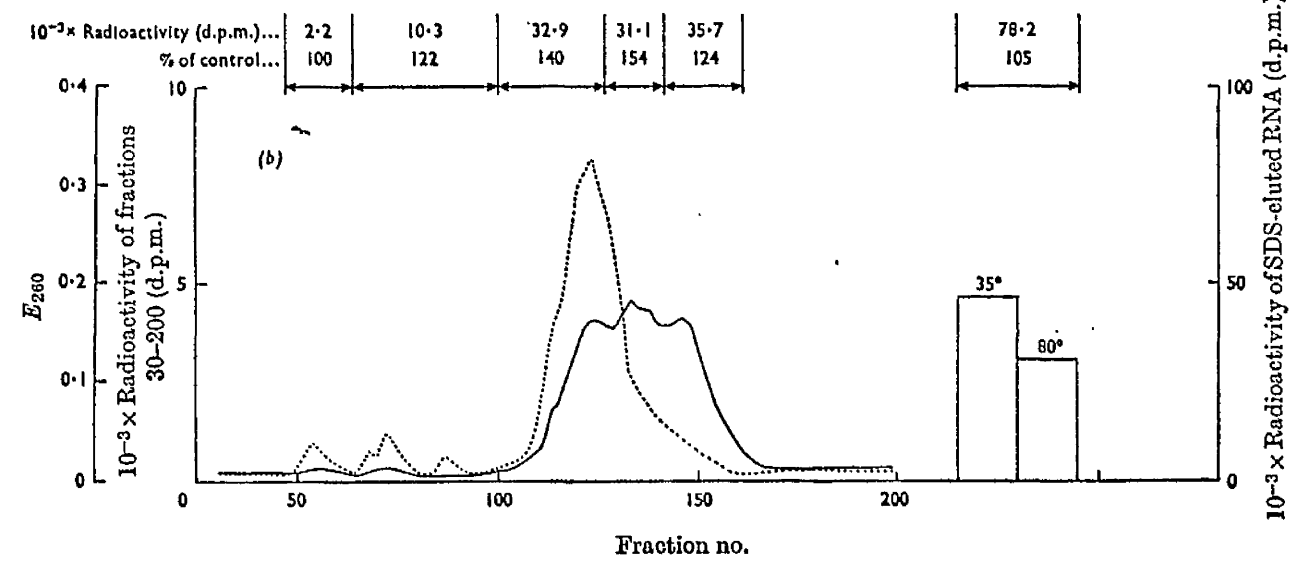
Fig. 21 pages 76 A and B shows the elution patterns obtained when RNA from control and oestradiol-treated rat uteri were fractionated on columns of kieselguhr coated with methylated albumin (MAK). The control pattern is shown in Fig. 21(a) page 76A and the patterns obtained from animals given oestradiol 30, 60, 120 and 360 min. before being killed are shown in Fig. 21(b), 21(c), 21(d) and 21(e) respectively (these are shown on pages 76A and 76B). No major changes in the E_{260} values were observed at the various time intervals.

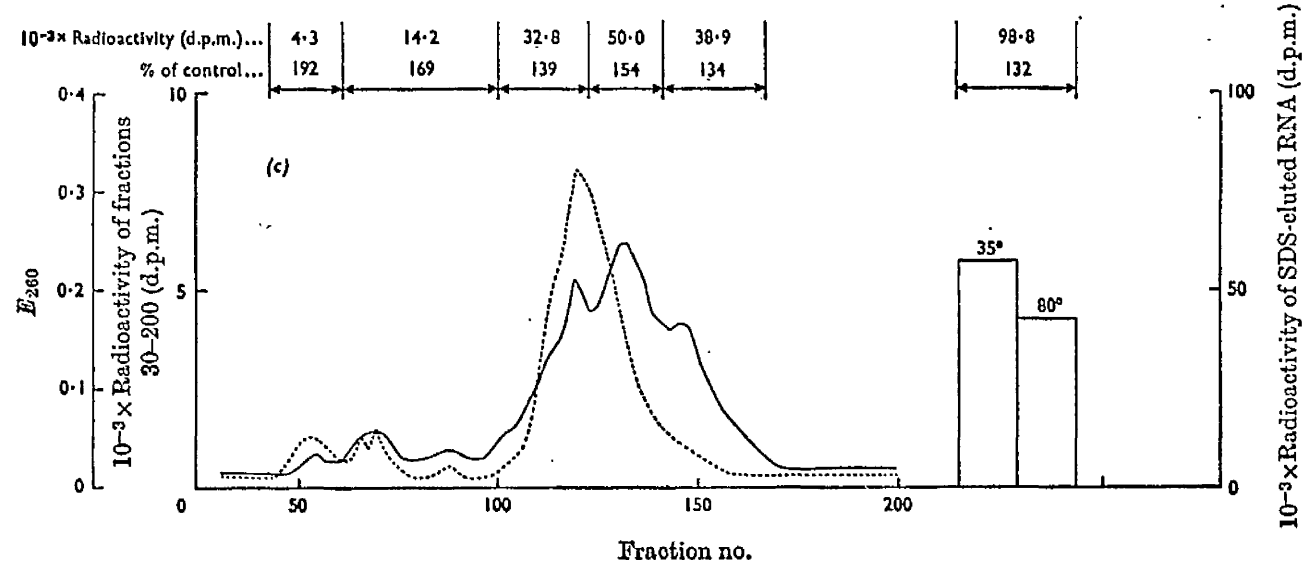
The total radioactivity of RNA extracted from the uteri increases with time after administration of oestradiol. The increases over the control are 22%, 48%, 85% and 144% at 30, 60, 120 and 360 min. respectively. The earliest increases occur in the ribosomal precursor (Ω1) fraction which rises to 54% over the control in the first 30 min. and to 145% over the control at 60 min. Thereafter the level of radioactivity in this fraction appears to stabilise. The mature ribosomal RNA fraction shows a slight increase of 40% over the control value at 30 and 60 min., but after 2 hr. and 6 hr. there is a dramatic rise in the radioactivity in this fraction with increases of 115% and 400% respectively.

The Ω2 fraction (50S - DNA like RNA) shows a slight increase of 24%

Fig. 21. Chromatography of RNA from rat uterus on columns of Kieselguhr coated with methylated serum albumin. In each experiment RNA from 14 uteri was pooled and 40 E_{260} units were applied to the column. All animals received 25 μc of ^{3}H uridine and 25 μc ^{3}H guanosine 30 min. before being killed. Controls (a) received 0.1 ml of 0.9% (w/v) NaCl 180 min. before killing while the test animals received 1 μg oestradiol-17β in 0.1 ml of 0.9% (w/v) NaCl at the following times before death (b) 30 min., (c) 60 min., (d) 120 min., (e) 360 min. ..... E_{260} ——— ^{3}H.


t-RNA
7s RNA + DNA
r-RNA
Q1-RNA
Q2-RNA
SDS-eluted RNA
10⁻³× Radioactivity (d.p.m.)... 2·2 8·4 23·6 20·2 28·9 74·7
% of control... 100 100 100 100 100 100
(a)
35°
80°
E260
10⁻³ × Radioactivity of fractions 30–200 (d.p.m.)
10⁻³ × Radioactivity of SDS-eluted RNA (d.p.m.)
Fraction no.


10⁻³× Radioactivity (d.p.m.)... 2·2 10·3 32·9 31·1 35·7 78·2
% of control... 100 122 140 154 124 105
(b)
35°
80°
E260
10⁻³ × Radioactivity of fractions 30–200 (d.p.m.)
10⁻³ × Radioactivity of SDS-eluted RNA (d.p.m.)
Fraction no.


10⁻³× Radioactivity (d.p.m.)... 4·3 14·2 32·8 50·0 38·9 98·8
% of control... 192 169 139 154 134 132
(c)
35°
80°
E260
10⁻³ × Radioactivity of fractions 30–200 (d.p.m.)
10⁻³ × Radioactivity of SDS-eluted RNA (d.p.m.)
Fraction no.

