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

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Acknowledgements

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Abbreviations

The abbreviations recommended by the Biochemical Journal in its Instructions to Authors, 1978, have been used throughout this thesis with the following additions:-

Oestradiol-17β	oestra-1,3,5(10)-trien-3,17β-diol
EBP	oestrogen binding plasma protein
IP	induced protein
Pre-rRNA	precursor of rRNA
BHK	baby hamster kidney fibroblast
ppGpp	guanosine tetraphosphate
PPO	2,5-diphenyloxazole
bis-MSB	p-bis (o-methylstyryl) benzene
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N' - tetramethylethylene diamine

The nomenclature of ribosomal proteins followed the convention proposed by McConkey et al.(1979).

The activity of radioactive isotopes has been expressed in curies (Ci) throughout this thesis, although the International Commission on Radiation Units and Measurement has recommended the adoption of the becquerel as the International System (SI) unit of activity.

(1 Ci = 3.7×10^{10} becquerels)

SUMMARY

An investigation into the effect of oestradiol-17β on the synthesis of the components of polyribosomes in the immature rat uterus has been carried out. Previous studies have shown that rRNA synthesis is stimulated 2-4hr after treatment with the hormone. This is preceded by, and dependent on, a stimulation of the synthesis of HnRNA, detectable 30 min after giving the hormone. It has been postulated that mRNA sequences present in the HnRNA must be translated before the stimulation of rRNA synthesis can occur.

A method for the preparation of ribosomes from immature rat uteri, taking precautions to minimise ribonuclease activity, is described. Polysome profiles have been analysed on sucrose density gradients in order to detect alterations in protein synthesis following hormone treatment. Ribosomes from control animals are mainly in the form of monomers and dimers, but following oestrogen treatment, there is an increase in the proportion of polysomes. This effect is first detectable 30 min - 1hr after giving hormone, reaching a maximum after 4 hr and declining after 16 hr.

The kinetics of the synthesis of the components of the polysomes in response to oestrogen have been studied. The RNA was extracted from the polysomes and fractionated on oligo (dT) cellulose in order to study the synthesis of mRNA and rRNA, whilst the ribosomes were dissociated into subunits and the subunits separated on sucrose density gradients in order to study the synthesis of ribosomal proteins. It has been shown that the aggregation of ribosomes into polysomes 2-4hr after hormone treatment occurs as a result of the association of newly-synthesised mRNA with pre-existing ribosomes. Despite the stimulation of rRNA synthesis 2-4hr after oestrogen administration, the incorporation of newly-synthesised rRNA into ribosomes is not maximal until 12hr after hormone treatment. The incorporation of newly-synthesised ribosomal proteins into ribosomes is also maximal at this time.

Fractionation of these newly-synthesised proteins by polyacrylamide gel electrophoresis has been carried out in order to confirm their identity as

ribosomal proteins. The kinetics of the incorporation of newly-synthesised ribosomal proteins into ribosomes follow the kinetics of the stimulation of total protein synthesis in the uterus by oestrogen. The peak of maximal incorporation of newly-synthesised mRNA into polysomes coincides with the peak of maximal protein synthesis.

These results are discussed in relation to the postulated mechanism of oestrogen-stimulated rRNA synthesis in the immature rat uterus. The involvement of protein synthesis in rRNA synthesis is also discussed.

INTRODUCTION

1 INTRODUCTION

1.1 Control systems in mammals

Many different cell types are present in an individual, each cell type carrying out its own specialised function. It follows that, in order for the different cells to function together effectively, control mechanisms must exist to coordinate their activities. In mammals, as in most eukaryotic organisms, there are two types of control system. There is a fast-acting, electrical control system, or nervous system, in which electrical impulses are transmitted along specific pathways known as neurones or nerves. There is also a relatively slower-acting chemical control system, or endocrine system. In this latter system, chemical control is achieved by the action of hormones, which may be defined as chemical compounds formed in one part of the body and transported in the circulatory system to other organs and tissues whose activity they influence (Best and Taylor, 1945). The first report of such a "secreted body" or hormone was published by Bayliss and Starling (1902). These authors were able to demonstrate an increase in the secretion of pancreatic juice upon introducing acid into a loop of the small intestine whose nervous connections had been severed, so that the only communication with the pancreas was through the bloodstream. In addition, an extract of the duodenal mucosa led to the same result when injected into an animal.

Within the endocrine system, two classes of hormone can be distinguished on the basis of their proposed mechanism of action. The first class includes the peptide hormones and catechol amines whilst the second class includes the steroid hormones and thyroid hormones. In general, the physiological response to the former group is quite rapid. In the example of the "secreted body", or secretin, described by Bayliss and Starling (1902), increased pancreatic secretion was observed within 2 min of the introduction of acid into

the small intestine and died away rapidly unless a second stimulus was given. It was formerly believed that the peptide hormones and catechol amines did not enter the target cells to exert their physiological effect but, instead, bound to a receptor in the cell membrane. This resulted in the activation of an intracellular messenger system, which, in most cases, appeared to be an adenyl cyclase which was stimulated to synthesise adenosine 3':5' - phosphate (Rall and Sutherland, 1958 ; Sutherland and Robinson, 1966). Evidence for the extracellular site of action of peptide hormones came from studies in which insulin was covalently linked to a high molecular weight polymer, which did not penetrate cell membranes, and the insulin was shown to retain its biological activity (Cuatrecasas, 1969; Armstrong et al., 1972). In addition, cell membrane receptors for polypeptide hormones have been described (Cuatrecasas, 1974). However, the validity of the experiments in which insulin was linked to a high molecular weight support has been questioned since, under the incubation conditions employed, the covalent link between the polymer and insulin is unstable, resulting in the release of sufficient free insulin to account for the biological activity of the Sepharose-insulin (Kolb et al., 1975). Intracellular binding sites for polypeptide hormones have also been reported (Goldfine and Smith, 1976). Therefore controversy surrounds the mechanism of action of the polypeptide hormones. They may exert their action at the surface of the cell and only subsequently enter the cell in order to be degraded or for the receptor to be recycled. Alternatively, it is possible that the hormone binds to the cell membrane and causes short term effects via a secondary messenger, and the long term effects of the hormone, if any, occur after its entry into the cell (Kolata, 1978).

Steroid and thyroid hormones belong to the second group of hormones. Although these hormones can also lead to responses in their target

tissues within minutes, their action is usually more prolonged, in contrast to the action of the polypeptide hormones and catechol amines. The action of steroid and thyroid hormones results in an alteration of the pattern of macromolecular synthesis of their target cells. The mechanism of action of steroid hormones will be discussed below.

1.2 Steroid hormones

Steroids are small, hydrophobic molecules derived chemically from a parent compound cholesterol (Figure 1).

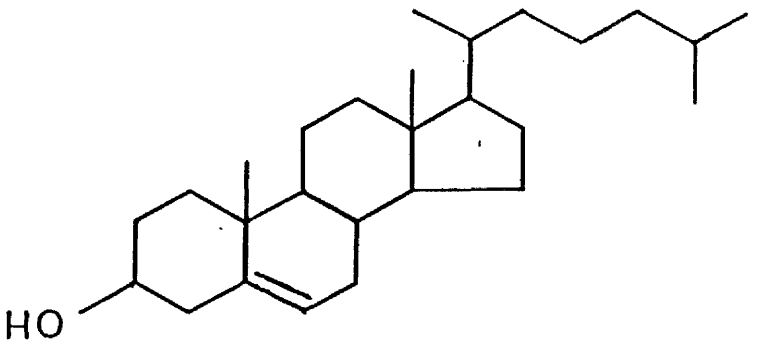


Figure 1. Chemical structure of cholesterol (cholest-5-ene-3β-ol)

This 27-carbon compound is converted to pregnenolone, a 21-carbon compound, by a series of biosynthetic steps common to all mammalian steroid hormones (Baird, 1972). Pregnenolone can be converted to the 21-carbon corticosteroids and progesterone, the 19-carbon androgens and the 18-carbon oestrogens.

Steroid hormones have been described in arthropods and vertebrates and the biosynthesis of steroidal oestrogens has also been demonstrated in plants (Young et al., 1977), although the physiological significance of steroid hormones in plants is not clear.

1.2.1 Mechanism of action of steroid hormones

In this section, the mechanism of action of steroid hormones in general will be outlined before going on to describe in detail the action of oestrogen in the uterus.

Nearly all steroid hormones induce the synthesis of one or more proteins in their target cells (Tomkins and Martin, 1970). This

was first suggested by the experiments of Knox and Auerbach (1955) who demonstrated that the activity of tryptophan pyrrolase, an enzyme present in the liver, was reduced in rats following adrenalectomy. Treatment with cortisone, one of the glucocorticoid hormones normally produced by the adrenal glands, led to an increase in tryptophan pyrrolase activity in both normal and adrenalectomised animals. Schimke et al. (1965) subsequently showed that this glucocorticoid-stimulated increase in tryptophan pyrrolase activity was a result of de novo synthesis of the enzyme. The induction of the synthesis of certain proteins appears to be a fundamental action of all steroid hormones (O'Malley et al., 1969; Tomkins and Martin, 1970).

It is believed that steroid hormones act by a two-step mechanism in which the hormone is bound by a cytoplasmic receptor protein before being translocated to the nucleus. This suggestion was made as a result of experiments on the cellular location of radioactively-labelled oestrogen carried out in the laboratories of Jensen (Jensen et al., 1968) and Gorski (Shyamala and Gorski, 1969). A simplified version of this two-step mechanism is shown in Figure 2.

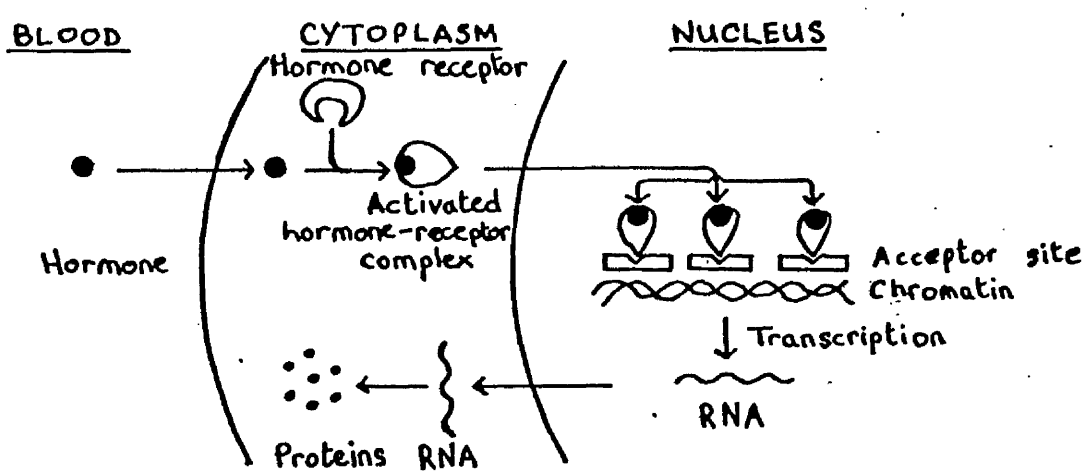


Figure 2. Two-step mechanism of steroid hormone action (From Leake (1976)).

In outline, the hormone is transported in the bloodstream from the endocrine organ to its target cell. It passes across the cell membrane into the cytoplasm of the cell where it becomes bound by a specific hormone receptor. This binding occurs with a very high affinity and, under physiological conditions, it results in a modification of the receptor known as activation. In this activated state, the hormone-receptor complex can pass into the nucleus. Once inside the nucleus, the hormone-receptor complex becomes associated with the chromatin at sites known as acceptor sites. This results in the induction of the responses characteristic of the hormone, such as the stimulation of ribonucleic acid (RNA) synthesis and protein synthesis. This two-step mechanism of action has been studied most extensively for oestrogens, but it appears to be applicable to all steroid hormones (Jensen and DeSombre, 1972; Gorski and Gannon, 1976).

Although this model of steroid hormone action has been the subject of very detailed examination and a great deal of evidence has been accumulated in support of its major concepts, a few aspects of the model are still poorly defined. In particular, the mechanism by which the activated hormone-receptor complex translocates to the nucleus, interacts with nuclear components and stimulates the expression of particular genes is far from clear.

The most attractive hypothesis to describe the effects of steroid hormones at the nuclear level suggests that the steroid induces the transcription of specific messenger RNA (mRNA) molecules from the deoxyribonucleic acid (DNA) template. The mRNA sequences then code for the synthesis of the particular proteins induced by the hormone. This hypothesis was originally based on an analogy with the lac repressor system in the bacterium Escherichia coli, first described by Jacob and Monod (1961). In this organism, the genes coding for

three enzymes concerned with the metabolism of lactose are transcribed as a single unit, known as an operon. The three enzymes are β-galactosidase, β-galactoside permease and galactoside acetylase, and the complete unit is known as the lac operon. In addition to these three structural genes, there is another region of DNA present in the operon, termed the operator region, which can bind a protein known as a repressor. When the repressor is bound, transcription of the structural genes is prevented. Lactose, however, can combine with the repressor and prevent its binding to the operator region, thus allowing transcription of the operon. Therefore, the presence of lactose in the medium induces, or de-represses, the synthesis of these three enzymes concerned in its metabolism.

An early line of evidence supporting the view that steroid hormones acted as specific de-repressors of genes came from studies on the action of the moulting hormone, ecdysone, in insects (Clever and Karlson, 1960; Karlson, 1963; Clever, 1966). The salivary glands of certain insects, such as Chironomus tentans, contain giant, polytene chromosomes which appear "puffed up" at certain sites along their length. These regions, known as chromosome puffs, are sites of RNA synthesis (Pelling, 1959). Treatment of larvae with ecdysone leads to a rapid alteration in the pattern of chromosome puffs, new puffs appearing whilst pre-existing puffs regress. These results led Clever and Karlson to postulate that the primary effect of ecdysone was to alter the activity of specific genes.

Such studies have now been extended by the direct demonstration of a hormone-induced increase in the number of mRNA sequences coding for a specific protein in a number of experimental systems. The increase in mRNA activity can be demonstrated either by extracting the RNA and translating it in an heterologous, cell-free protein synthesising system

or, more directly, by hybridisation of cellular RNA to a complementary DNA transcript of the particular mRNA species believed to be induced by the hormone. The most extensively studied system in which the hormone-dependent induction of specific mRNA species has been demonstrated by these techniques is the chick oviduct system. Treatment of female chicks with oestrogen results in differentiation of the oviduct which starts to synthesise the major egg-white proteins (O'Malley et al., 1969). In the case of three of these proteins, ovalbumin, conalbumin and ovomucoid, it has been shown that there is an increase in the level of the mRNA species in response to oestrogen (Means et al., 1972; Chan et al., 1973; Palmiter, 1973; Rhoads et al., 1973; McKnight et al., 1975; Palmiter et al., 1976; Mulvihill and Palmiter, 1977; S. Tsai et al., 1978). Progesterone has also been shown to have the same effect on the mRNA species coding for ovalbumin and conalbumin if it is administered to chicks which have been previously treated with oestrogen, so that differentiation of the oviduct has occurred, and then the oestrogen is withdrawn (McKnight et al., 1975; Palmiter et al., 1976). In addition, progesterone induces the mRNA coding for another egg-white protein, avidin, which is not induced by oestrogen alone (Chan et al., 1973; Sperry et al., 1976).

Other examples of the induction of specific mRNA species by a steroid hormone are known. Glucocorticosteroids stimulate the levels of the mRNAs coding for tyrosine amino transferase and tryptophan oxygenase in rat liver (Schutz et al., 1975; Ernest et al., 1978; Hoffer and Sekeris, 1978). Oestrogen induces the mRNA coding for the egg yolk protein precursor, vitellogenin, in the liver of roosters (Deeley et al., 1977; Gordon et al., 1977; Burns et al., 1978; Jost et al., 1978) and the liver of the clawed toad, Xenopus laevis, (Clemens, 1974; Baker and Shapiro, 1977; Farmer et al., 1978), and androgens induce the

mRNA coding for a number of proteins, including aldolase, in the ventral prostate of rats (Mainwaring et al., 1974; Parker and Scrace, 1978; Mezzetti et al., 1979).

These studies all provide evidence supporting the hypothesis that steroid hormones act by regulating the accumulation of specific mRNA molecules. However, in a number of cases, the synthesis of all classes of RNA is stimulated by the hormone (O'Malley et al., 1969; Tomkins and Martin, 1970). In the case of androgens acting on rat seminal vesicle, Higgins and Burchell (1978) have shown that the hormone controls the synthesis of two basic, secretory proteins by regulating the level of translatable mRNA coding for these two proteins. However, this effect of androgens is part of a general effect on mRNA and also on total cellular RNA, so that the hormone does not regulate the synthesis of the secretory proteins independently of general protein synthesis.

Other mechanisms by which steroid hormones may influence gene expression have been postulated. One possibility is that the hormone acts at the level of post-transcriptional processing and transport of the mRNA. Tomkins has proposed a post-transcriptional control model based on studies of the so-called paradoxical effect of inhibitors of RNA synthesis (Tomkins et al., 1972). In the liver, and in cultured hepatoma cells, tyrosine amino transferase is induced by glucocorticoids. Removal of the steroid leads to deinduction of the enzyme, but treatment with actinomycin D, an inhibitor of RNA synthesis, after induction of the enzyme has occurred, not only prevents deinduction but can also "superinduce" the enzyme beyond the levels elicited by the steroid. Tomkins has interpreted these results as being due to the presence of a labile, post-transcriptional repressor which prevents translation of the mRNA coding for tyrosine amino transferase and promotes its degradation. The steroid inhibits the activity of this repressor whilst actinomycin D

prevents transcription of the gene coding for the repressor, thus allowing translation of the more stable tyrosine amino transferase mRNA molecules which have accumulated under the influence of the steroid. More recent experiments have demonstrated superinduction of tyrosine amino transferase by protein synthesis inhibitors, suggesting that the post-translational repressor is a labile polypeptide (Ernest et al., 1978; Hofer and Sekeris, 1978).

Superinduction of egg-white proteins has also been demonstrated in the chick oviduct following treatment with actinomycin D. Palmiter and Schimke (1973) have proposed that this occurs as a result of competition at the translational level. The rate of ovalbumin synthesis does not decline as rapidly as the rate of synthesis of non-secretory proteins following treatment with actinomycin D. Palmiter and Schimke therefore suggested that those mRNA molecules which are superinduced have longer half-lives than other mRNA molecules, so will form a greater proportion of the mRNA population following inhibition of RNA synthesis. Therefore, they will be able to compete more effectively for rate-limiting factors for translation.

Palmiter had, in fact, previously suggested that oestrogen acts at the translational level, in addition to its effect on mRNA transcription, in the chick oviduct (Palmiter, 1972). Analysis of the rates of initiation and elongation of polypeptide chains suggested that treatment with oestrogen doubled the rate of initiation and also led to a small increase in the rate of elongation. Pennequin et al (1978) have confirmed these findings and have also noted similar effects of progesterone on oestrogen-treated chicks. However, in contrast to oestrogen which affects translation in a non-specific manner, progesterone appears to regulate preferentially the initiation of ovalbumin mRNA sequences.

These studies were carried out on the chick oviduct in which the major effect of the hormone is to regulate the rate of mRNA production.

An experimental system in which steroid hormones appear to regulate translation without affecting transcription is the chick blastoderm cultured in vitro. This tissue is stimulated by 5 β-reduced steroids to produce foetal haemoglobins, and it appears that the globin mRNA molecules are already present, but are not translated, prior to hormone stimulation (Irving et al., 1976). The steroid appears to act at the translational level by stimulating initiation (Irving and Mainwaring, 1976).

Another possible site of action of steroid hormones which has been proposed, is the cell membrane. Amphibian oocytes are arrested at the prophase of the first meiotic division. Administration of progesterone in vitro leads to meiotic maturation, that is meiosis proceeds to the metaphase of the second meiotic division and the first polar body is extruded. In this state, the oocyte is ready for fertilisation. The hormone is effective on enucleated oocytes, so it does not appear to be acting by the two-step mechanism (Baulieu et al., 1978). A progesterone analogue covalently linked to a polyethylene oxide polymer is also effective under conditions in which cleavage of the bond between the hormone and the polymer does not occur, suggesting that progesterone is acting at the cell membrane (Godeau et al., 1978). However, although Baulieu et al. (1978) did not detect progesterone receptors in the cytoplasm of the oocyte, Kalimi et al., (1979) have reported the presence of progesterone receptors. The interaction between progesterone and the cell membrane of the oocyte therefore remains controversial. Another example of a steroid hormone interacting with cell membranes, in this case oestradiol-17β and the surface of endometrial cells, has been described by Pietras and Szego (1976).

In conclusion, the evidence presented in this section is consistent with the major effects of steroid hormones being at nuclear sites within cells. The hormone interacts with the nucleus by a two-step mechanism

involving cytoplasmic receptor proteins and this appears to result in a modulation of transcription. In addition, the steroid hormone may also act at other, non-nuclear, sites to complement its effect at the nuclear level.

The action of oestradiol-17β on the rat uterus will be discussed in the next section.

1.2.2 Action of oestrogen on the immature rat uterus

In the adult, female rat, the oestrous cycle lasts approximately 4 days. As the Graafian follicles mature, they secrete increasing amounts of oestrogen. The level of oestrogen in the plasma reaches a peak of about 30 pg per ml just before the animal enters the oestrus phase of the cycle (Brown-Grant et al., 1970). One of the effects of this secretion of oestrogen is to initiate a process of growth and development in the uterus. The endometrium becomes vascularised and there is proliferation of the glandular elements. If corpora lutea are formed from the Graafian follicles, as a result of copulation, the level of progesterone secreted by the ovary will increase. This hormone appears to stimulate secretion by the endometrial glands. In this way, the uterus is prepared for the implantation and maintenance of the blastocyst should successful fertilisation occur.

The action of oestrogen is most easily studied in immature or ovariectomised animals, in which the level of free, circulating oestrogens should be negligible. Immature, female rats have been used in the experiments described in this thesis.

1.2.2.1 Transport of oestrogen to target organs

Oestrogen is transported in the bloodstream from the ovaries to the target organs. Approximately 90% of the steroid in the blood is bound to plasma proteins. In the adult rat, most of the oestrogen is bound to albumin, which is the plasma protein present in highest concentrations. Some oestrogen is also transported by corticosteroid binding globulin.

In the immature animal, another protein, known as α-fetoprotein, is also present in the blood. This is a glycoprotein belonging to the α-globulin class of plasma proteins. It is present in relatively high concentrations in foetal blood and its level declines during the first 5 weeks following birth, although it is still present at a concentration of 1mg/ml in 21 day-old animals (Uriel et al., 1976). This protein binds oestrogen with a high affinity, studies by Versee and Barrel (1978) having shown that 0.4 molecules of oestradiol are bound per molecule of α-fetoprotein at physiological pH. Because of its oestrogen-binding properties, α-fetoprotein is also known as oestrogen binding plasma protein or EBP (Baulieu, 1975; Germain et al., 1978). It is specific for oestrogen, unlike albumin which can bind a large number of substances in addition to oestrogen (Bennhold, 1966). However, albumin has a higher capacity since its concentration in the blood is 20-30 times that of α-fetoprotein.

It is not clear what the function of these transport proteins is. It is believed that only unbound hormone is biologically active. Since an equilibrium will exist between the free and protein-bound hormone, the latter could serve as a reservoir of hormone. Binding of a hormone to a carrier protein could also protect the hormone from degradation by the liver, thereby assisting its transport from the endocrine organ to the target organ.

1.2.2.2 Entry of oestrogen into target cells

It is believed that steroid hormones, being lipophilic molecules, enter cells by passive diffusion across the cell membrane. Therefore steroid hormones should be able to enter non-target cells as well as target cells, and this was first demonstrated for oestradiol by Jensen and Jacobson (1960). On treating immature rats with tritiated oestradiol, the label was taken up very rapidly by non-target tissues, such as muscle and kidney, as well as target tissues, such as the uterus and vagina. However,

only the target tissues were found to accumulate the hormone against a concentration gradient in the blood, so that the label was retained by these tissues for a period of several hours, whilst the label was lost by the non-target tissues as the level of hormone in the blood declined. This indicated that there was no permeability barrier to the entry of oestradiol into cells, but the hormone could pass out of the cells again unless retained by a receptor.

The presence of intracellular receptors for steroid hormones can lead to problems in the study of entry into cells since it is difficult to differentiate between transport across the cell membrane and intracellular binding by the cytoplasmic hormone receptor. Some authors have proposed that the entry of oestrogen into uterine cells occurs by a protein-mediated step on the basis of being able to distinguish between these two parameters (Baulieu, 1975; Uriel et al., 1976). On measuring the entry of oestradiol at a range of physiological hormone concentrations, Milgrom et al.(1973) found that entry was saturable at higher concentrations. In addition, these authors found that the specificity of oestrogen entry was different from that of binding to the cytoplasmic receptor and also that thiol-blocking reagents inhibited oestradiol entry under conditions in which the receptor was unaffected. However, Peck et al.(1973) were unable to confirm these findings. They found that the initial rate of cellular uptake of oestradiol was the same for the diaphragm, a non-target organ, and the uterus, was non-saturable, and was unaffected by N-ethylmaleimide, a thiol-blocking reagent not used by Milgrom et al.

The fact that uptake of oestrogen by a non-target organ occurs at the same initial rate as the uptake by a target organ, would appear to support the case for entry of the steroid by passive diffusion. However, experiments using intact uterine tissue may be subject to errors due to diffusion of the hormone through the various cell layers and binding of

the hormone to non-specific extracellular sites. In an attempt to avoid such errors, Muller and Wotiz (1979) used free uterine cell suspensions. These authors showed that the uptake of steroid was extremely rapid and was a linear function of the concentration of oestrogen in the medium, suggesting that entry is by passive diffusion.

Therefore, on balance, the evidence would appear to support entry of the hormone into the cell by passive diffusion rather than by a protein-mediated step. Whatever the mechanism, entry does not appear to be energy-dependent since it is unaffected by inhibitors of oxidative phosphorylation (Shyamala and Gorski, 1969).

1.2.2.3 Binding of oestrogen to the cytoplasmic receptor

In the preceding section, reference was made to the experiments of Jensen and Jacobson (1960) in which retention of oestradiol by the uterus and other target organs was observed. This suggested that a receptor for oestradiol was present in the uterine cells. This retention did not appear to be due to metabolism of oestradiol by the uterus since oestradiol was the major labelled substance recovered at the end of the experiment (Jensen and Jacobson, 1962). The oestrogen receptor was characterised by subjecting uterine cytoplasm from animals injected with tritiated oestradiol to sucrose density gradient centrifugation. Most of the radioactive label was bound to a substance with a sedimentation coefficient of 9.5 S (Toft and Gorski, 1966). No such receptors were found in non-target tissues. This binding was specific for oestrogens, since steroid and non-steroid oestrogens inhibited the binding of tritiated oestradiol whereas non-oestrogenic steroids did not (Noteboom and Gorski, 1965). Binding was also inhibited by treatment with a protease, and these results led Noteboom and Gorski to propose that the oestrogen receptor was a protein.

In addition to binding the hormone with a high specificity, the oestrogen receptor also displays a very high affinity for the steroid.

Measurement of the dissociation constant for theequilibrium between the uterine receptor and oestradiol-17β has given values of 10^{-9} - 10^{-10} M (Jensen and DeSombre, 1972; Baulieu, 1975) which are of the same order as the circulating levels of the hormone. Quantification of the number of receptors present has given values ranging from 6,000 - 20,000 per cell (Leake, 1976) and even as high as 100,000 per cell (Jensen and DeSombre,. 1972). This means that the receptor is present at a very low concentration in the cell, making its purification and subsequent characterisation very difficult. Most of the characterisation of the rat uterine oestrogen receptor has been concerned with the determination of its sedimentation coefficient on sucrose density gradients by comparison with marker proteins. A range of values has been obtained, depending on the ionic strength and protein concentration during the preparation (Chamness and McGuire, 1972; Stancel et al., 1973). In their initial experiments, Toft and Gorski (1966) used a low ionic strength, and, under these conditions, the sedimentation coefficient of the oestrogen receptor can vary from 7 S to 9.5 S, although 8 S is the commonest value reported. In 0.15 M potassium chloride, which is thought to approximate the intracellular ionic strength, values of 3.8 S - 7 S are obtained, whilst in 0.4 M potassium chloride, the receptor sediments at 3.8 S - 5.2 S, with 4 S being the commonest value reported. In addition to these effects of ionic strength, the receptor appears to undergo concentration-dependent aggregation (Stancel et al., 1973) . The 4 S form is believed to be the form present in the cytoplasm in vivo and this will be used when referring to the cytoplasmic oestrogen receptor throughout this thesis. When isolated under high salt conditions, the rat uterine cytoplasmic oestrogen receptor has a molecular weight of 76,200 (Notides and Nielsen, 1974).

In immature rats, the number of cytoplasmic oestrogen receptors per cell is very low at birth but increases during the first few days of life

until a maximum value is reached after 10 days, the number remaining fairly constant after this time until puberty (Clark and Gorski, 1970). In mature rats, it appears that the number of uterine cytoplasmic oestrogen receptors varies during the oestrous cycle, although conflicting reports have been published. Some authors have reported that the number is maximal at pro-oestrus, when the circulating level of oestrogen is highest (Feherty et al, 1970), others have reported that the number is lowest at pro-oestrus (Lee and Jacobson, 1971), whilst White et al. (1978) could find no changes in the concentration of cytoplasmic receptor throughout the cycle.

Although most studies on the cytoplasmic oestrogen receptor have been carried out on the immature rat uterus, it appears that uterine oestrogen receptors from other mammals have similar properties. Sica and Bresciani (1979) have purified the oestrogen receptor from calf uterus to apparent homogeneity, a single band of molecular weight 70,000 being observed on polyacrylamide gel electrophoresis. In addition, a calcium-derived oestrogen receptor has been reported to occur in the cytosol fraction of this same tissue (Molinari et al., 1977). This is produced by the action of a calcium-dependent protease on the 4 S receptor (Puca et al., 1977). This calcium-derived receptor still sediments at 4 S but it does not aggregate on lowering the ionic strength. The significance of this calcium-derived receptor is not clear. It may be an experimental artifact or it may represent the way in which the oestrogen receptor is metabolised by the uterine cell.

1.2.2.4 Translocation of the hormone-receptor complex to the nucleus

On giving tritiated oestradiol to immature rats, it was shown, by sub-cellular fractionation (Noteboom and Gorski, 1965) or autoradiography (Stumpf, 1968), that approximately 60% of the label was localised in the nuclei of the uterine cells. The hormone could be extracted from the nuclei with 0.3 M potassium chloride, when it was found

to be complexed with a protein of sedimentation coefficient 5 S (Gorski et al., 1968; Jensen et al., 1968). A temperature-dependent transformation of the 4 S hormone-receptor complex to this 5 S hormone-receptor complex was shown to occur if the cytosol fraction of the uterus were incubated at 25°C with oestradiol (Brecher et al., 1970). This temperature-dependent conversion is known as activation and it appears to result from the addition of an extra subunit of molecular weight 50,000 to the 4 S receptor (Notides and Nielsen, 1974; Yamamoto, 1974). Activation can take place in the absence of nuclei in vitro (Brecher et al., 1970; Jensen et al., 1971), suggesting that it normally occurs in the cytoplasm in vivo. No 5 S hormone-receptor complex is formed if nuclei are incubated with tritiated oestradiol in the absence of cytosol (Jensen et al., 1968; Shyamala and Gorski, 1969). If, however, uteri are incubated with tritiated oestradiol at 0°C, when the cytoplasmic 4 S hormone-receptor complex will be formed, and then transferred to a hormone-free medium and incubated at 37°C, the 5 S hormone-receptor complex will be formed and will translocate to the nucleus, with concomitant depletion of the 4 S hormone-receptor complex in the cytoplasm (Shyamala and Gorski 1969; Giannopoulos and Gorski, 1971). Such a hormone-induced translocation can also be demonstrated if a physiological dose of tritiated oestradiol-17β is given to immature rats in vivo (Jensen et al., 1969; Shyamala and Gorski, 1969).

These experiments provided the initial evidence upon which the two-step theory of steroid hormone action was based. This theory explains the fall in the level of the 4 S hormone-receptor complex in the cytoplasm accompanied by the rise in the level of the 5 S hormone-receptor complex in the nucleus by saying that the 5 S receptor is derived from the 4 S receptor. Further evidence that the two forms of receptor are related comes from the fact that they show similar specificities of binding hormone agonists and antagonists (Jensen and DeSombre, 1972) and that,

in the presence of urea, the 5 S hormone-receptor complex sediments at 4 S (Notides and Nielsen, 1974). In addition, an antiserum raised against calf uterine nuclear receptor cross-reacts with both cytoplasmic and nuclear receptors from rat uterus (Greene et al., 1977). This last result may, however, be fortuitous since Fox (1978) reported that antisera raised against several different antigens contained a component that could interact with the nuclear oestrogen receptor.

Very little is known about the mechanism of translocation of the hormone-receptor complex from the cytoplasm to the nucleus. The original studies of Shyamala and Gorski (1969) showed that the process was not energy-dependent, nor was it inhibited by cycloheximide, suggesting that it was not linked to protein synthesis. One interesting hypothesis, proposed by Gorski and Gannon (1976), suggests that translocation can occur as a result of differences between the water spaces in the nuclear and cytoplasmic compartments of the cell. This hypothesis is based on the observation that radioactively-labelled inulin, injected into frog oocytes, becomes concentrated in the nuclei although it does not bind to any nuclear components (Horowitz and Moore, 1974). It appears that inulin is excluded from the water space of the cytoplasm because of the interaction of water with cytoplasmic macromolecules, making a large proportion unavailable as a solvent. According to Gorski and Gannon, it is possible that the oestrogen receptor exists in the cytoplasm in some way loosely bound to other components, and interaction with the hormone releases the receptor so that it can equilibrate with the larger water space in the nuclear compartment.

The nuclear 5 S hormone-receptor complex has a molecular weight of 132,700 (Notides and Nielsen, 1974) and, like the 4 S hormone-receptor complex, it aggregates in the absence of salt. Although nuclear receptors are usually extracted with potassium chloride, not all the nuclear bound oestrogen is removed by this treatment (Clark and Peck,

1976; Barrack et al., 1977; Traish et al., 1977). This would suggest that some oestrogen-receptor complexes are bound more tightly than others (Clark and Peck, 1976), although Traish et al (1977) claimed that the resistance to salt extraction was a result of physical entrapment in the nuclear pellet and therefore probably not of physiological significance.

Nuclear binding of oestrogen appears to be of physiological importance in the uterotrophic response to the hormone (Leake, 1976). Macromolecular synthesis is stimulated almost immediately after the hormone has entered the nucleus. One of the earliest responses in the rat uterus is the stimulation, after about 40 min, of the synthesis of a protein known as induced protein (IP). The degree of induction of this protein is proportional to the amount of nuclear bound oestradiol (Katzenellenbogen and Gorski, 1972). Later responses to the hormone, such as the stimulation of DNA synthesis after 24 hr , require the long term retention of oestrogen-receptor complexes in the nucleus. Thus, oestradiol-17β , if administered as a single injection, will be retained in the nucleus at a low level for up to 24 hr and will elicit the long term responses to the hormone, whilst a single dose of oestriol, which is lost from the nucleus after 6hr, will only elicit the early responses (Anderson et al., 1972, 1975; Stormshak et al., 1976; Clark et al., 1977). As well as demonstrating the physiological significance of nuclear binding, these experiments, with two different oestrogens which bind to the same receptor, also show that the structure of the hormone is important for the uterotrophic response. The function of the steroid, therefore, involves more than transporting the receptor protein to the nucleus. Other factors are required, in addition to nuclear binding, for the response to the hormone to occur. Although nuclear binding of exogenous oestradiol can be demonstrated in the uteri of rats at birth, the capacity to respond to the hormone is not aquired until 1-2 weeks after birth (Somjen et al., 1973).

The amount of oestrogen-receptor complex in the nucleus declines fairly slowly, following the initial translocation of the cytoplasmic hormone-receptor complex, until the control level is reached after 24hr. The level of cytoplasmic receptors at this time is elevated above control values (Clark et al., 1973), and this would suggest that oestrogen induces the synthesis of its own receptor. Additional evidence comes from studies on adult rats showing that, although nuclear receptor levels are highest at pro-oestrus, there is no depletion of cytoplasmic receptor levels (Feherty et al., 1970; White et al., 1978). The replenishment of cytoplasmic receptor levels commences some time before 24 hr after hormone treatment and is inhibited by cycloheximide, an inhibitor of protein synthesis, and actinomycin D, an inhibitor of RNA synthesis, suggesting that replenishment is due to synthesis of receptors (Jensen et al., 1969; Sarff and Gorski, 1971). However, it is possible that some recycling of the nuclear receptor occurs. The anti-oestrogen nafoxidine has some oestrogenic activity in rats. It binds to the oestrogen receptor, resulting in translocation to the nucleus, but the receptor is retained in the nucleus for up to 19 days. The normal uterine growth responses occur for the first 24 hr , but there is no replenishment of cytoplasmic receptors. Consequently, the uterus will be unresponsive to further injections of oestrogen (Clark et al., 1973). This would suggest that, under normal circumstances, some recycling of the receptor occurs in some protein synthesis-dependent fashion (Leake, 1976). It is possible that the cytoplasmic receptor levels are replenished, partially by recycling and partially by the synthesis of new receptors, the latter eventually leading to an elevated level of oestrogen receptors.

1.2.2.5 Nuclear acceptor sites

Once the oestrogen-receptor complex has entered the nucleus, it becomes bound at certain sites known as acceptor sites. A great deal of controversy surrounds the identity of the nuclear components engaged in the high affinity binding of hormone-receptor complexes. Nearly every component of the nucleus has been proposed as the acceptor site. Thus, DNA, histones, non-histone proteins, ribonucleoprotein particles, nuclear membranes and RNA polymerase have all been implicated in the binding of the hormone-receptor complex (Gorski and Gannon, 1976). The bulk of the evidence has favoured the attachment of hormone-receptor complexes to chromatin, in particular to the DNA and non-histone proteins.

Binding of radioactively-labelled oestradiol to uterine chromatin was shown to occur very rapidly following treatment of rats with the hormone (Teng and Hamilton, 1968). Similar studies carried out on the calf uterus have also demonstrated binding to the chromatin (Maurer and Chalkley, 1967). The oestrogen-receptor complex can be shown to be associated with nucleosomes following micrococcal nuclease digestion of chromatin from rat uterus (Senior and Frankel, 1978) and sheep uterus (Andre et al., 1978).

DNA has been proposed as the acceptor for the oestrogen-receptor complex in the rat uterus since it has been shown that the complex will interact with DNA (King and Gordon, 1972; Yamamoto and Alberts, 1974). However, this binding is non-specific; oestrogen-receptor complexes will bind, with a similar affinity, to double-stranded DNA from rat uterus, calf thymus, salmon sperm, the bacterium Escherichia coli, and even to the synthetic polymer poly (dA- ^{d}T) (Yamamoto and Alberts, 1974; Leake, 1976; Kallos and Hollander, 1978). It would appear, therefore, that DNA alone cannot act as the acceptor site for the oestrogen-receptor complex. There is also evidence which suggests that binding of the oestrogen-receptor complex to the non-histone proteins of the chromatin occurs. Such

experiments are, however, hampered by certain difficulties. The oestrogen-receptor preparations could contain substances which inhibit binding, resulting in an under-estimation of the number of acceptor sites (Gorski and Gannon, 1976). Alternatively, the tendency of the oestrogen-receptor complex to aggregate, particularly in the presence of polyanions, could lead to artifacts if the binding to putative acceptor sites is studied by a technique involving co-sedimentation or filtration on Millipore filters (Yamamoto and Alberts, 1974). Thus, the target tissue-specific binding of oestrogen receptors to uterine chromatin reported by Steggles et al.(1971), using Millipore filtration, may have resulted from receptor aggregation, possibly resulting from interaction with acidic polysaccharides in the nuclear extracts.

These problems of receptor aggregation can be circumvented if the nuclear components are covalently linked to an inert matrix and the retention of oestrogen receptor by columns of these materials is studied. By these techniques, it has been shown that binding occurs to basic, non-histone proteins in the calf uterus (Puca et al., 1974, 1975) and to a soluble, nuclear protein extract of rat uterus (Mainwaring et al., 1976). This binding is of very high affinity and is dissociated by potassium chloride. However, Puca et al. (1975) showed that similar acceptor sites were also present in non-target tissues, whereas Mainwaring et al.(1976) demonstrated that more binding of the oestrogen-receptor complex occurred to a nuclear extract from the uterus than to nuclear extracts from non-target tissues.

