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

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Abbreviations

The abbreviations recommended by the Biochemical Journal in its Instructions to Authors, 1981, have been used throughout this thesis with the following additions:-

Oestradiol-17β	1,3,5,(10)-estratriene-3,17β-diol
IP	Induced protein
hnRNA	heterogeneous nuclear ribonucleic acid
hnRNP	heterogeneous nuclear ribonucleoprotein
MDL	messenger-dependent rabbit reticulocyte lysate
PPO	2,5-diphenyloxazole
DMSO	dimethylsulphoxide
TEMED	N,N,N',N'-tetramethylethylene diamine
IEF	isoelectric focussing
NEpHGE	non-equilibrated pH gradient electrophoresis

SUMMARY

A number of previous studies have built up a picture of the early events in the oestrogenic response of the immature rat uterus. This consists of an overall growth and development of the tissue preparing it to support a pregnancy. The most important requirement in this chain of events is the massive increase in protein synthesis as the lining of the uterus differentiates and proliferates. This requires the synthesis of large numbers of new ribosomes. The evidence suggests that this synthesis is preceded by and is dependent on stimulated hnRNA synthesis which is detectable 30 minutes after giving the hormone. It is apparently vital that messenger sequences present in this hnRNA be translated before the stimulation of ribosomal RNA can occur. Hybridisation studies suggest that the proteins eliciting this response are encoded by a small number of moderately abundant messenger sequences, all of which appear in the polysome population between 2 and 4 hours after oestrogen administration.

This project set out to detect and characterise these proteins by radio-labelling immature rat uteri *in vitro* after short periods of oestrogen stimulation *in vivo* and analysing the protein species by two dimensional gel electrophoresis and fluorography.

The three approaches adopted were the labelling of whole uteri *in vitro*; the labelling of fractionated epithelial, stromal and myometrial cells *in vitro*; and the translation of uterine polysomes in cell free systems. These three methods yielded different information. Whole uteri labelled *in vitro* showed very little difference between the protein populations from control and stimulated animals, although analysis of their polysome profiles showed the translational statuses

of the two groups of uteri to differ greatly.

The study of fractionated cell type proteins revealed a differential response to the hormone. Epithelial cells showed relatively small quantitative differences between the control and hormone-treated uteri, but at least three new proteins were reproducibly detected as radioactive spots. Stromal protein synthesis was strongly stimulated after four hours of oestrogen treatment and in addition to the quantitative changes, some further qualitative changes were also detected. Nine new species were seen in response to oestrogen treatment and the labelling of a number of other species was strongly enhanced. Little data were collected on the response of the myometrium which showed lower levels of radioactive incorporation, and fewer protein species than the other two fractions examined.

Cell-free translation revealed a number of changes in the protein population during the first four hours of stimulation. The synthesis of at least ten proteins was stimulated 2 hours after oestrogen administration although only five of these were totally reproducible. Further changes were observed by 4 hours after treatment. Unfortunately, none of the new proteins detected by cell-free protein synthesis could be identified with oestrogen-induced species in intact, isolated cells. However, the possible identity of some of the proteins synthesised in increased quantities by polysomes from oestrogen-treated rats was investigated. These experiments established the presence of actin and IP, an induced protein, in the uterine protein population. None of the stimulated uterine protein species comigrated with a number of other candidate proteins, including non-histone chromatin protein and hnRNP particle protein, however, the presence of low levels of ribosomal proteins was established. The

results obtained by each method are assessed, discussed and compared with results from other systems. It is thought that the bulk of early induced proteins we sought to identify was present in quantities too small to be detected using the methods employed in this study.

INTRODUCTION

1. INTRODUCTION

1.1 Steroid Hormones

The complex processes of growth and differentiation in animals require control signals to coordinate these developments. Hormones are the chemical messengers which perform this function and in particular, it is steroid hormones which bring about long term changes in the macromolecular synthesis patterns of their target cells. This has led to a great deal of interest in the mechanism of steroid action as a handle to further the understanding of the control of gene expression.

Steroid hormones are derived from the 27-carbon compound cholesterol. The removal of a C_6 side chain results in the C_{21} compound pregnenolone, which is the precursor of progesterone. This in turn can be converted into the corticosteroids (glucocorticoids and mineralocorticoids) in the adrenal cortex, and the androgens which are made in the testis. Androgens are also made in the ovary where they are converted into the 18-carbon oestrogens by loss of a carbon atom.

1.2 The mechanism of action of steroid hormones.

Steroids are transported from their site of synthesis bound to plasma proteins in the blood. They do not appear to be excluded from any cell types in the body and enter the cell, probably by passive diffusion across the plasma membrane. They are retained only in the cells of their target tissues by binding to specific cytoplasmic receptor molecules (Jensen & DeSombre, 1972; Gorski & Gannon, 1976). In general the steroid binding molecules are heat labile and non dialyzable and their protein nature has been demonstrated by their sensitivity to proteolytic enzymes (Toft & Gorski, 1966). They are characterised by

high affinity for steroids (K_D 10^{-9}-10^{-12}M) and are found in both cytoplasm and nuclei of target cells (Jost, 1978). The hormone receptor complex is translocated to the nucleus where it binds to the chromatin and induces specific gene transcription. This two step mechanism of action was originally observed for oestrogens (Jensen et al., 1968; Jensen & DeSombre, 1972), but has been shown to be general and can be demonstrated for progesterone (Schwartz et al., 1976; Buller et al., 1976a), androgens (Mainwaring & Peterken, 1971; Bullock & Bardin, 1974) mineralocorticoids (Edelman et al., 1968) and glucocorticoids (Baxter et al., 1972; Higgins et al., 1973a; Rousseau et al., 1973).

In a number of target tissues, steroids stimulate the synthesis of major new secretory proteins. These are valuable markers for monitoring the control and kinetics of the response and have enabled specific probes to be made with which to study the genes that specify such proteins. The most extensively studied of these systems is the chick oviduct. Oestrogen treatment of immature female chicks causes development of the tubular gland cells of the oviduct and these secrete the major egg-white proteins, ovalbumin, conalbumin, lysosyme and ovomucoid (O'Malley et al., 1969). It has been shown that oestrogen treatment causes an increase in the level of the messenger RNA species coding for these proteins (Means et al., 1972; Palmiter, 1973; Rhoads et al., 1973; McKnight et al., 1975). Progesterone also stimulates the levels of conalbumin and ovalbumin messenger RNA in oestrogen-primed birds and induces another egg-white protein, avidin (Chan et al., 1973; McKnight et al., 1975; Palmiter et al., 1976)

Another much-studied system is the induction by oestrogen of the egg-yolk protein precursor, vitellogenin, in the livers of the toad

Xenopus laevis (Clemens, 1974; Tata, 1976) and chicken (Jost et al., 1978; Ryffel, 1978). Androgens have been shown to stimulate specific messenger RNA and protein synthesis in rat ventral prostate (Mainwaring et al., 1974; Parker & Scrace, 1978) and to stimulate both specific and total protein synthesis in the rat seminal vesicle (Higgins et al., 1976; Higgins & Burchell, 1978; Ostrowski et al., 1979). A number of secreted proteins have been shown to be regulated at the transcriptional level, and this occurs as part of a general stimulation of the synthesis of all classes of RNA and subsequently of general protein synthesis. This is similar to the effect of oestrogens on mammalian uteri where RNA and protein synthesis are stimulated and the organ hypertrophies. Oestrogens and progesterone have been shown to exert some translational control in the chick oviduct (Palmiter, 1972; Robins & Schimke, 1978; Pennequin et al., 1978), but the major effect is at the level of stimulated transcription. The following sections will expand on the mechanism of action as it is known for oestrogen, comparing this with other hormones.

1.3.1 The Oestrogen receptor.

This was first characterised by Toft & Gorski (1966) in uterine tissue. Cells of the newborn rat uterus contain receptor at low levels, which increase to a maximum at about day 10 and remain nearly constant until the onset of puberty at 28 days (Clark & Gorski, 1970; Barbanel & Assenmacher, 1980). There are about 15-20,000 cytoplasmic receptor sites per cell (Clark et al., 1978a), although estimates as low as 6,000 and as high as 100,000 per cell have been reported (Jensen & DeSombre, 1972; Leake, 1976). The protein is a single polypeptide with a molecular weight of approximately 80,000, having a single hormone binding site (Notides & Nielsen, 1974). Toft & Gorski

(1966) isolated the receptor in association with radioactively labelled oestradiol-17β on sucrose gradients of low ionic strength. Under these conditions, the sedimentation coefficient can vary from 7-9S, although 8S is the usually reported value. Sucrose density centrifugation generates a wide variety of sedimentation coefficients depending on the salt concentration used during extraction and sedimentation (Chamness & McGuire, 1972) but it is generally accepted that the cytoplasmic form of the oestrogen receptor exists in 4S form. In common with other steroid receptors it has a tendency to aggregate and to bind other proteins in tissue homogenates (Stancel et al., 1973).

1.3.2 Heterogeneity of oestrogen-binding sites

It has long been known that oestrogen binds to sites in the cell other than the classical receptor (Erdos et al., 1969; Best-Bolpomme et al., 1970; Steggles & King, 1970). Usually these have been discounted as serum albumin or α-fetoprotein (Michel et al., 1974). Further study by Clark and his co-workers, however, has characterised these sites in both cytoplasm (Eriksson et al., 1978) and nucleus of rat uterus (Markaverich & Clark, 1979). Type I sites represent the classical oestrogen receptor, having high affinity ($K_D \sim 1nM$) and low capacity (1pmol/uterus) in the cytosol and are translocated to the nucleus after an injection of oestradiol. Cytoplasmic Type II sites are present in four times the concentration and have an affinity for oestradiol about 40 times lower than that of the Type I sites ($K_D \sim$ 30nM). This means that they are undetected by low concentrations of tritiated oestradiol and release labelled hormone during sucrose gradient centrifugation. However, they can be detected by post labelling gradients and sediment at the 4S position (Clark et al., 1978b). Type II sites are not found in blood and persist in adult

rats; they are therefore distinct from the serum albumins and α-fetoprotein. Up to 50% of oestrogen bound in the uterus is distributed between these secondary sites (Peck et al., 1973). Although their function is not known it may be that Type II sites serve to concentrate oestrogen in the target cells.

Type II nuclear oestrogen-binding sites do not originate from either Type I or Type II cytoplasmic sites. They also have a lower affinity for oestradiol (K_D 10-30nM) and exist in higher concentrations than the Type I sites. They are stimulated by oestradiol and high levels may be maintained for up to 72 hours after a single oestradiol injection, whereas Type I sites decline to low levels by 24 hours (Markaverich & Clark, 1979). Markaverich & Clark observe that oestrogen stimulation of these sites is highly correlated with the long term uterine growth responses, but not with early responses. Their increase is dependent on long term (>6 hours) nuclear occupancy by Type I sites and thus weak oestrogens such as oestriol which do not elicit the full growth response, also do not stimulate either the long term retention of Type I sites or elevated levels of Type II sites. The observation that nuclear type II sites are absent from target tissues such as pituitary and hypothalamus, which do not grow in response to oestrogen, supports the premise that they are involved in growth responses (Kelner & Peck 1981).

Smith & Taylor (1981) have recently shown that an oestrogen-binding site distinct from the classical receptor, but which is translocated into the nucleus by oestrogen, mediates the effect of the receptor (X) on ovalbumin mRNA induction. This second receptor (Y) is absent from unstimulated oviduct and its disappearance from hormone-treated oviduct can be achieved by using the drug Danazol. In the presence of

Danazol, X is unaffected and oestrogen stimulates both its translocation and RNA polymerase II activity. The absence of Y however, is correlated with the decreased transcription of ovalbumin mRNA after oestrogen stimulation.

Secondary binding sites for oestrogen have now been described for rat and guinea-pig uterus (Capony & Rochefort, 1978; Sumida & Pasqualini, 1979), mouse and human mammary tissue (Watson & Clark, 1980; Panko et al., 1981), chick oviduct (Smith et al., 1979) and rabbit corpus luteum (Yuh & Keyes, 1979). Multiple binding sites have also been described for glucocorticoids (Barlow et al., 1979; Do et al., 1979), for progesterone (Giannopoulos & Munowitz, 1980) in a variety of tissues and for aldosterone (Farman et al., 1978). These findings suggest that secondary binding sites for steroids are a general phenomenon.

1.3.3 Binding of oestrogen to the cytoplasmic receptor

The binding of steroid hormones to their receptors results in increased affinity for nuclei, chromatin and DNA, which is believed to be caused by an allosteric effect (Jensen et al., 1969; Samuels & Tomkins, 1970; Yamamoto & Alberts, 1974). In the case of the oestrogen receptor only, there is also an increase in the sedimentation coefficient, from 4S to 5S. These changes are termed 'activation' or 'transformation' in the literature. To avoid confusion in the case of the oestrogen receptor, Gschwendt & Kittstein (1980) have suggested that 'activation' be reserved for the increase in affinity for nuclear components and that 'transformation' should be used to describe the increase in sedimentation coefficient. They have shown that for the oestrogen receptor in chick oviduct, activation, measured as increased affinity for DNA-cellulose, can be achieved by high salt treatment

with no accompanying increase in receptor size. Work on the rat uterine oestrogen receptor confirms that the two processes are separable and occur at different rates with different orders of reaction . 4S & 5S receptors recovered from gradients show equal proportions of activated receptors thus disproving the possibility that inactive 4S receptors are transformed to active 5S receptors in a single step (Bailly et al., 1980).

Activation is a temperature dependent process for all steroid hormone receptors. When the hormones are incubated at low temperatures (0-4^{o}C), either with target cells or with cytosols from such cells, they form 'non-activated' complexes that do not bind nuclei (King & Mainwaring, 1974). As shown originally with oestrogens (Jensen et al., 1972) and subsequently for glucocorticoids (Munck et al., 1972; Baxter et al., 1972), if these cytosols are warmed to 20-37^{o}C in the absence of nuclei the complexes become activated and will then bind nuclei even at low temperatures. Activation can also be brought about by increased ionic strength (Williams, 1974; Baxter et al., 1972; Higgins et al., 1973b; Kalimi et al., 1973), gel filtration, dilution (Goidl et al., 1977), and in the case of the oestrogen receptor, by dialysis (Sato et al., 1979). This last treatment is of interest since it does not require the presence of hormone, and suggests the oestrogen receptor is bound by a low molecular weight inhibitor of activation. Evidence for similar inhibitors of glucocorticoid (Goidl et al., 1977; Bailly et al., 1977), progesterone (MacDonald & Leavitt, 1982) and androgen (Sato et al., 1980) receptor activation have also been reported . This agrees with Notides' (1978) model of oestrogen receptor activation in which he predicts such an inhibitory molecule from kinetic data. It is not known if activation occurs immediately as a result of hormone binding <u>in vivo</u> or if a nonactive complex is

formed first. In the case of the glucocorticoid-receptor complex, Munck and Foley (1979) argue that a nonactive complex is an obligatory short-lived intermediate. This may indicate a step in which the inhibitor is released.