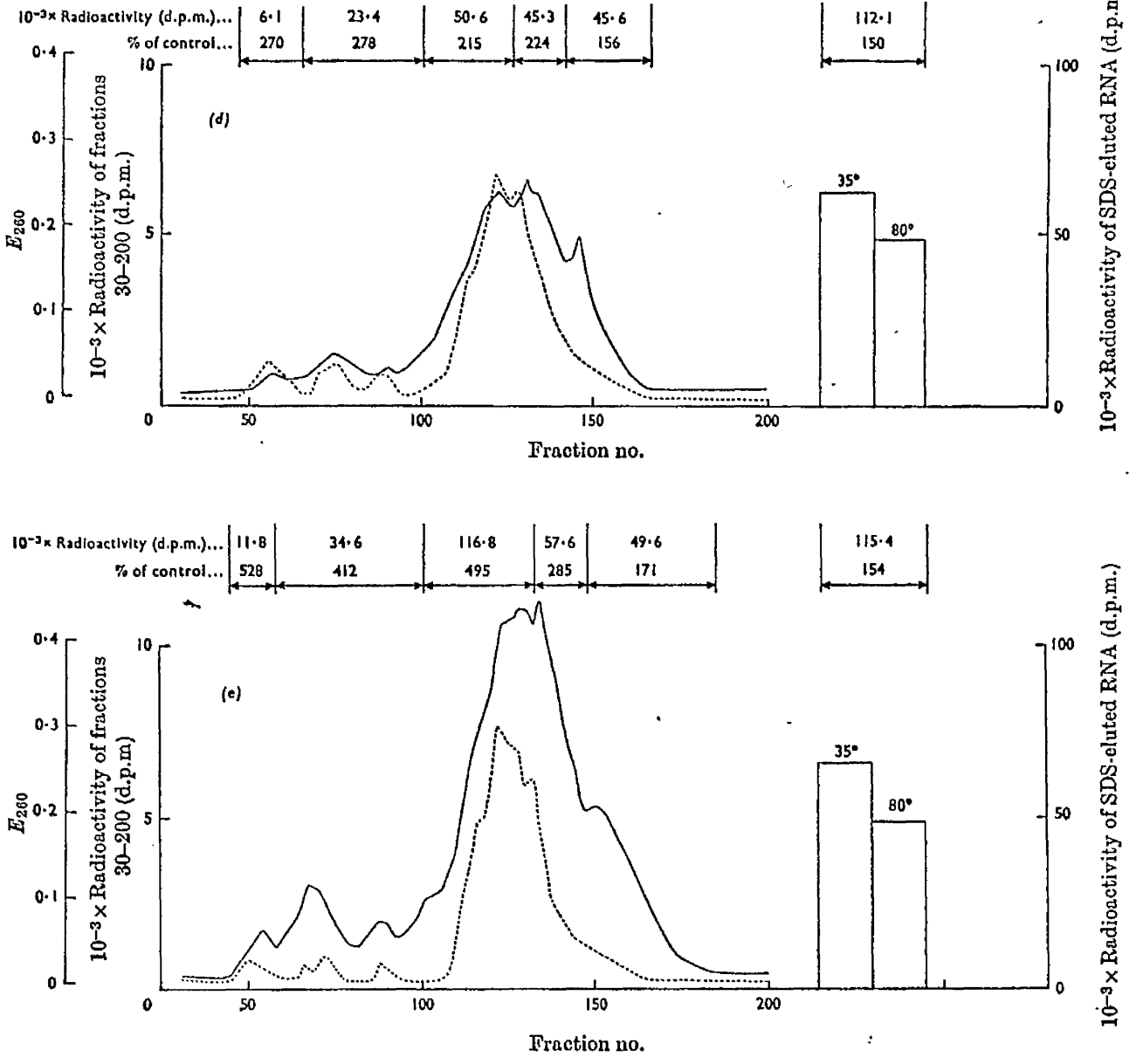


Fig. 2. Chromatography of RNA from rat uterus on MAK columns. In each experiment RNA from 14 uteri was pooled and 40 E_{260} units was applied to the column. All animals received 25 μc of [^{3}H]uridine and 25 μc of [^{3}H]guanosine 30 min. before being killed. Controls (*a*) received 0·1 ml. of 0·9% NaCl 3 hr. before being killed, and the test animals received 1 μg. of oestradiol-17β in 0·1 ml. of 0·9% NaCl at the following times before death; (*b*) 30 min.; (*c*) 1 hr.; (*d*) 2 hr.; (*e*) 6 hr. ····, E_{260}; ——, ^{3}H radioactivity.

over the control after 30 min. which has only risen to 52% over the control after 360 min.

The total radioactivity eluted with 0.2% SDS which has been shown to contain a 16S-18S DNA-like RNA shows no increase at 30 min., but increases to 24% at 60 min., and to 52% over the control after 360 min.

No increase occurred in the t-RNA fraction until 60 min. by which time it had risen to 92% over the control t-RNA level. Thereafter the radioactivity in this fraction rose rapidly until at 6 hr. it was 400% over the control value.

The nature of the peaks between t-RNA and r-RNA is not clearly known. The first one could be 7S RNA (Pene, Knight and Darnell, 1968) which is produced from 28S r-RNA by the method of RNA extraction employing hot phenol and SDS. The second peak is probably DNA of which only a small amount is extracted with this method of RNA preparation. The radioactivity in these fractions increases in a similar way to that in the ribosomal RNA fraction.

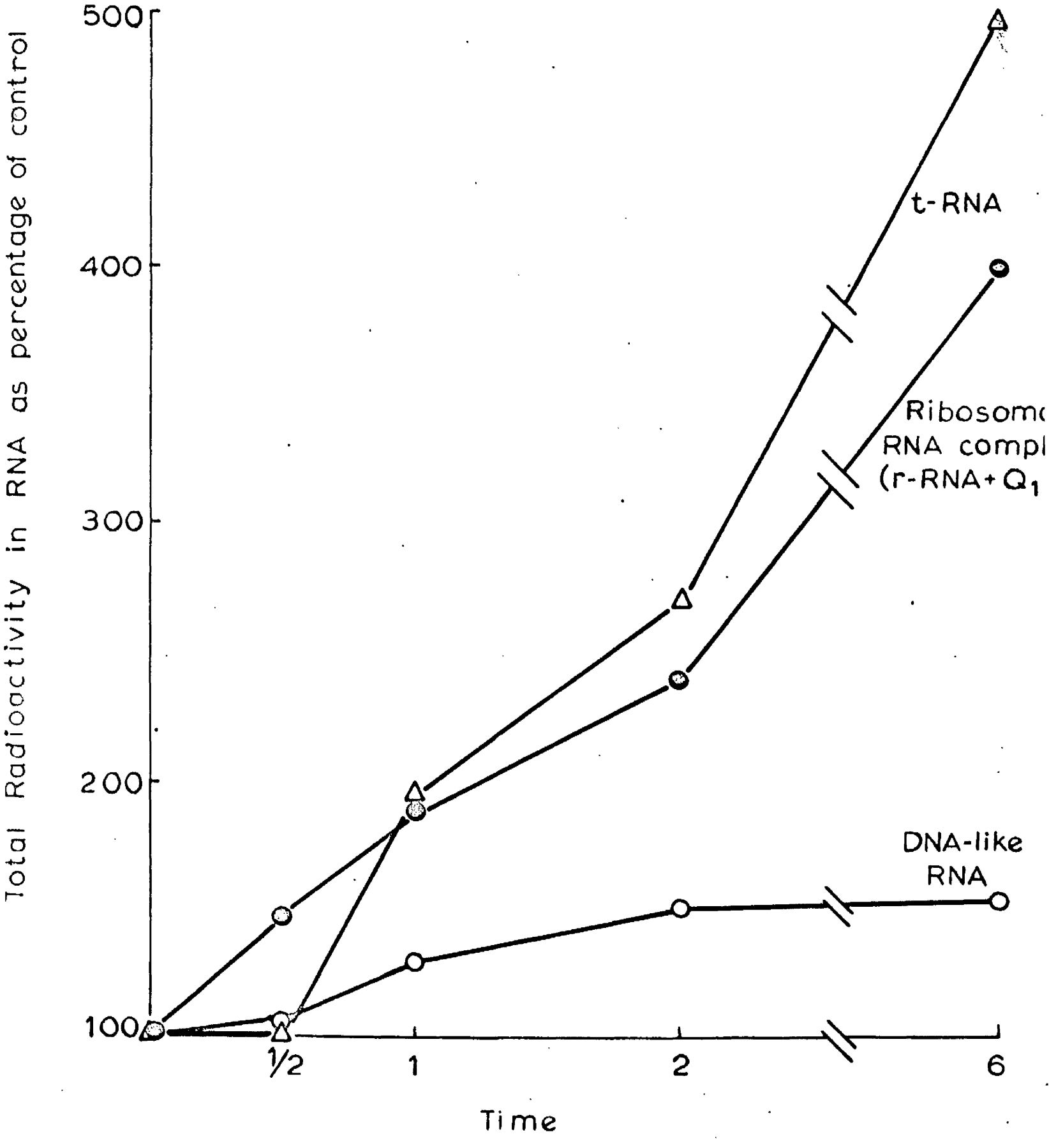
A time course of the changes in radioactivity of the principal RNA components is shown in fig. 22 page 77A. The labelling of the ribosomal complex (r-RNA and Q1 RNA) rises steadily over the 6 hr. period from the time of administration of oestradiol to about four times the control level. The t-RNA fraction, after a delay of 30 min. rises to about five

Fig. 22 Changes with time after the administration of oestradiol in the labelling of various RNA species from immature rat uterus. Experimental conditions as in Fig. 21

● ribosomal RNA complex (r-RNA + Q_1); Δ t-RNA;

○ DNA-like RNA

FIG 22


500
400
300
200
100
Total Radioactivity in RNA as percentage of control
t-RNA
Ribosomal
RNA compl
(r-RNA+Q1
DNA-like
RNA
1/2
1
2
6
Time

times the control level, but the DNA-like RNA fraction show only a slight increase of 50% and has reached a plateau at 2 hr.

3. Dual labelling technique used to distinguish the patterns of RNA synthesised in control and hormone-treated rat uteri

To avoid any differences in extraction procedure and chromatography the uteri from both control and hormone treated rats were pooled for the extraction of RNA. The RNA from control rats had been labelled with ^{3}H uridine and that from hormone-treated rats with ^{14}C uridine. Both groups of animals received the radioactivity 90 min. before death and the hormone treated group received 1.0 μg oestradiol 90 min. before death. The total ^{14}C recovered after allowing for the different specific activities of the precursors was 63% greater than the total ^{3}H. The pattern of elution of the RNA from MAK column is shown in fig. 23 page 78A. The largest increase (256%) was found in the Q1 fraction (ribosomal precursor). The ribosomal RNA fraction had increased by 130% but the Q2 and SDS eluted DNA-like RNA only increased by 58% and 38% respectively. Fig. 23 page 78A also shows the ratio of $^{14}C/^{3}H$. The largest values of the ratio occur in the fractions which show the largest increases following hormone treatment. These are the ribosomal RNA peak (3.2) and the Q1 fraction (2.5-3). The other fractions show a ratio of about 1.6

Fig. 23 Chromatography of rat uterine RNA on columns of kieselguhr coated with methylated serum albumin.

