THE EFFECTS OF OESTRADIOL-17 β ON THE SYNTHESIS OF RIBOSOMAL PROTEINS IN THE IMMATURE RAT UTERUS

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Abbreviations

The abbreviations recommended by the Biochemical Journal in its Instructions to Authors (Biochem. J. (1978) 169, 5-27), have been used throughout this thesis with the following additions:

bisacrylamide	N,N'-methylene bisacrylamide.
BSA	Bovine serum albumin.
cAMP	Adenosine 3'-5' cyclic monophosphate.
cpm	Counts per minute.
DMSO	dimethyl sulphoxide.
ELISA	Enzyme linked immunosorbant assay.
hnRNA	Hetrogenous nuclear ribonucleic acid.
MDL	Messenger dependent rabbit reticulocyte lysate.
oestradiol-17β	1,3,5, (10)-estratriene-3, 17β-diol.
PBS	phosphate buffered saline.
poly A(+) mRNA	polyadenylated mRNA species.
poly A(-) mRNA	Non-polyadenylated mRNA species.
PPO	2,5-diphenyloxazole.
rDNA	DNA encoding rRNA genes.
SDS	Sodium dodecylsulphate.
TEMED	N,N,N',N'-tetramethylethylene diamine.

The system of nomenclature for ribosomal proteins proposed by McConkey et al., (1979) was used in this thesis.

SUMMARY

The effects of oestrogen on the immature rat uterus may be summarised as follows. The hormone, circulating in the bloodstream enters uterine cells and binds to a receptor. The hormone receptor complex binds to chromatin and this leads to quantitative and qualitative changes in transcription. Newly synthesised hnRNA matures to mRNA and is translated by pre-existing ribosomes in the cytoplasm. A small number of new proteins are synthesised which are required for the subsequent stimulation of RNA and protein synthesis. These proteins are required for the transcription of new rRNA and mRNA encoding ribosomal proteins. New polysomes are then assembled and are responsible for protein synthesis in the developing uterus. Changes in RNA and protein bring about tissue hypertrophy which is followed by DNA replication and cell division. The resulting growth and development of the tissue initiates its preparation for the possible implantation of a fertilised ovum.

Studies of protein synthesis in the uterus responding to oestrogen have revealed that major changes in the gross protein population were not to be found. The changes oestrogens induced were altogether too subtle to be revealed by the crude analysis of total protein. Therefore, one group of oestrogen-induced proteins was selected for detailed study, namely the ribosomal proteins. Previous work had shown that the synthesis of these proteins coincided with the appearance of new polysomes *in vivo*. However, the small quantity of ribosomal proteins in the uterus relative to the whole body of the rat severely limited these studies, and thus it was decided to pursue further studies by the use of two differenct procedures *in vitro*.

The first of these procedures required the incubation, with radio-labelled precursors, of whole uteri *in vitro* after oestrogen

stimulation *in vivo*. The uteri, removed from immature rats after various periods of oestrogen treatment, were incubated with either [^{3}H]-leucine, [^{35}S]-methionine or [^{32}P]-phosphate. In this way, the synthesis of total ribosomal proteins, and the synthesis and post-translational modification of individual proteins could be analysed. It was found that oestrogen stimulated ribosomal protein synthesis *in vitro* in the same way as *in vivo* and that the maximal incorporation of new ribosomal proteins into ribosomes coincided with maximal polysome assembly. Incorporation of radioactivity into ribosomal proteins of the unstimulated rat uterus was insignificant, except in the case of the large-subunit protein L10. Oestradiol treatment of rats at various times before death was followed by stimulated incorporation of radio-labelled precursor into individual ribosomal proteins *in vitro*. This stimulation was maximal 12 hours after hormone administration and could not be accounted for by increased pool sizes. It was possible to quantify the effect of oestradiol on 17 small subunit proteins and 16 large subunit proteins. Response to the hormone was not uniform and varied from 3-fold stimulation of synthesis of L10 to an almost 5-fold increase in the synthesis of protein L19. Incubation of uteri with [^{32}P]-inorganic phosphate resulted in the labelling of 2 large subunit proteins and 1 protein of the small subunit. However, an apparent increase in the incorporation of label after oestrogen stimulation could be accounted for by changes in the size of precursor pools.

A second analytical approach involved the isolation of active uterine polysomes and their subsequent translation in a cell free protein synthesisiing system. The synthesis of total proteins increased from control levels until 8-12 hours after oestrogen

treatment and then declined to control levels once more. The proportion of ribosomal proteins synthesised in this system became nearly maximal at 2 hours and their synthesis remained a constant proportion of the total activity of uterine polysomes until polysome disassembly after 12 hours.

An anti-ribosomal protein antibody was raised in rabbits and used to determine the antigenicity of uterine homogenate after oestrogen treatment of rats. The results produced by this analysis were unexpected in that they revealed an apparent decrease in antigenicity after 12 hours. This conflicted with the current idea of ribosome turnover requiring many days, but could be rationalised in terms of a pool of free ribosomal proteins becoming less antigenic as they were incorporated into ribonucleoprotein particles.

These results were compared to other relevant work and discussed with a view to future studies.

INTRODUCTION

1.1. The Role of the Ribosome in Cell Metabolism

In all living cells, the major site of protein synthesis is the ribosome. As such the ribosome plays a crucial role in the changes in the rate of cell growth and development.

This introduction will begin with a short discussion of prokaryotic ribosomes. This is necessary because it is at this level that ribosome structure, assembly and function are best understood, but this section will be short in view of the limited relevance to this project. A description of the eukaryotic ribosome will follow and the synthesis and assembly of these ribosomes will be discussed in detail. Since a large number of components are required for protein synthesis, a further section will be devoted to what is known of the co-ordination of ribosome assembly and of protein synthesis in hormone responsive tissues.

As this project is on the effects of oestrogen on ribosome synthesis, a major part of the introduction will review specific and relevant examples of oestrogen induced protein synthesis. These examples include: 1) The model of Xenopus leavis liver where oestrogen induces the synthesis of one single protein in the absence of tissue differentiation. 2) The chick oviduct where oestrogen induces the synthesis of egg white proteins after tissue development. 3) The immature rat uterus, that does not synthesise major new proteins in response to oestrogen but undergoes an overall growth in preparation for implantation of a fertilised egg.

1.2. The Control of Ribosome Synthesis in E. coli

There are major differences between the ribosomes of prokaryotic and eukaryotic cells. In spite of this, a discussion of prokaryotic ribosome synthesis is valuable in light of the advances recently made in this field. The prokaryotic ribosome is

a ribonuclear-protein structure of sedimentation value 70S. Three rRNA molecules, of 23S, 16S and 5S, together with 55 proteins, comprising 21 small subunit and 34 large subunit proteins, make up this organelle. With the exception of proteins L7/L12 all the molecules are present as one copy per ribosome. Any model of r-gene regulation must account for this unity of expression since there is an absence of rRNA, and rProtein, pools in E. coli (Brimacombe et al., 1978).

Nomura's group of co-workers have made major advances in the study of ribosomal protein synthesis in E. coli. They have demonstrated that a few ribosomal proteins can control the translation of their corresponding mRNA (Fallon et al., 1979). Since these mRNA are usually polycistronic, a single ribosomal protein may regulate the synthesis of a group of others. For example, protein L4 regulates translation of an eleven gene operon (Zengel et al., 1981), protein L1 controls synthesis of both itself and L11 (Yates and Nomura, 1981). From experiments based on analysis in vivo and in vitro, Nomura and co-workers have suggested that, if no rRNA is available, these regulatory proteins bind to a leader sequence on the mRNA encoding the controlled proteins. This binding prevents further translation of that messenger. Structural studies have demonstrated extensive primary and secondary structural homology between the mRNA leader sequence and the protein binding site on the rRNA (Nomura et al., 1981; Olins and Nomura, 1981).

The model described above does not account for regulation of rRNA genes. What evidence there is suggests that rRNA is synthesised constitutively and, except under conditions of rapid growth, in excess. This excess is then degraded (Gausing, 1977). The detailed mechanism of this control is still unknown.

1.3. The Structure of the Eukaryotic Ribosome

There are many excellent reviews on this subject and the work of Bielka and Stahl (1978) and of Cox and Godwin (1975) are worthy of particular attention.

In a buffer of high monovalent cation concentration the ribosome separates into 2 subunits (Martin et al., 1969). Both of these subunits consist of RNA and protein. The larger subunit sediments on a sucrose density gradient at a velocity of 60S. It comprises 3 species of rRNA and about 40 proteins. The smaller subunit, which sediments at 40S, comprises 1 rRNA species and about 30 proteins (Bielka and Stahl, 1978).

Ribosomal RNA may vary in size between species but in higher vertebrates are generally 28S, 5.8S and 5S in the large subunit and 18S in the small subunit. These sizes correspond to molecular weights of 1.5×10^6, 0.09×10^6 and 0.085×10^6, and 0.65×10^6. In most plants and protozoa the molecular weights of the major rRNA species are 1.3×10^6 and 0.7×10^6 (Loening, 1968). All rRNA studied are methylated. There are about 110 methylation sites in HeLa pre-rRNA all of which are found in rRNA. Methylation usually occurs at the 2'-OH position of the ribose moiety, or in a few cases, on the bases (Vaughan et al., 1967). Thus apart from adenine, guanine, cytosine and uracil, rRNA also contains 1-methyl adenine, 7-methyl adenine, 7-methyl guanine, 5-methyl cytosine and 3-methyl uracil. The significance of these modifications is not clear. Methylation of the bases confers ribonuclease resistance which may be important during synthesis and maturation (to be discussed in detail later). Modifications of the bases may also be important in ensuring the correct secondary structure of the molecule. Recently computer analysis has been

used to compare primary and secondary structures of rRNA molecules from different species. It was considered that rRNA molecules with very different primary sequences would have equally different secondary structures. However it has become evident that although primary sequence may vary greatly between rRNA molecules from different species, the differences are limited in such a way as to conserve a common secondary structure. It would appear that base changes during evolution for different rRNA molecules have been subject to functional constraints. Ursi et al., (1982) have studied 11 different 5.8S rRNA molecules and concluded that all may have the same basic secondary structure. Indeed Nazar (1980) and Jacq (1981) have found sequences homologous to eukaryotic 5.8S rRNA at the 5' end of the prokaryotic 23S rRNA. Similarly Stiegler et al., (1981) have proposed a common folding pattern for all 16S and 18S rRNA molecules irrespective of origin. Work on 28S rRNA sequences has not progressed to the same level as that of 5.8S and 18S rRNA, however data are now accumulating concerning the primary and secondary structures of rRNA from many different species. The available evidence suggests that the differences in rRNA from different sources may not be as large as at first imagined. That is, although there may be sequence differences, these are self compensating so that similar base pairing and hence secondary structures are possible (Stiegler et al., 1981; Ursi et al., 1982).

Ribosomal RNA is associated with about 80 different proteins although the exact number is difficult to establish (McConkey et al., 1979). In rat liver, 33 proteins are associated with the small subunit (numbered S2-S30) and 45 with the large subunit (numbered L3-L39). The differences in the numbers of different ribosomal proteins reported in the literature has been due

in part to the different methods of preparation used by various workers (Welfle et al., 1972; Sherton and Wool, 1972). In addition some proteins, such as initiation and elongation factors, associate with the ribosome at different stages in protein synthesis (Weissbach and Ochoa, 1976). Finally there are a few species-specific differences (Delauney et al., 1973; Ramjoue and Gordon, 1977; Kuter and Rodgers, 1974).

Ribosomal proteins are distinguished by being smaller than the average cell protein (Welfle et al., 1978; Issinger and Beier, 1978; Martini and Gould, 1975). In rat liver the molecular weights of the large subunit proteins range from 11 x 10^3 to 50 x 10^3 whilst the proteins of the small subunit range from 11 x 10^3 to 42 x 10^3. Ribosomal proteins are largely basic due to the predominence of lysine and arginine over glutamic acid and aspartic acid (Collatz et al., 1976; Tsurugi et al., 1976: Tsurugi et al., 1977; Collatz et al., 1977). There are however at least 2 proteins of the large subunit that are acidic (L44/L45 or L7). It appears that each ribosomal protein within the ribosome is unique with the possible exception of these acidic proteins. Reconstitution and immunological experiments have shown that L44/L45 are very similar if not identical to proteins L7/L12 of E. coli, (Sanchez-Madrid et al., 1981; Stoffler et al., 1974). Ribosomal proteins L7/L12 of E. coli are identical except for the N terminal which is acetylated in the case of L7. However it appears that the E. coli ribosome contains 4 of these proteins, usually as two dimers although the exact relative proportion may depend on the nutritional status of the microorganism (Terhorst et al., 1972; Lee et al., 1981).

The proteins of eukaryotic ribosomes may be modified by methylation (Scolnik et al., 1979), acetylation (Terhorst et al., 1972), or phosphorylation. A large variety of ribosomal proteins

may be subject to phosphorylation, for example, one study revealed that L6, L14, L19, S2, S3a and S6 were labelled by radioactive phosphate *in vivo* (Roberts and Morelos, 1979). However, it is protein S6 that has been most frequently demonstrated to exist in the phosphorylated form (Gressner and Wool, 1974; Gressner and Wool, 1976; Thomas *et al.*, 1980).

1.4. The Synthesis of the Eukaryotic Ribosome

1.4.1. Ribosomal RNA

Due to certain unusual features of rDNA, the study of these genes has progressed more rapidly than the study of any other Eukaryotic genes. The features which have been responsible for this progress include localisation of the genes in the nucleolus, the redundancy of the genes, a very G:C rich primary sequence and the ease with which the gene products may be isolated.

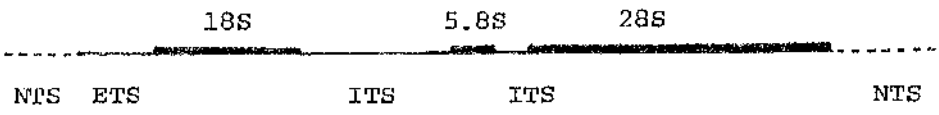
The 28S, 18S and 5.8S rRNA are synthesised solely in the nucleolus, and it is in this organelle that the genes are found (Brown and Gurdon, 1964; Ritossa and Spiegelman, 1965). Synthesis of 5S rRNA occurs in the nucleoplasm and will be discussed later. In higher organisms the genes in the nucleolus are highly redundant although the exact number varies between species (and the analysis of different workers). This number may be a little over 100 in fungi (Retel and Planta, 1972) or as many as several million in the Xenopus oocyte (Birnstiel *et al.*, 1971). Although this redundancy is unusual it is not unique, 5S rRNA genes, tRNA genes and histone genes are also highly redundant (Birnstiel *et al.*, 1972; Hatlen and Attardi, 1971; Childs *et al.*, 1981). The reasons for this redundancy are at least twofold. Unlike most genes, those encoding ribosomal RNA are transcribed to form the final product, there is no amplification of information through translation. Thus one

ovalbumin mRNA may be translated to form 40,000 to 50,000 protein molecules (Harris et al., 1975) whilst the rRNA primary transcript has a 1:1 relationship with the final product. In addition, ribosome synthesis and protein synthesis frequently increase as the result of a single stimulus (see later sections) and the cell requires the ability to synthesise large amounts of rRNA very rapidly, more rapidly than a single gene could satisfy.

The rDNA genes do not normally contain introns. That is, they do not include non-coding sequences which must be removed from within the coding sequence. Exceptions may be found in some ribosomal genes of Drosophila melanogaster (Dawid et al., 1981) or Tetrahymena thermophila (Kan and Gall, 1982), however although the genes of T. thermophila appear to be active (Din et al., 1979) those of D. melanogaster are not (Grabowski et al., 1981).

Ribosomal genes occur, as illustrated below, in transcriptional units which include one gene of each of the 18S, 5.8S and 28S rRNA (see Figure 1). These are separated by spacers which are transcribed and must be removed during the processing of pre-rRNA (a detailed discussion of this aspect follows later). Each transcriptional unit is separated by non-transcribed spacers. It is these spacers which give rise to much of the heterogeniety in mRNA between genus (Salim and Maden, 1980; Hall and Maden, 1980), species (Brown et al., 1982; Forsheit et al., 1974) or even individuals.

Figure 1 The rRNA Gene Cluster of Xenopus leavis taken from Long and Dawid (1980).



ITS Internal transcribed spacer

ETS External transcribed spacer

NTS Non-transcribed spacer.

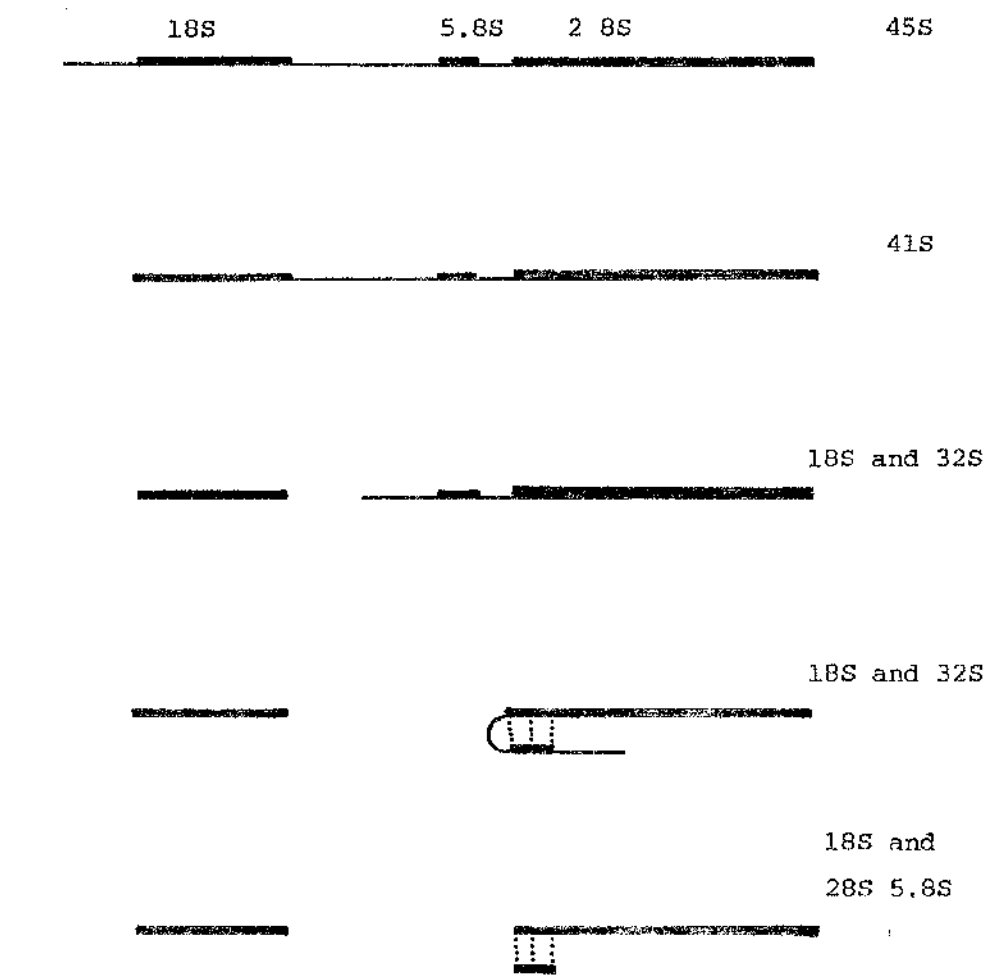
The primary sequence of rDNA is very rich in G, C residues. Indeed the non-transcribed spacers may show long tracts of these residues (Moss et al., 1980). The evolutionary significance of these residues is not clear, they may be important during recombination or gene multiplications.

The control of transcription within the nucleolus is poorly understood. Clearly because of the nucleolar location of rDNA, and the sole involvement of type A (I) RNA polymerase (Roeder and Ruttler, 1969; Roeder and Ruttler, 1970; Chambon, 1975), an increased level of this enzyme could result in preferential transcription of rDNA. What little evidence there is does not support this simple mechanism (Thompson et al., 1981; Courvalin et al., 1976). Deoxyribonuclease I has been used to study rDNA gene activity under different conditions of rDNA methylation of nucleolar chromatin histone acetylation. Differences in gene sensitivity to this enzyme were not detected (Macleod and Bird, 1982; Vavra et al., 1982). However, DNAase I may not be an accurate guide to gene

transcription (Stadler et al., 1978). It seems likely that studies of regions flanking the rRNA genes may prove more instructive. Bach et al., (1981) has sequenced the non-transcribed spacer region of rDNA from Xenopus laevis, X. clivii and X. borealis and have found 13 identical bases at this site. Similary, Salim and Maden (1980) have found 5 conserved bases at the spacer gene junction of X. leavis and Saccharomyces cerevisiae. If conservation of sequence implies functional significance then these invariant regions, within tracts of very variable sequence, may be important in the initiation of transcription.

Each rRNA cistron is transcribed as a complete unit. The gene product is an RNA molecule of 45S in higher animals (Scherrer and Darnell, 1962; Perry, 1962; Scherrer et al., 1963). Plants and lower animals appear to produce a smaller precursor (Loening et al., 1969). The precursor is cleaved at specific sites to produce, via a number of intermediates, the 18S, 28S and 5.8S rRNA (Scherrer et al., 1963; Penman, 1966; Greenberg and Penman, 1966; Weinberg et al., 1967). The intermediate steps in the maturation pathway have been summarised by Perry (1976), and are shown in figure 2.

Figure 2 The Intermediate Steps in the Maturation of rRNA of Yeast (Perry, 1976).



There are a number of possible mechanisms by which these steps may be regulated. Recently Cech's group have suggested that RNA may possess enzymic activity and have shown that the removal of introns from Tetrahymena rRNA is an autocatalytic process (Kruger et al., 1982). It remains to be shown whether this is an important and universal mechanism or whether the example they have studied is an unusual biochemical curiosity. A more simple and direct mechanism of processing would involve the action of a separate and specific ribonuclease catalysing each step. Perry has isolated a nucleolar ribonuclease that has a requirement for C residues (Winicov and Perry, 1974). In view of the large C content of rRNA and especially spacer RNA, these properties indicate that this enzyme may be involved in rRNA processing. Recently Denoya et al., (1981) have isolated 2 nucleolar ribonuleases that degraded 45S rRNA to 18S and 28S RNA. One of these enzymes possessed an RNA component that conferred specificity. As yet however both enzymes and their products remain poorly characterised. There are extensive areas of conserved primary sequence throughout the transcribed areas of rDNA but whether these play a role in rRNA maturation is unknown. Areas of conserved primary sequence would result in conserved secondary structure, however there is no direct evidence that the ribonucleases responsible for rRNA processing recognise either specific sequences or secondary structure.

Ribosomal RNA is extensively methylated and this methylation may play an important part in determining secondary structure. The 45S RNA of HeLa cells is methylated at approximately 110 sites and this is achieved almost exclusively as the precursor is being transcribed (Greenberg and Penman, 1966). The majority of these sites are at the 2' carbon of the ribose unit

although some base methylation occurs. Vaughan et al., (1967) presented evidence that methylation is necessary for the synthesis of stable rRNA. This group found that HeLa cells starved of methionine synthesised 45S RNA and 32S RNA but not 28S or 18S rRNA. The absence of complete ribosomal subunits was not due to a lack of ribosomal proteins since these were synthesised from amino acids from protein degradation (Maden et al., 1969). These workers suggested that methylation protected rRNA from random cleavage by ribonuclease although the group did not effectively explain the correct processing of 45S RNA to 32S RNA. Work by Maden and Salim (1974) has shown that all methylation of pre-rRNA is conserved during processing and that the transcribed spacer regions are entirely unmethylated. More recently, Maden (1980) and Salim and Maden (1981) have located and mapped all the methylated nucleotides within the complete sequence of X. laevis. The implication of this data is that methylation appears to protect rRNA from ribonuclease action in vivo but it is not clear how sites are selected for methylation. Selected primary sequences may be recognised by the enzymes responsible for methylation but how this is achieved at so many different sites is not known.

Nucleolar pre-ribosomal RNA is associated with many proteins. Although the majority of these proteins are apparently identical to those of cytoplasmic ribosomes, a number of non-ribosomal proteins are also present. These do not enter the cytoplasm and whether they play any role in rRNA processing is unknown. Detailed discussion of ribosomal proteins follows in the next section.

1.4.2. 5S RNA

Eukaryotic cells possess a fourth rRNA species, the 5S rRNA species. In recent years the synthesis of 5S rRNA has been extensively studied in Xenopus oocytes but very little information exists for any other system. Surprisingly, 5S rRNA synthesis appears to be totally unconnected with the synthesis of 45S RNA in these oocytes. The 5S rRNA genes which are highly reiterated are in the nucleoplasm (Millar, 1973) and are transcribed by polymerase C (III) before transcription of the 45S RNA (Ford, 1971). The latter are synthesised later in the nucleolus by polymerase A (I) from amplified genes. The 5S rRNA gene has a control region within the gene sequence. A factor, designated TF III A, binds specifically at this region and stimulates transcription. However TF III A also binds to 5S RNA itself forming a 7S RNP. Thus 5S rRNA transcription may be suppressed when TF III A is sequestered from the nucleus to the cytoplasm along with 5S RNA (Engelke et al., 1980; Honda and Roeder, 1980; Gottesfeld and Bloomer, 1982).

It must be emphasised that the 5S rRNA: TF III A model has, as yet, only been found in Xenopus oocytes. It may not exist in any other system. Indeed, whilst the general picture of rRNA synthesis is uniform throughout Eukaryotic species, each model studied may reveal subtle differences in control mechanisms, precursor sizes or maturation pathways (Long and Dawid, 1980b; Dudov et al., 1978).

1.4. The Synthesis of the Eukaryotic Ribosome

1.4.3. Ribosomal Proteins and the Assembly of Ribosomes

The use of recombinant DNA technology has led to the identification and isolation of a number of genes for ribosomal proteins. So far, relatively few genes have been cloned and they

are, as yet, uncharacterised with respect to the flanking regions which might contain sequences associated with the control of their expression. The construction of ribosomal protein gene clones has been reported for yeast, Drosophila, mouse and amphibian genes. Woolford et al., (1979) studied 4 yeast genes and Warner's group (Fried et al., 1981) have cloned genes representative of 15 of the 5S ribosomal proteins in yeast. Only one ribosomal protein gene from Drosophila has been cloned (Vaslet et al., 1980). Perry's group (Meyuhas and Perry, 1980) have cloned 8 mouse genes and Amaldi's group have isolated 6 Xenopus genes (Bozzoni et al., 1981). All clones were identified by co-fractionation of cell free translation products with ribosomal proteins on urea gels (Lastick and McConkey, 1976). Thus if a translation product had the same ionic charge and molecular weight as a ribosomal protein it would have been identified as that protein. This assumption may not be valid since cellular chromatin may encode a number of small basic proteins, and only small polyA(+) mRNA were used to probe total genomic libraries. It is possible that histones, hnRNP proteins or, most significantly, mitochondrial ribosomal proteins were also cloned. Only Amaldi's group (Bozzoni et al., 1981) performed protease peptide mapping of translation products (which did confirm the identity of their clones) but no group used antibody identification. Current ideas concerning ribosomal protein genes must be assessed in the light of these reservations.

Ribosomal protein genes have been shown to be repetitive in all species studied. In mammalian cells (L cells or HeLa cells) there average about 10 copies of each gene per diploid cell (Monk et al., 1981). There are 7-8 copies of genes encoding proteins S16 and L18 in mouse, whilst L10 was present in about 19-20 copies per

diploid cell. By extrapolation of this data, one would predict that mouse cells would contain approximately 750 ribosomal protein genes. Although amphibian ribosomal protein genes were also repetitive they were present in fewer numbers. Genes for proteins L1, L14 and S19 were each present at 2 copies per haploid genome whilst there were 4-5 copies of the genes for S1, S8 and L32 (Bozzoni et al., 1981). Similarly in yeast, ribosomal protein genes were present in multiple copies but the use of a novel protein numbering system prevented identification of the proteins (Fried et al., 1981; Mager et al., 1977). It seems likely that ribosomal protein genes in Drosophila are also repetitive (Vaslet et al., 1980). Whether all the genes are active and what role they play is unknown.

In general ribosomal protein genes are not closely linked, either to each other or to rRNA genes (D'Eustachio et al., 1981). Fried et al., (1981) found a clone that encoded two ribosomal protein genes, but this was an exception. It appears that ribosomal protein genes are widely and randomly dispersed throughout the genome.

The primary transcripts from these genes are larger than the mRNA encoding the ribosomal proteins. The transcripts from murine genes are 3-7 times longer than the mRNA (Meyuhas and Perry, 1980). Although yeast protein-encoding genes appear to contain introns less frequently than higher eukaryotes, their ribosomal protein genes do have introns (Fried et al., 1981) and are up to 1.7 times the length of their corresponding mRNA (Mager et al., 1977).

The transcripts are processed into small, polyadenylated, monocistronic mRNA (Woolford et al., 1979; Woolford et al., 1980; Bollan et al., 1980, 1972; Nabeshima et al., 1979). In mouse cells

these fall into the moderately abundant class with 300-900 copies of each per cell (Meyuhas and Perry, 1980). Recently it has become possible to measure the relative proportions of ribosomal protein mRNA within a population of polyA(+) mRNA. Changes in the percentage of mRNA encoding ribosomal protein synthesis has been studied in regenerating rat liver, during embryogenesis in Xenopus, and after serum stimulation of 3T3 cells. Liver regeneration (Feliks and Meyuhas, 1982) and embryogenesis (Amaldi et al., 1982) resulted in increased accumulation of ribosomal protein mRNA above the increase in total mRNA. In 3T3 cells, stimulated to grow with fresh serum, there was no increase in ribosomal protein mRNA, and there was a percentage decrease with respect to the total mRNA population (Geyer et al., 1982). The half-lives of these mRNA species were no different to that of other cellular mRNA. The half-life of yeast ribosomal protein mRNA was found to be 15-17 minutes (Warner and Gorenstein, 1977; Mager and Planta, 1976) and that of mouse L cells was 2.5 to 3 hours.

The mRNA of ribosomal proteins are translated on cytoplasmic polysomes (Craig et al., 1971) and in yeast and rat liver, these polysomes are probably free or loosely bound to the cytoplasmic membrane (Mager and Planta, 1975; Nabeshima et al., 1975). Due to the small size of ribosomal protein mRNA, these polysomes are smaller than the average cellular polysome (Mager and Planta, 1972).

The assembly of new ribosomes occurs predominantly in the nucleolus (Liau and Perry, 1969). It must be assumed that after synthesis in the cytoplasm, ribosomal proteins migrate into the nucleolus. How this process is achieved is unknown. A number of authors (Tsurugi et al., 1973; Kumar and Subramainian, 1975; Auger-Beundia and Longuet, 1978) have isolated pre-ribosomal

particles from the nucleolus. These particles have sedimentation values of 80S (containing 45S RNA) and 55S (containing 28S rRNA). Particles containing 18S rRNA were not isolated (Auger-Beundia and Tavitian, 1979; Liau and Perry, 1969). The proteins found associated with the 55S precursor particle are largely those of the 60S ribosomal subunit (Lastick, 1980; Auger-Beundia et al., 1979; Kruiswijk et al., 1978). Tsurugi et al., (1973) have demonstrated a direct precursor/product relationship between the proteins of pre-ribosomal particles and those of cytoplasmic ribosomes. In addition to the proteins found in both nucleolar and cytoplasmic particles, there are 4 proteins found only in the nucleolar precursor (Tsurugi et al., 1973) and 3 other proteins found only in the cytoplasmic subunits (Auger-Beundia and Tavitian, 1979). Clearly, the 4 nucleolar proteins may play an important role in rRNA processing and in subunit assembly but as yet there is no direct evidence for this.

It is probably subunits and not whole ribosomes that exit the nucleus to become associated with polysomes. Indeed the 40S subunits leave the nucleus (Liau and Perry, 1969) and are found in polysomes before the 60S subunits (Peterson et al., 1976). The mechanism by which pre-ribosomal particles leave the nucleus is not clear, however the nuclear matrix and the cytoskeletal framework have been implicated. Since subunits enter the polysome fraction before the monosome fraction mRNA may also be involved in the translocation. Penman's group have shown that active polysomes are attached by their mRNA to the cytoskeleton. Conversely monomers are not attached (Lenk et al., 1977; Cervera et al., 1981). In addition cytoskeletal preparations show a concentration gradient of polysomes decreasing from the nucleus towards the cell periphery (Fulton et al., 1980).

A number of ribosomal proteins do not migrate into the nucleus but associate with ribosomes in the cytoplasm. Kalthoff and Richter (1982) injected labelled proteins S6 and eL12 (the acidic phosphoprotein of the large subunit) into enucleated oocytes. They found that eL12 was incorporated into subunits independently of the nucleus whilst S6 was not. Auger-Buendia and Tavitian (1979) inhibited rRNA maturation with toyocamycin and found that some ribosomal proteins became labelled in the absence of de novo ribosome synthesis. Such results indicate not only that ribosomal proteins are added to ribosomes in the cytoplasm, but that they can also exchange between ribosomes. These results also imply a cytoplasmic pool of free ribosomal proteins.

There has been much discussion in the literature concerning the existence of free ribosomal protein pools. Various authors have suggested either very large (Dice and Schimke, 1972) or insignificant pools (Philps and McConkey, 1976). Lastick and McConkey (1976) have found 5 ribosomal proteins in HeLa cells that exchange between ribosomes and a soluble fraction. These were proteins L5, L10, L19, L28 and S2. There appeared to be an especially large pool of the protein L5. Cazillis and Houssais (1979) have found that 9 small subunit proteins and 7 large subunit proteins could be shown to exchange in L cells but they did not identify the proteins concerned.

The evidence for free ribosomal protein pools is too strong to be dismissed but their full significance is not yet understood.

1.5. The Co-ordination of Ribosome Synthesis

To assemble active ribosomes, the cell must synthesise approximately 80 different proteins and 4 different rRNA species. The co-ordinated accumulation of ribosomal components is tightly

controlled in all systems studied so far. In no case were excess rRNA or ribosomal proteins found in cellular pools. Even in mouse-hamster cell hybrids there was co-ordinated synthesis of ribosomal protein and rRNA, irrespective of gene dosage or origin (Wejksnora and Warner, 1981).