The hormone-binding capacity of receptors decreases after activation and it is possible that activation involves a conformational change in the receptor causing steric hindrance at the steroid binding site. This could slow down the dissociation of hormone from complexes and prevent the steroid-binding by free receptors. High salt or elevated temperature activation have been shown to inactivate free glucocorticoid receptors to a form incapable of binding free steroid (McBlain & Shyamala, 1980). The low molecular weight factors have been implicated in this process and some authors have suggested that they include a shortlived reducing activity (Leach et al., 1979, 1982; Nishigori & Toft, 1980; MacDonald & Leavitt, 1982). Reduced sulphydryl groups are required for optimal steroid-binding by some receptors and this is stabilised by reducing agents such as EDTA and dithiothreitol (Rees & Bell, 1975; Sando et al., 1979; MacDonald & Leavitt, 1982). The loss of steroid receptor hormone-binding capacity can be prevented by the metal oxyanion molybdate and in some cases by tungstate and vanadate (Leach et al., 1979; Nishigori & Toft, 1980; Noma et al., 1980; Shyamala & Leonard, 1980; Dahmer et al., 1981). In stabilising the nonactivated receptors these substances inhibit receptor transformation and may interfere with the dissociation of the low molecular weight inhibitors. An example is the case of the mouse mammary glucocorticoid receptor, where molybdate causes the nontransformed receptor to sediment at 7-8S instead of the 4S form usually found (McBlain et al., 1981).

Since molybdate, tungstate and vanadate are phosphatase inhibitors, it has been suggested that a phosphorylation process is involved in steroid-binding and the activation of receptors (Shyamala & Leonard, 1980; Nishigori & Toft, 1980). Recently Auricchio et al. (1981a) have demonstrated that the oestrogen-binding capacity of the receptor can be activated in vitro by an ATP-dependent enzyme isolated from uterine cytosol. A nuclear phosphatase causes the release of oestrogen in vitro, from both cytosolic and nuclear steroid-receptor complexes (Auricchio et al., 1981b,c). Their observation that the phosphatase is ineffective in releasing the antioestrogens nafoxidine and tamoxifen has led them to propose that this explains the long-term nuclear retention of non-steroidal antioestrogens in intact cells (Auricchio et al., 1981c). A dephosphorylating process which releases glucocorticoids from cytosolic receptor complexes has also been demonstrated (Wheeler et al., 1981). Furthermore, the chick progesterone receptor has been shown to be a substrate for heterologous phosphorylases, and phosphatase inhibitors prevent the loss of the receptor hormone-binding activity in vitro (Weigel et al., 1981). Thus a number of factors and processes may modulate the activation of all types of steroid hormone receptor.

The binding of the hormone may elicit a change in conformation that in part causes the transformation of the oestrogen receptor from the 4S to the 5S form. However much evidence has built up to show that this is a second order reaction involving another molecule as well as the hormone-receptor complex (Yamamoto & Alberts, 1972; Yamamoto, 1974; Thrower et al., 1976; Murayama et al., 1980). The binding of this factor increases the molecular weight of the complex by 50,000, from 80,000 to 130,000. This factor was recently characterised by Thampan & Clark (1981) in rat uterine cytosol. They reasoned that the receptor

activating factor might be similar to cytoplasmic proteins which stimulate eukaryotic RNA polymerases. These are basic proteins which are not adsorbed onto DEAE-cellulose and have a sedimentation coefficient of about 3S (Seifart et al., 1973). Indeed, the fraction of cytosol which did not bind DEAE-cellulose was found to contain a protein with these properties, which was shown to bind to the 4S oestrogen-receptor complex although it did not bind oestrogen alone. This protein caused activation and transformation to the 5S receptor form, which could then bind uterine nuclei, DNA-cellulose and native calf thymus DNA. It is possible that the function of the receptor is to translocate this factor to the nucleus where it stimulates transcriptional events (Thrower et al., 1976; Thampan & Clark, 1981). It is interesting that the binding of a similar factor has not been demonstrated for other steroid hormones. For example, the progesterone receptor isolated from exactly the same protein environment as the oestrogen receptor shows no increase in size when activated. This has been shown for rabbit and rat uterus, and in chick oviduct (Fleischmann & Beato, 1979; Bailly et al., 1980; Gschwendt & Kittstein, 1980).

It is not entirely clear whether oestrogen receptor transformation occurs in the cytoplasm or in the nucleus. Whilst transformation can occur in the absence of nuclei (Jensen et al., 1971) it occurs much more rapidly in the presence of DNA (Yamamoto, 1974). In addition, pulse chase experiments by Siiteri et al. (1973) have shown a nuclear 4S form which decreases as the 5S form increases. Kinetic evidence which shows the disappearance of cytoplasmic 4S hormone-receptor complexes, coincident with the appearance of nuclear 5S complexes, indicates that the 4S and 5S forms are related (Shyamala & Gorski, 1969; Giannopoulos & Gorski, 1971). This is confirmed by sedimentation

studies which show that the isolated 5S hormone-receptor complex sediments at 4S in the presence of urea (Notides & Nielsen, 1974). In addition, antisera raised against calf uterine nuclear receptor cross-react with both cytoplasmic and nuclear receptors from rat uterus (Greene et al., 1977).

1.3.4 Translocation of the steroid-receptor complex to the nucleus

Nuclear concentration of receptor-bound steroid was one of the earliest observations in the study of receptor function (Noteboom & Gorski, 1965; King & Gordon, 1966; Maurer & Chalkley, 1967; Stumpf, 1968a). However the translocation step in steroid action is poorly understood. There is an absolute requirement for the specific steroid and it is reported not to be energy dependent or protein synthesis dependent (Shyamala & Gorski, 1969). However there is some work which indicates that ATP is involved in alleviating the effects of a macromolecular inhibitor of translocation of glucocorticoid receptors (Horiuchi et al., 1981), and the level of nuclear binding of corticosteroids is related to the cellular ATP level (Sloman & Bell, 1976). Nuclear binding of the oestrogen-receptor complex is enhanced by incubating with ATP (King et al., 1971), and progesterone receptor complexes will bind ATP-sepharose (Miller & Toft, 1978; McBlain et al., 1981). Thus although its function seems unclear, ATP may be involved in steroid receptor function. There are frequent reports of factors (both macromolecular and of low molecular weight) inhibiting translocation and nuclear binding. Inhibitors of glucocorticoid and oestrogen receptor translocation have been described as both protein and RNA (Chamness et al., 1974; André & Rochefort, 1975; Milgrom & Atger, 1975; Simons et al., 1976; Cake et al., 1978; Isohashi et al., 1980; Feldman et al., 1981).

It has been suggested that steroid receptors are metalloproteins, and that activation causes an allosteric change, involving the altered availability of the metal ions, which are important in the translocation process (Schmidt et al., 1981). This hypothesis is based on the finding that 1,10-phenanthroline, a metal-ion chelating compound, inhibits the binding of glucocorticoid receptors to DNA-cellulose. The effect is not due to the chelation of free metal ions, or to damage to the DNA. Furthermore, the inhibitor causes activated receptor complexes to elute from DEAE-cellulose as if they are nonactivated. The results from this study suggest that the metal ions are integral parts of the receptor protein, and are directly associated with a DNA binding site or regulate it allosterically. There is also evidence that the oestrogen receptor is a metalloprotein (Shyamala, 1975) and that phenanthroline inhibits the binding of progesterone receptors to nuclei (Lohmar & Toft, 1975). It has been suggested that molybdate may stabilise steroid-free receptors by interaction with a heavy metal (Nishigori & Toft, 1980).

The exact mechanism of translocation is unclear, most authors attribute it to the increased affinity of the steroid-receptor complex for a nuclear component. Gannon et al. (1976) propose an elegant model based on the work of Horowitz & Moore (1974) on the intracellular movements of solutes. They hypothesised that not all of the water present in the cell may be available to solutes due to the existence of gels of macromolecular cellular constituents. These would make a large proportion of the cytoplasmic water unavailable as a solvent, and act as molecular sieves preventing free access of solutes to all areas of the cell. The cytoplasmic exclusion model suggests that the receptor is loosely bound to a cytoplasmic molecule preventing its movement into the nucleus. Binding of the hormone causes an allosteric

change releasing the receptors so that they can equilibrate with the larger water space available in the nucleus.

The amount of oestrogen-receptor complex in the nucleus declines slowly after translocation, to control levels at about 24 hours after hormone treatment. Cytoplasmic receptor levels are elevated above control values at this time (Clark et al., 1973) and this indicates that oestrogen is inducing the synthesis of its own receptor. Studies with actinomycin D, a transcriptional inhibitor, and cycloheximide, an inhibitor of protein synthesis, suggest that about 60% of the oestrogen receptors reappearing in the cytoplasm after their depletion by translocation are newly synthesised, because they are dependent on de novo RNA or protein synthesis (Jensen et al., 1969; Sarff & Gorski, 1971). The remaining 40%, however, are most likely replenished by recycling of the nuclear receptors. It seems likely that both de novo synthesis and recycling contribute to cytoplasmic receptor replenishment and Mester & Baulieu's findings (1975) that replenishment can be divided into an early phase which is not affected by inhibitors and a later protein-synthesis dependent phase is compatible with existing results. Kassis & Gorski (1981) report that after a single injection of the short acting oestrogen 16α-oestradiol, cytoplasmic receptor replenishment is complete within 4 hours and is not blocked by cycloheximide treatment. The disappearance of nuclear receptor corresponds to reappearance of receptor in the cytoplasm. Short acting oestrogens such as 16α-oestradiol and oestriol induce early oestrogenic responses such as the imbibition of water and stimulated protein synthesis, but fail to induce long term responses such as DNA synthesis and uterine growth. The long acting oestrogens such as 17β-oestradiol also lead to a decrease in total numbers of receptors by a loss of some nuclear molecules. This

"processing" (Horwitz & McGuire, 1978a) may involve modification or degeneration, but it is inhibited by actinomycin D (Horwitz & McGuire, 1978b).

Because it is characteristic of long acting oestrogens, receptor-modification may be important in long term responses in the uterus. Schoenberg & Clark (1979) have found an endogenous nuclease in immature rat nuclei which solubilises nuclear receptors and is also susceptible to actinomycin D inhibition. They subsequently found that nuclear-bound receptors resistant to exogenous DNAase treatment correspond to those sites which are processed (Schoenberg & Clark, 1981). This suggests that some of the nuclear receptors are involved in a function other than binding to chromatin sites and that only this subset of receptors is processed . In cultures of human breast cancer cells (MCF-7) processing of nuclear receptors after oestradiol treatment is associated with the induction of progesterone receptor. When anti-oestrogens such as tamoxifen or nafoxidine are added to the culture, they translocate the receptors to the nucleus but processing is only partial or absent. In this case no progesterone receptor is induced (Horwitz & McGuire, 1979c).

1.4 Nuclear acceptor sites.

Because of the steroid induction of specific gene expression, much effort has been put into the attempt to identify the particular sites at which the receptor complex binds in the nucleus, to elicit this response. These sites are known as 'acceptor' sites, and are expected to have a limited capacity and high affinity for the steroid-receptor complex. They should also be hormone and target tissue specific. The demonstration of nuclear acceptor sites has been difficult and controversial (Chamness et al., 1974; Buller et al., 1975). Several

investigators have shown nuclear binding to be a saturable phenomenon, thus suggesting that limited numbers of acceptors do exist (Fang & Liao, 1971; Mainwaring & Peterken, 1971; King & Gordon, 1972; O'Malley et al., 1972; Higgins et al., 1973a), while others claim that limited numbers of specific nuclear sites do not exist and that nuclear saturation is an artifact (Chamness et al., 1973; André & Rochefort, 1975). The problems associated with the interpretation of *in vitro* binding experiments was highlighted after a study by Higgins et al. (1973c) on the nuclear binding of glucocorticoid receptors in hepatoma cells and oestrogen receptors in immature rat uterus. It was shown that saturation of cells with hormone did not inhibit the subsequently isolated chromatin from binding further hormone-receptor complexes. This led to the proposal that the available sites *in vivo* are not identical to those *in vitro* and that results are dependent on the isolation procedure used. Yamamoto and Alberts (1975) have argued, however, that a small number of specific acceptor sites would be completely masked by large numbers of low affinity sites which may facilitate rapid transfer of receptor complex to the high affinity acceptors. They may also be necessary for multiple binding of steroid receptors which may function cooperatively in activating a transcription unit (Yamamoto & Alberts, 1976). This correlates with the work of Clark and others who have shown that the usual method of potassium chloride extraction of uterine nuclei does not remove all nuclear-bound oestrogen. This indicates that some oestrogen-receptor complexes are bound more tightly than others (Clark & Peck, 1976; Barrack et al, 1977; Traish et al., 1977). Clark's group have shown that only a limited number of nuclear binding sites, 1-3000 per cell, are involved in the production of maximal uterine growth (Anderson et al., 1972, 1973, 1975). These sites are retained for longer than 4 to 6 hours in the nucleus and equate in number to the sites resistant to

salt extraction. Clark & Peck propose these as the true acceptor sites for oestrogen-receptor complex. Salt-resistant nuclear oestrogen-binding sites are also found in the immature mouse uterus where 0.4M KCl exposes 3,500 salt resistant sites per cell (Korach, 1979).

The nuclear envelope (Jackson & Chalkley, 1974a,b), histone (King & Gordon, 1967; Slyser, 1969), non-histone protein (Spelsberg et al., 1971; King & Gordon, 1972; Liang & Liao, 1972; Lebeau et al., 1973; Puca et al, 1974; Mainwaring et al., 1976), DNA (Clemens & Kleinsmith, 1972; Musliner & Chader, 1972; Higgins et al., 1973a; Yamamoto & Alberts, 1975), ribonucleoproteins (Liao et al., 1973) and the nuclear matrix (Barrack et al.,1977) have all at different times been proposed as the acceptor sites.