The above results would therefore appear to implicate both DNA and non-histone proteins in forming the acceptor sites for oestrogen-receptor complexes in the uterus. However, the significance of such binding studies carried out in vitro has been questioned. Higgins et al.(1973) treated immature rats with oestrogen, thereby "pre-loading" the nuclei of the intact cells of the uterus. Uterine nuclei were then isolated and it

was found that they did not exhibit decreased binding of oestrogen-receptor complexes in a cell-free system compared to nuclei from untreated rats. This would suggest that the acceptor sites in isolated nuclei are topographically different from those in the intact cell (Higgins et al., 1973; Leake, 1976). Furthermore, the number of acceptor sites appears to be too high. Saturation of uterine chromatin with oestradiol-receptor complex corresponds to 8,000 molecules per cell, which is inconsistent with the role of steroid hormones as activators of a limited number of genes (Leake, 1976). Yamamoto and Alberts (1975, 1976) have postulated that there are a large number of low affinity acceptor sites which mask a small number of high affinity acceptor sites at which the physiological actions of the hormone occur. This hypothesis is based on an analogy with the lac repressor system in E. coli. The lac repressor protein binds with a very high affinity to DNA containing a specific base sequence normally present in the operator region of the lac operon, but it will bind to other regions of DNA with a low affinity. In a similar way, oestrogen-receptor complexes could bind to a large number of sites with a low affinity and as many as 1,000 high affinity acceptor sites could be masked. The low affinity sites could be necessary for co-operative binding of oestrogen-receptor complexes to occur in order to unmask a single gene (Yamamoto and Alberts, 1976), or they could "funnel" the hormone-receptor complexes to the high affinity sites (Jaffe et al., 1975).

There is evidence from other steroid hormone-dependent systems that nuclear binding of the steroid-receptor complex involves the non-histone protein component of the chromatin. Thus, in the rat prostate, the androgen receptor has been reported to bind to the non-histone proteins (Mainwaring et al. 1976; Wang, 1978) and characterisation of the acceptor sites has shown that they are basic, non-histone proteins (Mainwaring et al., 1976). In the chick oviduct, nuclear binding of progesterone receptors has been extensively studied. It has been shown that progesterone-receptor complexes

will bind more strongly to oviduct chromatin than to chromatin from non-target tissues (Spelsberg et al., 1971). Further experiments have demonstrated that the chromatin can be dissociated into its DNA, histone and non-histone protein components and reconstituted without loss of its binding characteristics. Spelsberg et al.(1971, 1972) were able to prepare hybrid chromatins, using components from different tissues, and the ability to bind the progesterone-receptor complex was found to reside in the non-histone, or acidic, protein component. Further analysis of the non-histone proteins showed that binding occurred to one particular fraction, designated AP3 (O'Malley et al., 1972; Spelsberg et al., 1972), and that other non-histone protein fractions could mask these sites, partially in the oviduct and totally in non-target tissues (Spelsberg et al., 1976). In this particular experimental system, the steroid receptor is unusual in that it is dimeric (Schrader and O'Malley, 1972). The A subunit on its own binds to naked DNA whilst the B subunit binds to chromatin. Both subunits have been extensively purified (Schrader et al., 1977; Coty et al., 1979) and it is now possible to obtain, by molecular cloning quanties of purified fragments of DNA containing the ovalbumin gene (Garapin et al., 1978; Kourilsky and Chambon, 1978; Woo et al., 1978; Dugaiczyk et al., 1979; Gannon et al., 1979). Using these purified components, it may be possible to study further the binding of the hormone-receptor complex to a gene whose function it controls.

The uterus differs from the chick oviduct system in that no evidence has been obtained to suggest that the 4 S cytoplasmic oestrogen receptor is dimeric. Moreover, Puca et al. (1974) showed that the acceptor sites in the calf uterus were basic, non-histone proteins, whereas Spelsberg et al.(1971, 1972) termed the oviduct acceptor sites acidic proteins. However, it is possible that the extraction procedures employed by Spelsberg et al.may have resulted in the inclusion of small amounts of basic proteins

in the acidic protein fractions.

In the rat uterus, the integrity of the acceptor sites appears to depend on the three-dimensional structure of the chromatin. Both DNA and non-histone proteins are strongly implicated in forming the acceptor site. It has been suggested that the non-histone proteins could play an active role, as the actual acceptor, or a passive role by modifying the interaction of the hormone-receptor complex with the DNA (King and Gordon, 1972; King and Mainwaring, 1974).

1.2.2.6 Transcriptional effects of oestrogen

The most striking effects of oestradiol -17β on the rat uterus involve alterations in the transcription of RNA from the DNA template. These transcriptional effects are amongst the earliest responses to the hormone. Nevertheless, there are some effects of oestradiol which do not appear to involve transcription.

Within 15 min of hormone administration, hyperaemia of the uterus can be detected which appears to lead to an increased accumulation of fluid by the organ (Mueller et al., 1958). It has been proposed by Szego (1965) that the hyperaemic effect is mediated by histamine released from the uterus. The increased uptake of water persists for up to 6hr (Mueller et al., 1958; Billing et al., 1969 a) and it is not abolished by actinomycin D, an inhibitor of DNA-directed RNA synthesis (Ui and Mueller, 1963). This increase in uterine permeability also leads to an increase in the uptake of the nucleoside precursors for RNA synthesis, so that if radioactively-labelled precursors are administered in vivo, oestrogen will lead to an increase in the specific activity of the precursor pools in the uterus (Billing et al., 1969 a). As with the increased fluid accumulation in the uterus, this stimulation of nucleoside uptake is not blocked by actinomycin D, showing that it is not a transcriptional response (Billing et al., 1969 a). It is not clear if these non-transcriptional effects of oestrogen occur as a result of interaction of the hormone with the

cell membrane, or whether formation of the oestrogen-receptor complex in the cytoplasm is necessary. Also, the role, if any, played by cyclic nucleotides, which act as secondary messengers for peptide hormones, is unclear.

Changes in the levels of cyclic nucleotides have been reported to occur rapidly in response to oestrogen. Goldberg's group has shown that oestrogen leads to an increase in the level of guanosine 3':5'-phosphate (cyclic GMP) in the uterus whilst decreasing the level of adenosine 3':5'-phosphate (cyclic AMP) (Kuehl et al., 1974; Nicol et al., 1974). In contrast to most effects on cyclic GMP levels, which are usually transient, the elevated cyclic GMP level in the uterus is maintained for up to 24 hr. This effect of the hormone is of interest because cyclic GMP has been implicated in a number of proliferative responses. Transcription appears to be necessary for the maintenance of the elevated cyclic GMP level, since the effect is inhibited by actinomycin D and protein synthesis inhibitors (Nicol and Goldberg, 1976). Furthermore, the increase in cyclic GMP content, like other uterotrophic responses, appears to involve binding of the hormone to oestrogen receptors with subsequent translocation to the nucleus (Flandroy and Galand, 1979).

The majority of the responses of the rat uterus to oestrogen are inhibited by actinomycin D and therefore appear to stem from an effect of the hormone on the transcription of RNA. Within 30 min of the administration of oestradiol-17β , increased RNA polymerase activity and increased RNA synthesis can be detected. This is followed by a stimulation of protein synthesis and, at later times, a stimulation of DNA synthesis. The amounts of RNA, protein and DNA in the immature rat uterus, determined chemically, thus increase in sequence during the first 30 hr following hormone treatment (Billing et al., 1969 b). The result of giving oestradiol -17β is therefore an overall hypertrophy and hyperplasia of the uterus.

The initial stimulation of RNA synthesis in the immature rat uterus has been shown to occur in two phases. 30 min after the administration of hormone, there is a stimulation of the synthesis of a species of RNA of very high molecular weight (Knowler and Smellie, 1971, 1973). Determination of the base composition, cellular location and rate of decay, and demonstration of the lack of methylation and the heterogeneous nature of this RNA have shown this species to be heterogeneous, nuclear RNA, or Hn RNA (Knowler and Smellie, 1973), a species of RNA believed to contain the precursors of m RNA. Further evidence supporting the identification of this RNA species as Hn RNA has come from the demonstration that it contains tracts of poly-adenylic acid, or poly (A) (Aziz and Knowler, 1978a). The poly (A) content has been used as the basis of fractionation of the Hn RNA on poly (U)-Sepharose columns into three classes. The synthesis of all three classes is stimulated by oestradiol but the degree and timing of the response is different for each, with the fraction having highest affinity for poly (U)-Sepharose showing the greatest response after 30 min (Aziz and Knowler, 1978a). This stimulation of Hn RNA synthesis is still detectable once the Hn RNA has been processed into ribonucleoprotein particles in the nucleus, although it is found to occur at slightly later times (Knowler, 1976).

This initial phase of stimulated Hn RNA synthesis is followed by an increase in the synthesis of ribosomal RNA (rRNA) and transfer RNA (tRNA) occurring 2-4 hr after hormone administration (Billing et al., 1969c; Knowler and Smellie, 1971). This stimulation of rRNA synthesis can be followed through from the 45 S precursor (Knowler and Smellie, 1971). Similar experiments using ovariectomised adult rats have also shown a stimulation of the synthesis of rRNA and, in addition, an increased rate of conversion of the rRNA precursors to the mature 28S and 18S rRNA species (Luck and Hamilton, 1972; Knecht and Luck, 1977).

Studies on the activities of uterine RNA polymerases have given results which correspond to the pattern of stimulated RNA synthesis. There is an increase in the activity of RNA polymerase II (B), the enzyme responsible for the synthesis of Hn RNA, 30-60 min after giving oestradiol to immature rats (Hardin et al., 1976), ovariectomised adult rats (Glasser et al., 1972) and rabbits (Borthwick and Smellie, 1975). The activity then declines after 1-2 hr and rises to a second peak at 3-4 hr, before returning to control levels after 24 hr (Clark et al., 1978). The initial rise in RNA polymerase II activity is followed by an increase in the activity of RNA polymerase I (A), the enzyme responsible for the synthesis of rRNA. The activity of this enzyme reaches a maximum 4 hr after giving hormone and is maintained at this level for up to 12 hr (Glasser et al., 1972; Borthwick and Smellie, 1975; Hardin et al., 1976). The activity of RNA polymerase III, the enzyme responsible for the synthesis of tRNA and 5S rRNA, also appears to be stimulated at the same time as the RNA polymerase I activity in isolated nuclei from the uterus of immature rats (Weil et al., 1977) and ovariectomised adult rats (Webster and Hamilton, 1976). The stimulation of RNA polymerase I activity is dependent on the initial stimulation of RNA polymerase II activity. Treatment with α-amanitin, a specific inhibitor of the latter enzyme, before giving oestradiol abolishes the hormone-stimulated increase in RNA polymerase I activity, and hence the increase in rRNA synthesis, whereas treatment with the inhibitor after the increase in RNA polymerase II activity has already occurred does not prevent the stimulation of rRNA synthesis (Raynaud-Jammet et al., 1972; Borthwick and Smellie, 1975). Treatment with cycloheximide, an inhibitor of protein synthesis, leads to similar results.

These findings have led to the suggestion that the stimulation by oestrogen of rRNA synthesis in the rat uterus, an important event in the uterotrophic response, is dependent on the initial stimulation of RNA polymerase II

activity (Glasser et al., 1972). This results in the synthesis of Hn RNA which contains mRNA sequences which must be translated to produce a small number of proteins necessary for the subsequent stimulation of rRNA synthesis (Baulieu et al., 1972; Baulieu, 1975; Knowler et al., 1975). Because these proteins, whose existence is suggested by the results of the inhibition experiments, could play a key role in initiating the trophic response of the uterus to oestrogen, they have been termed Key Intermediary Proteins by Baulieu (Baulieu et al., 1972; Baulieu,1975).

The nature of these Key Intermediary Proteins is unknown. They do not appear to be components of the RNA polymerases, since isolation of the enzymes has failed to show any differences between control and hormone-treated animals (Borthwick and Smellie, 1975; Weil et al., 1977) and an exhaustive search for enzyme factors has proved unsuccessful (Knowler et al., 1975). It is possible, therefore, that hormone treatment leads to an alteration in the chromatin template (Knowler et al., 1975). It has been reported that both the composition of uterine chromatin and its template capacity, determined using exogenous RNA polymerase from E. coli, are altered following hormone treatment, although the changes are found to follow the initial stimulation of RNA polymerase II activity (Glasser et al., 1972). In addition, there is an oestrogen-induced increase in the number of initiation sites on the chromatin for E. coli RNA polymerase, as measured in the presence of the antibiotic rifampicin which prevents re-initiation (Markaverich et al., 1978). Although these studies using bacterial RNA polymerase may not accurately reflect alterations in the transcription of a eukaryotic template by eukaryotic RNA polymerases, they are indicative of a structural alteration of the chromatin in response to the hormone (Johnson and Baxter, 1978).

Experiments carried out to investigate the synthesis of proteins at these early times following hormone stimulation have demonstrated the synthesis

of an oestrogen-induced protein, known as IP, 40 min after hormone treatment. This protein is detectable by electrophoresis of radioactively-labelled uterine proteins or by radioimmunoassay (Iacobelli et al., 1977). Its synthesis is stimulated by oestrogens, both in vivo (Notides and Gorski, 1966) and in vitro (Katzenellenbogen and Gorski, 1972), and inhibited by treatment with actinomycin D given up to 15 min after hormone administration (De Angelo and Gorski, 1970). No function has yet been assigned to this protein, although King et al. (1977) have shown that IP co-purifies with some factor able to stimulate DNA synthesis in cultured fibroblasts, suggesting that IP may play a role in the later stimulation of DNA synthesis in the uterus. Barker (1971) has also reported the stimulation of the synthesis of a non-histone protein associated with arginine-rich histones occurring soon after oestrogen treatment.

There is very little evidence, however, to suggest that either of these two proteins is a Key Intermediary Protein involved in the stimulation of rRNA synthesis in the uterus.

Attempts to detect specific, hormone-induced mRNA species in the uterus have led to controversy. An RNA species of sedimentation coefficient 15 S, whose synthesis is stimulated within 15 min of hormone treatment, has been reported (Baulieu, 1975), but Frolik and Gorski (1977) could not confirm this finding. It is possible that the high ribonuclease activity of the rat uterus can result in losses during the isolation of RNA (Glasser et al., 1972; Frolik and Gorski, 1977). Ribonuclease-catalysed degradation could also explain the failure to detect a stimulation of RNA polymerase II activity in some cases (Webster and Hamilton, 1976; Weil et al., 1977). It is also possible that the techniques used by Frolik and Gorski, involving the incorporation of labelled precursors into RNA in vitro followed by polyacrylamide gel electrophoresis of the RNA, are not sensitive enough to detect the

synthesis of specific mRNA species and that a more sensitive technique, such as molecular hybridisation, is necessary. This approach has been used by Aziz and Knowler (1978b) to demonstrate alterations in the mRNA population of the rat uterus 4 hr after hormone treatment and to estimate the diversity and complexity of the mRNA population (Aziz et al., 1979). It is of interest to note that Frolik and Gorski (1977) incubated rat uteri in vitro in order to label the RNA. Using this particular technique, it has been shown that oestrogen stimulates the synthesis of HnRNA incorporated into nuclear ribonucleoprotein particles (Knowler, 1976) and the synthesis of IP (Katzenellenbogen and Gorski, 1972), so that new mRNA species should be present. However, under these same conditions, the stimulated synthesis of rRNA cannot be detected, suggesting that, at least for the rRNA species, part of the transcriptional mechanism is sensitive to the in vitro incubation conditions (Knowler et al., 1975; Knowler, 1976).

A cascade phenomenom, whereby early transcriptional responses are a pre-requisite for later transcriptional responses, appears to occur in a number of other steroid hormone-dependent systems. The ecdysone-induced puffing of the giant chromosomes of insect salivary glands has already been mentioned in Section 1.2.1. In Drosophila, a distinction can be made between chromosome puffs which appear rapidly in response to the hormone and other puffs which only appear after an interval of some hours. The induction of the early puffs is independent of protein synthesis whereas the induction of the late puffs is blocked by inhibitors of protein synthesis, suggesting that the late puffs are controlled by a product resulting from the early puffs (Ashburner, 1972, 1974).

In rat liver, glucocorticoids stimulate rRNA synthesis and this stimulation is prevented by α-amanitin and cycloheximide (Yu and Feigelson, 1973), suggesting that a sequence of events similar to that occurring in the oestrogen-stimulated rat uterus is also occurring in

this tissue.

Also discussed in Section 1.2.1 was the induction by oestrogen and progesterone of the mRNA species coding for the egg white proteins in the chick oviduct. Although some effects of the hormones on translation have been found to occur, the major effects appear to be transcriptional. The number of initiation sites on oviduct chromatin for the RNA polymerase from E. coli can be estimated in a rifampicin challenge assay. It has been shown that there is an increase in this number, following oestrogen treatment, which can be correlated with the number of nuclear oestrogen-receptor complexes (S. Tsai et al., 1975; M. Tsai et al., 1976). Similar results have been obtained using purified progesterone-receptor complexes in a cell-free system (Schwartz et al., 1976; Buller et al., 1976). Experiments using homologous RNA polymerase II, and the antibiotic rifamycin to prevent re-initiation, have given the same results and it has also been shown that the homologous enzyme will compete with the bacterial enzyme for binding to the chromatin, suggesting that they are binding to the same initiation sites, although the former leads to more ovalbumin mRNA transcripts (M. Tsai et al., 1976). The actual number of extra initiation sites following progesterone treatment in vitro has been estimated to be 5,000 - 7,000 per cell (Schwartz et al., 1976; Buller et al., 1976). This figure seems greater than expected if the hormone were stimulating the transcription of only a small number of genes. Therefore it would appear that, as postulated by Johnson and Baxter (1978), the rifampicin-resistant binding of RNA polymerase to chromatin is an index of chromatin structure rather than an index of transcriptionally active genes.

The kinetics of induction of the egg white proteins in the chick oviduct are not identical for each protein. Oestrogen stimulates the synthesis of conalbumin almost immediately, whereas there is a lag of about 3 hr

before ovalbumin synthesis is stimulated, suggesting that intermediate steps must occur between the binding of the hormone-receptor complex to the chromatin and the induction of ovalbumin mRNA (Palmiter et al., 1976). Palmiter originally discounted the possibility that the synthesis of protein intermediates was required for ovalbumin mRNA induction since, although cycloheximide inhibited the induction of ovalbumin mRNA, emetine was found to have little effect. Cycloheximide appeared to be affecting RNA synthesis by a mechanism unrelated to its action on protein synthesis and, in fact, both cycloheximide and emetine eventually led to the death of the experimental animals, illustrating the problems of interpretation of experiments involving the administration of metabolic inhibitors in vivo. To explain the lag phase in the induction of ovalbumin mRNA, Palmiter proposed a receptor translocation model whereby oestrogen-receptor complexes bind to non-productive sites on the chromatin and then translocate to productive sites at which the genetic responses are initiated, the rate of receptor translocation determining the timing of the induction of the mRNA species (Palmiter et al., 1976). However, in a more recent publication from the same laboratory, McKnight (1978) has reported that primary cultures of chick oviduct will respond to oestrogen and progesterone in vitro. Inhibitors of protein synthesis prevent the induction of both ovalbumin and conalbumin mRNAs, suggesting that protein synthesis is necessary in order for the steroid-induced accumulation of these specific mRNA species to occur.

To conclude this section on transcription, mention should be made of recent discoveries on the structure of eukaryotic genes. As discussed in the preceding section, it is now possible to obtain copies of certain eukaryotic genes, including the ovalbumin gene, by molecular cloning. Treatment of the cloned ovalbumin gene with restriction endonucleases followed by mapping the sites of endonuclease cleavage has shown that, in common with a number of other eukaryotic genes, the DNA coding for

the ovalbumin mRNA is not continuous. The gene appears to consist of eight coding regions of DNA interrupted by seven regions of DNA of unknown function, referred to as intervening sequences, inserts or introns (Breathnach et al., 1977; Doel et al., 1977; Dugaiczyk et al., 1978, 1979; Kourilsky and Chambon, 1978). Intervening sequences have also been reported in the ovomucoid gene (Catterall et al., 1979). The function of these intervening sequences is unknown. They appear to be transcribed (Roop et al., 1978; Nordstrom et al., 1979; Swaneck et al., 1979) so there must be post-transcriptional mechanisms for their removal before the mature mRNA species enter the cytoplasm.

1.2.2.7 Effect of oestrogen on protein synthesis in the rat uterus

During the first hour following hormone treatment, there are alterations in the synthesis of RNA and phospholipids and in the metabolism of glucose in the uterus (Mueller et al., 1961, 1972; Jensen and DeSombre, 1972). A stimulation of protein synthesis appears to follow in the wake of these changes (Mueller et al., 1958, 1961, 1972; Billing et al., 1969b; Jensen and DeSombre, 1972). As discussed in the previous section, the stimulation of RNA synthesis is dependent on protein synthesis. This is also true for the early stimulation of phospholipid synthesis. Thus, protein synthesis inhibitors will block the oestrogen-induced increases in phospholipid and RNA synthesis (Mueller et al., 1961, 1972; Noteboom and Gorski, 1963; Gorski and Axman, 1964).

Although these results show that a stimulation of the synthesis of selective proteins appears to be necessary for the initial actions of oestrogen, a general increase in protein synthesis in the uterus is not detectable until 4 hr after hormone treatment (Mueller et al., 1961; Noteboom and Gorski, 1963), reaching a maximum after about 12 hr (Mueller et al., 1972). This general stimulation occurs in the nuclear, ribosomal and mitochondrial fractions of the cell (Noteboom and Gorski, 1963) and is dependent on the prior stimulation of RNA synthesis (Ui and Mueller, 1963).

As discussed in Section 1.2.2.4, the oestrogen receptor levels in the cytoplasm are replenished during this time, so the oestrogen receptor would appear to be one of the many proteins whose synthesis is stimulated (Jensen et al., 1969; Sarff and Gorski, 1971). Oestrogen also appears to stimulate the synthesis of progesterone receptors in the rat (Faber et al., 1972), guinea pig (Milgrom et al., 1970; Freifeld et al., 1974; Saffran et al., 1976) and rabbit uterus (Faber et al., 1972; Saffran et al., 1976).

This general stimulation of protein synthesis in the uterus is accompanied by an increase in the number of ribosomes in the cytoplasm (Teng and Hamilton, 1967b; Hamilton et al., 1968). It has also been reported that treatment with oestrogen leads to an increase in the protein-synthesising activity of the ribosomes, as measured in a cell-free system, in the rat (Greenman and Kenney, 1964; Teng and Hamilton, 1967b; Whelley and Barker, 1974) and guinea pig uterus (Suvatte and Hagerman, 1970; Shapiro et al., 1975). Shapiro et al.(1975) found that this increased activity was associated with the 60S ribosomal subunit and that a single extra band was present following polyacrylamide gel electrophoresis of 60S ribosomal proteins from hormone-treated animals.

It has been postulated by Tata (1967, 1976) that growth-promoting hormones, of which oestradiol is an example, lead to a coordinated stimulation of the synthesis of ribosomes and the membranes to which they are attached. The net result would be a new population of topographically-segregated ribosomes. The synthesis of ribosomes and their subsequent distribution in the cytoplasm could thus be an important event in the uterotrophic response to oestrogen.

1.2.2.8 Effect of oestrogen on DNA synthesis in the rat uterus

A stimulation of DNA synthesis follows the stimulation of RNA and protein synthesis in the rat uterus in response to oestrogen (Mueller et al., 1958; Billing et al., 1969b). It is first detectable 18hr after the administration of oestradiol and it reaches a maximum 24hr after hormone

treatment (Kaye et al., 1972; Stormshak et al., 1976). This stimulation of DNA synthesis is accompanied by an increase in the mitotic index of the uterine cells, leading to an increase in cell numbers. In the mature rat, oestradiol treatment, in the absence of progesterone, stimulates cell division almost exclusively in the endometrium (Leake et al., 1975). However, in the immature animal, oestradiol stimulates DNA synthesis and mitosis simultaneously in both the endometrium and myometrium (Kaye et al., 1972).

This stimulation of DNA synthesis results in hyperplasia of the uterus and would appear to be a long term oestrogenic response. Such long term responses result in uterine growth, as measured by an increase in the dry weight of the uterus. As discussed in Section 1.2.2.4, long term responses require the continued presence of hormone-receptor complexes in the nucleus (Anderson et al., 1972, 1975; Stormshak et al., 1976; Clark et al., 1977). This would suggest that the stimulation of DNA synthesis does not depend on the synthesis of intermediate factors initiated shortly after the oestrogen-receptor complex enters the nucleus. Thus, it would appear that a cascade phenomenom, similar to that occurring in the stimulation of rRNA synthesis, does not occur in the case of DNA synthesis. However, as mentioned in Section 1.2.2.6, a factor which co-purifies with IP can stimulate DNA synthesis in cultured fibroblasts, suggesting a possible role for IP in the stimulation of DNA synthesis in the uterus (King et al., 1977). Leake et al.(1975) have shown that the oestrogen-induced stimulation of DNA synthesis in the immature rat uterus is blocked by cycloheximide, an inhibitor of protein synthesis, suggesting that the synthesis of a protein intermediate is necessary for the stimulation of DNA synthesis. This protein intermediate appears to be degraded fairly rapidly, however, since cycloheximide is effective several hours after oestradiol treatment (Leake et al., 1975).

It is therefore possible that the oestrogen-induced stimulation of DNA

synthesis is dependent on the synthesis of Key Intermediary Proteins in a similar manner to the oestrogenic stimulation of rRNA synthesis. The continued presence of oestrogen-receptor complexes in the nucleus could be necessary because the proteins have a short half-life. The continued synthesis of these proteins would thus be required until shortly before DNA synthesis is stimulated.

1.2.2.9 Summary

In summary, the action of oestradiol-17β leads to a process of growth and development in the immature rat uterus, resulting in hypertrophy and hyperplasia of the organ. The stimulation of rRNA synthesis, and hence ribosome synthesis, is an important factor in uterine hypertrophy since there is a general increase in protein synthesis in response to the hormone. The subsequent hyperplasia of the uterus also appears to depend on the uterotrophic response. Therefore, the oestrogen-induced stimulation of ribosome synthesis appears to be an important early effect of the hormone.

The structure and function of mammalian ribosomes will be discussed in the next section.

1.3 Role of Ribosomes in Protein Synthesis

The demonstration of the role of ribosomes in protein synthesis was preceded by observations of a relationship between the amount of RNA in a cell and the rate of protein synthesis. An historical account of the involvement of RNA in protein synthesis has been presented by Watson (1963). Most of the RNA was found to be present in cytoplasmic ribonucleoprotein particles which could be observed in electron micrographs of both prokaryotic (Luria et al., 1943) and eukaryotic cells (Littlefield et al., 1955; Palade and Siekevitz, 1956). Experiments involving pulse labelling with amino acids followed by cell fractionation showed that these cytoplasmic, ribonucleoprotein particles were the sites of initial incorporation of amino acids into protein in eukaryotic cells (Littlefield et al., 1955). Similar observations were made on prokaryotic cells, using sucrose density gradient centrifugation to resolve the ribonucleoprotein particles (McQuillen et al., 1959). By this time, Roberts (1958) had given the name "ribosome" to these ribonucleoprotein particles.

The name ribosome includes the cytoplasmic ribonucleoprotein particles of both prokaryotic and eukaryotic cells. Structural differences do exist between prokaryotic and eukaryotic ribosomes, however: prokaryotic ribosomes are smaller and have a higher ratio of RNA to protein than their eukaryotic counterparts. The structure and function of prokaryotic and mammalian ribosomes will be discussed separately in the following sections.

1.3.1 Ribosomes from prokaryotic organisms

As stated in the preceding section, there are several differences between ribosomes from prokaryotic and eukaryotic organisms. These differences are summarised in Figure 3.

Ribosomes from prokaryotic organisms have a sedimentation coefficient of 70S. On lowering the concentration of magnesium ions, the ribosome

Prokaryotic ribosomes	Mammalian ribosomes
Sedimentation coefficient 70S	Sedimentation coefficient 80S
Dissociate into subunits of 50S and 30S	Dissociate into subunits of 60S and 40S
Approximately 64% by weight RNA and 36% by weight protein	Approximately equal amounts of RNA and protein by weight
23S, 16S and 5S rRNA species present	28S, 18S, 5.8S and 5S rRNA species present
34 proteins in larger subunit, 21 proteins in smaller subunit	Approximately 40 proteins in larger subunit and 30 proteins in smaller subunit
Synthesise proteins with N-formyl methionine at N-terminus	Synthesise proteins with methionine at N-terminus
Different supernatant factors required for translation	Different supernatant factors required for translation
Different translational inhibitors	Different translational inhibitors

Figure 3

Differences between prokaryotic and mammalian ribosomes

dissociates into two subunits of sedimentation coefficients 50S and 30S respectively (Watson, 1963). The larger subunit from Escherichia coli contains two rRNA molecules, one with a sedimentation coefficient of 23S and the other with a sedimentation coefficient of 5S, together with 34 different proteins (Brimacombe et al.,1978). The smaller subunit contains one rRNA species, of sedimentation coefficient 16S, and 21 proteins (Brimacombe et al.,1978). Because they are less complex than eukaryotic ribosomes, and also because of the relative ease with which they can be prepared and genetic studies carried out, extensive studies have been carried out on the ribosomes from this organism. These studies have been concerned with the functioning of the ribosome during protein synthesis, structural studies on the individual ribosomal components, and how the individual components are arranged relative to each other in space. The results of such studies have been reviewed by Brimacombe (1976), Kurland (1977) and Brimacombe et al.(1978).

The most rapid progress, in recent years, has been made on the arrangement in space of the ribosomal components, or ribosome topography. A number of different techniques have been used to study ribosome topography, including neutron scattering, fluorescence transfer between modified derivatives of ribosomal proteins, nuclease digestion of rRNA in the presence of ribosomal proteins, in vitro assembly, cross-linking with bifunctional reagents, and immune electron microscopy. The last two techniques have proved to be the most productive for studying the spatial relationships between the ribosomal proteins. In cross-linking studies, the intact ribosomal subunit is treated with a bifunctional reagent. This can result in the formation of cross-links between ribosomal proteins, and the cross-linked proteins can be isolated, following dissociation of the ribosomal subunit into its rRNA and protein components, and identified by means of specific antibodies against single ribosomal proteins. Identification of the cross-linked proteins can also be carried out if the

particular bifunctional reagent is cleavable. Cross-linking reagents with disulphide groups, cleavable with 2-mercaptoethanol, (Traut et al., 1973) or with vicinal hydroxyl groups, cleavable with periodate, (Lutter et al., 1974) have been developed for this purpose. The individual ribosomal proteins which have become cross-linked can be identified by polyacrylamide gel electrophoresis following chemical cleavage of the cross-link. In this way, it is possible to map protein "neighbourhoods" of the ribosomal subunits (Kenney and Traut, 1979). The information about the relative positions of the ribosomal proteins obtained from such experiments is limited, however. Many of the ribosomal proteins have an elongated shape and would therefore be expected to be in contact with several other ribosomal proteins in the intact ribosome (Brimacombe, 1976; Kurland, 1977; Brimacombe et al., 1978). Experiments can also be carried out to cross-link ribosomal proteins to rRNA by irradiation with ultra-violet light or by treatment with formaldehyde or periodate (Kurland, 1977; Brimacombe et al., 1978).

In the technique of immune electron microscopy, ribosomal subunits are treated with a purified antibody specific to one particular ribosomal protein. The bivalent antibody can bind two ribosomal subunits, thus forming dimers, joined by a single antibody molecule, which can be examined by electron microscopy. Since the ribosomal subunits have distinctive shapes, the point of attachment of the antibody can be determined, and hence the position of the antigenic determinant of the particular protein can be located. In this way, the locations of all 21 proteins from the 30S subunit and many of the proteins from the 50S subunit have been published (Tischendorf et al., 1975; Lake, 1977).

The various experimental approaches to ribosome topography have given results which are in fairly good agreement and a general picture of the ribosome from E. coli is emerging (Kurland, 1977; Brimacombe et al., 1978). The 30S subunit has an elongated structure with a "head" and a "body" region.

All 21 proteins appear to be associated with specific regions of the 16S rRNA. The 50S subunit has a shape resembling that of a kidney, the 30S subunit fitting into the groove at the top of the structure. Less information is available concerning the arrangement of the proteins and the two rRNA species.

In addition to studies on the structure of the prokaryotic ribosome, advances have been made in understanding the functioning of the prokaryotic ribosome during protein synthesis. Several lines of evidence have suggested that the rRNA, which makes up almost two thirds of the ribosome, is very important in ribosome function. Indeed, both Crick (1968) and Orgel (1968) have suggested that the primitive ribosome consisted entirely of rRNA, with proteins gradually taking over some of the functions, leading to an improved efficiency.

It has been shown that the 3' end of the 16S rRNA can form base pairs with purine-rich sequences near the initiation codon of mRNA and this has been proposed as a mechanism for the initiation of protein synthesis (Shine and Dalgarno, 1974; Steitz and Jakes, 1975). This interaction appears to be applicable to all prokaryotic mRNA species and is believed to play a role in the identification of the initiation codon of the message. In addition to this interaction between the 16S rRNA and mRNA species, the 5S rRNA of the larger ribosomal subunit has been implicated in the binding of transfer RNA (tRNA) whilst the 16S rRNA has also been implicated in the binding of one of the initiation factors, IF3, required for protein synthesis (Kurland, 1977; Brimacombe et al., 1978). This functional importance of the rRNA is reflected by the fact that the sequences have been conserved to a large extent during evolution (Brimacombe, 1976; Brimacombe et al., 1978). Ribosomal proteins are also involved in the binding of mRNA, tRNA and supernatant factors and it would appear that the functional sites of the prokaryotic ribosome consist of RNA and protein "domains" (Kurland, 1977).

1.3.2 Structure and function of mammalian ribosomes

As discussed in the preceding sections, eukaryotic ribosomes differ from prokaryotic ribosomes in that they are bigger and more complex. However, studies carried out on a range of eukaryotic organisms have shown that there is a phylogenetic variation in the ribosomes of the different organisms. The actual size of the ribosomal subunits, as judged from their sedimentation coefficients, does not appear to vary, but differences in the various components of the ribosome, in particular the rRNA, occur. The size of the rRNA shows a gradual increase going from the lower eukaryotes to the higher eukaryotes (Hadjiolov, 1977). The smallest rRNA species are thus found in plants, yeast, protozoans and slime moulds whilst the largest rRNA species occur in vertebrates. The ribosomes from the insect Drosophila appear to fit phylogenetically between these two groups (Levis and Penman, 1978).

In addition, the cells of eukaryotic organisms contain organelles, namely, mitochondria and chloroplasts, which contain their own ribosomes. These ribosomes are believed to synthesise certain proteins of the mitochondria and chloroplasts which cannot cross the membranes of these organelles. Synthesis of these proteins by cytoplasmic ribosomes followed by their transport into the mitochondria and chloroplasts would therefore be precluded. The ribosomes present in these organelles are smaller than the cytoplasmic ribosomes and resemble prokaryotic ribosomes. In fact, mitochondrial ribosomes have a sedimentation coefficient of 55S, dissociating into subunits of sedimentation coefficients 39S and 29S respectively, and have a higher protein content than prokaryotic ribosomes, consisting of approximately two thirds protein (Sacchi et al., 1977). The ribosomes from chloroplasts have been reported to have a sedimentation coefficient of 70S, although Margulies and Tiffany (1979) have suggested that, in the case of the chloroplast ribosomes from the unicellular alga, Chlamydomonas, such a sedimentation coefficient occurs as a result of an

experimental artifact. Taking precautions against dissociation of the ribosomes, these authors reported a value of 75S for the sedimentation coefficient of the undissociated chloroplast ribosome and values of 60S and 45S for the two subunits (Margulies and Tiffany, 1979).

The remainder of this discussion will be confined to the structure and function of mammalian, cytoplasmic ribosomes.

1.3.2.1 Structure of mammalian ribosomes

Mammalian ribosomes consist of nearly equal amounts of protein and rRNA (Bielka, 1978) and they are synthesised as two separate subunits which associate reversibly during protein synthesis (Falvey and Staehelin, 1970). These subunits have sedimentation coefficients of 60S and 40S respectively, re-associating to form the 80S ribosome (Watson, 1963). The large subunit contains one high molecular weight rRNA molecule, of sedimentation coefficient 28S, and one low molecular weight rRNA molecule, of sedimentation coefficient 5S (Hadjiolov, 1977; Bielka, 1978; Wool, 1979). There is also another rRNA species, of sedimentation coefficient 5.8S, hydrogen-bonded to the 28S rRNA. In addition to the rRNA species, the large ribosomal subunit contains approximately 40 different ribosomal proteins (Hadjiolov, 1977; Bielka, 1978; Wool, 1979). The small ribosomal subunit contains one rRNA molecule, of sedimentation coefficient 18S, and approximately 30 ribosomal proteins (Hadjiolov, 1977; Bielka, 1978; Wool, 1979).

The rRNA species have a high content of guanosine (G) and cytidine (C) (Scherrer et al., 1963; Bielka, 1978). They also contain the unusual ribonucleoside pseudouridine, or 5-ribosyluracil, which arises by post-transcriptional modification of an existing uridine ribonucleoside. Another modification of the rRNA which occurs is methylation. All the rRNA species are methylated and two classes of methylation can be distinguished. Most of the methyl groups occur on the 2'-hydroxyl group of the ribose, but some methylations of the bases occur (Maden et al., 1972;

Maden and Salim, 1974; Khan and Maden, 1978).

Studies of the protein complement of mammalian ribosomes have been based almost entirely on analysis of the proteins by two-dimensional polyacrylamide gel electrophoresis using a method such as that described by Sherton and Wool (1972). The number of protein spots resolved by this method ranges from 70 to 80, depending on the source of the ribosomes (Sherton and Wool, 1972, 1974; Collatz et al., 1977; Tsurugi et al., 1978; Schiffmann and Horak, 1978; Cazillis and Houssais, 1979). An estimation of the exact number of ribosomal proteins is made difficult by problems in defining true ribosomal proteins in eukaryotic organisms (Wool, 1979). It has also been suggested that some protein spots could arise by oxidation of ribosomal proteins during their isolation, thus leading to errors (Welfle et al., 1978).

The majority of the proteins are basic, with only approximately four proteins of the 80S ribosome being acidic. Each of the proteins appears to be unique and most are present in only one copy per ribosome (Bielka, 1978; Wool, 1979). Nearly all of the proteins have been isolated in a pure form and their amino acid compositions have been published (Collatz et al., 1977; Tsurugi et al., 1977, 1978; Goerl et al., 1978).

Because of the greater complexity of mammalian ribosomes compared to bacterial ribosomes, and of the greater difficulty in their preparation, knowledge of the topography and function of the individual ribosomal components is not so advanced. Progress is being made, however, using the same methods used for studying ribosome topography in prokaryotic ribosomes. Thus, experiments carried out in Wool's laboratory have demonstrated interactions between various proteins of the large ribosomal subunit and the 5S rRNA (Ulbrich and Wool, 1978) and the 5.8S rRNA species (Ulbrich et al., 1979). Two proteins of the small ribosomal subunit were also found to interact with the 5.8S rRNA (Ulbrich et al., 1979). Noll et al. (1978) have used immune electron microscopy to determine the

location and function of one of the proteins of the small ribosomal subunit. It is possible, therefore, to make tentative deductions about the roles played by some of the components of mammalian ribosomes. These deductions have been summarised by Bielka (1978) and Wool (1979).

The greater complexity of mammalian ribosomes as compared to bacterial ribosomes may be related to extra functional roles of the former, such as the regulation of protein synthesis at the level of translation. However, the basic function of the ribosome, that is, protein synthesis, is virtually unaltered in the two different types of organism. There is very little structural homology between the protein components, however. The ribosomal proteins appear to have different compositions and there is a low degree of immunological cross-reaction when antibodies raised against the individual proteins from E. coli are tested against the proteins from mammalian ribosomes (Brimacombe et al., 1978). The one exception is the antisera against the E. coli proteins L7 and L12 which cross-react with the mammalian proteins L40 and L41 (Stoffler et al., 1974; Leader and Coia, 1978c). In addition, the mammalian proteins can replace those in E. coli ribosomes without loss of activity, suggesting that these proteins have been conserved during evolution (Stoffler et al., 1974; Brimacombe et al., 1978). These two proteins have the same amino acid sequence in E. coli, L7 being an N-acetylated derivative of L12, and they appear to be the only proteins present in multiple copies.