This co-ordinated synthesis has been studied using three models, inhibition of synthesis by drugs, altered synthesis by mutants and during cell differentiation. When drugs were used to inhibit synthesis of one component there was no accumulation of the other. Thus inhibition of ribosomal protein synthesis with puromycin resulted in reduced rRNA accumulation (Soeiro et al., 1968). Inhibition of rRNA synthesis with actinomycin D resulted in reduced ribosomal protein accumulation (Warner, 1977; Craig et al., 1975). Drugs may alter gross cellular metabolism. However, Warner's group have isolated temperature sensitive yeast mutants in which rRNA or ribosomal protein synthesis was defective and found the situation was exactly the same. In the absence of one ribosomal component there is reduced accumulation of the other (Udem and Warner, 1972; Gorenstein and Warner, 1977).

The control of co-ordination does not appear to occur at the level of synthesis. Indeed in most cases inhibited synthesis of one component did not affect the synthesis of the other (Soeiro et al., 1968; Warner, 1977; Gorenstein and Warner, 1977). The reduced accumulation of the latter was due to increased degradation. It has been suggested that this degradation occurs in the nucleolus since ribosomal proteins migrate there in the absence of rRNA synthesis (Warner, 1979), however there is, as yet, little evidence to support this.

Kreuter et al., (1980) have studied the co-ordination of ribosome synthesis in L_6E_9 mouse myoblasts. These cells differentiate to myotubes with concomitant loss of protein synthetic activity. The loss of this activity is associated with reduced rRNA synthesis, but ribosomal protein synthesis carries on unabated. Once again co-ordination is controlled by degradation of unused proteins. This work would suggest that ribosome synthesis is limited by the level of rRNA synthesis whilst ribosomal proteins are synthesised in excess, and unused proteins are degraded. Whether this scheme is true of other models remains to be demonstrated. Indeed the reverse seems to be true of the immature rat uterus, as will be discussed later.

The assembly of ribosomes must also be co-ordinated with the accumulation of other components of protein synthesis. These other components include mRNA, initiation factors, elongation and release factors, and enzymes responsible for post-translational modifaction of newly synthesised proteins.

1.6. The Co-ordination of Protein Synthesis in Animal Tissues Responding to Hormonal Stimulation

Table 1 surveys a range of stimuli that have been shown to trigger polysome assembly. Most of this information has been based on polysome profile analysis, but where other supporting evidence has been presented this is included. Clearly major modulators of polysome assembly in animal tissues are the hormones, both steroidal and non-steroidal. The effect of oestrogen has been chosen for detailed discussion and three different target tissues have been selected which allow the effect of this hormone on ribosome synthesis to be reviewed in relation to its overall effects on the growth of its target tissues. The liver of Xenopus laevis has been

Table 1

Stimuli Associated with Polysome Assembly

Stimulus	Model	Supporting Evidence	Authors
Oestrogen	immature rat uterus	rRNA, r Protein synthesis	Merryweather and Knowler, 1980.
Oestrogen	immature chick oviduct	rRNA, r Protein synthesis	Means et al., (1971)
Oestrogen	chick liver	rRNA, rProtein synthesis	Bast et al., (1977)
Oestrogen	male Xenopus liver	rRNA, rProtein synthesis	Berridge et al., (1976)
Testosterone	castrated rat prostate	rRNA synthesis	Johnsonbaugh et al., (1976)
Prolactin	sheep mammary gland	rRNA synthesis	Gaye and Denamur (1969)
TSH	sheep thyroid cells	rRNA synthesis	Nilson et al., (1980)
FSH	immature rat testicle	cell free translation increase	Means and Hall (1969)
Growth Hormone	hypophasectomised rat liver	cell free translation increase	Clemens and Pain (1974)
Insulin	diabetic rat liver	cell free translation increase	Clemens and Pain (1974)
S. typhi antigen	mouse spleen	rRNA synthesis	Storb and Martin (1972)
Phytohaemoglutinin	human lymphocytes	rRNA synthesis	Kay et al., (1971)
Epidermal growth factor	chick embryo epidermis	no net synthesis	Cohen and Stastny (1968)
Development	amphibian embryo	rRNA synthesis	Woodland (1974)
Partial hepatectomy	rat liver	rProtein synthesis	Nabeshima et al., (1975)
Feeding	Liver of starved rat	rRNA synthesis	Finklestein et al., (1979)

selected because its study reveals how the expression of one gene may be stimulated in the absence of overall tissue growth. Conversely cell division and tissue differentiation are pre-requisite effects of the hormone on chick oviduct during primary stimulation of ovalbumin synthesis. Finally, the rat uterus responding to oestrogen does not synthesise any major secreted proteins but rather undergoes total growth, hypertrophy and hyperplasia in preparation for possible embryo implantation. In each of these tissues the response is accompanied by polysome assembly.

The initial changes brought about by oestrogen appear to be the same in all target tissues (Jensen and DeSombre, 1972; O'Malley and Means, 1974). A basic picture has emerged but this picture is fraught with uncertainties and each step is argued by different groups. The following discussion is of the consensus rather than the disputed details.

Oestrogens circulate in the bloodstream bound to a variety of different proteins the most important of which is serum albumin. Why the hormone exists bound to these proteins is not clear. Albumin may protect the hormone from degradation, however, it is the free hormone that passes through the cell membrane and into the cell. The entry of oestrogen into target cells is not dependent on energy and does not involve active transport (Peck et al., 1973; Muller and Wotiz, 1979). In view of the hydrophobic nature of steroid hormones it has always been assumed that oestrogens can pass freely through membranes. Attempts to demonstrate facilitated transport (Milgrom et al., 1973) have not been conclusive and oestrogen appears to pass into target and non-target cells with similar efficiency. The avidity with which oestrogens accumulate in target

tissues is due to the presence of a specific receptor in these tissues. Non-target tissues possess very few if any of these receptors. In the immature rat uterus, the receptors are present at a concentration of approximately 15 x 10^3 molecules per cell, and they possess an affinity for oestradiol-17β of between 10^{-9} 10^{-10}M (Toft et al., 1967; Giannopoulos and Gorski, 1971). The empty receptor has an approximate molecular mass of 76 x 10^3 and is characterised by a sedimentation co-efficient of 4S on sucrose density gradients. At appropriate concentrations and ionic strengths, this 4S protein forms an 8S aggregate. Which of these forms is found within the cell is the subject of dispute (Notides and Nielson, 1974; Clark et al., 1973). Similar oestrogen binding proteins have been detected in chick oviduct (Best-Belpomme et al., 1975; Sutherland and Baulieu, 1976) and in the liver of male Xenopus laevis (Westly and Knowland, 1978; Westly and Knowland, 1979). Indeed it would appear that probably all steroid hormones exert their influence through an intracellular protein receptor. These hormones include glucocorticoids (Rousseau, 1974; Wicks, 1974), progesterone (O'Malley and Means, 1974) androgens (Liau, 1974), mineralocorticoids (Rousseau et al., 1974), derivatives of vitamin D (DeLuca, 1981) and the ectosteroids in insects (Gilbert et al., 1980). On binding oestrogen, the receptor changes. These changes, referred to as activation, seem constant between tissues and species but their nature is still hotly debated. The activated oestrogen receptor sediments at 5S and not 4S. This change may be due to conformational changes, dephosphorylation, proteolysis, loss of an inhibitor or association with a second protein (Katzenellenbogen, 1980). Workers supporting this latter model claim to have isolated such a protein and determined its molecular

mass to be 50 x 10^3 (Notides and Nielson, 1974; Yamamoto, 1974). It is generally believed that these events occur in the cytoplasm and that the 5S form translocates into the nucleus (Jensen et al., 1968; Jensen and DeSombre, 1972; Jensen and DeSombre, 1973). However, the evidence is not conclusive. Linkie and Siiteri (1978) have suggested that the 4S receptor is normally found in the nucleus and it is there that oestrogen is bound. They suggest that cytoplasmic receptor is simply an artefact of the preparative methods. Sheridan et al., (1979) have suggested that the receptor partitions between the nucleus and the cytoplasm according to their respective water spaces. Thus 4S receptor may leak out of the nucleus during cell fractionation. Although these basic problems have not yet been resolved, it is clear that once in the nucleus the receptor complex binds to chromatin.

Some progress is now being made in the identification of the receptor complex binding sites. A number of groups have isolated and cloned DNA sequences upstream from genes inducible by steroid hormones although this work has not yet been done with the genes induced by oestrogen. Cochet et al., (1982) working with genes induced by glucocorticoids have discovered a region of homology, 21 base pairs long in similar regions upstream from genes encoding bovine proopiomelanocortin (POMC), rat growth hormone and in genes of mouse mammary tumour virus (MMTV). Payvar et al., (1981) have demonstrated that glucocorticoid receptor complex will bind to cloned regions of MMTV in vitro but have not yet sequenced those regions. A similar situation exists in the case of the progesterone receptor complex (Compton et al., 1982; Mulvihill et al., 1982). Competition binding assays between calf thymus DNA and cloned genes of ovalbumin, conalbumin, ovomucoid and of X and Y

genes (described in a later section) revealed a total of some 40 homologous regions near the transcriptional initiation site. Sequences of these regions revealed a 19 base pair concensus sequence found both upstream and downstream of the initiation site, indeed 2 of these sequences were found within exons. No one has yet published a study of these sequences in non-inducible genes. In addition, although Mulvihill et al., (1982) could not find the concensus sequence in the published sequences of globin genes, they only studied inducible genes in their experimental analysis. Until such time as suitable controls are performed, the specificity of the concensus sequences to progesterone-induced genes must remain in doubt.

1.7. The Effects of Oestrogen on Protein Synthesis in the Amphibian and Avian Liver

The maturation of the amphibian oocyte involves the uptake, by the cell, of large amounts of vitellogenin. Xenopus vitellogenin, a protein of approximately 200,000 molecular mass, is extensively modified by lipidation, glycosylation and phosphorylation. Once in the oocyte this protein is cleaved to lipovitellin and phosphvitin (Tata, 1976). After hormonal stimulation, vitellogenin may account for as much as 90% of protein secreted by cultured hepatocytes (Clemens et al., 1975).

Vitellogenin is synthesised in the livers of normal female frogs, but its synthesis can be induced in male frogs by injection of oestrogen. Vitellogenin is detectable in the blood 12 hours after a single injection of oestradiol-17β. The amount increases from 8 to 15 days after injection but declines to control levels by 30 to 40 days (Clemens et al., 1975; Green and Tata, 1976). About 12-16 copies of the genes encoding vitellogenin synthesis are

present in all hepatocytes, although less than half that number may be inducible (Wahli et al., 1979; Tata et al., 1981). The synthesis of vitellogenin appears to be dependent on the mRNA concentration within the cell. Barker and Tata (1975) found a 4-8 fold increase in total hepatic RNA in less the 12 hours of oestrogen treatment. Most of this was mRNA.

In conjunction with the synthesis of new mRNA, new polysomes were assembled (Lewis et al., 1976). An antibody specific to vitellogenin has been used by Tata and co-workers (Berridge et al., 1976) to quantitate the number of polysomes synthesising this protein. Polysome assembly was detectable after one day, and a maximal number were found in the liver 6-16 days after oestrogen treatment. This work was supported by polysome profile analysis and these results were later confirmed by Skipper and Hamilton (1977). Oestrogen may affect liver polysomes in other ways. Clemens and Tata (1973) and Ryffel et al., (1977) found that the activity of polysomes increased at a different rate to mRNA production over a period of 7-13 days. Until 7 days after oestrogen treatment protein synthetic activity increased in a manner dependent on new mRNA (Witliff and Kenny, 1972; Clemens and Tata, 1973; Ryffel et al., 1977) but protein synthesis continued to increase over 7 days while vitellogenin mRNA concentration did not. These workers suggested an intrinsic activation of ribosome activity resulting in a general stimulation of protein synthesis at later times. Little analysis has been made of the effect of oestrogen on the components necessary for protein synthesis in the stimulated frog liver. Witliff et al., (1972) demonstrated an increase in rRNA synthesis after oestrogen stimulation but did not study the synthesis of ribosomal proteins.

The male chick liver shows a very similar response to that of the amphibian liver. Vitellogenin, of 240,000 molecular mass, is secreted into the bloodstream of male chicks from the liver responding to oestrogen. The liver shows a peak of polysome assembly 4 days after oestrogen stimulation (Jost et al., 1975; Maenpaa, 1976; Bast et al., 1977) and these polysomes have been shown to synthesise vitellogenin by immunoprecipitation with anti-vitellogenin antibody (Bos et al., 1972). Van den Berg et al., (1974) and Maenpaa (1976) have demonstrated increased liver RNA polymerase A (I) activity in chicks receiving oestrogen thus suggesting increased rRNA synthesis and Lane-Smith et al., (1976) have shown that elongation factors 1 and 2 are synthesised co-ordinately with ribosomes.

1.8. The Effects of Oestrogen on Protein Synthesis in the Chick Oviduct

Although oestrogens may cause some stimulation of cell division in the liver, this is not a primary effect. Vitellogenin genes are expressed in the absence of cell proliferation (Green and Tata, 1976). In the chick oviduct exposed to oestrogen for the first time however, cell division and tissue differentiation precede the induced synthesis of ovalbumin and other egg white proteins. Oestrogen stimulates the differentiation of the immature chick oviduct to form three cell types, these are the ciliated cells, the goblet cells and the tubular gland cells. The ciliated cells are concerned solely with the passage of the ovum along the oviduct. The goblet cells show no further response to oestrogen, but synthesise avidin in response to progesterone. It is the tubular gland cells that synthesise ovalbumin, conalbumin, ovomucoid and

lysosyme in response to continued oestrogen treatments (Kohler et al., 1968; O'Malley et al., 1969; Socher and O'Malley, 1973). This synthesis does not occur until the tissues have differentiated.

The ovalbumin gene structure has been extensively studied by Chambon's group. Using a cosmid vector, they were able to clone not only the ovalbumin gene but also extensive flanking regions. They demonstrated that the ovalbumin gene co-exists with 2 pseudogenes. Figure 3 shows the structure of the ovalbumin gene family.

Figure 3 5' - X - Y - Ovalbumin - 3'

The two pseudogenes, X and Y, share extensive sequence homology with the ovalbumin gene. The genes are in the same orientation, as shown by restriction mapping, and all 3 contain 7 introns (Royale et al., 1979; Colbert et al., 1980; Dugaiczyk et al., 1978).

The effect of oestrogen on the transcription of the ovalbumin gene has been studied in detail. Cells of the unstimulated oviduct possess very few ovalbumin mRNA molecules. Treatment with oestrogen causes the accumulation of ovalbumin mRNA within the tubular gland cells (Means et al., 1972; Roads et al., 1973; Chan et al., 1973; Palmiter and Smith, 1973). The concentration of ovalbumin mRNA per tubular gland cell has been shown to change from less than one molecule per cell to 50,000 in response to oestrogen (Tsai et al., 1979; Harris et al., 1975; Cox et al., 1974). The primary transcript from the gene is probably 7.8 kilobases long. This is 4 times the length of the ovalbumin mRNA and 200 nucleotides longer than the gene. The transcript is

polyadenylated (Roop et al., 1980; Tsai et al., 1980). Harris et al., (1975) found that the half-life of ovalbumin mRNA was 60-80 hours and that each mRNA molecule was translated into 40,000 to 50,000 protein molecules. Primary control of ovalbumin gene expression is thus at the level of transcription. Whether or not mRNA processing plays a significant role is not known but turnover of mRNA is not affected by oestrogen (Cox, 1977).

Although the synthesis of ovalbumin is directly dependent on the synthesis of ovalbumin mRNA, there is also an increase in the other components necessary for protein synthesis. Thus, increases in tRNA and co-factors of protein synthesis are observed (Compstock et al., 1972; O'Malley et al., 1968) and oestrogen also induces the assembly of ribosomes (Palmiter et al., 1970). One day after primary oestrogen administration to chicks, polysomes are assembled from pre-existing ribosomes. Shortly afterwards, assembly of new ribosomes takes place and large polysomes are most abundant between 4 and 7 days of oestrogen stimulation (Means et al., 1971; Palacios et al., 1972). Total messenger activity is maximal at 3 days. Contrasting with the peak of protein synthetic activity at 4-7 days, are the data concerning the accumulation of ovalbumin-specific mRNA. Harris et al., (1975) demonstrated maximal cellular concentrations of mRNA for ovalbumin 18 days after hormone treatment. The late appearance of mRNA is rationalised in terms discussed at the beginning of this section. Maximal ovalbumin mRNA concentration coincides with the maximal numbers of tubular gland cells. Thus although the predominent effect of oestrogen is the induced synthesis of a few major proteins, this must be preceded by development of the previously undifferentiated tissue.

1.9. The Effects of Oestrogen on Protein Synthesis in the Immature Rat Uterus.

The effects of oestrogen on the immature rat uterus warrants a more detailed discussion than either the avian and amphibian liver or the chick oviduct. This is partly due to its relevance to this project and also because of the complexity of its response. Unlike the previous models of oestrogen action, the immature rat uterus does not synthesise any single protein as its major response to the hormone; instead oestrogen stimulates overall tissue growth and development. The effects of oestrogen on mRNA and protein synthesis in the uterus reflect the need for a broad spectrum of changes.

Once localised in the nucleus, the oestrogen receptor complex is responsible for profound alteration in the synthesis of RNA (Teng and Hamilton, 1968). These alterations result from increased template activity and changes in chromatin structure. A number of workers have tried to relate altered transcription to changes in the RNA polymerases involved. In fact no oestrogen induced changes in polymerase type, quantity or subunit structure have been found within the first 12 hours of oestrogen action (Courvalin *et al.*, 1976; Borthwick and Smellie, 1975). For example, Courvalin *et al.*, found that changes in RNA polymerase activity preceded increases in polymerase concentration by at least 6 hours. The rapid changes in transcription must be attributed to changes in the regulatory proteins associated with chromatin. There has been little detailed work in this area but Levitz *et al.*, (1974), Means and Hamilton (1966), Church and McCarthy (1970) and Teng and Hamilton (1968) have found a variety of changes in the composition of uterine chromatin after oestrogen stimulation.

Markerverich et al., (1978) found an increased rate of transcription, in vitro, of uterine chromatin by E. coli polymerase, after oestrogen treatment. Thus the changes in transcription that oestrogen induces can be explained in terms of changes in the template activity of the chromatin and not in terms of the polymerases involved.

The earliest detected changes in transcription are an increased transcription of hnRNA (Knowler and Smellie, 1971; 1973; Aziz and Knowler, 1978) also detectable as increased activity of RNA polymerase B in isolated nuclei (Hamilton et al., 1965; Glasser et al., 1972; Borthwick and Smellie, 1975). These changes are found as early as 15-30 minutes after oestrogen stimulation and appear to be a pre-requisite for the continued action of oestrogen (Mueller et al., 1961; Knowler and Smellie, 1971; Borthwick and Smellie, 1975).

Transcriptional activity increases again at about 2 hours after oestrogen administration. At this time, both polymerase A and B (I/II) are active. RNA polymerase B (II) activity increases until at least 12 to 16 hours while polymerase A (I) shows a peak of activity at 2-4 hours (Hamilton et al., 1968; Knowler and Smellie, 1971; Catellie and Baulieu, 1976).

Aziz and Knowler have studied the synthesis of hnRNA and mRNA in the rat uterus in some detail. They have demonstrated that the early increases in polymerase B (II) activity result in the synthesis of 3 classes of hnRNA, non-adenylated, moderately and highly adenylated classes (Aziz and Knowler, 1978; Orava et al., 1980). Oestrogen-induced synthesis of the highly adenylated class was most rapid. Sequences present in these hnRNA classes appear later in mRNA (Aziz and Knowler, 1980). Aziz et al., (1979b) went on to analyse the sequence complexity present in the polysomal mRNA

from uteri of immature rats treated with oestrogen for 4 hours and detected 32,000 to 53,000 different polyadenylated mRNA species depending on whether the assay was by cDNA:mRNA hybridisation or by unique DNA:mRNA hybridisation. These sequences were compared, by heterologous Rot hybridisation, with those of polysomal mRNA from unstimulated and 2 hour stimulated rats. Unfortunately, it was not possible to isolate enough mRNA from unstimulated rats to construct complete Rot curves but the partial curve obtained hybridised to only 11% of the 4 hour stimulated cDNA probe. Clearly, a high percentage of the polyadenylated mRNA present in the 4 hour stimulated animal was not present or was much less abundant in unstimulated animals. The mRNA preparation from 2 hour stimulated animals hybridised to 56% of the probe from 4 hour treated animals showing that there was still a substantial difference between the 2 hour and 4 hour mRNA populations. Aziz et al., (1979b) were able to analyse this difference using fractionated cDNA preparations and they concluded that the most dramatic changes were in sequences of the intermediate abundance class. It has been suggested that this class includes the ribosomal protein mRNA. In addition the results emphasise the lack of any single major protein synthesised by the uterus since the sequences of the highly abundant class showed only slight decrease with time.

The synthesis of rRNA is dependent on the translation of mRNA produced at early times in oestrogen-induced growth. The stimulation of rRNA synthesis is maximal between 2 and 4 hours after oestrogen administration (Knowler and Smellie, 1971a; Knowler and Smellie, 1973; Notides and Gorski, 1966; Catellie and Baulieu, 1976). However, Merryweather and Knowler (1980) have shown that the incorporation of rRNA into polysomes is maximal at 12 hours

after oestrogen administration. Thus there appears to be a delay of approximately 2-6 hours in the incorporation of the newly made rRNA into ribosomes. Whether this delay is real has not yet been proven. It is possible however that ribosome assembly is limited by the absence of sufficient ribosomal protein or by another component of protein synthesis. One of the aims of this project will be to analyse ribosomal protein synthesis with this problem in mind.

A single injection of oestrogen stimulates great changes in the protein synthetic apparatus of the immature rat uterus. The unstimulated uterus contains ribosomal monomers and subunits (Merryweather and Knowler, 1980). Within 1 to 2 hours of hormone injection, uterine cells assemble active polysomes. At this time there is little de novo synthesis of ribosomes and thus polysomes are assembled from pre-existing monomers. Inhibition of protein synthesis at this early stage prevents a continued response to oestrogen (Mueller et al., 1961; Nicolette and Babler, 1974; Borthwick and Smellie, 1975). Raynaud-Jammet et al (1972) and Borthwick and Smellie (1975) found that α amanitin, a poison specific to RNA polymerase B (II) would produce similar effects to the inhibition of early protein synthesis. Thus, if the fungal toxin was given before oestrogen, it not only inhbited hnRNA synthesis but also rRNA synthesis. If, however, it was given 30 minutes after the hormone, when stimulation of hnRNA synthesis had already been initiated, then rRNA synthesis was unaffected (Borthwick and Smellie, 1975). Similar time dependent effects in rRNA synthesis followed the administration of inhibitors of protein synthesis (Borthwick and Smellie, 1975). The early uterine

response to oestrogen requires transcription and subsequent translation of new mRNA to produce a small number of early proteins. What these proteins might be is unknown. They may include new oestrogen receptor, a group of induced proteins known as I.P. (Mueller et al., 1961; Skipper et al., 1980), and possibly ribosomal proteins. It is perhaps most likely that specific regulators of transcription are synthesised (Barker, 1971; Cohen and Hamilton, 1975) and that these control the continuing response to the hormone.

Following the initial stimulation of protein synthesis and the stimulated synthesis of rRNA and tRNA (Knowler and Smellie, 1971) there is a general stimulation of protein synthesis (Merryweather and Knowler, 1980). There is renewed synthesis of mRNA with a peak of incorporation into polysomes at 16 hours. A maximum number of large polysomes can be isolated from the uterus at 12 hours after oestrogen injection (Gasde et al., 1971; Merryweather and Knowler, 1980). At this time the incorporation of radioactivity labelled ribosomal proteins and rRNA into polysomes is also maximal. Whelly and Barker (1974) have shown that oestrogen may also increase the efficiency of uterine polysomes. They were able to demonstrate an increase in protein synthetic activity before an increase in polysome number.

Walker and Kaye (1981) have attempted to analyse the proteins being synthesised on these new uterine polysomes. They used a cell free protein synthesising system to label nascent polypeptides on uterine polysomes and separated these proteins on a 2D gel system (O'Farrell, 1975). With very few exceptions they were not able to find significant changes in the proteins synthesised before and after oestrogen treatment. Korach et al., (1981) used the same gel system to analyse proteins synthesised in

the mouse uterus responding to oestrogen. Again, only a few oestrogen induced changes could be found. The O'Farrell gel system is a very useful technique for separating a large number of proteins differing in isoelectric point and size. However, in eukaryotic cells, the system only visualises the most abundant proteins. Korach et al., (1981) were only able to identify 200 different proteins or less, Walker and Kaye (1981) found less than half that number. These proteins were probably encoded by highly abundant mRNA. Minor proteins such as those encoded by moderately abundant or rare mRNA sequences would not account for a sufficiently large part of protein synthesis for changes to be seen by gel analysis. Aziz et al., (1979a) found between 800 and 12,000 new mRNA sequences in the rat uterus responding to oestrogen. However, they also demonstrated that the largest changes occur in the moderately abundant and rare classes (Aziz et al., 1979b).

The total screening of cellular protein synthesis is not a very useful approach to the study of oestrogen-induced changes in the uterus. So changes must be analysed at the level of individual proteins, or groups thereof. That is by isolating each protein in question. Table 2 shows a range of uterine proteins that have been shown to alter in amount of activity during oestrogen-induced protein synthesis.

Following polysome assembly there are gross changes in uterine metabolism. After 6 hours cellular RNA content increases. From 14 hours there is a further increase in protein synthesis. Continued oestrogen stimulation results in a peak of DNA synthesis at 24-28 hours and cell division follows shortly thereafter (Billing et al., 1969; Kaye et al., 1972; Stormshak et al., 1976). The continued presence of oestrogen in the bloodstream ensures the continued growth and differentiation of the uterus.

Table 2: Oestrogen induced changes in uterine proteins.

Aspect	Change	Reference
Chromatin (N.H.P.	synthesis	Cohen and Hamilton (1975)
Chromatin (Histones	synthesis	Barker (1971)
Ribosomal proteins	synthesis	Merryweather and Knowler (1980)
Thymidine Kinase	increased activity	Yamada et al., (1980)
Creatine Kinase	increased activity	Notides and Gorski (1966)
Neurone specific Enolase	increased activity	Skipper et al., (1980)
Cathepsin	increased activity	Elangovan and Moulton (1980)
Hexokinase	increased activity	Baquer and MacClean (1972)
Glucose 6 Phosphate dehydrogenase	increased activity	Baquer and MacClean (1972)
Ribulose 5 Phosphate Isomerase	increased activity	Baquer and MacClean (1972)
Ig A	synthesis	Wira et al., (1980)

The Aims of this Project

As described in the last section, studies of protein synthesis in the immature rat uterus have met with varying degrees of success. In particular, analysis of the appearance of new proteins has not been successful. It is now necessary to select one group of proteins for detailed analysis where total analysis failed. The essential role of protein synthesis and the central role of ribosomes has already been discussed. Ribosomal proteins were selected for detailed analysis since their synthesis must play a vital part in the response of the immature rat uterus to oestrogen.

The aim of this project is to study the synthesis of new ribosomal proteins and their appearance in the polysomes of the rat uterus responding to oestrogen.

MATERIALS AND METHODS

2. Materials and Suppliers

2.1. Radiochemicals

All radiochemicals, and rabbit reticulocyte lysate, were supplied by Amersham International, Amersham, Bucks, U.K.

The radiochemicals were all of the following specific activities:

L[4,5 - ^{3}H] Leucine	52 Ci/mmol.
[^{32}P] Inorganic phosphate	10mCi/ml.
L[^{35}S] Methionine	500 mCi/mmol.
[^{35}S] Sodium sulphate	40 Ci/mg.

2.2. Hormones and Metabolites

Oestradiol-17 β and oestradiol benzoate ATP, ADP, GTP and NADP were all supplied by Sigma (U.K.) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

2.3. Immunological Reagents

Bovine Serum Albumin (BSA) and O-phenylenediamine (OPD) were obtained from Sigma Chemical Co. Ltd. Freunds adjuvants were obtained from Difco Laboratories, Central Avenue, East Molsey, Surrey, U.K. Antibody-enzyme conjugate was purchased from Miles-Yeda Ltd., Kiryat Weizmann, Rehovot, Israel. All precipitating sera were the gift of the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland, with the exception of rabbit antiribosomal protein antibody which was raised in the department.

2.4. Reagents for Electrophoresis

Reagents for electrophoresis were obtained from British Drug Houses (BDH) Ltd., Poole, Dorset, U.K., with the following exceptions: Bromophenol blue, TEMED and β-mercaptoethanol were obtained from Koch Light Laboratories, Colnbrook, Bucks, U.K.

2.5. Miscellaneous

Kieselguhr, trichloroacetic acid and diphenyloxazole were purchased from Koch Light Laboratories. Trisma base (Tris) was supplied by Sigma Chemical Co. Ltd. Agarose was purchased from Miles Laboratories Ltd., P.O. Box 37, Slough, U.K. Eagle's medium was purchased from Gibco Europe, Glasgow, Scotland. Hexokinase and Glucose-6-phosphate dehydrogenase (the ATP assay) were purchased from Boehringer Mannheim GmbH, P.O. Box 51, 68 Mannheim 31, West Germany. Protosol was purchased from New England Nuclear, 2 New Road, Southhampton U.K. and X-omat film was supplied by Eastman-Kodak Co., Rochester, New York, U.S.A.

Wherever possible all other reagents were AnalaR grade and purchased from BDH Ltd.

2.6. Apparatus

Although most glassware and apparatus was purchased through the departmental stores, some items of equipment were purchased separately. Corex centrifuge tubes were purchased from Corning Glassworks, New York, U.S.A. Cellulose nitrate ultracentrifuge tubes were purchased from Beckman Spinco Ltd., Palo Alto, California, U.S.A. Eppendorf Gerätbau, Hamburg, West Germany supplied low volume centrifuge tubes. ELISA microlitre plates were supplied by Becton, Dikinson and Co., 1950 Williams drive, Oxnard, California, U.S.A. Capillary tubing was supplied by A.R. Horwell, 2 Grangeway, Kilburn High Road, London, U.K.

3. METHODS

3.1. Experimental Animals

All animals were supplied by the departmental Animal House. The immature female rats used were derived from the Wistar

strain and were weaned at 18-21 days after birth. The weight of these animals was between 30 and 40 grams. All animals used fell into this age and weight group since after 28 days female rats begin to secrete their own oestrogen. Weanling rats were killed by cervical dislocation after ether anaesthesia.

The adult male rats used were also of the Wistar strain and weighed between 100g and 200g. These animals were killed by concussion.

The adult rabbits used for raising antibodies were of the New Zealand White strain and were bled from the ear vein.

All animals used were given free access to food (supplied by Labsure Animal Foods, Christor Hill Group Ltd., Poole, Dorset, U.K.) and to water. In addition all were on a fixed 16 hour day.

3.2. Administration of Hormone and Antigen

Administration of oestradiol-17β followed the method of Knowler and Smellie (1971b). Oestradiol-17β was stored in absolute alcohol at -20°C at a concentration of 2mg/ml. A volume of 0.25ml of stock solution was diluted in 15ml of 0.5M NaOH and titrated to neutrality with 0.5M HCl, the volume was then adjusted to 50ml with water thus creating solubilised oestradiol at 10µg/ml in 0.5M NaCl. This solution was prepared immediately prior to use and 0.1ml was injected intraperitoneally into immature female rats. Control animals received 0.1ml of carrier solution only. Oestradiol benzoate is not soluble in water and was therefore injected in an adjuvant. 50µl of a 2mg/ml solution in absolute alcohol was dissolved in 10µl of corn oil by rapid mixing for 5 minutes. Again the solution was prepared immediately prior to use and 0.1ml of solution was injected subcutaneously into the back of the neck. Control animals received 0.1ml of carrier solution only.

Immunisation of rabbits followed the method of Stoffler and Wool (1976). Ribosomal protein immunogen was dissolved in 8M urea and diluted to 1M urea with 0.9% (w/v) NaCl and 0.2% (w/v) KPO_3 (pH 7.1). The primary innoculum was mixed with an equal volume of Freunds complete adjuvant by brief sonication. Subsequent innocula were in an equal volume of Freunds incomplete adjuvant. Each innoculation was of about 1mg of protein, in 1ml, injected subcutaneously. The innoculation was at many sites injecting approximately 200 µl of adjuvant at each. Two rabbits were injected every week for four weeks and bled 3 days later.

3.3. Preparation of Glassware and Solutions

After use, all glassware was soaked in a strong solution of Haemo-sol, unless radioactive. If contaminated by radioactivity, glassware was soaked in a 5% solution of Decon 75. All glassware was then scrubbed, rinsed thoroughly with tap water and then deionised water. After drying in an oven (100^{o}C) the glassware was covered with tinfoil and sterilised by steam in an autoclave. Pipettes were sterilised in an oven at (180^{o}C) overnight.

In addition to the use of ribonuclease inhibitors, such as diethyl pyrocarbonate or heparin, all solutions for use with RNA were autoclaved.

These precautions ensured that all glassware and solutions were free of contamination by ribonuclease, stray radioactivity and damaging chemicals.

3.4. Incubations In Vitro

Incubations *in vitro* of immature rat uteri took place under conditions developed by Merryweather (1981) from the procedure of Knowler and Smellie (1973). Rats were killed by cervical dislocation under ether anaesthesia and the uteri rapidly removed. With the exception of a few preliminary experiments (see results),

uteri were slit along their length on a warm brass plate with a sharp scalpel blade. They were then collected in a 5ml conical flask containing 2ml of Eagle's medium (Glasgow modification) (Busby et al., 1964). The incubation was maintained at 37°C under an atmosphere of 95% O_2: 5% CO_2 (v/v). Incubation took place in a gently shaking water bath and was usually for a period of one hour. Glassware was sterilised as usual and all solutions were sterilised by filtration.

During labelling experiments the uteri were collected in 1ml of medium lacking the label to be incorporated (i.e. leucine-free, phosphate-free or methionine-free medium). The incubation was timed from the addition of the remaining 1ml containing the radioactive isotope ([^{3}H]-leucine, [^{32}P]-inorganic phosphate or [^{35}S]-methionine).

At the end of the incubation period, uteri were washed three times in a large volume (200ml) of ice cold saline (0.9% w/v). The uteri were then blotted dry and rapidly frozen in a bath of Drikold-methanol, and stored for less than 24 hours at -60°C.