Early DNA digestion experiments suggested that DNA might be involved in binding the steroid-receptor complex. Deoxyribonuclease (DNAase) destruction of DNA in rat uterine nuclei, prevented the formation of nuclear oestradiol-receptor complex upon subsequent incubation with cytosol containing activated complexes. The digestion did not release any component capable of binding either oestrogen or the receptor complex, and thus appears to destroy the acceptor (Musliner & Chader, 1971). DNAase also releases prebound receptor complexes from the nucleus (Harris, 1971). Schoenberg and Clark (1981), examining the rat uterus 1-12 hours after oestrogen stimulation, have shown a correlation between the levels of nuclear-bound receptors which can be solubilised by exogenous nucleases, and the numbers of salt resistant nuclear receptor sites they designate as acceptors. They also describe a class of receptor-binding sites resistant to nuclease digestion which decline rapidly during stimulation, and suggest that these are involved with processing and turnover of the receptor. This is in

contrast to a report on the glucocorticoid receptor, which appears to be successively present in a nuclease sensitive and then a nuclease-resistant site in hepatoma tissue culture (HTC) cell nuclei (Defer et al., 1981). The 5S receptor has been shown to bind a variety of heterologous and homologous DNAs with similar affinity (King & Gordon, 1972; Yamamoto & Alberts, 1974), including those from rat uterus, calf thymus, salmon sperm and Escherichia coli.

Much work has been done on the base sequence specificity of this binding for various steroid-receptor complexes. These studies make particular use of synthetic deoxyribopolymers; and halodeoxyuridine substitution of DNA, which in several cell systems has resulted in altered gene expression (Rutter et al., 1973). Of interest is the specific inhibition, by 5-bromodeoxyuridine, of an oestrogen-induced glycoprotein secreted by the human breast cancer cell line MCF-7. Inhibition is only effective when the bromodeoxyuridine is incorporated into the DNA and the synthesis of other proteins not regulated by oestrogens is unaffected. This suggests that the inhibition is due to interference with the recognition of chromatin acceptor sites by the oestrogen-receptor complex (Garcia et al., 1981). Although binding preferences have been reported for individual steroids, there is no concensus between studies; thus while oestrogen-receptor complex binds preferentially to AT-rich DNA and to halosubstituted DNA (Kallos & Hollander, 1978; Kallos et al., 1978, 1979), androgen receptor has a greater affinity for poly dGdC than poly dAdT and binding is unaffected by bromodeoxyuridine (Lin & Ohno, 1981). Even a study of the interaction of the chick oviduct progesterone receptor with cloned DNA coding for the ovalbumin it induces, has shown no sequence specificity for any part of the gene. In contrast, the three dimensional structure of the DNA was found to

be the most important parameter (Hughes et al., 1981). Recent studies have shown, however, that the DNA sequence at or close to the activated genes is important in hormone recognition. Thus, fluorescent derivatives of the insect steroid hormone ecdysone have been shown to bind specifically at the puffs on polytene chromosomes which mark the site of induced protein synthesis (Gronemeyer & Pongs, 1980). Furthermore, when cloned sequences of genes for glucocorticoid-induced proteins are introduced into non-target cells, their transcription becomes hormone-inducible (Buetti & Diggelman, 1981; Hynes et al., 1981; Kurtz, 1981).

Although steroid receptors bind DNA they do so with relatively low affinity (Buller & O'Malley, 1976). DNAase treatment of chromatin prevents receptor-binding and releases previously bound receptor (King & Gordon, 1972; Higgins et al., 1973a). However, this only suggests that DNA is an essential component of the final three dimensional acceptor site and does not indicate that DNA is the exclusive acceptor.

The chromosomal proteins also bind the hormone-receptor complex and there is evidence that it is the non-histone proteins which confer specificity of binding on the chromatin. O'Malley's group has shown that chick oviduct chromatin binds 3 to 9 fold more progesterone-receptor complex than does chromatin from non-target tissues such as spleen, heart or erythrocytes (Steggles et al., 1971a). Spelsberg has fractionated the chromosomal proteins from target and non-target tissues and carried out binding studies with heterologous reconstituted chromatins. This method showed that binding of labelled progesterone-receptor complex to DNA-histone was lower than to native chromatin, and that the source of histone did not affect the amount of

binding (Steggles et al., 1971b). A major class of acidic non-histone proteins, designated AP_3, was found to be responsible for the increased binding of the progesterone-receptor complex to chromatin (Spelsberg et al., 1971, 1972). Target cell chromatin in which the AP_3 fraction has been replaced with non-histone proteins from another tissue loses its ability to bind the complex (Spelsberg et al., 1976). Furthermore in a recent study Thrall & Spelsberg (1980) confirm that non-histone protein-DNA complexes show the characteristics of native acceptor sites and show that much of the reported DNA binding by progesterone receptors to pure DNA can be artificially induced by the assay conditions. It should be mentioned however, that chromatin reconstitution on which many of these studies depend, is a very controversial technique which has been shown to give rise to a number of artifacts (Biessman et al., 1976; Zasloff & Felsenfeld, 1977; Fulmar & Fasman, 1979; Stein, 1979).

Puca et al. (1974, 1975) have studied the interaction of oestrogen-receptor complexes with nuclear components immobilised on an inert matrix, agarose. A fraction of basic non-histone chromatin proteins was isolated from calf uterus, that showed very high affinity for the receptor, even in absence of nucleic acids. Basic non-histone proteins were also implicated as the acceptor sites for androgen receptors in rat ventral prostate (Mainwaring et al., 1976). Hiremath et al. (1980) report two distinct androgen acceptor sites - a soluble, loosely bound non-histone protein of molecular weight 14,000 and a second site, resistant to 2M salt extraction of the chromatin (Hiremath et al., 1981).

There is still much controversy as to the involvement, if any, of histone molecules. Whilst they cannot satisfy the specificity

requirement for acceptors, Kallos et al. (1981) claim that histones are important in stabilising the binding of oestrogen to DNA and may account for the salt-resistant binding sites reported by Clark & Peck (1976). They may also account for the salt-resistant androgen acceptor described by Hiremath et al.(1981). Although some authors advocate that the non-histones serve only to mediate the availability of the acceptor-DNA (King & Gordon, 1972), the collected evidence suggests that DNA and non-histone protein are both involved in defining the acceptor site for steroid-receptor complexes.

O'Malley & Schrader's model for the binding of the chick oviduct progesterone receptor (O'Malley & Schrader, 1976; Schrader et al., 1977) is based on their finding that this steroid-hormone receptor consists of a dimer of non-identical polypeptides. These each have a molecular weight of about 100,000 and a single progesterone-binding site, and can be isolated because they elute from DEAE-cellulose as two separate peaks of progesterone-binding activity (Kuhn et al., 1975). The A subunit binds DNA but will not bind chromatin, and the B subunit binds AP_3 non-histone chromatin proteins. The model suggests that the receptor binds chromatin through the B subunit after which the A subunit dissociates and binds DNA. This changes the chromatin structure allowing RNA polymerases to gain access to initiation sites. In subsequent work, Hughes et al. (1981) showed a binding preference of the A subunit for single-stranded DNA (ssDNA) and suggested it might be a helix-destabilising protein. These proteins have been associated with replication, recombination, repair and transcription - all processes involving transient single-stranded nucleic acid conformations (Champoux, 1978). Schrader proposes that since receptors for androgens, oestrogens and glucocorticoids can be separated into two peaks of hormone-binding activity upon DEAE-cellulose

chromatography, the model is applicable to all steroid receptors. However, the two forms of glucocorticoid receptor show a precursor-product relationship and are the nonactivated and activated complexes (Sakaue & Thompson, 1977; Munck & Foley, 1979). Although the 5S oestrogen receptor has a subunit structure there is no evidence for the pre-existence of a separate DNA-binding subunit. All other steroid receptors undergo an activation process from a DNA non-binding to a DNA-binding form without an increase in size. The fact that progesterone receptors from rabbit uterus undergo activation (Fleischmann & Beato, 1979) and that antibodies raised against them do not cross-react with chick progesterone receptors (Logeat et al., 1981), suggests that the avian progesterone receptor is a special case and argues against the model being applicable to all receptors (Gschwendt, 1980).

1.5 Transcriptional effects of oestrogen and other steroids.

1.5.1 Changes in chromatin structure.

The idea that the steroid-receptor complex in some way opens up the chromatin structure, thus facilitating transcription, is founded on studies using nuclease digestion of the DNA. Chromatin to which oestrogen-receptor complex has bound shows a highly increased sensitivity to DNAase 1 digestion, suggesting that the DNA is exposed (Chamness et al., 1974). Examination of endometrial chromatin by electron microscopy reveals that oestrogen treatment both *in vivo* and *in vitro* transforms condensed chromatin into dispersed chromatin within one hour of administration. Substances which block oestrogenic effects such as the transcriptional inhibitor actinomycin D, also prevent the ultrastructural change caused by the hormone (Vic et al., 1980), and this links the decondensation of chromatin to transcriptional activity.

A similar decondensation of chromatin occurs in insects. The salivary glands of Diptera such as Chironomus and Drosophila contain giant polytene chromosomes which are easily visualised by light microscopy. These respond to the ecdysteroid hormones in the insect by puffing up at certain sites along their length. These "puffs" are interpreted as the uncoiling of chromatin, and are sites of RNA synthesis (Pelling, 1959). The puffs are specific for a particular hormone and appear in a particular sequence for specific periods during development, and then regress (Clever, 1963; Clever et al., 1973).

Oestrogen can be shown to induce target-tissue-specific changes in the conformation of particular genes whose activities it stimulates. The presence of structural gene sequences can be monitored after digestion of nuclei with DNAase, by hybridisation of the remaining DNA to labelled specific gene probes. These are complementary DNAs (cDNAs) transcribed in vitro from the particular messenger RNA using the retroviral enzyme reverse transcriptase. DNAase treatment reduces exposed DNA to non-hybridisable fragments. Thus an increase in the DNAase sensitivity of the four Vitellogenin genes can be demonstrated in oestrogen-treated Xenopus liver cells, but this change does not occur in erythrocytes of the hormone-treated animal (Gerber-Huber et al., 1981). Similar results have been obtained in chick oviduct where oestrogen induces the egg-white proteins ovalbumin and ovomucoid with a concomitant increase in the DNAase sensitivity of their structural genes (Garel & Axel, 1976, 1977; Lawson et al., 1980).This change does not occur in non-target tissues such as liver.

The DNAase sensitivity must be mediated by a change in some property of the chromosomal proteins. Although the histones are thought to have

a mainly structural function many workers have investigated the possibility of a regulatory role. It was first suggested in 1950 that they served as repressors of gene transcription (Stedman & Stedman, 1950), and it has been shown since, that histones repress ovalbumin and globin gene activity (Axel et al., 1973; Tsai et al., 1976). This may be due to the inhibition of RNA polymerase (Butler & Chipperfield, 1967), or an interference in RNA chain elongation (Koslov & Georgiev, 1970), but a change in chromatin structure at active genes would also be consistent with a change in the availability of DNA to the RNA polymerases (Axel & Felsenfeld, 1973; Gilmour & Paul, 1973; Steggles et al., 1974). These changes could be caused by post-translational modifications of the histones (Ruiz-Carillo et al., 1975) and might include phosphorylation, methylation and acetylation. Acetylation particularly has been shown to be a characteristic of histones present in cells undergoing biological changes, and has been found associated with hormonal stimulation, embryonic development and cellular differentiation (Gottesfeld et al., 1975; Cohen et al., 1975; Riggs et al., 1977a,b; Johnson & Allfrey, 1978). Although it is uncertain whether histone acetylation is the cause or effect of gene activation it is probable that histones on active genes are hyperacetylated (Davie & Candido, 1978). DNAase 1 preferentially degrades active genes and releases a hyperacetylated fraction of the histone population (Vidali et al., 1978). Chromatin which has been acetylated chemically becomes very sensitive to DNAase, as does chromatin from butyrate treated cells (Bonner et al., 1978). Sodium butyrate is an inhibitor of histone deacetylation and interestingly, it has been shown to block the induction of egg-white protein genes by steroid hormones (McKnight et al., 1980). The authors suggest that oestrogen receptors normally bind to highly acetylated regions of chromatin and that butyrate blocks protein induction by creating a large number of non-productive

binding sites for the receptor. However, this presupposes that acetylation is a causal agent, rather than an effect of receptor binding - a point which at present is unclear. Transient increases in histone acetylation have been observed in the uterus and are maximal 5 to 10 minutes after oestrogen administration. Similar changes are noted in response to corticosteroid treatment of kidney and lymphocytes. The increased acetylation is unaffected by RNA or protein synthesis inhibitors given prior to hormone and the turnover of acetyl groups is extremely rapid (Libby, 1972, 1973; Jackson et al., 1975).

The non-histone proteins are more likely candidates as regulators of gene transcription because of their diversity and their specific composition in different tissues. They are present in elevated levels in actively transcribing tissue, whilst amounts of histone remain constant (Dingman & Sporn, 1964), and they can be shown to stimulate the in vitro transcription of chromatin from both homologous and heterologous tissues (Kostraba et al., 1975; Wang & Kostraba, 1977). They have also been shown to bind specifically to different DNA regions (Chytil & Spelsberg, 1971; Teng et al., 1971; Sevall et al., 1975). Like the histones, post-translational modifications by phosphorylation, methylation and acetylation occur and have been observed accompanying changes in gene expression in response to steroid hormones (O'Malley & Means, 1974).

The high mobility group (HMG) of non-histone proteins, so called because of their migration characteristics on polyacrylamide gels, are strongly implicated in defining the structure of actively transcribing genes. The HMG proteins were first discovered by Goodwin et al. (1973), as loosely bound chromosomal proteins extractable in buffers of low salt concentration. Although similar, they are distinct from

the histones by virtue of an unusual amino acid composition. There are four major proteins, the high molecular weight HMG 1 & 2, and the low molecular weight HMG 14 & 17. These are all released by DNAase digestion of chromatin in conditions chosen to selectively digest active genes (Vidali et al., 1977; Levy-Wilson et al., 1977; Levy-Wilson & Dixon, 1978; Weisbrod & Weintraub, 1979; Georgieva et al, 1981). Significantly, the selective digestion of the chick globin gene in erythrocytes can be abolished if HMG proteins are previously extracted in 0.35M salt. Reconstitution experiments show that the presence of HMG 14 & 17 alone is sufficient to restore DNAase sensitivity to the chromatin (Weisbrod & Weintraub, 1979; Gazit et al., 1980). These two proteins have also been shown to preferentially bind single-stranded DNA (Isackson & Reeck, 1981), and to be components of the nucleosomes of active chromatin (Weisbrod et al., 1980).