Elution pattern of E_{260} and of ^{3}H and ^{14}C radioactivity obtained on chromatography of ^{3}H - labelled RNA from the uteri of six control rats mixed with ^{14}C - labelled RNA from the uteri of six oestradiol-treated rats; 15 E_{260} units were applied to the column. Control animals received 0.1 ml. of 0.9% NaCl and 100 μc of ^{3}H uridine 90 min. before they were killed. Test animals received 1 μg of oestradiol-17β in 0.1 ml. of 0.9% and 25 μc of ^{14}C uridine 90 min. before they were killed. The ratio of ^{14}C to ^{3}H for the major components is plotted in the upper section of the figure.

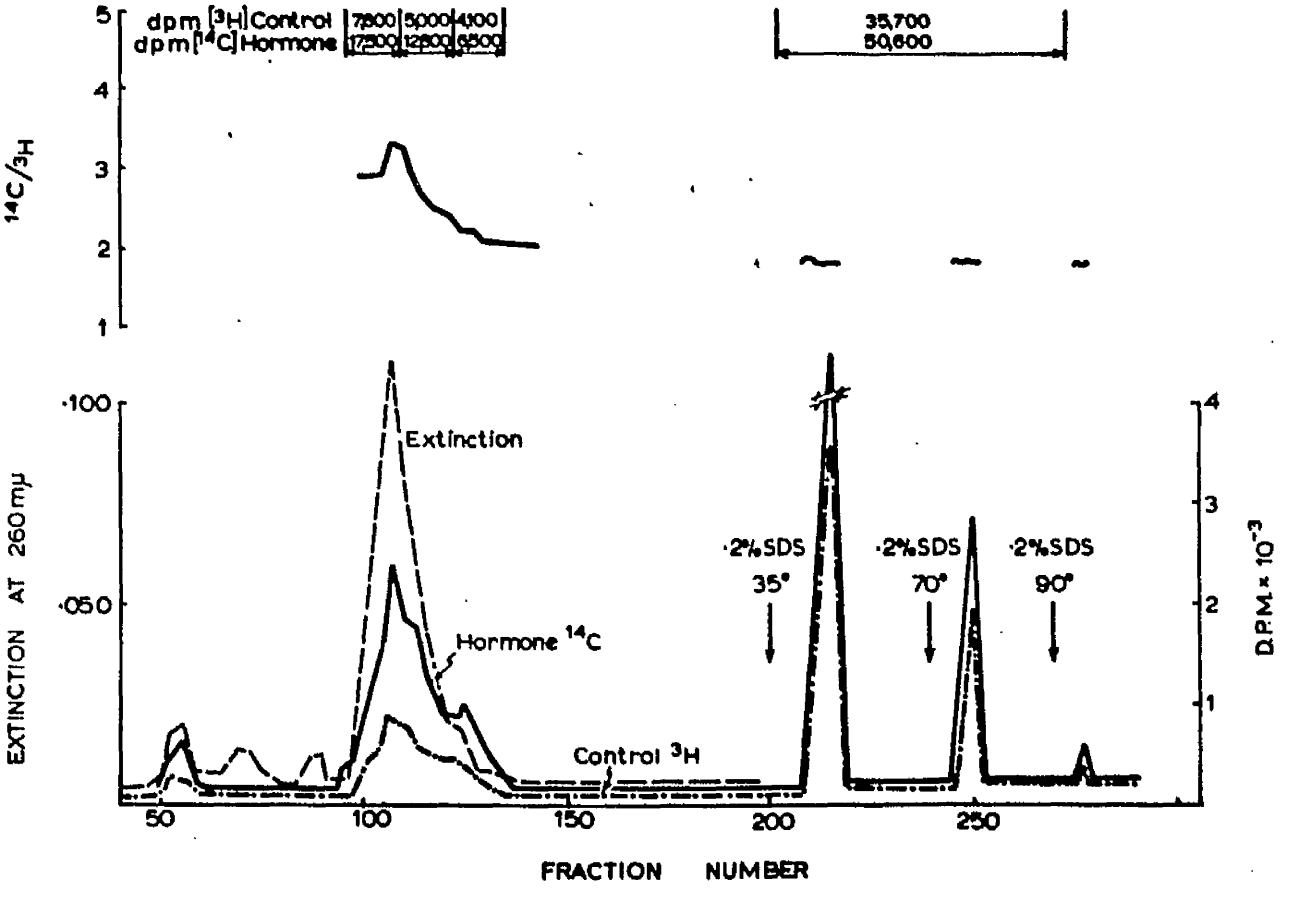
——— ——— E_{260}

-·-·-·-·-·- ^{3}H radioactivity

——————— ^{14}C radioactivity

FIG 23

SEPARATION ON M.A.K. COLUMNS OF RNA FROM THE UTERI OF CONTROL AND HORMONE TREATED RATS – 90 min HORMONE TREATMENT 90 min PULSE DUAL LABEL TECHNIQUE



4. Dual labelling technique to show the base ratios of the various fractions of RNA separated by MAK chromatography

The base ratios of the various RNA fractions eluted from MAK columns have been determined by Ellem (1966). He labelled the RNA with ^{32}P and hydrolysed each fraction to its component nucleotides. These were analysed by paper chromatography. With the purpose of trying a new and quicker method of doing this and to find the relative base ratio of the fractions from uterine RNA we labelled the RNA with ^{3}H guanosine and ^{14}C uridine. The ratio of $^{3}H/^{14}C$ in each fraction gave an indication of the relative amounts of guanine and uracil in the RNA in each fraction.

Fig. 24 page 79 A shows that the ratio of $^{3}H/^{14}C$ is similar (.5) for Q1, r-RNA, "7S" RNA and t-RNA but that it is much lower (.33) in the Q2 and SDS eluted RNA species. This agrees with the results of Ellem (1966) showing that Q2 and the SDS-eluted RNA species have a lower guanine (+ cytosine) content and thus are more like DNA in base ratio than Q1, r-RNA, "7S" RNA and t-RNA species.

5. The Transport of RNA precursors into the uterus following stimulation by oestradiol

Fig. 25 page 79B shows the uptake of a mixture of labelled adenosine, guanosine, cytidine and uridine from the blood stream into acid-soluble and acid-insoluble fractions of rat uterus at various times

Fig. 24 Chromatography of RNA from rat uterus on a column of Kieselguhr coated with methylated serum albumin. Rats were injected with 1 μg of oestradiol-17β 3 hr. and 15 min. before they were killed. Isolated uteri were incubated in vitro with a mixture of 10 μc of ^{14}C uridine and 5 μc of ^{3}H guanosine for 45 min., the RNA extracted and applied to the column.

.....E_{260}; - - - - - ^{14}C; _____ ^{3}H.

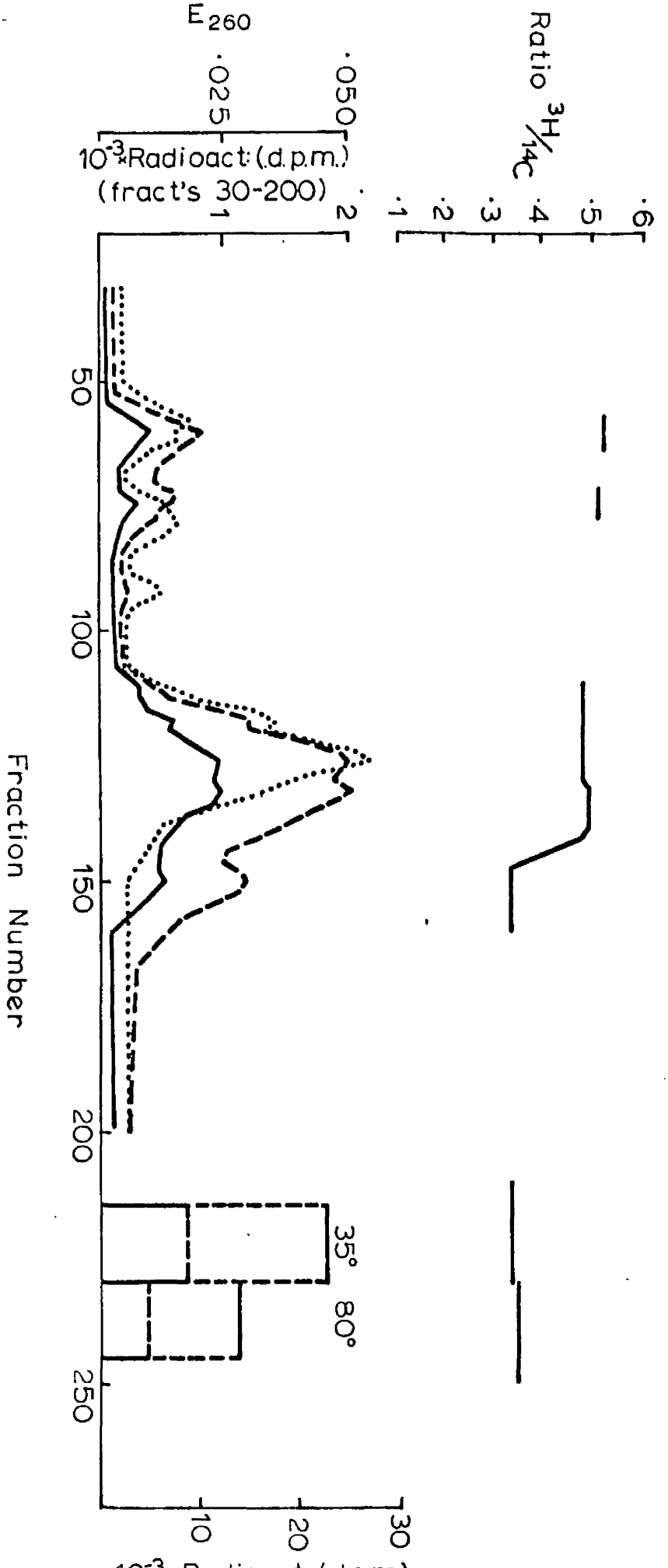

E260
.025
.050
10-3*Radioact: (d.p.m.)
(fract's 30-200)
1
2
Ratio 3H/14C
.1
.2
.3
.4
.5
.6
50
100
150
200
250
Fraction Number
35°
80°
10
20
30
10-3× Radioact: (d.p.m.)
(fract's 215-245)