The rRNA species may have similar functions in the two different types of organism. It has been suggested that the 5.8S rRNA species in mammalian ribosomes is homologous to the 5S rRNA in bacterial ribosomes (Wrede and Erdmann, 1977). One difference between mammalian and bacterial ribosomes, however, is that no direct evidence has yet been obtained for the formation of base pairs between the 18S rRNA in mammalian ribosomes and the 5' end of mRNA species. A purine-rich sequence at the 3' end of the 18S rRNA in eukaryotes has been conserved during evolution and it has

been proposed that this interacts with the 5' end of the mRNA in an analogous way to the 16S rRNA of prokaryotes (Hagenbuchle et al., 1978 ; Both, 1979). However, the ribosome binding regions from a number of different mRNA species do not share common features, nor do they appear to contain regions complementary to the 3' end of 18S rRNA (Rose, 1977). In fact, the only sequence which appears to be conserved at the 5' end of a number of different eukaryotic mRNA species is the AUG initiation codon (Kozak, 1977; Baralle and Brownlee, 1978). It has been proposed that the interaction between the rRNA and mRNA species in prokaryotes is replaced by an interaction with a methylated guanosine residue linked by a 5'-5' triphosphate, a so-called cap structure, at the 5' end of many eukaryotic mRNA molecules (Kozak, 1977; Clemens, 1979). According to this model, initiation would occur only at the AUG sequence nearest the capped 5' end of the mRNA. This would predict that in a polycistronic message, cap-dependent translation would be restricted to the 5'-proximal cistron, and this has been demonstrated experimentally by Rosenberg and Paterson (1979) using polycistronic, prokaryotic mRNA molecules modified by the addition of a 5' cap structure. This theory still leaves a number of questions unanswered, however. In particular, it does not explain why some viral mRNA species appear to lack a 5' cap nor why translation of some mRNA species is still possible following removal of the cap (Rose and Lodish, 1976).

1.3.2.2 Synthesis of mammalian ribosomes

In mammals, synthesis of the rRNA and its assembly, with the ribosomal proteins, into ribosomes takes place in the nucleolus (Perry, 1967; Maden, 1971). The involvement of the nucleolus in ribosome synthesis was first suggested by experiments with actinomycin D. This antibiotic can inhibit all DNA-directed RNA synthesis, but, if a sufficiently small dose is administered, the synthesis of rRNA only will be inhibited. Under these circumstances, the incorporation of radioactively-labelled uridine into

RNA in the nucleolus will also be inhibited (Perry, 1963).

Further evidence for the nucleolus being the site of ribosome synthesis in mammals has come from experiments showing that isolated nucleoli contain all the components necessary for rRNA synthesis. It has been shown that nucleoli isolated from rat liver contain precursors of the rRNA species, as well as small amounts of the mature rRNA species themselves (Dabeva et al., 1978). Isolated nucleoli have also been shown to contain pre-ribosomal ribonucleoprotein particles (Higashinakagawa and Muramatsu, 1974) and to be enriched in the genes coding for rRNA (McConkey and Hopkins, 1964). Furthermore, nucleoli from hepatoma cells will synthesise rRNA in vitro, and chromatin isolated from such nucleoli will act as a template for the synthesis of rRNA (Matsui et al., 1977).

The genes coding for rRNA are transcribed by the enzyme RNA polymerase I (Matsui et al., 1977; Hadjiolov, 1977). The 18S rRNA of the small ribosomal subunit and the 28S rRNA, together with the 5.8S rRNA, of the large subunit are transcribed initially as part of the same precursor molecule(Scherreret al, 1963; Maden et al., 1972; Hadjiolov, 1977; Bielka, 1978). The synthesis of rRNA has been studied in HeLa cells by pulse chase experiments carried out in both the absence and presence of actinomycin D which prevents incorporation of radioactively-labelled precursors from the large nucleotide pools of the cells during the cold chase (Penman, 1966; Penman et al., 1966). The initial precursor of the rRNA species has a sedimentation coefficient of 45S. This precursor rRNA (pre-rRNA) molecule then undergoes modification by methylase enzymes and cleavage by specific endonucleases to generate the mature 18S and (28S + 5.8S) rRNA species. A number of intermediate pre-rRNA molecules have been identified and processing pathways for the maturation of the 45S pre-rRNA to the mature rRNA molecules have been proposed. There appears to be some flexibility in the sequence of endonuclease attacks on the pre-rRNA molecules, so that two or more processing pathways can exist in parallel (Hadjiolov, 1977; Dudov et al., 1978). Weinberg and Penman (1970) have suggested that the predominant maturation pathway in HeLa cells is as shown in Figure 4.

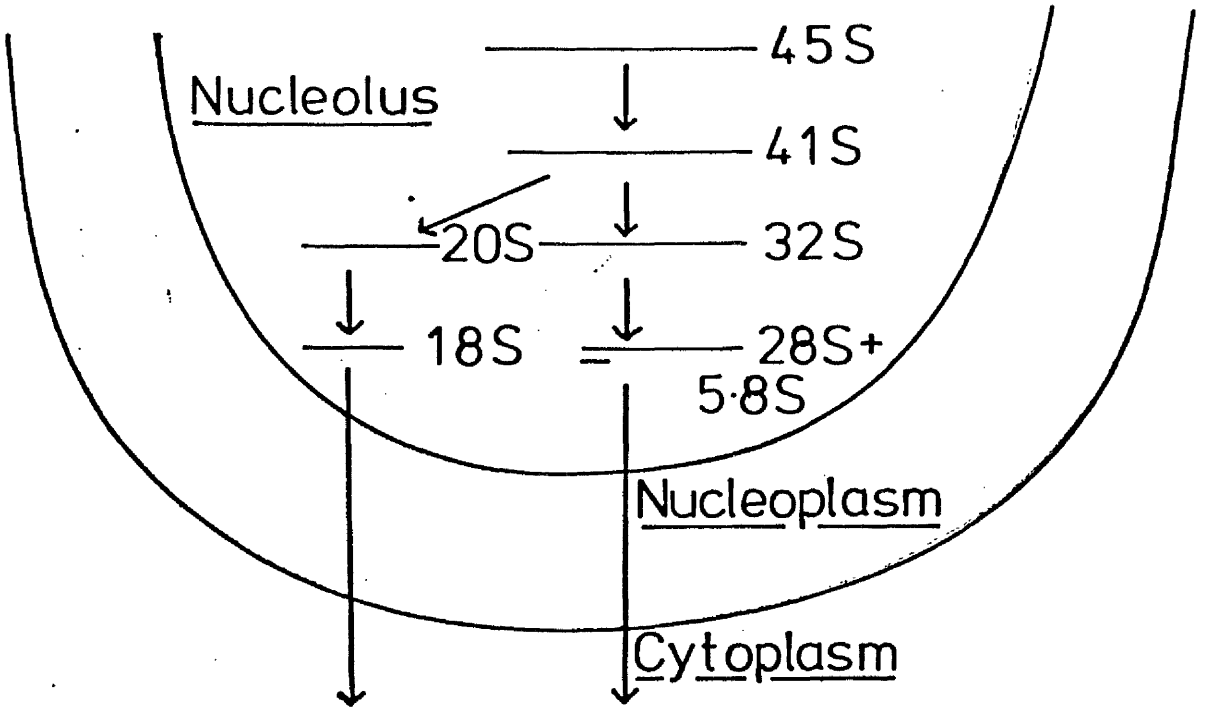


Figure 4. Proposed maturation pathway for pre-rRNA in HeLa cells according to Weinberg and Penman (1970)

It should be stressed that this is not the exclusive maturation pathway, other pathways existing simultaneously with this particular one. Experiments in Hadjiolov's laboratory have demonstrated the existence of 39S and 36S pre-rRNA species in rat liver, in addition to those illustrated in Figure 4, and have also led to the suggestion that other pre-rRNA species may exist (Dabeva et al., 1978; Dudov et al., 1978).

Evidence for the precursor-product relationship between the pre-rRNA and the mature rRNA species has come from hybridisation experiments, comparison of the base compositions of the various RNA molecules, and comparison of the patterns of methylation of the RNA molecules. 18S and 28S rRNA will both compete with 45S pre-rRNA for hybridisation back to the DNA, but only the 28S rRNA will compete with 32S pre-rRNA, and no cross-competition will occur between the 18S and 28S rRNA species

(Jeanteur and Attardi, 1969). Comparison of the base compositions of the various RNA species has shown that they all have a high (G+C) content (Scherrer et al., 1963). The high (G+C) content of the mature rRNA species was mentioned in the previous section. These observations would imply that the (G+C) - rich regions of the pre-rRNA molecules are conserved to a large extent during processing (Hadjiolov, 1977).

The extensive methylation of the rRNA species was also mentioned in the previous section. The methylations occur mainly in the 2'-hydroxyl position. The pre-rRNA species are also methylated, and analysis of the methylated nucleotides has shown that 110 out of the total 117 methyl groups present in the mature rRNA molecules occur in pre-rRNA (Maden and Salim, 1974). The methylated sequences of 45S pre-rRNA and 41S pre-rRNA appear to be identical and to correspond to a mixture of 18S and 28S rRNA (Maden et al., 1972). Furthermore, the methylated nucleotides present in 32S pre-rRNA are identical to those present in 28S rRNA whilst the methylation patterns of 20S pre-rRNA and 18S rRNA also appear to be identical (Maden et al., 1972). These results have led Maden et al. (1972) to conclude that the 45S and 41S pre-rRNA species contain both rRNA sequences; 32S pre-rRNA contains the 28S rRNA sequence only, and 20S pre-rRNA contains the 18S rRNA sequence only. These findings are in agreement with the maturation pathway illustrated in Figure 4. The reasons for the methylation of pre-rRNA are unclear. Almost all the methyl groups of 45S pre-rRNA are retained during maturation, so that the unmethylated regions of the molecule are removed (Maden, 1971; Maden et al., 1972; Maden and Salim, 1974). It is possible that methylations of the 2'-hydroxyl group of the ribose could protect particular phosphodiester bonds from cleavage during processing of the pre-rRNA (Hadjiolov, 1977). This does not, however, explain the role of methylations of the bases.

Studies of the structural organisation of the 45S pre-rRNA have shown the 18S rRNA sequence to be at the 5' end of the molecule and the 28S rRNA to be at the 3' end of the molecule (Liau and Hurlbert, 1975; Bielka, 1978). Newly-synthesised 18S rRNA appears in ribosomal subunits in the cytoplasm earlier than newly-synthesised 28S rRNA (Girard et al., 1965; Penman, 1966). However, this faster appearance of newly-synthesised 18S rRNA in the cytoplasm in the form of small ribosomal subunits does not appear to result from a small pool of this rRNA molecule in the nucleus (Dabeva et al., 1978). Instead, Dudov et al., (1978) have proposed that there are nuclear pools of small and large ribosomal subunits and that the former is in rapid equilibrium with the cytoplasmic pool of 40S subunits. This model explains the asynchronous appearance of the 18S and 28S rRNA species in the cytoplasm, despite the fact that they are synthesised as part of the same precursor molecule. It also explains the lower specific activity of the nuclear 18S rRNA compared with the nuclear 28S rRNA in radioactive labelling experiments.

Although there is considerable flexibility in the sequence of endonuclease cleavage of the pre-rRNA molecules, the 32S pre-rRNA species is believed to be the main precursor of 28S rRNA whilst 20S pre-rRNA (termed 21S by Hadjiolov) is the main precursor of 18S rRNA. Therefore, it appears that the relevant phosphodiester bonds are protected from endonuclease attack until the formation of the 32S and 20S pre-rRNA molecules respectively (Dudov et al., 1978).

The 5S rRNA present in the large ribosomal subunit is not contained within the 45S pre-rRNA molecule and does not appear to be synthesised in the nucleolus. Thus, low doses of actinomycin D, which inhibit the synthesis of pre-rRNA without inhibiting nuclear RNA synthesis, do not inhibit the synthesis of 5S rRNA (Perry and Kelley, 1968). There does not appear to be a precursor of 5S rRNA in mammals, the molecule found

in ribosomes bearing a triphosphate group at its 5' end and appearing to be a primary transcript (Levis, 1978).

The pre-rRNA molecules are associated with proteins in the nucleolus, forming ribonucleoprotein particles (Vaughan et al., 1967; Maden, 1971). This is because maturation of the pre-rRNA occurs concomitantly with assembly of the ribosomes, so the 18S and 28S rRNA enter the cytoplasm in the form of substantially complete ribosomal subunits (Girard et al., 1965). It is possible, therefore, that some of the proteins in the ribonucleoprotein particles are involved in specifying the sites for the specific endonuclease cleavages of the pre-rRNA during maturation (Hadjiolov, 1977). The ribosomal proteins are synthesised in the cytoplasm (Craig and Perry, 1971) and they migrate to the nucleolus, where they associate with the pre-rRNA and the 5S rRNA to form the precursor ribonucleoprotein particles.

The most nascent ribonucleoprotein particle has a sedimentation coefficient of 80S and contains the 45S pre-rRNA species (Warner and Soeiro, 1967). Analysis of the proteins by two-dimensional polyacrylamide gel electrophoresis has shown that there are approximately 60 different proteins present, the electrophoretic pattern being very similar to that of a mixture of cytoplasmic 40S and 60S ribosomal subunits (Auger-Buendia and Longuet, 1978; Auger-Buendia et al., 1979). However, several additional protein spots are present on electrophoresis of the proteins from 80S nucleolar ribonucleoprotein particles. Moreover, a number of proteins present in the mature ribosomal subunits, particularly the small subunit, are absent from the 80S precursor particles (Auger-Buendia and Longuet, 1978).

The 80S particle undergoes maturation, resulting in the small ribosomal subunit and a nucleolar ribonucleoprotein particle which appears to be the immediate precursor of the large ribosomal subunit. In HeLa cells, this precursor particle has a sedimentation coefficient of 55S (Warner

and Soeiro, 1967; Kumar and Subramanian, 1975), although values of 60S have been reported for the corresponding precursor particles from rat liver (Higashinakagawa and Muramatsu, 1974) and cultured mouse leukaemia cells (Auger-Buendia and Longuet, 1978). The sedimentation coefficient does appear to vary with the concentration of magnesium ions, however. These precursor particles contain the 32S pre-rRNA species (Warner and Soeiro, 1967; Auger-Buendia and Longuet, 1978), although Higashinakagawa and Muramatsu (1974) reported that the RNA extracted from rat liver 60S precursor particles consisted of 28S rRNA hydrogen-bonded to a low molecular weight RNA species. It is possible that differences in the associated pre-rRNA molecules are related to differences in the predominant maturation pathway of the pre-rRNA in the different cell types. As well as containing the precursor to the 28S rRNA, the 55-60S precursor particles contain the 5S rRNA species (Warner and Soeiro, 1967; Higashinakagawa and Muramatsu, 1974). Analysis of the protein complement of the nucleolar 55-60S precursor particles has shown that nearly all the proteins of the large ribosomal subunit are present, only 5-10 proteins being absent (Warner and Soeiro, 1967; Higashinakagawa and Muramatsu, 1974; Kumar and Subramanian, 1975; Auger-Buendia and Longuet, 1978). In addition, the 55-60S ribonucleoprotein particle has been shown to contain some extra proteins not present in the large ribosomal subunit (Warner and Soeiro, 1967; Higashinakagawa and Muramatsu, 1974; Kumar and Subramanian, 1975). Two-dimensional polyacrylamide gel electrophoresis has shown that some of these non-ribosomal proteins co-migrate with the non-ribosomal proteins of the 80S nucleolar precursor particle (Auger-Buendia and Longuet, 1978). Using this method of analysis, Auger-Buendia and Longuet (1978) were unable to demonstrate the presence of any of the proteins of the small ribosomal subunit in the 55-60S precursor particle. Evidence for the 55-60S ribonucleoprotein particle's being the precursor of the large ribosomal subunit has come from pulse chase

experiments (Warner and Soeiro, 1967). A maturation pathway for nucleolar ribonucleoprotein particles in HeLa cells, similar to that for pre-rRNA, can thus be represented. This is shown in Figure 5.

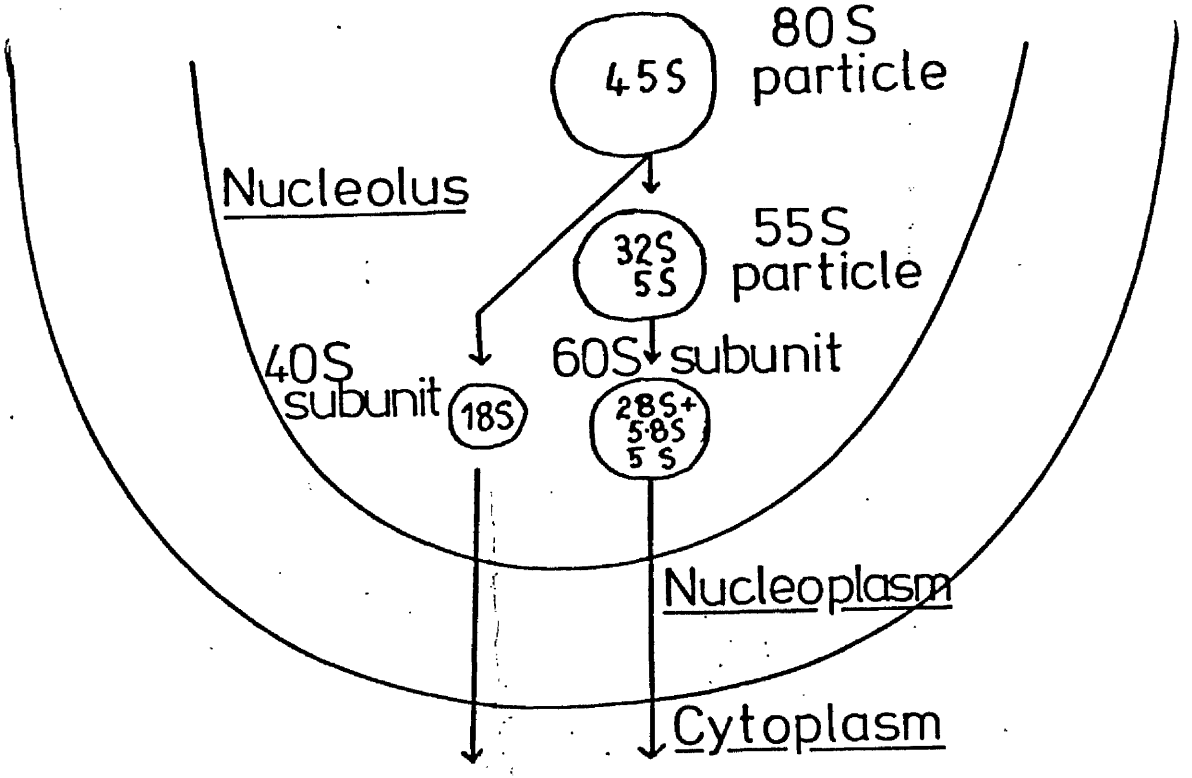


Figure 5. Proposed maturation pathway for nucleolar ribonucleoprotein particles in HeLa cells. The figures inside the particles refer to the sedimentation coefficients of the associated RNA species.

Again, it should be stressed that this may not be the exclusive maturation pathway and that different maturation pathways may predominate in other cell types. Also, it is not clear whether intermediate precursor particles, containing other pre-rRNA molecules, exist. The net result of these maturation processes is that cleavage of the pre-rRNA occurs concomitantly with changes in the protein complement of the precursor particles in the nucleolus, resulting in the mature ribosomal subunits.

At first sight, the synthesis of rRNA and ribosomal proteins in the cell would appear to be closely linked. With the exception of a very small number of proteins, to be discussed below, newly-synthesised ribosomal proteins do not become incorporated into ribosomal subunits if the synthesis of rRNA is inhibited with actinomycin D (Warner, 1966; Craig, 1971). However, using a crude cell extract of HeLa cells, Warner (1977) has shown that a group of proteins which co-migrate with authentic ribosomal proteins on two-dimensional polyacrylamide gel electrophoresis, and therefore presumed to be ribosomal proteins, are synthesised when pre-rRNA synthesis has been blocked for more than 24hr by actinomycin D. This could occur either because the synthesis of the mRNA species coding for the ribosomal proteins is independent of the synthesis of pre-rRNA or because the ribosomal protein mRNA species have an exceptionally long half-life. Warner (1977) has suggested that the former explanation is the more likely of the two. He has also proposed that these newly-synthesised ribosomal proteins accumulate in the nucleolus but are degraded extremely rapidly in the absence of pre-rRNA synthesis. They do not, therefore, become incorporated into ribosomal subunits if pre-rRNA synthesis is resumed. These findings have been extended by studies using toyocamycin, an analogue of adenosine, which is incorporated into 45S pre-rRNA, altering its structure so that processing to the mature 28S and 18S rRNA molecules does not occur (Weiss and Pitot, 1974). Under these conditions, newly-synthesised ribosomal proteins associate with the 45S pre-rRNA in the nucleolus, forming 80S precursor particles (Auger-Buendia and Tavitian, 1979). Therefore, the synthesis of ribosomal proteins does not appear to depend on the transcription of pre-rRNA nor on its processing.

Similarly, the transcription of 45S pre-rRNA does not appear to depend on ribosomal protein synthesis. Blocking protein synthesis by treatment with cycloheximide does inhibit the formation of mature ribosomes (Warner et al., 1966). However, if a sufficiently low dose of cycloheximide is

given, 45S pre-rRNA continues to be transcribed but appears to undergo aberrant processing (Stoyanova and Hadjiolov, 1979). It is possible that, under these conditions, the excess rRNA is degraded, as appears to be the case in resting lymphocytes (Cooper, 1969).

The results of these inhibition experiments have led Hadjiolov (1977) to suggest that ribosome synthesis is subject to some form of control during maturation of the precursor ribonucleoprotein particles in the nucleolus. If the supply of pre-rRNA is limiting, the ribosomal proteins continue to be synthesised whilst if the supply of ribosomal proteins is limiting, the transcription of pre-rRNA continues. The excess components are degraded, resulting in what appears to be coordinate synthesis of the ribosomal components that appear in the cytoplasm in the form of ribosomal subunits. Once in the cytoplasm, the structural components of the ribosomes appear to be degraded as a unit with a half-life of several days (Tsurugi et al., 1974; Lastick and McConkey, 1976). The ribosomal components therefore have a high stability when they are assembled to form the complete ribosome.

It was mentioned that, amongst the ribosomal proteins, there were some exceptions to this rule of assembly and degradation of the ribosome as a unit. Some ribosomal proteins appear to be added to the ribosomes in the cytoplasm. This addition still continues if the synthesis of the ribosomal precursor particles and their subsequent maturation is inhibited with actinomycin D, suggesting that it occurs as a result of exchange between the ribosomes and cytoplasmic pools of the ribosomal proteins. The question of ribosomal protein exchange has been the subject of some controversy. Dice and Schimke (1972) have suggested that mammalian cells contain large pools of ribosomal proteins which are in equilibrium with the ribosomes. However, Warner (1966) could detect the labelling of only three proteins of the 60S subunit by exchange in the presence of actinomycin D. These findings have been confirmed by Lastick

and McConkey (1976) who were able to identify the proteins, from their positions on two-dimensional polyacrylamide gels, as being L10, L19, and L24, according to the nomenclature system of McConkey et al., (1979). Other workers have suggested that a greater number of newly-synthesised ribosomal proteins become incorporated into ribosomes in the presence of actinomycin D (Aiello et al., 1977; Cazillis and Houssais, 1979) or toyocamycin (Auger-Buendia and Tavitian, 1979). It is not clear why such differences in the number of exchangeable ribosomal proteins should occur.

Lastick and McConkey (1976) measured the stability of the ribosomal proteins and found that two of the three exchangeable proteins which they could detect had a reduced stability compared to the high stability of the bulk of the ribosomal proteins. This would be expected since exchangeable ribosomal proteins would not be associated permanently with the ribosomes and would therefore be more liable to degradation whilst part of a cytoplasmic pool. The third exchangeable protein did not have a reduced stability, possibly because of a slow rate of exchange. Only one other ribosomal protein had a reduced stability and exchange of this protein could not be detected. It is possible that this protein was being degraded whilst on the ribosomal subunit (Lastick and McConkey, 1976).

These experiments depend on the assumption that large, free pools of most of the ribosomal proteins do not exist, in contrast to the findings of Dice and Schimke (1972). Using immunochemical methods, Wool and Stoffler (1976) have shown that the pool of free ribosomal proteins in rat liver cytoplasm is very small. In fact, since most authors agree that very few of the ribosomal proteins synthesised in the presence of actinomycin D become associated with ribosomes in vivo, yet the synthesis of all the ribosomal proteins continues in the presence of the antibiotic, it seems highly unlikely that there are pools of ribosomal proteins which are in equilibrium with the mature ribosomes. Sherton and Wool (1974) have suggested that the use of sodium dodecyl sulphate (SDS) by Dice and

Schimke (1972) for the extraction of ribosomal proteins may have led to erroneous results on polyacrylamide gel electrophoresis.

Post-translational modifications of the ribosomal proteins have been shown to occur. These modifications include phosphorylation and methylation, together with other forms of modification. Phosphorylation is the form of modification which has been studied most extensively. It appears to be specific to eukaryotic ribosomes. Phosphorylation of ribosomal proteins in vivo was demonstrated initially in rabbit reticulocytes (Kabat, 1970) and rat liver (Loeb and Blat, 1970). These early studies employed electrophoresis of the ribosomal proteins on one-dimensional polyacrylamide gels, a technique in which only approximately twenty protein bands are resolved. Greater resolution is possible with two-dimensional polyacrylamide gels, which allows the phosphorylated proteins to be identified. Such studies have shown that the major phosphorylated product from a variety of mammalian tissues is the ribosomal protein S6, according to the nomenclature of McConkey et al., (1979). Phosphorylation of S6 in intact cells has been demonstrated in rat liver (Gressner and Wool, 1974), baby hamster kidney fibroblast (BHK) cells (Leader et al., 1976), mouse ascites cells (Rankine et al., 1977; Leader and Coia, 1978 b), HeLa cells (Kaerlein and Horak, 1976, 1978; Blair and Horak, 1977; Schiffmann and Horak, 1978), rabbit reticulocytes (Traugh and Porter, 1976) and rat brain (Roberts and Ashby, 1978; Roberts and Morelos, 1979). Covalent attachment of the phosphate group to the protein is suggested by the fact that, if the phosphorus is radioactively-labelled, treatment with alkaline phosphatase will remove the label (Gressner and Wool, 1974; Rankine et al., 1977) and labelled phosphoserine will be recovered from an acid hydrolysate of ribosomal proteins (Gressner and Wool, 1974; Rankine et al., 1977; Leader and Coia, 1978b; Kaerlein and Horak, 1978). The phosphorylation of S6 is increased under conditions of rapid cellular growth, such as those found in regenerating rat liver (Gressner and Wool,

1974) and pre-confluent BHK cells (Leader et al., 1976), and also in HeLa cells infected with vaccinia virus (Kaerlein and Horak, 1976, 1978) and adenovirus (Blair and Horak, 1977). This increased phosphorylation possibly may be related to an increase in the rate of protein synthesis. Another possibility is that phosphorylation of S6 occurs during ribosome assembly in the nucleolus and the phosphate group is removed in the cytoplasm. The increased phosphorylation observed in rapidly growing cells could thus result from a greater proportion of newly-synthesised ribosomes (Gressner and Wool, 1974). The role of phosphorylation of S6 is still not clear, however. Other proteins of the small subunit have been reported to be phosphorylated under certain conditions, these proteins being S2 (Rankine et al., 1977; Leader and Coia, 1978b; Kaerlein and Horak, 1976, 1978; Roberts and Ashby, 1978; Roberts and Morelos, 1979), S3 (Leader and Coia, 1978b; Roberts and Ashby, 1978; Roberts and Morelos, 1979), S5 (Roberts and Morelos, 1979) and S16 (Kaerlein and Horak, 1978).

The phosphorylation of proteins of the large ribosomal subunit is a more contentious issue. Leader's laboratory has reported the phosphorylation in vivo of an acidic protein, designated L8, of the large ribosomal subunit of mouse Krebs II ascites cells (Rankine et al., 1977; Leader and Coia, 1977, 1978a, b, c) and BHK cells (Leader and Coia, 1978 a, c). This protein appears to be equivalent to rat liver proteins L40 and L41, on the basis of its electrophoretic mobility. In addition, immunochemical experiments have shown that both the ascites protein and rat liver L40 and L41 are related to the acidic protein L12 from E. coli (Leader and Coia, 1978 c). Phosphorylation in vivo of L40/L41 has also been reported in HeLa cells (Horak and Schiffmann, 1977; Schiffmann and Horak, 1978) and mouse L cells (Houston, 1978). However, other workers have not been able to detect phosphorylation of any of the proteins of the large ribosomal subunit under conditions in which the phosphorylation of S6 occurs (Gressner and Wool, 1974; Traugh and Porter, 1976). As with the phosphorylation of S6,

the significance of any possible phosphorylation of acidic proteins of the large ribosomal subunit is not clear. In fact, the relationship, if any, between phosphorylation of ribosomal proteins and protein synthesis still remains to be established.

Another modification of ribosomal proteins is acetylation, which has been reported to occur in rat liver in vivo (Liew and Gornall, 1973). It has been mentioned that the mammalian ribosomal proteins L40 and L41 are immunologically and functionally related to the E. coli proteins L7 and L12. In the prokaryotic organism, these proteins are involved in translocation, providing the binding site for initiation, elongation and termination factors, and participating in the hydrolysis of GTP. They differ only in their N-terminal amino acid, which is acetylated in L7. It is not known whether a similar N-acetylation occurs in the mammalian proteins L40 and L41. Horak and Schiffmann (1977) have postulated that the phosphorylation of these proteins in mammalian ribosomes is homologous to the N-acetylation which occurs in bacterial ribosomes.

Methylation of ribosomal proteins in vivo occurs in both prokaryotes and eukaryotes. A number of ribosomal proteins have been reported to be methylated in HeLa cells on either arginine or lysine residues (Goldenberg and Eliceiri, 1977; Chang et al., 1978; Scolnik and Eliceiri, 1979). A further post-translational modification of ribosomal proteins reported in mammalian ribosomes is the cyclisation of glutamic acid to form γ-carboxyglutamic acid (Van Buskirk and Kirsch, 1978).

1.3.2.3 Aim of project

The aim of the experimental work described in this thesis is to investigate the relationship between the synthesis of the various components of polyribosomes in the oestrogen-stimulated immature rat uterus. It was discussed, in Section 1.2.2.6, that treatment of immature rats with oestradiol-17β leads to a stimulation of RNA synthesis in the uterus followed by a stimulation of protein synthesis and then a stimulation of DNA synthesis.

For the synthesis of ribosomes, both rRNA and ribosomal proteins are required. It is not known whether the ribosomal proteins are synthesised at the same time as the rRNA or whether the stimulation of ribosomal protein synthesis in response to oestrogen occurs at the same time as the general increase in protein synthesis in the uterus. Also, the exact relationship between the synthesis of mRNA, and the so-called Key Intermediary Proteins, and the later stimulation of rRNA synthesis is not clear.

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Experimental animals

Female, albino rats, derived from the Wistar strain, were bred in the departmental animal house. All the animals used were 18-21 day-old weanlings and, for most experiments, their weights were in the range 30-40g.

Throughout the course of the experiments, the rats were given free access to water and pelleted diet supplied by Labsure Animal Foods, Christor Hill Group Ltd., Poole, Dorset, U.K.

Oestradiol-17β [oestra-1,3,5 (10)-trien-3,17β-diol], obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., was dissolved in absolute ethanol to give a stock solution of 2mg/ml and stored at -20°C. Radioactive precursors were purchased from the Radiochemical Centre, Amersham, Bucks., U.K., and were diluted to the required concentration with 0.9% (w/v) saline before use. The administration of oestradiol -17β and radioactive precursors was carried out as described by Knowler and Smellie (1971). Oestradiol -17β was given as a single, intraperitoneal injection in 0.1ml 0.9% (w/v) saline containing 0.5% (v/v) ethanol. Control animals received 0.1ml saline-ethanol alone. Radioactive precursors were administered by intravenous injection via the lateral tail vein in 0.2ml 0.9% (w/v) saline whilst the animal was under ether anaesthesia.

2.2 Reagents and glassware

Unless otherwise specified, all chemicals used were, as far as possible, supplied by B.D.H. Chemicals Ltd., Poole, Dorset, U.K. and were AnalaR grade.

All homogenisation steps were carried out in glass homogenisers with motor-driven Teflon pestles. The homogenisers were either obtained from Jencons, Hemel Hempstead, Herts., U.K. or were made in the Chemistry Department of the University of Glasgow.

Corex glass centrifuge tubes were purchased from Corning Glass Works, New York, U.S.A. Cellulose nitrate tubes, for ultracentrifugation, were supplied by Beckman Spinco Ltd., Palo Alto, California, U.S.A. All other glassware was obtained from the laboratory stock.

The glassware for all sub-cellular preparations was sterilised at 160°C for 14hr before use. Plastic tips for automatic pipettes were steam autoclaved. Any further precautions taken against ribonuclease activity will be described in the relevant section.

Dialysis tubing was boiled in 0.01M EDTA (ethylenediaminetetra-acetate), pH 7.0, and stored in 10% (v/v) ethanol before use.

2.3 Reagents for liquid scintillation counting

The reagents used for liquid scintillation spectrometry were: 2,5-diphenyloxazole (PPO), obtained from International Enzymes Ltd., Windsor, Berks., U.K.; scintillation grade p-bis (o-methylstyryl) benzene (bis-MSB), supplied by Eastman-Kodak Co., Rochester, New York, U.S.A.; Triton X-100, supplied by Rohm and Haas (U.K.) Ltd., Croydon, Surrey, U.K.; AnalaR grade toluene, obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

The scintillation fluids used were PPO-toluene, consisting of 0.5% (w/v) PPO in toluene, and Triton-toluene, consisting of 0.5% (w/v) PPO, 0.05% (w/v) bis-MSB and 35% (v/v) Triton X-100 in toluene.

2.4 Experimental methods

2.4.1 Preparation of acid-soluble and acid-insoluble fractions from immature rat uteri

Acid-soluble and acid-insoluble fractions were prepared from the uteri of immature rats that had received radioactive precursors 30 min before death as described by Knowler and Smellie (1971).

For this part of the experimental work, the rats were limited to a weight range of 22-30g. The radioactive precursors used were [5-^{3}H] uridine, of specific activity 5 Ci/mmol, and L [U-^{14}C] leucine, of

specific activity 324 mCi/m mol.

The animals were killed and their uteri removed, dissected free of adipose tissue, washed in saline and snap-frozen in a solid CO_2-methanol bath. The uteri were stored individually at -60°C for up to four days.

The subsequent steps in the preparation were carried out at 0-4°C. Each uterus was allowed to thaw, chopped finely with scissors and homogenised in 2.5ml distilled water. The homogeniser was washed with a further 2ml distilled water and the combined homogenate and washings were added to 0.5ml 50% (w/v) trichloroacetic acid. After allowing the mixture to stand for 15 min, one quarter was removed into a separate tube and both portions were centrifuged at 800g for 5 min. The supernatant from the smaller fraction was discarded, retaining the pellet for subsequent DNA assay. The supernatant from the larger fraction was retained and the pellet was resuspended in 3ml 5% (w/v) trichloracetic acid and re-spun at 800g for 5 min. The supernatant was added to the supernatant from the previous centrifugation, the combined supernatants constituting the acid-soluble fraction. The acid-insoluble pellet was resuspended in 2ml 5% (w/v) trichloroacetic acid, and 2ml of a 2% (w/v) suspension of kieselguhr (Hyflo Super Cell, Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) in 5% (w/v) trichloroacetic acid was added. The resuspended pellet, bound to kieselguhr, was filtered on a pad of kieselguhr, using a Millipore filtration unit, and washed three times with 15ml 5% (w/v) trichloracetic acid, once with 15ml methanol and once with 15ml diethyl ether. The acid-insoluble pellet was digested for 10 min at 60°C with 0.5ml of a 1M solution of hyamine hydroxide in methanol (Fisons Scientific Apparatus, Loughborough, Leics., U.K.). Radioactivity was measured, after the addition of 10ml PPO-toluene scintillant, in a Philips liquid scintillation counter. This constitutes the acid-insoluble fraction. The radioactivity in 0.3ml samples of the acid-soluble fraction was measured after the addition of 2.7ml Triton-toluene scintillant. The counting efficiency, estimated by the external

standard channels ratio method, was approximately 20% for ^{3}H and 50% for ^{14}C for both the acid-soluble and acid-insoluble fractions. DNA estimation was carried out on the smaller, acid-insoluble pellet that had been retained.

2.4.2 Preparation of polyribosomes

Ribosomes were prepared from immature rat uteri by a method based on that of Berridge et al. (1976). This method, describing the preparation of frog liver ribosomes, was scaled down to suit the smaller amount of tissue available. A further modification was that diethyl pyrocarbonate was omitted from the buffers.

Uteri were removed from the immature rats and snap-frozen in a solid CO_2-methanol bath. The uteri were not stored at this stage but were used immediately for the preparation of ribosomes. Twelve uteri were used for each preparation.

The uteri were broken up, whilst still frozen, with a glass rod and the resulting fragments were allowed to thaw. All subsequent steps in the preparation were carried out at 0-4°C. The uteri were homogenised in a total of 5ml 0.2M Tris-HCl (2-amino-2-hydroxymethyl-propane-1,3-diol, supplied as "Trizma Base" by Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.), 0.05M KCl, 0.015M $MgCl_2$ buffer, pH 8.5, containing 5µg/ml cycloheximide (Sigma) and 7mM 2-mercaptoethanol. The homogenate was adjusted to 2% (w/v) Triton X-100, by the addition of 0.55ml 20% (w/v) Triton X-100 (Rohm and Haas), and centrifuged at 10,000g for 10 min in a Sorvall SS 34 (fixed angle) rotor. The supernatant was removed, layered over a 1ml cushion of 0.05M Tris-HCl, 0.05M KCl, 0.015M $MgCl_2$ buffer, pH 8.5, containing 1M sucrose, 5 µg/ml cycloheximide and 7 mM 2-mercaptoethanol, and centrifuged at 47,000 rev/min for 90 min at 2°C in a Beckman SW 50.1 rotor (g_{av} = 200,000 g). The resulting supernatant was discarded and the inside of the centrifuge tube was wiped with a clean tissue, taking care not to disturb the pellet. The pellet was rinsed with a small

amount of 0.05M Tris-HCl, 0.25M KCl, 0.005M $MgCl_2$ buffer, pH 7.6, containing 5 μg/ml cycloheximide and 50 μg/ml heparin (supplied at a concentration of 5,000 U/ml in a pyrogen-free, sterile solution by Evans Medical Ltd., Liverpool, U.K.). The pellet was then resuspended in 0.4ml of this same buffer, using a motor-driven, Teflon-glass homogeniser operating at the slowest possible speed, and carefully layered over a 5ml, linear, 15-45% (w/v) sucrose density gradient made up in the same buffer. The gradient was centrifuged at 50,000 rev/min for 40 min at 2°C in a Beckman SW50.1 rotor (g_{av} = 230,000g). The absorbance at 260nm of the gradient was measured by upwards displacement with 50% (w/v) sucrose through a flow cell attachment for the Gilford 240 spectrophotometer.

In addition to sterilising the glassware, all the solutions were sterilised by steam autoclaving; cycloheximide, 2-mercaptoethanol and heparin being added to the solutions, where necessary, after autoclaving.

2.4.3 Determination of ribonuclease activity

The ribonuclease activity of uterine homogenates was assayed by a method based on that of Ingebretsen et al. (1972) using a radioactively-labelled substrate.

All solutions were sterilised by steam autoclaving, with cycloheximide and 2-mercaptoethanol, where necessary, being added after autoclaving.

Uteri were removed from immature rats, dissected free of adipose tissue, and snap-frozen in a solid CO_2-methanol bath. Two uteri were used for the preparation of each homogenate. The uteri were allowed to thaw and chopped finely with scissors. They were homogenised in 1ml 0.2M Tris-HCl, 0.05M KCl, 0.015M $MgCl_2$ buffer, pH 8.5, containing 5 μg/ml cycloheximide and 7mM 2-mercaptoethanol. The homogenates were stored at -70°C for up to 2 days.

The substrate for the ribonuclease assay was ^{32}P-labelled 28S rRNA from HeLa cells, from which the 5.8S rRNA species had been removed, provided by Mr J.M. Kelly, of this department, in the form of a suspension in ethanol.

The RNA was pelleted by centrifuging at 2,000 g for 30 min. The pellet was dried in a gentle stream of nitrogen and dissolved in 1ml 0.1M Tris-HCl, 0.06M KCl buffer, pH 8.5. The RNA was precipitated three times from this buffer with ethanol, to remove any low molecular weight contaminants, and dialysed against 0.1M Tris-HCl, 0.06M KCl, pH 8.5. The final product gave only one peak on polyacrylamide gel electrophoresis in the position expected of 28S rRNA. The concentration of RNA in the final preparation, as measured by the absorbance at 260nm, was 56 µg/ml and its specific activity was estimated to be 1.7 mCi/g. This solution was stored at -20°C.