Some specific modifications of the HMG proteins have been reported. HMG 14 & 17 can be phosphorylated (Bhorjee, 1981; Linnala-Kankkunen & Mäenpää, 1981) and glycosylated (Reeves et al., 1981) and HMG 1 & 2 are acetylated. In the presence of butyrate the deacetylation of these proteins, as well as of histones, is inhibited (Sterner et al., 1979). HMG 14 & 17 have been found to partially inhibit histone deacetylase in active chromatin (Reeves & Candido, 1980).

HMG protein levels are related to the hormonal status of a target tissue, for example, prostatic chromatin from androgen-treated rats has a higher HMG protein content than the corresponding chromatin from castrated animals (Hiremath et al., 1981). In the same system Lesser & Elliot (1981) have described a "specific DNA-binding activity" present in the non-histone protein fraction which is dependent on the presence

of androgen. In addition it has recently been reported that levels of HMG 1 increase in chick oviduct after oestrogen treatment (Teng & Teng, 1981).

These proteins, then, are likely to be involved in the early stages of the steroid-induced response by participating in the change of chromatin structure necessary to facilitate transcription.

The DNA of active and inactive genes may also be expected to exhibit some differences. Thus, the degree of methylation of both transcribed and flanking sequences of genes, has been implicated in the control of transcription (Desrosiers et al., 1979; Marcaud et al., 1981; Weintraub et al., 1971). DNAase-sensitive, active gene sequences are under-methylated (Naveh-Many & Cedar, 1981), and recently Compere & Palmiter (1981) have shown that glucocorticoid induction of metallothionein only occurs when the gene for the protein is not methylated. It is not known at present, whether steroid hormones exert any control over the extent of methylation of the genes which they stimulate.

1.5.2 Oestrogen-induced transcription in chick oviduct.

Before describing in detail the transcriptional responses in immature rat uteri, it is useful to describe some of the work on the avian oviduct which illustrates the oestrogenic control of specific gene activity as previously outlined in Section 1.2.

Administration of oestrogen to immature chicks stimulates the synthesis of egg-white proteins in the oviduct. The use of the inhibitor rifampicin in in vitro transcription assays has shown that the number of nuclear initiation sites in this tissue rises and falls

in conjunction with the number of hormone-receptor complexes present in the nucleus (S.Y. Tsai et al., 1975; Schwartz et al., 1976; Kalimi et al., 1976). The assay is done in such a way that the inhibitor limits RNA synthesis to a single round of transcription, and the number of RNA molecules transcribed, which should then equal the number of initiation sites, is estimated from the radioactive incorporation and the average length of an RNA molecule synthesised under those conditions of assay (M.-J. Tsai et al., 1975; Schwartz et al., 1976). Most of the assays are done using excess, exogenous, bacterial RNA polymerase, and there is some doubt as to the fidelity and efficiency with which this enzyme recognises eukaryotic initiation sites. This means that numerical data obtained with bacterial polymerase must be viewed with caution. A recent study showed that hormonally induced transcription by this enzyme was attributable to contaminating messenger RNAs, however these authors confirmed an oestrogen-stimulated increase in activity of endogenous RNA polymerases (Palmiter & Lee, 1980). This emphasises the dangers of using crude systems which may cause template independent stimulation of RNA synthesis as pointed out by O'Malley's group (Buller et al., 1976b).

The oestrogen-induced synthesis of the egg-white proteins ovalbumin, ovomucoid, conalbumin and lysosyme is preceded by the accumulation of their messenger RNAs in the tubular gland cells (Harris et al., 1975, 1976; Palmiter et al., 1976; Hynes et al., 1977; McKnight, 1978). The fine control exerted on the production of the proteins is indicated by the dose responses and the kinetics of induction of ovalbumin and conalbumin. Conalbumin mRNA begins to accumulate within 30 minutes of oestrogen administration, whereas there is a lag of 3 to 4 hours before ovalbumin mRNA begins to accumulate (Palmiter et al., 1976).

The rate of conalbumin mRNA synthesis is directly proportional to the concentration of nuclear receptors and half maximal induction occurs when nuclear receptor levels are 50% of maximum. Half maximal induction of ovalbumin mRNA is not reached until nuclear receptor levels are 80% of saturated (Mulvihill & Palmiter, 1977). Both Thomas & Teller (1981) and Palmiter et al. (1981) have put forward a model to explain these results. They suggest that the interaction of hormone-receptor complex at a single site on the gene is sufficient to induce conalbumin, whereas synthesis of ovalbumin mRNA requires interaction of the complexes at multiple sites on the ovalbumin gene. This is supported to some extent by the fact that increasing dosage and using a direct route of administration of hormone to the lumen of the oviduct can induce maximal ovalbumin mRNA synthesis within 1 hour (Swaneck et al., 1979a). Palmiter et al. (1981) have extended their model by proposing that different types of steroid receptor interact at the same chromatin sites with different affinities. This could explain the different synergistic and antagonistic effects of oestrogen and progesterone on ovalbumin and conalbumin synthesis. They further propose that receptor-binding is coupled to transcriptional activation by unstable intermediary proteins. The activity of these could be regulated by covalent modifications, such as acetylation, which could explain the effect of butyrate on egg-white protein induction.

Oestrogen stimulates the number of ovalbumin mRNA molecules per chick oviduct cell, during primary stimulation, from near zero to 48,000 per cell. If hormone is withdrawn this declines to near zero again over 12 days (Harris et al., 1975). Monahan et al. (1976) have estimated the number of different polyadenylated mRNA sequences per cell as over 25,000. They stated that after 12 days of hormone withdrawal the

sequence complexity (the number of different sequences) in chick oviduct had fallen by 50% and that the highly abundant sequences present in laying hen & stimulated chick oviduct were absent. Mizuno & Cox (1979) claim that 95% of the unique DNA sequences transcribed in the stimulated oviduct are still represented in the nuclear RNA population up to 6 days after withdrawal and suggest that the loss in cytoplasmic sequences coded for by unique DNA is due to a hormone-dependent change in the processing and export from the nucleus of these sequences rather than a cessation of transcription. This seems unlikely in view of the short half-lives of the hnRNA molecules, although oestrogen does have a stabilising effect on ovalbumin mRNA (Palmiter & Carey, 1974), and withdrawal decreases the half-life (Cox, 1977). A recent study on the ribonucleoprotein particle population of chick oviduct nuclei shows that the number of ovalbumin messenger sequences in these complexes rises and falls in response to oestrogen stimulation and withdrawal (Thomas et al., 1981).

Molecular cloning of the conalbumin, ovalbumin and ovomucoid genes has shown that in common with other eukaryotic genes, the coding regions of the DNA are interrupted by non-coding regions known as intervening sequences, inserts, or introns. In the ovalbumin gene there are seven such sequences interrupting eight coding regions (Breathnach et al., 1977; Doel et al., 1977; Dugaizyck et al., 1978, 1979). There are at least six introns in the ovomucoid gene (Catterall et al., 1979), and the conalbumin gene has at least sixteen introns (Cochet et al., 1979). The function of these regions is unknown but they are transcribed in response to oestrogen (Roop et al., 1978; Swaneck et al., 1979b; Nordstrom et al., 1979). These sequences are processed out of the primary transcript before its export from the nucleus as mature messenger RNA.

Utilisation of the cytoplasmic messenger RNAs is affected by both progesterone and oestrogen. Progesterone enhances the initiation of translation of ovalbumin messenger RNA, and both hormones stabilise the messenger in the cytoplasm. Oestrogen may be involved in the translation process itself since translational efficiency cannot be maintained if the hormone is withdrawn (Robins & Schimke, 1978).

1.5.3 The oestrogenic response in immature rat uterus.

In the mammalian uterus oestrogen does not stimulate the production of major new proteins but initiates a chain of events leading to overall growth and development of the tissue. This may involve a multiplicity of new minor proteins, preparing the uterus for the implantation of a fertilised egg. The majority of the uterine responses are inhibited by actinomycin D and therefore appear to arise from an effect of the hormone on the transcription of RNA. The synthesis of all classes of RNA is stimulated by oestrogen, and this is followed by increased protein synthesis, and later in the response by stimulated DNA synthesis. In the immature rat uterus this sequence of events is characterised by an overall hypertrophy followed by hyperplasia of the organ (Billing et al., 1969).

A number of early studies demonstrated oestrogen-induced stimulation of the chromatin template activity in immature and ovariectomised rat uteri. The increased ability of the chromatin DNA to act as a template for bacterial RNA polymerase (Hamilton, 1968) coincides with the early increase in endogenous RNA polymerase activities (Teng & Hamilton, 1968; Church & McCarthy, 1970). These findings correlate with the increased number of nuclear initiation sites measured by the rifampicin challenge assay (Markaverich et al., 1978).

Glasser et al. (1972) showed that the earliest detectable response to oestradiol-17β administered to ovariectomised rats was an increase in RNA polymerase II activity. This polymerase is responsible for transcribing the large heterogeneous nuclear RNA species assumed to be precursors of messenger RNA. The increase in RNA polymerase II activity occurs 15 minutes after oestrogen administration, peaks at 30 minutes and falls to control levels at 1 to 2 hours after hormone stimulation. A second increase occurs over 2 to 12 hours, and this declines to control levels by 24 hours (Glasser et al., 1972; Borthwick & Smellie, 1975; Clark et al., 1978c).

Increased synthesis of ribosomal RNA (rRNA) has been shown by an increase in RNA polymerase I activity in the uterus of rat and rabbit. Activity increases from 1 to 2 hours after oestrogen administration and remains constant from 2 to 12 hours (Widnell & Tata, 1966; Hamilton et al., 1968; Borthwick & Smellie, 1975; Hardin et al., 1976; Weil et al., 1977). The activity of polymerase III which transcribes small RNAs such as tRNA and 5S ribosomal RNA also increases at about the same time as that of polymerase I, to a maximal level between 3 and 4 hours (Webster & Hamilton, 1976). The stimulation by oestradiol of these RNA polymerase activities was shown to be template dependent by Borthwick & Smellie (1975) who could find no direct effect of the hormone on the isolated enzymes. However, oestrogen may control the population of polymerase molecules as in rooster liver, where stimulated polymerase II activity is in direct proportion to an increase in the number of polymerase molecules per nucleus (Kastern et al., 1980).

The increased polymerase II activity accounts for the early oestrogen-induced synthesis of nuclear RNAs in immature rats reported by many workers (Means & Hamilton, 1966a, b; Hamilton, 1968; DeAngelo & Gorski, 1970; Wira & Baulieu, 1971). It also confirms the observation that the earliest detectable transcriptional response to oestrogen in immature rat uteri is an increase in the synthesis of heterogeneous nuclear RNA (hnRNA) seen at 30 minutes after the administration of oestradiol-17β (Knowler & Smellie, 1971, 1973; Aziz & Knowler, 1978). This RNA is identified on the basis of its high molecular weight, base composition, polyadenylation, a low degree of methylation, nuclear location and rapid synthesis and decay. Its synthesis is stimulated two-fold, 30 minutes after oestrogen administration and reaches a peak ten times control values by 2 hours after injection. Differential elution from polyuridylate-sepharose columns using different formamide concentrations allows the fractionation of this hnRNA into three classes, non-adenylated, oligo-adenylated and polyadenylated. Stimulated synthesis of the polyadenylated class accounts for most of the early response whilst synthesis of non-adenylated species increases dramatically at later times (Aziz & Knowler, 1978). Maturation of the hnRNA can be followed kinetically by its appearance in extractable hnRNP particles, by detection of mRNA sequences in the hnRNA and by entry into the cytoplasm of the newly synthesised mRNA (Knowler, 1976; Aziz & Knowler, 1980; Merryweather & Knowler, 1980).

Studies using α-amanitin, an inhibitor of transcription, show that if the early peak of RNA polymerase II activity does not occur the subsequent increase in polymerase I activity is abolished (Raynaud-Jammet et al., 1972; Borthwick & Smellie, 1975). Inhibitor administered after the 30 minute peak in polymerase II activity is ineffective in preventing the increased polymerase I activity. Exactly

the same results are obtained if cycloheximide, a protein synthesis inhibitor, is used in place of α-amanitin. This work shows that new ribosome synthesis cannot occur without the prior synthesis of some proteins coded for by the hnRNA transcribed as early as 30 minutes after stimulation by oestrogen (Baulieu et al., 1972; Baulieu, 1975; Knowler et al., 1975). Protein synthesis inhibitors have also been shown to block the glucocorticoid-stimulated synthesis of ribosomal RNA (Yu & Feigelson, 1973; Feigelson & Kurtz, 1978) and the induction of specific messenger RNAs by oestrogen and glucocorticoid (McKnight, 1978; Chen & Feigelson, 1979). This may mean that early protein synthesis is a prerequisite for the continuation of all steroid-induced responses. The hormone-receptor complexes may primarily interact with genes specifying intermediary regulatory proteins, and it may be these that mediate the steroid-induced response.

This scheme may also apply to the early and late transcriptional responses in Drosophila. The early chromosome puffs induced by ecdysone are independent of protein synthesis, whereas the puffs which appear after 5 hours of hormone treatment can be blocked by various inhibitors of protein synthesis (Ashburner, 1974). This has led to the suggestion that ecdysone-receptor complex regulates the appearance of the early puffs, and that a protein product of these then regulates the induction of the late puffs (Ashburner & Richards, 1976). The possible nature of intermediary regulatory proteins is discussed later in this section.