FIG 24

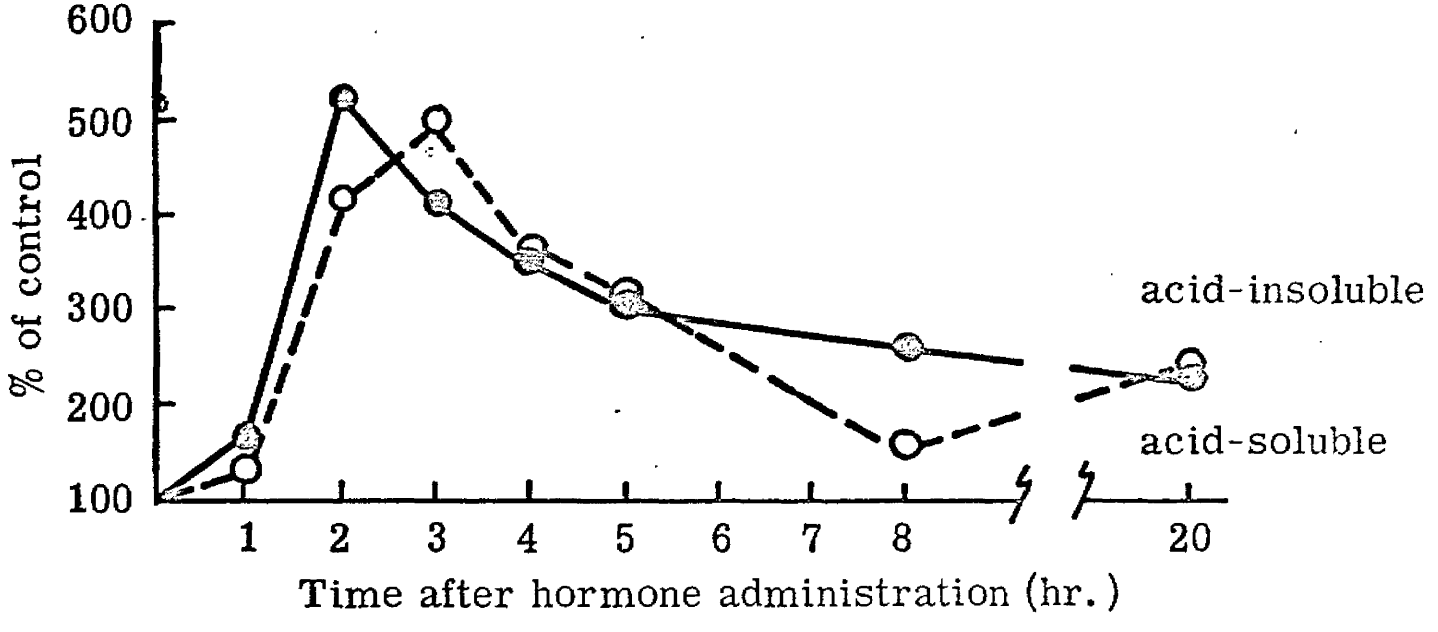
Fig. 25 The effect of a single dose of oestradiol-17β on the incorporation of all 4 ^{3}H ribonucleosides into acid-soluble and acid-insoluble material in the uterus of immature rats.

All animals received 1 μg of the hormone intraperitoneally at zero time (controls being injected with 0.9% NaCl solution) and a mixture containing 12.5 μc each of ^{3}H adenosine, ^{3}H guanosine, ^{3}H cytidine and ^{3}H uridine intravenously 30 min. before killing. Data are expressed as percentages of control values. Every point represents the average of at least five animals. Control values are 8,500 d.p.m. for acid-insoluble material and 12,000 d.p.m. for acid-soluble material.

●——● insoluble d.p.m. O——O soluble d.p.m.

FIG 25

Uptake of [^{3}H]-Nucleosides into Immature Rat Uterus under action of Oestradiol-17β



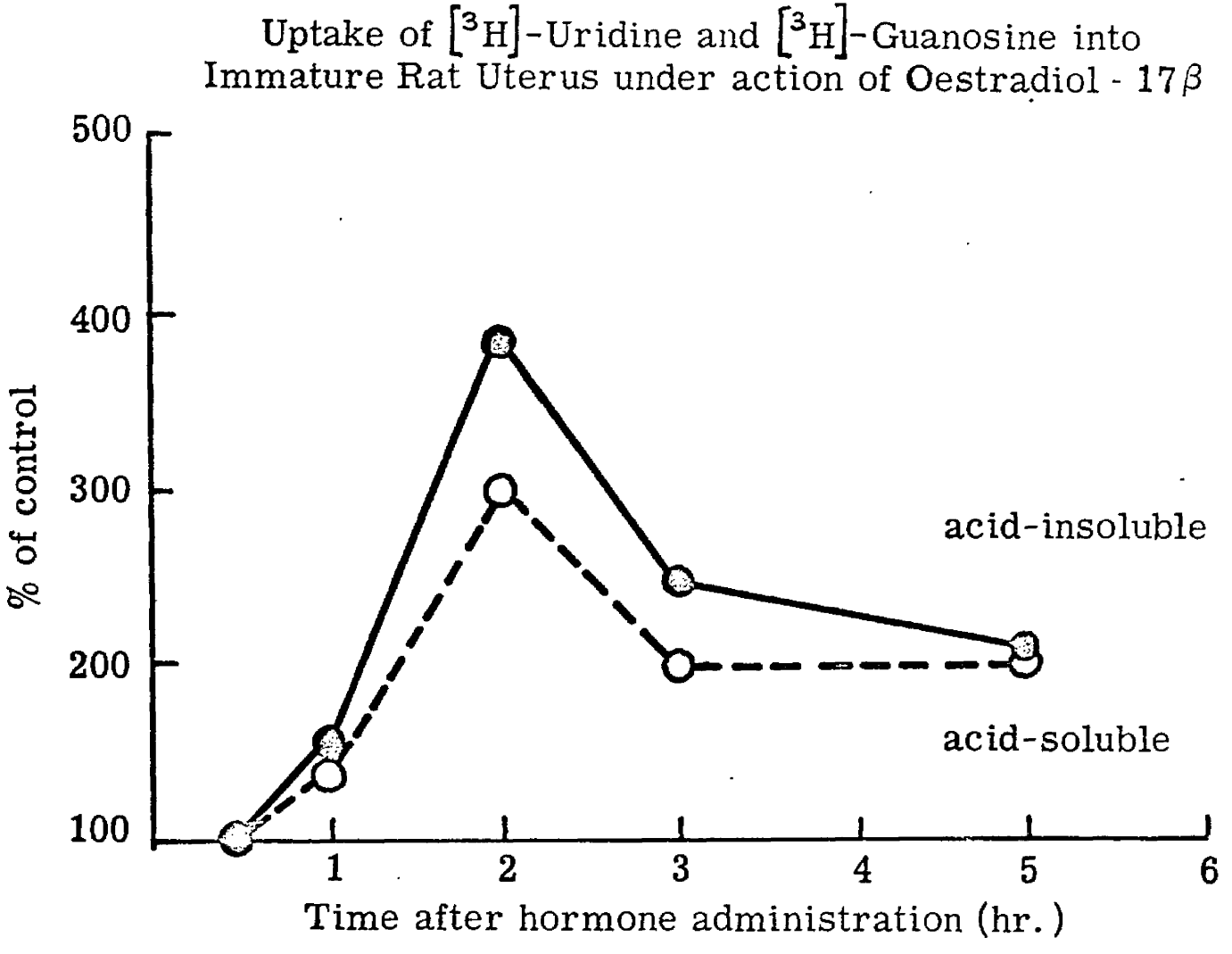
following administration of oestradiol-17β. The general pattern of uptake of these precursors into acid-insoluble material corresponds quite closely to the pattern of incorporation into the acid-soluble material. More detailed consideration of these results shows that there is only a slight increase in the uptake into both fractions 1 hr. after the administration of oestradiol, but by 2 hr. there is a dramatic increase in both fractions. The uptake into the acid-insoluble fraction reaches a maximum of 530% of the control value at 2 hr. while the uptake into the acid-soluble fraction at this time is 416% of the control value. By 3 hr. the radioactivity in the acid-insoluble fraction has fallen to 412% and thereafter it declines slowly reaching a value of 220% after 20 hr. of hormone action. The uptake into the acid-soluble fraction reaches a maximum of 500% of the control at 3 hr. after hormone administration and it also declines at longer time intervals reaching a minimum of 150% of the control value at 8 hr.