3.5. Preparation of [^{35}S] -methionine

[^{35}S]-methionine was prepared from [^{35}S]-sodium sulphate by the method of Bretscher and Smith (1972). E. coli M2308 were inoculated into growth medium containing the following:

Growth Medium
- 60mM Na_2HPO_4
- 25mM K H_2PO_4
- 10mM NaCl
- 20mM NH_4Cl
- 1mM $MgCl_2$
- 0.05mM Na_2SO_4
- 1% Glucose

After eight hours of incubation at 37°C a sample was transferred to 20ml of similar medium containing 20mCi of [^{35}S]-Na_2SO_4. Growth was allowed to continue for a further 16 hours at 37°C.

Cells were then collected by centrifugation at 10,000g (12,000 rpm) for 10 minutes in a Sorval RC-5 centrifuge. The pellet was washed by resuspension in 1ml of water and the cells collected by recentrifugation. After washing, the pellet was resuspended in 0.5ml of water and then 0.5ml of 13M HCl and 0.1ml of 6M thioglycolic acid were added. This mixture was then transferred to a thick-walled glass 5ml pipette and frozen in a bath of Drikold-methanol. The pipette was sealed under a vacuum at both ends. Cells were then hydrolysed (under vacuum) overnight at 108°C. After digestion, the slurry was refrozen, the glass seal broken and the sample was dried down using a freeze-drier. After drying the sample was resuspended in 0.1mM β-mercaptoethanol. The hydrolysate was streaked across a 10cm strip of 3MM paper (at least 15cm wide) and subjected to descending chromatography in n-butanol: acetic acid: water (4:1:5 v/v/v), overnight.

The methionine was detected with a mini-monitor at an Rf value of about 0.55. The strip corresponding to methionine was cut out with a scalpel and radioactivity was eluted from it with 0.1mM β-mercaptoethanol. Over 95% of the radioactivity is eluted in the first 100 µl of the solvent, and 5-10mCi of [^{35}S]-methionine were usually obtained. Bretscher and Smith suggest that the specific activity of methionine obtained by this method is 15-18,000 Ci/mol. The pH of the eluent was adjusted to approximately pH8 with KOH and was aliquated into 1mCi amounts. These were stored at -60°C.

3.6. Ribosome and Polysome Preparations

3.6.1. Preparation of Ribosomes from HeLa cells

Ribosomes from HeLa cells were prepared by the method of Ascione and Arlinghaus (1970). The following solutions were required for the preparation:

PBS (Phosphate Buffered Saline)

170mM NaCl

1.8mM KH_2PO_4

10mM Na_2HPO_4 (pH 7.4)

Swell Buffer	Lysis Buffer	TKME 1
20mM Tris-HCl (pH 7.5)	0.5mM Sucrose	10mM Tris-HCl (pH 7.5)
50mM KCl	0.55M KCl	10mM KCl_2
5mM $MgCl_2$	10mM $MgCl_2$	1mM $MgCl_2$
5mM $CaCl_2$	5mM $CaCl_2$	0.1mM EDTA
	1mM EDTA	
	40mM Tris-HCl (pH 7.5)	
	1% Triton X100	

The growth medium (Glasgow modification of Eagle's medium) was poured from two burlers containing HeLa cells which were just entering the exponential growth phase (that is about 1-2 days after seeding the medium). The growth medium was replaced with medium containing 90% of the required phosphate concentration, and 500μCi of [^{32}P]-inorganic phosphate. The cells were then allowed to grow for another 2 days.

After growth, the medium was poured from the two burlers and the cells were washed twice with ice-cold PBS. The cells were then scraped off the sides of the glass into 10ml of ice-cold PBS. The suspension was centrifuged at 1000g (1000 rpm) for five minutes in a Beckman benchtop centrifuge, at 0^{o}C. The pellet was gently

resuspended in cold PBS and the cells collected once again by centrifugation.

The pellet was then resuspended in 3ml of hypotonic swell buffer, and left on ice for 10 minutes. An equal volume of lysis buffer was then added and mixed well. This was left for a further 5 minutes on ice. The suspension was then homogenised by 6 strokes of a tight fitting Teflon-glass homogeniser. The homogenate was centrifuged at 1000g, (1000 rpm) for 10 minutes at $0^{o}C$, to remove cell debris, nuclei, etc. Sodium deoxycholate (as a fresh 10% solution in water) was added to a final concentration of 0.5% (i.e. $^{1}/_{20}$th volume) and the solution mixed. The suspension was then centrifuged for 2 hours at 165,000g (50,000 rpm) in a Ti50 rotor of a Beckman L2 65B ultracentrifuge, at $4^{o}C$.

The pellet was resuspended in 8.7ml of TKME 1 and layered over a 7ml cushion of 2M Sucrose in this buffer. This was centrifuged overnight (16 hrs) at 65,000g (25,000 rpm) in an SW40 rotor of a Beckman L2 65B at $4^{o}C$. The surface of the pellet was gently washed with TKME 1 and the pellet stored at $-20^{o}C$.

3.6.2 Rat Uterine Polysomes

Rat uterine polysomes were prepared using the method of Merryweather and Knowler (1980), based on the procedure of Berridge et al., 1976. The following solutions were used:-

Polysome Buffer 1	Polysome Buffer 2
0.2M Tris-HCl (pH 8.5)	0.05M Tris-HCl (pH 8.5)
0.05M KCl	0.05M KCl
0.015M $MgCl_2$	0.15M $MgCl_2$
7mM β-mercaptoethanol	7mM β-mercaptoethanol
	1M Sucrose

Diethyl pyrocarbonate, a potent ribonuclease inhibitor, was added to a concentration of 0.02% (w/v) if polysomes were not required to be active in protein synthesis.

3-12 uteri were excised from immature rats, cleaned of fatty tissue, and rapidly frozen in a bath of Drikold-methanol. The uteri were then ground with a footed glass rod. All following procedures were performed at 2-4^{o}C using sterilised solutions and glassware.

The ground uteri were homogenised with 20 strokes of a motor-driven teflon-glass homogeniser, in 4.5ml of polysome buffer solution 1. 0.5ml of a 20% (w/v) solution of Triton X100 in water was added to the homogenate. The solution was well mixed by vortex mixing for at least 15 seconds. Cell debris, mitochondria and nuclear material were removed from the homogenate by centrifugation at 20,000g (12,000 rpm) for 10 minutes in an HB4 rotor of a Sorvall RC-5 centrifuge, at 4$^{\emptyset}$C.

The supernatant was layered over a 1ml cushion of polysome buffer solution 2. The polysomes were collected as a pellet after centrifugation at 200,000g (47,000 rpm) in an SW50.1 rotor of a Beckman L2 65B for 90 minutes at 4^{o}C.

3.6.3. Rat Liver Polysomes

Rat liver polysomes were prepared from groups of 5-12 adult male rats weighing 100g-200g that had been starved for 16 hours prior to death. The following solutions were required:-

Liver (homogenisation) buffer 1	Liver buffer 2
0.25M Sucrose	0.5M Sucrose
0.05M Tris-HCl (pH 7.5)	0.05M Tris-HCl (pH 7.5)
0.08M KCl	0.98M KCl
0.0125M $MgCl_2$	0.0125M $MgCl_2$

The rats were killed by concussion and the livers removed to 50ml of ice cold Liver buffer 1. The livers were minced and washed free of blood and then homogenised in 4 volumes of the same buffer with ten strokes of a teflon-glass homogeniser. The homogenate was centrifuged twice at 20,000g (12,000 rpm) in an HB4 rotor of a Sorval RC-5 for 20 minutes at 4°C to remove cell debris and mitochondria. The supernatant was filtered through glass wool and centrifuged at 176,000g (50,000 rpm) in a 60Ti rotor of a Beckman L2 65B ultracentrifuge for 85 minutes at 4°C. The supernatants were discarded and the pellets resuspended in 8ml Liver buffer 1 containing 0.5% w/v of freshly prepared sodium deoxycholate. This was layered over 5.7ml of the Liver buffer 2. The polysomes were collected as a pellet by centrifugation at 140,000g (40,000 rpm) in an SW40 rotor of a Beckman L2 65B for 120 minutes at 4°C. Finally, the surface of the pellets were carefully washed with a little buffer 1.

3.7. Polysome Profile Analysis

The size distribution of polysomes was analysed by the method of Merryweather and Knowler (1980). Polysomes were resuspended in 200µl of a buffer consisting of:-

0.05M Tris-HCl (pH 7.6)
0.25M KCl
0.005M $MgCl_2$
0.02% (w/v) Diethylpyrocarbonate

This solution was also prepared with 15% and 40% sucrose.

The polysomes were separated on sucrose gradients containing 15%-40% sucrose. These gradients were centrifuged at 230,000g (50,000 rpm) in the SW50.1 rotor of a Beckman L2 65B ultracentrifuge at 4°C for 40 minutes. The profiles were

analysed by upward displacement through a gradient scanning attachment for a Gilford 240 spectrophotometer.

3.8. Subunit Preparation

The ribosomes, prepared as polysomes in the preceeding sections, still contained nascent and adventitiously attached proteins (Warner, 1966). The procedure of Leader and Wool (1972) was used to remove these proteins. Ribosomes were dissociated into constitutive subunits by the presence of puromycin, followed by the separation of the subunits on sucrose density gradients containing a high concentration of monovalent cations. HeLa cell, rat uterine and rat liver ribosomes were separated in the same way. Subunits used for the preparation of ribosomal protein immunogen were separated after incubation in a solution of raised cation concentration. The following solutions were used:-

Subunit Separation Buffer	Gradient Buffer	Ribosome Suspension Buffer
0.01M Tris-HCl (pH 7.5)	0.01M Tris-HcL (pH 7.5)	0.01M Tris-HCl (pH 7.5)
0.8M KCl *(0.93M KCl)	0.5M KCl	0.08M KCl
0.005M $MgCl_2$	0.005M $MgCl_2$	0.012M $MgCl_2$
20mM β-mercaptoethanol	20mM β-mercaptoethanol	7mM β-mercaptoethanol
0.1mM puromycin	10%-30% Sucrose	

Ribosomes were incubated in the subunit separation buffer at 37°C for 15 minutes. When subunits were to be used as a source of ribosomal protein immunogen, the concentration of KCl was 0.93M*. This resulted in a more rigorous washing and although some ribosomal protein may have been lost it ensured that no non-ribosomal proteins were associated with the immunogen (Leader and Wool, 1972).

Immediately after incubation 1.5ml of suspension was layered onto 15% to 30% sucrose density gradients in gradient

buffer. Gradients were centrifuged at 96,000g (27,000) rpm) for 4 hours in an SW27 rotor of a Beckman L2 65B ultracentrifuge. The temperature was maintained at 28°C to prevent the association of 40S subunits into dimers. The rotor was allowed to coast to a rest without the use of the brake.

The gradients were scanned by continuous monitoring through the 0.25cm path length flow-through cell of a Gilford 240 spectrophotometer. Fractions corresponding to 60S and 40S subunits were collected, diluted with ribosome suspension buffer and pelleted by ultracentrifugation in a 60Ti rotor of a Beckman L2 65B at 175,000g (50,000 rpm) for 16 hours at 4°C.

3.9. Extraction of Ribosomal Protein

Proteins were extracted from ribosomal subunits by the method of Hardy et al., (1969), employing the modifications of Sherton and Wool (1972). This method used a solution of 66% (w/v) glacial acetic acid containing 66mM magnesium acetate at pH 7.7. The method required a high concentration of subunits to be effective and to achieve this, radioactive uterine subunits were extracted in the presence of unlabelled liver subunits. Even so, the final concentration of subunit nucleic acid was only adjusted to 10 A_{260} units/ml rather than the recommended 1,000 A_{260} units/ml. This was necessary to ensure that the specific activity of the extracted proteins remained high enough to avoid overloading of the subsequent polyacrylamide gels. The extraction was found to give a recovery of 95% of radioactivity in subunit protein. Ribosomal RNA was removed by centrifugation at 20,000g (12,000 rpm) in an HB4 rotor of a Sorval RC-5 centrifuge.

Ribosomal proteins used for immunisation were acetone precipitated and re-extracted to ensure the complete removal of rRNA. Proteins to be analysed in polyacrylamide gels were precipitated with trichloroacetic acid.

3.10 Preparation of Ribosomal Protein for Gel Separation

The method used was that of Howard et al., (1975). The protein containing supernatant from which rRNA had been removed was stirred on ice while 10-15 volumes of 5% (w/v) ice-cold trichloroacetic acid was added dropwise. The solution was stirred on ice for a further one hour and the precipitate was collected by centrifugation at 1,000g (1,000 rpm) for 10 minutes. After careful drying the sample was resuspended in the appropriate sample buffer at 60°C for 10 minutes.

3.11 Preparation of Ribosomal Proteins for Immunisation

Since trichloroacetic acid may damage the integrity of ribosomal proteins, proteins for immunisation were prepared by acetone precipitation. Barritault et al., (1976) have shown that this treatment maintained the activity of prokaryotic ribosomal proteins. Proteins were precipitated by the direct addition of 5 volumes of ice-cold acetone and stored overnight at -20°C. The precipitate was collected by centrifugation and resuspended in 8M urea and 10mM triethanolamine. After reprecipitation, as above, the pellet was redissolved in 8M urea and diluted to 1M urea with 0.9% (w/v) NaCl and 0.2% (w/v) KPO_3 (pH 7.1). This solution was mixed with Freunds adjuvant by sonication, as described in an earlier section.

3.12 The Electrophoretic Separation of Ribosomal Proteins

3.12.1. Separation in One Dimension

Separation of ribosomal proteins in one dimension was by Sodium dodecyl sulphate polyacrylamide gel electrophoresis. This was based on the method of Laemmli (1970) using the procedure of Le Stourgen and Bayer (1977). The concentration of polyacrylamide was adjusted to 12.5% rather than the 8.75% recommended in the text. This achieved better separation of the relatively small ribosomal proteins.

The following solutions were required:

Resolving Gel	Stacking Gel	Sample Buffer
12.5% Acrylamide	3% Acrylamide	0.0625 Tris-HCl (pH 6.8)
0.33% Bisacrylamide	0.05% Bisacrylamide	2% SDS
0.4M Tris-HCl (pH 8.8)	0.2M Tris-HCl (pH 6.8)	10% Glycerol
1% SDS	1% SDS	5% β-mercaptoethanol
10 μl/40ml TEMED	10 μl/ml TEMED	0.001% Bromophenol blue
4mM Ammonium persulphate	3mM Ammonium persulphate	

Running buffer

25mM Tris (pH adjusted by glycine)

0.2M glycine

1% SDS

Polymerisation of the gels was achieved by the addition of TEMED and ammonium persulphate. The gels were prepared as slabs and run using our own equipment. Samples were run at 20mA per gel through the stacking gel but this was increased to 40mA per gel when the sample had entered the resolving gel. The gel was run until the blue tracker dye (bromophenol blue) had reached the bottom of the gel; usually in about 5 hours. Electrophoresis was toward the anode.

3.12.2. Separation in Two Dimensions

Ribosomal proteins were separated in two dimensions by the method of Lastick and McConkey (1976) based on the techniques of Kaltschmidt and Wittman (1970).

Separation in the first dimension employed the following solutions:-

1st D Gel	1st D Running Buffer
6M Urea	3mM EDTA
4% Acrylamide	75mM Boric acid
0.13% Bisacrylamide	0.06M Tris-NaOH (pH 8.6)

1st D Gel continued	Sample Buffer
0.01M EDTA	6M urea
0.26M Boric acid	0.02M Tris (pH 8.0)
0.01M Tris-unbuffered (pH 8.7)	0.01M EDTA
0.2% TEMED	26mM Boric acid
4mM Ammonium persulphate	5% (v/v) β-mercaptoethanol

Gels were poured into 3.5mm glass-tubes that had been coated with "Repelcoat". Polymerisation took place overnight at room temperature. Proteins were run towards the cathode, at 3mA per gel for 3 hours. The coloured protein, cytochrome c was used as a marker and was run to 80% of the length of the gel. Gels were removed from their tubes by water pressure and soaked for 2 minutes in interdimensional soaking solution. The first dimension was annealed to the second dimension by the addition of 2ml of warm annealing solution (60^{o}C). The annealing solution solidified as it cooled. The following solutions were used in the second dimension:

Interdimensional Soaking Solution	Annealing Solution
6M Urea	6M Urea
0.33M Acetic acid	0.33M Acetic acid
5% (v/v) β-mercaptoethanol	1% (w/v) Agarose
	5% (v/v) β-mercaptoethanol

2nd D Gel	2nd D Running Buffer
6M Urea	10mM glycine
15% (w/v) Acrylamide	12mM Acetic acid
0.5% (w/v) Bisacrylamide	pH 4.05
0.42M Acetic acid	
25mM KOH	
0.5% (v/v) TEMED	
20mM Ammonium persulphate	
pH 4.5	

Tracking Dye

10mM glycine

14mM Acetic acid

50% (w/v) Glycerol

Saturated with $FeCl_3$

The second dimension, cast as a slab gel, was run overnight at 20mA per gel, again towards the cathode. In this case $FeCl_3$ was used as tracking dye (Bernabeu et al., 1979).

3.13. The Treatment of Gels

3.13.1. Staining

Gels were stained in 0.5% Coomassie blue G, 50% methanol, 10% acetic acid for 3 hours or more. The background was destained by diffusion in 10% methanol, 10% acetic acid.

Gels were dried with a Biorad gel drier.

3.13.2. Autoradiography and Fluorography

Autoradiography was performed with Kodak X-omat film.

Fluorography was performed by the method of Bonner and Laskey (1974). Gels to be fluorographed were soaked in three changes of dimethyl sulphoxide, for at least 30 minutes each change, and then soaked for 3 hours in 22% (w/v) diphenyloxazole in dimethyl sulphoxide. The diphenyloxazole was precipitated into the gel by thorough washing under running water for at least one hour. The gel was then dried and fluorographed with Kodak X-omat R film at $-70^{\circ}C$.

3.13.3. Scintillation counting

Scintillation counting of proteins within gel slices was achieved by digesting the excised spot with 300µl of New England Nuclear Protosol at $60^{\circ}C$ overnight. Since Protosol digested proteins within the gel without dissolving the acrylamide, 1ml of methoxyethanol was shaken with the digested gel slice to leach out radioactivity and 10ml of 5g/L diphenyloxazole in toluene was added for scintillation counting.

3.14. Cell Free Protein Synthesis

Cell free protein synthesis was performed by the method of Pelham and Jackson (1976) using Rabbit reticulocyte lysate supplied by Amersham International. Endogenous messenger activity in this lysate has been destroyed using a calcium dependent ribonuclease so the incorporation of radioactive precursor into a protein is solely dependent on exogenously added mRNA.

To 20µl of rabbit reticulocyte lysate was added [^{35}S]-methionine, of specific activity 14.14 mCi/ml, to a final concentration of 1µCi/µl. To this mixture was added 0.2 absorbance units (at 260nm) of polysomes in 1-2µl of sterile water and the final volume was adjusted to 25µl with sterile distilled water. Assays were incubated at 30^{o}C in a gently shaking water bath for 80 minutes after which the reaction was stopped by the addition of 1 µl of 10mM puromycin dihydrochloride and continuation of the incubation for a further 10 minutes. Samples were rapidly frozen and stored at -20^{o}C. All steps were performed under ribonuclease-free conditions.

3.15. Antibody Preparation and Purification

Immunological techniques used either crude serum (diluted in PBS) or a purified gamma-globulin fraction.

Blood from rabbits was allowed to clot overnight at 4^{o}C. The clot was loosened and removed by centrifugation at 1,000g (1,000 rpm) in a Beckman benchtop centrifuge. The serum was then centrifuged at 20,000g (12,000 rpm) for 30 minutes in a Sorval RC-5 at 4^{o}C. The supernatant was aliquoted and frozen to -20^{o}C, although the aliquot in use was stored at 4^{o}C.

A purified gamma-globulin fraction was prepared by ammonium sulphate precipitation. Just prior to precipitation a 30ml volume of saturated $(NH_4)_2SO_4$ was adjusted to pH 6.5 by the addition

of 2N NaOH. A 2ml sample of serum was stirred at room temperature whilst 1ml of $(NH_4)_2SO_4$ solution was slowly added. When the serum sample had been made 33% with respect to $(NH_4)_2SO_4$ saturation, the suspension was stirred at room temperature for a further 30 minutes. The gamma-globulins were collected by centrifugation at 1,000g (1,000 rpm) in a Beckman benchtop centrifuge and redissolved in 2ml of 0.9% (w/v) saline. The precipitation was then repeated to ensure the removal of all unwanted proteins. The final precipitate was then redissolved in PBS and dialysed against this buffer overnight at 4°C to remove all ammonium sulphate. Finally the solution was centrifuged at 20,000g (12,000 rpm) in a Sorval RC-5 (HB4 rotor) to ensure the removal of any insoluble precipitates.

3.16. Optimal Proportions Test

The optimal proportions test depends on the ability of antibody and antigen to form an insoluble immune complex. Maximum precipitation occurs when antigen and antibody are present in equivalent amounts. Since the technique requires large amounts of both antigen and antibody this technique was reserved for double immunoprecipitations.

Doubling dilutions of antiserum in PBS were "spotted" onto parafilm in 20µl volumes. An equal volume of antigen solution was placed separately on the parafilm. The antiserum was drawn into a 50µl capillary followed by the antigen solution. The end of the capillary tube was then plugged with plasticine. This was performed for each dilution. The tubes were stored overnight at 4°C and inspected for a maximum quantity of precipitate. Capillary tubes could be centrifuged at 1,000g (1,000 rpm) for 15 minutes for a quantitative measurement of immunoprecipitation. The greatest quantity of precipitate occurred in the tube containing optimal proportions of antigen and antibody.

3.17. Double Immunoprecipitation

Ribosomal proteins labelled by cell free protein synthesis were removed from the reticulocyte lysate by a double immunoprecipitation technique. The proteins were bound by an excess of anti-ribosomal protein antibody. This antibody was then precipitated by addition of an anti-rabbit antibody at optimal proportions (Kusel et al., 1975).

Before any of this could be done, however, ribosomal subunits, which would interfere with the reaction, had to be removed from the protein synthesising mixture. The mix was diluted to 5ml with detergent supplimented PBS (containing PBS, 0.05% BSA, 0.02% Triton X100, 0.02% Sodium deoxycholate) and the ribosomal subunits were removed from suspension by centrifugation at 230,000g (50,000 rpm) in an SW50.1 rotor of a Beckman L2 65B ultracentrifuge, for 4 hours at 4°C. Aliquots of the supernatants were diluted to 1ml with the above buffer and to each was added 10μl of normal mouse serum, followed by 10μl of sheep anti-mouse gamma-globulin antibody. The samples were incubated for 4 hours at 4°C and the resulting precipitate was removed by centrifugation for 15 minutes in an Eppendorf centrifuge. Almost all material that bound to antibodies non-specifically was removed by this procedure. (See results section.)

To the supernatant fraction was added 20μl of a purified gamma-globin of specific rabbit anti-ribosomal protein antibody. This was then incubated at 37°C for one hour, with mixing. Subsequently, 20μl of a purified gamma-globulin containing donkey anti-rabbit gamma-globulin antibody was added to effect precipitation, achieved by incubation overnight (16 hours) at 4°C, after which the precipitate was collected by centrifugation for 15 minutes in an Eppendorf centrifuge. The pellet was washed three

times with detergent supplimented PBS (described above). Finally the pellet was digested with 300 µl of Protosol overnight at 60°C, and the radioactivity solubilised by the digestion was measured in a Beckman LS 8100 scintillation counter, using toluene:PPO (5g/L) as a scintillant.

3.18. The Enzyme-Linked Immunosorbant Assay (ELISA)

The ELISA system can be used to measure either antibody or antigen (Voller et al., 1976). Initially the system was used to monitor serum levels of anti-ribosomal protein antibody but thereafter was used to measure ribosomal protein levels in the rat uterus.

To measure antibody levels the antigen, in 100µl of PBS, was allowed to bind to the bottom of a well of a Falcon microtitre plate for 2 hours at 4°C. The plate was then washed free of excess antigen with washing buffer (PBS, 0.05% BSA, 0.02% Tween, 0.02% New born calf serum). After washing 200µl of washing buffer containing 0.5% BSA was added to the wells to block any remaining binding sites. This step was also carried out at 4°C for 2 hours, and again the plate was washed with washing buffer. Serum, diluted from $^{1}/1000$ to $^{1}/1000,000$ in 100µl of washing buffer was placed in the wells, again for 2 hours at 4°C. Once again the plate was washed. A sample of goat anti-rabbit gamma-globulin conjugated to horse radish peroxidase was then diluted $^{1}/500$ in washing buffer and 100µl added to each well. After 2 hours, at 4°C, excess conjugate was washed away and the plate soaked in washing buffer for one hour at 4°C. This final washing helped to reduce the final background of non-specifically bound conjugate.

The quantity of peroxidase activity bound to the plate was determined spectrophotometrically using 100µl per well of the following assay solution:

0.04% O-phenylene diamine (OPD)

0.04% H_2O_2

36mM Citric acid

128mM $NaPHO_4$

The assay was allowed to proceed in the dark for 20 minutes at room temperature. After this period it was stopped by the addition of 50µl of 4N H_2SO_4. The absorbance at 492nm was determined for each well using a Titertek multicscan spectrophotometer. The absorbance in each well was proportional to the total rabbit antibody bound.

A modification of this procedure (the ELISA competition assay) was used to determine antigen concentration. A 100µl volume of test solution was incubated with an equal volume of diluted serum for 2 hours at 4°C in a silicon coated glass tube. The unbound antibody was determined as described above and measured by comparison with a series of standards containing known quantities of antigen. The serum was diluted 1:1000 in order to give assay values of 1.8 absorbance units at 492nm in the absence of any competing antigen.

3.19. Analysis of Phosphate Pools in Uterine Cells

3.19.1. After incubation in vitro the uteri were carefully washed three times in 200ml of ice-cold saline and rapidly frozen in a bath of Drikold-methanol, they were then ground with a footed glass rod. The powder was homogenised in 1.2ml of 0.4M perchloric acid with a ground-glass tissue homogeniser. The homogenate was left to stand on ice for thirty minutes and then centrifuged at 1,000g (1,000 rpm) for 5 minutes in a Beckman benchtop centrifuge. The precipitate was washed and collected on a kieselguhr pad for scintillation counting as described in a later section.

The supernatant was titrated to neutrality with 0.6ml of 0.72M KOH in 0.16M $KHCO_3$. Again the samples were left to stand on ice for 10 minutes and then centrifuged, at 1,000g (2,000 rpm) in a Beckman benchtop centrifuge, to remove the $KClO_4$ precipitate. Samples of supernatant were removed for scintillation counting and used to estimate uptake of precursor into the acid soluble fraction.

The remaining supernatant was used for the determination of the specific activity of ATP.

The Specific Activity of Uterine ATP.

The specific activity of ATP was measured by the method of Eastabrook et al., (1967). ATP concentration was determined using a coupled enzyme reaction involving Hexokinase and Glucose-6-phosphate dehydrogenase. The reaction mixture contained the following:

0.1M Tris-HCl (pH 7.6)

0.1M $MgCl_2$

0.04M Glucose

0.04M NADP

and 10µl of enzyme mixture.

The resultant changes in NADPH concentration were measured by fluorimetry on an Aminco-Bowman spectrophotofluorometer using an excitation wavelength of 340nm and an emission wavelength reading of 460nm.

The radioactivity incorporated into ATP was determined by chromatographically separating ATP from other nucleotides on P.E.I. cellulose. The separating buffer was 0.85M KH_2PO_4 (pH 3.4). The chromatogram was dried and analysed by autoradiography to locate the labelled nucleotides. Spots corresponding to standard ATP, ADP, GTP and inorganic phosphate were cut out and analysed in a scintillation counter, using 5g/l diphenyloxazole in toluene as scintillant.

3.20. Acid Soluble and Acid Insoluble Fractions

Acid soluble and acid insoluble fractions were prepared by the method of Knowler and Smellie (1971). After incubation *in vitro* uteri were washed three times in ice-cold saline and frozen in a bath of Drikold-methanol. The uteri were broken into small pieces with a footed glass rod and homogenised in 2.5ml of ice-cold distilled water. The homogeniser was washed with a further 2ml of water and both homogenate and washings were added to 0.5ml of ice-cold 50% (w/v) trichloroacetic acid, with mixing. The precipitation was allowed to proceed for 15 minutes and the acid insoluble fraction was removed by centrifugation at 1,000g (1,000 rpm) for 15 minutes in a Beckman benchtop centrifuge. The supernatant was collected and the pellet was washed with a further 2ml of 5% (w/v) trichloroacetic acid. Samples were centrifuged as previously and the supernatants pooled. These supernatants comprised the acid soluble fraction and aliquots were removed for scintillation counting. The method of counting the supernatant, and that of the pellet or acid insoluble fraction is described in the following section.

3.21. Scintillation Counting

3.21.1. Aqueous Samples

300 µl of aqueous solutions were mixed with 2ml of methoxyethanol. Subsequently 8ml of 5g/l diphenyloxazole in toluene was added. The samples were well mixed by shaking and analysed in a Beckman LS 8100 scintillation counter.

3.21.2. Small Samples Containing Radioactive Proteins

Small quantities of the sample, in aqueous solution, were spotted onto Whatman 3MM paper discs and were left to stand for 15 minutes in cold 10% (w/v) trichloroacetic acid. These were then transferred to boiling trichloroacetic for another 15 minutes.

The discs were washed once with cold trichloroacetic acid, then with methanol, methanol:ether mixed in a 1:1 ratio, and finally ether. The discs were dried and analysed for radioactivity in 10ml of 5g/l diphenyloxazole in toluene using a Beckman LS 8100.

3.21.3. Large Acid Insoluble Precipitates

The pellets from acid insoluble analyses were mixed with 2ml of 2% (w/v) kieselguhr in 5% (w/v) trichloroacetic acid and collected, under vacuum, on a Whatman 3MM paper disc. The disc was washed with 15ml 5% (w/v) trichloroacetic acid three times, then with 15ml methanol and 15ml methanol ether mixed in a 1:1 ratio and finally twice with 15ml of ether. After washing with ether the pad was dried overnight at 60°C. One millilitre of hyamine hydroxide was then added and digestion allowed to proceed at 60°C for 16 hours. Finally 2ml of methoxyethanol and 8ml of 5g/l diphenyloxazole in toluene was added for scintillation counting.

3.22. Protein Measurements

Routine measurements of protein concentration used the method of Lowry et al., (1951). However β-mercaptoethanol interferes with this assay and the method of Bramhall et al., (1969) was used if this reagent was present.

3.23. RNA Measurements

Routine measurements of RNA concentration used the method of Kerr and Seraidarian (1945).

RESULTS

4.1. Isolation of Rat Uterine Polysomes and Ribosomal Proteins

4.1.1. Isolation of Polysomes

Initial work on this project centered on the preparation of uterine polysomes and a satisfactory procedure for preparing polysomes was required before their subsequent analysis could begin. High molecular weight polysomes from uteri are unstable, being susceptible to degradation by ribonuclease. Uterine ribonuclease levels are high (Greenman and Kenney, 1964; McGregor et al., 1981) and steps had to be taken to prevent the effects of this enzyme. One of the most potent ribonuclease inhibitors presently available is diethyl pyrocarbonate and this was included in all solutions used during polysome preparation. However, since diethyl pyrocarbonate causes total inactivation of almost all proteins, this inhibitor was excluded if polysomes active in protein synthesis were required. If active polysomes were required, heparin was used in the place of diethyl pyrocarbonate.

Figure 4 shows the effect of a single injection of oestradial-17β on the size of rat uterine polysomes. Figure 4 shows that few or no polysomes could be isolated from the unstimulated rat uterus. Evidence to be presented in Figure 5 will show that this profile reveals only ribosomal subunits and monomers. After a short period of oestrogen treatment (4 hours) a small number of low molecular weight polysomes could be isolated. With increasing duration of oestrogen stimulation there is an increase in both the size and number of polysomes. A maximal number of large polysomes could be isolated from the immature rat uterus after 12 hours of oestrogen treatment (Figure 4C). After this period the polysomes decreased in molecular weight until 24 hours after a single injection of oestrogen, when only monomers and

Figure 4

The Effects of Oestradiol-17β on the Size Distribution of Rat Uterine Polysomes.

Groups of six immature female rats were injected with 1µg of oestradiol-17β in saline at various times before death. Polysomes were prepared from the rat uteri and analysed on 15-40% sucrose density gradients as described in the Methods section (3.6.2. and 3.7.). The gradients were scanned at 260nm by upward displacement through a Gilford 240 spectrophotometer and the resulting profiles are presented in figure 4.