In the hypertrophic responses of the uterus, the most important requirement will be the huge increase in protein synthetic activity. Incorporation of radiolabelled precursors reveals that messenger RNA, derived from the hnRNA synthesised at 30 minutes after oestrogen

stimulation, accumulates in the cytoplasm and brings about an increased aggregation of pre-existing ribosomes into polysomes (Merryweather & Knowler, 1980). This is detectable between 30 minutes and 1 hour after oestradiol administration and is maximal after 2 to 4 hours (Teng & Hamilton, 1967; Merryweather & Knowler, 1977). The aggregation precedes the oestrogen-induced increase in rRNA synthesis, which is detectable at 1 hour and peaks 10 to 12 times control values, 2 to 4 hours after oestrogen administration (Knowler & Smellie, 1971). There is also some early stimulation by oestrogen of the maturation of 28S and 18S ribosomal RNA from their precursor molecules, which may involve the increased efficiency of the base methylation or cleavage (Knecht & Luck, 1977).

The accumulation of this new rRNA into ribosomes does not occur until 12 hours after hormone injection, when the incorporation of newly synthesised ribosomal proteins is also maximal. The synthesis of ribosomal proteins is not stimulated until 8 to 12 hours after treatment with oestradiol, at the same time as the stimulation of total uterine protein synthesis. Thus pools of newly synthesised rRNA must accumulate in the cells over 2 to 12 hours following hormone treatment. The peak of new ribosome synthesis at 12 hours is followed by the stimulation of total protein synthesis, marked by a second peak of incorporation of newly synthesised mRNA into polyribosomes (Merryweather & Knowler, 1980).

Support for the precursor-product relationship of the early stimulated hnRNA and the cytoplasmic mRNA sequences present after 4 hours of oestradiol stimulation comes from hybridisation studies (Aziz & Knowler, 1980). The bacterial enzyme reverse transcriptase is used to make copies of polysomal polyadenylated messenger RNA which is

labelled using radioactive ribonucleotides. There is some complementarity between polyadenylated hnRNA from rat uterus after both 30 minutes and 2 hours of oestrogen treatment, and the abundant sequences of complementary DNA (cDNA) transcribed from polyadenylated mRNA from 4 hour treated uteri. These abundant cDNA sequences are more common in the hnRNA from rats receiving 2 hours of oestrogen treatment, than 30 minutes of oestrogen treatment.

Analysis by homologous hybridisation of cDNA to its template mRNA has been used to compare the complexity and diversity of polysomal poly(A)- containing mRNA sequences in rat uteri in differing hormonal states. This method generates an estimate of 36,000 different sequences expressed in the adult uterus at pro-oestrus as against 8,000 in the immature rat uterus responding to 4 hours of œstrogen treatment. The results suggest that at the earlier stage in oestrogen-induced differentiation, there is a greater percentage of highly abundant RNA species than is found in the mature animal. The mRNA population in the adult is also shown to have a greater base sequence complexity in each abundance class than the 4 hour stimulated animal (Aziz et al., 1979a).

cDNA-mRNA hybridisation may give underestimates of the number of unique sequences, therefore the sequence complexities have been recalculated by a second method. Saturation hybridisation of total tritiated unique DNA by excess mercurated poly(A)-containing mRNA yields hybrids which can be isolated using their affinity for thiol-sepharose columns. This method may give over-estimates due to the presence of introns in gene sequences, or the estimates may reflect differences in mRNA size distribution since rare mRNA sequences may be longer than abundant species. The relative merits of the two methods

remain disputed. The second method gives estimates of 53,000 different mRNA sequences in the adult uterus and 12,000 in the 4 hour treated animal. As in many other published analyses the method gives higher values than cDNA-mRNA hybridisation but it confirms the four-fold difference in sequence number between the adult and immature rats. The figures for the adult uterus are very high, and may reflect the large number of different cell types in the uterus.

cDNA-mRNA hybridisation can be used to analyse changes in mRNA complexity by examining the degree to which a cDNA copy of one population will hybridise to a different mRNA population (heterologous hybridisation). In this way, Aziz et al. (1979b) showed that mRNA from untreated immature rat uterus was only able to hybridise with 11% of the cDNA prepared from the mRNA of 4 hour treated rat uteri. The uterine mRNA of 2 hour treated rats could hybridise to 56% of the same 4 hour cDNA, though at a much slower rate than in the homologous hybridisation. The slower rate of hybridisation showed that sequences common to both populations were less abundant in the 2 hour preparation while the incomplete hybridisation showed that some sequences, present 4 hours after oestrogen administration, were not present at earlier times in the response. These latter sequences, particularly those appearing in the uterine population between 2 and 4 hours after oestradiol administration, may well be important in the control of rRNA synthesis which is stimulated at this time. They were further investigated and quantified using fractionated cDNA.

4 hour cDNA was fractionated into samples enriched in abundant and rare sequences respectively. Abundant sequences were prepared by large batch hybridisation of 4 hour cDNA to an excess of its template, to a

low $r_o t$ value. Hydroxylapatite column chromatography was used to isolate the double stranded hybrids containing the abundant cDNA sequences, followed by recovery of the cDNA strand by alkaline hydrolysis. Rare sequences were prepared by extending the hybridisation to a high $r_o t$ value and separating the single strands enriched in rare sequences.

Heterologous hybridisation showed considerable homology between abundant fractions of cDNA from 4 hour treated rats and total polyadenylated mRNA from 2 hour treated rats. The rarest sequences were also very similar in the 2 hour and 4 hour populations but a contaminant of intermediate abundance in the 4 hour cDNA preparation appeared to have no complementary component in the 2 hour mRNA. Attempts to isolate this intermediate abundant cDNA by traditional fractionation procedures failed but the sequences of interest were isolated and analysed as follows. cDNA to 4 hour mRNA was hybridised to high $r_o t$ values against mRNA from 2 hour stimulated uteri. The cDNA not forming hybrids under these conditions was isolated using hydroxylapatite chromatography. Hybridisation of this fraction to its own template, namely mRNA from 4 hour stimulated uteri, showed that the sample was considerably enriched in sequences of intermediate abundance. The number found was 150, which agreed exactly with the total number of sequences of intermediate abundance in 4 hour polyadenylated mRNA. This means that all of these sequences are produced between 2 and 4 hours after oestrogen stimulation. In contrast, of the 9 abundant and 7,800 rare sequences present in 4 hour mRNA, only 1 or 2, and 2,500 respectively are not present by 2 hours after oestrogen administration . Thus the uterus differs from other systems such as chick oviduct and rat prostate, where the most profound steroid-dependent changes observed have been in the most

abundant messenger sequences (Hynes et al., 1977; Parker & Mainwaring, 1977; Parker & Scrace, 1978). The only report of a similar system indicates that the major effect of castration on the protein population of rat seminal vesicle is a large decrease in the moderately abundant messengers (Higgins et al., 1979). This may reflect the function of the proteins coded for by these sequences; the overall growth process of the uterus may necessitate moderate quantities of proteins coded for by messengers in the intermediate abundance class. Ribosomal proteins may fall into this category as it is between 2 and 4 hours after oestradiol-17β administration to immature rats that their synthesis begins to increase (Merryweather & Knowler, 1980).

Other proteins possibly encoded in this class of mRNA are specific non-histone chromatin proteins. It is possible, for example, that stimulation of rRNA synthesis depends on specific newly synthesised proteins associating with the ribosomal DNA. Such a repetitive part of the genome may require proteins in quantities necessitating fairly abundant mRNAs to code for them. This role for non-histones as intermediary regulatory proteins in steroid hormone action has been suggested frequently (O'Malley et al., 1977; Schrader & O'Malley, 1978; Lesser & Elliot, 1981) and several authors have reported the early appearance of oestrogen-stimulated acidic proteins in the nuclear fraction of rat uterus, although none of these has been assigned a definite function (Barker, 1971; King et al., 1974; Cohen & Hamilton, 1975a,b).

A third group of proteins which might be coded for by the intermediate abundant mRNA, are hnRNP and mRNP particle proteins. The mRNAs appear in the polysomes at a time when uterine hnRNA synthesis is 10 times

control values (Aziz & Knowler, 1978) and can be extracted in hnRNP particles (Knowler, 1976). The requirement for the proteins associated with these particles may necessitate fairly abundant messengers. The proteins may participate in processing and post-transcriptional modifications of hnRNA and mRNA. They may control the excision of intervening sequences and splicing of the coding regions of the message, and chemical modifications such as capping and polyadenylation (Reviewed by Knowler & Wilks, 1980). Other functions must include those concerned with transport of hnRNA sequences from the nucleus and there is evidence of functional masking and the preservation of mRNA sequences in the cytoplasm. A specific example is the translational repression of globin mRNA in free cytoplasmic ribonucleoprotein complexes (Civelli et al., 1980).

Other possible candidates for the early induced proteins are receptor protein, which is replenished during the oestrogenic response (Mester and Baulieu, 1975; Clark et al., 1978a), the type II nuclear receptors such as the Y protein in chick oviduct (Smith & Taylor, 1981), and possibly structural or secreted proteins. Oestrogen has been shown to induce secretion of several proteins in cell cultures of breast cancer cells (Westley & Rochefort, 1979, 1980a,b). However, the time between induction, synthesis and secretion is not clear since cultures were exposed to hormone for 2 days.

Much interest has accrued over the years to an oestrogen-induced protein known as IP, which is detectable after 40 minutes of oestrogen treatment in immature rat uterus (Notides & Gorski, 1966). It also occurs in brain (Walker et al., 1979) where it is constitutively synthesised. However, the protein was recently identified as an isoenzyme of creatine kinase and it is interesting primarily as a

rapidly responding and early marker of oestrogen action in rat uterus which is not involved in gene action (Reiss & Kaye, 1981). It is probably needed to process the excess of creatine phosphate resulting from the early increased uptake and metabolism of glucose, induced by oestrogen (Smith & Gorski, 1968). This would ensure a plentiful supply of energy for the processes of cell growth and division.

1.6 Differential responses of uterine cell types to oestrogens.

It was demonstrated in early studies that oestradiol is not evenly concentrated by the different cell types in the uterus. The location of tritiated oestradiol was monitored using autoradiography of tissue sections freeze-dried after oestrogen treatment, and this showed that 86% of the hormone became localised in the epithelial tissue of immature rat uteri (Stumpf, 1968b). In more recent studies, McCormack & Glasser (1980) fractionated cell types from immature rat uteri and measured the amounts and concentration of oestrogen receptor present in each cell type. Epithelial and stromal cells from untreated animals contain higher concentrations of nuclear oestrogen receptor than myometrial cells and epithelial cells contain the highest concentration of cytoplasmic receptor. Although myometrial cells have the lowest concentrations they contain 84-89% of the total uterine oestrogen receptors, by virtue of the preponderance of myometrium in whole uterus. Epithelium and stroma account for 5% and 10% of receptors respectively. Following oestrogen treatment cytoplasmic receptors increase in all cell types. The distribution of receptor in untreated whole uterus is found to be in the ratio 90% cytoplasmic to 10% nuclear, in agreement with the existing literature, but all the isolated cell types exhibit exactly the opposite partitioning, of 10% cytoplasmic to 90% nuclear receptor in untreated tissue, and no experimental procedure could be found to account for this unusual

distribution. This may mean that care should be exercised in interpreting responses to steroids of cell culture systems, particularly cancer derived cultures such as the human MCF-7 cells which show a similar receptor distribution (Lippman et al., 1977; Zava & McGuire, 1977; Edwards et al., 1980).

Work on the immature mouse uterus shows a biphasic translocation of oestrogen receptors from cytoplasm to nucleus. Following injection of tritiated oestradiol, a major peak of nuclear radioactivity occurs at 1 hour, followed by a rapid decline to control levels by 4 to 5 hours. A second increase in nuclear radioactivity occurs at 7 to 8 hours after the injection, and reaches about 20% of the maximum level in the initial increase (Korach & Ford, 1978; Korach, 1979). Further, autoradiographic studies of labelled steroid uptake have shown that the initial sequestration of steroid is in the nuclei of stroma and glandular epithelial cells. The luminal epithelial cells do not show appreciable nuclear accumulation of label until 7 to 8 hours after hormone injection (Korach & Lamb, 1981). This may indicate the requirement of an additional stimulus from stromal tissue to luminal epithelium, such as a factor influencing steroid uptake.

This biphasic uptake is not seen in the rat but there are other differential responses in the cells. McCormack & Glasser (1980) report a stimulated increase in the incorporation of tritiated cytidine into RNA of epithelial cells 2 hours after oestrogen administration. This increased nucleotide incorporation is restricted to the epithelium and does not occur in either stromal or myometrial cells This effect agrees with the early increase of precursor incorporation into total uterine RNA seen by Knowler & Smellie (1971). Subsequently, oestrogen stimulates the incorporation of ^{14}C-lysine into acidic nuclear

proteins, four to six-fold, 4 hours after injection, and incorporation declines rapidly thereafter. This occurs only in the epithelia of ovariectomised mouse uteri and and stroma does not respond unless pretreated with progesterone (Smith et al., 1970; Martin et al., 1976; Pollard & Martin, 1977).

One further observation from the autoradiographic analysis of tissue sections is that hypertrophy occurs in the stroma and myometrium, while hyperplasia occurs in the luminal epithelium of oestrogen-treated mouse uteri (Martin et al., 1973). Thus our current knowledge of the differential responses of uterine cell types to oestrogen treatment is somewhat sketchy, and further investigations may help to explain some of the ambiguous results obtained from studies of whole uterus.

1.7 The aim of the project.

This project is to investigate the nature of the proteins synthesised early in response to oestrogen stimulation of immature rat uterus. From the work in this laboratory on sequences found in the mRNA populations during the first 4 hours of the response, it seems likely that oestrogen induces a small number of relatively abundant proteins which are produced between 2 and 4 hours after stimulation. These are vital to the mobilisation of the protein synthetic machinery of the cell for continuation of the response. In an attempt to detect these proteins, the techniques of *in vitro* translation have been developed, together with analysis of the products by two dimensional gel electrophoresis.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

MATERIALS

2.1 Suppliers

Unless otherwise specified, all chemicals used were, as far as possible, AnalaR grade supplied by B.D.H. Chemicals Ltd. Where chemicals and equipment were obtained from other sources, this is indicated in the text and a list of the names and addresses of the suppliers is given below.

B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

Beckman Spinco Ltd., Palo Alto, California, U.S.A.

Bio-Rad Laboratories Ltd., Watford, Herts., U.K.

Corning Glass Works, New York, U.S.A.

Eastman-Kodak Co., Rochester, New York, U.S.A.

Eppendorf Gerätebau, Hamburg, W. Germany

Evans Medical Ltd., Speke, Liverpool, U.K.

Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

*Gurr, Hopkin & Williams Ltd., Chadwell Heath, Essex, U.K.

*Gurr, Searle Ltd., High Wycombe, Bucks., U.K.

Jencons Ltd., Hemel Hempstead, Herts., U.K.

Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Labsure Animal Foods, Christor Hill Group Ltd., Poole, Dorset, U.K.

L.K.B. Instruments Ltd., South Croydon, Surrey, U.K.

Pharmacia Fine Chemicals AB, Uppsala, Sweden

Radiochemical Centre, Amersham, Bucks., U.K.

Rohm & Haas (U.K.) Ltd., Croydon, Surrey, U.K.

Schleicher & Schüll, Dassel, W. Germany.

Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

*Gurr, Hopkin & Williams, and Searle companies are now subsidiaries of B.D.H. Chemicals Ltd.

2.2 Experimental animals

Female, albino rats, derived from the Wistar strain, were bred in the departmental animal house. All animals used were 18 to 21 day-old weanlings weighing 30-40g. At all times prior to death the rats had free access to pelleted feed (CRM diet, Labsure Animal Foods) and water.

Oestradiol-17β (1,3,5,(10)-estratriene-3,17β-diol) (Sigma) was stored at -20°C as a stock solution of 20mg/ml in absolute ethanol. Immediately before use, a solution of 10μg/ml oestradiol-17β in 0.5% (v/v) ethanol, 0.15M NaCl, was made as described by Roberts & Szego (1947), and hormone was injected intraperitoneally as a single dose of 1μg hormone in 0.1ml carrier per rat. Control animals received carrier only.

2.3 Glassware

Glass homogenisers with motor driven Teflon pestles were purchased from Jencons Ltd. and Corex glass centrifuge tubes were obtained from Corning Glassworks. All other glassware was obtained from laboratory stocks. Pipettes, glass centrifuge tubes, test tubes, flasks and other glass items were sterilised at 160°C for at least 4 hours before use. Plastic items such as tips for automatic pipettes, millipore filter assemblies and capped 1.5ml reaction tubes (Eppendorf), and most solutions were autoclaved.

Cellulose nitrate tubes for ultracentrifugation were obtained from Beckman Spinco Ltd. and were ethanol washed and rinsed in sterile distilled water before use.

2.4 Radiochemicals and Liquid Scintillation Counting

All radioactive isotopes were purchased from the Radiochemical Centre, Amersham. AnalaR grade toluene and PPO (2,5 diphenyloxazole) were obtained from Koch-Light Laboratories Ltd.

Preparation of samples is described in the appropriate sections on experimental procedures. All samples were counted in 10mls 0.5% PPO in toluene, in disposable plastic vials in a Beckman LS8100 scintillation counter.

EXPERIMENTAL METHODS

2.5 Preparation of samples

2.5.1 The preparation of radioactively labelled proteins in whole uteri.

Animals were killed by cervical dislocation and their uteri removed. Each organ was dissected free of adipose tissue, slit longitudinally and collected in 5mls Eagles medium at 37°C. During dissection 95% O_2/5% CO_2 was continuously bubbled through this medium. When all uteri had been collected, groups of 6 were blotted to remove excess medium and transferred to a 5ml conical flask containing 1ml Eagles medium with one thousandth the usual concentration of amino acids, and either 20µCi L-[U-^{14}C] amino acid mixture (57mCi/mAtom carbon), or 40µCi L-[^{35}S]-methionine (800-1450 Ci/m.mol). Flasks were sealed and incubated at 37°C in a gently shaking water bath for 3 hours. The atmosphere in the flasks was flushed with 95% O_2/5% CO_2 every 10 minutes.

Incubated uteri were washed 3 times with ice-cold 0.9% saline, blotted to remove excess moisture and used immediately to prepare total

protein or cytoplasmic and nuclear fractions.

For total protein samples, groups of 6 labelled uteri were homogenised in 2mls Buffer A (0.01M Tris/HCl pH 7.4, 0.01M NaCl, 0.0015M $MgCl_2$. Tris (2-amino-2-hydroxymethyl-propane-1,3-diol), was supplied as Trizma base by Sigma). This and subsequent steps were conducted at 0-4°C. Insoluble debris was removed by centrifugation at 10,000g for 5 minutes in a Sorvall SS34 fixed-angle rotor and the resulting supernate was precipitated with 9 volumes acetone containing 0.1M HCl at -20°C overnight. The precipitate was pelleted by centrifugation, washed with acetone, dried in a stream of nitrogen and dissolved in lysis buffer for specific activity determination and two-dimensional gel electrophoresis.

Uterine nuclear and cytoplasmic fractions were prepared using the method of Knowler (1976). After the labelling incubation, groups of 6 uteri were homogenised in 2mls of Buffer A, containing 1% w/v Triton X-100 (Rohm & Haas), using an Ultra-Turrax homogeniser run at 60V for 20 seconds. The homogenisation and all subsequent steps were carried out at 0-4°C. The homogenate was filtered through two thicknesses of muslin and the homogeniser and muslin washed with a further 2mls of Buffer A. Crude nuclei were collected by sedimentation at 300g for 5 minutes in a Sorvall HB4 rotor (1200 rpm). The supernatant was precipitated with 9 volumes of acetone, 0.1M HCl, at -20°C overnight. Nuclei were resuspended in 2mls of Buffer A, which was then made 0.25M with respect to sucrose by addition of an equal volume of 0.5M sucrose in Buffer A. This was underlaid with 2mls of 0.32M sucrose in Buffer A. Clean nuclei were collected by centrifugation at 800g (2000 rpm) in a Sorvall HB4 rotor for 5 minutes and samples were checked for purity using phase contrast microscopy. The pellets were resuspended in 1ml

of Buffer A and precipitated as above with acidified acetone. Cytoplasmic and nuclear precipitates were pelleted by centrifugation, washed with acetone and dried in a stream of nitrogen. Both samples were dissolved in lysis buffer for specific activity determination and two-dimensional gel electrophoresis.

2.5.2 Determination of radioactivity in protein samples.

Duplicate 5µl samples were each added to 0.5mls of an aqueous solution of 200µg/ml bovine serum albumin. 0.5mls 20% w/v trichloroacetic acid was added and the precipitation allowed to proceed for 20 minutes at room temperature. Precipitates were collected onto 2.5cm Whatman glass fibre filters (Fisons), rinsed with 2x5mls 5% w/v trichloroacetic acid, dried and counted.

2.5.3 Protein assay.

Protein was determined by the method of Bramhall et al (1969). This method is best for samples containing not more than 50µg of protein. Its advantage lies in the lack of interference by substances such as mercaptoethanol and ampholines, both of which occur in lysis buffer, which is used to load samples for the first dimension gels in the O'Farrell two-dimensional gel system (see section 2.6.2). Duplicate aliquots of 5µl were spotted onto 2.5 cm Whatman No. 1 filter discs and dried. These were then put into cold 7.5% w/v trichloroacetic acid to fix, and this was then heated to 80°C and maintained at this temperature for 30 minutes. The discs were then drained and washed twice in 7.5% trichloroacetic acid. The protein on the discs was stained in a solution of 10mg/ml Xylene Brilliant Cyanin G (Gurr; Searle) in 7% v/v acetic acid at 50°C for 15 minutes. Excess dye was removed by washing with several changes of hot (50°C) 7% v/v acetic acid until the background was almost white. The filters were drained

and dried. 5mls of destaining fluid (66ml methanol, 34mls water, 1ml 0.88% ammonia) was added to each filter disc in a test tube and whirlimixed to release the dye. The absorbance of the solution was measured at 610nm, in a Gilford 240 spectrophotometer.

2.5.4 Fractionation of uterine cell types.

This followed the method of McCormack and Glasser (1980) who used it to study the differential response of individual cell types from immature rats (see 1.6). They showed that the cells separated by this method were viable in culture and retained their physiological integrity. The procedure is an adaptation of an earlier one used to separate embryonic epithelial cells by digestion of their basement membranes.

All enzyme solutions were made up in Dulbecco's phosphate buffered saline, pH 7.2, lacking calcium and magnesium (Grand Island Biological Co., GIBCO) and sterilised by filtration through Selectron nitrocellulose filters, pore size 0.45 micron (Schleicher & Schüll) which had been autoclaved. Pancreatin (GIBCO) was supplied as a crude lyophilised extract of pig's pancreas, and solutions required several filtrations before the final sterilisation. 1.25g pancreatin was added to 50mls phosphate buffered saline and stirred on ice for twenty minutes. The resulting extract was passed twice through double thicknesses of muslin. Aliquots of the solution were passed through Selectron GF92 glass fibre filters, pore size 50 micron (Schleicher & Schüll) in a Buchner funnel. The filtrate was then passed twice through Selectron nitrocellulose filters (pore size 0.45 micron). Trypsin (Sigma, type III) was added to 0.5%, and the solution refiltered through Selectron filters, sterilised and stored in 6ml aliquots at -20^{o}C. Trypsin (0.05%)-EDTA (ethylene diamine tetra-acetic acid, 0.02%) was frozen in 5ml aliquots, and deoxyribonuclease I

(DNAase, Sigma) at 400 units/ml and collagenase (Type I, Sigma) at 200 units/ml were stored in 1ml amounts, all at -20°C.

Foetal calf serum (GIBCO) was heat inactivated by incubating at 56°C for 30 minutes with frequent mixing. After the incubation it was cooled to 0°C and 0.25 volumes of dextran-coated charcoal (0.15% Norit A activated charcoal (Sigma), 0.0015% Dextran T-70 (Pharmacia Fine Chemicals), 0.25M sucrose, 1.5mM EDTA, 10mM Hepes (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid, Hopkin & Williams), pH 7.4) was added. The mixture was stood on ice for 15 minutes, vortexing every 3 minutes. Charcoal was removed by centrifugation at 2,500 rpm at 4°C for 5 minutes in a Sorvall HB4 rotor. The stripped serum was aliquoted and stored at -20°C.

Groups of 24 uteri were dissected, trimmed and kept on ice in phosphate buffered saline. When the dissection was completed, the horns of each uterus were separated at the vagina, slit longitudinally, blotted and placed in a vial of ice-cold trypsin/pancreatin solution. Vials were held at 0-4°C for one hour, and then placed at room temperature for a further hour. Each vial was then vortexed for 10 seconds and the supernate containing epithelial cells aspirated into a clean tube. The tissue was washed with 2 x 4.5mls phosphate buffered saline and the washes added to the tube containing the epithelial cells. 0.1 volumes of heat inactivated, charcoal stripped, foetal calf serum was added to the cells and they were held on ice whilst preparing the stromal and myometrial cells.

Uteri lacking epithelial cells were resuspended in a mixture of 2.5mls trypsin-EDTA (0.05%, 0.02%), 0.5mls DNAase I (200 units) and 6, 6mm glass beads were added to the vial. The tissue was incubated at 37°C

in a shaking water bath for 30 minutes, and the vial was vortexed for 5 seconds at 15 minutes and 30 minutes after the start of the incubation. The turbid supernate containing stromal cells was aspirated into a clean tube and the tissue washed with 2 x 5mls of phosphate buffered saline. The washes and 0.1 volumes of foetal calf serum were added to the cells and they were kept on ice whilst preparing myometrial cells.

The uterine tissue and glass beads were resuspended in a mixture of 2.5mls trypsin/EDTA (0.05%, 0.02%), 0.5mls DNAase I (200 units) and 1ml collagenase (200 units). This mixture was incubated at 37°C, with shaking, for 1 hour and was vortexed for 5 seconds at 30 minutes and 1 hour after the start of the incubation. The turbid supernate was recovered from the mixture by filtering through muslin and the residue was washed with 5mls of phosphate buffered saline. The myometrial cells were collected in a tube containing 0.1 volumes of foetal calf serum.

Cell suspensions were cleaned up by filtration over glass bead columns. Three 10ml disposable syringes were filled to the 5ml mark with 6mm glass beads. The cell suspensions were mixed by inversion, and poured over the columns. The suspensions were collected in centrifuge tubes. Each column was washed with a further 5mls of phosphate buffered saline. Cells were pelleted by centrifugation at 500rpm for 10 minutes at 4°C in a Beckman TJ-6 bench-top centrifuge. The supernate was aspirated off and discarded. Each cell pellet was resuspended in 5mls of Eagles medium containing 5% foetal calf serum and incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 4 hours.

2.5.5 Microscopic examination of cell samples.

Cell samples were examined for purity and damage using phase contrast microscopy, immediately after separation, and after resting. Samples were also examined by transmission electron microscopy which was performed by Mr. I Montgomery of the Physiology Department, University of Glasgow.

Briefly, samples supplied as sedimented pellets in Eppendorf tubes were fixed in glutaraldehyde-osmium tetroxide, embedded in plastic and cut sections were supported on grids. These were stained with lead and uranium for visualisation in the electron beam.

2.5.6 Labelling of uterine cell-type proteins

Cells 'rested' in Eagles medium for 4 hours were pelleted and resuspended in 1ml Eagles medium lacking methionine but containing 70μCi L-[^{35}S]-methionine and 5% foetal calf serum. The cells were incubated for 90 minutes at 37^{o}C under an atmosphere of 95% O_2/ 5% CO_2. After the incubation the cells were pelleted at 1000 rpm for 5 minutes in the Beckman TJ-6 bench-top centrifuge and the medium discarded, the pellets were washed twice in 5mls phosphate buffered saline and repelleted. Pellets were resuspended in 1ml isotonic saline, homogenised, and the solution precipitated with 9 volumes of acetone, 0.1M HCl overnight at -20^{o}C. Precipitates were washed with acetone, dried under nitrogen and dissolved in lysis buffer.

2.5.7 Isolation of uterine polyribosomes

Ribosomes were prepared from immature rat uteri by a method based on that of Berridge et al. (1976).