Fig. 26 page 20A shows the uptake of ^{3}H guanosine and ^{3}H uridine into the acid-insoluble and acid-soluble fractions of rat uterus after an intraperitoneal injection of these precursors. The pattern is essentially the same as that obtained in fig. 25 there being a close correlation between the changes in the acid-insoluble material and the

Fig. 26 The effect of a single dose of oestradiol-17β on the incorporation of ^{3}H uridine and ^{3}H guanosine into acid-soluble and acid-insoluble material of immature rat uterus. 25 μc each of ^{3}H guanosine and ^{3}H uridine were injected intraperitoneally 30 min. before killing. Other experimental details as in fig. 1. Every point represents the average of at least five rats. Control values are 27,000 d.p.m. for acid-insoluble material and 43,000 d.p.m. for acid-soluble material.

●——● acid-insoluble d.p.m., O——O acid-soluble d.p.m.

FIG 26


Uptake of [3H]-Uridine and [3H]-Guanosine into
Immature Rat Uterus under action of Oestradiol - 17β
500
400
300
200
100
% of control
acid-insoluble
acid-soluble
1
2
3
4
5
6
Time after hormone administration (hr.)

changes in the acid-soluble material. The peak of incorporation into both fractions occurs 2 hr. after administration of the hormone and thereafter radioactivity in both fractions declines steadily up to 5 hr.

Table 1 page 81 A shows how the E_{260} and the radioactivity of the nucleoside and the nucleotide components of the acid-soluble fraction change in response to hormone action. The size of the total acid-soluble nucleotide pool, measured by the E_{260} of the eluate from a Dowex-1 column, shows only little change over the first five hr. By 8 hr. it has increased to 30% over the control level, and by 20 hr. it has increased to 76% over the control level. The total uptake of RNA precursors into the uterus shows a maximum between 2 and 3 hr. after administration of oestrogen. Thereafter there is a decline to a minimum at 8 hr. followed by a further increase between 8 and 20 hr. The specific activities of the acid-soluble nucleotides follow a pattern similar to that observed for the total uptake of radioactive precursors. The proportions of the total radioactivity occurring as acid-soluble nucleosides, acid-soluble nucleotides and acid-insoluble nucleotides are shown in columns A, B and C. The value for the acid-soluble nucleosides falls with time after hormone treatment reaching a minimum at 8 hr. and then rises again by 20 hr. The percentage of the total

Table I. Uptake of labelled ribonucleosides into immature rat uteri under the influence of oestradiol-17β and the distribution of radioactivity in the acid-soluble nucleoside and nucleotide fractions and in the acid-insoluble fraction. Groups of 12 or 14 three week old immature rats were injected at zero time with 1 µg. of oestradiol-17β 30 min. before death. Four animals in each group received an intravenous injection of a mixture containing 12.5 µc each of [^{3}H] adenosine, [^{3}H] guanine, [^{3}H] cytosine, [^{3}H] uridine. The uteri were pooled and the acid-soluble and acid-insoluble fractions isolated as described in the methods section. The acid-soluble fraction was applied to a Dowex 1 Chloride ion exchange resin. Data represent the average of at least two separate experiments.

time after oestradiol-17β administration	A_{260} mµ of total acid-soluble nucleotides	Radioactivity				Specific Activity of acid-soluble nucleotides
		total uptake	acid-soluble nucleosides A	acid-soluble nucleotides B	acid-insoluble nucleotides (RNA) C	
hr.	A units/ uterus	d.p.m./uterus	% of total uptake			d.p.m./A_{260} m unit
0	.540	20,500	21.5	37.1	41.4	14,100
1	.550	30,000	20.6	32.7	46.6	17,800
2	.540	95,000	13.7	38.9	47.3	68,500
3	.610	95,000	11.6	51.6	36.8	80,300
5	.610	60,000	15.8	41.7	42.5	41,000
8	.700	40,000	6.9	38.7	54.4	22,100
20	.950	50,500	12.9	47.5	39.6	25,200

radioactivity occurring as nucleotides in the acid-soluble fraction falls during the first hour after hormone treatment, rises again to a maximum about 3 hr., falls to a minimum at 8 hr. and then begins to increase again by 20 hr. The proportion of total radioactivity occurring as acid-insoluble nucleotides increases during the first 2 hr. after hormone treatment, falls to a lower value at 3 hr. after which time it increases to a maximum at 8 hr. before falling again by 20 hr.

Table II page 82 A shows the effect of actinomycin D on the uptake of RNA precursors into the uterus 2 hr. after administration of oestradiol. In the absence of actinomycin D administration of oestradiol increases the total uptake of RNA precursor into the uterus by 383%. When actinomycin D is present oestradiol still causes an increase in total uptake but this is limited to 212%. Under the conditions employed oestradiol treatment causes a 447% increase in incorporation of labelled ribonucleosides into the acid-insoluble fraction. Actinomycin D, in the absence of treatment with oestradiol caused 80% inhibition of this incorporation. In animals which had been treated with actinomycin D and which then received oestradiol the radioactivity in the acid-insoluble fraction was 325% higher than in animals treated with actinomycin D alone.

Table 2 Effect of actinomycin D on the oestrogen-stimulated uptake of ^{3}H ribonucleosides and their incorporation into acid-insoluble material in immature rat uterus.

Rats were injected intraperitoneally with 150 µg. of actinomycin D. After 30 min. the animals received 1 µg. of oestradiol-17β or saline and after a further 2 hr. the animals were killed. All animals were injected intravenously with mixture containing 12.5 µc each of all 4 ^{3}H ribonucleosides 30 min. before death. Data are given as d.p.m./uterus and represent the mean of at least 5 separate experiments.

Treatment of animals	acid insoluble d.p.m.	% of control	total uptake d.p.m.	% of control
Control	7,500	100	17,500	100
Oestradiol-17β	41,000	547	84,500	483
Actinomycin-D	1,550	20	15,950	91
Actinomycin-D + Oestradiol-17β	6,400	85	49,700	284

6. The rate of synthesis of RNA following oestradiol stimulation

Fig. 27 page 83 A shows the amount of radioactivity taken up into immature rat uterus between 3 and 11 hr. after a single intraperitoneal injection of ^{3}H adenosine. It can be seen that the total amount of radioactivity in the tissue is fairly constant over this time period in the controls and in rats which had been given oestradiol 3 hr. after the injection of ^{3}H adenosine.

Although the total uptake of ^{3}H adenosine into the uteri of control and oestradiol stimulated animals is the same from 3 to 11 hours after administration of the labelled precursor, the proportion which has been incorporated into RNA changes (fig. 28 page 83 B). The level of acid-insoluble radioactivity (RNA) in control rat uteri rises only slightly from 16% to 20% of the total radioactivity between 3 and 11 hr. after administration of the precursor. The level of acid-insoluble radioactivit in rats treated with oestradiol showed only a slight increase over the control level during the first 3 hr. of hormone treatment, but after 5 hr. this begins to increase rapidly until at 8 hr. after hormone treatment the proportion of the total radioactivity incorporated into RNA is 42% compared to a control value at this time of 20%.

7. Turnover rate of the tenaciously bound DNA-like RNA in immature rat uterus

Table 3 shows the effect of increased length of labelling time

Fig. 27 Uptake of ^{3}H adenosine into immature rat uterus under the action of oestradiol-17β.

Each rat was injected intraperitoneally at zero time with 50 μc of ^{3}H adenosine and 3 hr. later they received 1 μg. of oestradiol-17β in 0.9% NaCl or 0.9% NaCl alone. The animals were killed at various times up to 8 hr. after hormone administration and total radioactivity in the uteri (O-control, ●-hormone-treated) and in the acid-soluble fractions (Δ -control, ▲ -hormone-treated) were measured. Results are expressed as d.p.m./ uterus.