The ribonuclease assay mixture consisted of 10 µl of this ^{32}P-labelled RNA substrate, 0.2ml 0.1M Tris-HCl, 0.06M KCl buffer, pH 8.5, and sufficient distilled water to bring the volume to 0.55 ml. The mixture was pre-incubated at 37°C for 15 min and the reaction initiated by the addition of 50 µl uterine homogenate. The mixture was incubated at 37°C for 30 min and the reaction was terminated by cooling to 0°C and adding 5ml of 5% (w/v) trichloroacetic acid, together with 0.1ml of a 5mg/ml solution of yeast RNA to act as a co-precipitant. Acid-insoluble radioactivity was determined by allowing the tubes to stand for at least 15 min before spinning at 12,000 g for 10 min in a Sorvall SS 34 rotor. The supernatants were retained and the pellets were resuspended in 2ml 5% (w/v) trichloroacetic acid and filtered on 2.5cm diameter GF/C glass fibre filters (Whatman, Maidstone, Kent, U.K.). The filters were washed three times with 5ml 5% (w/v) trichloroacetic acid, once with 5ml methanol and once with 5ml diethyl ether, and allowed to dry. 5ml PPO-toluene scintillant was added to each filter and the radioactivity was measured in a Philips liquid scintillation counter. To check the recovery of radioactivity, the activity in 0.3ml aliquots of the supernatants was also measured after the addition of 2.7ml Triton-toluene scintillant.

Control incubations were carried out in which the 50 µl uterine homogenate was replaced by water. Incubations were also carried out using 50 µl of a mixture of pancreatic ribonuclease (Sigma) and T_1 ribonuclease (Calbiochem Ltd., Bishops Stortford, Herts., U.K.), both at a concentration of 2 µg/ml in water, to act as standards.

The protein concentration of the uterine homogenates was measured by the method of Bramhall et al.(1969).

2.4.4 Preparation of rat liver ribonuclease inhibitor

A crude supernatant fraction from rat liver was prepared, in order to provide a source of ribonuclease inhibition, as described by Roth (1958). The actual method adopted differed from the original one described by Roth (1958) in that, instead of two centrifugation steps at the same speed, a low speed spin, to remove nuclei and mitochondria, was carried out followed by a high speed spin to pellet ribosomes.

One adult, male rat weighing approximately 200 g was killed and its liver was removed and weighed. The liver was chopped finely with scissors and homogenised in sterilised, distilled water, using 5ml water per g liver. The homogenate was centrifuged at 12,000g for 10 min at 4°C in a Sorvall SS 34 rotor. The pellet was discarded and the supernatant was centrifuged at 25,000 rev/min for 2 hr at 4°C in a Beckman SW 40 rotor (g max = 110,000 g). The supernatant was decanted and stored frozen at -20°C.

The effectiveness of this supernatant preparation as a ribonuclease inhibitor was determined by including 50 µl in a standard ribonuclease assay, as described in Section 2.4.3, using a mixture of pancreatic and T_1 ribonucleases, both at a concentration of 2 µg/ml.

2.4.5 Preparation of cytoplasmic RNA

Cytoplasmic RNA was prepared from the post-mitochondrial supernatant of immature rat uteri by a method based on that of Berridge et al.(1976).

All buffer solutions were sterilised by steam autoclaving, with heparin and polyvinyl sulphate being added, where necessary, after autoclaving.

Phenol was re-distilled, collecting the fraction distilling at 182°C, and stored at 4°C before dilution with the appropriate buffer.

The animals were killed and their uteri were removed, dissected free of adipose tissue, washed in saline and snap-frozen in a solid CO_2-methanol bath. The uteri were not stored at this stage but were used for the preparation of RNA immediately. The preparation was carried out using twelve uteri.

The uteri were broken up with a glass rod whilst still frozen. The fragments were allowed to thaw and homogenised in a total of 5ml 0.2M Tris-HCl, 0.05M KCl, 0.05M $MgCl_2$ buffer, pH 8.5, containing 50 µg/ml heparin (Evans Medical Ltd., Liverpool, U.K) and 10 µg/ml polyvinyl sulphate (Eastman-Kodak Co., Rochester, New York, U.S.A.). 0.55ml 20% (w/v) Triton X-100 was added, to give a concentration of 2% (w/v) Triton X-100, and the homogenate was centrifuged at 10,000g for 10 min at 4°C in a Sorvall HB 4 (swing-out) rotor. The supernatant was removed and its volume measured. One ninth vol. 0.1M Tris-HCl, 0.1M EDTA buffer, pH 7.6, was added, thus bringing the concentration of EDTA in the post-mitochondrial supernatant to 0.01M. 0.5 vol. 88% (v/v) phenol in 0.01M Tris-HCl, 0.01M EDTA buffer, pH 7.6, containing 1% (w/v) SDS was then added and the mixture was shaken for 15 min at room temperature (20-24°C). 0.5 vol. chloroform was added and the shaking was continued for a further 20 min at room temperature. The mixture was centrifuged at 10,000 g for 10 min at 20°C in a Sorvall HB4 rotor and the lower, phenol phase was removed and discarded. The aqueous phase was re-extracted with an equal volume of 88% (v/v) phenol-chloroform (1:1 v/v) by shaking for 30 min at room temperature. The mixture was re-centrifuged at 10,000 g for 10 min at 20°C. The aqueous layer was removed and its volume measured. 0.1 vol.1.65M NaCl was added to give a NaCl concentration of 0.15M and the RNA was precipitated by the addition of 2 volumes absolute ethanol and stored at -20°C.

This method was used to prepare radioactively-labelled RNA and also unlabelled RNA to act as a carrier. When the latter was carried out, the RNA suspension in ethanol was spun down by centrifuging at 2,000 g for 30 min. The pellet was dried in a gentle stream of nitrogen and dissolved in sufficient sterilised, distilled water to give a concentration of 1mg/ml, as determined from the absorbance at 260nm. Samples were also taken for electrophoresis on polyacrylamide gels to check the integrity of the rRNA. A typical preparation from twelve immature rat uteri resulted in approximately 1mg RNA which gave peaks of rRNA on electrophoresis. The RNA solution was stored frozen at -20°C as 1ml aliquots. The rRNA was stable, as judged by electrophoresis, for up to 4 months.

2.4.6 Extraction of polyribosomal RNA

Polysomal RNA was prepared from the uteri of immature rats that had received [^{3}H] uridine 30 min before death.

[5,6 - ^{3}H] Uridine was supplied by the Radiochemical Centre, Amersham, Bucks., U.K. at a specific activity which varied from 43 Ci/m mol to 58 Ci/m mol. Unlabelled uridine (Sigma) was added to bring the specific activity to 5 Ci/m mol and the solution was diluted to the required concentration with 0.9% (w/v) saline. The animals received 100 µCi of the isotope 30 min before death.

Polysomal RNA was prepared from groups of twelve uteri by a modification of the method described by Berridge et al.(1976). Solutions were sterilised by steam autoclaving and heparin added, where necessary, after autoclaving.

Polysomes were prepared and sedimented on a sucrose density gradient as described in Section 2.4.2. The region of the gradient containing the ribosomes, from the ribosomal subunits to the largest polysomes, was collected. The ribosomes were precipitated by the addition of 2 volumes absolute ethanol and stored at -20°C overnight. The following day, the precipitated ribosomes were pelleted by centrifuging at 2,000 g for 30 min. The pellet was dried in a gentle stream of nitrogen and resuspended in

0.25ml 0.05M Tris-HCl, 0.25M KCl, 0.005M $MgCl_2$ buffer, pH 7.6, containing 50 µg/ml heparin (Evans Medical Ltd.), and 5ml 0.01M Tris-HCl, 0.01M EDTA buffer, pH 7.6, containing 1% (w/v) SDS was added. 2.5ml 88% (v/v) phenol in 0.01M Tris-HCl, 0.01M EDTA buffer, pH 7.6, containing 1% (w/v) SDS was then added and the mixture was shaken at room temperature for 15 mins. 2.5ml chloroform was added and the shaking was continued for a further 20 min at room temperature. The mixture was centrifuged at 10,000 g for 10 min at 20°C in a Sorvall HB4 rotor. The lower, phenol layer was removed and discarded whilst the aqueous layer was re-extracted with 5ml 88% (v/v) phenol-chloroform (1:1 v/v) by shaking for 30 min at room temperature. The mixture was centrifuged at 10,000 g for 10 min at 20°C in a Sorvall HB4 rotor. The aqueous layer was removed and its volume was measured. 0.1 volume 1.65M NaCl was added, to give a NaCl concentration of 0.15M, and the RNA was precipitated by the addition of 2 volumes absolute ethanol and stored at -20°C.

2.4.7 Affinity chromatography of polyribosomal RNA on oligo (dT) - cellulose

Polysomal RNA, prepared as described in the preceding section, was fractionated by chromatography on oligo (dT) - cellulose by the method of Aviv and Leder (1972), modified according to McGrath (1978).

All the reagents, except for the oligo (dT) - cellulose, were autoclaved. In addition to the usual precautions of sterilising the glassware, the pasteur pipettes into which the oligo (dT) - cellulose columns were going to be poured, together with the glass wool plugs, were soaked in 0.02% (v/v) diethyl pyrocarbonate and dried in an oven.

Oligo (dT) - cellulose, type T-2, was purchased from Uniscience Ltd., Cambridge, U.K. 0.07 g was suspended in 5ml water and the suspension was poured into a pasteur pipette with a glass wool plug. After settling, the resulting column of oligo (dT) - cellulose was 1.5cm high with a bed volume of approximately 0.5ml. The column was maintained at room temperature throughout the experimental procedures. It was washed with

5ml 2% (w/v) SDS followed by 5ml loading buffer (0.5 M LiCl, 0.001 M EDTA, 0.01M Tris-HCl, pH 7.5, containing 0.1% (w/v) SDS).

The extracted polysomal RNA, stored as a suspension in ethanol, was pelleted by centrifuging at 2,000g for 30 min. The pellet was dried with nitrogen and dissolved in 1ml loading buffer. The amount of RNA present was determined by measuring the absorbance at 260nm, using yeast RNA as a standard. The solution of polysomal RNA was warmed to 60°C for 10 min, to disaggregate the RNA, and applied to the column. The eluate was collected and re-applied to the column twice more to ensure complete binding of all the poly (A)-containing RNA. The final eluate was collected and retained.

The column was washed with 5ml intermediate buffer (0.1M LiCl, 0.001M EDTA, 0.01M Tris-HCl, pH 7.5, containing 0.1% (w/v) SDS), collecting the eluate as 1ml fractions whose absorbance at 260nm was measured. 5ml of this buffer was usually sufficient to reduce the absorbance to below 0.05. If the absorbance was still greater than 0.05, the column was washed with further 1ml aliquots of intermediate buffer until it had fallen to below 0.05. The fractions eluted by this procedure were pooled and combined with the final eluate resulting from the application of the original RNA solution to the column. The pooled eluates were designated the unbound fraction.

RNA bound to the column was eluted with 5ml eluting buffer (0.001M EDTA, 0.01M Tris-HCl, pH 7.5). The resulting eluate was designated the bound fraction.

The volumes of both fractions were measured and the amount of RNA present was determined by measurement of the absorbance at 260nm, using yeast RNA as a standard. 1.65M NaCl was added to both fractions to a concentration of 0.15M, followed by 0.1ml of a 1mg/ml solution of carrier, uterine RNA. The RNA in the two fractions was precipitated with 2 volumes absolute ethanol and stored at -20°C for up to one week.

The amount of acid-insoluble radioactivity in the two fractions was determined following precipitation with trichloroacetic acid. The RNA

suspensions in ethanol were pelleted by centrifuging at 2,000g for 30 min. The pellets were dried with nitrogen and dissolved in 2.5ml water. 0.1ml of a 5mg/ml solution of bovine serum albumin was added as a co-precipitant followed by 2.5ml 10% (w/v) trichloroacetic acid. After standing at 0°C for 30 min, the mixtures were filtered on 2.5cm diameter glass fibre filters (Whatman) and the filters were washed three times with 5ml 5% (w/v) trichloroacetic acid, once with 5ml methanol, once with 5ml diethyl ether, and dried. 10ml PPO-toluene scintillant was added to each filter and the radioactivity was measured in a Searle liquid scintillation counter.

The oligo (dT) - cellulose columns were discarded after a single use and were not regenerated with dilute alkali as described by Aviv and Leder (1972).

2.4.8 Analysis of RNA

2.4.8.1 Polyacrylamide gel electrophoresis of RNA

Purified RNA was separated on 2.7% polyacrylamide gels as described by Loening (1969) with modifications (Knowler and Smellie, 1971).

Acrylamide (specially purified for electrophoresis) was purchased from B.D.H. Chemicals, Ltd., Poole, Dorset, U.K. and ethylene diacrylate was supplied by Kodak Ltd., Kirkby, Lancs., U.K. A stock solution containing 27% (w/v) acrylamide and 2.5% (v/v) ethylene diacrylate was prepared and stored at 4°C in the dark. A ten-fold concentrate of the electrophoresis buffer was prepared, using orthophosphoric acid to adjust the pH, so that a 1 in 10 dilution would have the composition 0.036M Tris-HCl, 0.03M NaH_2PO_4, 0.001M EDTA, pH 7.8. N,N,N',N' - tetramethylethylene diamine (TEMED) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. and stored at 4°C.

The gels contained 2.7% acrylamide, 0.25% ethylene diacrylate and 0.1% TEMED in electrophoresis buffer. Polymerisation was initiated by the addition of sufficient of a freshly-prepared solution of 10% (w/v)

ammonium persulphate to give a final concentration of 0.1%. 3ml aliquots of the solution were poured into Plexiglass tubes, of internal diameter 6mm, each with a polypropylene washer and a filter paper disc at the bottom to prevent the gel falling out of the tube when polymerised. The solution was overlayered carefully with water and allowed to polymerise, resulting in a gel approximately 10cm long. The gel was placed in the electrophoresis tank, the tank filled with electrophoresis buffer to which SDS had been added to a concentration of 0.2% (w/v), and pre-electrophoresis of the gel was carried out at 5mA per gel for 1hr.

The RNA sample, in the form of a suspension in ethanol, was pelleted by centrifuging at 2,000g for 30 min. The pellet was washed by resuspending in 5ml ethanol and re-centrifuging. The final pellet was dried with nitrogen and dissolved in 0.06ml of a solution of electrophoresis buffer containing 10% (w/v) sucrose and 0.2% (w/v) SDS that had been sterilised by autoclaving. The sample was layered on to the gel and electrophoresis was carried out at 5mA per gel for 2½ hr. After electrophoresis, the gel was removed from the tube, soaked in distilled water for 30 min and scanned at 260nm using a gel-scanning attachment for the Gilford 240 spectrophotometer. The positions of the peaks were compared with markers of 18S and 28S rRNA from HeLa cells, provided by Mr J.M.Kelly, and tRNA from Escherichia coli, provided by Dr J.P.Goddard, both of this department.

2.4.8.2 Sucrose density gradient centrifugation of RNA

Labelled, cytoplasmic RNA, extracted from immature rat uteri, was analysed on sucrose density gradients as described by Maden and Robertson (1974).

All solutions were sterilised by steam autoclaving.

The RNA sample, in the form of a suspension in ethanol, was spun down by centrifuging at 2,000g for 30 min. The pellet was dried with

nitrogen and dissolved in 0.5ml LETS buffer (0.1M LiCl, 0.001M EDTA, 0.01M Tris-HCl, pH 7.4, containing 0.2% (w/v) SDS). This solution was layered carefully over a 13ml, 10-25% (w/v), linear sucrose gradient made up in LETS buffer. The gradient was centrifuged at 25,000 rev/min for 16 hr at 20°C in a Beckman SW40 rotor (g_{av} = 77,000g). It was fractionated by pumping it through a flow cell attachment for the Gilford 240 spectrophotometer, using a peristaltic pump, and the absorbance at 260nm was monitored. 0.5ml fractions were collected in tubes containing an equal volume of 10% (w/v) trichloroacetic acid. 0.1ml of a 5mg/ml solution of bovine serum albumin was added to each fraction followed by 5ml 5% (w/v) trichloroacetic acid. Acid-insoluble radioactivity was determined as described in Section 2.4.7.

2.4.9 Preparation of ribosomal subunits from rat uterus

Ribosomes were dissociated into subunits in a buffer of high ionic strength containing puromycin as described by Blobel and Sabatini (1971).

Immature, female rats were given either 200 µCi L-[G - ^{3}H] tryptophan (Radiochemical Centre, Amersham, Bucks., U.K.), of specific activity 11 Ci/m mol or 100 µCi L - [4,5 - ^{3}H] leucine (Radiochemical Centre), of specific activity 1 Ci/m mol, by intravenous injection in 0.2ml saline 1hr before death. The uteri were removed, dissected free of adipose tissue, washed in 0.9% (w/v) saline and frozen in a solid CO_2 - methanol bath.

Ribosomes were prepared from the pooled uteri from twelve rats as soon as possible after removal of the uteri, following the method described in Section 2.4.2 up to the ultracentrifugation step to yield the ribosomes as a pellet. The pellet was resuspended in 0.6ml 0.01M Tris-HCl, 0.08M KCl 0.005M $MgCl_2$ buffer, pH 7.6, using a motor-driven, Teflon-glass homogeniser, and 0.3ml 1.25M KCl was added, bringing the KCl concentration to 0.5M. 0.02ml 1M 2-mercaptoethanol was then added followed by 0.01ml 0.01M puromycin (Sigma), resulting in concentrations of 0.02M 2-mercaptoethanol and 0.1mM puromycin. The mixture was incubated at 37°C for 15 min and then layered

immediately over sucrose density gradients. The preparation was divided equally between two 5ml, 15-30% (w/v), linear sucrose density gradients made up in 0.01M Tris-HCl, 0.5M KCl, 0.005M $MgCl_2$ buffer, pH 7.6, containing 0.02M 2-mercaptoethanol. The gradients were centrifuged at 50,000 rev/min for 90 min at 28°C in a Beckman SW50.1 rotor (g_{av} = 230,000g). The absorbance at 260nm of the gradients was measured by upwards displacement with 50% (w/v) sucrose through a flow cell attachment for the Gilford 240 spectrophotometer. The portions of the gradients containing the 40S and 60S ribosomal subunits were collected separately, the corresponding fractions from the two gradients pooled, and the ribosomal subunits were precipitated by the addition of 2 volumes absolute ethanol and stored at -20°C for up to 3 days.

The amount of radioactivity in the ribosomal subunits was determined following precipitation with trichloroacetic acid. The ethanol precipitates were centrifuged at 2,000g for 30 min. The pellets were dried with nitrogen and dissolved in 2ml distilled water. Two 0.1ml (1/20) aliquots were removed from each fraction and placed on Whatman, 2.5cm diameter paper filters (Grade 1) for subsequent protein assay. To the remaining 9/10 of each fraction was added 0.1ml of a 5mg/ml solution of bovine serum albumin, to act as a co-precipitant, followed by 2ml 10% (w/v) trichloroacetic acid. Acid-insoluble radioactivity was determined as described in Section 2.4.7.

2.4.10 Preparation of ribosomal subunits from rat liver

Ribosomal subunits were prepared from rat liver to provide material to act as a carrier during the extraction of proteins from the uterine ribosomal subunits. The method used was essentially a scaled-up version of the method described in the previous section for the preparation of uterine ribosomal subunits.

One adult, male rat weighing 200g was starved overnight and killed the following morning. The liver was removed and chopped finely with scissors.

All subsequent steps up to the dissociation of the ribosomes into subunits were carried out at 0-4°C.

The chopped liver was rinsed in a small volume of 0.2M Tris-HCl, 0.05M KCl, 0.015M $MgCl_2$ buffer, pH 8.5, containing 5 µg/ml cycloheximide and 7mM 2-mercaptoethanol. Sufficient of this same buffer was added to the chopped liver to bring the total volume to 30ml and the liver was homogenised in a Teflon-glass homogeniser with a motor-driven pestle. 3.3ml 20% (w/v) Triton X-100 (Rohm and Haas) was added, to give a concentration of 2%, and the homogenate was centrifuged at 10,000g for 20 min in a Sorvall SS34 rotor. 11ml aliquots of the supernatant were layered over 3ml cushions of 0.05M Tris-HCl, 0.05M KCl, 0.015M $MgCl_2$ buffer, pH 8.5, containing 1M sucrose, 5 µg/ml cycloheximide and 7mM 2-mercaptoethanol and centrifuged at 40,000 rev/min for 2hr (g_{av} = 200,000g). The resulting supernatants were poured off, the insides of the centrifuge tubes wiped with a clean tissue, and the pellets were rinsed carefully with a small volume of 0.01M Tris-HCl, 0.08M KCl, 0.005M $MgCl_2$ buffer, pH 7.6. One pellet was used for the preparation of ribosomal subunits whilst the remainder were stored frozen at -20°C until more ribosomal subunits were required.

The pellet that was to be used for the preparation of ribosomal subunits was resuspended in 2ml 0.01M Tris-HCl, 0.08M KCl, 0.005M $MgCl_2$ buffer, pH 7.6, using a motor-driven, Teflon-glass homogeniser. 1ml 1.25M KCl was added, to bring the concentration of KCl to 0.5M, and the mixture was clarified by centrifuging at 800g for 5 min. A small amount of material was spun down by this centrifugation. The supernatant was decanted and 0.06ml 1M 2-mercaptoethanol and 0.03ml 0.01M puromycin (Sigma) were added, giving concentrations of 0.02M 2-mercaptoethanol and 0.1mM puromycin. The mixture was incubated at 37°C for 15 mins and 1ml aliquots were layered on to 13ml, 15-30% (w/v) linear, sucrose density gradients made up in 0.01M Tris-HCl, 0.5M KCl, 0.005M $MgCl_2$ buffer, pH 7.6, containing 0.02M

2-mercaptoethanol. The gradients were centrifuged at 40,000 rev/min for 2hr at 28°C in a Beckman SW40 rotor (g_{av} = 200,000g).

The absorbance at 260nm was monitored whilst the gradients were pumped through a flow cell attachment for the Gilford 240 spectrophotometer using a peristaltic pump. The portions of the gradients corresponding to the 40S and 60S ribosomal subunits were collected separately and the corresponding fractions from the three gradients were pooled. The two fractions were dialysed against 1 litre 0.05M Tris-HCl, 0.08M KCl, 0.0125M $MgCl_2$ buffer, pH 7.6, containing 0.01M 2-mercaptoethanol, overnight at 4°C with one change of dialysis buffer. The volumes of the two dialysed fractions were measured, 0.2 volumes of a ten-fold concentrate of the dialysis buffer was added and the ribosomal subunits were precipitated by the addition of 2 volumes absolute ethanol and stored overnight at -20°C.

The following day, the fractions were spun down by centrifuging at 2,000g for 30 min and the pellets were dried with nitrogen. The pellets were dissolved in 1ml distilled water and the absorbances at 260nm and 280nm measured. The amount of ribosomal protein present was calculated, using the relationship that 1 absorbance unit at 260nm over a path length of 1cm is equivalent to 42.4 µg/ml ribosomal protein (Nieuwenhuysen et al., 1978). The yield from one rat liver was approximately 4mg 60S ribosomal subunit protein and 1.5mg 40S ribosomal subunit protein. The ratio of the absorbance at 260nm to that at 280nm was in the range 1.4 - 2.1.

1.65M NaCl was added to a concentration of 0.15M and the ribosomal subunits were re-precipitated by the addition of 2 volumes absolute ethanol and stored at -20°C overnight. The following day, they were pelleted by centrifuging at 10,000g for 10 min in a Sorvall HB4 rotor. The pellets were dried in a gentle stream of nitrogen and each fraction was dissolved in a sufficient volume of 0.01M Tris-HCl, 0.6M magnesium acetate buffer, pH 7.7, to give a solution containing 600 µg/ml ribosomal protein. The two fractions were frozen and stored at -20°C.

2.4.11 Incubation of rat uteri in vitro

Immature rat uteri were incubated in Eagle's HeLa medium (Eagle, 1955), modified according to Busby et al., (1964). Leucine was omitted during the preparation of the medium and [^{3}H] leucine was added where appropriate. The medium was filtered through a Nalgene filter unit with a membrane filter of 0.2 µm pore size (Sybron Corporation, Rochester, New York, U.S.A.) before use and all manipulations were carried out under sterile conditions. The incubation conditions used were as described by Knowler and Smellie (1973).

Twelve immature female rats were killed by cervical dislocation and their uteri were removed, without removing any adhering adipose tissue at this stage. The uteri were collected in leucine-free Eagle's medium during the dissection. The medium was maintained at 37°C and a mixture of 95% O_2, 5% CO_2 (v/v) was passed through it. When all 12 uteri had been collected, they were transferred to 2ml of the same medium containing 50 µCi/ml L - [4,5-^{3}H] leucine (Radiochemical Centre), of specific activity 53Ci/m mol, in a 10ml, stoppered, conical flask. The flask was incubated for 1hr at 37°C under an atmosphere of 95% O_2, 5% CO_2 (v/v) in a shaking water bath. At the end of the incubation period, the medium was poured off and the uteri were washed five times with ice-cold 0.9% (w/v) saline. Any adhering adipose tissue was removed and the uteri were snap-frozen in a solid CO_2-methanol bath.

Ribosomal subunits were prepared from the uteri as described in Section 2.4.9 except that the volumes used for the dissociation of the ribosomes into subunits were reduced to one half of their original value. Thus, the ribosomal pellet resulting from the first ultracentrifugation step was resuspended in 0.3ml 0.01M Tris-HCl, 0.08M KCl, 0.005M $MgCl_2$ buffer, pH 7.6. 0.15ml 1.25M KCl was added, followed by 0.01ml 1M 2-mercaptoethanol and 0.005ml 0.01M puromycin (Sigma). Following incubation for 15 min at 37°C, all the material was layered on to a single, 5ml, 15-30% (w/v), linear

sucrose density gradient. The gradient was centrifuged at 50,000 rev/min for 90 min at 28°C in a Beckman SW50.1 rotor (g_{av} = 230,000g). The absorbance at 260nm of the gradient was measured by upwards displacement with 50% (w/v) sucrose through a flow cell attachment for the Gilford 240 spectrophotometer. The portions of the gradient containing the 40S and 60S ribosomal subunits were collected separately and dialysed against 500ml 0.05M Tris-HCl, 0.08M KCl, 0.0125M $MgCl_2$ buffer, pH 7.6. Dialysis was carried out overnight at 4°C with one change of dialysis buffer.

The following morning, the volumes of the two fractions were measured and 0.2 volumes of a ten-fold concentrate of the dialysis buffer was added, followed by 2 volumes absolute alcohol to precipitate the ribosomal subunits. The fractions were stored at -20°C for up to two weeks before fractionation of the ribosomal proteins.

2.4.12 Fractionation of ribosomal proteins

2.4.12.1 Fractionation of ribosomal proteins by one-dimensional polyacrylamide gel electrophoresis

The ribosomal subunits were subjected directly to electrophoresis on 12.5% SDS-polyacrylamide disc gels using the discontinuous buffer system of Laemmli (1970).

Acrylamide and N,N'-methylene bis-acrylamide (both specially purified for electrophoresis) were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. TEMED was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. and stored at 4°C. Bovine serum albumin and cytochrome c, for use as marker proteins, were supplied by Boehringer, Mannheim, Germany, with a third marker protein, bovine pancreatic ribonuclease, being supplied by Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Glass tubes with an internal diameter of 6mm were siliconised using Siliclad, a water-soluble silicone concentrate supplied by Clay Adams, Parsipanny, New Jersey, U.S.A. This was carried out to prevent the polyacrylamide gels shattering inside the tubes during polymerisation.

A stock solution containing 28% (w/v) acrylamide and 0.735% (w/v) N,N'-methylene bis-acrylamide was prepared. This solution was stored at 4°C in the dark over Amberlite monobed resin MB1 (BDH) to absorb hydrolysis products.

The separation gels contained 12.5% acrylamide, 0.33% N,N'-methylene bis-acrylamide, 0.03% TEMED and 0.1% SDS in 0.38M Tris-HCl buffer, pH 8.5. Polymerisation was initiated by the addition of a freshly-prepared 0.2% (w/v) solution of ammonium persulphate to give a final concentration of 0.05% ammonium persulphate. The mixture was poured into the siliconised glass tubes to a height of 8cm. The solution was overlayered carefully with water and left for 1hr to polymerise and set. When polymerisation was complete, the water overlayer was removed with a tissue and the polyacrylamide stacking gel was prepared. The stacking gel contained 3% acrylamide, 0.078% N,N'-methylene bis-acrylamide, 0.03% TEMED and 0.1% SDS in 0.12M Tris-HCl buffer, pH 7.0. Polymerisation was initiated by the addition of a freshly-prepared 0.2% (w/v) solution of ammonium persulphate to a final concentration of 0.1% and aliquots were poured over the separation gels to give a height of stacking gel of 1.5cm. The solution was overlayered carefully with water and left for 1hr to polymerise and set.

The suspensions of ribosomal subunits in ethanol, prepared as described in the preceding section, were centrifuged at 2,000g for 30 min. The pellets were washed by resuspending them in 5ml ethanol and re-centrifuging at 2,000g for 30 min. The pellets were dried with nitrogen and dissolved in 100 µl SDS-gel sample buffer (0.05M Tris-HCl, pH 7.0, containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol). The samples were heated at 100°C for 2 min, to release the ribosomal proteins from the RNA and to ensure complete reduction and denaturation of the proteins, and one drop 0.05% (w/v) bromophenol blue (L. Light and Co. Ltd., Colnbrook, Bucks., U.K.) was added as a marker dye.

The polyacrylamide gels were placed in the electrophoresis tank which

was filled with electrophoresis buffer (0.025M Tris base, 0.19M glycine, pH 8.5, containing 0.1% (w/v) SDS). The samples were applied to the gels and electrophoresis was carried out at 4mA per gel until the bromophenol blue front was 1cm from the bottom of the gels (this usually took approximately 2hr).

At the end of the run, the gels were removed from their tubes and fixed overnight in methanol: acetic acid: water (5:1:5 v/v). They were stained for 2hr at 37°C in 0.1% (w/v) Coomassie brilliant blue (Gurr, Searle, High Wycombe, Bucks., U.K.) in the same solvent, and destained by diffusion at room temperature in 5% methanol, 7.5% acetic acid (v/v) in water. The gels were scanned at 600nm using a gel scanning attachment for the Gilford 240 spectrophotometer. The gel patterns of uterine ribosomal proteins were compared with those of 40S and 60S ribosomal proteins from BHK cells provided by Dr D.P. Leader of this department. Standard proteins to act as molecular weight markers were also electrophoresed on a separate gel. The marker proteins used were cytochrome c (mol. wt. 11,300), bovine pancreas ribonuclease (mol.wt. 13,500) and bovine serum albumin (mol. wt. 65,400).

If the ribosomal subunits were radioactively-labelled, the gel was frozen, after scanning, with solid CO_2 and sliced into 1mm sections using a Mickle gel slicer. The individual gel slices were digested with hydrogen peroxide, as described by Diener and Paetkau (1972), except that digestion was carried out overnight instead of for 1hr. The gel slices were dried for 2hr at 60°C. 0.3ml 30% (w/v) hydrogen peroxide was added and the slices were digested at 60°C overnight. The following morning, 10ml Triton-toluene scintillant was added to each slice and the radioactivity was measured in a Searle liquid scintillation counter.

2.4.12.2 Fractionation of ribosomal proteins by two-dimensional polyacrylamide gel electrophoresis

Before fractionation of the ribosomal proteins on two-dimensional polyacrylamide gels could be carried out, the ribosomal proteins had to be

extracted from the subunits since, under the conditions of the electrophoretic system used, the ribonucleoprotein particles would not have been disaggregated completely. Extraction of the ribosomal proteins was carried out with 67% (v/v) acetic acid as described by Sherton and Wool (1972), except that the concentration of magnesium acetate was increased from 0.033M to 0.2M in an attempt to increase the yield.

All steps were carried out at 0-4°C. The suspensions of ribosomal subunits in ethanol, prepared as described in Section 2.4.11, were pelleted by centrifuging at 10,000g for 10 min in a Sorvall HB4 rotor. The pellets were dried in a gentle stream of nitrogen. 0.5ml of the solution of carrier, liver 40S ribosomal subunits in 0.01M Tris-HCl, 0.6M magnesium acetate buffer, pH 7.7, prepared as described in Section 2.4.10, was added to the uterine 40S fraction. Similarly, 0.5ml of the solution of carrier, liver 60S ribosomal subunits in the same buffer was added to the uterine 60S fraction. Since the two solutions of liver ribosomal subunits had a concentration of 600 µg/ml ribosomal protein, 300 µg carrier, liver ribosomal protein would have been added to the respective uterine fractions in 0.5ml 0.01M Tris-HCl, 0.6M magnesium acetate buffer, pH 7.7. The pelleted uterine ribosomal subunits were dissolved in this solution and 1ml glacial acetic acid was added to each fraction. The mixtures were shaken for 1hr and centrifuged at 15,000g for 10 min in a Sorvall HB4 rotor to spin down rRNA. The supernatants were retained whilst the pellets were re-extracted by dissolving them in 0.5ml 0.01M Tris-HCl, 0.6M magnesium acetate buffer, pH 7.7, adding 1ml glacial acetic acid and shaking for a further 1hr. The mixtures were re-centrifuged at 15,000g for 10 min and the supernatants were added to the supernatants from the previous extraction. 10ml ice-cold acetone was added to the combined supernatants and the mixtures were allowed to stand at -20°C overnight to ensure complete precipitation of the ribosomal proteins. The following morning, the two fractions were spun down by centrifuging at 15,000g for 10 min. The

pellets were washed by resuspending them in 5ml ice-cold acetone and re-centrifuging at 15,000g for 10 min. The washing was repeated by resuspending the pellets in a further 5ml acetone and the two fractions were stored, as a suspension in acetone, at -20°C for up to four days.

Two-dimensional polyacrylamide gel electrophoresis of the acetone-precipitated ribosomal proteins was carried out by Mr I.M. Kennedy, of this department, using the standard procedure of Kaltschmidt and Wittmann (1970) with modifications (Lastick and McConkey, 1976). The apparatus used has been described previously (Leader, 1975).

In outline, the two protein fractions, in the form of a suspension in acetone, were spun down by centrifuging at 15,000g for 10 min in a Sorvall HB4 rotor. The pellets were dried in a very gentle stream of nitrogen, taking extreme care not to disturb the flaky material at the bottom of the tube. The pellets were dissolved in 100 µl sample buffer (8M urea, 0.001M 2-mercaptoethanol, 0.01M bicarbonate buffer, pH 8.3) and layered over 5cm x 0.4cm, 4% polyacrylamide disc gels containing 6M urea at a pH of 8.7. One drop of a 0.5% solution of Pyronin Y was added as a tracker dye and electrophoresis was carried out at 3mA per gel for 3hr, the direction of migration being towards the cathode. The tank buffer used was a Tris-borate buffer with a pH of 8.6. The first dimension gels were removed from their tubes and equilibrated for 1hr in acetate-urea buffer, pH 5.2, with three changes of buffer, to bring their pH down to a value approaching that of the second dimension gels. Each first dimension gel was laid on top of a 7cm x 7cm x 0.4cm, 18% polyacrylamide slab gel, containing 6M urea at a pH of 4.5. The first dimension gel was annealed into place with 1% (w/v) agarose. Electrophoresis was then carried out at 10mA per gel for 18hr, with migration again being towards the cathode. The tank buffer used for the second dimension was a glycine-acetate buffer with a pH of 4.0. When electrophoresis was complete, the gels were stained for 3hr at 37°C in 0.1% (w/v) Coomassie brilliant blue in methanol: acetic acid: water (5:1:5 v/v)

and destained at 37°C by diffusion in 7.5% (v/v) acetic acid.

The radioactivity of individual protein spots was determined by cutting out the spots using a dental scalpel. The gel pieces were transferred to scintillation vials and digested with hydrogen peroxide as described in the previous section. Radioactivity was measured in a Searle liquid scintillation counter after the addition of 10ml Triton-toluene scintillant.

2.4.13 Extraction of RNA from ribosomal subunits

RNA was extracted from ribosomal subunits from rat uteri by a method based on that of Joel and Hagerman (1969), except that a lower temperature was used and the treatment with deoxyribonuclease and second phenol extraction at pH 7.5 were omitted.

Bentonite, obtained from BDH Chemicals Ltd., Poole,Dorset, U.K., was purified by the method of Fraenkel-Conrat et al.(1961), the final pellet being resuspended in 0.05M sodium acetate buffer, pH 5.2, at a concentration of 20mg/ml.

Ribosomal subunits were prepared as described in Section 2.4.9. The portions of the sucrose density gradients corresponding to the 40S and 60S ribosomal subunits were collected separately and precipitated with 2 volumes absolute ethanol. After storing overnight at -20°C, the two fractions were spun down by centrifuging at 2,000g for 30 min. All subsequent steps were carried out at 0-4°C. The pellets were dried with nitrogen and resuspended in 2ml 0.05M sodium acetate buffer, pH 5.2, containing 1mg/ml bentonite. To this were added 0.2ml 10% (w/v) SDS and 2.2ml 88% (v/v) phenol, both in the same buffer, and the mixtures were shaken for 10 min. They were then centrifuged at 27,000g for 6 min in a Sorvall SS34 rotor and the lower, phenol layer was removed and discarded. 1ml 88% (v/v) phenol in 0.05M sodium acetate buffer, pH 5.2, was added to the aqueous phase and the mixture was shaken and centrifuged as before. The aqueous layer was removed, with a wide-tipped pipette, added to 0.15ml of a 20mg/ml suspension of bentonite in

0.05M sodium acetate buffer, pH 5.2, and retained at 0°C. The phenol layer that remained was extracted by adding 1ml 0.05M sodium acetate buffer, pH 5.2, and 0.1ml 10% (w/v) SDS in the same buffer, and re-shaking for a further 10 min and centrifuging as before. The aqueous phase from this extraction was removed and added to the aqueous phase from the previous extraction. The combined aqueous phases were centrifuged at 27,000g for 15 min to sediment the bentonite. The supernatant was decanted and the RNA present was precipitated by the addition of 1.65M sodium chloride to a concentration of 0.15M followed by 2 volumes absolute ethanol. The precipitated RNA was stored at -20°C before subsequent analysis on 2.7% polyacrylamide gels as described in Section 2.4.8.1.

2.4.14 Chemical determinations

Protein concentrations were determined by the dye method of Bramhall et al.(1969). Samples containing up to 40 μg protein were spotted on to 2.5cm diameter paper circles (Whatman, Grade 1) and dried under a heat lamp. The paper discs were placed in 7.5% (w/v) trichloroacetic acid, heated to 80°C and maintained at this temperature for 30 min. The discs were then washed three times with 7.5% trichloroacetic acid, once with methanol:diethyl ether (1:1 v/v), once with diethyl ether and allowed to dry in a stream of air. This treatment would have removed all the non-proteinaceous material from the paper discs.

The protein on the discs was stained for 15 min at 50°C in a solution of xylene brilliant cyanin G (Microchrome no.1224, obtained from E. Gurr, London, U.K.) at a concentration of 10 mg/ml in 7% (v/v) acetic acid. The paper discs were washed with several changes of 7% (v/v) acetic acid at 50°C until the background was almost white. The discs were then allowed to drain and transferred to individual test tubes. The dye was leached from the paper by the addition of 5ml destain solution (66ml methanol, 34ml water, 1ml 0.88% ammonia). The absorbance at 610nm of the resulting supernatant solution was measured. A standard curve was prepared from 0-40 μg using

bovine serum albumin (Sigma).

RNA concentrations were determined by measuring the absorbance at 260nm and comparing it with the absorbance of a standard solution of yeast RNA.

DNA was determined by the diphenylamine method of Burton (1956), using highly polymerised calf thymus DNA (Sigma) as a standard.

RESULTS

3 RESULTS

3.1 Effect of oestradiol - 17β on protein synthesis

3.1.1 Effect of oestradiol - 17β on total uterine protein synthesis

The effect of oestradiol - 17β on the incorporation of a radioactively-labelled protein precursor into acid-precipitable material of the immature rat uterus was investigated. The radioactive precursor chosen was L-leucine, an amino acid that does not act as a precursor for as many anabolic pathways as some of the other amino acids. After a 30 min pulse with [^{3}H] leucine, therefore, nearly all the acid-insoluble material labelled with the isotope will be protein.

For the experiments described in this section, the rats were limited to a weight range of 22-30g. This was because it had been shown previously that the degree of hormonal stimulation of the incorporation of precursors into RNA decreased with increasing rat weight (Knowler and Smellie, 1971; Knowler et al., 1975), and these findings had been confirmed in preliminary experiments. For the remainder of the experiments described in this thesis, the rats were treated in groups rather than individually. Because of the larger number of animals involved, it was not possible to confine their weights to such a small range. Consequently, animals weighing up to 40g were used.