Uteri were collected in groups of 6-12 in plastic tubes immersed in a solid-CO_2/methanol bath and were broken into small fragments with a footed glass rod. Whilst still frozen, uteri were transferred to a sterile homogeniser and homogenised in 4.5 mls Buffer 1 (0.2M Tris/HCl pH 8.5, 0.05M KCl, 0.015M $MgCl_2$, containing 5 µg/ml cycloheximide (Sigma) and 7mM 2-mercaptoethanol (Koch-Light)). The homogenisation and all subsequent steps were carried out at 0-4°C using sterile glassware and solutions. The homogenate was adjusted to 2% (w/v) Triton X-100, by addition of 0.5 ml of a 20% (w/v) solution and centrifuged at 10,000g (9,000 rpm) in a Sorvall HB4 swing-out rotor for 10 minutes. The resulting supernatant was layered over a 1ml cushion of Buffer 2 (0.05M Tris/HCl, pH 8.5, 0.05M KCl, 0.015M $MgCl_2$, containing 1M sucrose, 5µg/ml cycloheximide & 7mM 2-mercaptoethanol) and centrifuged at 47,000 rpm for 90 minutes at 4°C in a Beckman SW50.1 rotor (200,000g). The resulting supernatants were discarded and the inside of the centrifuge tubes were wiped with a clean tissue, taking care not to disturb the pellets.

Where sucrose density gradients were to be run for polysome profiles, a small amount of post mitochondrial supernatant was run in a separate tube to give a separate small ribosome pellet.

Large pellets were washed twice with a small amount of sterile distilled water, and then resuspended in 50-100µl sterile distilled water. The optical density of the preparation was adjusted to between 0.25-0.5 A_{260} optical density units per microlitre, and aliquoted for storage at -70°C.

Small polysome pellets were rinsed twice with a small amount of Buffer 3 (0.05M Tris/HCl, pH 7.6, 0.25M KCl, 0.005M $MgCl_2$ containing 5µg/ml

cycloheximide and 50µg/ml heparin (supplied as a pyrogen-free sterile solution at a concentration of 5000 U/ml by Evans Medical Ltd.)). The pellet was then resuspended in 0.4ml of this buffer using a 1ml Teflon/glass homogeniser operated by hand, and carefully layered over a 5ml, linear, 15-45% (w/v) sucrose gradient made up in Buffer 3. The gradient was centrifuged at 50,000 rpm for 40 minutes at 4°C in a Beckman SW50.1 rotor (230,000g). The absorbance at 260nm of the gradient was measured by upwards displacement with 50% (w/v) sucrose through a flow cell attachment for the Gilford 240 spectrophotometer.

2.5.8 The preparation of polyribosomes from fractionated uterine cell types.

The methods of cell fractionation (2.5.4) and polyribosome preparation (2.5.7) were combined in this procedure, but were adapted to include further precautions against ribosome degradation. In addition to ribonuclease inhibitors, protease inhibitors were used to minimise any damage caused by the enzymes in the cell fractionation process being carried over into the homogenisation step.

In a pilot experiment 44 rats were given 12 hours of oestrogen treatment, and their uteri were processed in 2 groups. The epithelial cells were pooled and used to prepare polyribosomes. Stromal and myometrial cell types were not isolated. Polyribosomes were prepared from all three cell types in the subsequent experiment. 132 rats were injected with oestradiol-17β 4 hours before death. The uteri were dissected and the horns slit longitudinally under as near sterile conditions as possible. The uteri were divided into five groups for the cell fractionation which was performed as described in section 2.5.4.

As each cell type was isolated, the cells were pooled in a sterile tube stood in ice. All subsequent steps were conducted at 0-4°C. The cells were pelleted by gentle centrifugation. The pellet was resuspended and washed in 5mls of sterile Dulbecco's phosphate buffered saline, containing 1mM benzamidine hydrochloride (Sigma), 5mg/ml soybean trypsin inhibitor (Sigma) & 1mM PMSF (phenylmethyl sulphonyl fluoride (Sigma)). The cells were repelleted and immediately used to prepare polyribosomes. After the washing step, cells were resuspended in 4.5mls of polyribosome Buffer 1 (see section 2.5.7) containing 1mM benzamidine hydrochloride, 1mg/ml soybean trypsin inhibitor and 1mM PMSF. Triton X-100 was added to a concentration of 2% (w/v) to aid cell disruption. The homogenisation and all subsequent steps were performed as described in section 2.5.7.

2.5.9 Cell free translation assays.

Rabbit reticulocyte lysate which had been treated with micrococcal nuclease to destroy endogenous messenger RNA was purchased from the Radiochemical Centre, Amersham. The lysate is sold supplemented with all the ingredients necessary to ensure ideal translation conditions with high incorporation of labelled amino-acids into protein, and low background blanks. Commercial lysates are prepared by a modification of the method described by Pelham & Jackson (1976).

The lysate was aliquoted and stored under liquid nitrogen. Just prior to use it was thawed and an assay mix of 80% lysate and 20% L-[^{35}S]-methionine (by volume) was made, such that the concentration of label was 1µCi/µl. Assays were mixed on ice in sterile 1.5ml capped plastic tubes (Eppendorf). Each tube contained 10µl of assay mix and 1 µl of distilled water (blank) or 1µl of a polysome preparation under test. The incubation was started by transferring the tubes to a water bath

at 30°C. The assays were incubated for 90 minutes after which they were rapidly cooled to 0°C.

Incorporation into protein was assayed as trichloroacetic acid precipitable counts, measured as follows. 1µl of the translation assay was diluted with 9µl of distilled water and mixed. Duplicate 5µl aliquots were spotted onto 2.5cm Whatman 3MM filter discs (Fisons) and dried. The discs were then placed in ice cold 10% (w/v) trichloroacetic acid (TCA) for 10 minutes to precipitate the protein, boiled in 10% (w/v) TCA containing 10 mM L-methionine (Sigma) for 15 minutes and washed twice in cold 5% (w/v) TCA. Discs were rinsed in ethanol, dried and counted in 10mls 0.5% w/v PPO in toluene. The remainder of the assays were stored at -70°C until analysis of the translation products by two-dimensional gel electrophoresis.

2.5.10 Preparation of protein samples for cofractionation with labelled uterine proteins on two-dimensional gels.

a) Rabbit skeletal muscle actin

Rabbit muscle acetone powder was a gift from Dr. J. Dow of this department. 2.5 grammes of the powder was added to 50ml of sterile distilled water and stirred on ice for 30 minutes. The mixture was vacuum filtered through Whatman No.1 paper and the residue was reextracted by stirring with a further 25mls of sterile distilled water, on ice. The extracts were combined and spun at 34,000 rpm in a Beckman Ti50 rotor at 4°C for 60 minutes. The supernatant was retained and stored at -20°C. Samples were lyophilised, weighed and dissolved in lysis buffer at a concentration of 2mg/ml. Aliquots containing 20µg were loaded onto first dimension (isoelectric focussing) gels, either alone, or mixed with radioactive uterine proteins.

b) Rat leg muscle actin.

A lyophilised pure sample of rat leg muscle actin was a gift from Dr. J. Elce of the Department of Biochemistry, Queen's University, Kingston, Canada. This had been extracted from acetone powders in a similar way to that described for rabbit actin, but had been purified by polymerisation and depolymerisation. (Spudich & Watt, 1971; Elce et al., 1981). The actin sample was dissolved and used as described for rabbit actin, above.

c) Induced protein (IP, creatine kinase).

This was a gift from Dr. A. Kaye of the Weizmann Institute of Science, Rehovot, Israel, and was supplied as a lyophilised sample. Full details of the isolation and purification have been described by Reiss and Kaye (1981). Briefly, cytosol fractions were prepared from homogenates of rat brain. Proteins precipitated at 40-65% ammonium sulphate were collected and subjected to DEAE-cellulose chromatography firstly using step gradient elution and secondly using linear salt gradient elution. The creatine kinase was finally purified by chromatography on a Reactive Blue 2-Agarose column. IP was dissolved in lysis buffer at a concentration of 1mg/ml and aliquots containing 10µg were loaded onto first dimensional gels, either alone or mixed with labelled uterine samples. The solution was stored at -20°C.

d) Uterine chromatin proteins.

Uterine nuclei from 24 rats were prepared as described in section 2.5.1. The nuclear pellet was resuspended in 50 volumes of 0.08M NaCl, 0.02M EDTA pH 6.3, in a Teflon/glass homogeniser. The pestle was pulled hard on the upward stroke, to create a vacuum to break the nuclei. The solution was centrifuged for 5 minutes at 12,000 rpm in a Sorvall HB4 rotor (23,500g). The suspensions and homogenisation were

repeated twice, examining a sample under the microscope after each spin, for breakage of nuclei. The NaCl/EDTA extractions remove large amounts of RNA and protein that are not firmly bound to the chromatin. The pellet was resuspended in 50 volumes of 0.35M NaCl and centrifuged as before. This helps to remove contaminating ribosomal RNA and acidic proteins of the nucleoplasm and cytoplasm. The chromatin was dissolved in 50 volumes of 2mM Tris/HCl pH 7.5, 0.1M EDTA, and stirred on ice for 30 minutes. This solution was centrifuged for 10 minutes at 12,000 rpm. The pellet was drained well, and dissolved in lysis buffer. The concentration of the solution was determined and it was stored at -20°C until use. Where non-histone proteins were to be prepared, the pellet from the Tris-EDTA extraction was resuspended in 10 volumes of cold 0.4N sulphuric acid and stirred for 15 minutes at 4°C. The solution was centrifuged at 5,000 rpm (HB4 rotor) for 10 minutes at 4°C, resuspended in 0.4N sulphuric acid and recentrifuged. The pellet was drained well and resuspended in a large volume of 0.1M Tris/HCl, pH 7.5, 2mM $MgCl_2$, 2mM $CaCl_2$ (TMC). The dehistonised chromatin was repelleted (5000 rpm) and resuspended in TMC buffer. The pH of this solution was checked and if it was below 7.0 the wash was repeated. The washed pellet was then resuspended in 250µl TMC buffer containing 25µg DNAase I (Sigma) and incubated in a 30°C waterbath for 30 minutes. The solution was then mixed with an equal volume of lysis buffer, a protein determination was carried out and the solution stored at -20°C until required.

e) Rat liver hnRNP particle proteins.

Livers of adult male rats, weighing 200-250g were homogenised in 9 volumes of 2.3M sucrose, 10mM $MgCl_2$. Aliquots of 30ml were layered over 7ml cushions of 2.3M sucrose containing 10mM $MgCl_2$ and the nuclei were pelleted by centrifugation for 1 hour at 40,000g (15,000rpm) in a

Beckman SW27 rotor at 4°C. HnRNP particles were extracted from the purified nuclei by the method of Samarina et al. (1968). Briefly, the nuclei were subjected to extraction in a pH 7.0 buffer at 0°C after which they were repelleted and the extract discarded. The nuclei were then extracted 3 times in a pH 8.0 buffer, on ice, and the pooled extracts were fractionated on a linear 15-30% (w/v) sucrose density gradient. The gradients were scanned at 260nm by pumping through a flow cell attachment to a Gilford 240 spectrophotometer. The 30S hnRNP monomer peak was collected and precipitated in 2.5 volumes of absolute ethanol at -20°C in the presence of 0.15M salt. Precipitates were pelleted by centrifugation, dried in a stream of nitrogen, dissolved in lysis buffer and aliquots removed for analysis of protein. Solutions were then adjusted to 5mg protein/ml and stored at -20°C. Aliquots containing 100-150µg protein alone, or mixed with labelled uterine proteins, were fractionated on two-dimensional gels.

f) Ribosomal proteins.

Samples of ribosomal subunit proteins prepared from rat ascites tumour cells were a gift from Mr. M. McGarvey of this department. In outline, ribosomal preparations were dissociated into subunits in a Tris/HCl buffer containing mercaptoethanol and puromycin, and the 40S and 60S fractions separated on 15-30% (w/v) linear sucrose density gradients. Subunit fractions were dialysed to remove sucrose and precipitated with ethanol at -20°C. Subunits were pelleted and resuspended for protein extraction. Ribosomal RNA was removed by precipitation with acetic acid and the precipitate was reextracted. Ribosomal protein extracts were pooled and dialysed against 1N acetic acid, lyophilised and dissolved in sample buffer at a concentration of 1mg/ml, for two-dimensional gel electrophoresis (section 2.6.5).

2.6 Two-dimensional gel electrophoresis of samples.

2.6.1 The O'Farrell gel system.

The gel system of O'Farrell (1975) is a two-dimensional polyacrylamide gel separation devised for the high resolution of proteins from complex biological sources. Proteins are separated according to isoelectric point, by isoelectric focussing in the first dimension, and according to molecular weight by sodium dodecyl sulphate (SDS) electrophoresis in the second dimension. Since these two parameters are unrelated, it is possible to achieve an almost uniform distribution of protein spots across a two-dimensional gel. The technique can resolve over 1000 different components in a highly reproducible manner and therefore is very suitable for use in comparing the total protein populations of the immature rat uterus in different hormonal states.

2.6.2 First dimension isoelectric focussing gels.

A stock solution of 30% acrylamide (28.38% acrylamide, 1.62% bis-acrylamide (N,N'-methylene bis-acrylamide)) was prepared and stored at $4^{o}C$ in the dark. Both constituents were supplied by B.D.H., specially purified for electrophoresis. A 10% (w/v) stock solution of the detergent Nonidet P-40 (NP40) was also made.

For 10mls of gel mixture, 5.5g of Aristar urea (B.D.H.) was weighed into a universal bottle and dissolved in 2ml NP40 stock, and 1.97mls of distilled water. The mixture was made 4% with respect to acrylamide and 2% with respect to ampholines, by addition of 1.33ml of acrylamide stock; 0.3ml of pH range 5-7 ampholines and 0.2ml of pH range 3.5-10 ampholines (both supplied as a sterile solution of 40% (w/v) ampholines by L.K.B.). After thorough mixing and degassing under vacuum, polymerisation was initiated by addition of 10µl of a freshly

made 10% (w/v) solution of ammonium persulphate, and 7µl TEMED (N,N,N',N'-tetramethylethylenediamine, Koch-Light). The mixture was immediately poured into glass tubes 14cm long, with an internal diameter of 3mm. 0.8-1.0 ml was used per tube giving a gel 10cm long. Each tube was sealed at the base using the rubber caps from Portex sterile surgical canulae (Fisons). The canulae were used with a 1ml tuberculin syringe to deliver the gel solution into the base of the gel tubes without trapping air bubbles. Each gel was overlaid with distilled water and left to polymerise for 2 hours. The rubber seals were then removed and the water overlay replaced with 20µl of lysis buffer (9.0M Aristar urea, 2% w/v ampholines, (comprised of 1.2% pH range 5-7 ampholines, 0.8% pH range 3.5-10 ampholines), 5% (v/v) 2-mercaptoethanol) which was stored as frozen aliquots. The gels were then pre-electrophoresed for 30 minutes at 250V and 30 minutes at 400V, to remove any isocyanate ions which could cause protein carbamylation and thus artifacts in the separation. The cathode reservoir (upper) contained 0.02M NaOH and the anode reservoir (lower) contained 0.01M H_3PO_4. After the prerun, the NaOH and lysis buffer were removed from the surface of the gels and samples loaded using an automatic pipette. Wherever possible, samples did not exceed 50µl or 150µg of proteins as either excess volume or excess protein may cause loss of resolution.