FIG 27

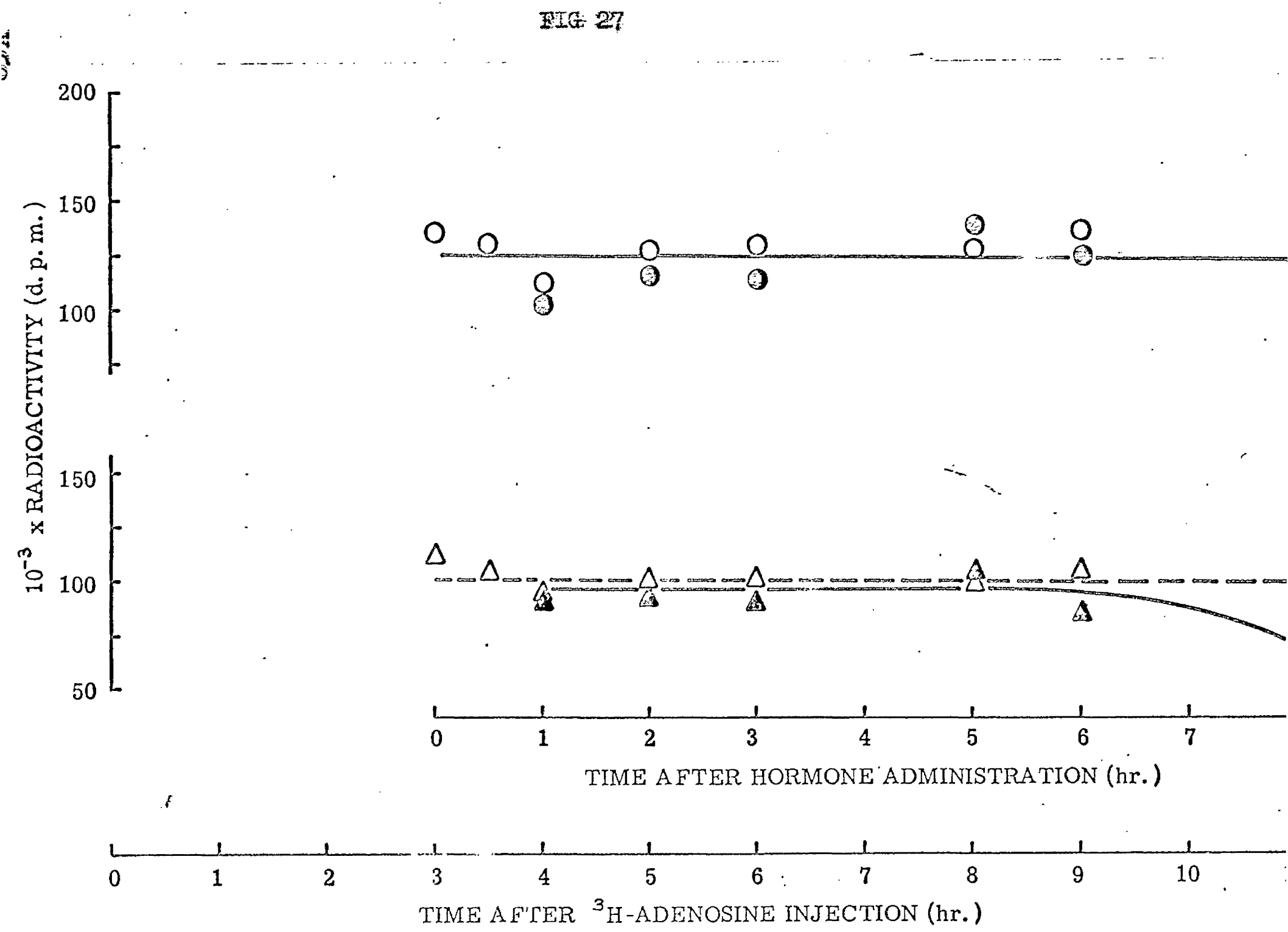

200
150
100
150
100
50
10^{-3} x RADIOACTIVITY (d.p.m.)
0
1
2
3
4
5
6
7
TIME AFTER HORMONE ADMINISTRATION (hr.)
0
1
2
3
4
5
6
7
8
9
10
TIME AFTER ^{3}H-ADENOSINE INJECTION (hr.)

Fig. 28 Incorporation of ^{3}H adenosine into acid-insoluble material of immature rat uterus under action of oestradiol-17β.

Experimental details are described in Fig. 27and in the text. Data are expressed as percentage of total uptake and represent the average of at least 4 different experiments. O——O control rats, ●——● hormone-treated rats.

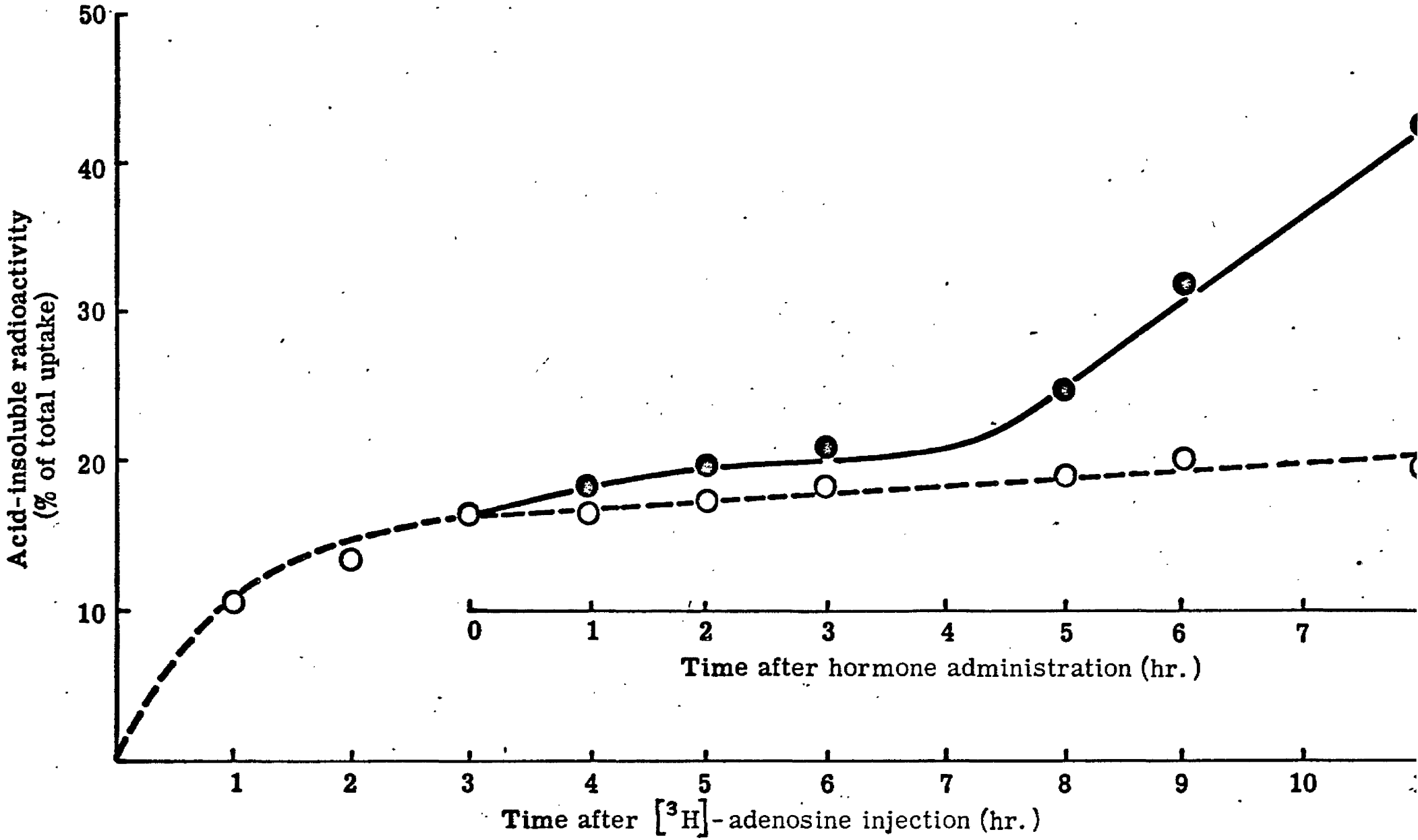

Incorporation of [3H]-adenosine into acid insoluble material of immature rat uterus under action of oestradiol - 17β
Acid-insoluble radioactivity (% of total uptake)
50
40
30
20
10
0
1
2
3
4
5
6
7
Time after hormone administration (hr.)
1
2
3
4
5
6
7
8
9
10
Time after [3H]-adenosine injection (hr.)

Table 3. The effect of increased length of labelling time on the uptake of labelled precursors into total RNA (and into TD RNA) from untreated rat uterus.

Seven immature female rats were each given intraperitoneally a mixture of 25 μc ^{3}H uridine and 25 μc ^{3}H guanosine ½, 4 and 27 hr. before death. The uteri from these animals were pooled with seven more from untreated rats for the extraction of RNA. The RNA was fractionated on a MAK column into a salt-eluted fraction and a SDS eluted fraction. The amount of radioactivity in the SDS eluted fraction (TD RNA) was expressed as a percentage of the radioactivity in the total RNA.

Length of labelling time (hr.)	½	4	27
radioactivity in total RNA (d.p.m.)	158,000	302,000	210,000
radioactivity in TD RNA (% of total)	47	27	15

on the amount of radioactivity in total uterine RNA and in the proportion of this which is present as TD-RNA. Half an hour after the administration of labelled precursor the amount of radioactivity in uterine RNA is 158,000 d.p.m. and the proportion of this in TD-RNA is 47%. Four hours after the administration of the labelled precursor the amount of radioactivity in the total RNA extracted has risen to 302,000 d.p.m. but the percentage of this in TD-RNA has fallen to 27%. Twenty seven hr. after the administration of precursor the radioactivity in total RNA has declined to 210,000 d.p.m., but the proportion of this in TD-RNA has decreased even further to 15%.

DISCUSSION

As I have mentioned in the general introduction the mode of action of hormones is not understood. Many of the experiments done in recent years on early effects of oestrogens have been misinterpreted giving a wrong impression of the role of RNA and protein synthesis in oestrogen action. From the results presented in this thesis the early effects of oestrogens on the synthesis of the major cell constituents have been measured, and although the precise initiation process is not yet apparent a much clearer picture of the early events occurring in the uterus after oestrogen stimulation has been obtained.

1. Changes in the composition of the major constituents of immature rat uterus following oestrogen treatment

From fig. 18 (page 74A) and 19 (page 74B) the order of the changes in the major constituents of the uterus can be seen. The RNA content is the first to increase followed by protein and then DNA. This is the order of events that would be expected from current theories of protein synthesis, and cell division. This could imply that the change in DNA synthesis is controlled by the amounts of protein (enzymes) in the cell, and that the increase in protein synthesis is controlled by the levels of RNA in the cell. Although there is no direct evidence for this it could be quite possible that

oestradiol initiates this series of events by causing an increase in RNA synthesis and thereafter the hormone plays no further role in subsequent events. This could also lead to the possibility that cancer of the uterus (and maybe other forms of cancer) could be caused by an uncontrolled RNA synthesis.