Figure 6 shows the time course of the effect of oestradiol - 17β on the incorporation of [^{3}H] leucine into immature rat uteri.

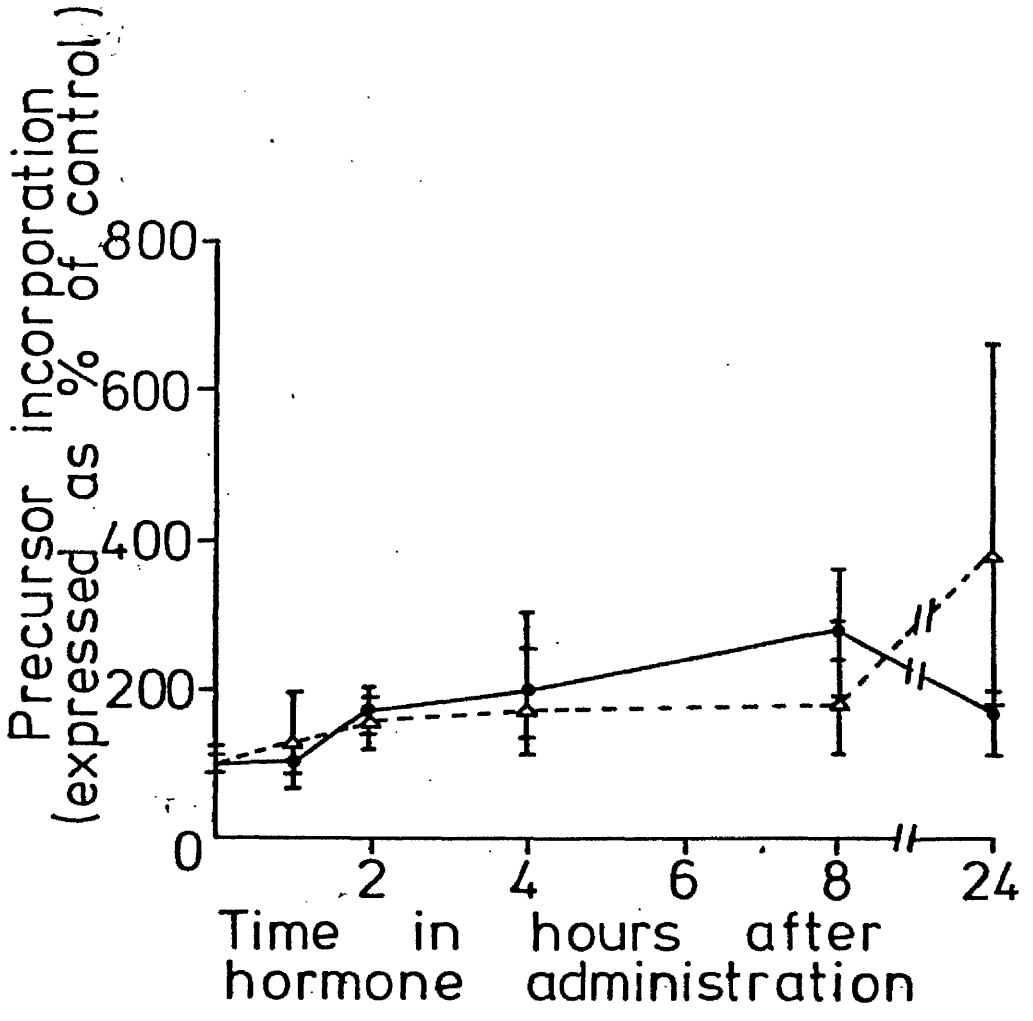
The incorporation into the acid-insoluble fraction is stimulated at 2hr and increases to a maximum, at nearly three times the control level, 8hr after hormone treatment. After 24 hr, the incorporation has returned almost to the control level. The incorporation into the acid-soluble precursor pool was also stimulated following hormone treatment, but, for most of the experiment, this stimulation did not exceed two-fold. The stimulation of incorporation into the acid-insoluble fraction exceeded this level, although

Figure 6

Time Course of incorporation of L-[4,5-^{3}H] leucine into immature rat uteri in response to oestradiol-17β

18-21 day-old female rats were given 1 μg oestradiol-17β or saline carrier by intraperitoneal injection at the times before death indicated. All the animals received 20 μCi L-[4,5-^{3}H] leucine by intravenous injection 30 min before death. Acid-soluble and acid-insoluble fractions were prepared from the uteri of the animals, as described in Materials and Methods. The results were calculated as d.p.m./μg DNA and are expressed as percentages of the incorporation into uteri from animals that did not receive hormone. Each point represents the mean of at least three animals and the error bars indicate the range of the results. ●——● , acid-insoluble fraction; Δ- - -Δ , acid-soluble fraction.

Incorporation of [^{3}H] leucine



it can be seen that the error bars for the two sets of results overlap. Thus, it would appear that there is stimulation of protein synthesis, but the uptake of precursor may play a role in this stimulation. After 24hr, the stimulation of incorporation into the acid-soluble fraction is greater than that of the acid-insoluble fraction. The reason for this is not understood.

To investigate the relationship between this stimulation of protein synthesis and the previously-reported stimulation of RNA synthesis, a double-labelling experiment was carried out using uridine as an RNA precursor and leucine as a protein precursor. The effect of oestradiol - 17β on the incorporation of $[^{3}H]$ uridine and $[^{14}C]$ leucine into immature rat uteri is shown in figure 7.

The radioactivity of standards containing $[^{14}C]$ leucine only was measured and no spill-over of counts into the channel set for ^{3}H was detected. Figure 7 shows that treatment with oestradiol - 17β results in a stimulation of the incorporation of $[^{3}H]$ uridine into acid-precipitable material from immature rat uteri, first detectable after 1hr. This stimulation is maximal after 2hr at nine times the level of incorporation of control animals. It then declines fairly rapidly, although it is still twice the control level after 24hr. The stimulation of incorporation into the acid-insoluble fraction is much greater than the stimulation of incorporation into the acid-soluble precursor pool, which is only two-fold at maximum, so would appear to be due to a true increase in RNA synthesis. This stimulation of RNA synthesis following hormone treatment is in agreement with previous results of Knowler and Smellie (1971) and Knowler et al.(1975), although the time of maximum stimulation is slightly earlier, at 2hr after hormone administration instead of 4hr. This may be a result of variations in the animals used.

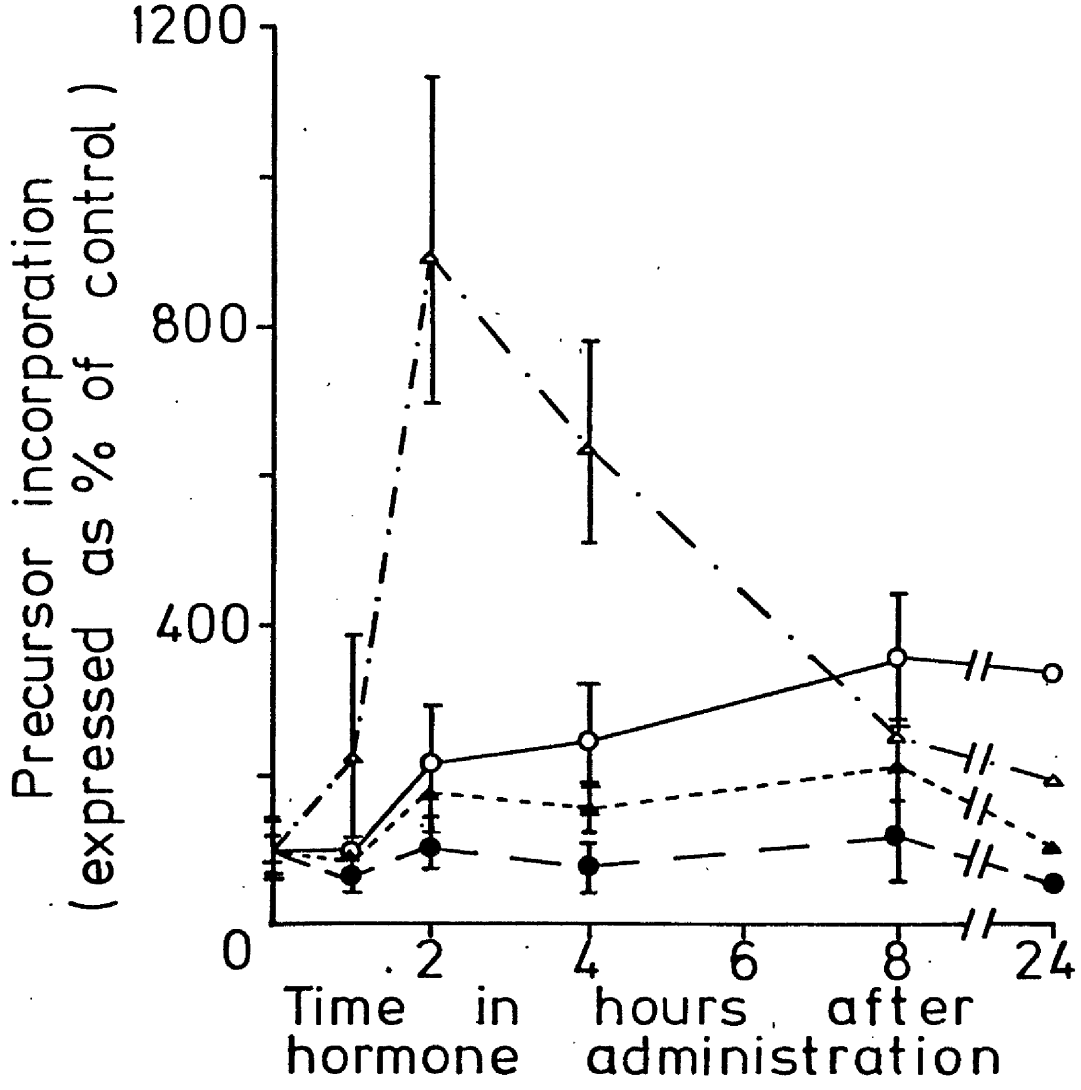
The results for the incorporation of $[^{14}C]$ leucine are in good agreement with the results of the incorporation of $[^{3}H]$ leucine shown

Figure 7

Time course of incorporation of [5-^{3}H] uridine and L-[U-^{14}C] leucine into immature rat uteri in response to oestradiol-17β

18-21 day-old female rats received 1 μg oestradiol-17β or saline carrier at the times before death indicated. All the animals received 20 μCi [5-^{3}H] uridine and 1 μCi L-[U-^{14}C] leucine by intravenous injection 30 min before death. Acid-soluble and acid-insoluble fractions were prepared from the uteri of the animals. The results were calculated as d.p.m./ μg DNA and are expressed as percentages of the incorporation into uteri from control animals. Each point represents the mean of at least three animals, except the 24hr values which were taken from one animal. The error bars indicate the range of the results. Δ—·—·—Δ, ^{3}H acid-insoluble radioactivity; ▲- - - ▲, ^{3}H acid-soluble radioactivity; o——o, ^{14}C acid-insoluble radioactivity; ●— — —●, ^{14}C acid-soluble radioactivity.

Incorporation of [^{3}H] uridine and [^{14}C] leucine



in figure 6. The stimulation of incorporation into the acid-insoluble fraction is even more marked, being almost four-fold by 8hr after hormone treatment. Furthermore, the incorporation of the precursor into the acid-soluble fraction is appreciably less than the incorporation into the acid-insoluble fraction. In contrast to the previous experiment (figure 6), no stimulation of incorporation into the acid- soluble fraction was observed after 24 hr.

Therefore, the results of this experiment show that hormone treatment results in a stimulation of both total RNA and total protein synthesis in the immature rat uterus. The stimulation of RNA synthesis precedes the stimulation of protein synthesis, being detectable after 1hr and maximal 2-4hr after oestrogen treatment. The stimulation of protein synthesis is detectable after 2hr and continues to increase up to 8hr after treatment with the hormone, declining slightly after 24hr. This is in agreement with previous findings of Mueller et al.(1961, 1972) and Noteboom and Gorski (1963).

3.1.2 Effect of oestradiol - 17β on uterine polyribosomes

As discussed in Section 1.2.2.6, studies using inhibitors of protein synthesis have suggested that, within the first hour following hormone treatment, certain so-called Key Intermediary Proteins must be synthesised for the later uterotrophic effects of the hormone to occur (Baulieu et al., 1972; Baulieu, 1975; Knowler et al.,1975). In the experiments described in the preceding section, however, a stimulation of protein synthesis was not detectable until 2hr after hormone treatment. This is probably because the technique of incorporation of radioactively-labelled precursors into total, acid-precipitable material is not sensitive enough to detect small changes resulting from the synthesis of a limited number of proteins.

A more sensitive technique is the analysis of the profiles of polyribosomes on sucrose density gradients. Ribosomes were prepared, therefore, from immature rat uteri and polysome profiles were analysed to

determine whether a stimulation of protein synthesis could be detected at early times following hormone treatment. Initially, the preparation of uterine ribosomes proved to be difficult. Various methods were followed, including published methods for the preparation of ribosomes from rat uterus (Teng and Hamilton, 1967a), guinea pig uterus (Suvatte and Hagerman, 1970) and rat skeletal muscle (Florini and Breuer, 1966) but with little success. The main problem appeared to be ribonuclease-catalysed degradation during the preparation, resulting in no ribosomes being observed after sucrose density gradient centrifugation and no rRNA being detected following extraction of the RNA and subsequent analysis on polyacrylamide gels. No such problems were encountered in the preparation of ribosomes from rat liver, presumably because of the presence of an endogenous ribonuclease inhibitor in the supernatant fraction of the liver (Roth, 1958), resulting in a lower ribonuclease activity in this tissue compared with the uterus. The instability of uterine polysomes has been noted previously by Teng and Hamilton (1967a).

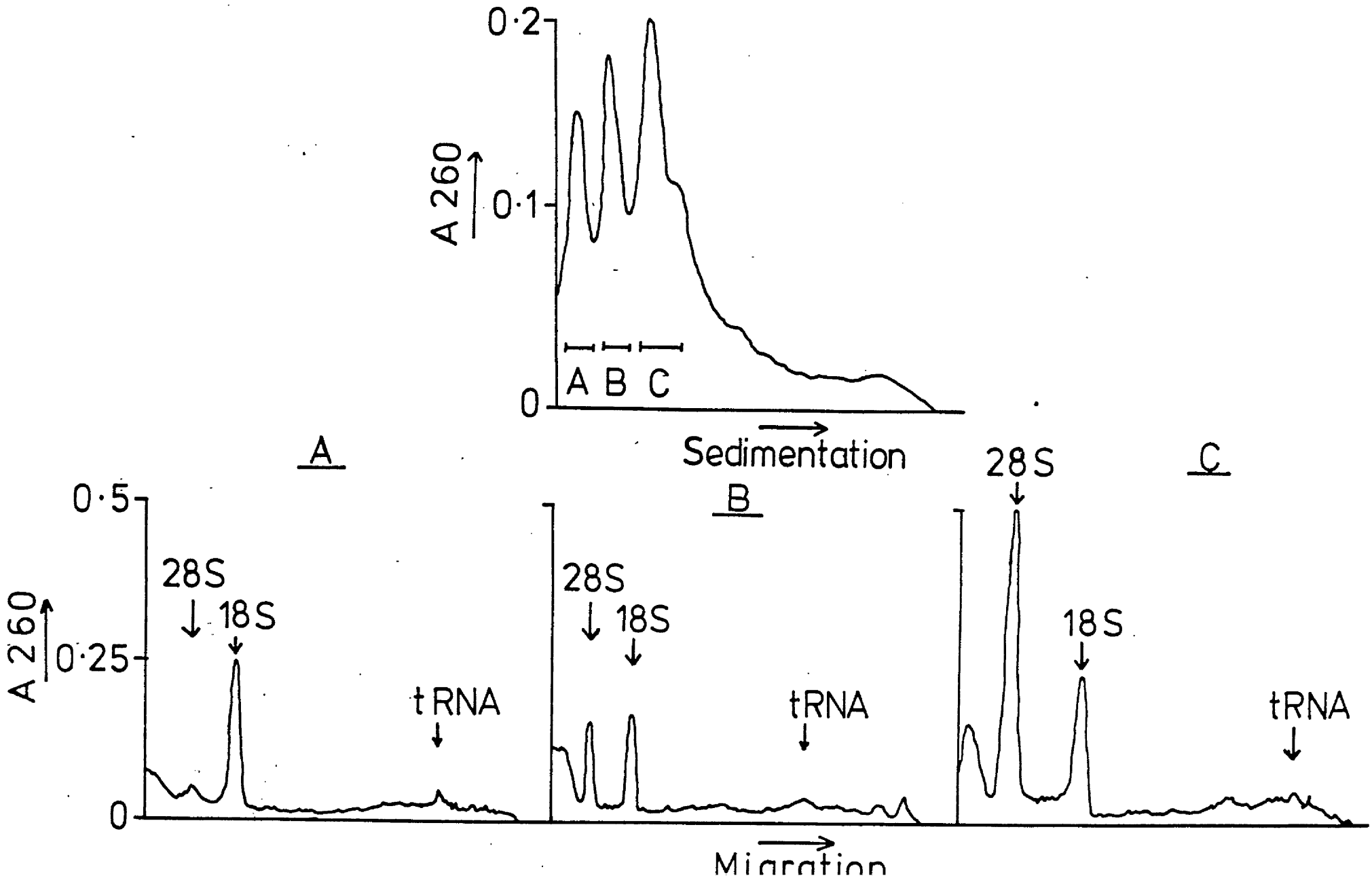
The method of Berridge et al.(1976), describing the isolation of frog liver ribosomes, was found to be more successful. This method uses a high pH (8.5) and a moderately high ionic strength during the homogenisation steps, to minimise ribonuclease activity, and also utilises heparin as a ribonuclease inhibitor during analysis of the polysomes. It was found that polysome profiles from immature rat uteri could be analysed using 5ml sucrose density gradients. Figure 8 shows the results from a typical preparation obtained from the uteri of animals that had not been treated with hormone. Three peaks were observed at the top of the sucrose density gradient. The ribosomal pellet obtained from the uteri of twelve such untreated animals usually contained 200 μg RNA and the ratio of the absorbances at 260 nm and 280 nm was 1.6 - 1.7. Also shown in figure 8 are the results of collecting the three peaks separately from the sucrose gradient and analysing the RNA on polyacrylamide gels. The slowest-sedimenting

Figure 8

Polysome profile of immature rat uterus

Uteri were removed from twelve immature rats that had not received hormone. Ribosomes were prepared and analysed on a 15 —45% sucrose density gradient as described in Materials and Methods. The upper part of the figure shows the polysome profile obtained. The trace has been corrected for the absorbance of a blank gradient.

Fractions from the gradient were pooled, as indicated, and the RNA was extracted and analysed on 2.7% polyacrylamide gels. The densitometer traces of the gels are shown in the lower part of the figure. The arrows indicate the positions of 28S rRNA, 18S rRNA and tRNA markers which were electrophoresed on parallel gels.


0·2
0·1
0
A 260
A B C
Sedimentation
A
B
C
0·5
0·25
0
A 260
28S
18S
tRNA
28S
18S
tRNA
28S
18S
tRNA
Migration

peak from the sucrose gradient, peak A, contains mainly 18S rRNA, suggesting that this peak corresponds to 40S ribosomal subunits. The other two peaks from the sucrose gradient both contain 28S and 18S rRNA. This suggests that peak B consists of a mixture of 60S subunits and monomeric ribosomes whilst the fastest-sedimenting peak, peak C, corresponds to dimeric ribosomes. The sucrose density gradient shows that very few polysomes are present in the uteri from untreated animals, the ribosomes being present mainly as monomers. This is to be expected of a tissue which is not growing very rapidly in the immature animal.

The effect of giving oestradiol - 17β was investigated. The rats were given two injections of hormone, one 24hr and the other 4hr before death, in order to obtain as marked an effect as possible. The results are shown in figure 9.

Oestradiol - 17β leads to an increase in the proportion of polysomes. This effect was reproducible, provided the sucrose density gradient centrifugations were carried out the same day the animals were killed and the ribosomes prepared. Figure 10 shows the effects of storing the resuspended ribosomal pellet at -20^{o}C.

On comparing the polysome profiles from the control animals, shown in figure 9, with those shown in figure 10, it can be seen that storing for 1 day at -20^{o}C results in a partial loss of the three peaks at the top of the gradient and after 2 days at -20^{o}C, there is complete loss of these peaks. In the preparation from the animals that received two injections of oestradiol - 17β , the faster-sedimenting polysomes are lost after 1 day at -20^{o}C, whilst almost all the peaks are lost after 2 days at this temperature. These losses could result from aggregation of the ribosomes during storage, so that they sediment to the bottom of the sucrose gradient, or from ribonuclease-catalysed degradation of the ribosomes. The fact that only the polysomes are lost after storing the preparation from the hormone-treated animals for 1 day would suggest that the latter explanation

Figure 9

Effect of oestradiol-17β on polysome profiles of immature rat uteri

Immature female rats were given two intraperitoneal injections of 1 μg oestradiol-17β 24hr and 4hr before death. Polysomes were prepared from the uteri and analysed on sucrose density gradients as described in Materials and Methods. Control animals received saline in place of hormone. The traces have been corrected for the absorbance of a blank gradient.

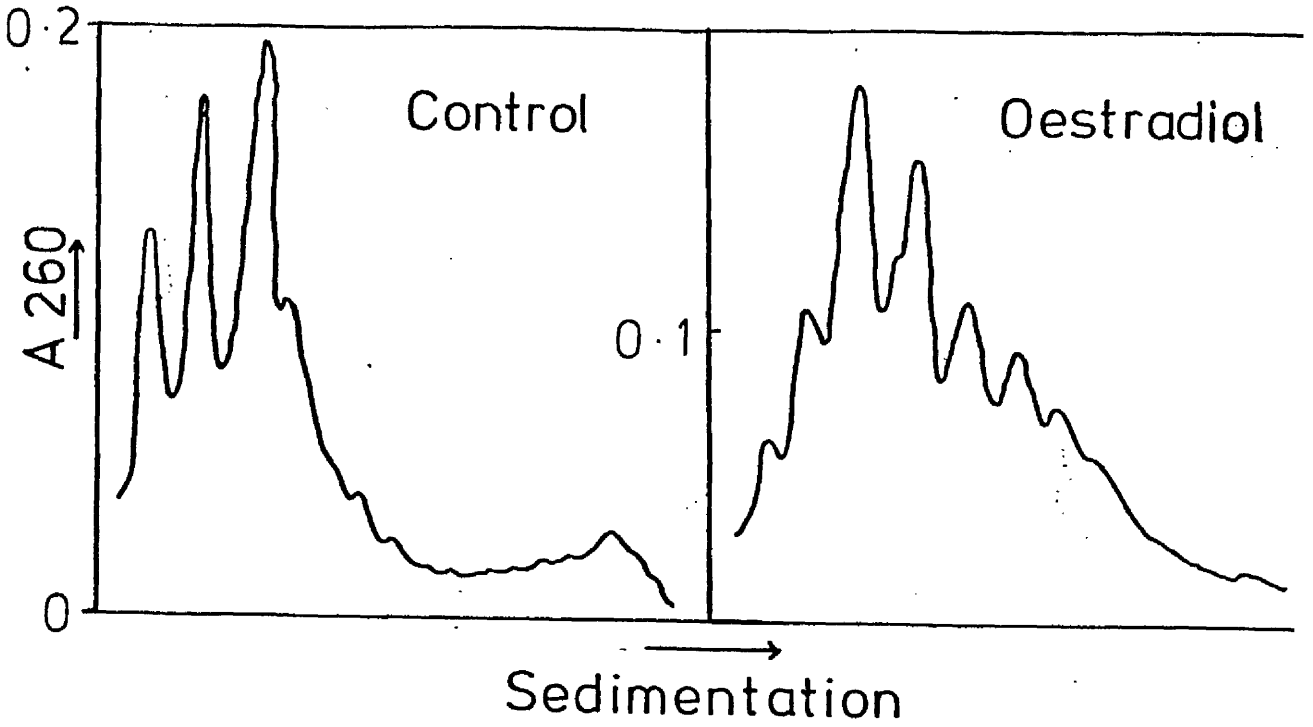
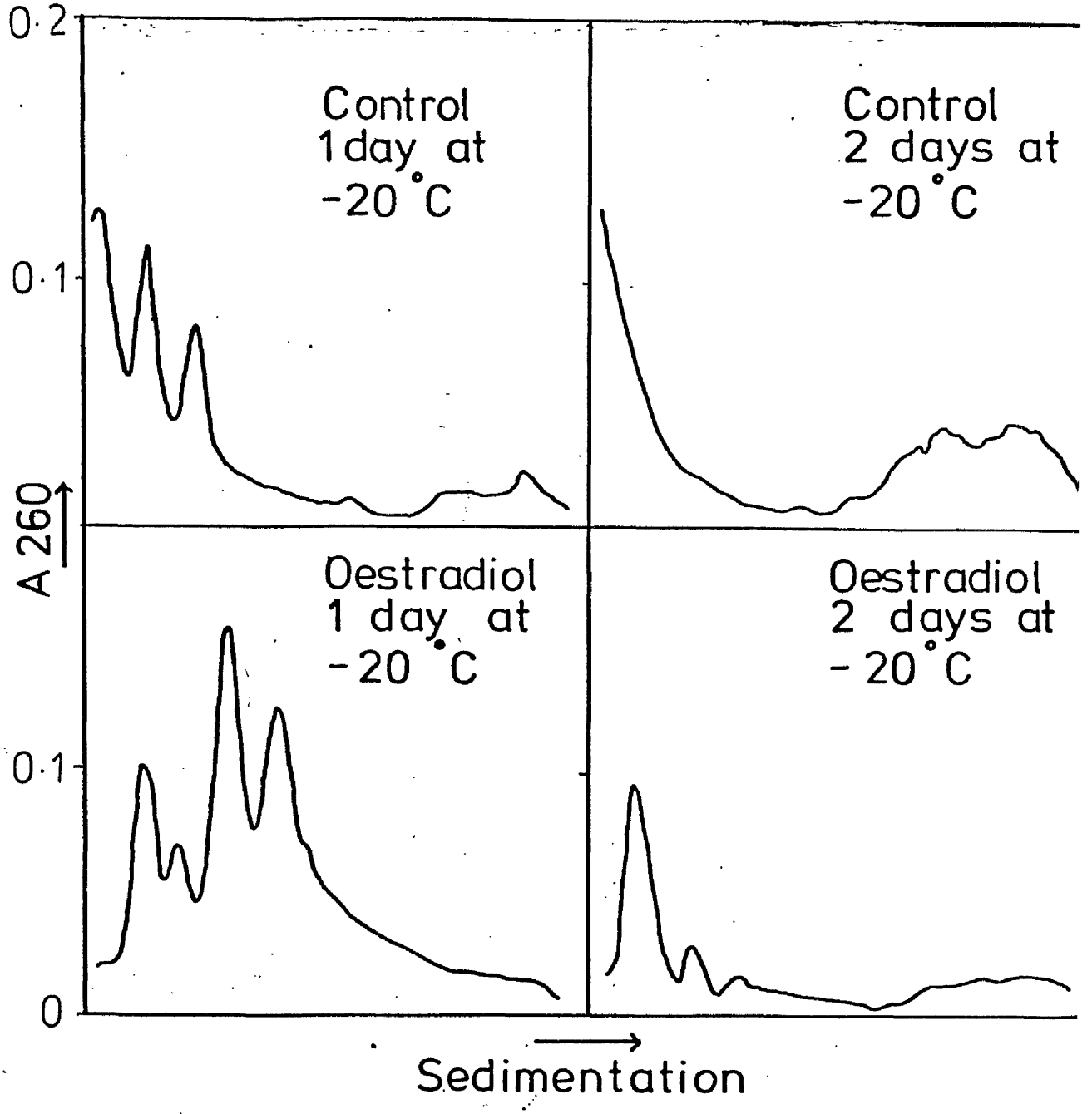

0·2
Control
Oestradiol
A 260
0·1
0
Sedimentation

Figure 10

Effect of storing at -20°C on polysome profiles from immature rat uteri

The remainder of the preparations used to obtain the polysome profiles shown in Figure 9 were frozen in the form of the resuspended ribosomal pellet and stored at -20°C. Samples were taken for sucrose density gradient centrifugation after 1 day at -20°C and after 2 days at -20°C.

For experimental details, see legend to Figure 9.


0·2
0·1
A 260
Control
1day at
-20 °C
Control
2 days at
-20 °C
Oestradiol
1 day at
-20 °C
Oestradiol
2 days at
-20 °C
0·1
0
Sedimentation

is more likely. The mRNA linking the ribosomes to form polysomes would be expected to be the RNA species most sensitive to the effects of ribonuclease. Berridge et al.(1976), in fact, reported that partial degradation of vitellogenin and albumin-synthesising polysomes could be detected in preparations carried out by this method. They also found that polysomes prepared by this method were unstable during repeated freezing and thawing. Therefore, although the method of Berridge et al.(1976) overcame the problem of ribonuclease-catalysed degradation during the preparation of uterine ribosomes, ribonuclease activity was still present. Because of this, uteri removed from the immature rats were not stored but were used for the preparation of ribosomes immediately.

One of the precautions taken by Berridge et al.(1976) against ribonuclease activity was the use of diethyl pyrocarbonate in the buffers. This was used in some of the preliminary preparations of rat uterine ribosomes, but it was found that the diethyl pyrocarbonate could be omitted without any deleterious effects on the polysome profiles (results not shown). This is in agreement with the findings of Berridge et al.(1976), who reported that diethyl pyrocarbonate was not necessary if a high concentration of Tris-HCl, at a pH of 8.5, were used in the buffers. Since diethyl pyrocarbonate has been reported to react with proteins (Wolf et al., 1970) and nucleic acids (Ehrenberg et al., 1976), and so could possibly interfere with subsequent analysis of the rRNA and ribosomal proteins, it was decided to omit it from all further preparations.

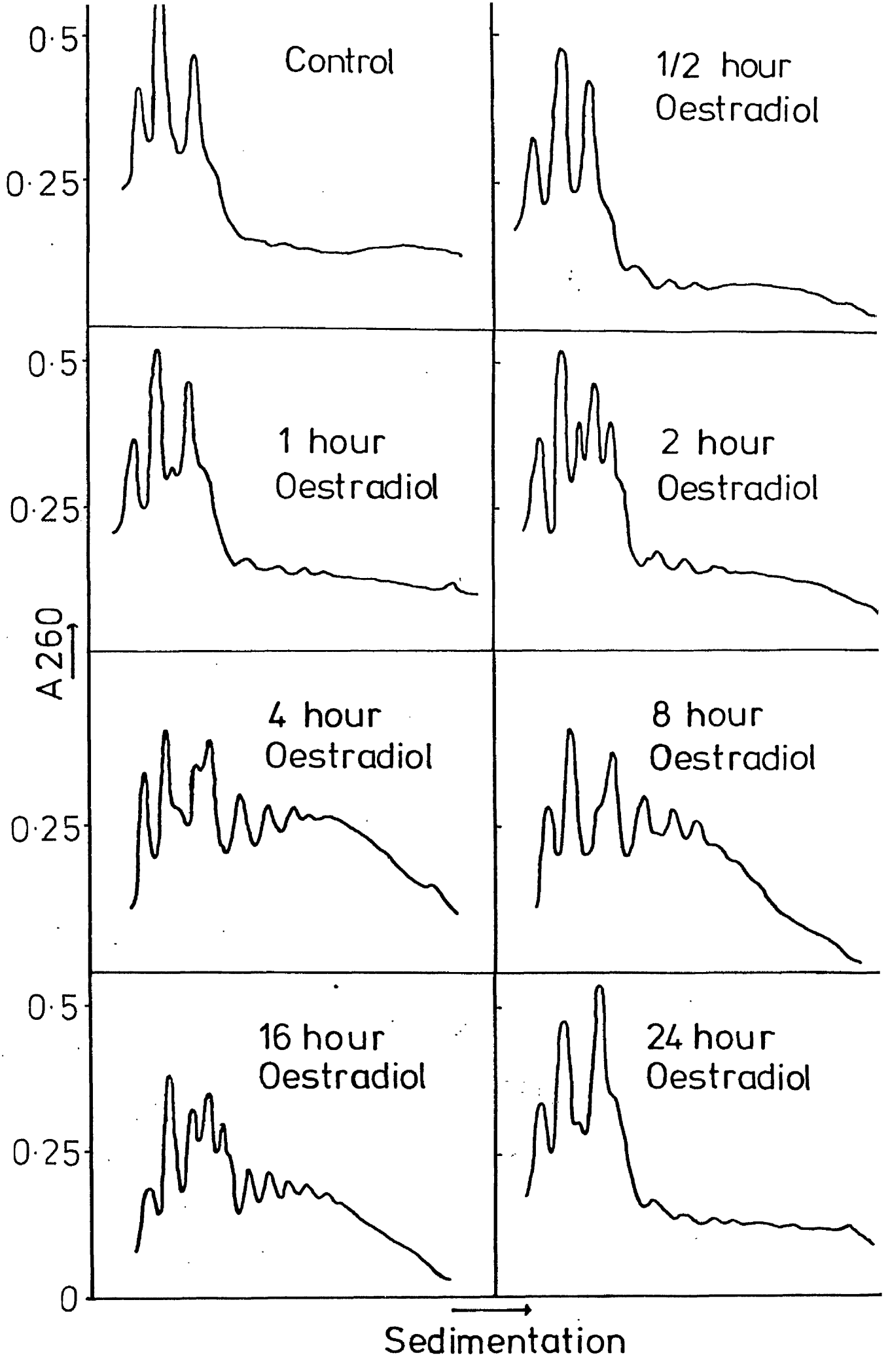
Polysomes prepared from immature rat uteri by this method were found to be active in a cell-free translation system (J.T. Knowler, unpublished observations). Treatment of immature rats with two injections of oestradiol - 17β had led to a marked increase in the proportion of polysomes on sucrose density gradient centrifugation (figure 9). The time course of the changes in the polysome profile, resulting from primary stimulation of immature rats with oestradiol - 17β , was therefore followed, the results are shown in figure 11.

Figure 11

Polysome profiles of immature rat uterus responding to oestradiol-17β

18-21 day-old rats were given 1 μg oestradiol-17β by a single, intraperitoneal injection at the times before death indicated. Control animals received saline in place of hormone

For experimental details, see legend to Figure 9.


0·5
0·25
Control
1/2 hour
Oestradiol
0·5
0·25
1 hour
Oestradiol
2 hour
Oestradiol
A 260
0·25
4 hour
Oestradiol
8 hour
Oestradiol
0·5
0·25
0
16 hour
Oestradiol
24 hour
Oestradiol
Sedimentation

As noted previously, ribosomes from control animals are mainly in the form of monomeric ribosomes and subunits. Treatment with oestradiol-17β leads, initially, to a slight increase in the proportion of polysomes. This effect is detectable ½ hr after giving the hormone and is observed for up to 2 hr. After 4hr, the effect of oestradiol-17β on the polysome profile is even more marked. This effect is maintained up to 16 hr and by 24 hr, it is beginning to decline. The profiles shown in figure 11 were reproducible except for the profile obtained ½ hr after hormone treatment. In two out of three experiments, the polysome profile was identical to that observed 1 hr after giving hormone, as shown in figure 11, whereas in the third experiment, the profile was similar to that of control animals. This may be due to variations in the experimental animals.

This effect of oestradiol-17β on the organisation of monomeric ribosomes into polysomes in the uterus may be interpreted by saying that the hormone results in a stimulation of protein synthesis in this organ. This could occur possibly because of an increased availability of mRNA sequences for translation in the cytoplasm.

These results are in contrast to those of Teng and Hamilton (1967 b), who concluded that administration of oestradiol to ovariectomised rats did not lead to changes in the profiles of uterine polysomes. Differences could arise because of the type of experimental animal used. A similar stimulatory effect of oestrogen on the organisation of ribosomes into polysomes has been reported by Berridge et al.(1976) for Xenopus liver. Similar results have also been obtained studying the effects of oestrogen and progesterone on chick oviduct (Palmiter et al., 1970; Means et al., 1971) and oestrogen on cockerel liver (Bast et al., 1977). In all these experiments, however, the alterations in the polysome profiles were not observed until several hours, or even days, after hormone treatment. The effect of oestradiol on the polysome profiles shown in figure 11 after ½-1hr

would appear to agree with the results of Whelley and Barker (1974). These authors detected an increase in the rate of protein synthesis by uterine ribosomes 1hr after giving oestradiol to ovariectomised rats.

Another possible interpretation of these results is that treatment with oestrogen leads to a decrease in the ribonuclease activity of the uterus, resulting in an improved recovery of polysomes. This has been reported to occur in rooster liver (Dijkstra et al., 1978). Treatment with oestradiol led to an increase in the level of an endogenous ribonuclease inhibitor, and hence an increase in the recovery of polysomes. A new class of large polysomes did appear, however, which were probably responsible for the synthesis of vitellogenin.

Several experiments were carried out to determine whether oestradiol-induced changes in the profiles of polysomes from the rat uterus were a result of alterations in the ribonuclease activity. The first series of experiments involved the actual measurement of the ribonuclease activity of uterine homogenates. The ribonuclease assay of Ingebretsen et al.(1972) was chosen on the basis of preliminary experiments which showed that this method gave the best recovery of the radioactive label. Also, the pH at which the assay was carried out was the same as the pH of the buffers used at the beginning of the polysome preparation. Further preliminary experiments showed that, although ribonuclease activity was present in all the sub-cellular fractions of the uterus, most activity was present in the soluble fraction. It was decided to carry out the ribonuclease assay on a crude uterine homogenate, again to approximate as closely as possible the conditions of the polysome preparation, and also because of the relative ease with which a large number of samples could be prepared for subsequent assay.

The ribonuclease activity of uteri from control animals was compared with the activity of uteri from animals that had received oestradiol-17β 4 hr before death. The time of hormone treatment was chosen as 4 hr since this

was the time at which the effect on the polysome profile was maximal. The results are shown in figure 12.

The radioactivity in both the acid-insoluble and acid-soluble fractions was measured for each sample, so the recovery of the label could be calculated. Samples which gave a low recovery were discarded. The acid-insoluble counts were used for the calculation of ribonuclease activity. In all of the samples shown in figure 12, greater than 80% recovery of the label was obtained. Approximately 50% solubilisation of the labelled substrate was found to occur in those incubations carried out with uterine homogenates and approximately 70% solubilisation occurred in the tubes to which 0.1µg of each of pancreatic ribonuclease and T_1 ribonuclease had been added. No digestion of the substrate occurred in the control incubations.

There is very little difference between the activities from control and hormone-treated animals, any difference being insignificant ($P > 0.25$ by Student's t test). These results should be considered in the light of the finding that the rat uterus contains a ribonuclease inhibitor (Ginesi and Knowler, unpublished observations). However, as can be seen from figure 12, free ribonuclease activity is still present in the rat uterus, being the same in both control and hormone-treated uteri. This is in contrast to the results of Zan-Kowalczewska and Roth (1975), who found no detectable, free ribonuclease activity in the immature rat uterus. These authors reported that ribonuclease activity was present, but it was complexed with an inhibitor, from which it was released by treatment with p-chloromercuribenzene sulphonic acid.

In the second series of experiments to determine whether hormone-induced changes in the polysome profiles resulted from alterations in the ribonuclease activity, the effect of adding rat liver supernatant, which contains an endogenous ribonuclease inhibitor (Roth, 1958), to uterine ribosomal preparations was investigated. The preparation of the rat liver

Sample	Ribonuclease Activity (c.p.m. solubilised/mg protein)	
	Control	4hr Oestradiol
1	10098	4640
2	7775	7902
3	5769	5500
4	7031	4716
5	5964	4841
6	4693	
7	6304	
8	8728	
9	4247	
10	3822	
Mean	6443	5520
S.D.	2004	1374

Figure 12

Effect of oestradiol-17β on the ribonuclease activity of uterine homogenates

Immature rats were given 1 μg oestradiol-17β by a single, intraperitoneal injection 4hr before death. Control animals received saline in place of hormone. The uteri were removed from the animals and the ribonuclease activity was determined as described in Materials and Methods. The results have been calculated as c.p.m. solubilised per mg protein.

supernatant fraction, described in Materials and Methods, differed from the original method of Roth (1958) in that a low speed centrifugation step followed by a high speed centrifugation was used. The purpose of this was to spin down nuclei and mitochondria and then to sediment the ribosomes, so that the final preparation should be suitable for addition to a uterine polysomal preparation without interference with the optical density trace of the uterine ribosomes. 50 µl of the supernatant fraction prepared in this way inhibited the activity of a mixture of 0.1 µg of each of pancreatic and T_1 ribonucleases by 25%.