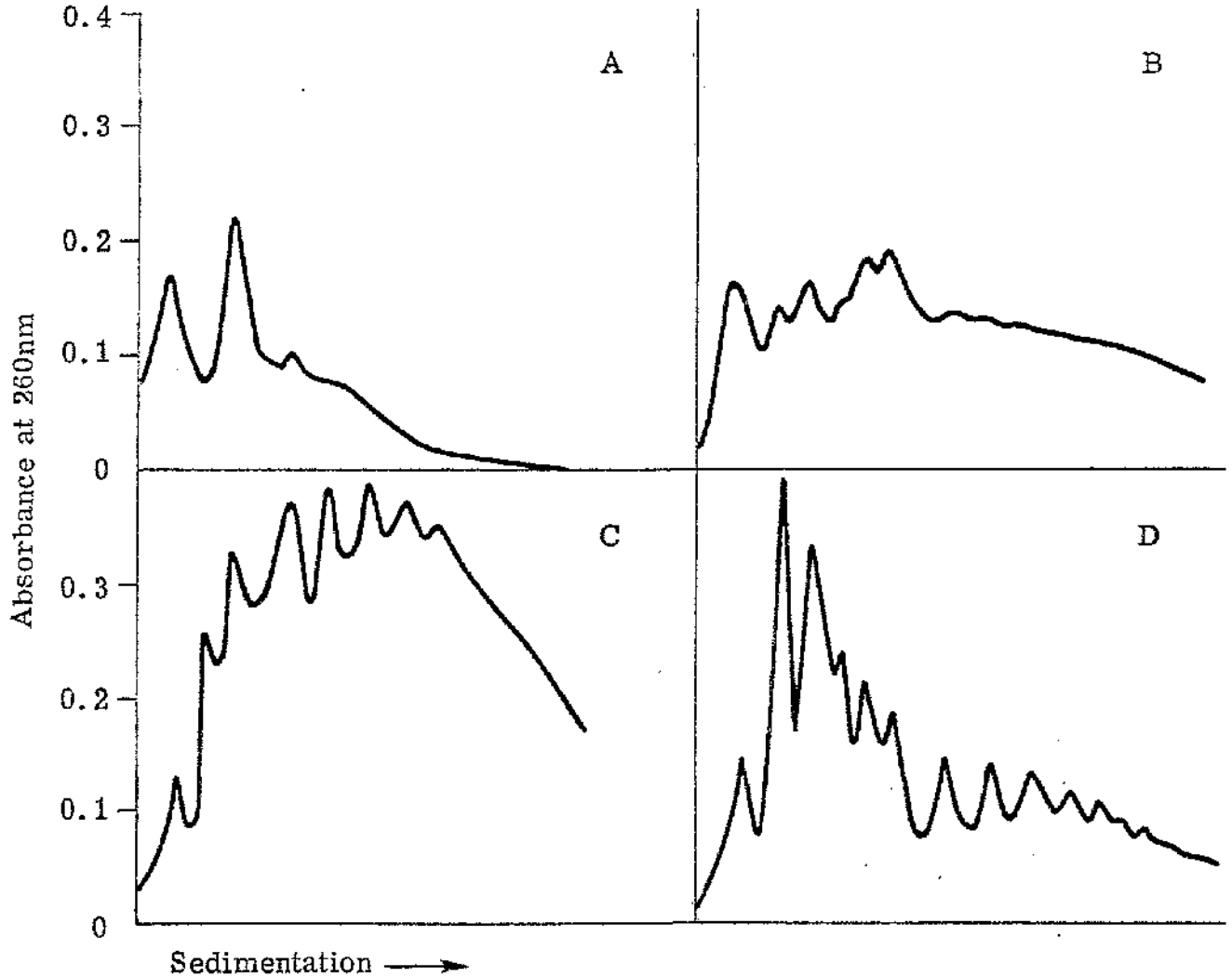
A. Polysomes from untreated uteri.

B. Polysomes from 4 hour treated uteri.

C. Polysomes from 12 hour treated uteri.

D. Polysomes from 16 hour treated uteri.

The Effect of Oestradiol-17β on the Size Distribution of Rat Uterine Polysomes



dimers could be isolated and the profile again resembles that in Figure 4A. The results shown in Figure 4 are supported by work at other times of oestrogen treatment although these results are not shown.

Since the polysomes were prepared in the presence of a detergent (Triton X100) it is highly unlikely that the aggregation seen with oestrogen treatment was of a non-specific nature. In addition, ribonuclease treatment of polysomes from 12 hour stimulated uteri resulted in a profile indistinguishable from that of unstimulated rat uteri (Figure 4A). It remained possible that the changing numbers of large polysomes isolated were due to changes in ribonuclease activity present in the uterine homogenate. McGregor et al., (1981) have demonstrated that ribonuclease activity changes with hormonal stimulation. However, these changes resulted in greater ribonuclease activity. Thus, it would appear that the changes shown in Figure 4 were due to increased assembly of polysomes and not due to decreased degradation. Figure 5 shows an analysis designed to identify the precise nature of the ribosomal peaks in the uterus of the immature rat. HeLa cell ribosomes were labelled to a high specific activity with [^{32}P]-inorganic phosphate. Subunits from these ribosomes were prepared on sucrose density gradients. Since subunits were totally separated on these gradients, it was possible to isolate 60S subunits free from contamination by 40S subunits and vice versa. Monomer ribosomes were also prepared on separate sucrose gradients in a low potassium ion concentration and in the absence of puromycin. In this case only one peak of absorbance was recorded from scanning the gradient. The labelled subunits (and monomers) were then added to preparations of polysomes from unstimulated rat uteri analysed on

Figure 5

The Co-fraction of Radioactively Labelled Ribosomal Subunits with Polysomes from Unstimulated Uteri

Radioactively labelled ribosomal subunits were prepared by the incubation of 2 burlers of HeLa cells with Eagle's medium containing 5 μCi/ml of [^{32}P]-inorganic phosphate for 24 hours at 37^{o}C. Polysomes were prepared from these cells (Methods 3.6.1.) and some of these polysomes were separated into subunits (Methods 3.8.). The remaining polysomes were also loaded onto a 15-40% sucrose density gradient under non-dissociating conditions, that is in the absence of puromycin or a high salt concentration. After centrifugation at 96,000g (27,000 rpm) for 4 hours at 28^{o}C the fractions which corresponded to 40S, 60S and 80S particles were isolated from the respective gradients.

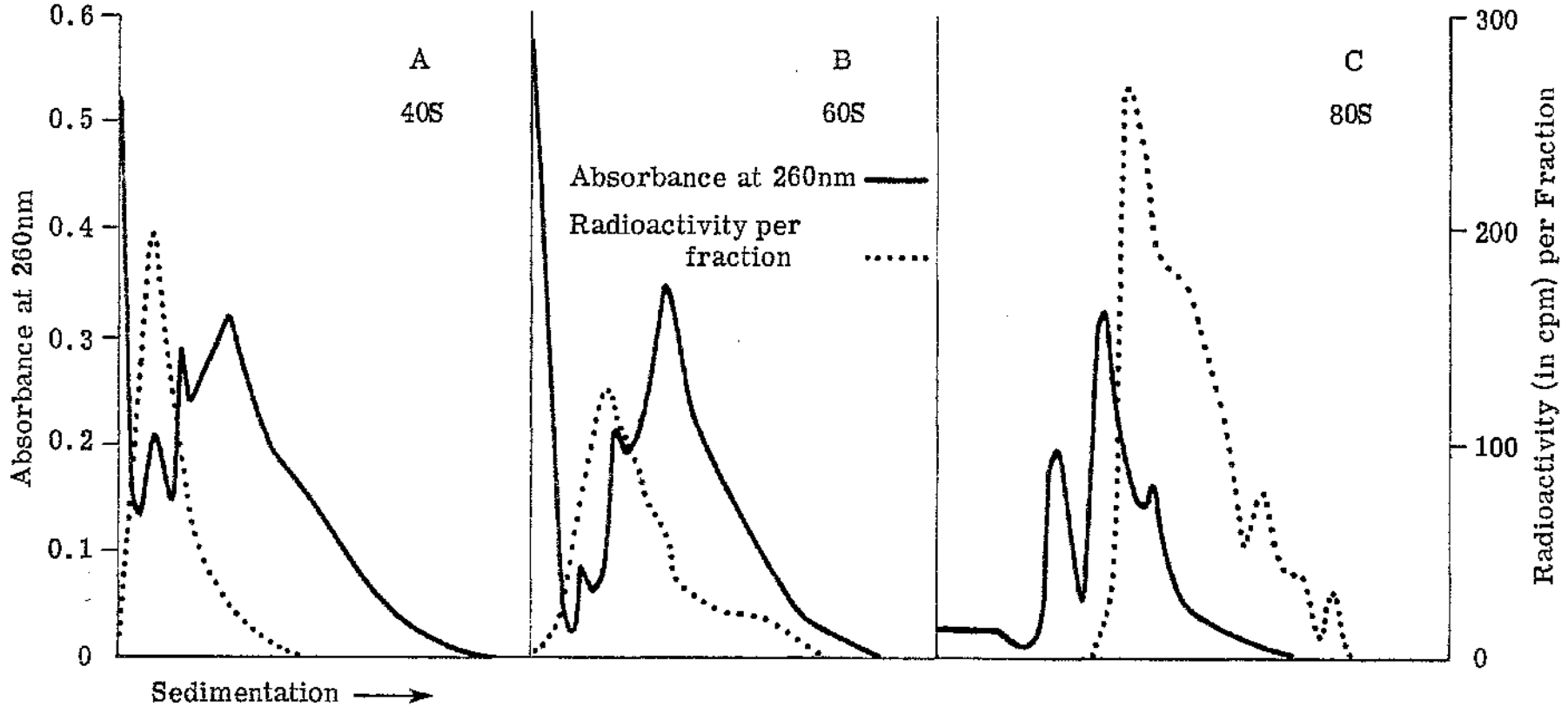
Polysomes from unstimulated rat uteri were also prepared and analysed as described in figure 4. However, just prior to analysis 1,000 counts per minute of radioactively labelled HeLa cell subunits, or monomers, were added to the polysomes. The sucrose density gradients were analysed after centrifugation at 234,000g (50,000 rpm) for 40 minutes at 4^{o}C by upward displacement through a spectrophotometer. The absorbance at 260nm and the [^{32}P]-radioactivity in the gradient are shown in figure 5 as follows:

A. Polysomes from unstimulated rat uteri co-fractionated with radioactively labelled 40S subunits.

B. Polysomes from unstimulated rat uteri co-fractionated with radioactively labelled 60S subunits.

C. Polysomes from unstimulated rat uteri co-fractionated with radioactively labelled monomer ribosomes.

The Cofractionation of Radioactively Labelled Ribosomal Subunits with Polysomes from Unstimulated Uteri



sucrose density gradients. The added HeLa cell ribosomes contained 2×10^3 cpm and less than 1µg of rRNA. This material did not therefore contribute to the A_{260} profile of the sucrose density gradient but could be measured by scintillation counting. By comparing the distribution of [^{32}P]-radioactivity and the uterine ribosomal profile (as an absorbance at 260nm) it was evident that the three peaks in increasing order of bouyant density in sucrose gradients corresponded to 40S, 60S and 80S ribosomal subunits.

4.1.2. The Separation of Ribosomal Subunits from Rat Livers

To study ribosomal proteins, it was necessary to remove from the ribosome nascent and adventitiously attached proteins. To achieve this, polysomal ribosomes were incubated with puromycin in the presence of a high potassium ion concentration. Puromycin, an inhibitor of protein synthesis, exerts its effect by mimicking an amino-acyl tRNA and combining with the nascent polypeptide. The ribosome then releases the peptide and this is accompanied by the separation of the two subunits. The separation is supported by the presence of a high potassium ion concentration. The ions also cause dissociation of non-ribosomal proteins that may be adventitiously attached to the subunits.

Figure 6A shows the absorbance profile at 260nm of a gradient loaded with ribosomes from an adult rat liver, in this case the total gradient was collected for preparation of total ribosomal proteins as an immunogen. This profile is accompanied by a photograph of a urea polyacrylamide gel loaded with total ribosomal protein prepared in this manner (Figure 7A).

4.1.3. Preparation of Ribosomal Subunits from Rat Uteri

Figure 6B shows a fractionation of uterine ribosomal subunits on a sucrose density gradient. The arrows (Figure 6B)

Figure 6

Fractionation of Ribosomes into 60S and 40S Subunits

Polysomes were prepared from the liver of one 200g male rat or the uteri of 12, 18-21 day old, immature female rats, as described in parts 3.6.2. and 3.6.3. of the Methods section. The polysomes were dissociated into subunits as described in part 3.8. of the Methods section and fractionated on 27ml, 10-30% sucrose density gradients by sedimentation at 96,000g (27,000 rpm) for 4 hours at 28°C. The gradients were analysed by downward displacement through a continuously reading spectrophotometer.

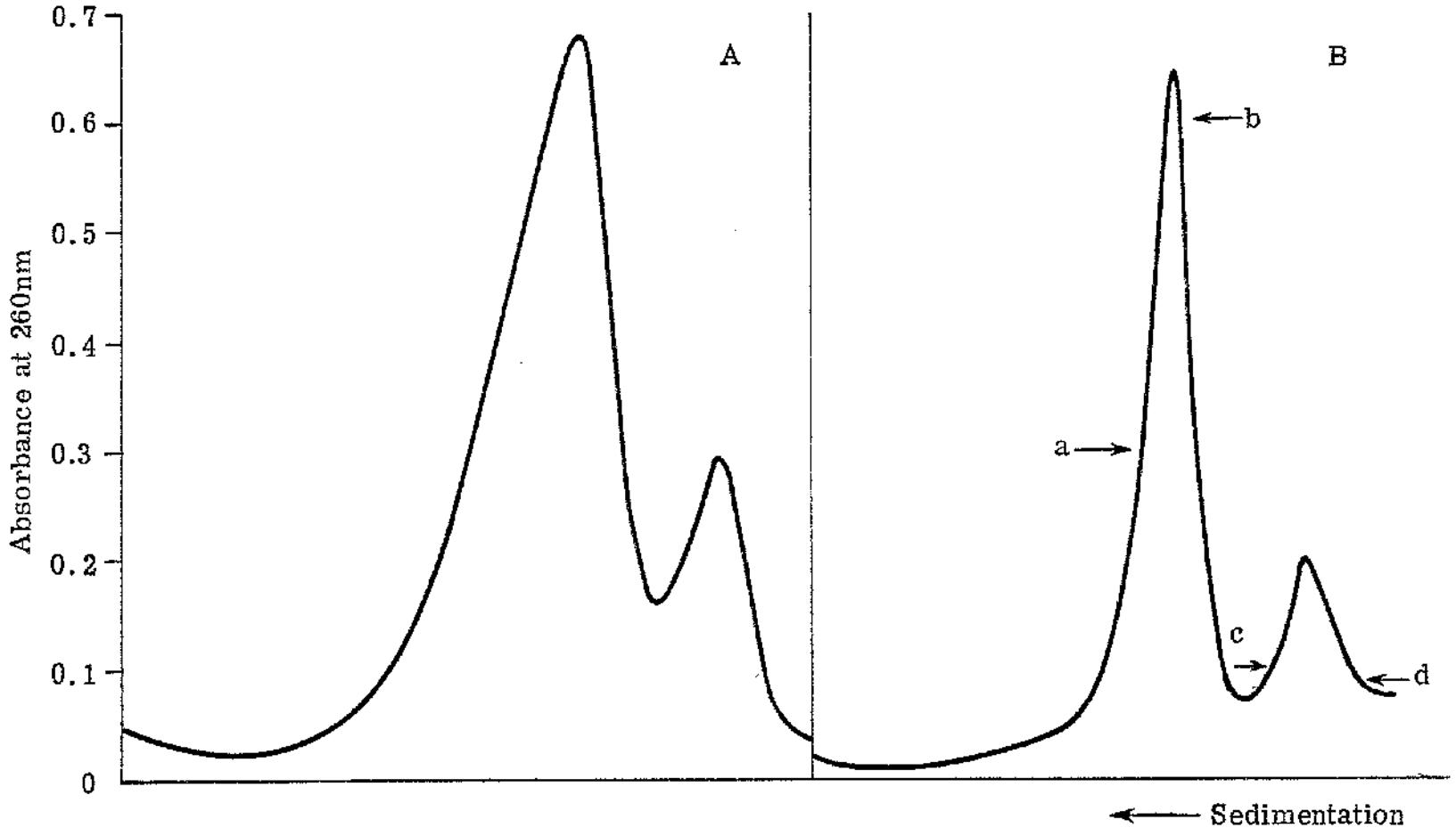
A. Rat liver ribosomal subunits.

B. Rat uterine ribosomal subunits. The arrows mark the portions of each peak collected for subsequent analysis,

a - b = 60S

c - d = 40S.

The Fractionation of Ribosomes into 60S and 40S Subunits



indicate the portions of the 60S and 40S peaks which were collected for the preparation of ribosomal protein. The trailing edge of the 60S peak was likely to be contaminated with 40S dimers and was discarded. The beginning of the 60S peak was also discarded as this could be contaminated with undissociated ribosomes.

Figure 7B is a photograph of a typical gel fluorogram of radioactively labelled ribosomal proteins from immature rat uteri. Since it was not possible to isolate sufficient rat uterine ribosomal protein for urea polyacrylamide gel fractionation, the efficient recovery and fractionation of uterine ribosomal protein required the addition of unlabelled carrier protein. Routinely, 800μg of rat liver ribosomal protein, as subunits, was mixed with the subunits isolated from 12 rat uteri prior to protein extraction. The liver proteins were identified by staining with coomassie blue while the profile of radioactive spots is due to the uterine proteins.

Although the evidence in Figures 7A and B demonstrated that ribosomal proteins could be isolated, it did not effectively show that they were uncontaminated. Most contaminating non-ribosomal protein would not be basic and therefore would not appear on the gel system employed. However, Figure 7C shows that when ribosomal subunits were co-fractionated with proteins labelled by cell free translation, the non-ribosomal proteins were left on the top of the sucrose density gradient.

4.1.4. Labelling of Ribosomal Protein in vitro

M.J. Merryweather, of this laboratory, has previously shown that uterine ribosomal protein synthesis in vivo was stimulated after oestrogen administration to immature female rats. He also made the preliminary observation that the stimulation of ribosomal

Figure 7

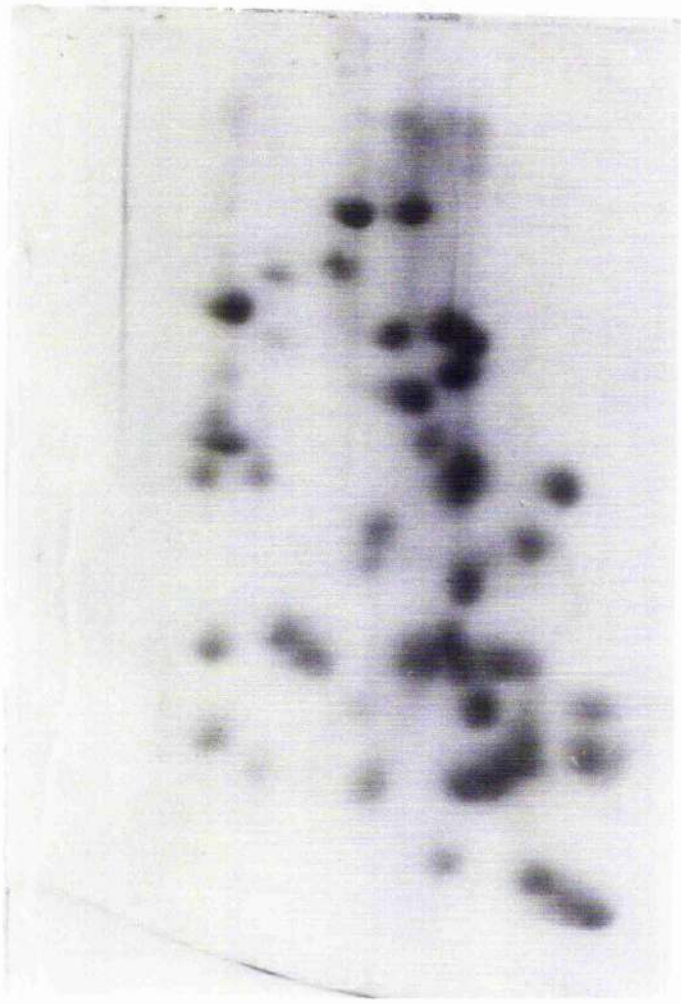
The analysis of Ribosomal Proteins on Urea-Polyacrylamide Gels

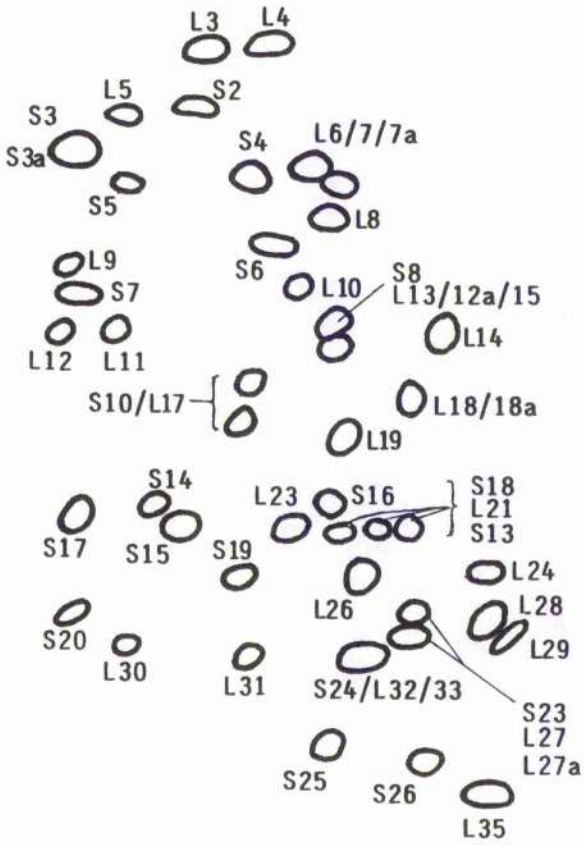
Ribosomal subunits from rat livers and rat uteri were prepared as described for figure 6. The proteins were extracted from the subunits (Methods section 3.9.) and precipitated with ice-cold trichloracetic acid.

The proteins were analysed by electrophoresis at 3 mA per gel for 3 hours in the first separation and at 16 mA per gel for 16 hours in the second separation. Electrophoresis was toward the anode. The stained pattern of rat liver proteins (a) and the fluorographic analysis of rat uterine proteins (b) labelled with radioactive methionine (described in detail in figure 17) are presented with a diagramatic representation of proteins. The numbering of these proteins is according to McConkey et al., (1979).

Figure 7C shows the co-fractionation of unlabelled uterine ribosomal subunits with 2,000 cpm of non-ribosomal proteins labelled by cell free translation (Methods section 3.14.). The subunits were prepared and analysed by the methods described for figure 6 and radioactivity was measured in 1ml fractions.

A

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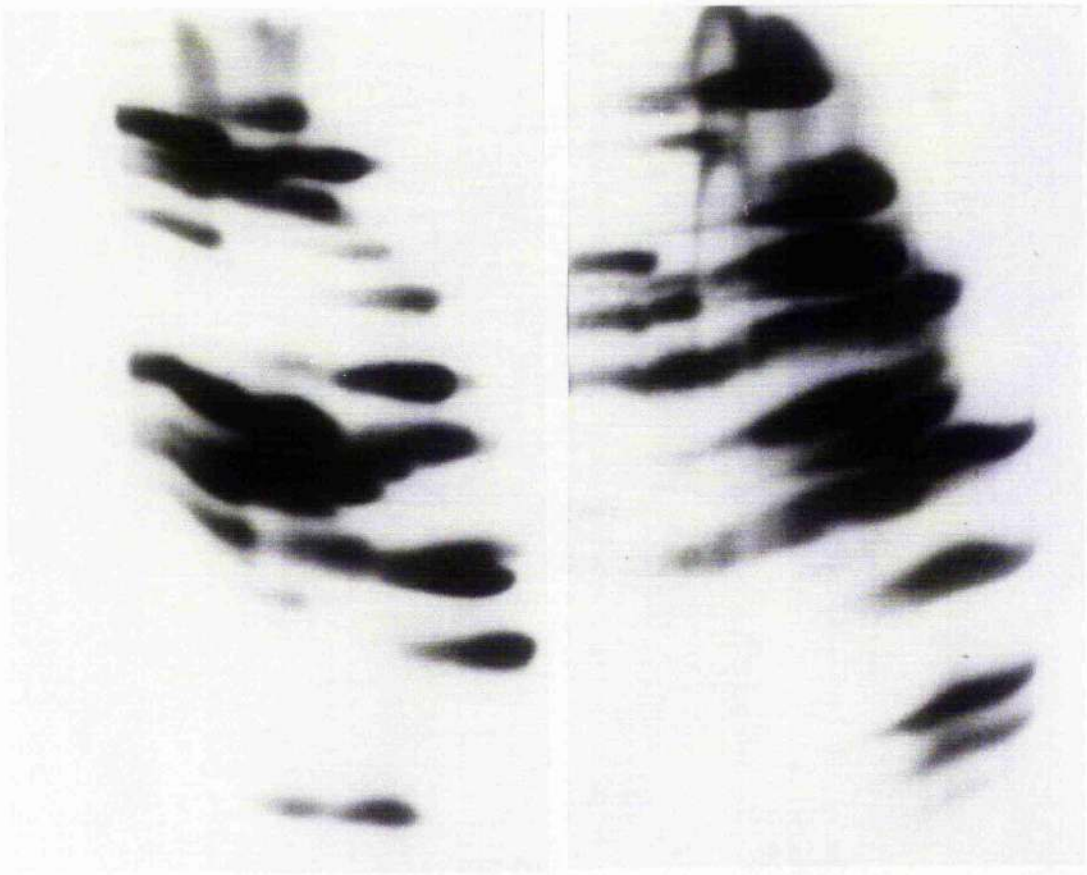

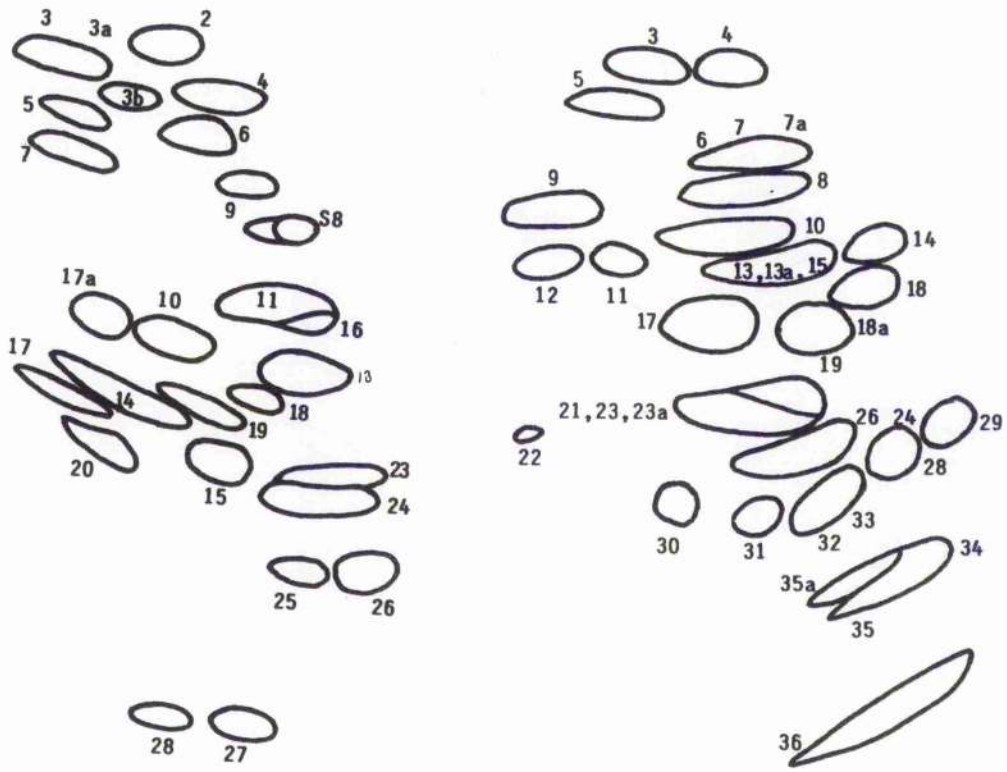
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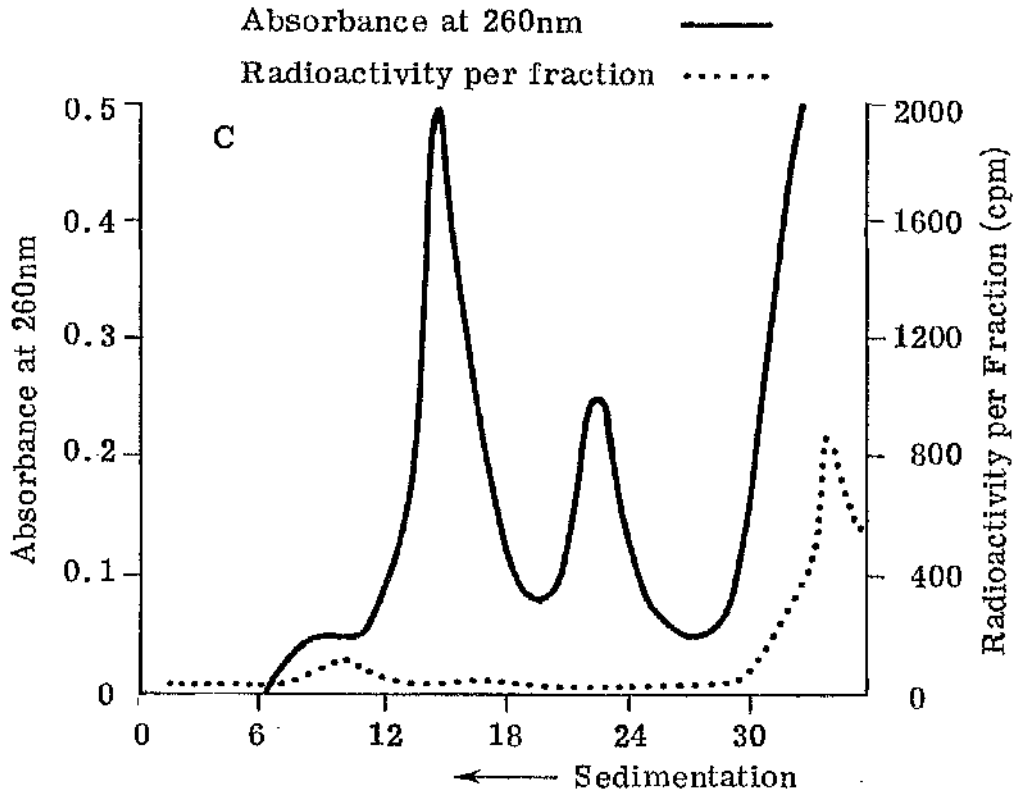
Cofractionation of Ribosomal Subunits with Radioactivity Labelled Non-Ribosomal Proteins



protein synthesis could be observed in vitro if uteri from oestrogen treated rats were incubated with radioactive precursor. This finding raised the possibility of labelling proteins to sufficient specific activity for investigating the synthesis and modification of individual proteins. Previous attempts, by Dr. Merryweather, to perform these experiments in vivo were not successful.

Figure 8 shows the specific activity of ribosomal proteins isolated from uteri which had been incubated in vitro for various lengths of time after excision from hormone treated rats. All rats had been treated with oestrogen for the same 12 hour period prior to incubation. The incorporation of radioactivity into uterine ribosomal proteins increased with the length of incubation period over $1\frac{1}{2}$ hours. After $1\frac{1}{2}$ hours the rate of incorporation and the total amount of recoverable radioactivity fell. An interesting and unexpected feature of these results was that the incorporation of radioactivity into both subunits was almost identical. In view of the earlier appearance of 40S subunits in the cytoplasm a faster incorporation of radioactivity into these subunits might have been expected. However, these results do not stand in isolation and will be discussed in more depth later.

Figure 9 shows the polysome profiles of twelve hour stimulated uteri incubated in complete Eagle's medium for various lengths of time. Even after half an hour of incubation extensive degradation of polysomes was evident. By one hour of incubation almost no polysomes were present. All subsequent incubations in vitro were limited to one hour to minimise the effects of degradation.

Since the option of increasing the incorporation of radioactive label into ribosomal proteins by increasing the

Figure 8

The Specific Activity of Ribosomal Proteins Labelled In Vitro for Various Lengths of Time

Four groups of 12 immature female rats were injected with 1 µg/rat of oestradiol-17β in saline, 12 hours before death. The uteri were removed to incomplete Eagle's medium and incubated with 50 µ Ci/ml of L[4,5-^{3}H]-leucine for varying periods of time. After incubation, polysomes were prepared and separated into subunits as described in the Methods section (3.6.2. and 3.8.). The ribosomal subunits were collected by centrifugation for 16 hours in a 60Ti rotor at 254,000g (50,000 rpm) at 4^{o}C. Typical recoveries of ribosomal protein were between 75 µ g and 100 µg from 12 uteri.