The level of DNA in the uterus increases rapidly by 50% about 30 hr. after oestrogen administration and it remains at this level for at least 24 hr. The fact that the level of DNA in the uterus increases by only 50% could mean that only half the uterine cells have responded to oestrogen and the fact that the increase remains at the same level for 24 hr. could mean most of the cells that have responded are in a synchronous state one to another. If this is true then this would provide a synchronous <u>in vivo</u> system which would be useful for example in the study of DNA synthesis. It may also represent the response of only one type of uterine cell to oestradiol. To clarify this point we must await experiments which will separate the responses of the various cell types in the uterus. It is quite possible that, for example, the myometrium responds to oestrogens in a different manner to the endometrium.

The earliest major constituent to change following oestrogen stimulation is the uterine water content. This begins to increase

rapidly at about 1½ - 2 hr. after hormone treatment. As this change represents a transport phenomenon it will be discussed in more detail in the later section on the discussion of transport of precursors into the uterus.

From fig. 19 it can be seen that the acid-soluble ribose content of the uterus (which contains RNA precursors) does not increase before the RNA content increases, but when the latter starts to decline again the acid-soluble ribose is not declining. This seems to indicate that the changes in the acid-soluble ribose pool are secondary to changes in RNA.

2. Changes in the patterns of synthesis of RNA in immature rat uterus in response to oestradiol-17β

Gorski and Nelson (1965) studied the changes in pattern of RNA synthesis in immature rat uterus within the first hour after treatment with oestradiol. Using sucrose density-gradient centrifugation they found no qualitative change in the RNA synthesised, but these methods have the drawback of not being able to account for DNA-like RNA which represents 60% of the radioactive RNA in a 30 min. pulse.

When the uterine RNA from periods up to 6 hr. after hormone administration are analysed by MAK column chromatography qualitative as well as quantitative differences in the rate of incorporation of labelled

precursors into the various species are observed (fig. 21, 22, 23). The first RNA species to show an increase in labelling is Q1, but 2 hr. after hormone administration the amount of radioactivity in this fraction begins to level off. This pattern is consistant with Q1 being a precursor of ribosomal RNA.

The major increases in labelling occur in the mature ribosomal species of RNA and in t-RNA which have increased by 395 and 428% respectively 6 hr. after hormone administration. The rate of incorporation of precursors into Q2 and TD-RNA increase over the first 2 hr. and then level off at 71 and 59% respectively over the control rate. This suggests that (following oestrogen treatment) there are large increases in the rate of synthesis of uterine ribosomal and t-RNA, but the corresponding increases in DNA-like RNA are very much smaller.

How can these changes in RNA synthesis cause an increase in protein synthesis? If it is assumed that at least part of the DNA-like RNA fractionated by MAK chromatography represents messenger RNA then these results (fig. 21, 22, 23) could be interpreted to mean an increase in synthesis of m RNA is not essential for increased synthesis of protein. In this case the increase in protein synthesis in the uterus following oestrogen treatment would be achieved solely through increases

in the synthesis of r RNA and t-RNA. This explanation would require the cytoplasmic m-RNA template to be quite stable.

Ellem (1966) has shown that both TD-RNA and Q2 DNA-like RNA turn over rapidly. Table III page 83C confirms this by showing that uterine TD-RNA also has a rapid turnover.

In recent experiments on liver we have shown that neither Q2 or TD-RNA enter the cell cytoplasm. This evidence suggests that there is a more complex explanation of the results from the MAK chromatography of uterine RNA following oestrogen treatment than the one given above. The main difficulty in explaining these results is that not enough is known about RNA metabolism in general, and this makes it even more difficult to say how the hormone is influencing this situation.

Since finishing these experiments on uterine RNA, I have performed some experiments on liver (which is an easier organ to work with) to gain some more information about the function of Q2 and TD-RNA. Without going into detail about these experiments, I would like to present another explanation, but a tentative one, of the pattern of RNA synthesis under early oestrogen stimulation.

The turnover rate and distribution pattern of TD-RNA and Q2-RNA suggest they could be precursors of another form of RNA. This would

explain why these species do not increase after oestrogen stimulation of uterine RNA synthesis. The mature form of DNA-like RNA could be combined with RNA which has a ribosomal-like base ratio, and which is eluted as one of the "ribosomal" RNA species, Georgiev et al (1963). The fact that 18S "ribosomal" RNA seems to contain 40% DNA-like RNA Hadjiolov (1967) supports this idea. This would mean that all species of RNA including m-RNA are synthesised in the uterus under hormone stimulation. This explanation seems to fit the observed facts about DNA-like RNA metabolism better than the first explanation given.

So far this discussion of the changes in the pattern of incorporation of precursors into RNA has not taken into account the changes in specific activity of the precursor pool of ribonucleotides. How these changes are related to the real changes in the rate of RNA synthesis will be discussed in detail in the next section. It was found that most of the increased incorporation of precursors into uterine RNA during the first 5 hr. of oestrogen action could be accounted for by increases in specific activity of the ribonucleotide pool. At 6 hr. after hormone administration there is a real net increase in uterine RNA synthesis. Therefore if it is assumed that there is a common ribonucleotide precursor pool for the synthesis of all RNA species and the changes in specific activity of the pool is taken into account, then there is a net

increase in r-RNA and t-RNA but there is probably a decrease in DNA-like RNA, after 6 hr. of hormone stimulation. This point does not invalidate any of the above discussion or conclusions.

3. The transport of RNA precursors and water into the uterus following stimulation by oestradiol

Fig. 25 and 26 (pages 79A and 80A) respectively show that in the first few hours after administration of oestradiol increases in the incorporation of radioactive precursors into RNA are in general matched by similar increases in the uptake of these precursors into the uterine acid-soluble pool. These results suggest that the observed changes in incorporation of precursors into the acid-soluble pools and into RNA are directly related to one another.

From Figs. 25 and 26 and Table 1 page 81A it can be seen that at 2 hr. and 8 hr. after hormone administration the increases in the incorporation of precursors into RNA are somewhat greater than the increases in radioactivity in the acid-soluble fraction and it may be that at these particular times there is a true increase in the rate of synthesis of RNA. Further experiments however are required to clarify this point.

It has been suggested that the increase in the radioactivity of the acid-soluble pool occurs as a consequence of RNA synthesis (Means and Hamilton, 1966; Hamilton 1968). This could happen if a large

increase in RNA synthesis were to deplete the pool of ribonucleotide precursors. This in turn would cause increased transport of precursors into the uterus. This does not seem to be the case because there is no detectable fall in the ribonucleotide pool, and because even when RNA synthesis is inhibited by 80% with actinomycin D there is still a three fold increase in the uptake of precursor into the uterus (Table 2 page 82A). It is therefore most unlikely that the increased transport of labelled precursors into the uterus can be caused by depletion of the precursor pool. Rather it seems that there is a more direct effect of oestradiol on transport of precursors into the uterus. This would cause an increase in the specific activity of the ribonucleotide pool which would in turn account for the greater part of the observed increase in the incorporation of labelled precursors into RNA.

Changes in the rate of increase in wet weight with time (Fig. 20 page 75A) after oestrogen administration show a similar pattern to the changes in uptake of nucleosides into the acid-soluble and acid-insoluble fractions after oestrogen administration (fig. 25). This suggests that the pattern of uptake is similar for water and small precursor molecules such as nucleosides. It may be that between 1.5 and 2 hr. after oestradiol administration, there is an increase in uterine permeability

to water and other small molecules. Further increases in wet weight at later times may correspond with increases in the amount of uterine water and protein.

Among the earliest known effects of oestrogens are those associated with the uterine membrane and histamine. Within 15 sec. oestradiol increases the level of 3'-5' cyclic AMP (Szego and Davis 1967) which is associated with the uterine membrane. The release of histamin from the uterus is another early effect of oestradiol (Spaziani and Szego, 1958; Szego, 1965). It has been shown that histamine will cause the inhibition of water into the uterus (Hamilton, Widnell and Tata, 1968). These events could be linked in some way which is not yet understood to explain the early effect of oestrogen on water inhibition. There is also the possibility that the effect of oestradiol on the increased transpor of nucleosides is mediated via a specific RNA species, the synthesis of which is not blocked by actinomycin D.

The purpose of this increased inhibition of water is unknown but it must be important because of its early and dramatic nature. It seems unlikely that the water inhibition is responsible for the general increase in RNA synthesis which occurs at a later time of hormone action, but it could provide a mechanism for the accumulation in the uterus of small molecule precursors required for the subsequent synthesis of proteins

and nucleic acids.

It is known that oestrogens are bound specifically to a uterine nuclear protein (Noteboom and Gorski, 1965; King and Gordon, 1967) and that there is an increase in DNA dependent RNA polymerase activity (Gorski 1964) in the uterus following hormone treatment. These findings suggest a more direct effect of oestrogens at the genome level, and in the following section, I will discuss changes in the rate of synthesis of RNA in uteri responding to oestradiol.

4. The changes in rate of synthesis of RNA in uterus following stimulation by oestradiol

One of the major problems in studies on the mode of action of hormones in relation to the synthesis of nucleic acids and proteins is the difficulty of distinguishing between changes that may occur in the sizes or specific activities of pools of precursors and changes in the actual rates of synthesis of nucleic acids and proteins. It is difficult to achieve the perfect solution to this problem. However it seemed possible that the level of radioactivity in the adenine nucleotide and nucleoside pool, which constitutes quantitatively the greatest part of the pool of acid-soluble precursors of nucleic acids, might reach a level which would remain reasonably constant over a period of some hours. In such circumstances it should be possible to investigate changes in the incorporation of adenine nucleotides from the precursor

pool into RNA and that this would be independent of the processes of transport of ^{3}H adenosine into the uteri if administration of the hormone were delayed until the radioactivity in the adenine nucleotide pool had reached a steady level.