1ml of this preparation was added to a uterine polysome preparation from control animals. If the polysome profile from control animals observed in figure 11 were a result of ribonuclease-catalysed degradation of most of the polysomes, then the addition of a ribonuclease inhibitor might be expected to result in a polysome profile with a greater proportion of polysomes. However, as can be seen in figure 13, the presence of rat liver supernatant made little difference to the polysome regions of the sucrose density gradients of ribosomes from control animals. This would suggest that the polysome profile from control animals is not caused by ribonuclease-catalysed degradation during the preparation of the ribosomes.

In the final experiment on uterine ribonuclease activity, a preparation in which uteri from control and hormone-treated animals were mixed was carried out. The results are shown in figure 14.

The profiles of the control and hormone-treated polysomes prepared separately show few polysomes and a greater proportion of polysomes respectively. The profile from the preparation from mixed control and hormone-treated uteri shows polysomes similar to those of the hormone-treated preparation. The peaks in the monosome region of the gradient are slightly larger, probably because of the presence of the control uteri, whose ribosomes will be mainly in the form of monosomes. Therefore, it would appear that the polysome profile observed in control uteri does not result from the action

Figure 13

Effect of rat liver supernatant on polysome profiles of uteri from control rats

Twelve uteri were homogenised in 5ml homogenisation buffer, as described in Materials and Methods, and a further twelve were homogenised in 4ml homogenisation buffer to which 1ml rat liver supernatant had been added.

For further experimental details, see legend to Figure 9.

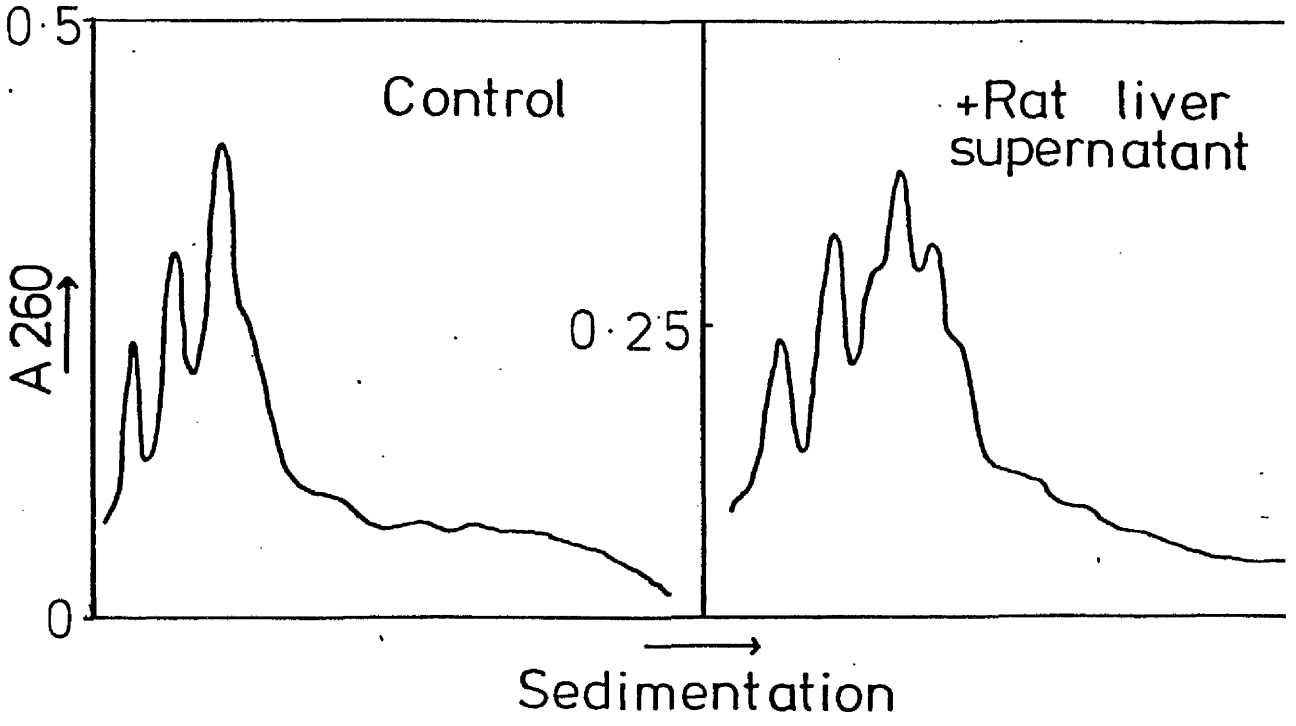
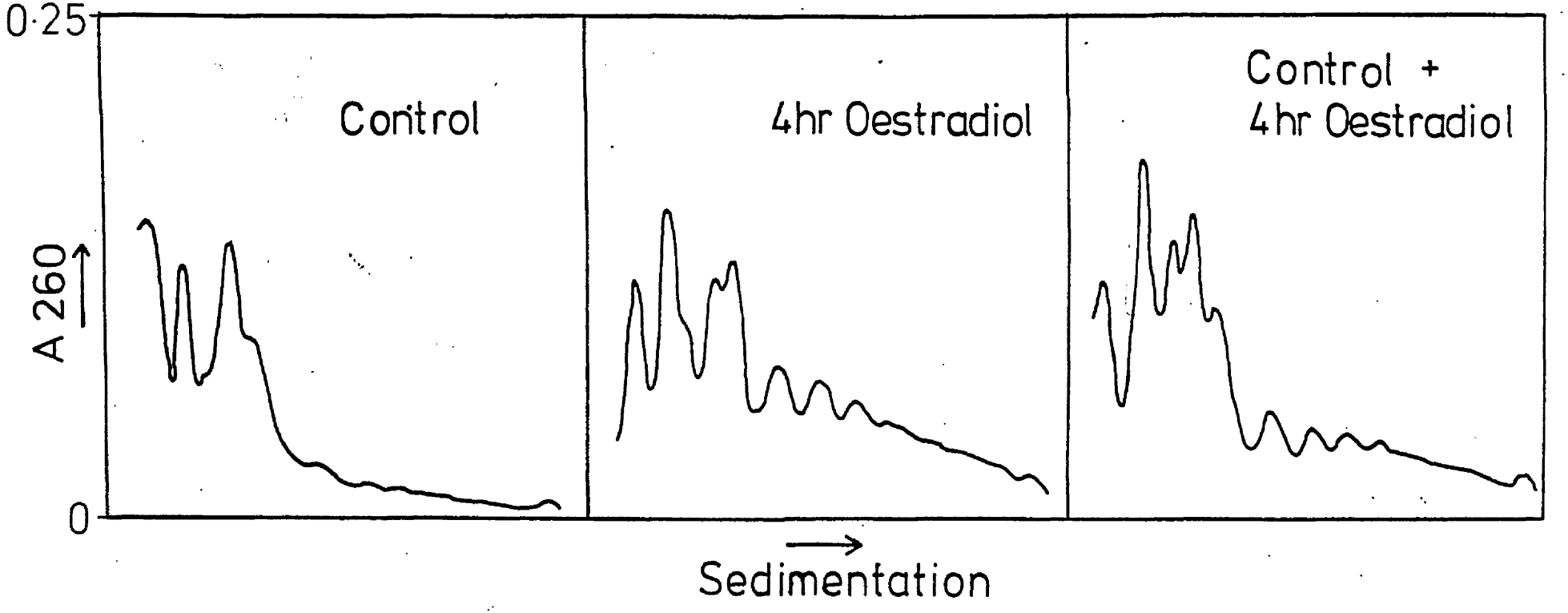

0·5
Control
+Rat liver
supernatant
A 260
0·25
0
Sedimentation

Figure 14

Effect of mixing uteri from control and hormone-treated rats on the polysome profile

Six uteri from animals that had received 1 μg oestradiol-17β by a single, intraperitoneal injection were mixed with six uteri from animals that had received saline in place of hormone. Polysomes were prepared and analysed on sucrose density gradients as described in Materials and Methods. Also shown are the polysomes from the control and hormone-treated animals prepared separately. The traces have been corrected for the absorbance of a blank gradient.


0·25
0
A 260
Control
4hr Oestradiol
Control +
4hr Oestradiol
Sedimentation

of a ribonuclease during the isolation of the ribosomes. This confirms the result of the preceding experiment.

Taken on its own, the result of the ribonuclease assay (figure 12) does not provide strong evidence against an effect of alterations in the free ribonuclease activity during the isolation of uterine ribosomes on the recovery of the faster-sedimenting polysomes. Firstly, the assay measured the fairly extensive degradation of rRNA, to produce acid-soluble fragments, whereas single-stranded nicks of mRNA is all that would be necessary to degrade polysomes. Secondly, it is possible that the ribonuclease activity and polysomes are compartmentalised within the uterine cell, so that they do not normally come into contact. However, the two other lines of evidence help to make out a strong case against the effect of oestradiol on the uterine polysomes being a result of changes in the free ribonuclease activity during the isolation of the polysomes.

This section can be concluded by saying that treatment of immature rats with oestradiol-17β results in a stimulation of protein synthesis in the uterus which is detectable $\frac{1}{2}$ - 1 hr after giving the hormone, although it does not become maximal until later times. Therefore, using polysome profiles as an index of protein synthesis would appear to be a more sensitive technique than measuring the incorporation of radioactively-labelled precursors into acid-precipitable material.

3.2 Effect of oestradiol-17β on uterine RNA synthesis

The experiments on the effect of oestradiol-17β on uterine RNA synthesis reported by Knowler and Smellie (1971) and Knowler et al.(1975) were carried out on total uterine RNA. In the preceding section, results were described which would suggest that oestrogen leads to an increase in the amount of mRNA available for translation in the uterus. This mRNA subsequently becomes associated with the ribosomes. To determine whether the increase in the proportion of polysomes to monosomes within the first hour of hormone treatment occurs as a result of the association of newly-synthesised mRNA with

pre-existing ribosomes, experiments were carried out to measure the incorporation of a radioactively-labelled precursor into poly (A)-containing and non-poly (A)-containing polysomal RNA.

Preliminary experiments showed that sufficient radioactivity was incorporated into polysomal RNA if each animal was given 100 μCi [5, 6 - ^{3}H] uridine and that all the label in the polysomes was present in an acid-insoluble form. The length of time between the injection of the radioactive precursor and the death of the animal appeared to make little difference to the actual level of incorporation. A pulse length of 30 min was chosen in order to detect rapid hormonal responses.

For the fractionation of polysomal RNA into poly (A)-containing and non-poly (A)-containing species, two methods were considered; fractionation on poly (U) Sepharose, and fractionation on oligo (dT) cellulose. The latter method was chosen since good recoveries of RNA could be achieved. Also, analysis of the fractionated RNA on polyacrylamide gels showed that the unbound, non-poly (A)-containing RNA consisted of rRNA and tRNA, with very little apparent degradation of the rRNA species, and the bound RNA, presumed to be polyadenylated mRNA, contained no detectable rRNA. In contrast, problems were encountered during fractionation of the polysomal RNA on poly (U) Sepharose columns. The columns took a long time to run and this appeared to result in some degradation of the rRNA in the unbound fraction. Furthermore, the bound RNA fraction appeared to contain some rRNA (results not shown).

An experiment was carried out, therefore, to determine the effect of hormone on the incorporation of tritiated uridine into uterine polysomal RNA that had been fractionated on oligo (dT) cellulose. The results are shown in figure 15. This figure shows the amount of RNA recovered in each fraction at various times following hormone treatment, the acid-precipitable radioactivity incorporated and the specific activity

Figure 15

Effect of oestradiol-17β on the incorporation of [^{3}H] uridine into uterine polysomal RNA

Groups of twelve 18-21 day-old female rats received 1 μg oestradiol-17β or saline carrier at the times before death indicated. All the animals received 100 μCi [5,6-^{3}H] uridine 30 min before death. Polysomes were prepared from the uteri of each group and the polysomal RNA was extracted with phenol-chloroform and fractionated on oligo (dT) cellulose as described in Materials and Methods. Acid-insoluble radioactivity was determined for the fraction retained by oligo (dT) cellulose (bound fraction) and the fraction not retained by oligo (dT) cellulose (unbound fraction).

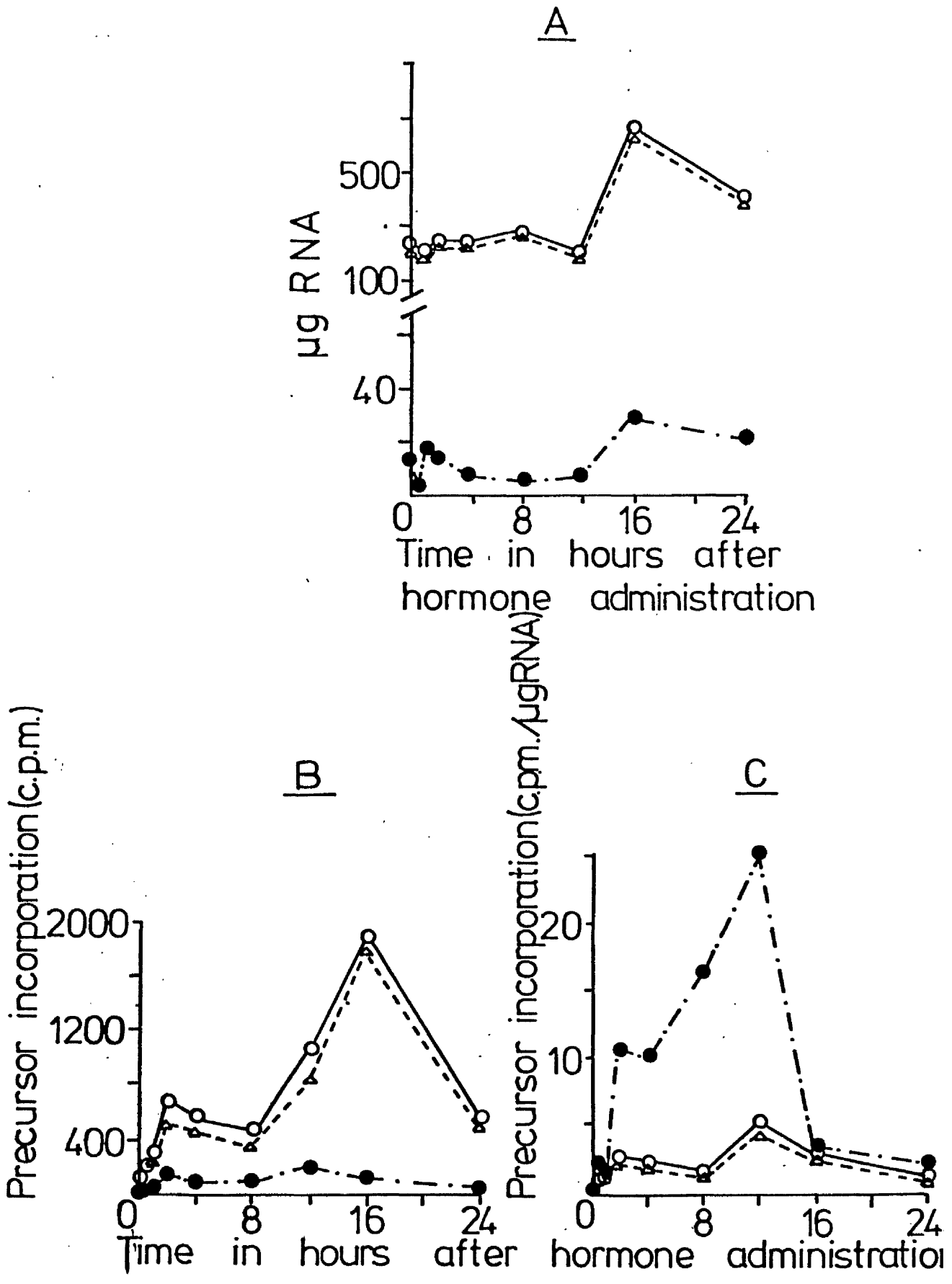
A. Amount of RNA

B. Acid-insoluble radioactivity

C. Specific activity of RNA

o——o , total polysomal RNA; ●—·—·—● , bound polysomal RNA; Δ- - - -Δ , unbound polysomal RNA.

N.B. The results for the points at 8hr and 12hr were obtained by Dr. J.T. Knowler.


A
500
100
40
µg RNA
0
8
16
24
Time in hours after hormone administration
B
2000
1200
400
Precursor incorporation (c.p.m.)
Time in hours after
C
20
10
Precursor incorporation (c.p.m./µgRNA)
hormone administratio

of each fraction. The results for the points 8 hr and 12 hr after hormone treatment were obtained by Dr J.T. Knowler and are included with his permission. The values at each time point have not been plotted as percentages of the control incorporation since, in the case of the bound fraction, hardly any radioactivity was incorporated by the control animals.

From the results of the amount of RNA recovered from uterine polysomes, it can be seen that the bound RNA, presumed to represent polysomal, polyadenylated RNA, represents approximately 2-6% of the total polysomal RNA, depending upon whether the animals had received hormone or not. The amount of RNA recovered in both fractions increases 16 hr after hormone treatment, and then falls off after 24 hr. This peak at 16 hr may be, in part, an artifact caused by the increased water uptake of the uterus, making the tissue easier to homogenise and therefore leading to a greater recovery of ribosomes. The amount of extractable RNA would be expected to increase, but should not exceed 200% of control levels over 24 hr of oestrogen treatment (Billing et al., 1969b).

The incorporation of radioactivity into the bound RNA is very low up to 1 hr after hormone treatment, after which it increases and appears to be maintained at this level. The incorporation into the unbound RNA shows a slight increase 2 hr after hormone treatment and a large increase after 16 hr, the effect declining after 24 hr. However, because of the possibility that the increased radioactive incorporation observed after 16 hr is partly a result of an increased recovery of RNA at this time, the results are better expressed as specific activity of RNA.

When this is done, it is seen that there is a slight stimulation of incorporation into the unbound RNA 2 hr after hormone treatment at approximately four times the control incorporation. This level of incorporation is maintained until 8 hr and is followed by a further stimulation of incorporation which reaches a maximum 12 hr after oestrogen

administration at approximately ten times the control level. The incorporation into unbound RNA then gradually declines.

Figure 15 also shows the results of the incorporation into the bound RNA expressed as the specific activity of the RNA. Caution should be exercised in the interpretation of these results since, as can be seen, both the amounts of RNA recovered and the levels of acid-insoluble radioactivity are low. Consequently, small alterations in either of these parameters could result in large changes in the specific activity of this RNA fraction. Thus, changes in the specific activity occurring within the first hour of hormone treatment are probably a result of experimental artifacts. However, there does appear to be a stimulation of incorporation 2 hr after hormone treatment which reaches a maximum at 12 hr and then declines.

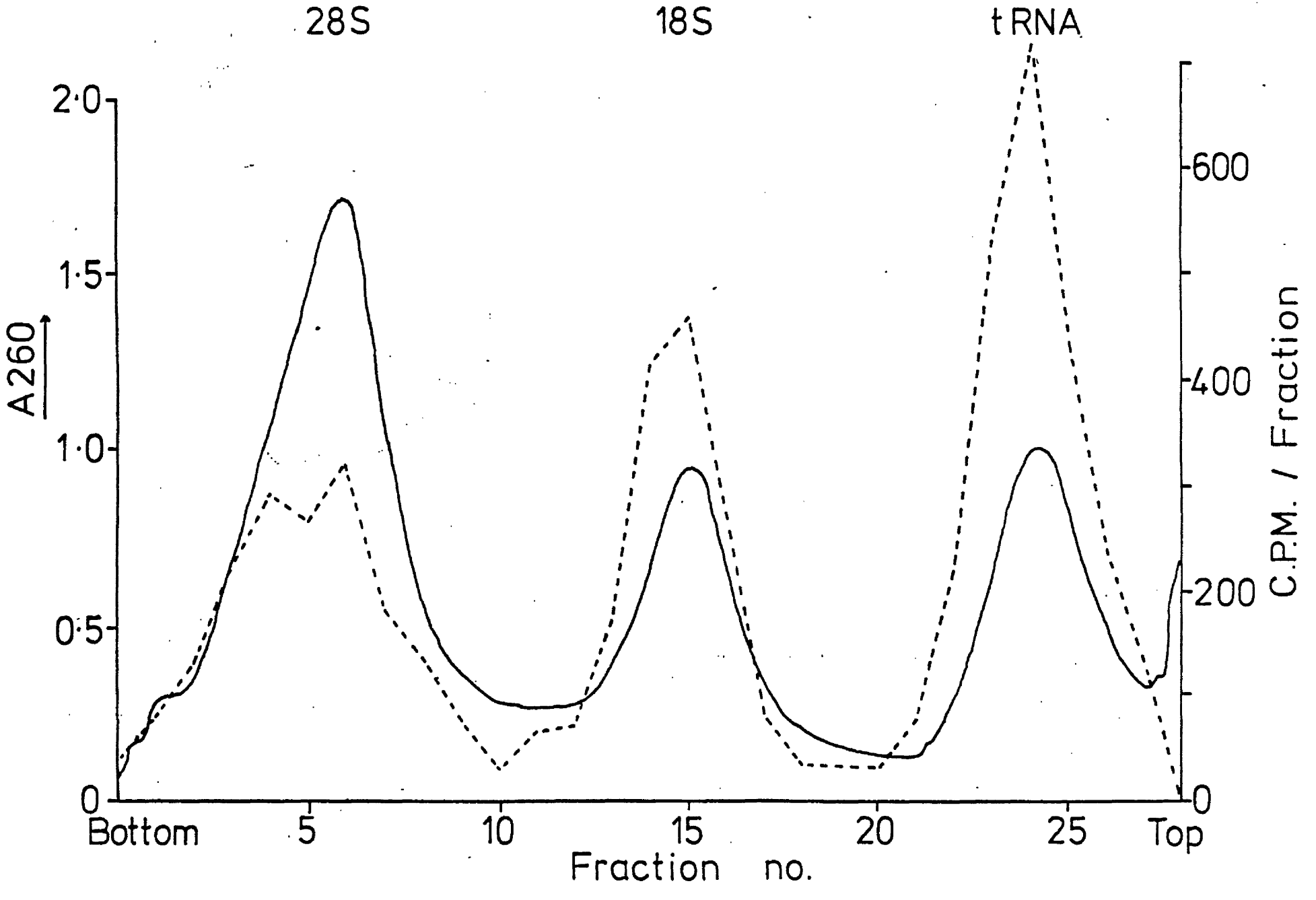
Although polyacrylamide gel electrophoresis had shown the unbound RNA to consist of rRNA and tRNA, it was not known whether administration of the radioactively-labelled precursor 30 min before death would result in the cytoplasmic rRNA and tRNA species becoming labelled. Therefore, RNA was extracted from the post-mitochondrial supernatant of uteri from hormone-treated animals that had been given [^{3}H] uridine 30 min before death. The RNA was analysed on sucrose density gradients, in preference to polyacrylamide gels, to allow a greater sensitivity of detection of radioactively-labelled RNA. The result is shown in figure 16.

Three peaks were observed on the sucrose gradient, corresponding to 28S rRNA, 18S rRNA and tRNA respectively. All three RNA species were radioactively-labelled, with tRNA being labelled to the greatest extent and 28S rRNA having the lowest activity, this being a reflection of the relative rates of synthesis and transport to the cytoplasm of the three RNA species. However, although the tRNA was labelled to a greater extent than either of the rRNA species taken singly, when the activity present in the two rRNA peaks was summed, the total activity was 25% greater than the activity of the tRNA.

Figure 16

Incorporation of radioactive precursor into uterine RNA

Twelve immature female rats were given 1 μg oestradiol-17β 16hr before death. Each animal also received 200 μCi [5,6-^{3}H] uridine 30 min before death. RNA was extracted from the post-mitochondrial supernatant and analysed by sucrose density gradient centrifugation as described in Materials and Methods. Fractions were collected from the sucrose gradient and the acid-insoluble radioactivity in each fraction was determined. ——— , A260; ----, radioactivity (c.p.m.) per fraction.


28S
18S
tRNA
2·0
1·5
1·0
0·5
0
A260
600
400
200
0
C.P.M. / Fraction
Bottom
5
10
15
20
25
Top
Fraction no.

Moreover, the RNA for this experiment was extracted from the post-mitochondrial supernatant of the uterus, so would include any cytoplasmic tRNA. In polysomes, there will be only two tRNA species per ribosome, so the labelled, unbound polysomal RNA would be expected to consist mainly of rRNA with some tRNA present. As stated previously , the bound polysomal RNA contained no detectable rRNA and, in fact, no discrete RNA species smaller than rRNA were detected on polyacrylamide gels, and no species larger than rRNA were detected on sucrose gradients under conditions where the rRNA remained at the top of the gradient.

Therefore, the stimulation of the incorporation of labelled, non-polyadenylated RNA into uterine polysomes would appear to result mainly from the incorporation of newly-synthesised rRNA, but also some tRNA, into the polysomes. There is also a stimulation of the incorporation of radioactivity into the polysomal RNA fraction retained by oligo (dT) cellulose, presumed to be polyadenylated mRNA.

It is not possible, from these experiments, to say whether the aggregation of ribosomes into polysomes in the uterus 30 min - 1 hr after treatment with oestrogen results from the association of newly-synthesised mRNA with the ribosomes. However, the increase in the proportion of polysomes to monosomes after 4 hr would appear to result from the association of newly-synthesised mRNA with mainly pre-existing ribosomes. Newly-synthesised ribosomes are beginning to appear in the polysomes at this time, although the incorporation of newly-synthesised rRNA into uterine polysomes is not maximal until 12hr after hormone treatment. By this time, the incorporation of newly-synthesised mRNA into polysomes also appears to be maximal.

3.3 Effect of oestradiol-17β on uterine ribosomal protein synthesis

3.3.1 Incorporation of radioactively-labelled precursors into ribosomal subunits

In the preceding section, results were described which showed that oestrogen stimulated the incorporation of newly-synthesised rRNA into uterine ribosomes.

In addition to rRNA, ribosomal proteins are required for the assembly of complete ribosomes. Experiments were carried out, therefore, to study the effect of oestradiol-17β on the incorporation of newly-synthesised ribosomal proteins into uterine ribosomes.

One major problem in studying the synthesis of ribosomal proteins concerns the definition of a ribosomal protein. Ribosomes isolated from eukaryotic cells are usually contaminated with cytoplasmic proteins. If the ribosomes are associated with mRNA to form polysomes, there will be nascent polypeptide chains present also. Contaminating proteins can be removed, to a large extent, if the ribosomes are dissociated into subunits and the subunits separated on a sucrose density gradient in the presence of a high concentration of potassium chloride. This will also simplify subsequent analysis of the ribosomal proteins. Preliminary experiments were carried out, therefore, to determine the best conditions for dissociation of the ribosomes into subunits. Figure 17 shows the results of incubating the ribosomes with a high concentration of potassium chloride in the presence of either puromycin or EDTA.

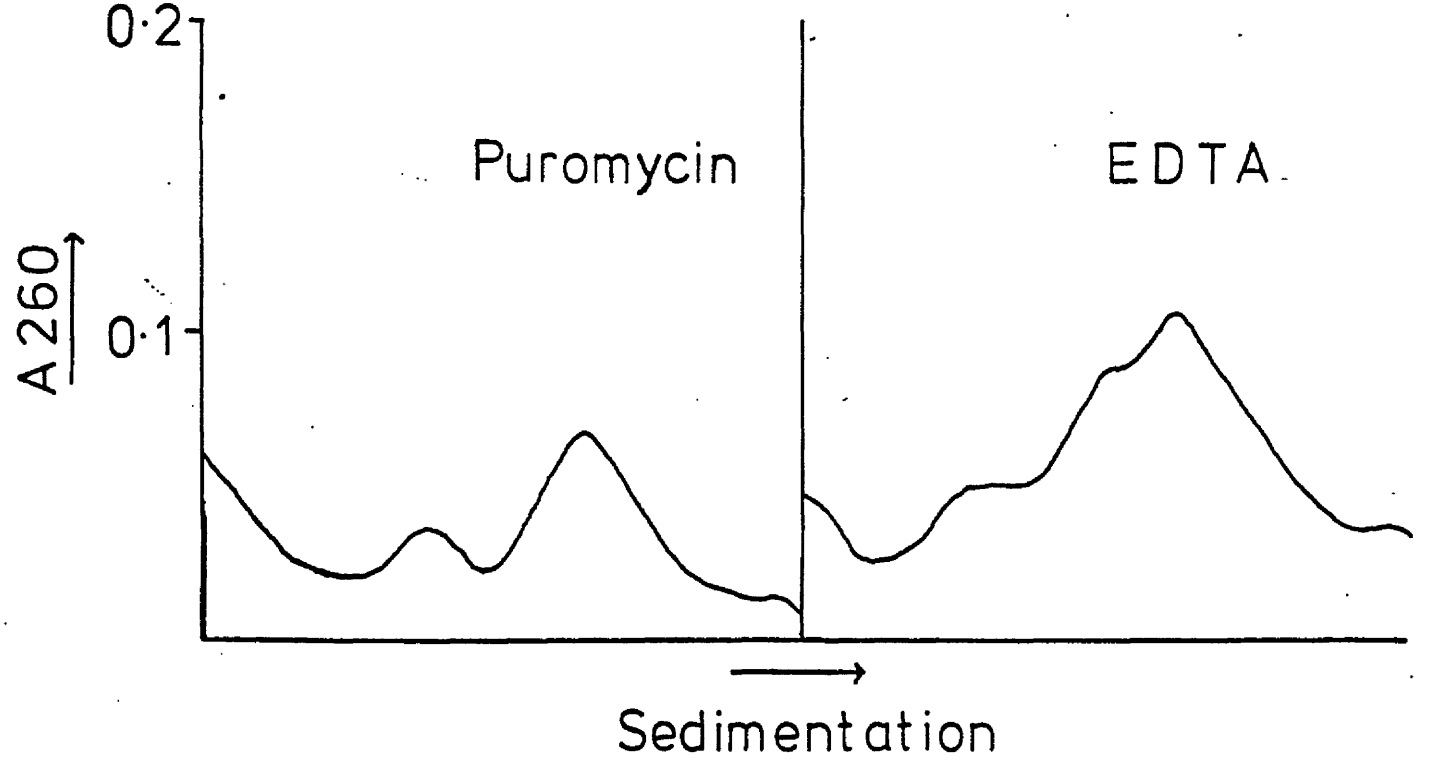
In the sample treated with puromycin, two resolved peaks can be seen on the sucrose gradient, which would appear to correspond to 40S and 60S ribosomal subunits respectively. The sucrose gradient of the EDTA-treated sample does not show such good resolution. The 40S subunits do not appear to be resolved from the faster-sedimenting 60S subunits, and there appears to be more than one component in the 60S peak. Dissociation therefore appears to be more complete in the presence of puromycin.

Dissociation of ribosomes is favoured by high potassium concentrations and low magnesium concentrations. It will thus be favoured by magnesium-chelating agents, such as EDTA. This has been reported to lead to biologically inactive subunits, however (Blobel and Sabatini, 1971). Puromycin appears to promote dissociation by leading to the release of the peptidyl-tRNA from the ribosome. The peptide chain affords some protection

Figure 17

Dissociation of uterine ribosomal subunits

Uterine ribosomes were prepared from two groups of twelve immature rats as described in Materials and Methods. Both ribosomal pellets were resuspended separately in 0.67ml 0.01M Tris-HCl, 0.08M KCl, 0.005M $MgCl_2$ buffer, pH 7.6, and 1.25M KCl was added to give a final concentration of 0.5M. To one of the resuspended pellets was added puromycin to 0.1mM and 2-mercaptoethanol to 0.02M. To the other pellet was added EDTA, pH 7.0, to 10mM. Both resuspended pellets were incubated at 37°C for 15 min before centrifugation on 15-30% linear, sucrose density gradients as described in Materials and Methods. Both traces have been corrected for the absorbance of a blank gradient.


0·2
0·1
A 260
Puromycin
EDTA
Sedimentation

against dissociation by a high concentration of potassium ions. Under conditions of a high ionic strength and a relatively high puromycin concentration such as 0.1mM, the peptidyl-tRNA will be released from both the peptidyl site and the acceptor site on the ribosome (Skogerson and Moldave, 1968). For the present work, release of nascent peptide chains is an added advantage of using puromycin. This antibiotic was used in all subsequent experiments on ribosomal subunits.

The two peaks observed on the sucrose gradient were collected separately and the RNA was extracted and analysed on polyacrylamide gels. The results are shown in figure 18. The slower-sedimenting peak contains both 28S and 18S rRNA, although it is enriched in 18S rRNA (the heights of the peaks corresponding to 28S and 18S rRNA are usually in the ratio 2 to 1 for undissociated ribosomes, as can be seen in figure 8). This suggests that this peak consists mainly of 40S ribosomal subunits with some contaminating 60S subunits present. The faster-sedimenting peak on the sucrose gradients contains 28S rRNA only, showing that it consists of 60S ribosomal subunits. Therefore it is possible to prepare pure 60S subunits, as judged by analysis of the rRNA, by this method, but it would appear that the 40S subunit fraction is contaminated with 60S subunits. Even when only the light side of the 40S peak from the sucrose gradient was collected, some 28S rRNA was still present on extraction and analysis of the rRNA (not shown). This is in contrast to the situation reported for rat liver ribosomes. When ribosomes from this tissue are dissociated and the subunits separated on a sucrose density gradient, the 40S subunit fraction is usually pure, whereas it is difficult to obtain pure 60S subunits. This is due to contamination on the light side of the 60S peak with aggregated 40S subunits and on the heavy side of the peak with undissociated monomeric ribosomes (Martin et al., 1971). The reason for such a difference between two organs from the same animal is not known.

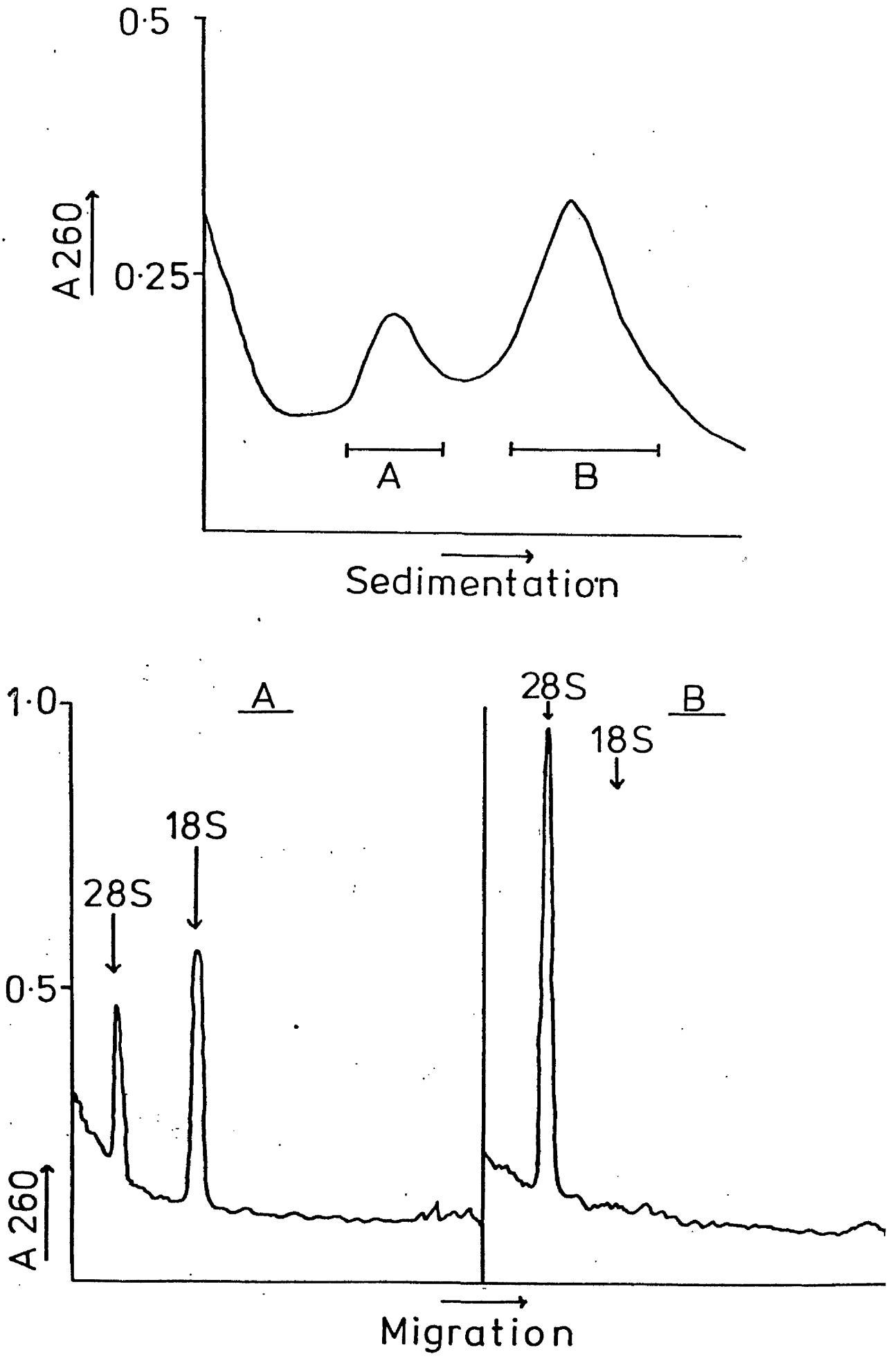
It was decided to collect the whole of the two peaks on the sucrose gradient

Figure 18

Analysis of the RNA of uterine ribosomal subunits

Uterine ribosomes were prepared from twelve immature rats, dissociated into subunits in the presence of puromycin, and the subunits were separated on a sucrose density gradient as described in Materials and Methods. The absorbance trace of the sucrose gradient, corrected for the absorbance of a blank gradient, is shown in the upper part of the figure.

The regions of the sucrose gradient indicated were collected separately. The RNA was extracted and separated on 2.7% polyacrylamide gels as described in Materials and Methods. The densitometer traces of the gels are shown in the lower part of the figure. The arrows indicate the positions of 28S and 18S rRNA which were electrophoresed on a separate gel.


0·5
A 260
0·25
A
B
Sedimentation
1·0
A
28S
B
18S
18S
28S
0·5
A 260
Migration

in subsequent experiments carried out to determine the effect of oestrogen on the incorporation of newly-synthesised proteins into uterine ribosomes. Immature rats were given 100 μCi L-[4,5-^{3}H] leucine and ribosomal subunits were prepared and precipitated with ethanol. Aliquots were removed for protein assay, in order that the specific activity of the labelled protein could be calculated, and the acid-insoluble radioactivity of the remainder was determined. The results are shown in figure 19, which shows the amount of protein recovered in the two subunit fractions at various times following hormone treatment, the radioactivity incorporated, and the specific activity of each fraction. A typical preparation from the uteri from twelve control animals yielded approximately 70 μg ribosomal protein, with approximately 300 c.p.m. incorporated. Both the amount of protein and radioactivity in the 60S subunit fraction was twice that in the 40S subunit fraction.

The total amount of protein recovered from the ribosomal subunits increases following hormone stimulation, being first detectable between 4-8hr after oestrogen treatment. The recovery of protein continues to increase until, after 24hr, the total amount recovered is four times the control value. For the same reason that the amount of polysomal RNA recovered from the uterus increases following hormone treatment, the increase in the amount of ribosomal protein could occur because of greater ease in homogenising the tissue, as well as an hormone-stimulated increase in the number of ribosomes.