Specific Activity of Ribosomal Proteins
against Time of Labelling in vitro

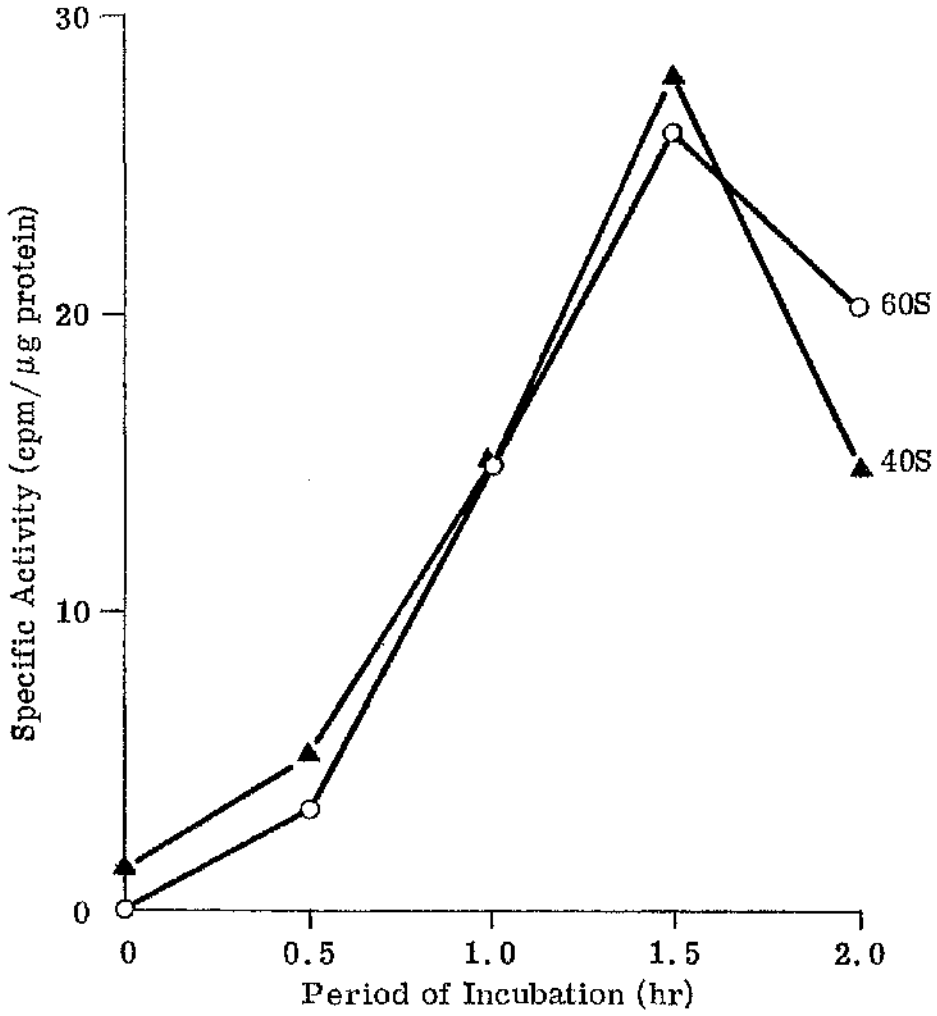


Figure 9

Polysome Profile Analysis of Uteri Incubated In Vitro for Different Periods of Time

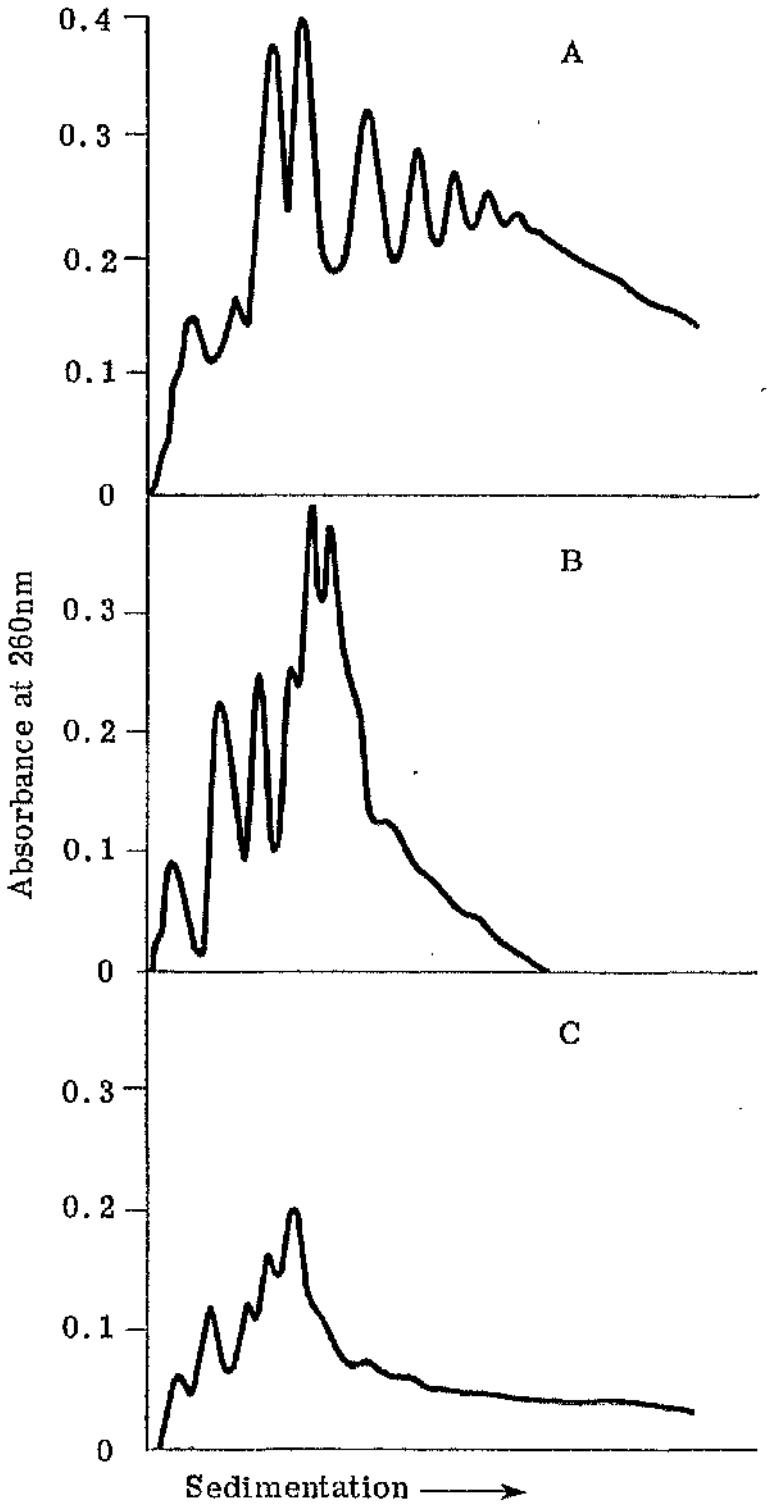
Three groups of 3 immature female rats were injected with 1 µg/rat of oestradiol-17β 12 hours before death. The uteri were then removed to complete Eagle's medium and incubated for various periods of time. After incubation, polysomes were prepared and analysed on 15-40% sucrose density gradients (Methods sections 3.6.2. and 3.7.). The gradients were scanned, using a Gilford 240 spectrophotometer, at 260nm.

A. Polysome profile from 12 hour stimulated rat uteri - not incubated.

B. Polysome profile from 12 hour stimulated rat uteri - incubated, for a further $^{1}/2$ hour, in vitro.

C. Polysome profile from 12 hour stimulated rat uteri - incubated, for a further 1 hour in vitro.

Polysome Profile Analysis of Uteri Incubated in vitro for Different Periods of Time



incubation time was not available, other methods were sought. Cutting uteri into 5 pieces produced no increase in the specific activity of labelled proteins. Cutting each uterus into 1 millimeter segments resulted in a decreased incorporation of label. Slicing the uteri laterally did result in some increase in incorporation and, because this could be performed quickly, was used routinely in subsequent experiments. Incubation of uteri with cold leucine added to a concentration of three times that of labelled leucine produced an equivalent drop in incorporation. Use of 90% phosphate Eagle's medium (instead of totally deficient medium) with [^{32}P]-inorganic phosphate resulted in no incorporation. The details of these results are not shown.

4.2. The Effects of Oestrogen on Ribosomal Protein Synthesis

4.2.1. The Effect of Oestrogen on the Incorporation of Radiolabelled Precursor into the Proteins of Ribosomal Subunits

Using the incubation procedure discussed above, uteri, subjected to various degrees of stimulation by oestrogen, were analysed. Figure 10A shows the changes in specific activity of ribosomal proteins after oestrogen stimulation of uteri *in vivo* followed by a 1 hour incubation *in vitro* with radiolabelled leucine. The control value (unstimulated) was an average of two separate determinations. The time intervals of treatment were chosen as four hours to give good coverage of the 24 hour period over which the effects of oestrogen are most noticable.

Oestrogen stimulated the incorporation of radioactive leucine into ribosomal proteins. The stimulated incorporation was maximal at 12 hours after oestrogen treatment, being 5-7 times the

Figure 10

The Effects of Oestrogen on Uptake of Precursor into Ribosomal Protein, Total Cell Protein and Precursor Pools

a) Ribosomal Protein Synthesis:

Nine groups of 12 immature female rats were injected with 1 µg/rat oestradiol-17β at various times before death. The uteri were then excised and incubated for 1 hour at 37°C in leucine-deficient Eagle's medium containing 50 µCi/ml L[4,5-^{3}H]-leucine. After incubation, the uteri were used to prepare polysomes and thereafter ribosomal subunits. The specific activity of the subunits was determined as cpm per µg of ribosomal protein and is presented here as a percentage of control values. Typical recoveries ranged from 10 µg of protein from unstimulated uteri to 100 µg from 16 hour stimulated uteri.

B. Total Uterine Wet Weight and Precursor Pools

Groups of 3 immature female rats were treated in a manner similar to that described above. However, from these rats, uterine acid soluble and acid insoluble fractions were prepared and analysed as described in the Methods section (3.20.).

The relative wet weights were determined by weighing groups of 12 rats and their excised uteri. The uterine weight was divided by the total body weight and expressed as a percentage of control values.

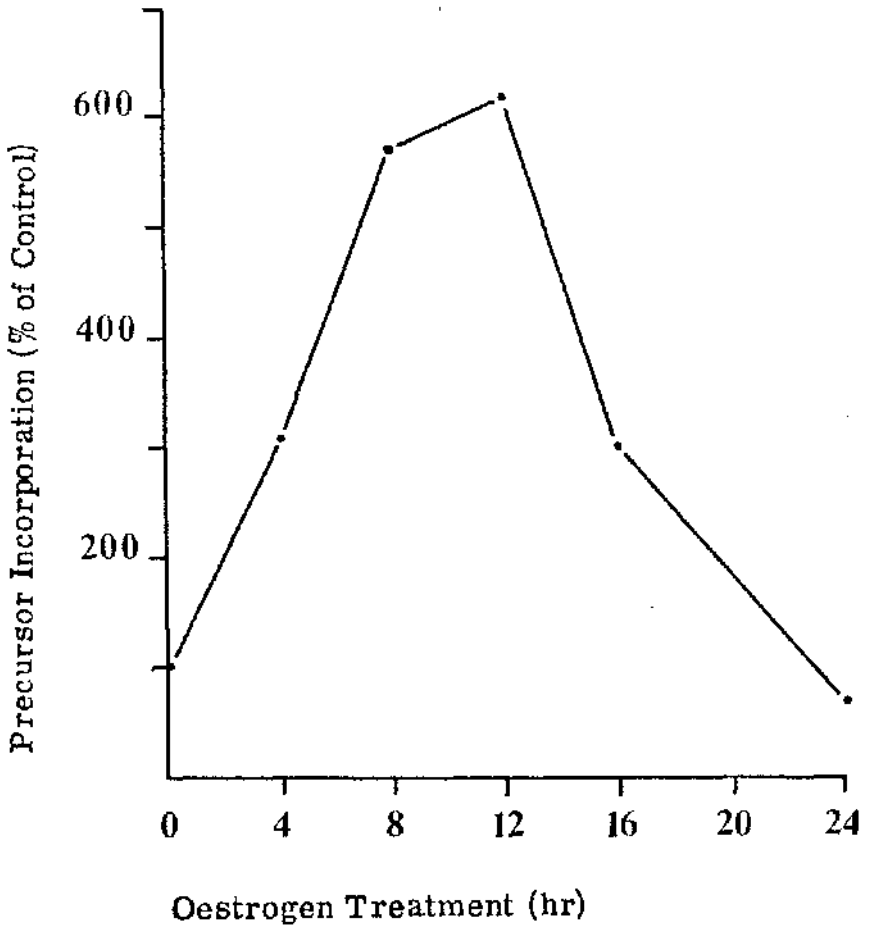
For reasons of clarity, standard deviations have not been included in the figure but each data point represents a mean of at least 3 groups of rats and the standard deviations ranged as follows:-

Time	Soluble	Insoluble
0 hours	77%	35%
12 hours	20%	5%

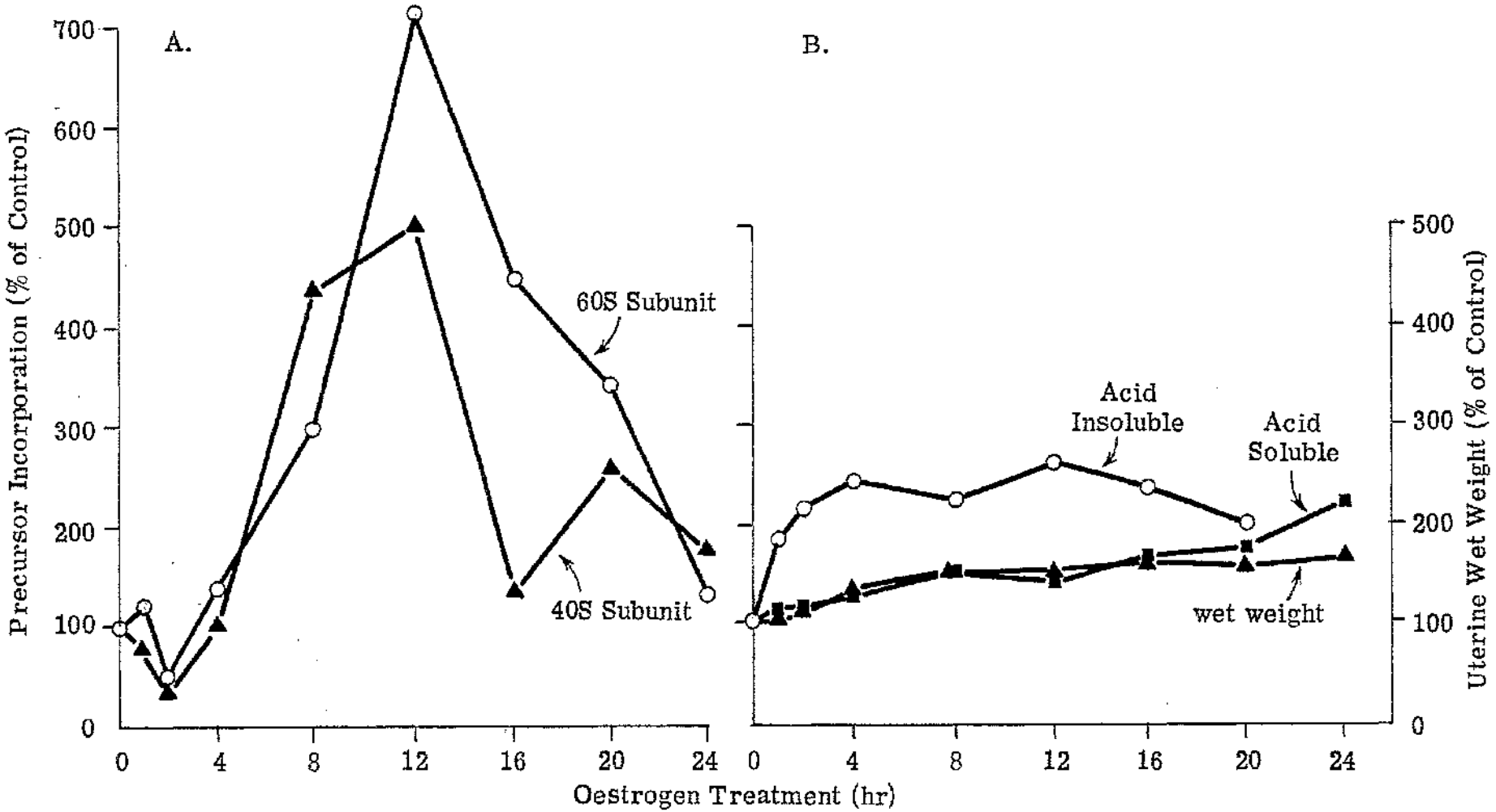
Figure 10C

The Effects of Oestrogen on Uptake of Precursor into Ribosomal Protein In Vivo

Six groups of 12 immature rats were injected with 1µg/rat oestradiol-17β at various times before death. Incorporation of newly synthesised ribosomal proteins into uterine polysomes was estimated as described in section a), by measurement of incorporation into purified ribosomal subunits of [4,5-^{3}H]-leucine administered at 100 µCi/rat 1 hour before death. These data are taken from Merryweather and Knowler (1980).



The Effects of Oestrogen Treatment on Uptake of Precursor into Ribosomal Protein, Total Cell Protein, and Precursor Pools



rate of incorporation into proteins of the unstimulated uterus. Afer 12 hours the rate of incorporation of radioactivity decreased until, by 24 hours, it had almost returned to control levels. It was evident that at most time points the rate of incorporation into 60S subunits was equal to or greater than that of the 40S subunits. Further discussion of this aspect may be found later.

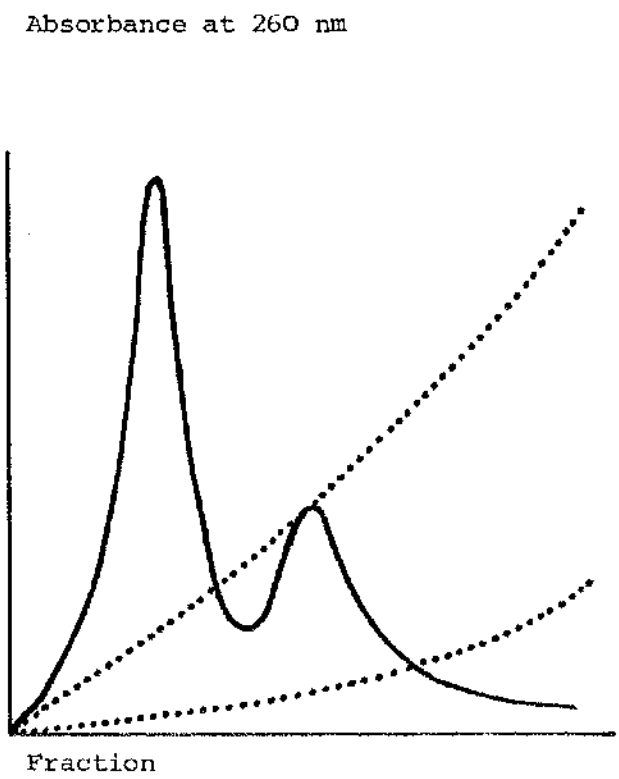
Since these results were obtained by analysis in vitro it is of value to compare them with the results obtained by Merryweather and Knowler (1980) after labelling in vivo. Figure 10C reproduces their data for comparative purposes and it can be seen that the time courses in vivo and in vitro are very similar.

The only significant difference between the two results was that the stimulated incorporation in vivo was initiated at an earlier time than that in vitro. Thus at 2-4 hours there was a significant increase in incorporation of radioactivity which was not seen in the analysis in vitro.

It is also useful to compare the incorporation of radioactivity into precursor pools with incorporation into ribosomal proteins. This may be done in two ways. First, an analysis of radioactivity in acid soluble pools should reveal any changes in the intracellular levels of leucine which could explain the increased uptake of the precursor. Alternatively, the increased incorporation into ribosomal proteins could merely be one facet of a general stimulation of protein synthesis. Analysis of incorporation of radioactivity into acid precipitable material would reveal if this were the case. Figure 10B shows the changes in incorporation of [^{3}H]-leucine into acid soluble and acid insoluble fractions in uteri treated with oestrogen for various times before labelling in vitro. If the stimulated incorporation of

radioactivity in ribosomal proteins were due to changes in the intracellular concentration of leucine, this would be reflected in a measurement of total uterine radioactivity soluble in acid. Clearly, this is not the case, as acid soluble radioactivity does not show a peak but a slow increase towards 24 hours of oestrogen treatment. By this time uptake is only twice that of control uteri. Interestingly, increased acid soluble radioactivity closely follows the increase in relative wet weight of uteri. The increases in labelling of the acid insoluble fraction are more difficult to analyse since this fraction accounts for a large variety of different molecules. It is clear that oestrogen treatment of the rats does stimulate total protein synthesis *in vitro* but to a smaller extent and with different kinetics to the stimulation of ribosomal protein synthesis.

It was realised that the results obtained in Figure 10A could be due to an artefact brought about by a spillover of precursor incorporation into nascent protein. Thus, in the separation of subunits, nascent protein could be separated to remain near the top of the gradient, as revealed earlier in Figure 4C. Nevertheless, it could be in the form of a peak with a long tail extending into the area of the subunit peak. The diagram below demonstrates this hypothetical situation.



Possible distribution of nascent protein on gradients of ribosomal subunits from oestrogen treated rats.

Possible distribution of nascent protein on gradients of ribosomal subunits from control rats.

Since only the subunit fractions were analysed in Figure 10A a label distribution as indicated above would lead to the assumption that synthesis of ribosomal proteins was stimulated. To exclude this possibility the effect of oestrogen was re-examined in all fractions of the gradient in which subunits were separated. The results of these analyses for oestrogen treated rats (killed 12 hours after injection) and for untreated rats are shown in Figure 11.

Figure 11 demonstrates that although there was a small background level of precursor throughout the gradient, this was insignificant compared to the amount of labelled protein found in the ribosomal subunit peaks. The background is most noticeable in the gradient separating subunits from control rats since the peaks of radioactivity corresponding to the subunits, were small. However, there was little or no increase in the background of gradients separating subunits of oestrogen treated rats, despite the considerable increase in radioactivity corresponding to the subunit peaks.

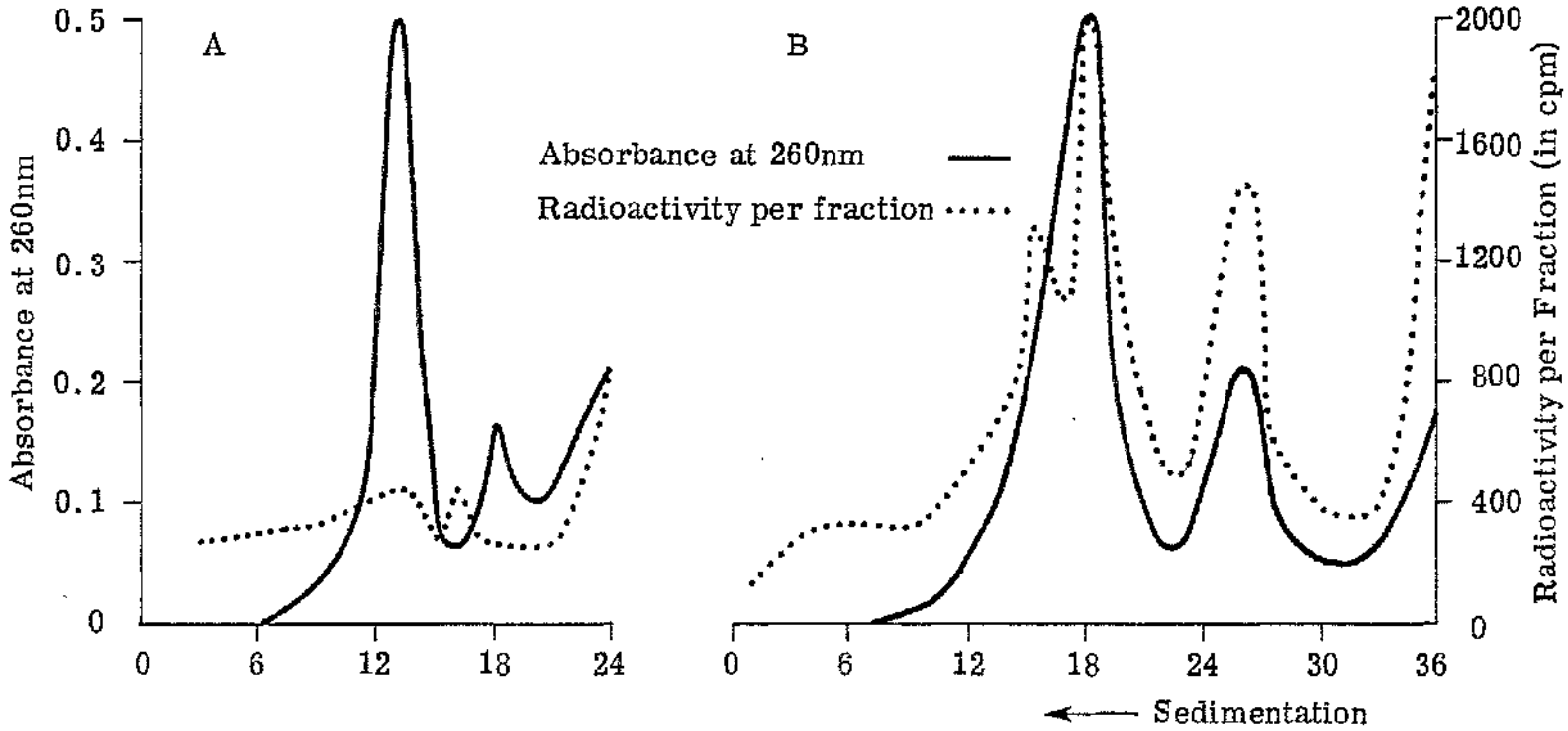
Figure 11

The Distribution of Precursor Incorporation in Ribosomal Subunits Fractionated on Sucrose Density Gradients

A. The uteri from 12 immature female rats were excised and removed to 2ml of leucine-deficient Eagle's medium. This medium contained 50 μCi.ml of L[4,5-^{3}H]-leucine and the uteri were incubated at 37^{o}C under 95% O_2:5% CO_2 for 1 hour. Uterine polysomes were isolated and used to prepare ribosomal subunits by fractionation on sucrose density gradients (Methods sections 3.6.2. and 3.8.). The gradients were continuously monitored at 260nm during downward displacement through the flow-through cell of a Gilford 240 spectrophotometer. Radioactivity was determined in 1ml fractions.

B. Twelve rats received 1 μg/rat of oestradiol-17β, 12 hours before death. All subsequent analysis was as above.

The Distribution of Precursor Incorporation into Ribosomal Subunits Fractionated on Sucrose Density Gradients



4.2.2. The Effect of Oestrogen on the Synthesis of Individual Ribosomal Proteins

Having studied the synthesis of total subunit proteins *in vitro*, it was decided to extend this procedure to an analysis of individual proteins. As a preliminary experiment, proteins labelled by incubation *in vitro* with [^{35}S]-methionine were separated on a sodium dodecylsulphate polyacrylamide gel with the hope of extending the procedure to 2 dimensional gel analysis later. The advantages in the former method of analysis lay in the efficiency of the gel system which could be used to study very small quantities of protein. In addition the ribosomal proteins did not require prior extraction (with its inevitable losses) that was needed for gels that did not contain sodium dodecylsulphate. Figure 12 shows the ribosomal proteins of subunits from unstimulated and 12 hour stimulated rats labelled *in vitro*. Again these time points were selected to show maximum differences in incorporation. In the case of the unstimulated rats there was inadequate incorporation of radioactivity for detection in this system. In fact there is only one band, barely visible, in the analysis of 60S proteins. (This band is not detectable in the photograph of Figure 12 but its position is arrowed.) The two lanes containing ribosomal proteins from rat uteri treated for 12 hours with oestrogen show strong labelling. There are at least 14 bands visible in the 40S preparation and at least 22 visible in the 60S group. Some proteins are represented by very dark bands while other bands are much fainter. This was the first indication that the labelling of ribosomal proteins was not uniform.

The experiment presented in Figure 11 also revealed that 50 μCi/ml of [^{35}S]-methionine in the incubation medium would not

Figure 12

The Effects of Oestrogen on the Incorporation of Radioactive Methionine into Individual Ribosomal Proteins In Vitro 1) Separation in One Dimension.

A group of 12 immature female rats received 1μ g/rat oestradiol-17β 12 hours before death, whilst a second control group of animals received saline only. The uteri from these animals were incubated in 2ml of methionine deficient Eagle's medium containing 50 μCi/ml of L[^{35}S]-methionine at 37°C in an atmosphere of 95% O_2:5% CO_2 for 1 hour. After incubation, polysomes were prepared from the uteri and the subunits of these polysomes were isolated (Methods sections 3.6.2. and 3.8.).

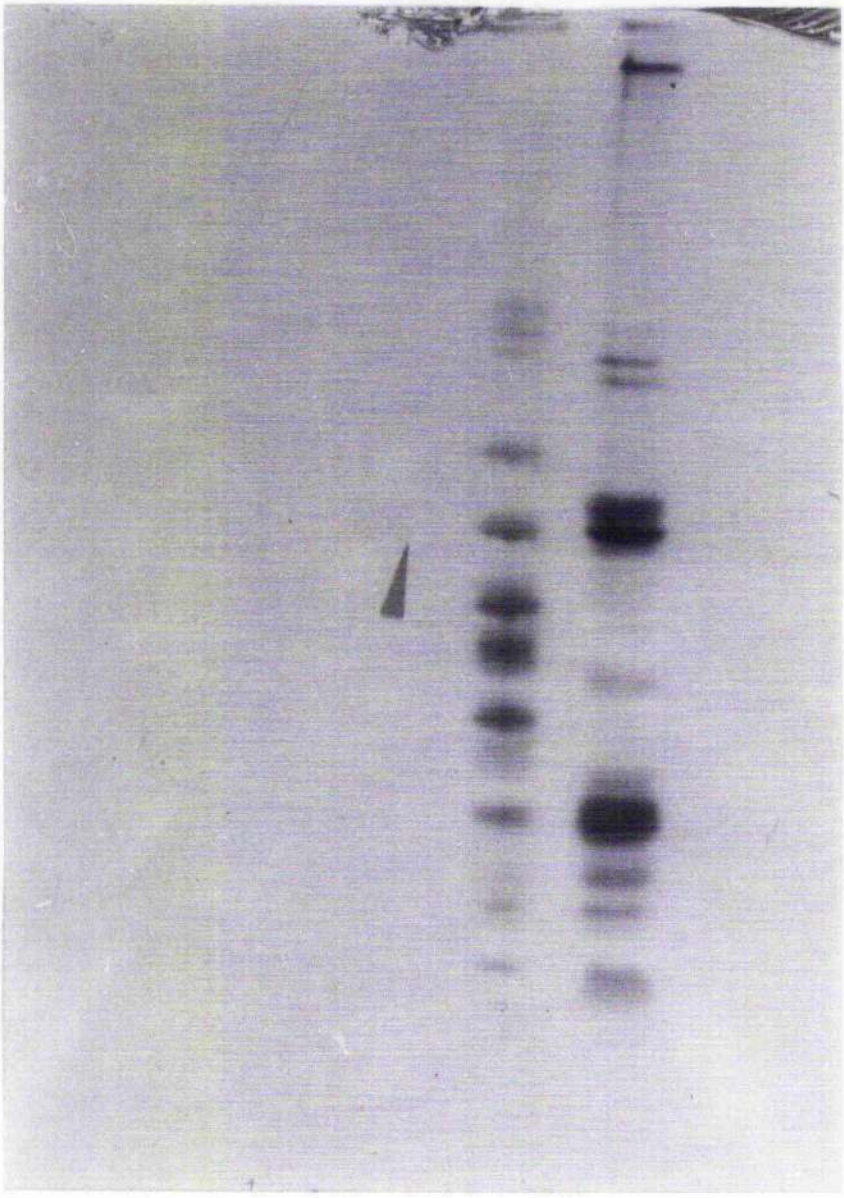
Subunits containing 100μg of ribosomal protein were analysed by gel electrophoresis on sodium dodecylsulphate polyacrylamide gels (Methods 3.12.1.) and the proteins were identified by staining with Coomassie blue and by fluorography (Methods 3.13.). Figure 12 is a photograph of this fluorogram.

Lane A, separated 40S proteins from unstimulated uteri. There are no bands visible.

Lane B, separated 60S proteins from unstimulated uteri. One band is visible in the fluorograph and its position is arrowed in the figure.

Lane C, separated 40S proteins from oestrogen stimulated uteri.

Lane D, separated 60S proteins from oestrogen stimulated uteri.



Lane A B C D

be enough for 2 dimensional gel analysis. An attempt was made to analyse proteins from uteri incubated with 250 μCi/ml. Whilst this resulted in ample labelling of the proteins of 12 hour time points, very little appeared in the proteins of control or 2 hour times. The use of 500 μCi/ml resulted in significant labelling of all subunits except those of the 40S subunit at control times. In order that the use of such large quantities of [^{35}S]-methionine could be justified in experiments with multiple time points, the radioactive amino acid was prepared from E. coli grown on medium containing 20 mCi of sodium [^{35}S]-sulphate and isolated by the method used by Amersham International to prepare the commercially available precursor (see Methods section).

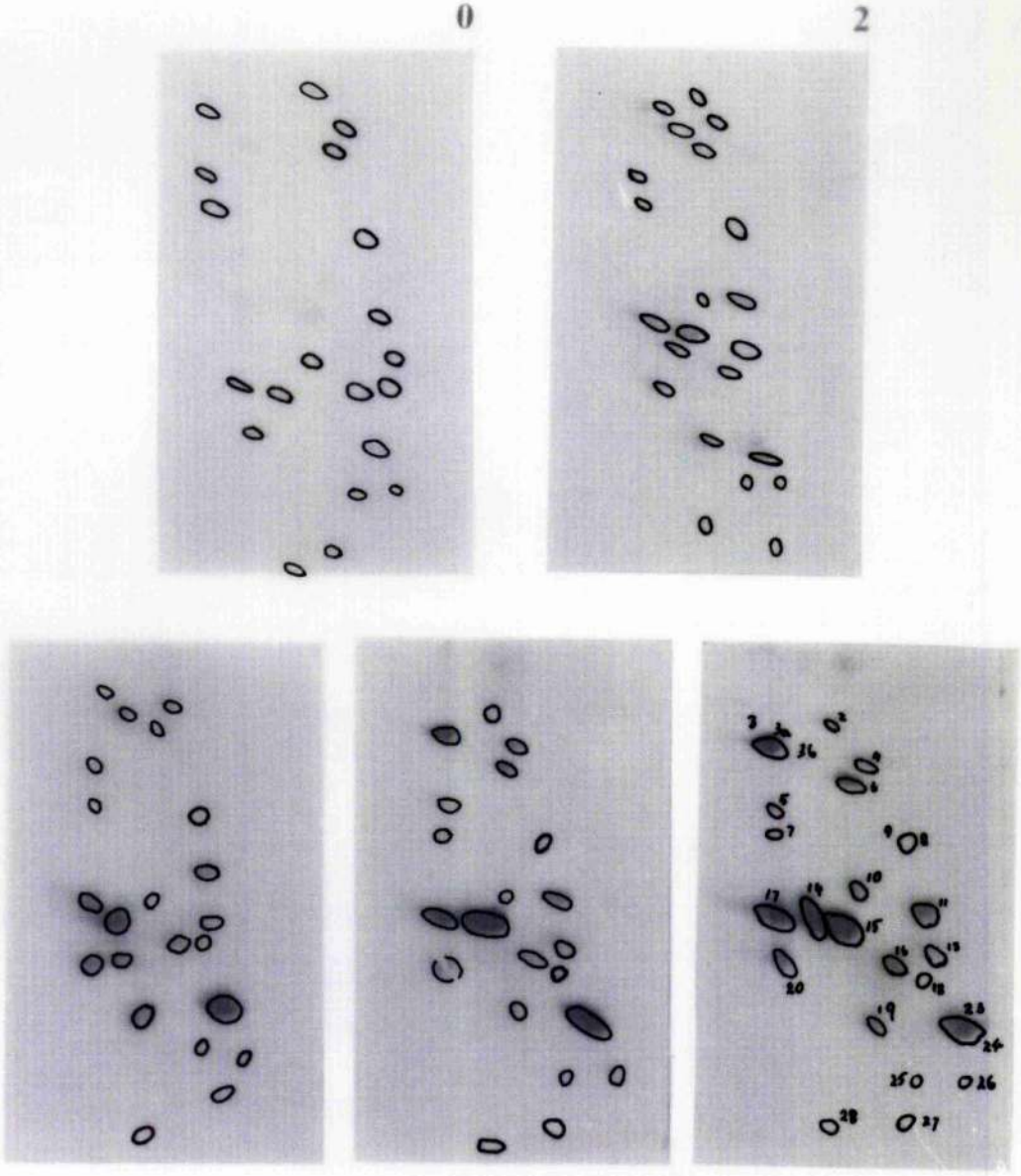
The effect of oestrogen on the labelling of individual ribosomal proteins from the small and the large subunit are shown in Figures 13 and 14 respectively. The periods of oestrogen stimulation were 2, 4, 8 and 12 hours, with a control zero hour time point. All the time points were repeated at least once, (the 12 hour time point twice) and the control was performed on at least six separate occasions. In all cases the results were completely reproducible. After fluorography, the proteins in the gel were located by aligning the fluorogram with the stained liver carrier protein. The protein spots were cut from the gels and digested for scintillation counting. The radioactivity recovered from the gels, in counts per minute, is shown in Tables 3A and 3B. Also presented in the table is the percentage of methionine found in each of the proteins, this data being derived from 1) Collatz et al., (1976); 2) Tsurugi et al., (1976); 3) Collatz et al., (1977); 4) Tsurugi et al., (1977).