From the experiments shown in Fig. 27 page 83 A it is evident that from 3 to 11 hours after the administration of ^{3}H adenosine the total radioactivity in the uteri of control and hormone treated rats is similar and quite constant, that the total acid-soluble radioactivity in the controls remains quite constant and that in the test animals the total acid-soluble radioactivity remains constant until about 5 hr. after hormone treatment when it begins to decline. This fall in acid-soluble radioactivity in the treated animals can be accounted for by a corresponding rise in acid-insoluble radioactivity (Fig. 28 page 83B) of the total acid-soluble adenine nucleotide pool reflects the radioactivity of the adenine nucleotide precursors of RNA, these results can be interpreted as indicating that there is only a slight increase in the rate of synthesis of RNA in the uterus during the first 5 hr. after administration of oestradiol but that a substantial increase in the rate of synthesis commenc after about 6 hr.

These findings are consistent with observations on the changes in the amount of RNA in rat uterus after oestradiol treatment (Fig 18 page 74A) from which it is evident that the RNA content of the organ only

begins to increase after about 7 hr. while the protein and DNA content only begin to increase after 14 and 28 hr. respectively.

While the major quantitative changes in the rate of RNA synthesis in response to oestradiol do not appear until after 5-6 hr., there is some evidence from incorporation experiments (Fig. 24, 25, 26 and Table 1 pages 83B, 79A, 80A and 81A respectively) of a small increase in the rate of RNA synthesis at 2 hr. More precise experiments will be necessary to clarify this point but, if genuine, such a change in the synthesis of RNA could represent a "trigger" mechanism necessary for the initiation of subsequent response to oestradiol.

5. Initiation of the increase in RNA synthesis in rat uterus following oestrogen administration

The question to be discussed in this final section is by what mechanism does oestradiol bring about the increase in RNA content which is first seen seven hours after hormone administration? This increased RNA content could be due to either an increased RNA synthesis or to a decreased RNA breakdown. Fig. 27 page 83A and fig. 28 page 83B shows that there is an increased incorporation of precursors into RNA immediately preceding the increase in RNA content. This suggests that there is an increased synthesis of RNA,

but a decreased breakdown of newly synthesised RNA cannot be ruled out.

The work of Gorski (1964) and Hamilton, Widnell and Tata (1965) showing an increase in RNA polymerase activity was described in the general introduction, but it was also suggested that this kind of experiment did not prove conclusively that there was only an increase in synthesis of RNA involved. The polymerase system employed would also contain ribonuclease which would interfere with the results. Careful experiments will have to be performed to elucidate the role of ribonuclease in the uterus.

It seems unlikely from a consideration of the energy wastage involved that in immature rat uterus the low level of RNA is maintained by the continuous degradation of newly synthesised RNA by ribonuclease while in the oestrogen stimulated organ this ribonuclease is inhibited. A more plausable explanation is that the actual synthesis of RNA is increased in some way. This could be achieved by (1) an increased synthesis of RNA polymerase (2) an increased activity of RNA polymeras (3) an increased availability of template (DNA) for transcription. The latter would assume that part of the DNA template in the resting uterus is covered by a repressor and that oestrogen eliminates this repressor activity. This is the theory presented by Jacob and Monod (1962) for

the control of enzyme synthesis in bacteria. Although it is a general synthesis of all RNA species (and all protein species) that is required for uterine growth rather than specific species this mechanism could account for the synthesis of a specific RNA species which would in turn cause the general increase in RNA synthesis. If ribosomal RNA were responsible for the removal of messenger RNA from the DNA template and then its subsequent transport into the cytoplasm then an increased synthesis of ribosomal RNA alone would give a general increase in RNA synthesis.

Speculation in this area of oestrogen action could be continued, but further experiments are needed to give the discussion more foundation.

The work presented in this thesis has shown that these experiments must distinguish between the effect of oestrogens on transport and its effect at the genome on RNA synthesis. If this is kept in mind, although it will involve detecting very small changes in the uterus, progress in elucidating the mechanism by which oestrogen initiates increased uterine RNA synthesis should be made in the future.

SUMMARY

1. Uterine cells from immature female rats have been grown in culture medium. Although most cells died after 8 or 9 days in culture, autoradiographic studies showed that at earlier times the cells were synthesising both RNA and DNA.

2. No effect on the growth of the cells was observed when oestrogen was added to the culture medium, but increased growth occurred when the hormone was administered to the rat before death.

3. Autoradiographic localisation of ^{3}H oestradiol showed that in cultured uterine cells the hormone was spread throughout the cell, but in smeared uterine cells which were taken directly from the animal there was evidence of localisation in the nucleus. This agrees with binding studies of other workers.

4. After treatment of immature rats with either diethylstilboestrol or oestradiol-17β the uterine content of the following parameter begin to increase at the following times after hormone administration: water, $1\frac{1}{2}$ - 2 hr.; RNA, 7 hr.; protein, 14 hr.; DNA, 28 hr. This is the order one would expect for RNA protein and DNA considering current ideas of protein synthesis and cell division.

5. The type of RNA synthesised under early oestrogen action was fractionated on MAK columns. Ribosomal and transfer RNA species were the main types to be synthesised. The amount of incorporation of radioactive precursors into DNA-like RNA species did not increase significantly. Although this could mean there is no increase in messenger RNA, a more plausible explanation might be that DNA-like RNA is a precursor of messenger RNA which is eluted with the ribosomal species when it is in the mature form. The fact that DNA-like RNA in untreated uteri has a rapid turnover supports this latter view.

6. The uptake of labelled RNA precursors into the immature rat uterus has been measured. There is a close correlation over the first 5 hr. of hormone action between the uptake of precursors into the uterus and the amount incorporated into RNA.

7. There is a close correlation between the rate of water uptake into the uterus and the rate of uptake of RNA precursors into the uterus.

8. We conclude that one of the primary effects of oestrogens is to cause an increased transport of RNA precursors into the uterus. This reaches a maximum 2 - 3 hr. after hormone administration which corresponds to a maximum in the uptake of water into the uterus.

9. If the changes in specific activity of the uterine ribonucleotide pool are taken into account there are no major increases in RNA synthesis over the first 6 hr. of hormone treatment.

10. This is in agreement with the changes in uterine RNA content as measured by chemical methods, but in disagreement with other workers in the field who have not accounted for changes in the specific activity of the precursor pools.

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Investigations into the Mechanism of Action of Oestradiol-17β

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Cells proliferated from explants of immature rat uterus when the latter were placed in a specific culture medium. Although most of the cells died after 8 or 9 days, autoradiographic studies showed that at earlier times the cells were synthesising both RNA and DNA. There was no detectable change in the growth pattern when oestrogen was added to the culture medium, but increased growth occurred when the hormone was administered to the rat before death.

Autoradiographic localisation of ^{3}H oestradiol-17β showed that in the cultured cells the hormone was spread throughout the cells, but in smeared uterine cells taken directly from the animal there was evidence of localisation in the nucleus. This is in agreement with other workers who have found that oestradiol-17β is preferentially bound to a nuclear fraction of uterus.

This system of cell culture of uterus proved very limited, and as a result all further experiments on the mechanism of oestrogen action were performed *in vivo* on whole animals.

After administration of a single dose of oestradiol or the synthetic oestrogen diethylstilboestrol the uterine content of the following parameters began to increase at the following times after hormone treatment: water 1½ - 2 hr.; RNA, 7 hr.; protein, 14 hr.; DNA, 23 hr. This is the order that would be

expected from current ideas of protein synthesis and cell division.

The type of RNA synthesised over the early period of oestrogen action was analysed by chromatography on columns of kieselguhr coated with methylated albumin. This method, which accounts for all the species of RNA, showed that the rate of synthesis of ribosomal RNA and transfer RNA were increased after hormonal stimulation, but DNA-like RNA was not. One interpretation of these results is that there is no increase in messenger RNA following oestrogen stimulation. Another and perhaps more plausible interpretation is that DNA-like RNA is a precursor of another form of messenger RNA which is eluted from the column with the ribosomal RNA fraction. This would mean that all the major species of RNA were increased after hormone stimultion. The rapid turnover rate of DNA-like RNA supports this latter interpretation.

The change in rate of RNA synthesis was measured by the amounts of incorporation of labelled precursors into RNA. When changes in the specific activity of the ribonucleotide pool were taken into account it was found that the rate of synthesis of RNA did not increase until 5 to 6 hr. after administration of oestradiol. This is in agreement with the increased amount of RNA in the uterus about 7 hr. after hormone administration.

It was found that 1½ - 2 hr. after hormone administration there was a 5 fold increase in the transport of labelled RNA

precursors into the uterus, and that this corresponded to a large increase in water uptake into the uterus. This explains the increased incorporation of precursors into RNA at this time which other workers in the field have interpreted as increases in RNA synthesis.

Two sites of action of oestradiol on the uterus have been proposed. One site is located at the periphery of the cell causing an increased transport of water and small precursor molecules into the uterus at 1½ - 2 hr. after hormone administration. The other site is located in the nucleus causing an increasing RNA synthesis about 5 - 6 hr after hormone administration.