The incorporation of radioactivity can be seen to be very similar for the 40S and 60S subunit fractions. Hormone treatment results in a stimulation of incorporation into both fractions detectable after 4hr and maximal after 12hr, declining by 24hr. The maximum level of radioactive incorporation into total ribosomes appears to be 18 times the control level. However, because of the possibility that the hormone-stimulated increase in water uptake by the uterus could lead to the tissue being more easily broken up,

Figure 19

Effect of oestradiol-17β on the incorporation of [^{3}H] leucine into uterine ribosomal subunits

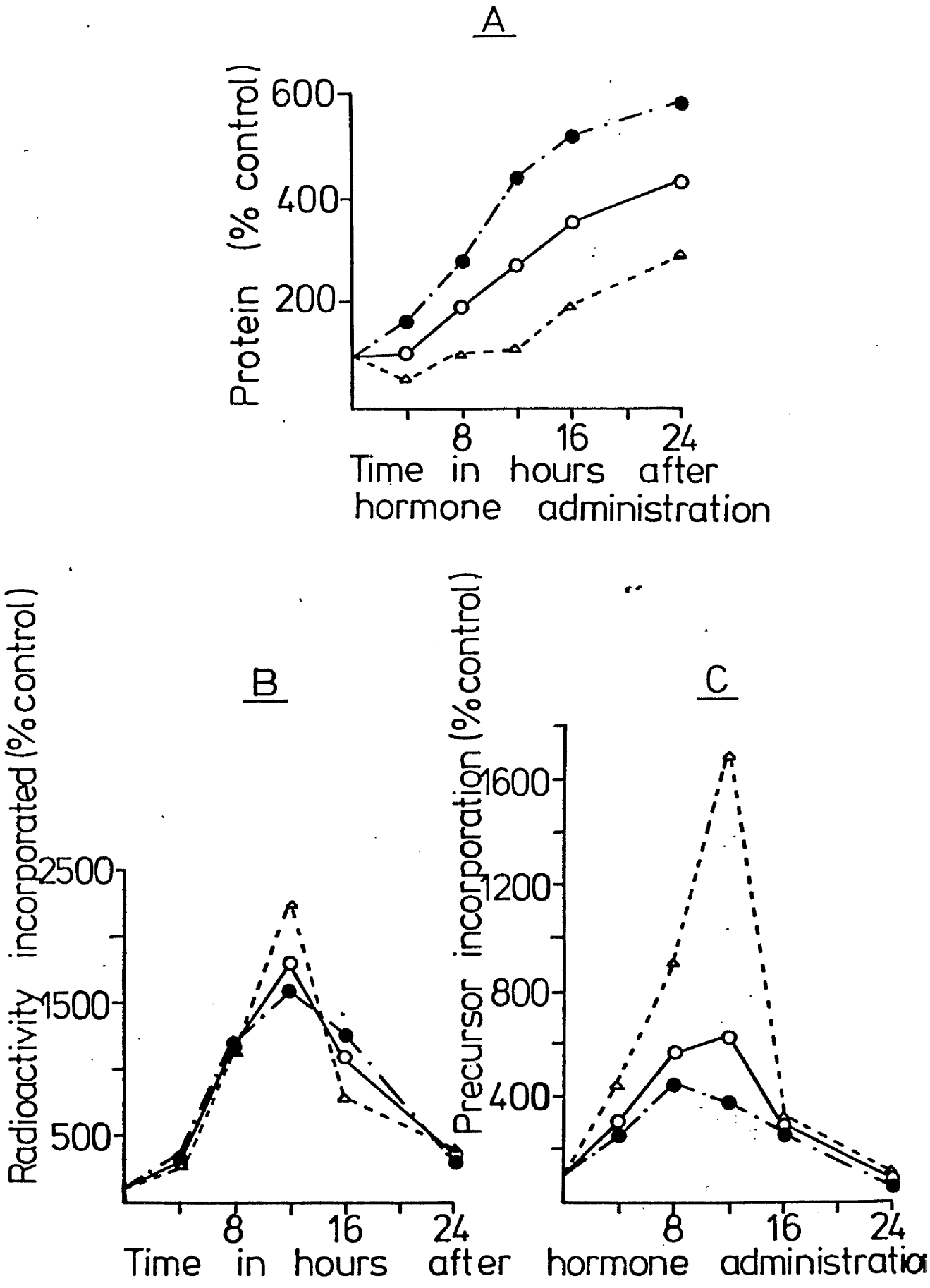
Groups of twelve immature female rats received 1 μg oestradiol-17β or saline carrier at the times before death indicated. All the animals received 100 μCi L-[4,5-^{3}H] leucine 1hr before death. Ribosomal subunits were prepared from the uteri of each group as described in Materials and Methods. 1/20 aliquots were removed for protein estimation and the remainder was precipitated with trichloroacetic acid and acid-insoluble radioactivity was determined for the 40S and 60S subunit fractions.

A. Amount of protein (measured in μg)

B. Acid-insoluble radioactivity (measured in c.p.m.)

C. Specific activity of protein (calculated as c.p.m./μg protein)

The results have been expressed as percentages of either the amount of protein or the incorporation by animals that received saline in place of hormone. o———o , total ribosomal subunits; ●—·—·—●, 60S ribosomal subunits; Δ- - -Δ, 40S ribosomal subunits.


A
Protein (% control)
600
400
200
8
16
24
Time in hours after hormone administration
B
Radioactivity incorporated (% control)
2500
1500
500
8
16
24
Time in hours after
C
Precursor incorporation (% control)
1600
1200
800
400
8
16
24
hormone administratio

resulting in an increased recovery of ribosomes, the results are expressed better as specific activity of protein.

When this is done, it is seen that there is a stimulation of incorporation into total ribosomes which is still detectable after 4hr and is maximal 8-12hr after hormone treatment at 6 times the control level. By 24hr after hormone treatment, the incorporation has returned to the control level. However, the results appear to be different for the two ribosomal subunits. The stimulation of incorporation into the 40S fraction is much greater than the stimulation of incorporation into the 60S, the maximum level being reached after 12hr at 17 times the control value. Incorporation into the 60S subunit fraction appears to reach a maximum 8hr after hormone treatment at 4 times the control level. This is unusual because it would be expected that the stimulation of incorporation of newly-synthesised protein into the two ribosomal subunits should be the same since the rRNA species present in the subunits are synthesised as part of the same precursor molecule. Consequently, a stimulation of the synthesis of rRNA should result in an equal stimulation of the synthesis of the rRNA of both the 40S and 60S subunits, and, presumably, this would be accompanied by an equal stimulation of the synthesis of the ribosomal proteins of both subunits. One reason for the apparently greater stimulation of incorporation into the 40S subunit fraction could be that this fraction, as isolated from sucrose density gradients, is contaminated with 60S subunits. Since the amount of protein in the 60S ribosomal subunit is greater than that in the 40S subunit, a low level of contamination could result in marked changes in the amount of radioactivity incorporated. This level of contamination could be altered in different preparations. It is therefore best to consider the sum of the amounts of protein and radioactivity recovered from the two ribosomal subunits, giving the results for complete ribosomes, which have thus been stripped of nascent polypeptides. The results for the specific activity of total ribosomes from figure 19 can be combined with

the results for the incorporation of [^{3}H] leucine into total uterine acid-insoluble material from figure 6. This is shown in figure 20. The results for the incorporation into total uterine protein 12hr and 16hr after hormone treatment were obtained by Dr. J.T. Knowler and are included with his permission.

The stimulation of incorporation of newly-synthesised proteins into uterine ribosomes in response to oestrogen shows the same kinetics as the stimulation of total protein synthesis in the uterus. Thus, a stimulation of incorporation into both total and ribosomal protein is detectable after 4hr, maximal after 12hr and has declined by 24hr. The stimulation of incorporation of newly-synthesised protein into ribosomes appears to be slightly greater than the stimulation of incorporation into total uterine protein. Similar results were obtained in an experiment measuring the incorporation of L-[G-^{3}H] tryptophan into ribosomal subunits (results not shown). 200 µCi of this precursor was given to each animal, but the radioactivity incorporated was about 1/10 that incorporated when 100 µCi [^{3}H] leucine was given as the radioactive precursor. The results using [^{3}H] tryptophan again showed that no stimulation of precursor incorporation into the ribosomes was detectable before 4hr after hormone administration.

These results would therefore appear to confirm the finding of the previous section that the increase in the proportion of polysomes in the uterus 4hr after hormone treatment results from the association of newly-synthesised mRNA with pre-existing ribosomes. After this time, newly-synthesised proteins become incorporated into ribosomes, maximum incorporation occurring 8-12hr after hormone treatment. Furthermore, the results shown in figure 20 strongly suggest that the stimulation of ribosomal protein synthesis in response to oestrogen occurs at the same time as the hormone-stimulated increase in general protein synthesis in the uterus.

Figure 20

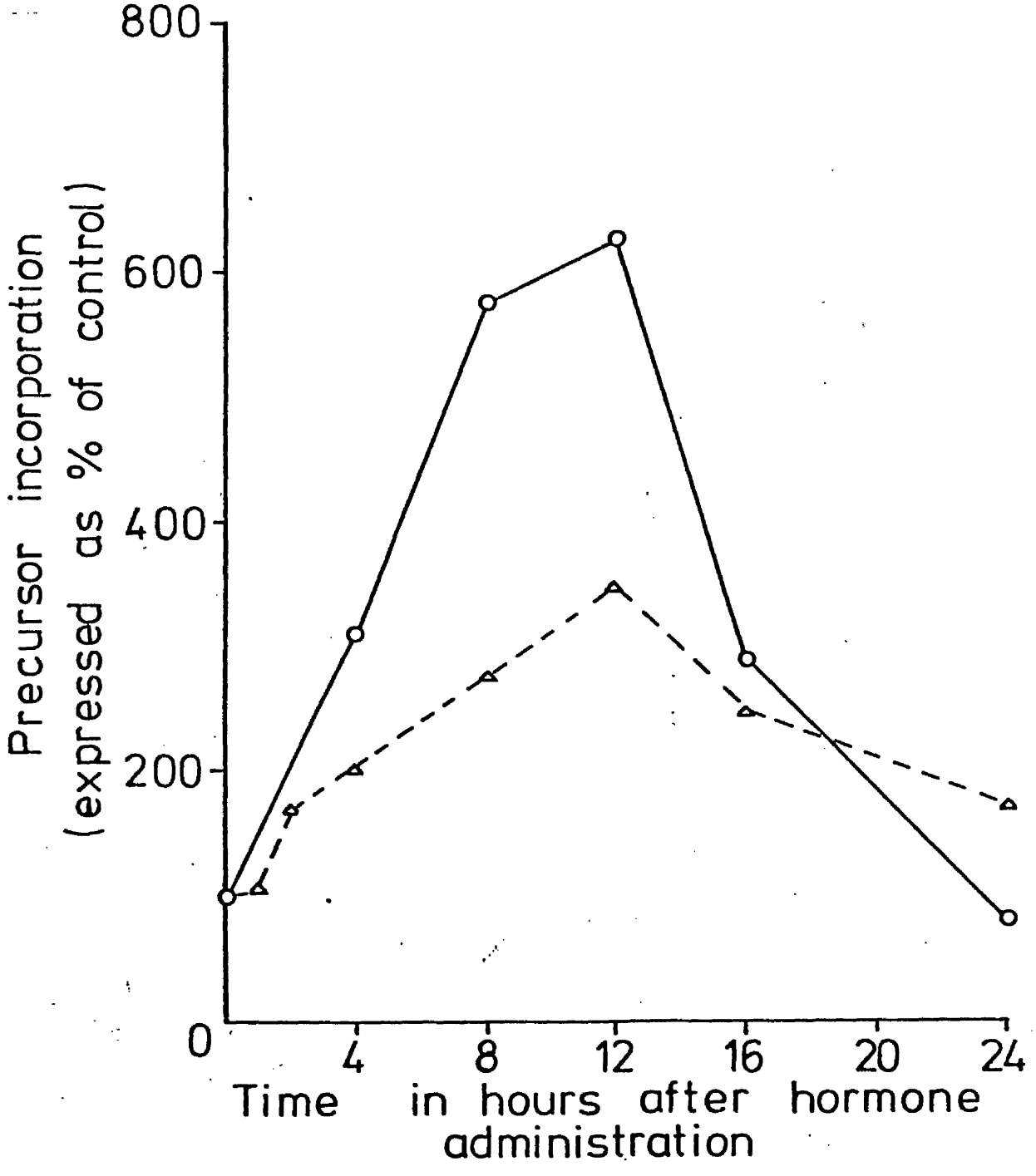
Comparison of the effects of oestradiol-17β on the incorporation of [^{3}H] leucine into uterine ribosomal protein and total protein

Immature rats were given 1 μg oestradiol-17β or saline carrier at the times before death indicated. Measurement of the incorporation into total uterine acid-insoluble material (Δ- - -Δ) was carried out as described in the legend to Figure 6. Animals received 20 μCi L-[4,5-^{3}H] leucine 30 min before death. The results were calculated as d.p.m./ μg DNA and are expressed as percentages of the incorporation into uteri of animals that did not receive hormone. Each point represents the mean of at least three animals.

N.B. The results for the points at 12hr and 16hr were obtained by Dr J.T. Knowler.

Incorporation into ribosomes (o———o) was carried out as described in the legend to Figure 19. Animals received 100 μCi L-[4,5-^{3}H] leucine 1hr before death. The results were calculated as c.p.m./ μg protein incorporated into total ribosomal subunits and have also been expressed as percentages of the incorporation by control animals.

Incorporation of [^{3}H] leucine into uterine ribosomal protein and total protein



3.3.2 Incorporation of radioactive precursors into uterine ribosomal proteins in vitro

3.3.2.1. Effect of oestradiol-17β on incorporation in vitro

In the preceding section, it was shown that radioactively-labelled amino acids administered to immature rats in vivo were incorporated into acid-precipitable material of uterine ribosomal subunits and that this incorporation was stimulated by oestradiol-17β . However, it was necessary to show that this incorporation was into ribosomal proteins. This can be done by fractionating the proteins by polyacrylamide gel electrophoresis and determining whether they have become radioactively-labelled.

The incorporation of radioactivity achieved in vivo was not sufficient to allow the detection of labelled proteins on polyacrylamide gels. Experiments were carried out, therefore, to determine whether it were possible to achieve sufficient labelling of the ribosomal subunits by incubating the uteri from the animals in vitro in a medium containing a radioactively-labelled amino acid. This would allow verification of whether the ribosomal proteins were becoming labelled.

Preliminary experiments were carried out to determine the amount of label in the medium and the length of incubation time required to achieve a sufficiently high level of incorporation. These showed that incubation in a medium containing L-[4,5-^{3}H] leucine, at a concentration of 50 μCi/ml, for 1hr resulted in the incorporation of approximately 10,000 c.p.m. into the ribosomes of the uteri from twelve control rats. This was considered sufficient for subsequent fractionation of the proteins by polyacrylamide gel electrophoresis. The degree of radioactive incorporation was, in fact, found to increase with increasing incubation times, but 1hr was chosen as the greatest length of time before which the uteri started showing signs of anoxia. It was found to make very little difference to the level of radioactive incorporation if the [^{3}H] leucine were added to leucine-free

Eagle's Medium or to Medium 199 (Gibco, Bio-Cult Laboratories Ltd., Paisley, U.K.), a more complete medium containing unlabelled DL-leucine at a concentration of 120 µg/ml. However, it was decided to use leucine-free Eagle's Medium in all subsequent experiments.

These experiments showed that it was possible to incorporate label into acid-insoluble material of ribosomal subunits using an in vitro incubation technique. However, as discussed in Section 1.2.2.6, when uteri are incubated in vitro, the oestrogen-stimulated synthesis of rRNA cannot be detected. This suggests that some step in the synthesis of ribosomes is sensitive to the in vitro incubation conditions (Knowler et al., 1975; Knowler, 1976). It was therefore considered necessary to demonstrate a stimulatory effect of oestradiol-17β on the incorporation of [^{3}H] leucine into ribosomal subunits in vitro. This would show whether the particular step sensitive to the incubation conditions included the synthesis of ribosomal proteins. Figure 21 shows the results of an experiment to determine the effect of oestradiol-17β, administered in vivo, on the in vitro incorporation of [^{3}H] leucine into ribosomal subunits.

It can be seen that hormone treatment in vivo results in a stimulation of incorporation into the ribosomal subunits. The stimulation of radioactivity incorporated is approximately ten-fold for both the 40S and 60S subunits. When the results are expressed as specific activity of protein, there is still a ten-fold stimulation of incorporation in the 40S fraction, but the stimulation of incorporation into the 60S fraction is only two-fold. Thus, as in the previous section, there appears to be a greater stimulation of incorporation into the 40S fraction than into the 60S fraction, probably resulting from incomplete separation of the ribosomal subunits on the sucrose gradients.

This experiment therefore shows that oestradiol-17β does stimulate the in vitro incorporation of [^{3}H] leucine into acid-precipitable material

Sample		c.p.m.	µg protein	c.p.m./µg protein
Control	40S Fraction	3022	34	89
	60S Fraction	6159	34	181
12hr Oestradiol	40S Fraction	31525	37	852
	60S Fraction	70156	170	413

Figure 21

Effect of oestradiol-17β on the incorporation of [^{3}H] leucine into uterine ribosomal subunits in vitro

Twelve immature rats were given 1 µg oestradiol-17β 12hr before death whilst another group of twelve animals received saline in place of hormone. The uteri from each group of animals were incubated in Eagle's Medium containing 50 µCi/ml L-[4,5-^{3}H] leucine for 1hr at 37°C as described in Materials and Methods. Ribosomal subunits were prepared and the fractions from the sucrose gradients were precipitated with ethanol. Aliquots were removed from each fraction for protein assay and the acid-insoluble radioactivity of the remainder of each fraction was determined.

of uterine ribosomal subunits. If the radioactively-labelled leucine is, indeed, becoming incorporated into the ribosomal proteins, the results of this experiment would appear to suggest that the particular step in the synthesis of uterine ribosomes that is sensitive to the in vitro incubation conditions is an early one. Thus, if the synthesis of rRNA has already been stimulated in vivo by treatment with the hormone, then it may be possible to obtain a stimulation of the synthesis of ribosomal proteins in vitro.

The experiments described in the next sections were designed to investigate whether the ribosomal proteins became labelled following in vitro incubation of rat uteri with [^{3}H] leucine.

3.3.2.2 Fractionation of ribosomal proteins on one-dimensional polyacrylamide gels

Ribosomal subunits, prepared from the uteri of twelve immature rats that had received oestradiol 12hr before death, were precipitated with ethanol and subjected to electrophoresis on 12.5% SDS-polyacrylamide gels. Figure 22 compares the gel patterns obtained with those of 40S and 60S ribosomal proteins from BHK cells.

Unfortunately, the gel to which the uterine 60S subunit fraction was applied is overloaded. However, it can be seen that there is very good agreement between the gel patterns of uterine ribosomal subunits and ribosomal proteins from BHK cells. In the case of the 40S subunits, the correspondence is excellent. Extra bands were present in both the uterine ribosomal subunit fractions, mainly at the top of the gels. These bands at the top of the gels are common to both the 40S and 60S subunit fractions from the rat uterus, and they appear to have a higher molecular weight than any of the BHK cell ribosomal proteins, indicating that they are not true ribosomal proteins. This would suggest that the ribosomal subunits from the rat uterus are still contaminated with some high molecular weight proteins. A further possible source of contamination of the 40S ribosomal proteins from the rat uterus may be proteins of the 60S ribosomal subunit. The

Figure 22

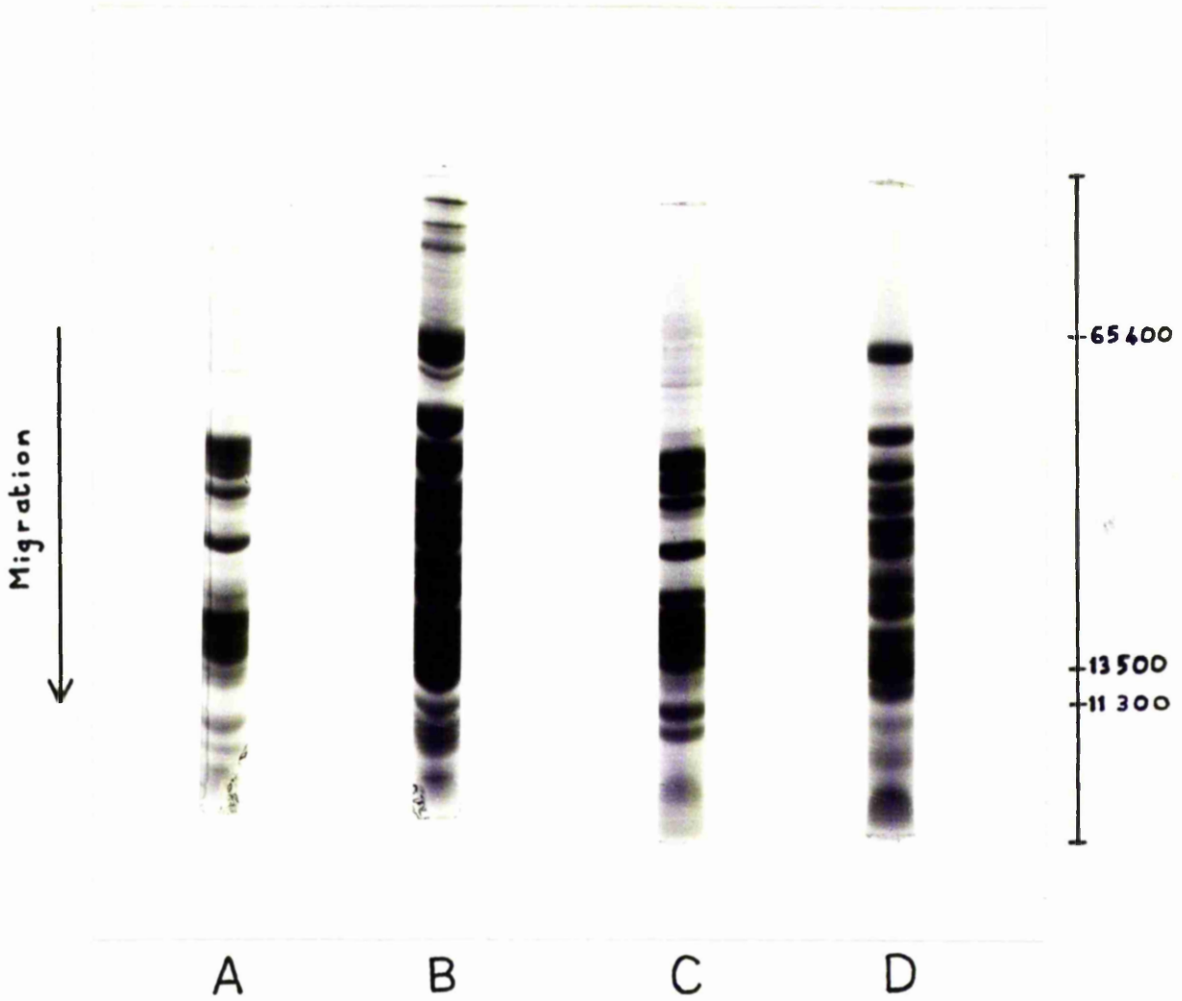
Electrophoresis of ribosomal proteins on SDS-polyacrylamide gels

Ribosomal subunits were prepared from the uteri of twelve immature rats that had received 1 μg oestradiol-17β 12hr before death. The 40S and 60S subunits were collected separately, dialysed against 500ml 0.05M Tris-HCl, 0.08M KCl, 0.0125M $MgCl_2$ buffer, pH 7.6, containing 0.01M 2-mercaptoethanol, and precipitated with ethanol.

The precipitated subunits were dissolved in 100 μl SDS-gel sample buffer, heated at 100°C for 2 min and electrophoresed on 12.5% SDS-polyacrylamide gels according to the method of Laemmli (1970). Electrophoresis was at 4mA per gel until the bromophenol blue front was 1cm from the bottom of the gels. The gel patterns are compared with those of 40S and 60S ribosomal proteins from BHK cells which were electrophoresed on separate gels.

Left to right: A, Uterine 40S subunits; B, Uterine 60S subunits; C, BHK 40S proteins; D, BHK 60S proteins.

Also shown are the positions of marker proteins, bovine serum albumin (mol. wt. 65,400), bovine pancreas ribonuclease (mol. wt. 13,500) and cytochrome c (mol. wt. 11,300) which were electrophoresed on a separate gel.


Migration
65400
13500
11300
A
B
C
D

resolution possible on one-dimensional gels was not sufficient to show whether this was occurring. Nevertheless, this does show that uterine ribosomal proteins can be fractionated on one-dimensional SDS-polyacrylamide gels.

Ribosomal subunits were prepared from immature rat uteri that had been incubated with $[^{3}H]$ leucine in vitro, and subjected to electrophoresis. The gels were then sliced and the radioactivity in the individual slices was determined. The results are shown in figures 23 and 24.

From the actual gel patterns shown in figure 23, it can be seen that hormone treatment does not lead to the appearance of any new bands nor to the disappearance of any bands. The only detectable differences are alterations in the staining intensity of some of the bands. In particular, one band in the gel pattern of 60S subunits from animals treated with oestrogen 12hr before death is much more intense than in any of the other gel patterns of 60S subunits. This band co-migrates with bovine serum albumin. It is probably an artifact, however, since it is not present in the gels shown in figure 22, which are also of ribosomal subunits from animals treated with hormone for 12hr, showing it is not an effect that is observed consistently.

The fact that no extra bands resulting from hormone treatment could be observed is in contrast to the findings of Shapiro et al.(1975). These workers reported the presence of an extra band on polyacrylamide gel electrophoresis of uterine 60S ribosomal proteins from ovariectomised guinea pigs treated with oestradiol-17β for three weeks. It could be that this hormone-induced extra band is observed in a different animal or that it only results from prolonged hormone treatment.

Figure 24 shows the results of plotting out the profiles of radioactivity along the gels. There is excellent correspondence between the peaks of radioactivity and the stained protein bands in all the gels. This shows that these proteins, which co-migrate with ribosomal proteins from BHK cells,

Figure 23

Electrophoresis of uterine ribosomal proteins on SDS-polyacrylamide gels

Groups of twelve rats were given 1 μg oestradiol-17β at various times before death. Control animals received saline in place of hormone. The uteri from each group of animals were incubated in Eagle's Medium containing 50 μCi/ml L-[4,5-^{3}H] leucine as described in Materials and Methods. Ribosomal subunits were prepared from each group of uteri and electrophoresed on 12.5% SDS-polyacrylamide gels, as described in the legend to Figure 22.

A, Control 40S subunits; B, Control 60S subunits; C, 2hr oestradiol 40S subunits; D, 2hr oestradiol 60S subunits; E, 4hr oestradiol 40S subunits; F, 4hr oestradiol 60S subunits; G, 12hr oestradiol 40S subunits; H, 12hr oestradiol 60S subunits.

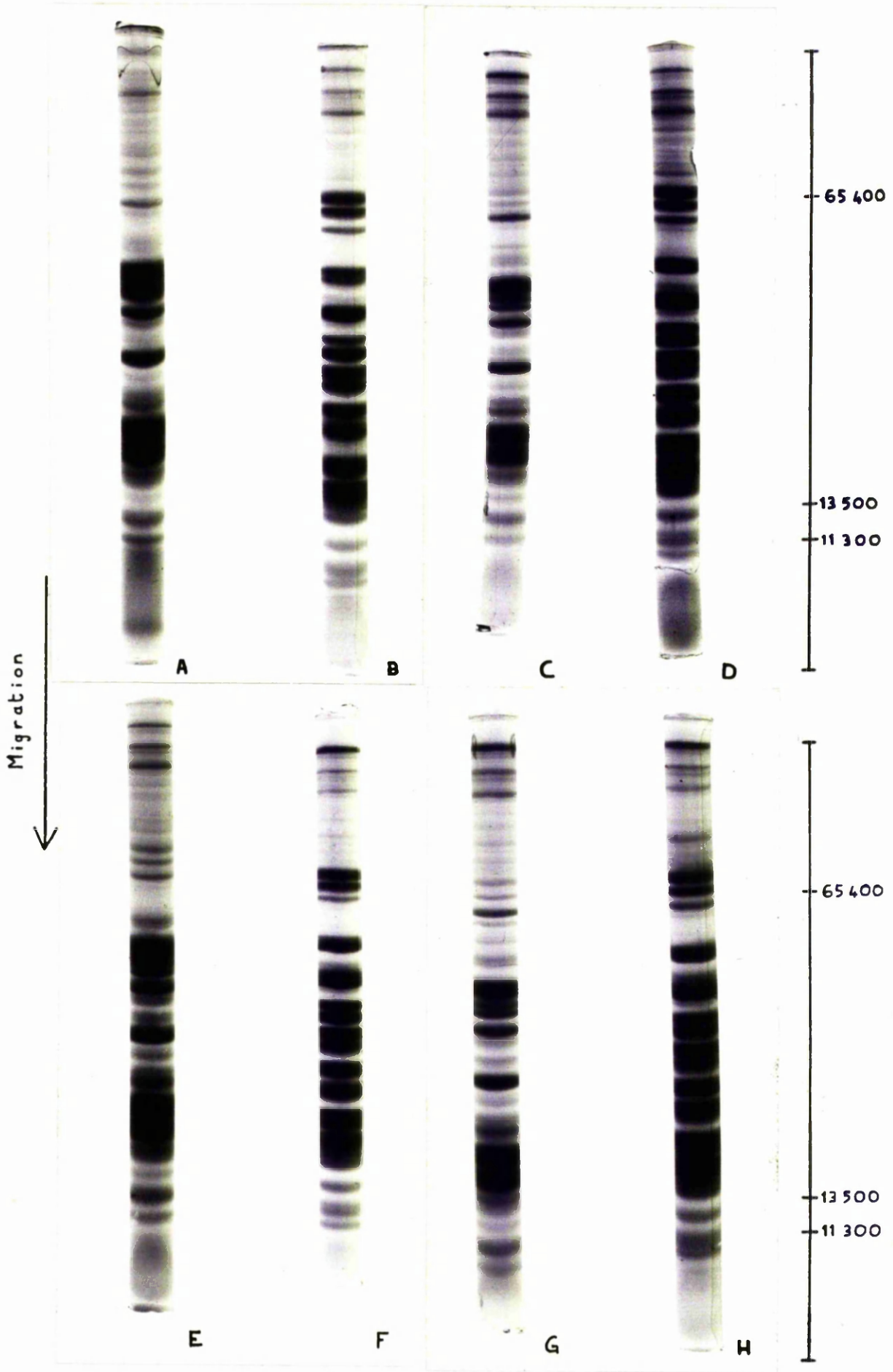
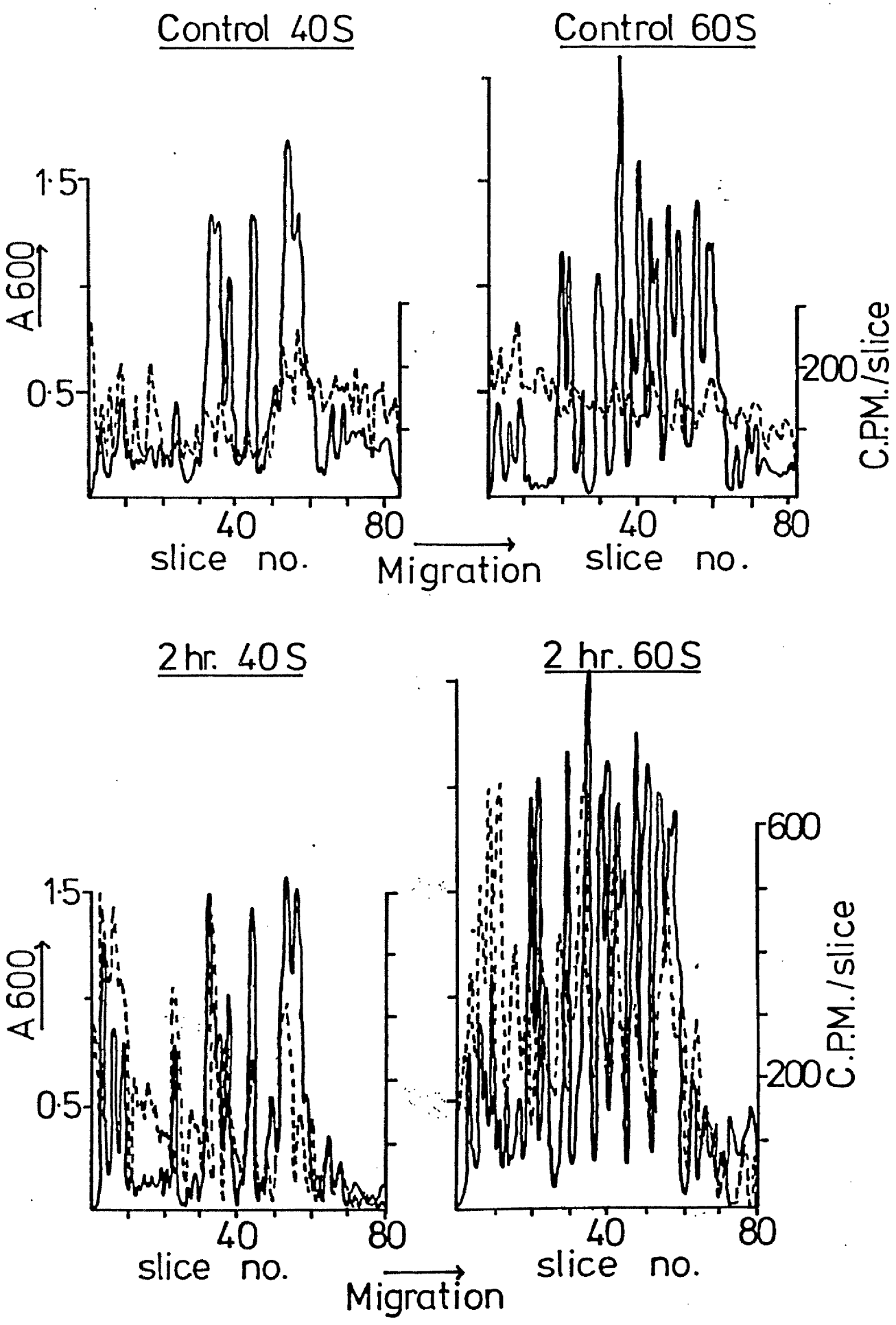

65 400
13 500
11 300
Migration
A
B
C
D
E
F
G
H

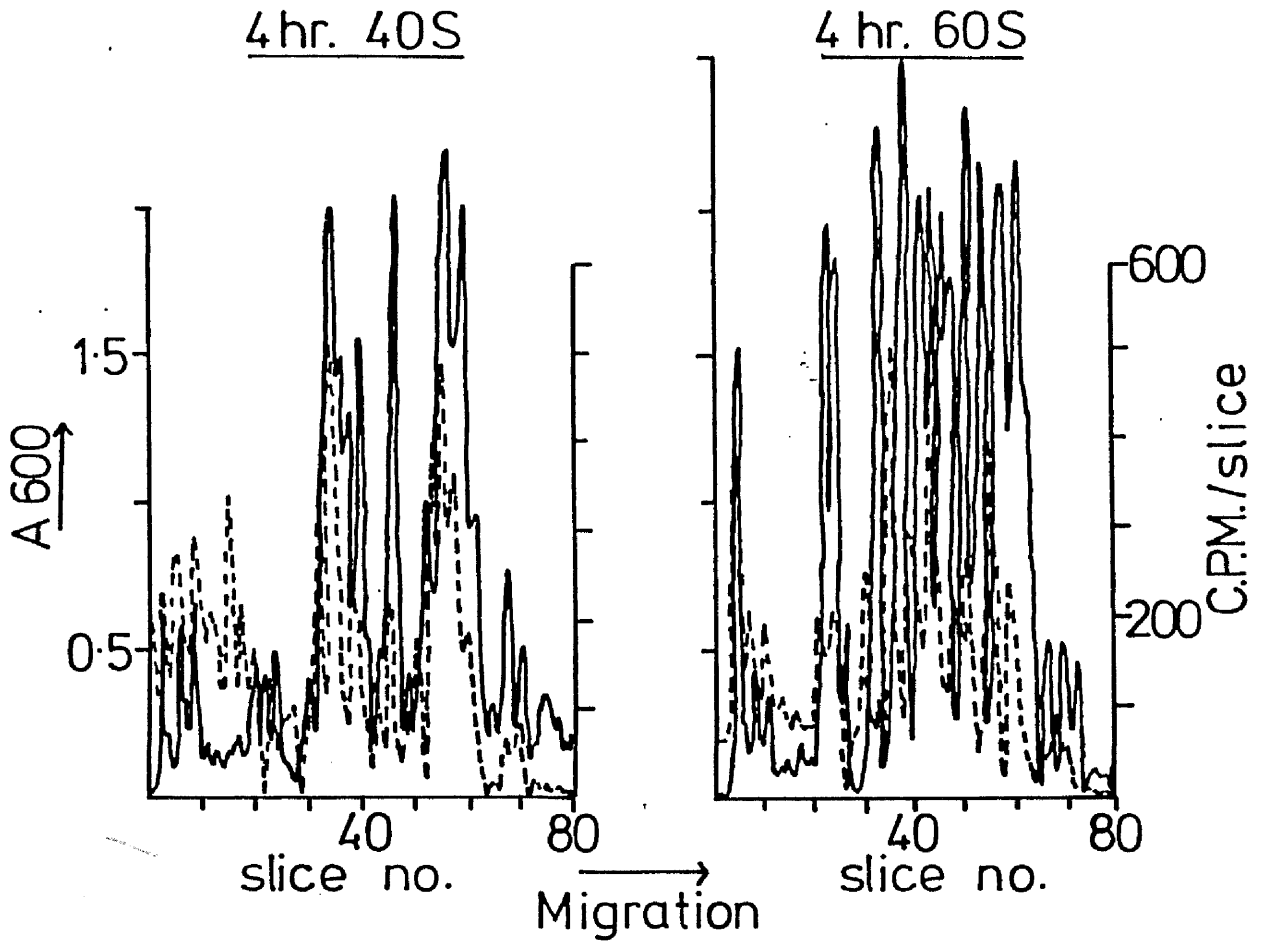
Figure 24

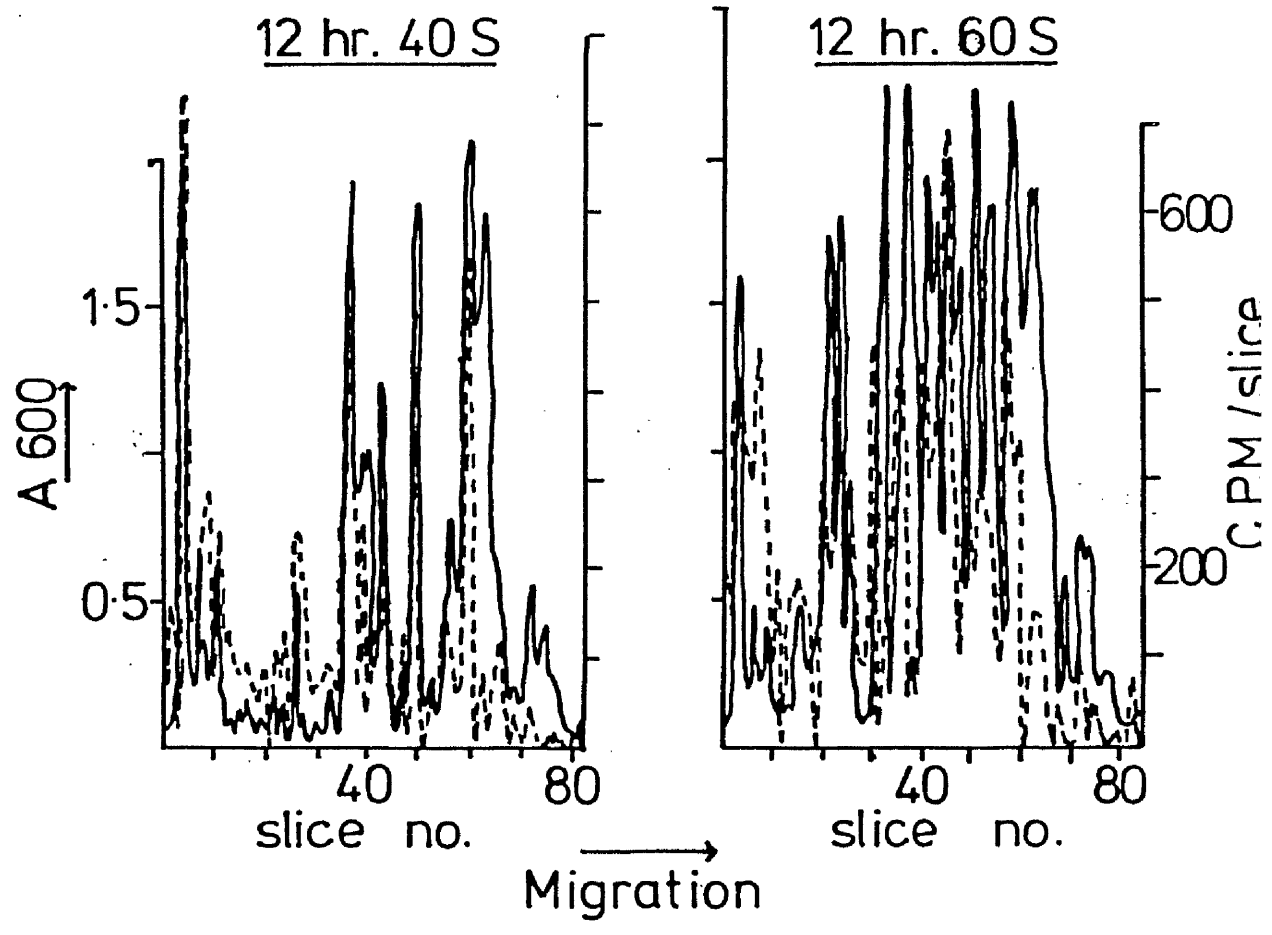
Electrophoresis of uterine ribosomal proteins on SDS-polyacrylamide gels

The gels shown in Figure 23 were scanned at 600nm. They were then sliced and the radioactivity in each slice was determined as described in Materials and Methods. ——— , A600; - - - -, radioactivity (c.p.m.) per slice.