Figure 13

The Effects of Oestrogen on the Incorporation of Radioactive Methionine into Individual Ribosomal Proteins In Vitro 2) Separation in Two Dimensions.

Groups of 12 immature female rats received 1 µg/rat of oestrogen at various times before death, whilst one group of 12 received carrier only. The uteri from these animals were incubated with 500 µCi/ml of L[^{35}S]-methionine in methionine-deficient Eagle's medium at 37^{o}C under 95% O_2: 5% CO_2 for 1 hour. Ribosomal subunits were then prepared from the purified uterine polysomes as described in the Methods (sections 3.6.2. and 3.8.). Proteins were extracted from the ribosomal subunits and fractionated on urea-polyacrylamide gels, these gels separated proteins in 2 dimensions (Lastick and McConkey, 1976). After staining and drying the gels were analysed by fluorography for 40 days.

Figure 13 shows the effects of oestrogen on the incorporation of radioactive methionine into individual ribosomal proteins of the small subunit in vitro.



Oestrogen treatment (hr)

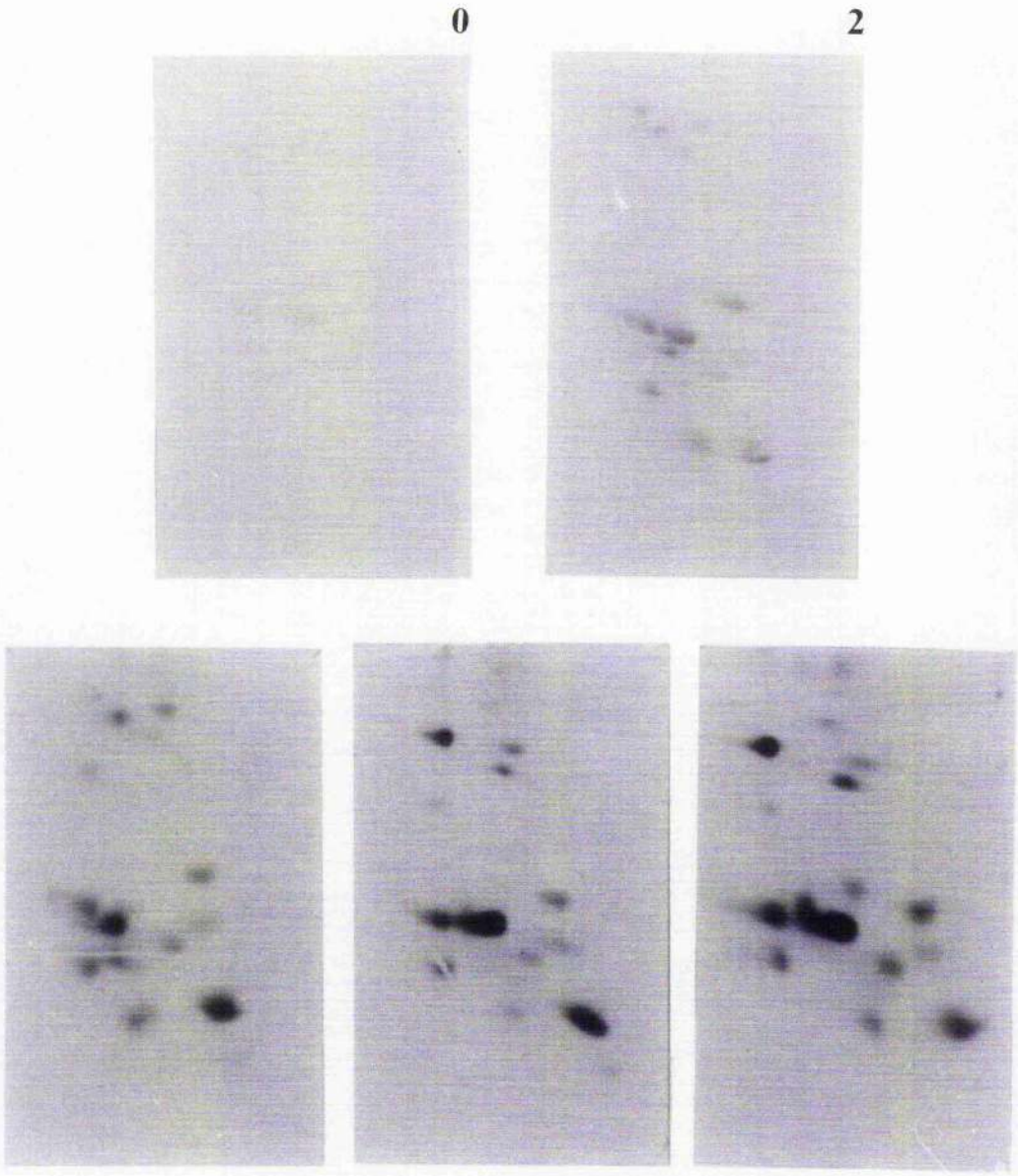
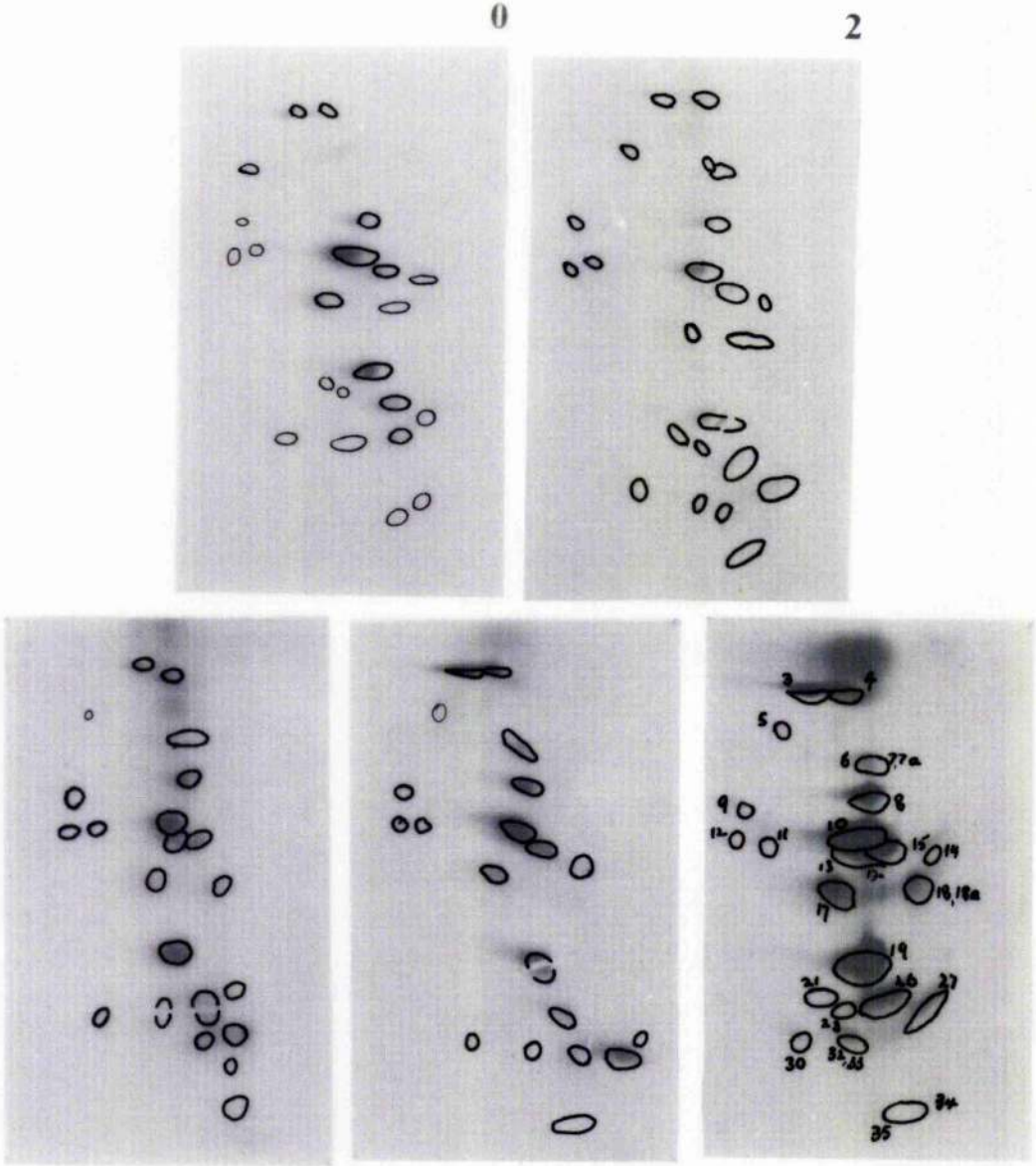

0
2
4
8
12
Oestrogen treatment (hr)

Figure 14

The Effects of Oestrogen on the Incorporation of Radioactive Methionine into Individual Ribosomal Proteins In Vitro 2) Separation in Two Dimensions.

Figure 14 shows the effects of oestrogen on the incorporation of radioactive methionine into individual ribosomal proteins of the large subunit *in vitro*. Experimental details are the same as those for figure 13.


0
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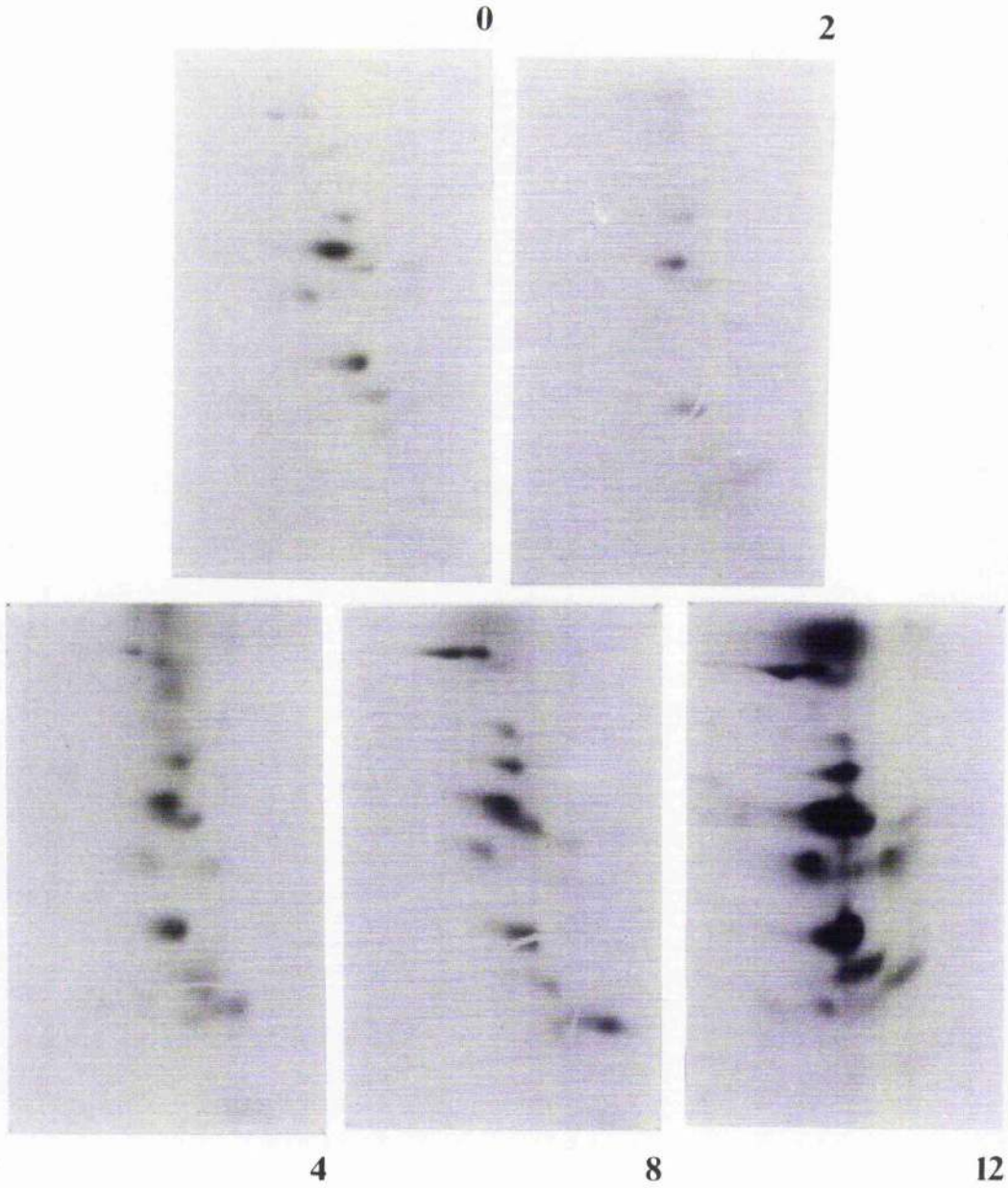
12
Oestrogen treatment (hr)



Oestrogen treatment (hr)

Table 3A

Incorporation of Radioactive Methionine into Ribosomal Proteins of the Small Subunit

Time of Oestrogen Treatment	0	2	4	8	12	% Methionine
Protein						
S2	9	01	48	32	69	1.6
S3	7	6	0	0	0	2.2
S3a	66	44	0	81	238	3.2
S3b	3	5	2	6	0	2.9
S4	-	32	17	19	60	1.8
S5,5a	0	0	2	23	87	2.2, 2.3
S6	0	22	7	18	-	1.3
S7	0	0	27	0	-	1.2
S8	0	0	1	3	0	0.3
S9	0	0	0	9	0	1.8
S10	25	10	0	15	77	2.0
S11	0	12	55	86	131	1.9
S13	11	0	0	21	13	0.6
S14	56	0	2	22	94	1.0
S15, 15a	25	195	149	339	539	4.3, 1.9
S16	11	30	36	33	125	0.8
S17, 17a	25	137	-	146	199	1.8
S18	36	37	62	12	68	0.8
S19	12	39	55	73	101	1.1
S20	13	70	69	78	96	1.1
S23	0	0	0	167	55	) 1.3
S24	2	107	159	168	133	)
S25	0	0	6	5	0	-
S26	0	0	26	32	0	0.9
S27	0	0	9	4	4	1.1
S28	7	18	1	2	18	0.7
Background Count	43	44	14	14	49	
Average Value	12	29	29	54	88	1.6
Total cpm	308	773	724	1294	2107	

Table 3B

Incorporation of Radioactive Methionine into Ribosomal Proteins of the Large Subunit

Time of Oestrogen Treatment	0	2	4	8	12	% Methionine
Protein						
L3	0	20	40	91	227	1.7
L4	34	-	48	117	229	2.1
L5	9	0	3	16	0	1.7
L6	0	11	33	65	69	0.8
L7, 7a	31	0	51	21	66	3.0, 0.3
L8	61	82	76	96	258	2.5
L9	4	23	0	3	16	0.7
L10	112	188	137	168	459	2.5
L11	0	24	14	14	152	0.6
L12	10	8	4	2	22	1.3
L13, 13a	3	10	5	48	223	1.9
L14	43	48	3	5	53	
L15	0	0	67	72	62	0.7
L17	84	0	44	70	191	2.5
L18, 18a	28	44	32	56	149	1.6
L19	168	102	106	147	741	2.1
L21	22	19	8	16	21	2.6
L23, 23a	48	27	13	-	148	1.5, 0.6
L26	93	43	38	67	22	2.3
L27, 27a	33	68	73	95	90	1.3, 2.2
L28	0	50	78	5	13	2.7
L29	0	0	2	1	0	1.6
L30	1	0	3	35	51	0.1
L31	14	54	30	2	22	1.5
L32	27	4	49	11	75	1.4
L33	6	4	37	93	70	0.1
L34	4	32	15	23	13	1.6
L35	15	23	46	30	15	1.1
Background Count	43	42	12	12	49	
Average Value	31	33	37	46	123	1.6
Total cpm	840	884	1055	1369	3457	

In the unstimulated uterus there were only 11 large subunit proteins and 3 or 4 small subunit proteins that acquired sufficient radioactivity to be detected by fluorography. In all the experiments L10 and L19 were most strongly labelled.

Ribosomal proteins isolated from rats which had received oestrogen 2 and 8 hours before death, included 14 large subunit proteins and 14 small subunit proteins which could be identified on fluorographic film. By 12 hours of oestrogen treatment there were 17 radioactivity labelled proteins from each subunit. Clearly, in the one hour labelling period, less than half of the 35 small subunit proteins and 45 large subunit proteins were becoming labelled. It is also evident, especially in the case of the large subunit proteins, that significant changes in the incorporation of radioactivity occured between 4 and 12 hours of oestrogen treatment. This confirmed the findings with precursor incorporation into non-fractionated proteins by in vitro methods as shown in Figure 10. Different proteins showed different trends in precursor incorporation over the oestrogen time course. Incorporation into proteins S17, S17a, S24, L27, L27a and L31 increased rapidly during the first 2 hours of oestrogen treatment but showed little increase in incorporation later. Conversely, S3a, S15, S15a, S16, L3, L4, L10 and L19 showed greatest increases in incorporation between 8 and 12 hours of oestrogen treatment. The rate and extent of labelling of different proteins is further illustrated by graphical representation of selected examples in Figure 15.

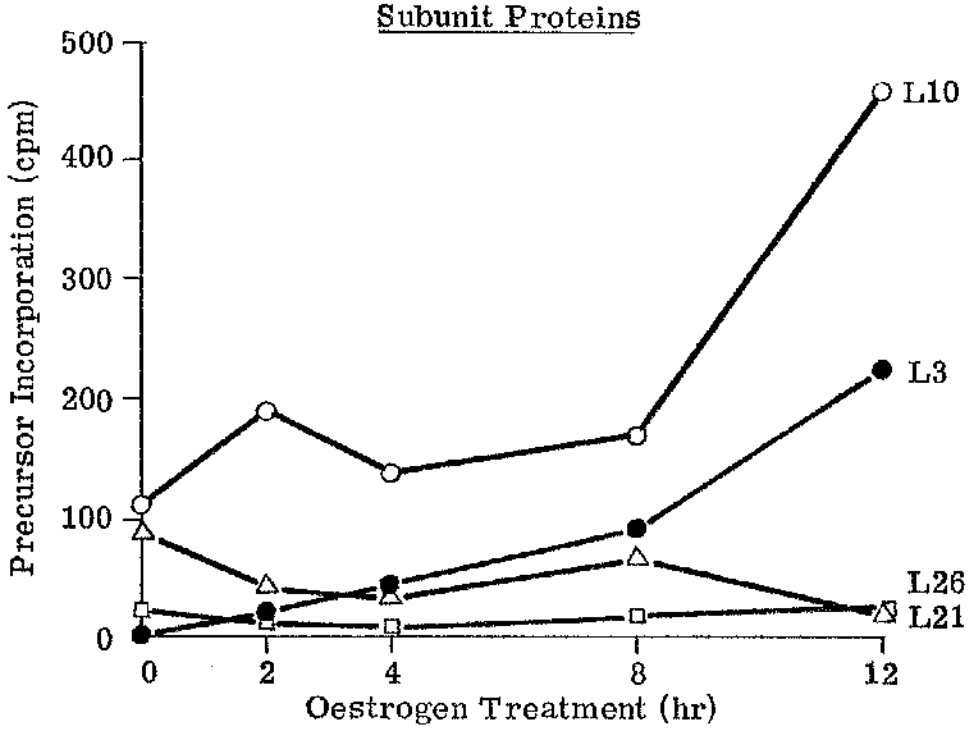
The reasons for the differential labelling of proteins from the same time point are not clear. This may have been in part due to the differences in methionine content of various proteins.

Figure 15

The Effects of Oestrogen on the Incorporation of Labelled Precursor into Four Large Subunit Proteins

After fluorography the radioactive spots in the gels of figures 13 and 14 were excised and digested with Protosol to release their radioactivity (described in detail in part 3.13.3. of the Methods section). The amount of released radioactivity was analysed by scintillation counting. The radioactive counts from 4 large subunit proteins, L3, L10, L26 and L21 are shown graphically in figure 11, the remainder of this data is presented in table 3.

The Effect of Oestrogen on the Incorporation of Labelled Precursor into Four Large Subunit Proteins



However, this was certainly not always the case. For example, protein L4 and L19 are reported to have the same methionine content (Tsurugi et al., 1976; Tsurugi et al., 1977) but the recovery of radioactivity in L19 was more than 3 times that in L4 at various time points. It was possible that differential extraction of the various proteins may have contributed to the result. However, the stainable pattern of rat liver protein added as a carrier and extracted at the same time did not reveal any significant selective differences. Furthermore, the pattern of labelled protein separated on sodium dodecylsulphate polyacrylamide gels (Figure 12) also showed preferential labelling. In this method no separate extraction procedure was needed.

At all times the 60S proteins incorporated a greater quantity of radioactive precursor than the 40S proteins. This was not simply due to the larger number of proteins as shown by the average radioactivity of each protein. Nor was it due to differing methionine contents since the average methionine content of the two subunits is the same. The difference is most noticable in the control uterus and in the uteri of 12 hour stimulated rats and was also evident from Figure 10A.

It seemed possible that the apparently anomalous rate of synthesis of different ribosomal proteins could be explained in terms of different turnover rates for different proteins or subunits. For example, if the turnover rate of some of the proteins was faster than for others more radioactivity would be expected in these proteins. A chase type experiment was planned to analyse the disappearance of radioactivity from ribosomal proteins when uteri were transferred from an incubation medium containing [^{35}S]-methionine to one containing non-radioactive methionine.

Experiments of this type have been very useful in the past for analysis of protein turnover in cultured cells. However, such experiments are very much more difficult and less reliable when performed with isolated tissue. Nevertheless, preliminary experiments were performed to assess whether suitable conditions for chase experiments could be devised. Figure 16 shows the changes in amount of radioactivity that were recovered in the acid insoluble and acid soluble fractions of uteri when a 1 hour pulse of radioactive methionine was chased with unlabelled methionine for a further 2 hours.

During the first hour of incubation, the amount of radioactivity in the acid insoluble fraction increased steadily to 6×10^5 counts per minute. After the change to unlabelled medium there was a further increase of 16% within the first half hour but there was no significant change in protein specific activity thereafter. The data presented as incorporation of radioactivity into the acid soluble fraction are less easy to interpret. These data reveal a very rapid rise of incorporation of radioactivity into acid soluble pools within the first 20 minutes. Thereafter the radioactivity recovered declined, even before transfer to fresh medium with unlabelled methionine. The rapid decrease of radioactivity in the acid soluble pools and the reduction of increasing activity in the acid insoluble pools suggested that a pulse chase experiment could be effective.

Figures 17 and 18 show the radioactivity incorporated into fractionated ribosomal proteins of oestrogen-treated uteri after they had been incubated for one hour in the presence of [^{35}S]-methionine and then for various periods of time in the presence of unlabelled methionine. The prolonged incubation period

Figure 16

The Effects of Unlabelled Methionine on the Incorporation of Radioactive Precursors into Acid Soluble and Insoluble Pools

A group of 18 immature female rats were injected with oestrogen 12 hours before death. The uteri from these animals were placed in 5ml of methionine-free Eagle's medium containing 10 μCi/ml of L[^{35}S]-methionine and incubated at 37°C under 95% O_2:5% CO_2 at 37°C for 1 hour. During this period 3 uteri were removed from the incubation at 20 minute intervals. After the first hour the remaining uteri were washed with complete Eagle's medium (at 37°C and under 95% O_2:5% CO_2) and transferred to a new incubation vessel containing complete Eagle's medium. The incubation proceeded for a further 2 hours and uteri were removed at regular intervals.

Once removed from the incubation the uteri were well washed and were then used for the preparation of acid soluble and insoluble material, as described in the Methods (section 3.20.).

Figure 16 A Acid insoluble fraction

B Acid soluble fraction.

The Effects of Unlabelled Methionine on the Uptake of Radioactive Precursor into Acid Soluble and Insoluble Pools

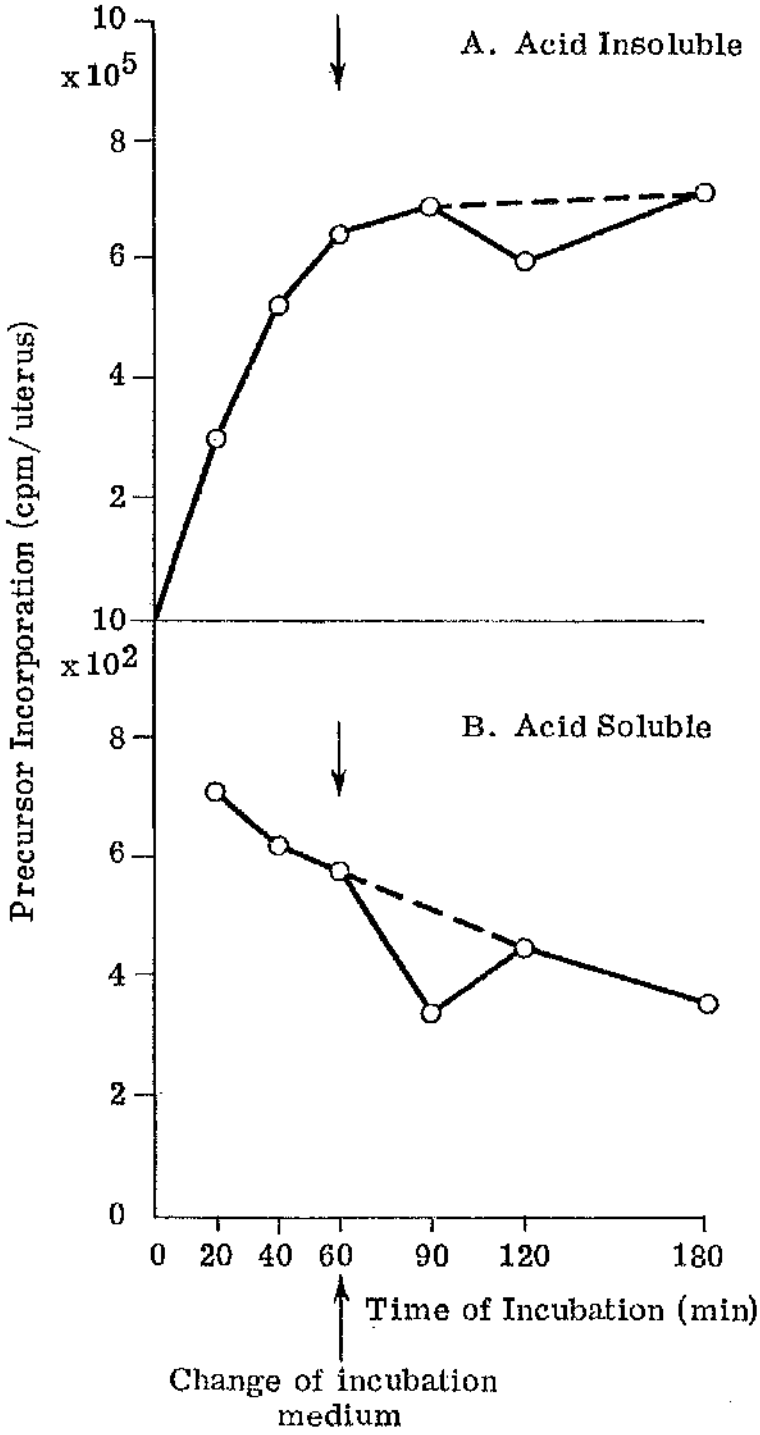


Figure 17

The Effects of Unlabelled Methionine on the Uptake of Radioactive Precursors into Ribosomal Proteins

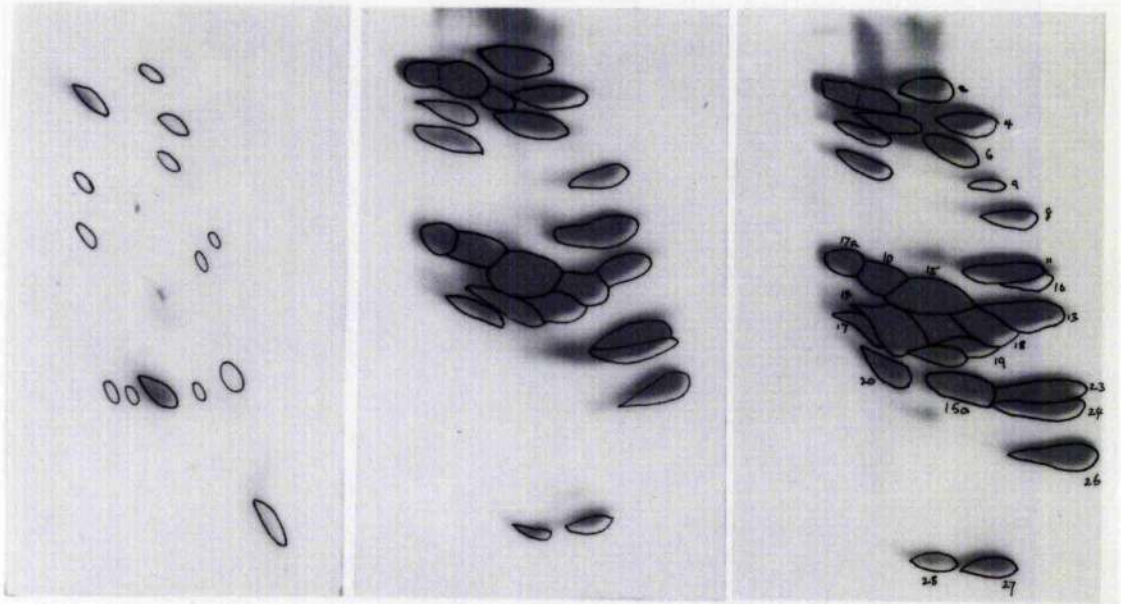
Groups of 12 immature rats each received 1 μg/rat of oestradiol-17β in saline, 12 hours before death. The uteri from these rats were incubated with 250 μCi/ml of L [^{35}S]-methionine in methionine-deficient Eagle's medium at 37°C under 95% O_2:5% CO_2. After one hour of incubation the uteri were washed with warm, oxygenated medium and the incubation was continued in complete Eagle's medium. After various periods of time the uteri were washed with ice-cold saline and used for the preparation of ribosomal proteins as described in the Methods section. The proteins were fractionated on urea-polyacrylamide gels and analysed by fluorography for 40 days.

Figure 17 shows the proteins of the small subunit:

i) 40S proteins from uteri incubated for one hour in the presence of labelled methionine.

ii) 40S proteins from uteri incubated for one hour in the presence of labelled methionine and chased for one hour with unlabelled methionine.

iii) 40S proteins from uteri incubated for one hour in the presence of labelled methionine and chased for two hours with unlabelled methionine.



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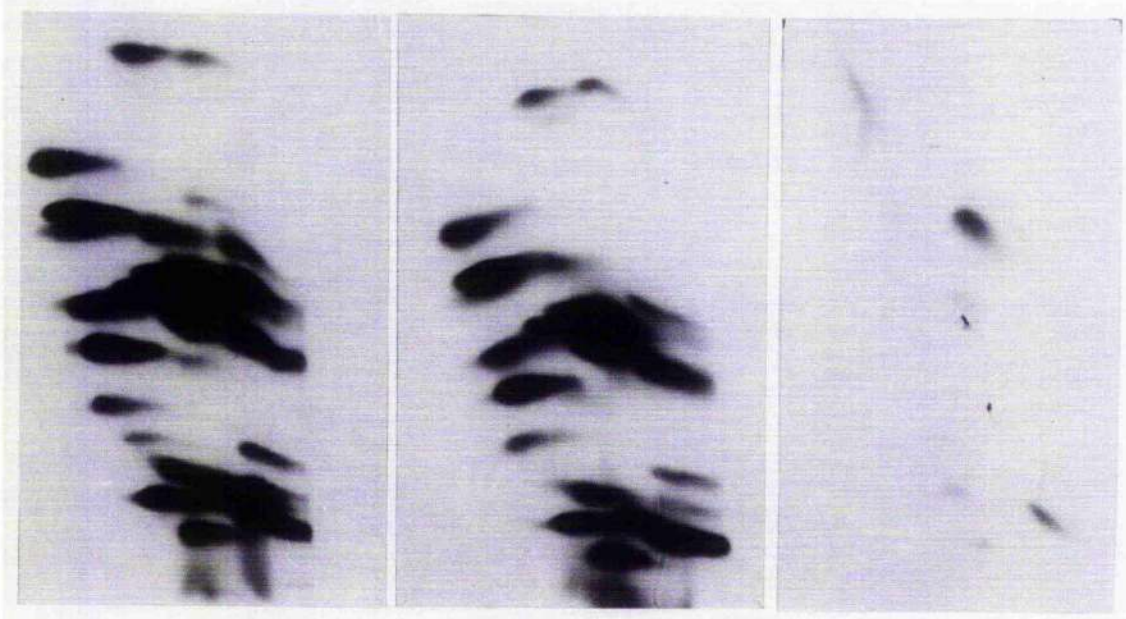


Figure 18

The Effects of Unlabelled Methionine on the Uptake of Radioactive Precursors into Ribosomal Proteins

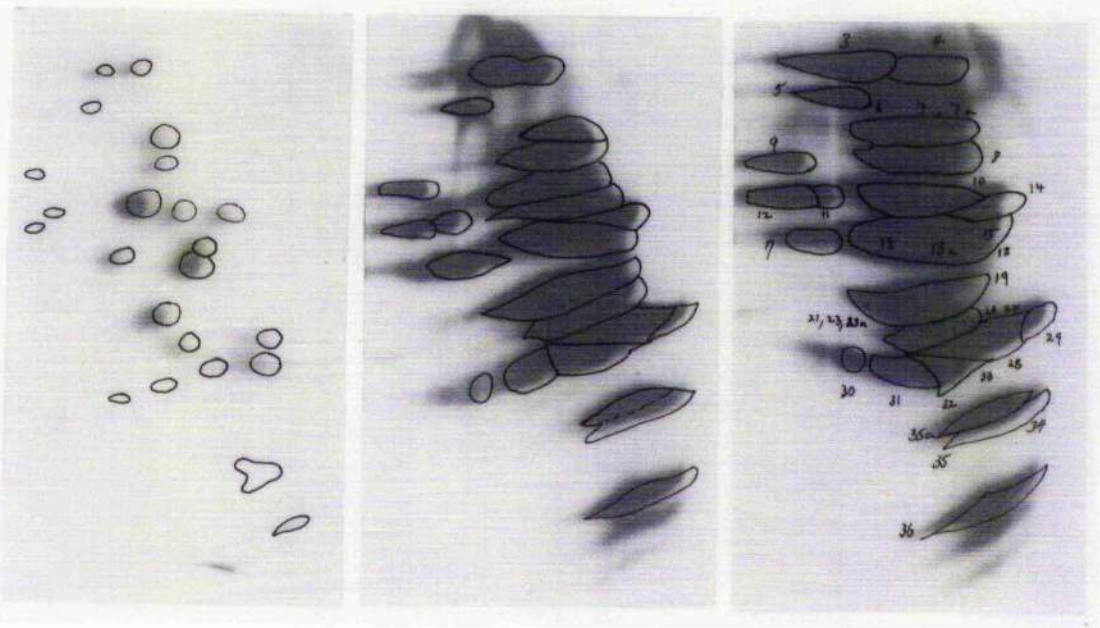
Figure 18 shows the analysis of proteins from the large subunit. Experimental details were the same as those for figure 17.

Figure 18 shows the proteins of the large subunit:

i) 60S proteins from uteri incubated for one hour in the presence of labelled methionine.

ii) 60S proteins from uteri incubated for one hour in the presence of labelled methionine and chased for one hour with unlabelled methionine.

iii) 60S proteins from uteri incubated for one hour in the presence of labelled methionine and chased for two hours with unlabelled methionine.



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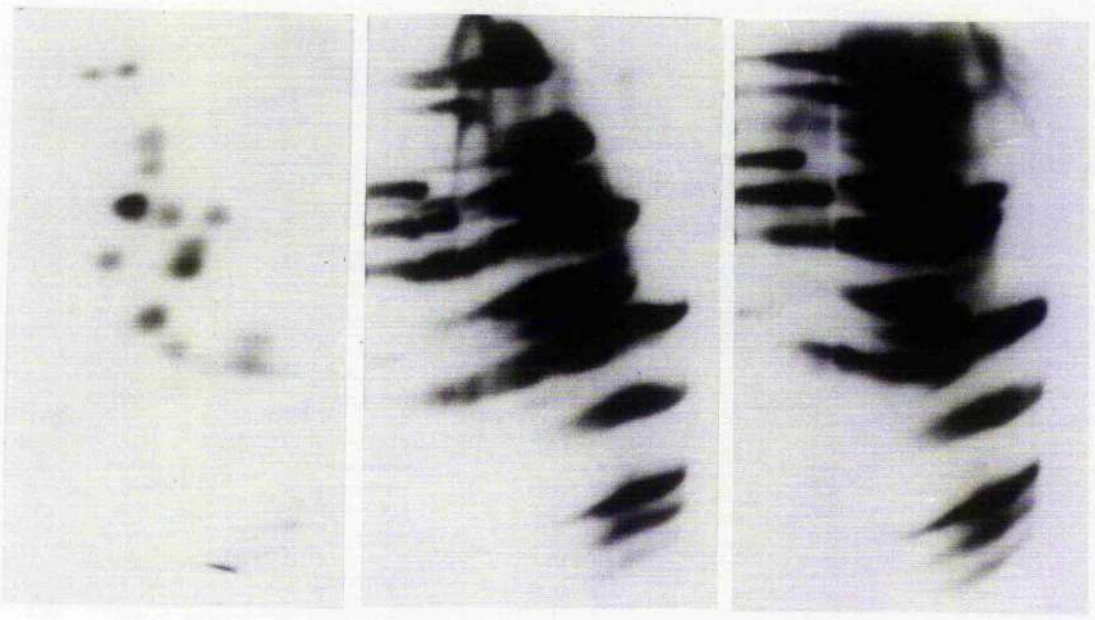


i ii iii

resulted in increased radioactive labelling of all the ribosomal proteins. Very many more proteins were sufficiently labelled to be detected on the fluorograms. However, quantitative measurement of radioactivity in these proteins revealed that the relative incorporation of radioactivity into each protein showed very little change. Table 4 reveals that L10 and L19 still were labelled to a greater extent than most of the other proteins. Proteins L9 and L11 again incorporated comparatively little radioactivity. The only clear exceptions were proteins L3 and L4 which were labelled to an unusual extent. However, the fluorograms had a high background level of radioactivity and this may have influenced the results with these two proteins. In very few cases (S36 and L35), was there a significant drop in incorporation with prolonged incubation. This would indicate that turnover or degradation rates do not play a significant role in the appearance of radioactively labelled precursor in ribosomal proteins synthesised in these experiments.

4.3. The Synthesis of Ribosomal Proteins in a Cell Free Protein Synthesising System.

Studies of the synthesis of ribosomal protein that have been performed so far involved the isolation of the protein from polysomes. Ribosomal proteins occupy other cell fractions such as the cytoplasm or the nucleus. Pools of ribosomal proteins in these other fractions may have significant effects on the incorporation of ribosomal proteins into the ribosome. Furthermore, there must be a time lag between the synthesis and the appearance in polysomes of the ribosomal proteins which first move into the nucleus and associate with pre-ribosomal particles. In order to differentiate between the effects of oestrogen on synthesis and any effect due to

Table 4

The Effects of Unlabelled Methionine on the Incorporation of Radioactive Precursors into Individual Ribosomal Proteins

	Small Subunit				Large Subunit		
Time of Oestrogen Treatment	12	12	12		12	12	12
Time of Incubation	1+0	1+1	1+2		1+0	1+1	1+2
Protein				Protein			
S2	24	167	307	L3	105	468	944
S3	18	56	43	L4	144	440	1198
S3a	–	–	51	L5	111	118	201
S3b	91	125	40	L6	55	142	286
S4	24	341	310	L7, 7a	208	142	435
S5, 5a	13	53	85	L8	180	620	481
S6	23	92	250	L9	38	125	175
S7	20	65	139	L10	335	446	937
S8	4	43	142	L11	31	159	188
S9	9	0	81	L12	39	152	233
S10	30	91	186	L13, 13a	–	438	638
S11	23	420	383	L14	219	410	–
S13	0	38	194	L15	162	454	335
S14	–	214	–	L17	210	155	625
S15, 15a	50	422	523	L18	260	375	590
S16	–	248	221	L19	312	557	740
S17, 17a	26	151	292	L21	38	111	) 784
S18	12	270	286	L23, 23a	119	296	)
S19	–	495	427	L26	133	320	527
S20	17	123	165	L27, 27a	123	479	171
S23	28	249	248	L28	54	200	602
S24	–	406	308	L29	–	272	83
S25	7	57	73	L30	41	102	193
S26	3	210	236	L31	43	176	215
S27	–	1	157	L32)			
S28	–	0	103	L33	48	330	271
				L34	49	154	257
				L35	37	123	83

protein pools or subcellular movement, it was necessary to study ribosomal protein translation. Figure 19A shows that uterine polysomes could be translated in vitro to yield acid precipitable material. The yield was maximal using polysomes from uteri treated with oestrogen for 8-12 hours. The translation mixture had been optimised for all the components necessary for protein synthesis with the exception of mRNA concentration. This mRNA was added as a constant quantity of ribosomal material (as 0.2 A_{260} units). Thus increased incorporation of [^{35}S]-methionine into acid precipitable material probably reflected a greater proportion of ribosomes attached to mRNA, that is in the form of polysomes. Thus, Figure 19A suggests that a maximal proportion of ribosomes exist as polysomes in the rat uterus after 8-12 hours of oestrogen treatment. This result confirms work performed by polysome profile analysis.

To determine what proportion of these new polysomes were synthesising ribosomal proteins it was necessary to isolate these proteins from the translation mixture. To this end an anti-ribosomal protein antibody was raised in a rabbit. The properties of this antibody will be discussed in the next section. At first many problems of non-specific antibody binding were encountered. A cell free translation mixture may contain a wide variety of complete and incomplete proteins, all labelled to a very high specific activity. Many of these proteins may have bound to antibody in a non-specific manner. The anti-ribosomal protein antibody would also bind to the ribosomes of the translation mixture some of which may still have been attached to highly labelled nascent peptides. For this reason many precautions were taken to ensure that non-specific binding did not occur. Puromycin was used

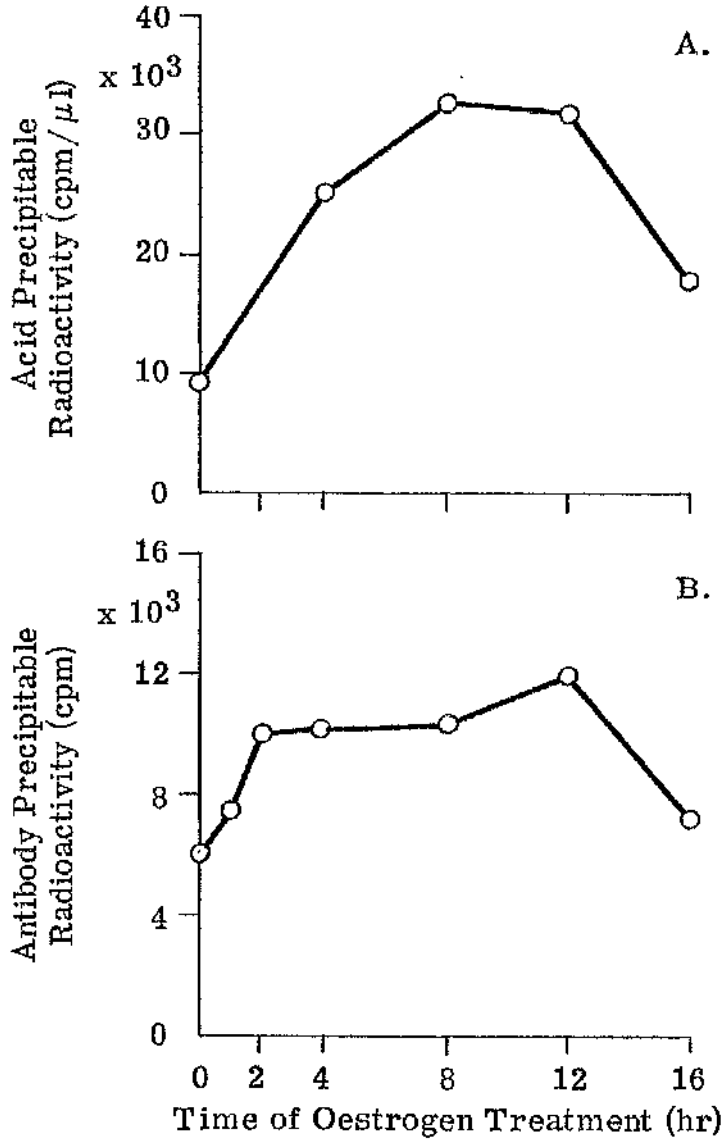
Figure 19

The Effects of Oestrogen on the Synthesis by Uterine Polysomes of a) Total Protein; b) Ribosomal protein

a) Groups of six immature female rats were injected with oestradiol-17βat various times before death. Polysomes were prepared from the uteri of these animals, as described in Methods section 3.6.2., and 0.2 A_{260}nm units were incubated in 25µl of messenger dependent lysate (Amersham International) for ninety minutes at 30°C (Methods 3.14.). Duplicate microlitre aliquots of incubation mixture were taken for measurement of acid precipitable radioactivity. Incorporation by polysomes from untreated animals was times the background level.

b) 10^6 counts per minute of acid precipitable material from incubation mix was precipitated with rabbit anti-ribosomal protein antibody as described in the Methods section (3.17.).

The Effect of Oestrogen on the Synthesis, by Uterine Polysomes of
a) Total Protein b) Ribosomal Proteins



to ensure the release of all nascent proteins. Ribosomes were then removed by centrifugation.

Since crude serum may contain many binding proteins, a gamma-globulin fraction was used in the presence of powerful detergents (see Methods section). Finally, an initial immunoprecipitation was performed in which normal mouse serum, lacking antibodies to ribosomal proteins, was used to bind and remove non-ribosomal gamma-globulin binding radioactive elements. Radioactivity precipitated with rabbit preimmune gamma-globulin accounted for less than 2% of radioactivity precipitated with post immunisation gamma-globulin.

Figure 19B shows the effect of oestrogen on the synthesis of ribosomal proteins by uterine polysomes. A constant total of acid precipitable material was analysed at each time point. Thus, the results appear to reflect a change in the proportion of mRNA that encodes ribosomal protein. The synthesis, and translation of ribosomal protein mRNA began at an early time after oestrogen treatment and continued at a constant level from 2 hours onwards. After 12 hours the quantity of these messengers being translated fell to control levels.

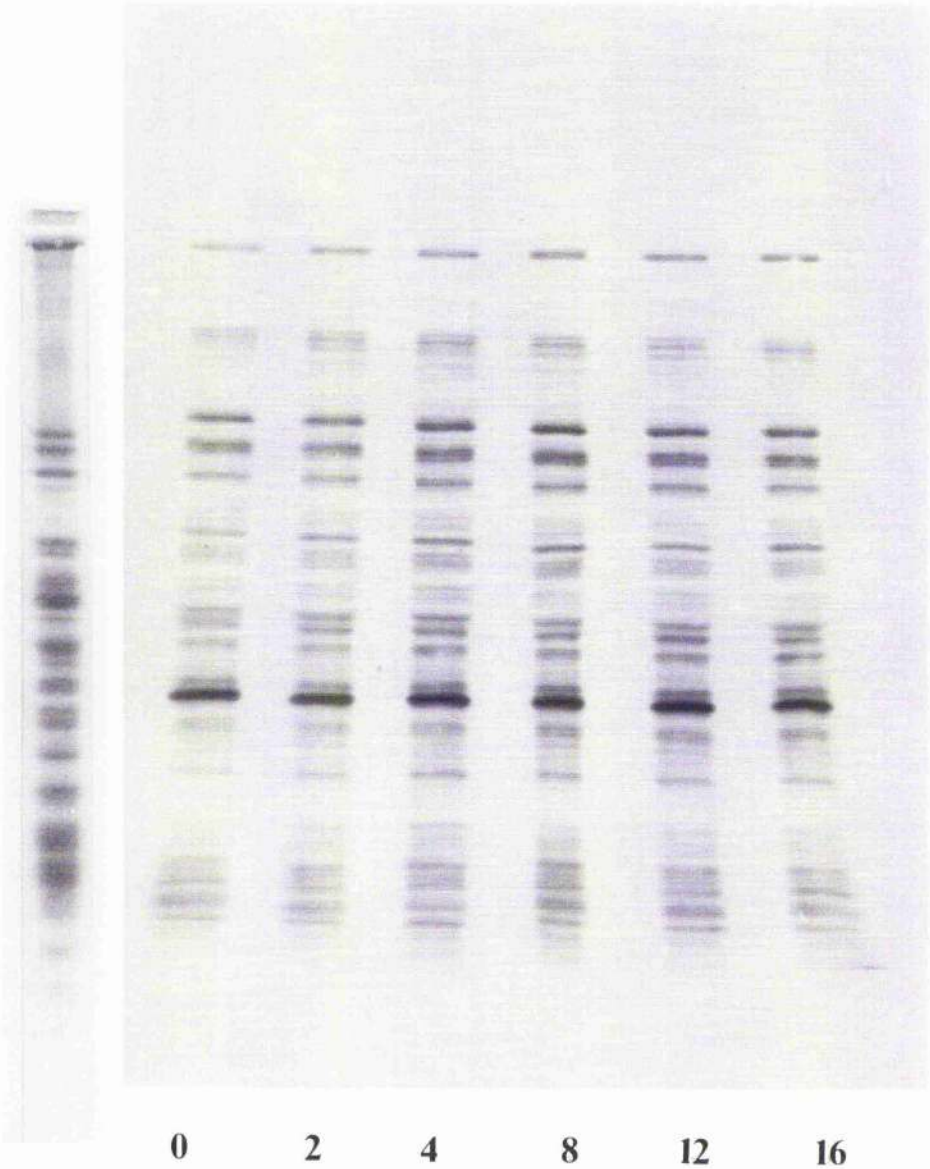
In view of the problems of non-specific binding it was desirable to confirm that the antibody was indeed binding solely to ribosomal proteins. To this end immunoprecipitate was co-fractionated with ribosomal proteins on sodium dodecylsulphate polyacrylamide gels. The pre-immune precipitate (normal rabbit gamma-globulin) was also analysed in this way. Figure 20 shows the co-electrophoresis of carrier ribosomal proteins with the immune precipitate of cell free translation products. Very little radioactivity was co-precipitated with normal rabbit serum despite

Figure 20

Analysis of Immunoprecipitates from Cell Free Translation Products

Polysomes were prepared from untreated and oestrogen treated rat uteri, and subsequently translated in a rabbit reticulocyte lysate. The products were reacted with serum containing anti-ribosomal protein antibody or normal rabbit serum. Antigen-antibody complexes were precipitated with a donkey anti-rabbit gamma globulin antibody as described in section 3.17 of the Methods section. The immune precipitate was analysed on sodium dodecylsulphate polyacrylamide gels which were subjected to fluorography for seven days at -70°C. Also included in figure 20 is a stained gel pattern of 80S ribosomal proteins.

Control immunoprecipitates, using rabbit normal serum did not bind sufficient radioactively labelled protein to be detected by this method.



Stained gel

the analysis of a quantity of immune precipitate exactly equal to the post-immune sample. This effectively demonstrates that there was an absence of non-specific binding of radioactive proteins by either pre-immune serum, or the anti-rabbit precipitating antibody. In contrast, the post-immunisation serum precipitated much radioactively labelled protein. The appearance of bands on the gel coincided with the non-radioactive ribosomal proteins that were added as a stainable carrier. Included in Figure 20 is part of a stained gel loaded with ribosomal proteins.

4.4. The Analysis of Ribosomal Protein Pools in the Rat Uterus Responding to Oestrogen

Although the anti-ribosomal protein antibody was primarily raised to analyse the synthesis of radioactive proteins in a reticulocyte lysate, it was also possible to use this antibody to monitor the appearance of unlabelled proteins in the uterus. The advantage of using an antibody, rather than radioactive labelling, for these studies lay in the fact that antigenic titre was not influenced by precursor pool sizes. An antibody could be used to assay absolute quantities of antigen.

Some consideration was given to the assay chosen for these studies. Initial work involved immunoprecipitation both in agarose and in capillary tubing. However, this method required large quantities of antigen and undiluted antisera. In addition, the results achieved were of a qualitative nature and quantitative immunoprecipitation was found to be unreliable and inaccurate. Complement fixation was chosen as an alternative method but it soon became apparent that rRNA would fix complement in the absence of an antigen-antibody complex. Since it was impossible to ensure the

total absence of RNA from samples this method was discarded. The enzyme linked immunosorbant assay (ELISA) was finally selected for its sensitivity and reliability.

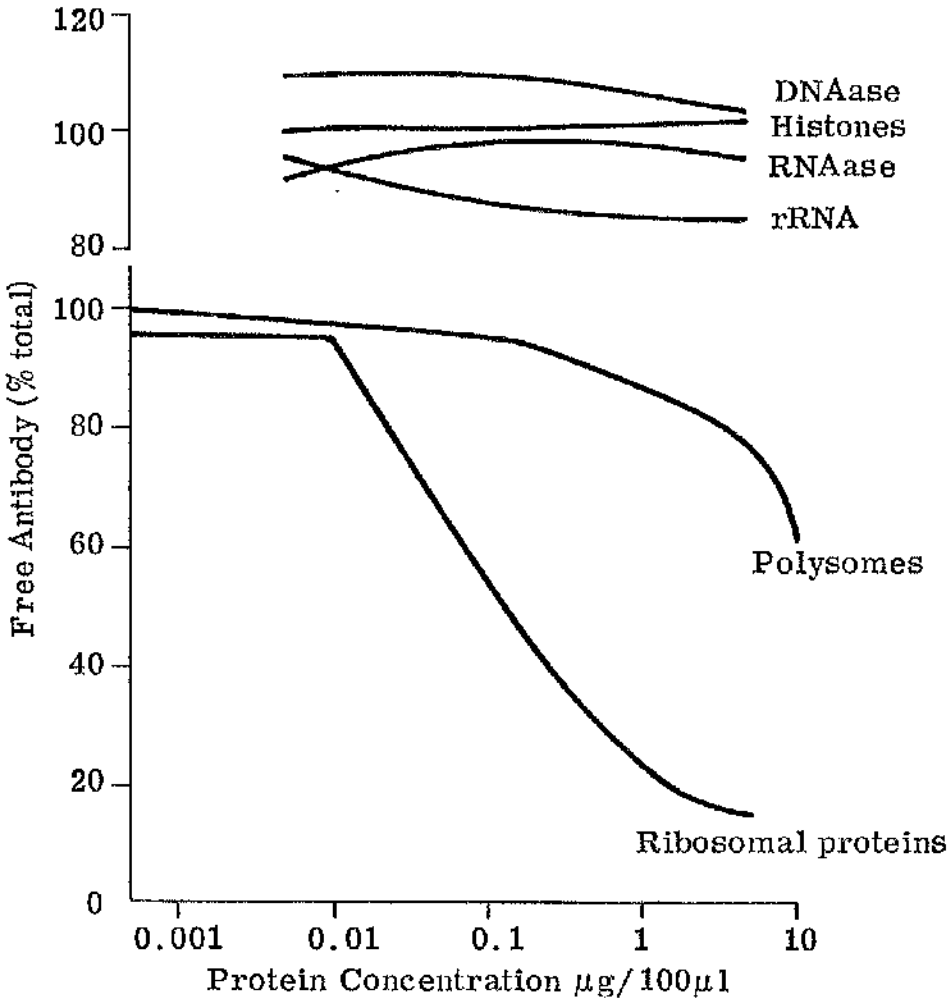
Figure 21 shows the binding properties of various macromolecules incubated with rabbit anti-ribosomal protein antibody. Ribosomal proteins were able to bind strongly to the antibody removing in excess of 80% of the specific gamma-globulin used in this assay. As little as 10 to 50ng of ribosomal protein in 100μl of buffer could be detected by this method. Intact ribosomes could also be detected by this procedure. However, the ability of purified ribosomal proteins to compete for antibody was frequently 5 times that of whole polysomes. Since the antibody was raised against purified proteins this would indicate that many of the ribosomal protein antigenic determinants were hidden by the tertiary and quaternery structure of the ribosomes. Histones, RNAase and DNAase did not show any significant binding on anti-ribosomal protein antibody. Indeed, DNAase caused an ELISA colour reaction of greater than 100% indicating that it stimulated the peroxidase or itself produced a colour reaction. No explanation for this has been discovered, however, the enzyme clearly had very little effect on antibody binding. Ribosomal RNA appeared to bind to antibody to a limited extent, indicating that there was some specificity within the serum for this macromolecule. Since it proved very difficult to remove all traces of rRNA from the immunogen used to raise the antibody this limited reaction was not totally unexpected. However, it was also possible that the rRNA sample (produced by phenol extraction) was still contaminated with ribosomal proteins. In both cases the ribosomal protein immunogen and the rRNA test sample were demonstrated to be

Figure 21

A Companion of the Antibody Binding Properties of Different Macromolecules

A competition ELISA assay was performed using different macromolecules to compete against extracted ribosomal proteins for antibody. Various proteins with a basic isoelectric point were selected for analysis as well as rRNA. Whole polysomes were also analysed. Those macromolecules that did not show extensive binding properties are shown on a separate graph for added clarity.

A Comparison of the Antibody Binding Properties of Different Macromolecules



pure by chemical assay. However, such determinations would be far less sensitive than immunoassay and could not be taken as an absolute measure of purity.

The first antigenic analysis of the appearance of ribosomal proteins in uterine tissue produced unexpected and anomalous results. The ELISA technique was used as a direct assay of the antigenicity of a fixed quantity of protein in uterine homogenate. Figure 22 shows the effects of oestradiol-17β and oestradiol benzoate on this antigenic titre. In both cases there was an increase in titre over the first 12 hours as would be expected in the light of current ideas of ribosomal protein synthesis in the uterus. However, after 12 hours the oestradiol stimulated uterus revealed a disappearance of this antigenicity. The uteri stimulated with the long lived oestradiol derivative, oestradiol benzoate, did not reveal this disappearance. The significance of these results will be discussed in a later section but they imply that over a very short period of time total ribosomal protein levels (as distinct from newly made proteins), rise and then fall. Such findings had not been predicted by previous work on ribosomes in the rat uterus or in any other tissue.

In an attempt to resolve this problem the experiment was performed in a slightly different manner using the ELISA competition method. This second method was considered less prone to potential problems with non-specific binding of antibody. Figure 23 shows that exactly the same result was achieved with this alternative method. Why these results were unexpected and possible reasons for these anomalies will be discussed later.

Figure 22

The Effects of Oestrogen on the Antigenic Titre of Uterine Homogenate A).

Uteri from rats treated for varying periods with either oestradiol or oestradiol benzoate, were homogenised in polysome buffer 1, and subjected to centrifugation at 10,000g (12,000 rpm) in a Sorval RC-5, at 4^{o}C for 10 minutes. The supernatant was carefully decanted and volumes containing 10µg of protein were assayed for antigenic titre by a direct ELISA.

The Effects of Oestrogen on the Antigenic Titre of Low Speed Supernatant of Uterine Homogenate

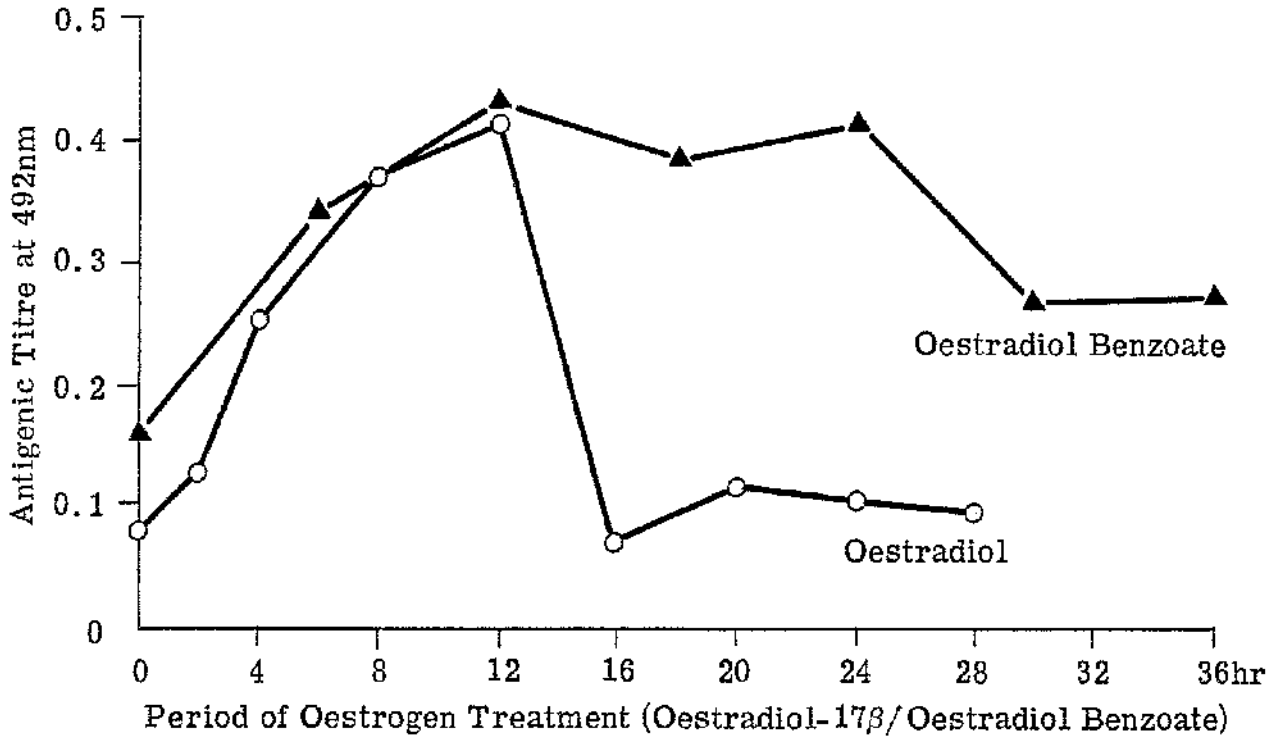
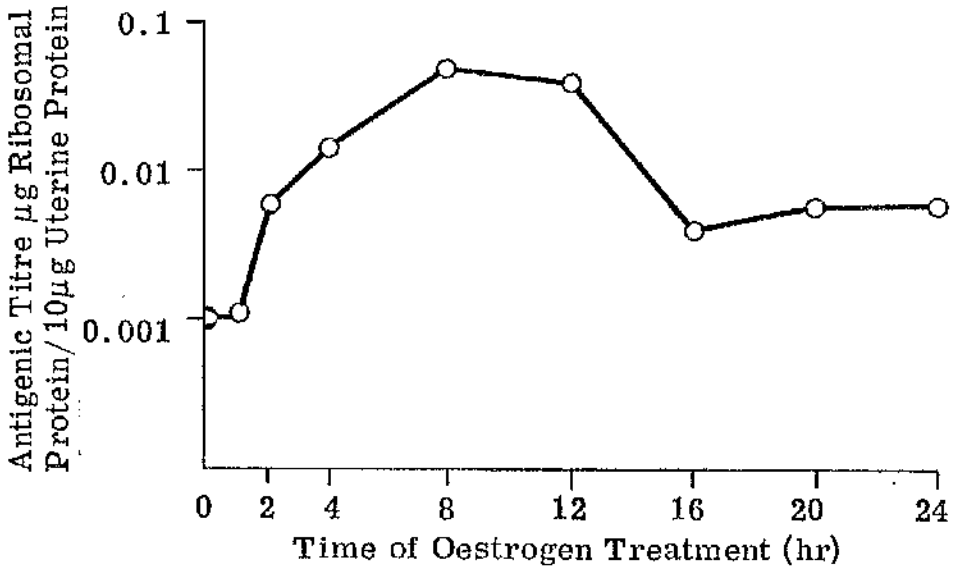


Figure 23

The Effects of Oestrogen on the Antigenic Titre of Uterine Homogenate B).

Uteri from oestradiol-17β treated rats were homogenised in polysome buffer 1, (from the method of polysome preparation 3.6.2.) and subjected to centrifugation at 10,000g in a Sorval RC-5, at $4^{o}C$ for 10 minutes. The supernatant was carefully decanted and assayed for antigenic titre by the ELISA competition method.

The Effects of Oestrogen on the Antigenic Titre of Uterine Homogenate



4.5. The effects of Oestrogen on the Phosphorylation of Ribosomal Proteins.

There now exists a large body of evidence to suggest that hormones may alter the type and extent of phosphorylation of ribosomal proteins. However, no analysis of the effect of oestrogen on phosphorylation of rat uterine ribosomal proteins has been reported. Initial experiments in this laboratory revealed that [^{32}P]-phosphate was indeed associated with isolated ribosomal proteins after uteri were incubated with [^{32}P]-inorganic phosphate. In addition, the specific activity of these proteins increased with oestrogen stimulation of the uterus.

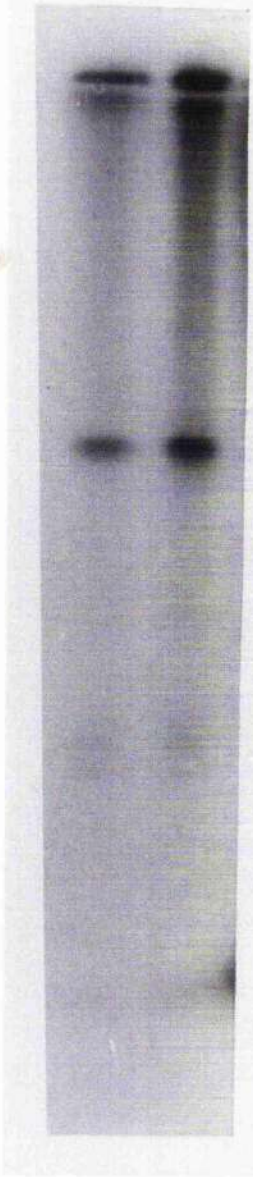
However, since [^{32}P]-inorganic phosphate would be incorporated into rRNA to a far greater extent than into ribosomal proteins any contaminating rRNA would mask the radioactivity in the ribosomal proteins. Although the extraction procedure of Sherton and Wool (1972) is the best available method, it is not 100% efficient. To clarify the results the proteins of ribosomal subunits were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis.

Figure 24 shows an autoradiograph of an investigation of the effects of oestrogen on the phosphorylation of ribosomal proteins which were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis. Incorporation of radioactive inorganic phosphate was achieved by one hour's incubation of uteri *in vitro* after treatment *in vivo* of the rats with oestrogen. The autoradiogram shows three distinct bands corresponding to phosphorylated proteins. The labelling of the proteins from stimulated uteri was more intensive than labelling of proteins from control uteri. Separation of the ribosomal subunits before

Figure 24

The Effects of Oestrogen on the Phosphorylation of Rat Uterine Ribosomal Proteins.

A group of 12 rats were injected with 1µg of oestradiol-17 β 6 hours before death. A group of control animals received carrier only. The uteri were removed to phosphate-deficient Eagle's medium containing 50 µCi/ml [^{32}P]-inorganic phosphate. After incubation for 1 hour, ribosomal subunits were prepared from purified polysomes, treated with sodium dodecylsulphate and the proteins fractionated on a sodium dodecylsulphate polyacrylamide gel (Methods section 3.12.1.). The gel was dried and analysed by autoradiography for 14 days.