For experimental details, see legend to Figure 23.


Control 40S
Control 60S
1·5
A 600
0·5
200
C.P.M./slice
40
80
slice no.
Migration
2 hr. 40S
2 hr. 60S
600


4 hr. 40S
4 hr. 60S
A 600
1·5
0·5
600
200
C.P.M./slice
40
80
slice no.
slice no.
Migration


12 hr. 40 S
12 hr. 60 S
A 600
1·5
0·5
600
200
C. PM /slice
40
80
slice no.
slice no.
Migration

have become labelled during the in vitro incubation with [^{3}H] leucine. Treatment with oestradiol-17β results in an increase in the level of radioactive labelling of the bands. This increase appears to occur in all the protein bands, with more label being incorporated after longer times of hormone treatment. There is some incorporation by control animals, however, suggesting that there is a low rate of synthesis of these proteins in the uterus in the absence of hormone.

One interesting result from these experiments concerns three bands at the tops of the gels. These bands were present in gels of both 40S and 60S uterine ribosomal subunits but were absent from gels of BHK ribosomal proteins. They also migrated slower than bovine serum albumin. Since the molecular weights of the proteins of the 40S ribosomal subunit range from 8,000 to 35,500, whilst those of the 60S ribosomal subunit range from 11,000 to 55,500 (Welfle et al., 1978), none of the ribosomal proteins should migrate slower than bovine serum albumin (mol. wt. 65,400). These proteins are therefore contaminants present in the uterus. They were found to be labelled following hormone treatment, although the degree of labelling was variable. The identity of these proteins is not known. From their positions in the gels, their molecular weights can be estimated to be 300,000, 125,000 and 90,000 respectively. Proteins in the uterus whose synthesis is stimulated by oestradiol include induced protein, IP, (Notides and Gorski, 1966; Katzenellenbogen and Gorski, 1972) and oestrogen receptor (Jensen et al., 1969; Sarff and Gorski, 1971). None of the three proteins would appear to correspond to IP, which has a molecular weight of 40,000 (King et al., 1977) but it is possible that the fastest-migrating band results from contamination of the ribosomes with oestrogen receptor. Another possibility is that the bands are due to aggregated forms of ribosomal proteins.

These experiments therefore show that electrophoresis of ribosomal subunits from immature rat uteri results in bands which co-migrate with

ribosomal proteins from BHK cells and that these bands are radioactively-labelled following incubation in vitro with [^{3}H] leucine. Hormone treatment stimulates the incorporation of label but it does not result in the appearance of any new bands detectable on one-dimensional gels. These results strongly suggest that the oestrogenic stimulation of the incorporation of radioactively-labelled amino acids, administered in vivo, into uterine ribosomal subunits, described in Section 3.3.1, occurs as a result of a stimulation of the incorporation of newly-synthesised ribosomal proteins into ribosomes. However, although the bands observed on electrophoresis of uterine ribosomal subunits co-migrated with ribosomal proteins from BHK cells, the resolution achieved on one-dimensional gels was not sufficient to separate all the ribosomal proteins. Furthermore, extra bands were present in the gels of ribosomal subunits from immature rat uteri, indicating the presence of contaminating cytoplasmic proteins. Experiments were therefore carried out using a two-dimensional electrophoresis system in order to obtain better resolution of the ribosomal proteins.

3.3.2.3 Fractionation of ribosomal proteins on two-dimensional polyacrylamide gels

In the experiments described in the preceding section, the precipitated ribosomal subunits could be layered directly on to the one-dimensional SDS-polyacrylamide gels since, under the denaturing conditions employed, all the proteins would be released from the rRNA. The rRNA would then remain at the top of the gel. This would not occur under the conditions of the two-dimensional electrophoretic system. It was necessary, therefore, to extract the ribosomal proteins prior to electrophoresis. Preliminary experiments to extract the proteins from ribosomal subunits from the uteri of twelve immature rats with acetic acid by the method of Sherton and Wool (1972) resulted in a recovery of only 1% of radioactively-labelled material, even when the concentration of magnesium acetate was raised in an attempt to increase the yield. The recovery of label was not greatly improved when the extraction

of ribosomal proteins was carried out by the Bearden precipitation method (Bearden, 1974), in which the ribosomal subunits are solubilised in guanidine hydrochloride and the rRNA precipitated with ethanol, retaining the proteins in solution (results not shown). The poor recoveries may result from the small amount of material present in the ribosomal subunits from the uteri of immature rats. This will mean that the concentration of ribosomal proteins will be very low during the extraction procedure.

Since fairly large (approximately 300 μg) amounts of protein are required in order to obtain visible spots on the two-dimensional polyacrylamide gels, it was decided to prepare liver ribosomal subunits to act as a carrier. The liver subunits could then be added to the radioactively-labelled uterine subunits prior to extraction with acetic acid. The disadvantage of this is that any effects of the hormone on the two-dimensional gel pattern of the uterine ribosomal proteins would not be detected. However, since it was not possible to extract sufficient protein from the uterine ribosomal subunits to give visible spots on the two-dimensional gels, there was no other alternative.

Rat liver ribosomal subunits were prepared by a scaled-up version of the method used for the preparation of uterine ribosomal subunits. Aliquots, estimated to contain 300 μg ribosomal protein, were removed from both subunit fractions and the ribosomal proteins were extracted by the acetic acid method of Sherton and Wool (1972) using an increased concentration of magnesium acetate. Acetic acid has been reported as being the most efficient method for extracting ribosomal proteins, with up to 90% recovery being achieved (Sherton and Wool, 1974). The extracted proteins were precipitated with acetone and subjected to two-dimensional polyacrylamide gel electrophoresis. The electrophoresis was carried out by Mr I.M. Kennedy, working under the supervision of Dr D.P. Leader of this department. The results are shown in figure 25.

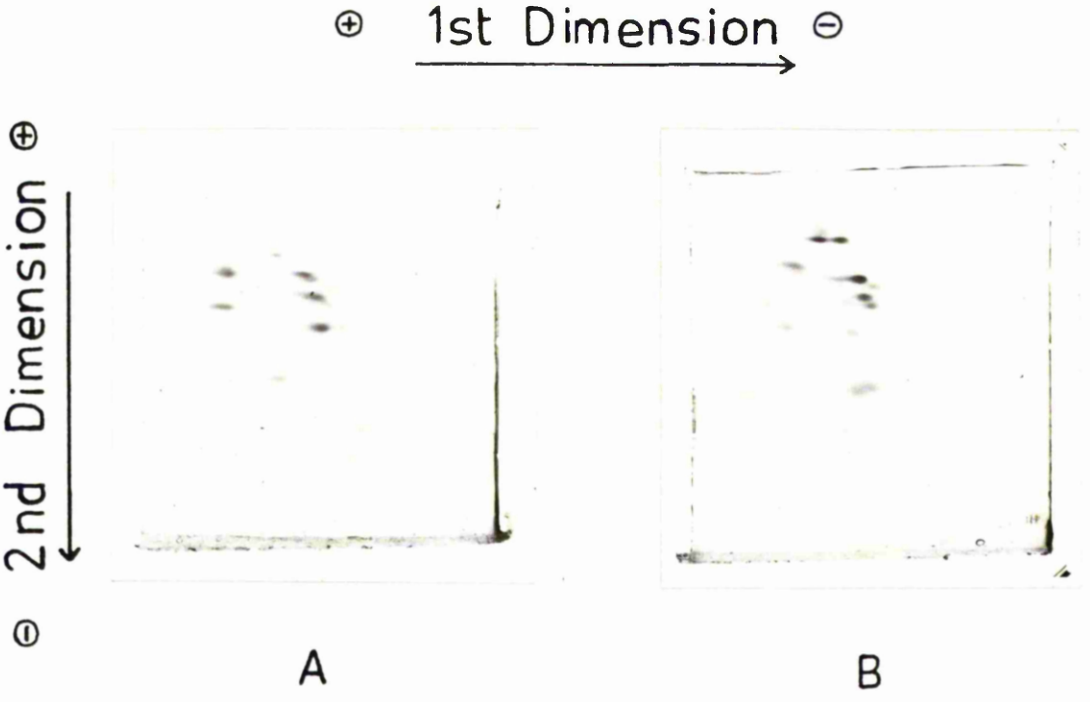
In this two-dimensional gel system, the basic ribosomal proteins are fractionated mainly on the basis of their charge in the first dimension and

Figure 25

Electrophoresis of rat liver ribosomal proteins on two-dimensional polyacrylamide gels

Ribosomal subunits were prepared from the liver of an adult male rat and the proteins were extracted and precipitated with acetone as described in Materials and Methods. The proteins were subjected to two-dimensional polyacrylamide gel electrophoresis by the method of Kaltschmidt and Wittmann (1970) with modifications (Lastick and McConkey, 1976). The electrophoresis was carried out by Mr I.M. Kennedy as described in Materials and Methods. The direction of migration was from left to right in the first dimension and from top to bottom in the second dimension.

A, 40S ribosomal proteins; B, 60S ribosomal proteins.


⊕ 1st Dimension ⊖
⊕ 2nd Dimension ⊖
A
B

their size in the second dimension. The few acidic ribosomal proteins do not enter the gel in the first dimension. Electrophoresis of the liver ribosomal proteins resulted in gel patterns similar to published patterns of rat liver ribosomes (Leader, 1975; Welfle et al., 1978). Twenty four spots were visible on the gel of 40S ribosomal proteins and thirty spots could be seen on the gel of the 60S ribosomal proteins. There appeared to be some proteins of high molecular weight on both gels which did not migrate very far in the first dimension. These could be due either to contamination of both ribosomal subunits with cytoplasmic proteins, or to aggregated ribosomal proteins. Therefore, the gel patterns obtained on two-dimensional electrophoresis of the liver ribosomal proteins were satisfactory, showing that the liver preparation could be used as a source of carrier protein for the extraction of uterine ribosomal proteins.

Ribosomal subunits were prepared from immature rat uteri that had been incubated with $[^{3}H]$ leucine in vitro. Liver ribosomal subunits were added to the corresponding uterine subunit fractions such that 300 μg liver ribosomal protein was added to each uterine subunit fraction. Ribosomal proteins were extracted and subjected to two-dimensional electrophoresis. Figure 26 shows the gels obtained when uterine ribosomal subunits were prepared at various times following hormone treatment.

Unfortunately, the gel patterns were not as good as those obtained on electrophoresis of liver ribosomal proteins alone, although exactly the same amounts of liver ribosomal subunits were taken for protein extraction. Only faint spots were observed on some of the gels of uterine ribosomal proteins, extracted in the presence of carrier liver ribosomal proteins, and several spots were absent altogether. However, the gel patterns that were obtained all showed similarities and also the spots appeared to correspond to spots obtained on electrophoresis of liver ribosomal proteins alone. Because the Coomassie brilliant blue staining is due to the carrier liver ribosomal proteins, it is not possible to say whether hormone treatment

Figure 26

Electrophoresis of uterine ribosomal proteins on two-dimensional polyacrylamide gels

Groups of twelve immature rats were given 1 μg oestradiol-17β at various times before death. Control animals received saline in place of hormone. The uteri from each group of animals were incubated in Eagle's Medium containing 50 μCi/ml L-[4,5-^{3}H] leucine as described in Materials and Methods. Ribosomal subunits were prepared from each group of uteri, liver ribosomal subunits were added to act as a carrier and the proteins were extracted. Two-dimensional gel electrophoresis was carried out by Mr I.M. Kennedy as described in the legend to Figure 25.
A, Control 40S subunits; B, Control 60S subunits; C, 2hr oestradiol 40S subunits; D, 2hr oestradiol 60S subunits; E, 4hr oestradiol 40S subunits; F, 4hr oestradiol 60S subunits; G,12hr oestradiol 40S subunits; H, 12hr oestradiol 60S subunits.


⊕ 1st Dimension ⊖
⊖ 2nd Dimension ⊕
A
B
C
D
E
F
G
H

results in the appearance of any new protein spots.

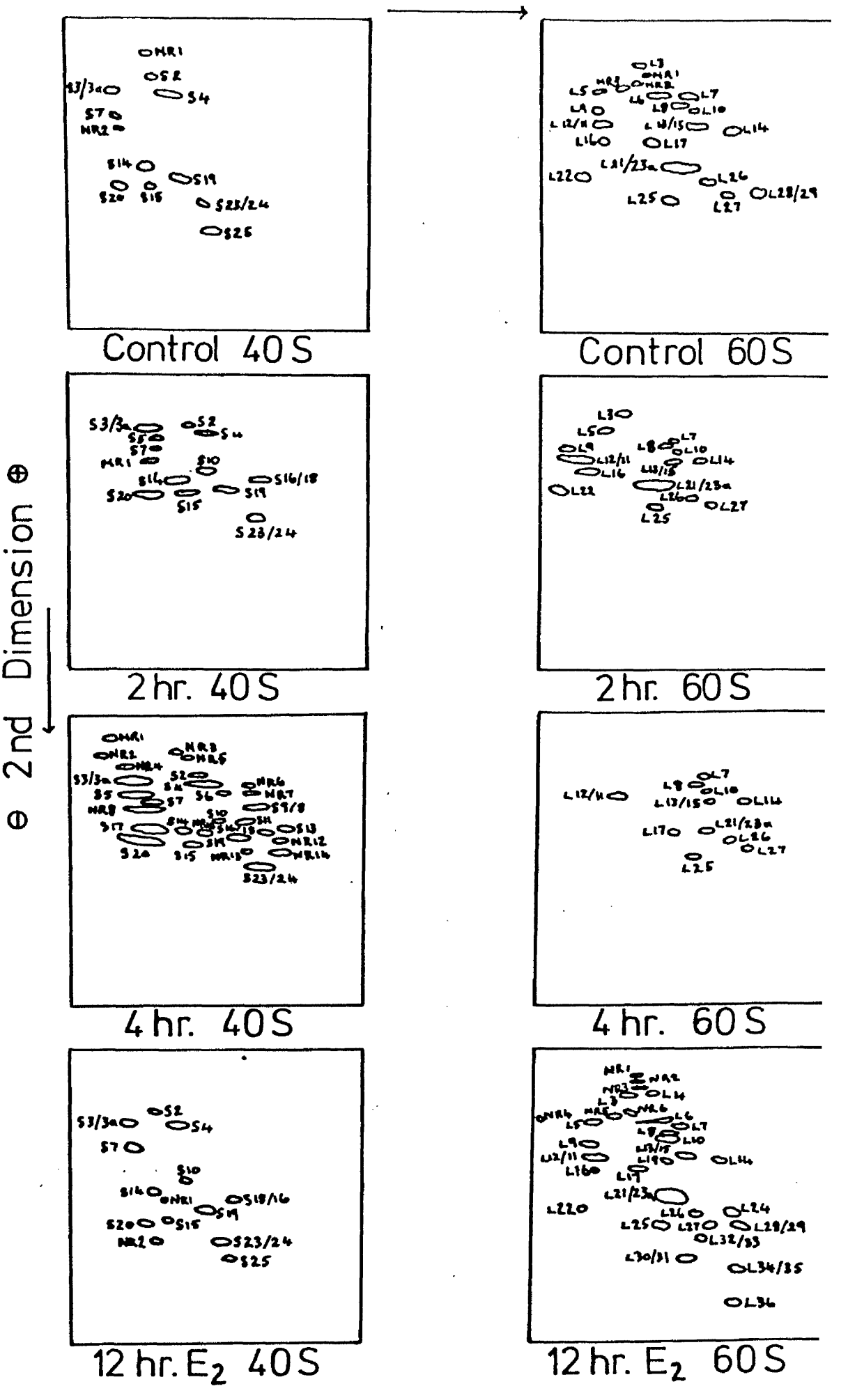
Schematic representations of the gels are shown in figure 27. The protein spots on the gels were tentatively identified and assigned numbers according to a new nomenclature system proposed by McConkey et al.(1979). In this system, the proteins of the small ribosomal subunit are assigned the prefix S followed by a number whilst the proteins of the large ribosomal subunit are given the prefix L followed by a number. Identification of some of the proteins proved difficult, particularly on those gels in which many of the proteins were missing, so that there were few points of reference. However, it was ensured that proteins which migrated to the same position in different gels were assigned the same number so that direct comparisons could be made. A few extra spots were present which could not be identified. These were given the prefix NR followed by a number, implying that they are not ribosomal proteins, although it is possible that they are aggregated forms of ribosomal proteins. In fact, some of the extra spots present on the gels of 40S ribosomal proteins appear to correspond to proteins of the 60S ribosomal subunit. This would suggest that there was some contamination of the liver 40S ribosomal subunits with 60S subunits. Some of the extra spots did not appear to correspond between different gels, however, and no comparison was made between them.

Individual spots were cut out and the amount of radioactivity was measured. Although the visible staining is due to the liver ribosomal proteins, any radioactivity present will be due to the uterine ribosomal proteins. This assumes that there is co-migration of the ribosomal proteins from the two different organs. For the majority of the proteins, this assumption is justified since mammalian ribosomal proteins from a number of sources give identical patterns on two-dimensional gel electrophoresis (Auger-Buendia and Longuet, 1978; Lastick and McConkey, 1976; Schiffmann and Horak, 1978; Cazillis and Houssais, 1979; McConkey et al., 1979). Minor differences have been observed, however (Lastick and McConkey, 1976; Schiffmann and

Figure 27

Schematic representations of two-dimensional polyacrylamide gels of ribosomal proteins

For experimental details, see legend to Figure 26.


Control 40 S
Control 60 S
2 hr. 40 S
2 hr. 60 S
4 hr. 40 S
4 hr. 60 S
12 hr. E2 40 S
12 hr. E2 60 S
⊖ 2nd Dimension ⊕

Horak, 1978; McConkey et al., 1979). In particular, any hormone-induced modifications of the uterine proteins resulting in an alteration in the net charge of a protein, such as phosphorylation, will result in an alteration of the position to which the protein migrates. The amount of radioactivity in each protein spot following treatment with oestrogen is shown in figure 28. The incorporation of radioactivity into all of the spots that could not be identified as true ribosomal proteins was very low and the results have not been included in figure 28.

It can be seen that the amount of radioactivity incorporated into the individual protein spots is less than that incorporated into the protein bands on the one-dimensional gels. This is probably because the ribosomal subunits were layered directly on the one-dimensional gels, whereas further purification of the ribosomal proteins, which could have resulted in losses, was necessary for two-dimensional gel electrophoresis. In addition, some of the bands on the one-dimensional gels would have consisted of more than one protein. On looking at the effect of hormone treatment on the incorporation of radioactivity into the individual protein spots on the two-dimensional gels, it would appear that the incorporation of newly-synthesised ribosomal proteins into ribosomal subunits from control animals is very low. In fact, the radioactivity incorporated is hardly above background. Treatment with oestrogen results in a stimulation of incorporation. However, differences in the amount of radioactivity incorporated between different gels may be caused by differences in the efficiency of extraction and subsequent recovery of the ribosomal proteins from the gels. The gels of the 40S proteins from animals given hormone 4hr before death and of the 60S proteins from animals treated 12hr before death gave the most clearly-defined patterns. This would suggest that the recovery of liver carrier protein was best in these two gels and, by implication, the recovery of radioactive label from the uterine ribosomal proteins would have been best. This, in fact, was observed. The gel of

Small Subunit

Protein	c.p.m. incorporated			
	Control	2hr Oestradiol	4hr Oestradiol	12hr Oestradiol
S2	0	28	31	1
S3/3a	4	16	38	0
S4	4	4	28	7
S5	-	0	28	-
S6	-	-	24	-
S7	6	17	27	0
S9/8	-	-	18	-
S10	-	11	16	0
S11	-	-	0	-
S13	-	-	17	-
S14	7	29	49	32
S15	1	0	29	11
S16/18	-	24	44	8
S17	-	-	27	-
S19	3	29	72	20
S20	0	0	28	16
S23/24	0	9	63	4
S25	0	-	-	13

Large Subunit

Protein	c.p.m. incorporated			
	Control	2hr Oestradiol	4hr Oestradiol	12hr Oestradiol
L3	0	24	-	28
L4	-	-	-	23
L5	0	26	-	26
L6	1	-	-	133
L7	7	0	14	60
L8	0	34	22	122
L9	0	31	-	10
L10	7	12	22	29
L12/11	9	40	2	46
L13/15	5	21	24	32
L14	0	25	26	31
L16	0	0	-	0
L17	0	-	22	25
L19	-	-	-	16
L21/23a	0	0	19	151
L22	7	36	-	29
L24	-	-	-	7
L25	0	3	11	14
L26	4	13	15	0
L27	9	20	16	0
L28/29	7	-	-	40
L30/31	-	-	-	11
L32/33	-	-	-	0
L34/35	-	-	-	4
L36	-	-	-	12

Figure 28

Effect of oestradiol-17β on the incorporation of radioactivity into uterine ribosomal proteins.

The protein spots from the gels shown in Figures 26 and 27 were cut out and the radioactivity of each spot was measured as described in Materials and Methods. A dash indicates that the particular protein could not be observed on a gel.

the 60S proteins from control animals also gave fairly well-defined spots, but the radioactive incorporation was low. This would suggest that there is a stimulatory effect of the hormone on the incorporation of newly-synthesised ribosomal proteins into ribosomes.

It was observed that, 12hr after hormone treatment, the incorporation of label was high for three of the spots, L6, L8, and L21/23a. This does not appear to be a reflection of the leucine content of the proteins since, although these particular proteins have a fairly high leucine content, there are other proteins of the small and large ribosomal subunits with an equally high, or even higher, leucine content (Tsurugi et al., 1977; Collatz et al., 1977; Goerl et al., 1978) which are not labelled to such a great extent. Moreover, no label was detected in three spots, S11, L16 and L32/33 in any of the gels, even though these proteins also have a fairly high leucine content (Tsurugi et al., 1977; Collatz et al., 1977; Goerl et al., 1978). However, differences in the labelling of individual protein spots could arise from differences in the recovery of a particular protein. In addition, absence of radioactivity from a protein spot could result from loss of the uterine protein during the extraction and subsequent electrophoresis, failure of the uterine protein to co-migrate with a liver ribosomal protein, or even complete absence of the uterine ribosomal protein.

It is not known why, in this particular experiment, the addition of the same quantity of carrier liver ribosomal subunits before protein extraction should give such different results on two-dimensional electrophoresis. Because of these differences in the recoveries of the ribosomal proteins, it is not possible to compare realistically the labelling of the proteins following different periods of hormone treatment or to compare the labelling of different proteins. The conclusion that can be drawn from the results of this experiment, however, is that treatment of immature rats with oestradiol-17β results in the incorporation of radioactive label into individual proteins that co-migrate with authentic ribosomal proteins on two-dimensional

gel electrophoresis. These results therefore corroborate the findings of the previous section, namely that in vitro incubation of immature rat uteri with [^{3}H] leucine results in the ribosomal proteins becoming labelled and that treatment with oestrogen stimulates the incorporation of label into these proteins.

Therefore, the use of an in vitro incubation technique has confirmed that the oestrogen-stimulated incorporation of radioactively-labelled amino acids, administered in vivo, into acid-insoluble material of uterine ribosomes occurs because of the incorporation of newly-synthesised ribosomal proteins into ribosomes.

DISCUSSION

4 DISCUSSION

The purpose of this discussion is to summarise the conclusions reached at the end of each experimental section and to set objectives for further research.

4.1 Incorporation of newly-synthesised RNA and protein into ribosomes in the immature rat uterus

Any experiments designed to study the effect of oestrogen on the synthesis of the various components of ribosomes will be dependent on the preparation of the ribosomes. The method used to prepare ribosomes in the experimental work described in this thesis would appear to be satisfactory by a number of criteria. The isolated ribosomes contain intact rRNA; they are active in a cell-free translation system; they can be dissociated into dissimilar subunits. Furthermore, electrophoresis of the proteins on one-dimensional polyacrylamide gels in the presence of SDS resulted in bands very similar to those obtained on electrophoresis of authentic ribosomal proteins from BHK cells. A more rigorous demonstration of the presence of ribosomal proteins in the uterine preparations would have been electrophoresis on two-dimensional polyacrylamide gels. This would have served to distinguish ribosomal proteins from other cytoplasmic proteins, particularly the proteins of messenger ribonucleoprotein particles which have different electrophoretic properties (Bag and Sells, 1979). Unfortunately, the two-dimensional electrophoretograms obtained were not very satisfactory. Nevertheless, there was co-migration of some of the proteins present in the uterine preparations with ribosomal proteins from rat liver.

The hypothesis of Key Intermediary Proteins, which must be synthesised before the oestrogen-stimulated synthesis of rRNA in the immature rat uterus can occur, was discussed in Section 1.2.2.6. This hypothesis poses a number of questions. Apart from the problem of the identity of these particular proteins, there is the question of whether a stimulation of

protein synthesis can be detected at early times following treatment with oestradiol. The analysis of polysome profiles of immature rat uteri responding to oestradiol-17β shows that an increase in protein synthesis, as judged by an increase in the proportion of polysomes to monosomes, can be demonstrated 30min - 1hr after giving the hormone. This result is in agreement with the results of Whelley and Barker (1974) who detected an increased rate of incorporation of $[^{14}C]$ leucine by uterine ribosomes incubated in vitro 1hr after giving oestradiol to ovariectomised rats. The timing of the increase in the proportion of polysomes to monosomes in the immature rat uterus also agrees with the prediction of the Key Intermediary Proteins model. The identity of the proteins synthesised by the polysomes is not known, however, although it is possible that at least one of the proteins is the specific, oestrogen-induced protein IP.

The fact that a hormone-induced stimulation of protein synthesis can be demonstrated at such an early stage by the analysis of polysome profiles, but not by measuring the incorporation of radioactively-labelled amino acids into acid-insoluble material, is a reflection of the greater sensitivity of the former technique. A stimulation of protein synthesis is only detectable by radioactive incorporation measurements at the beginning of the general increase in protein synthesis in response to oestrogen. By this time, the effect of the hormone on the polysome profiles is even more marked.

According to the Key Intermediary Proteins model, this stimulation of protein synthesis after 30 min - 1hr should result from a stimulation of the synthesis of the mRNA molecules coding for these proteins. When the polysomal RNA was extracted and fractionated by affinity chromatography on oligo (dT) cellulose, a bound fraction, presumed to represent polysomal, polyadenylated mRNA, was obtained. A stimulation of the incorporation of a radioactively-labelled precursor into this RNA fraction was detectable 2hr after hormone treatment. However, at earlier times following hormone treatment, the incorporation of radioactivity into this fraction was very

low and it is not possible to state, with certainty, whether the aggregation of ribosomes into polysomes within the first hour involves newly-synthesised mRNA. It is especially important that absolute reliability can be placed on any data obtained at these early times in view of the controversy surrounding the identification of specific, hormone-induced mRNA species in the uterus by radioactive incorporation experiments. This controversy has been discussed by Frolik and Gorski (1977) and is discussed also in Section 1.2.2.6. Nevertheless, there is a great deal of circumstantial evidence to suggest that oestrogen does induce the synthesis of specific mRNA species in the uterus. This has come from the results of experiments using inhibitors of RNA and protein synthesis, studies on the activities of RNA polymerases and studies on HnRNA synthesis. Furthermore, oestrogen-induced synthesis of specific mRNA species has been demonstrated in other target tissues, such as avian oviduct and liver. In the uterus, it is tempting to speculate that HnRNA, rapidly synthesised in response to oestrogen, contains mRNA species which pass to the cytoplasm and lead to the aggregation of ribosomes into polysomes (Aziz and Knowler, 1978a).

A more sensitive technique, possibly molecular hybridisation, is necessary to confirm this hypothesis. Encouraging results have already been obtained by this technique using polyadenylated, polysomal RNA from immature rats responding to 4hr hormone treatment (Aziz et al., 1979). However, there are technical difficulties in obtaining polysomal, polyadenylated RNA from the uteri of unstimulated animals or animals responding to 1hr hormone treatment because of the very small proportion of polysomes in the tissue at these times.

The incorporation of radioactivity into the bound fraction of polysomal RNA was maximal 12hr after hormone treatment. This concurs with the time at which the general stimulation of uterine protein synthesis in response to oestrogen is occurring. It is likely, therefore, that maximal labelling

of the bound RNA fraction 12hr after hormone treatment results from the synthesis of the mRNA sequences necessary for the stimulation of total protein synthesis at this time.

When the incorporation of a radioactively-labelled precursor into the unbound fraction of the polysomal RNA was studied, a stimulation of incorporation was observed 2hr after treatment with oestrogen. This represents the incorporation mainly of newly-synthesised rRNA into uterine polysomes, although some tRNA was also shown to be present in this fraction. This level of incorporation was maintained until 8hr after hormone treatment, when there was a further stimulation such that by 12hr after oestrogen treatment, precursor incorporation was stimulated maximally at approximately ten-fold over the control levels.

Studies were also carried out on the incorporation of newly-synthesised ribosomal proteins into uterine ribosomes in response to oestradiol. These experiments showed that there was a stimulation of incorporation of newly-synthesised proteins into ribosomes similar to the hormone-stimulated incorporation of newly-synthesised rRNA into ribosomes. Thus, maximum incorporation occurred 12hr after hormone treatment at six times the control level, although, unlike the oestrogen-stimulated rRNA incorporation, the stimulation was approaching its maximum level after 8hr. Furthermore, the stimulation of incorporation of newly-synthesised proteins into ribosomes showed the same kinetics as the stimulation of total protein synthesis in the uterus. This stimulation of incorporation could be reproduced if isolated uteri were incubated in vitro with the radioactively-labelled precursor. Electrophoresis of the proteins associated with the ribosomes on one-dimensional gels showed that protein bands, which co-migrated with authentic ribosomal proteins from BHK cells, incorporated radioactivity under these conditions. Although electrophoresis of the proteins from uterine ribosomes on two-dimensional gels was less successful, some proteins which co-migrated with authentic liver ribosomal proteins were

shown to be radioactively-labelled. Current definitions of eukaryotic ribosomal proteins are based on the sedimentation of the dissociated ribosomal subunits through sucrose gradients in the presence of high salt concentrations, and on the electrophoretic properties of the proteins on two-dimensional polyacrylamide gel electrophoresis. By the first criterion, the radioactively-labelled bands on the one-dimensional gels are ribosomal proteins. However, the fact that some high molecular weight contaminants were observed at the top of the gels would indicate that this identification must remain tentative until more complete information on the electrophoretic properties of the proteins on two-dimensional gels is available. Nevertheless, the fact that some uterine proteins which had incorporated label co-migrated with liver ribosomal proteins suggests that newly-synthesised ribosomal proteins are incorporated into uterine ribosomes and that it is this incorporation that is being measured in the experiments conducted in vivo.

On comparing the results of the incorporation of newly-synthesised mRNA into uterine polyribosomes with those of the incorporation of newly-synthesised rRNA and ribosomal proteins, it can be concluded that the aggregation of ribosomes into polysomes 2-4hr after oestrogen treatment involves the association of newly-synthesised mRNA with pre-existing ribosomes. The time of maximum stimulation of the incorporation of newly-synthesised rRNA and ribosomal protein into uterine ribosomes may not have been predicted to be as late as 12hr by the original experiments on rRNA synthesis of Knowler and Smellie (1971). In these experiments, total uterine RNA was separated on polyacrylamide gels and the incorporation of precursor into rRNA was stimulated by ten-fold over the control level 2-4hr after treatment with oestradiol. This was confirmed by the results of the experiment shown in figure 7. However, the results shown in figure 20 demonstrate that ribosomal protein synthesis is not stimulated until 8-12hr after hormone treatment, at the same time as the oestrogenic stimulation of

total uterine protein synthesis. Therefore, the synthesis of ribosomal proteins, as judged by the incorporation of newly-synthesised ribosomal proteins into ribosomes, does not precede the general increase in uterine protein synthesis in response to hormone. Furthermore, the results shown in figure 15 suggest that the incorporation of newly-synthesised rRNA into polysomes is not maximally stimulated until the incorporation of new ribosomal proteins is also maximal at 12hr after oestrogen treatment, despite the ten-fold stimulation in rRNA synthesis after 2-4hr (Knowler and Smellie, 1971). These results imply that, between 2hr and 12hr following hormone treatment, a pool of rRNA, not associated with cytoplasmic ribosomes, accumulates in the uterine cells. A small fraction of this rRNA could be incorporated into ribosomes, resulting in the slight stimulation observed after 2hr. However, it would appear that it is not until this pool of rRNA is depleted by association with newly-synthesised ribosomal proteins between 8hr and 12hr after oestrogen treatment that the incorporation of newly-synthesised rRNA into ribosomes reaches a maximum.

These conclusions would appear to be in contrast to the findings discussed in Section 1.3.2.2 that, although the synthesis of rRNA and ribosomal proteins can occur separately, the resulting products are not incorporated into ribosomes but are degraded rapidly. Many of the experiments on which these findings are based were carried out on cells cultured in vitro. It is possible that the uterus, which, as part of its normal metabolism, undergoes cyclical periods of growth and development, may show different properties. Thus, it could be that, in this tissue, components of ribosomes could accumulate independently of their assembly into mature ribosomes.

4.2 Role of protein synthesis in rRNA synthesis

The results described in this thesis lend support to the concept of a cascade phenomenon to describe the stimulation of ribosome synthesis in the uterus in response to oestrogen. Thus, the HnRNA synthesised 30min after

oestrogen treatment is proposed to contain mRNA sequences which must be translated, resulting in certain Key Intermediary Proteins required for the stimulation of rRNA synthesis. Evidence for a stimulation of protein synthesis 30 min - 1hr after oestrogen administration is presented in this thesis, although it is not possible to say whether this occurs because of the availability of newly-synthesised mRNA at this time.

Protein synthesis appears to be necessary for rRNA synthesis in all living cells. The prokaryotic organism, E. coli, undergoes a response, known as a stringent response, to the deprivation of an amino acid. This response is dependent on the rel A gene. In so-called stringent (rel^+) strains, the ribosomes synthesise guanosine tetraphosphate (pp G pp) during amino acid starvation in association with the product of the rel A gene known as stringent factor. Mutants lacking this gene, so-called relaxed (rel^-) strains, do not synthesise pp G pp. This product appears to mediate the inhibition of stable RNA synthesis, so that rRNA is not synthesised during amino acid deprivation. It also mediates the inhibition of the synthesis of the mRNA species coding for the ribosomal proteins (Dennis and Nomura, 1975). Under these conditions, the synthesis of ribosomes is said to be under stringent control. O'Farrell (1978) has suggested that the stringent response could protect cells from the effects of translational errors, because of substrate limitation, during amino acid starvation, resulting in an adaptation to a lower growth rate.

Eukaryotic cells are not known to have the equivalent of a stringent factor and they do not synthesise pp G pp. Nevertheless, yeast cells do show a stringent response to amino acid starvation which involves the parallel inhibition of the synthesis of rRNA and the mRNA coding for ribosomal proteins, but excludes the synthesis of both tRNA and mRNA for the bulk of the cells' proteins (Warner and Gorenstein, 1978). The regulatory mechanism for this response is not known.

In mammals, it has been postulated that ribosome synthesis is controlled by a mechanism linked to protein synthesis in a similar way to stringent

control in E. coli (Coupar et al., 1978). The inhibition of ribosome synthesis by protein synthesis inhibitors was discussed in Section 1.3.2.2. In addition, deprivation of an essential amino acid can inhibit ribosome synthesis (Maden, 1971). Again, the mechanism by which this inhibition occurs is not known, although hypotheses have been put forward. Stoyanova and Hadjiolov (1979) have suggested that low doses of cycloheximide, whilst not initially affecting the transcription of 45S pre-rRNA, cause drastic alterations in the processing pathways of the 45S pre-rRNA leading to an inhibition of the synthesis of 18S rRNA and a marked reduction in the synthesis of 28S rRNA. Evidence has been obtained by Lindell's laboratory to suggest that the transcription of rRNA is under the control of mRNA transcription. Thus, low doses of actinomycin D, which appear to inhibit exclusively rRNA synthesis, actually do so by inhibiting the synthesis of those mRNA species involved in the control of rRNA transcription. Experiments have shown that the same doses of actinomycin D which are effective in selectively inhibiting rRNA synthesis, also inhibit RNA polymerase II in isolated nuclei (Lindell, 1976) and when administered in vivo (Lindell et al.,1978). In the latter case, there is also an inhibition of the synthesis of rapidly-labelled nuclear proteins (Lindell et al., 1978). These observations have led Lindell to suggest that actinomycin D has an extranucleolar mechanism of action on rRNA synthesis. The antibiotic α-amanitin also inhibits RNA polymerase II activity, but its failure to inhibit rRNA synthesis may be due to its inability to penetrate the cell membrane of all cells (Lindell, 1976; Lindell et al., 1978). This hypothesis is not incompatible with that of Stoyanova and Hadjiolov (1979). It is possible that the proteins coded for by the mRNA sensitive to low doses of actinomycin D, postulated by Lindell, are necessary for the correct processing of the 45S pre-rRNA molecule. The prediction of both hypotheses is that these proteins will have a very high rate of turnover and a very small pool size, so that rapid alterations in the rate of rRNA synthesis can occur.

The similarity between the hypothesis of Lindell, describing the control of rRNA synthesis under normal conditions, and the Key Intermediary Proteins hypothesis, describing the control of rRNA synthesis under conditions of oestrogen-stimulated growth in the immature rat uterus, is striking. According to both hypotheses, the synthesis of rRNA is dependent on the synthesis and subsequent translation of mRNA species. The formation of mature ribosomes in eukaryotic organisms would therefore appear to be dependent upon a continuous supply of proteins. However, the proteins which appear to control ribosome synthesis may not be the same in both circumstances. It is possible that a new set of Key Intermediary Proteins is necessary for the stimulated synthesis of ribosomes during the oestrogen-induced hypertrophy of the immature rat uterus. The identification of these proteins is essential for a greater understanding of the effect of oestradiol-17β on the synthesis of ribosomes in the immature rat uterus.

4.3 Further experimental work suggested by these studies

There are a number of additional experiments suggested by the results described in this thesis. One possible experimental approach is suggested by the results of the analysis of polysome profiles following hormone treatment. It should be possible to isolate the polysomes and incubate them in a cell-free translation system. The polysomes should then complete the synthesis of the proteins they were synthesising in the uterus, since they will be attached still to the mRNA molecule. The proteins synthesised at various times following oestrogen administration can then be identified. The identification of the oestrogen-induced protein, IP, would be facilitated by use of the antiserum described by Iacobelli et al.(1977). Of particular interest would be those proteins synthesised 30 min-1hr after hormone treatment since they should include any possible Key Intermediary Proteins.

Another experimental approach would be to use immunological techniques to identify the synthesis of ribosomal proteins in the uterus. In the experimental work described in this thesis, the incorporation of newly-

synthesised ribosomal proteins into mature ribosomes was studied. However, the possibility that there is a considerable lag between the time of synthesis of the ribosomal proteins and the time of their incorporation into mature, cytoplasmic ribosomes cannot be ruled out. Using antibodies raised against ribosomal proteins, it may be possible to measure the synthesis of these proteins directly following hormone treatment. This approach has been used by Smith and Barker (1974) to demonstrate the oestrogen-stimulated synthesis of glucose-6-phosphate dehydrogenase in the uterus of ovariectomised rats. In the case of ribosomal proteins, it must be ensured that nascent proteins are separated from the ribosomes to avoid reactions between the antibodies against the ribosomal proteins and the mature ribosomes. Antibodies raised against total ribosomal proteins, ribosomal proteins of the 40S subunit, ribosomal proteins of the 60S subunit and individual ribosomal proteins have been described (Wool, 1979).

The experiments on fractionating the uterine ribosomal proteins by two-dimensional polyacrylamide gel electrophoresis were only partially successful, although they did show that some of the protein spots were radioactively-labelled. One of the problems was the low yield of ribosomal proteins from the immature rat uterus. A miniaturised version of the two-dimensional polyacrylamide gel electrophoresis system of Sherton and Wool (1972), requiring only 20-30µg protein and giving comparable resolution, has been described (Lin et al., 1976). Using this method, it should be possible to fractionate the proteins of uterine ribosomes, possibly without the necessity for liver ribosomal proteins to be added as a carrier.

The identification of the radioactively-labelled proteins could also be improved if sufficient radioactivity were incorporated into the uterine ribosomes to allow the use of fluorography, as described by Laskey and Mills (1975), to detect the labelled proteins. This method of detection was not used in the experiments described in this thesis because it was considered that the individual protein spots would contain insufficient radioactivity.

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