Phosphoprotein of the small subunit

Phosphoproteins of the large subunit

0 6

Oestrogen treatment (hr)

analysis on these gels revealed that one of the proteins was associated with the small subunit whilst the other two were associated with the large subunit (results not shown).

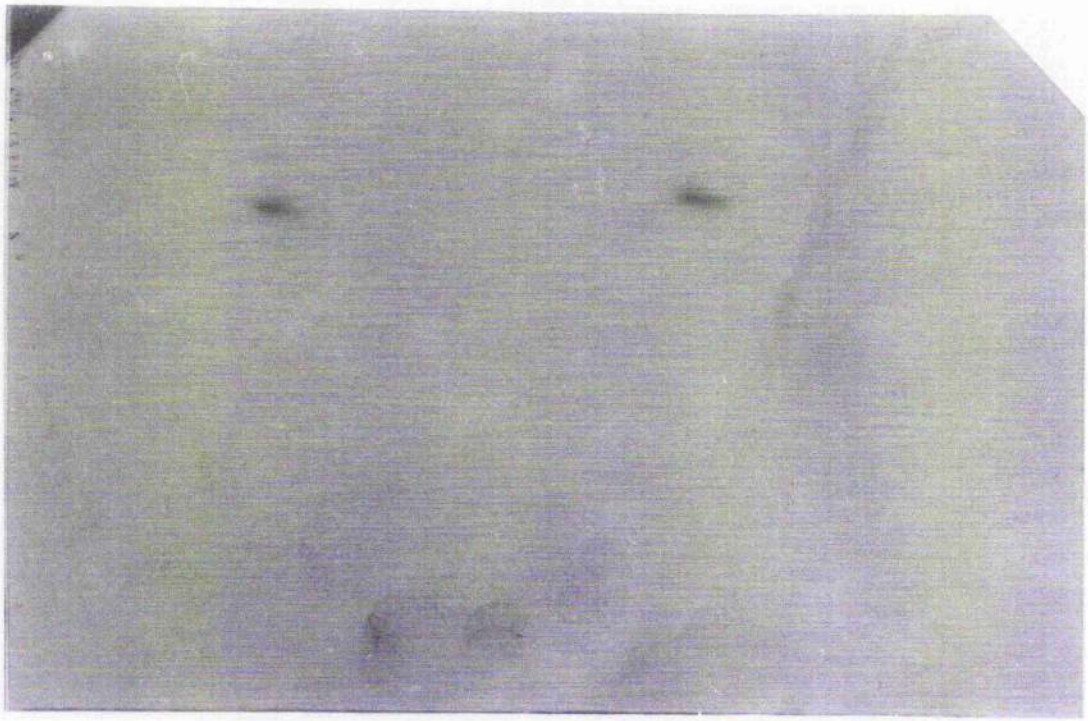
Figure 25 shows similarly labelled proteins from the small subunit separated on urea polyacrylamide gels. The incorporated radioactivity, in both the stimulated and unstimulated uteri, is clearly shown to co-fractionate with protein S6 from rat liver. No radioactivity was detectable in any other small subunit protein. A similar gel separating only basic proteins of the large subunit demonstrated that no radioactivity was associated with any of these. Unfortunately, conducting sweep type gels (Leader and Coia, 1978) in an attempt to include the acidic proteins L40 and L41 (or L8) which are known to be phosphorylated did not reveal any radioactivity either. Thus, protein S6 appeared to exist in the uterine ribosome in a phosphorylated form and its phosphorylation was slightly but reproducably stimulated by oestrogen. It remained possible however that the stimulated phosphorylation was an effect of the hormone on the precursor pool.

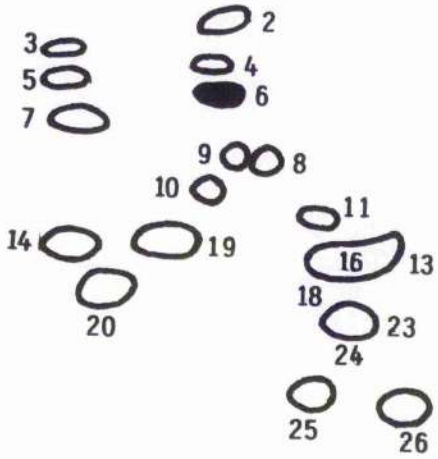
To determine if the changes in phosphorylation were due to changes in the availability of [^{32}P]-phosphate the specific activity of ATP in the uteri was determined at different times after oestrogen treatment. Figure 26 shows that there was a five fold increase in the specific activity of ATP during oestrogen treatment of rat uteri incubated in vitro with [^{32}P]-inorganic phosphate. The increase in ATP specific activity was partly reflected in an increase in radioactivity in the acid soluble fraction of uteri. There was little change in the incorporation of radioactivity into the acid insoluble fraction. The changes in specific activity of ATP after oestrogen treatment could easily account for the increases in phosphorylation of S6.

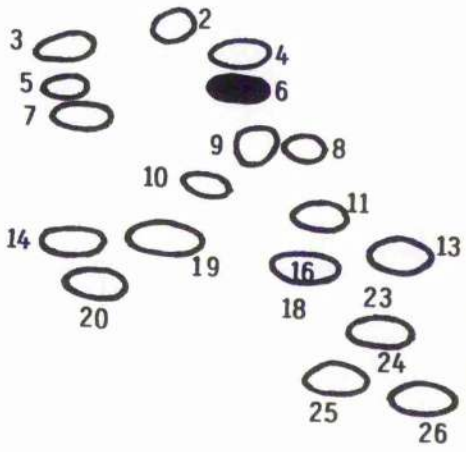
Figure 25

The Effects of Oestrogen on the Phosphorylation of Proteins in the Small Ribosomal Subunit of the Rat Uterus

A group of 12 rats each received an injection of 1µg of oestradiol-17β 12 hours before death, 12 control animals received carrier only. The uteri were removed to phosphate-deficient Eagle's medium containing 500 µCi/ml of [^{32}P]-inorganic phosphate. After incubation for 1 hour, ribosomal subunits were prepared from purified uterine polysomes, and the proteins extracted from the subunits were fractionated on urea-polyacrylamide gels (Methods section 3.12.2.). The gels were dried and analysed by autoradiography. The figure shows the results obtained with the 40S subunits. No phosphorylated protein was detected when this gel system was used to analyse 60S subunits.







0 6

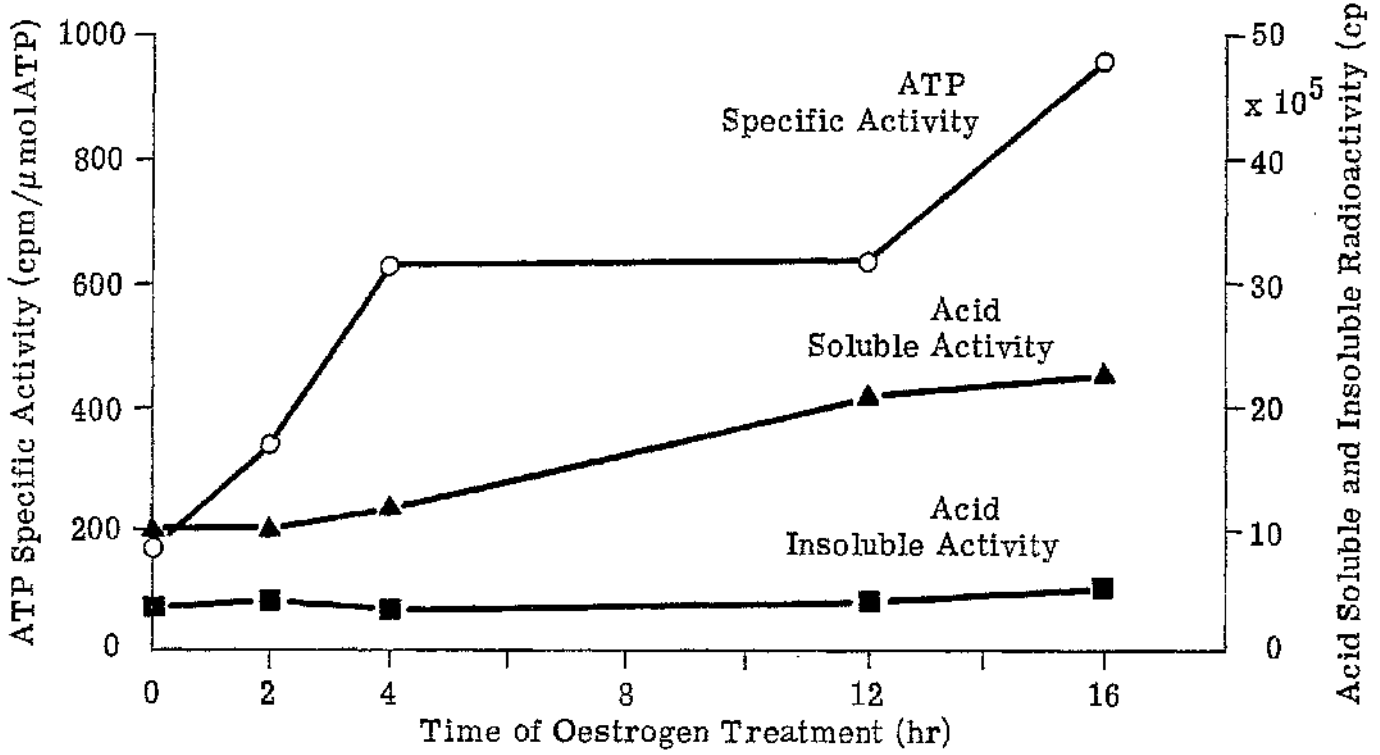
Oestrogen treatment (hr)

Figure 26

The Effects of Oestrogen on Incorporation of [^{32}P]-Inorganic Phosphate into Uterine Pools

Groups of 3 rats were injected with oestrogen at various times before death. Subsequently, the excised uteri were incubated *in vitro* in phosphate-deficient Eagle's medium containing 50 μCi/ml [^{32}P]-inorganic phosphate. After incubation the specific activity of the total uterine ATP was determined. The radioactivity in the acid soluble and acid insoluble fractions were also determined for comparison (Methods section 3.20.).

The Effect of Oestrogen on Incorporation of ^{32}P -phosphate into Uterine Pools



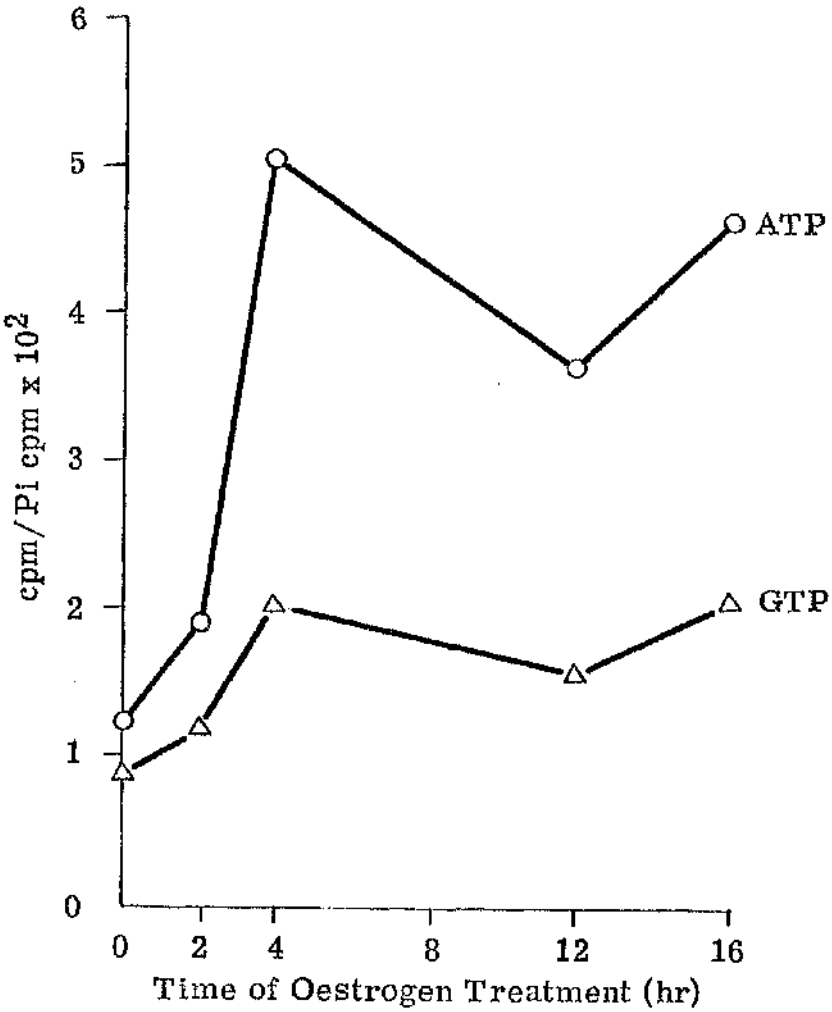
Increases in ATP specific activity may have been due to a change in the pool of inorganic phosphate as a precursor or may have been due to increased synthesis of ATP. A comparison of the radioactivity in ATP with that in the phosphate pool should reveal any changes in relative concentration. Figure 27 shows the radioactivity isolated in ATP and GTP plotted as counts per minute of [^{32}P]-phosphate in nucleotides divided by counts per minute in [^{32}P]-inorganic phosphate in the acid soluble fraction of the uterus. After uteri from oestrogen-stimulated rats were incubated with [^{32}P]-inorganic phosphate in vitro, ATP showed a five fold increase in specific activity over the increase due to uptake of [^{32}P]-inorganic phosphate. The synthesis of GTP was stimulated to a smaller extent.

Figure 27

A Comparison of the Effect of Oestrogen on Incorporation of Radioactivity into ATP, GTP and Inorganic Phosphate

Groups of 3 rats were injected with 1 μg of oestradiol-17β at various times before death. Uteri from these animals were removed to phosphate deficient Eagle's medium containing 50 μCi/ml [^{32}P]-inorganic phosphate. After 1 hour of incubation the uteri were used for the preparation of an acid soluble fraction. This fraction was analysed (as described in the Methods section 3.19.2.) by thin layer chromatography and the spots corresponding to inorganic phosphate and ATP were removed for scintillation counting. The counts per minute in the ATP were divided by those in the inorganic phosphate and are shown in figure 27. The radioactivity in GTP was treated in a similar way.

A Comparison of the Effect of Oestrogen on Incorporation of Radioactivity into ATP, GTP and Inorganic Phosphate



DISCUSSION

The oestrogen induced synthesis of ribosomal proteins in the immature rat uterus was selected for study for the following reasons: As part of a discrete unit, ribosomal proteins are easily isolated and there is now some standardisation of analytical techniques and nomenclature (McConkey et al., 1979). In addition, these proteins play an important role in the uterus, being necessary for the increase in protein synthesis following oestrogen treatment. Work by Merryweather and Knowler (1980) and Aziz et al., (1979b) indicated that profound changes in this group of proteins may be expected after oestrogen administration.

Figures 4 and 5 reveal that oestrogen induces the assembly of new high molecular weight polysomes in the rat uterus and that these polysomes could be isolated intact for further analysis. These results confirm the work of Gasde et al., (1971) and Merryweather and Knowler (1980) in showing that a maximal number of large polysomes could be isolated 12 hours after oestrogen treatment. The concept of polysome assembly in response to oestrogen is consistent with current views on the mode of action of oestrogenic and other steroid hormones, (see Table 1).

Although it was possible to isolate ribosomal proteins from the rat uterus (figure 7), it was soon evident that the yield was very small. Thus, a number of analytical techniques were to be used to aid identification of these proteins. These techniques included isotopic labelling in vitro, the use of rat liver carrier material and fluorography of ^{35}S labelled proteins. Some attempts had been made to study uterine ribosomal protein synthesis in vivo (Merryweather and Knowler, 1980). However, because of the diluting effect of the whole rat body, isotopic labelling of uterine ribosomes met with limited success. It was decided to extend

further studies in the direction of labelling *in vitro* after oestrogen treatment of the intact animal. Such techniques have been successfully used to study protein synthesis in the rat uterus (Notides and Gorski, 1966), mouse uterus (Korach *et al.*, 1981), Xenopus liver (Green and Tata, 1976) and chick oviduct (Palmiter and Schimke, 1973). Incubation of rat uteri in Eagle's medium under O_2:CO_2 (95%:5%) at 37°C with tritiated leucine resulted in an accumulation of isotopic label in ribosomal proteins (figure 8).

Melvin *et al.*, (1980) found maximal incorporation of radioactivity into mouse kidney ribosomal proteins over a period of $1\frac{1}{2}$ to 2 hours after nephrectomy. The results shown in figure 8 also indicate that a $1\frac{1}{2}$ to 2 hour incubation resulted in maximal incorporation into uterine proteins. However, the fall-off in incorporation after 2 hours and the morphological appearance of the uteri after prolonged incubation (Knowler, 1972) raised some doubts concerning the condition of these uteri during incubation. In addition, work by Dickson and Pogson (1980), and Grant and Black (1974) revealed some degradation of hepatocyte polysomes may occur after 2-3 hours of incubation *in vitro*. Figure 9 shows the effect of incubation on the polysome profiles of rat uteri and after one hour there was some decrease in the average size of the polysomes. Incubation periods of less than one hour resulted in low recoveries of radioactivity and 1 hour was therefore chosen as the optimal time for incubation. Attempts to increase the incorporation of radioactivity without increasing incubation time were unsuccessful.

Having selected the incubation conditions, the incorporation of radioactivity into uterine ribosomal proteins, after various periods of oestrogen treatment *in vivo*, were studied. Figure 10 shows that the incorporation of radioactivity

into ribosomal proteins was maximal at 12 hours of oestrogen stimulation and that the time course closely resembled that found by Merryweather and Knowler when following precursor incorporation *in vivo*. The only significant difference was the earlier response *in vivo* but this was not analysed further and its significance is unknown. Figure 10 also shows that the incorporation of radioactivity into ribosomal proteins did not resemble incorporation into either acid soluble or insoluble fractions of the rat uterus. There would appear to have been a faster accumulation of radioactivity in the 40S subunit than in the 60S subunit at 8 hours but this situation was reversed at 12 hours. It is possible that this was due to the more rapid appearance of the 40S subunit in the cytoplasm (Liau and Perry, 1969; Peterson *et al.*, 1976).

Most other workers have analysed the appearance of ribosomal proteins in whole ribosomes and used chemical rather than radiochemical methods of determination. Means *et al.*, (1971) isolated a maximal quantity of ribosomal protein at 7 days after diethylstibesterol treatment of chick oviduct. Bast *et al.*, (1977) determined peak ribosomal protein synthesis in chick liver to be 4 days after oestrogen treatment and maximal assembly of polysomes in Xenopus liver occured after 6 days (Berridge *et al.*, 1976). The action of oestrogen on the rat uterus would seem to be more rapid than on other oestrogen-responsive tissues, showing peak ribosomal protein synthesis at 12 hours after a single injection of Oestradiol-17 β. Tsurugi *et al.*, (1971) found a peak of rat liver ribosomal protein synthesis 12 hours after partial hepatectomy.

Analysis of the sucrose density gradients, used to fractionate ribosomal subunits (figure 11) revealed that incorporation of precursor into the ribosomal proteins of the rat

uterus was stimulated by oestrogen and that the proteins analysed were indeed ribosomal in nature and not nascent proteins.

The experimental procedures used above were adapted to analyse the incorporation of radioactive precursor into individual proteins. At first, problems were encountered due to insufficient incorporation of isotope but the use of [^{35}S]-methionine with fluorographic analysis of gels made the identification of many proteins possible. Figures 13 and 14 show the incorporation of precursor into individual ribosomal proteins and these results have been presented quantitatively in tables 3A and B and in figure 15. Incorporation of label into ribosomes from control uteri was very low, especially in the case of the 40S subunit. However, by 2 hours after the administration of oestrogen, precursor incorporation was stimulated and continued to increase over a period of 12 hours (Tables 3A and 3B).

Incorporation into the different ribosomal proteins was not uniform and the differences could not be explained by variation in methionine content. Also, it was unlikely that differential extraction was the cause of the variation since different extraction methods produced the same result and there was no evidence of differential extraction from the stained protein pattern. Some authors have shown that very short periods of labelling result in the preferential labelling of certain ribosomal proteins, for example with HeLa cells (Lastick, 1980) or in mouse L cells (Auger-Buendia and Tavitian, 1979). These authors suggested that short incubation periods, such as 15 minutes, resulted in the labelling of a class of late proteins, that is those that associated with the ribosome in the cytoplasm rather than in the nucleus. Longer periods of incubation, such as 75 minutes for example, did

not show this effect. Lastick (1980) and Auger-Buendia (Auger-Buendia and Tavitian, 1979) have both identified the proteins believed to associate with the ribosome in the cytoplasm in HeLa cells and mouse L cells. Comparable results were obtained for 5 proteins S3, S15, L10, L24 and L28. In the results of figures 13 and 14 it can be seen that S3, S15 and L10 incorporated significantly more label than many other proteins, however, the labelling of the uterine proteins did not resemble the labelling found in HeLa or L cells. This suggests that differential labelling was not due to insufficient incubation time. Futhermore, Merryweather and Knowler (1980) have shown that within 30 minutes of the administration of [^{3}H]-uridine, labelled rRNA can be detected in purified polysomes. Thus the labelling times used in the above study should be adequate to follow the association of all the ribosomal proteins with newly made rRNA. Finally, increasing the labelling time did not change the differences in the extent to which the different proteins were labelled (figures 13 and 14).

No analysis of extra-ribosomal pools of ribosomal proteins has been described for the rat uterus. Such pools would affect the incorporation of proteins into the ribosome. Nor has any analysis been made of ribosomal protein mRNA frequencies either as mRNP or within polysomes. Attempts to analyse the mRNA population of uterine polysomes will be discussed later.

It was also possible that the labelling pattern of ribosomal proteins shown in figures 13 and 14 were the result of differential turnover of ribosomal proteins. For this reason a pulse chase experiment was performed in an attempt to identify any proteins that may have been subject to rapid turnover. Conducting pulse chase experiments with intact tissue is very difficult.

Unlike experiments using cell monolayers the culture medium would not be in close contact with all the cells of a tissue. There may therefore be a significant delay between a change of medium and the effects of the new medium. However, figure 16 indicated that fresh Eagle's medium containing unlabelled methionine would affect the incorporation of radioactively labelled methionine into uterine proteins. There would also exist the difficulty of not knowing to what extent continued incorporation of precursor represented continued protein synthesis as distinct from movement of previously labelled particles from the nucleus into the polysomal fraction. Thus, one possible outcome of an effective pulse chase experiment would be that previously poorly labelled proteins might eventually incorporate as much precursor as strongly labelled species. Such a result would support the possibility that the differential labelling observed in figures 13 and 14 merely reflected a difference in the rate at which the different proteins were incorporated into polysomal ribosomes. Alternatively, if radioactivity was lost from the highly labelled proteins this would indicate rapid turnover.

In fact, figures 17 and 18 show that almost all proteins continued to be labelled at similar rates. Thus, proteins S2, S10, S15,15a, L3, L4, L10 and L19 continued to incorporate significantly more radioactivity than other proteins. Only proteins S36 and L29 may have lost radioactivity with prolonged incubation in the presence of unlabelled precursor. This result therefore tends to support the data of figures 13 and 14 which indicate a differential effect of oestrogen on the synthesis of uterine ribosomal proteins but such conclusions must be tempered by the reservations expressed above concerning pulse chase experiments with intact tissue.

Most work on the synthesis of ribosomal proteins has been performed on single cells such as yeast (Gorenstein and Warner, 1976), HeLa cells (Lastick, 1980), mouse L cells (Auger-Buendia and Tavitian, 1979) and frog oocytes (Pierandrei-Amaldi and Beccari, 1980). However, in all these cases analysis of individual ribosomal proteins has revealed differential labelling. Tsurugi et al., (1973) examined ribosomal protein synthesis in regenerating rat liver and they also found variation in the incorporation of labelled precursor into ribosomal proteins. Lieberman's group studied the effects of insulin on chick fibroblast cells in culture where insulin treatment resulted in a fourfold stimulation of ribosome synthesis (dePhilip et al., 1980). Again differential labelling of individual proteins was evident. Further studies by this group suggested that the control of ribosomal protein synthesis lay not in the transcription of new mRNA but in the efficiency with which pre-existing messengers were translated (Ignotz et al., 1981). Variations in the efficiency of mRNA translation may have explained the differential incorporation of radio-label into different proteins. Although the mechanism of action of insulin may be very different from that of oestrogen it was possible that similar translational effects occured in the rat uterus. Thus, it was considered important to analyse ribosomal protein mRNA populations in polysomes using a cell free translation system.

Cell free protein synthesising systems from various hormone responsive tissues have been described. Oestrogen stimulates cell free protein synthesis by polysomes from immature rat uterus (Gasde et al., 1971), ovariectomised rat uterus (Whelly and Barker, 1974), guinea pig uterus (Shapiro et al., 1975), chick oviduct (Means et al., 1971) and Xenopus liver (Shapiro et al., 1976). Insulin and

growth hormone stimulate protein synthesis *in vitro* by liver polysomes of diabetic and hypophosectomised rat respectively (Clemens and Pain, 1974) as does FSH on rat prostate (Johnsonbaugh *et al.*, 1976). Figure 19 shows the effect of oestrogen on cell free protein synthesis by the polysomes of the immature rat uterus. Since the translation mixture was messenger dependent and a constant number of ribosomes were added to the mixture it is possible that the synthesis of acid precipitable material reflected the amount of message and therefore the state of polysome aggregation. There are other interpretations to explain the increased translational activity after oestrogen treatment shown in figure 19. For example, oestrogen may have had an effect on the ribosomes in the uterus leading to increased translational activity (Whelly and Barker, 1974), however it is unlikely that this would be seen in a cell free system derived from rabbit reticulocytes. Alternatively there may have been oestrogen induced changes in the messages themselves (Aziz *et al.*, 1979a, 1979b) leading to more efficient translation *in vitro*. However, since the mRNA was added as polysomes, and not mRNP for example, it is likely that any changes observed *in vitro* had the same effects *in vivo*. Figure 19 appears to confirm the results of an earlier experiment (figure 4) in demonstrating maximal recovery of uterine polysomes 8-12 hours after oestrogen treatment. It may be that the aggregation of polysomes became maximal at 8 hours and it was probably ribosome synthesis that accounted for the greater yield of polysomes at 12 hours (Merryweather and Knowler, 1980).

Also shown in figure 19 is an immunological analysis to determine the extent to which the polysomes were synthesising ribosomal proteins. The labelling *in vitro* of ribosomal proteins

increased from control levels until 2 hours, after which synthesis remained constant. It thus appeared that the accumulation in uterine polysomes of mRNA's encoding ribosomal proteins was induced before 2 hours but remained a constant proportion of the total mRNA population thereafter. This stimulation was similar to that of the regenerating rat liver (Faliks and Meyuhas, 1982) and during Xenopus embryogenesis (Pierandrei-Amaldi et al., 1982). In both cases, analysis revealed the appearance of mRNA encoding ribosomal proteins in response to a growth stimulus. However, 3T6 cells, stimulated to grow by incubation in fresh serum, did not synthesise new ribosomal protein mRNA but assembled new polysomes with pre-existing mRNA (Geyer et al., 1982).

A number of different groups have employed gel electrophoresis to analyse cell free translation products for basic proteins. In this way differential labelling of ribosomal proteins was observed in Ascites tumour cells (Hacket et al., 1978), Xenopus oocytes (Pierandrei-Analdi and Beccari, 1980), yeast (Bollan et al., 1980) and regenerating rat liver (Nabeshima et al., 1979). Attempts to fractionate cell free translation products from rat uterine polysomes on basic urea-polyacrylamide gels met with very little success. Polysomes from 12 hour stimulated uteri were able to synthesise just enough labelled ribosomal protein to be detected by fluorography. However, these fluorograms did not photograph well enough for reproduction here. It may be that ribosomal protein mRNA did not constitute a large enough fraction of uterine mRNA to be identified by crude means. Greater success was achieved with sodium dodecylsulphate polyacrylamide gel analysis of specific immune precipitates. Figure 20 shows the separation of ribosomal proteins precipitated from a translation mixture and analysed on a

gel of 8.75% polyacrylamide. Use of this unusually low concentration of polyacrylamide (see Methods section) revealed that although many low molecular weight proteins were bound by the antibody, no high molecular weight proteins were precipitated. This is further proof of the specificity of this antibody. Furthermore, the radioactive bands correspond with the stainable bands of unlabelled ribosomal protein. It was not possible to analyse immune precipitates by separation in 2 dimensions because of the necessity of including sodium dodecylsulphate to dissociate the immune complex.

The antibody was also used to titrate the appearance of ribosomal protein in the uterus in terms of quantity rather than by isotopic labelling. Although the results in figure 22 confirm previous work on the first 12 hours of oestrogen action, the data for times after 12 hours were unexpected. The accumulation of new ribosomal protein over the first 12 hours was predicted by polysome profile analysis (figure 4), by incorporation of radio-labelled precursors into ribosomes (figures 13 and 14) and by the appearance of specific mRNA in active polysomes, (figure 19). However, ribosomal proteins have an estimated half life of between 3 and 6 days in rat liver (Tsurugi et al., 1974; Kawada et al., 1977; Conde and Franze-Fernandez, 1980) and the most rapid turnover of ribosomes seems to occur in BHK cells with a half life of 18 hours (Melvin and Kier, 1978). The ribosomal protein antigenic titre of the uterus fell rapidly after 12 hours of oestrogen treatment indicating ribosome degradation. This disappearance of ribosomal protein was not confirmed by protein assay or RNA assay of polysome pellets or by sodium dodecylsulphate polyacrylamide gel analysis of different cell fractions. Such analysis would not reveal the disappearance

of one protein and if the antiserum had been monovalent this might have explained the anomalies in figures 22 and 23. However, figure 20 had already confirmed the polyvalent nature of the serum. It was also possible that the loss of antigenicity was an artefact of the experimental technique. It is known that general protein synthesis is stimulated at about this time (Merryweather and Knowler, 1980) and since a constant amount of total cell sap protein was assayed the increasing cellular protein may have produced a drop in the proportion of ribosomal protein in the assayed samples. However, the results of oestradiol-benzoate treatment refute this, since this long acting oestrogen also stimulated protein synthesis but did not show the same loss of ribosomal protein antigenicity. The answer to this problem may reside in the results of figure 21 which show that isolated ribosomal proteins were able to bind approximately 5 x as much antibody as proteins in polysomes. This was presumably due to the loss of antigenic sites when the proteins associated with ribosomal particles. Thus, the loss of antigenicity revealed in figures 22 and 23 may have been due to the movement of newly synthesised ribosomal proteins from the cytoplasm into ribonuclear protein particles. Tsurugi and Ogata (1977) found that the half life of unbound ribosomal proteins in the rat liver cytoplasm was between 20 and 40 minutes. It would appear that no other groups have used an anti-ribosomal protein antibody to study the synthesis of these proteins although these antibodies have been used for immunological comparisons of proteins from various species (Leader and Coia, 1978; Fischer et al., 1978) and for the analysis of extra ribosomal protein pools (Wool and Stoffler, 1976).

Figure 24 shows the isotopic labelling of ribosomal proteins of uteri after incubation in vitro in the presence of [^{32}P]-phosphate. One of these proteins was shown to be S6 and

the others tentatively identified as the acidic phosphoproteins of the large subunit. Indications in figure 24 that there was stimulation in the incorporation of inorganic phosphate into protein S6 as a result of oestrogen administration was shown to be due to a variety of reasons. These reasons included changes in precursor pools and the increased synthesis of protein S6 (figures 26 and 13 respectively). The increased phosphorylation could not therefore be solely attributed to the activation of a specific protein kinase, although such activation was not ruled out. Many attempts have been made to correlate ribosomal protein phosphorylation to the aggregation state of polysomes and both glucagon (Gressner and Wool, 1976) and insulin (Lastick and McConkey, 1981) increase S6 phosphorylation and polysome assembly. However, it was not possible to make this correlation in the rat uterus responding to oestrogen.

The main conclusions of the above work provide a number of indications towards future experimentation. They may be enumerated as follows:

1. The synthesis of ribosomal proteins in vitro permitted an analysis of the effects of oestrogen on the production of individual proteins and indicated a differential effect on incorporation of different protein species. It would be desirable to determine whether this was a result of differential transcription, translation, or turnover of synthesised proteins. Time did not permit any analysis in the present study of the effects of oestrogen on the synthesis of mRNA's encoding ribosomal protein. The most likely explanation of the increased translation of ribosomal proteins from a fixed quantity of uterine polysomes is that they contained an increased proportion of ribosomal protein

mRNA but that mRNA could have come from increased transcription or from previously inactive message stored as mRNP. Cloned cDNA's for specific ribosomal proteins would provide the best tool to investigate this possibility. Similarly, mono-typic or preferably monoclonal antibodies to selected ribosomal proteins would provide the best means by which to further examine the possibility of differential translation or turnover of different proteins in the oestrogen stimulated uterus.

2. The difference in timing and magnitude, between oestrogen stimulated translation of ribosomal proteins and the appearance of the proteins in the ribosomal particles (figures 10 and 19) warrants further investigation as they indicate effects on the turnover and pools of ribosomal proteins. Specific monoclonal antibodies, preferably to antigenic sites shown to be equally available in the free and polysome bound proteins, would provide the best system with which to further study this phenomenon.

3. The use of cloned cDNA sequences and monoclonal antibodies would also permit the further analysis of another major problem, namely the co-ordination of ribosome assembly. Thus, by using the recombinant sequences to follow the transcription and processing of the mRNA's for ribosomal processing and monoclonal antibodies to follow their translation and processing, it would be possible to obtain an integrated picture of the production of specific ribosomal proteins and to relate this to the production and maturation of rRNA.

4. The uterus is comprised of different cell types which may well differ in their response to oestrogen. McCormack and Glasser (1980) described a fractionation of uterine tissue into three major cell types and Beaumont and Knowler (1983) have used the

technique to investigate the effects of oestrogen on protein synthesis in uterine epithelium, endometrium (stroma) and myometrium. The greater sensitivity which could result from the use of cloned cDNA to ribosomal proteins and of monoclonal antibodies would allow the study of oestrogen effects on ribosomal protein synthesis to be investigated in the different cell types.

Future research on the effect of oestrogen on the synthesis of ribosomal proteins in the rat uterus would therefore appear to require the joint approach of recombinant DNA technology and monoclonal antibodies.

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