The samples were carefully overlaid with 20µl of lysis buffer and the tubes filled with 0.02M NaOH. A blank gel overlaid with lysis buffer only, was run at the same time as the samples, and was used to determine the pH gradient across the gels after isoelectric focussing. The gels were run overnight at 400V for a minimum of 5,000 volt hours, and a maximum of 10,000 volt hours. After the run, gels were extruded using a syringe attached via a short piece of tubing, and were frozen

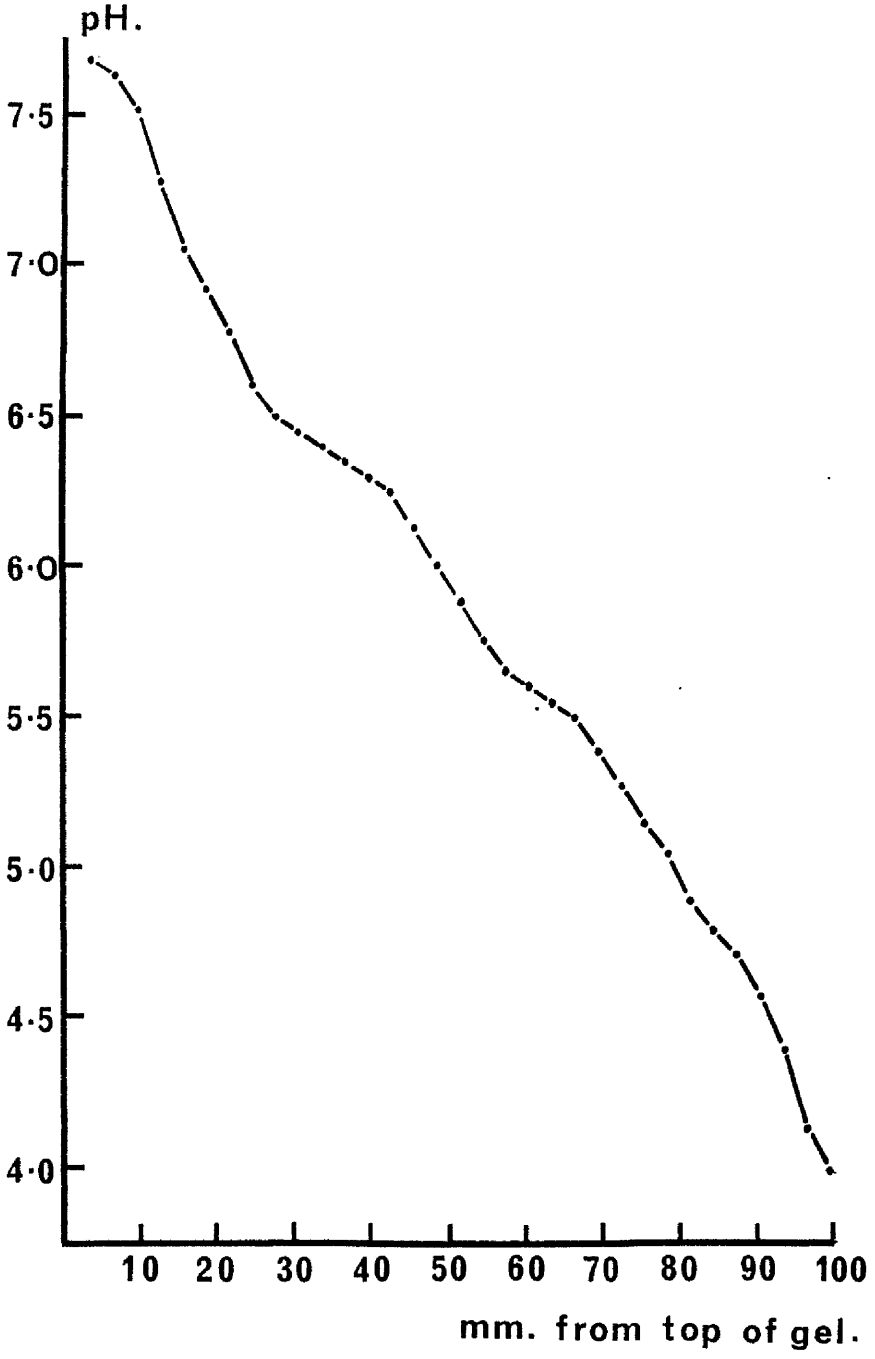


FIGURE (i)

pH gradient of an isoelectric focusing gel.

immediately in SDS sample buffer (10% w/v glycerol (B.D.H.), 5% v/v 2-mercaptoethanol, 2.3% w/v SDS, 0.01% w/v Bromophenol blue (Gurr, Searle). Blank gels were extruded, frozen in dry ice and sliced into 1mm sections using a Mickle gel slicer. Groups of three slices were placed into small tubes, each containing 0.5mls freshly degassed 0.01M KCl, and left to stand for 10 minutes. The pH of each fraction was measured using a combination electrode. A typical pH gradient is shown in Fig. (i) opposite.

2.6.3 First dimension non-equilibrated pH gradient electrophoresis.

Although basic ampholytes extending to pH 11 are commercially available, the basic end of ampholine generated pH gradients is not generally stable over long periods of electrophoresis. For this reason O'Farrell et al. (1977) devised the technique of non-equilibrated pH gradient gel electrophoresis, or NEpHGE, to separate mixtures of proteins containing basic components. The first dimension separation uses the same basic recipe as for the isoelectric focussing gel, but the ampholines used are pH range 3.5-10 (L.K.B.) only.

For 10 mls of gel mixture the following recipe was used: 5.5g Aristar urea (B.D.H.), 2ml, 10% w/v NP40 stock solution, 1.33mls 28.38% w/v acrylamide, 1.62% w/v N,N'-methylene bis-acrylamide stock, and 0.5mls pH range 3.5-10 ampholines. The solution was degassed and polymerised as described in section 2.6.2. Samples were prepared and loaded as for isoelectric focussing but gels were not pre-electrophoresed. After overlaying samples with 20µl of lysis buffer the tubes were filled with 0.01M H_3PO_4. The upper reservoir was filled with 0.01M H_3PO_4 and the lower reservoir with 0.02M NaOH. The gels were then run at 500V for 4 hours (a total of 2000 volt hours). This ensured that even the most basic proteins entered the gel but did not reach their

isoelectric points. For many proteins their isoelectric points would not lie within the gel if electrophoresis were continued to equilibrium. This method of separation was used to examine heterogeneous nuclear ribonucleoprotein (hnRNP) particle proteins for which it was adapated by Wilks & Knowler (1980).

2.6.4 Second dimension sodium dodecyl sulphate, polyacrylamide gels.

The discontinuous polyacrylamide gel system used in the second dimension was basically that of Laemmli (1970). The recipes given below are those of LeStourgeon & Beyer (1977). Stock solutions of acrylamide (30% w/v acrylamide, 0.8% w/v N,N'-methylene bis-acrylamide) and resolving gel buffer (Tris base (Sigma) 180g/l, SDS, 4g/l, HCl to pH 8.8) were stored at 4°C in the dark. The running buffer (Tris base 3g/l, glycine 14.4g/l., SDS 1g/l) was made up as a 10x concentrate and stored at room temperature.

10% polyacrylamide gels containing 0.1% SDS were made as follows. For each gel to be poured, 13mls distilled water, 10mls resolving gel buffer and 16.7mls acrylamide stock were mixed in a conical flask and degassed under vacuum. For each gel, 150µl of a freshly prepared 10% w/v ammonium persulphate solution and 10µl TEMED (Koch-Light) were added, mixed and the gels poured immediately. For 8.75% gels 18mls distilled water, 10mls resolving gel buffer and 11.7 mls acrylamide stock were mixed for each gel.

The gel formers consisted of 2 glass plates 6mm thick and 18x21 cm. One of these had a notch 2.5cm deep and 15cm wide in the short edge. The plates were separated by two 0.8cm wide, 1.5mm thick perspex strips placed at the sides, and by 2mm silicon tubing along the side and bottom edges. The plates were clipped together with bulldog clips,

with the notched edge uppermost. The gel solution was poured to within 0.5cm of the notch, overlaid with isobutanol, and left to polymerise for 1-2 hours. Before use the silicon tubing was removed and the isobutanol washed from the upper surface of the gel, which was then rinsed with running buffer.

First dimension gels were thawed and laid along the upper surface of the SDS gel ensuring contact along its entire length. A small sample well was formed at one end of the gel, by pouring molten 1% w/v agarose around a piece of silicon tube pushed between the glass plates, and allowing it to cool for 2-3 minutes. This was used for the application of molecular weight standards (Pharmacia, low molecular weight range 14,400 - 94,000). Loaded second dimension slab gels were run in pairs on a double apparatus. The gel plates were clipped to the upper reservoir of the apparatus, which had notches at each side, corresponding to those in the gel plates. The lower reservoir was a trough surrounding the bases of both gels. Wherever possible first dimension gels which had been run together were applied to second dimension gels poured from the same batch of solution and were run in pairs. Up to 6 slab gels could be run at one time. Gels were run at 35mA per gel until the dye front reached the base of the gel. For a 15x15cm, 1.5mm thick slab, this was usually between 3 and 4 hours. At the end of the run gels were placed in 0.25% w/v Coomassie Blue R250 (Gurr, Hopkin & Williams), 50% v/v methanol, 10% v/v acetic acid and left overnight to stain. Gels were destained in several changes of 50% v/v methanol, 10% v/v acetic acid.

2.6.5 Two-dimensional polyacrylamide gel electrophoresis of ribosomal proteins.

In outline, ribosomal proteins extracted from purified ribosomal subunits were dissolved in sample buffer (8M urea, 0.001M 2-mercaptoethanol, 0.01M $NaHCO_3$ pH 8.3) at a concentration of 1mg/ml. 100µg ribosomal protein (in 100µl) was loaded per gel. Two-dimensional polyacrylamide gel electrophoresis of ribosomal proteins was carried out by Mr. I.M. Kennedy of this department, using the standard procedure of Kaltschmidt and Wittman (1970), with modifications (Lastick & McConkey, 1976). The apparatus used has been described previously (Leader, 1975).

For cofractionation, 100µl aliquots were mixed with messenger dependent lysate translation assays directed by uterine polysomal messengers. The translation assay contained approximately 10^5 cpm [L-^{35}S]-methionine (in trichloroacetic acid-precipitable counts), in a maximum of 5 to 10µl. The exact counts and volume depended on the source of messenger used in different assays. Samples were layered over 5cm x 0.4cm, 4% polyacrylamide disc gels containing 6M urea at a pH of 8.7. Electrophoresis was carried out at 3mA per gel for 3 hours, the direction of migration being towards the cathode. The tank buffer used was a Tris-borate buffer with a pH of 8.0. Pairs of first dimension gels were removed from their tubes and laid on top of a 7cm x 7cm x 0.4cm, 15% polyacrylamide gel containing 6M urea at a pH of 4.5. The first dimension gels were annealed into place with 1% w/v agarose. Electrophoresis was then carried out at 10mA per gel for 16 hours, with migration again being towards the cathode. The tank buffer used for the second dimension was a glycine-acetate buffer at a pH of 4.05. After the run, gels were stained for 3 hr at 37°C in 0.1% (w/v) Coomassie Brilliant Blue R250 (Gurr, Hopkin & Williams Ltd.) in

methanol:acetic acid:water (5:1:5) and destained at 37°C in 7.5% (v/v) acetic acid.

2.6.6 Fluorography.

Fluorography was routinely used because it improves the efficiency of detection of beta-emitting isotopes such as ^{35}S and ^{14}C by at least tenfold. Gels were prepared according to the method of Bonner & Laskey (1974), with modifications. Gels were washed in tap water to remove excess destaining solution and soaked in three successive 250ml dimethylsulphoxide (DMSO, Koch-Light) washes for 30 minutes each. The gels were then immersed in 4 volumes of 22.2% w/v 2,5-diphenyloxazole (PPO) in DMSO, and were shaken gently for 2 hours. All stages involving DMSO were conducted in an airtight box. Following their impregnation with PPO, gels were left under running tap water for 1 hour to precipitate the PPO into the gel matrix. The gels were dried onto Whatman 3MM paper under vacuum, at 80°C for about one hour on a Bio-Rad gel drier. Dried gels were exposed to RP X-Omat X-ray film (Eastman-Kodak) pressed between 2 glass plates at -70°C for 4 to 6 weeks.

RESULTS

3. RESULTS

3.1 The detection of labelled uterine proteins from immature rats.

This project utilised the technique of labelling proteins of the immature rat uterus *in vitro*, with various high specific activity radioactive isotopes, after which they were subjected to two-dimensional gel electrophoresis. The proteins were visualised by fluorography of the dried second dimension sodium dodecyl sulphate (SDS)-polyacrylamide gels.

A number of approaches were used in the attempt to detect differences between the protein population of the unstimulated rat uterus and that seen in the early stages of oestrogen stimulation. Firstly, whole uteri were labelled *in vitro* with a high specific activity mixture of ^{14}C-labelled amino acids. Both total protein, and subcellular fractions were prepared and examined using the techniques described in the Methods section. Subsequently these techniques were extended to the study of the protein population present in three different uterine cell types:- epithelium, stroma and myometrium. In a third approach uterine proteins were labelled by the cell-free translation, *in vitro*, of uterine polysomal messenger RNA, derived from both whole uteri and from isolated cell types. Finally, a number of known proteins were cofractionated with labelled uterine preparations on two-dimensional gels, to try to identify some of the components in the spectrum of uterine proteins.

3.2 The investigation of proteins synthesised in vitro in whole uteri.

The specific activities of total uterine protein samples varied from 1000 to 2000 cpm/μg protein. Fig. 1 shows gels of ^{14}C-labelled proteins from unstimulated animals, separated by isoelectric

Figure 1

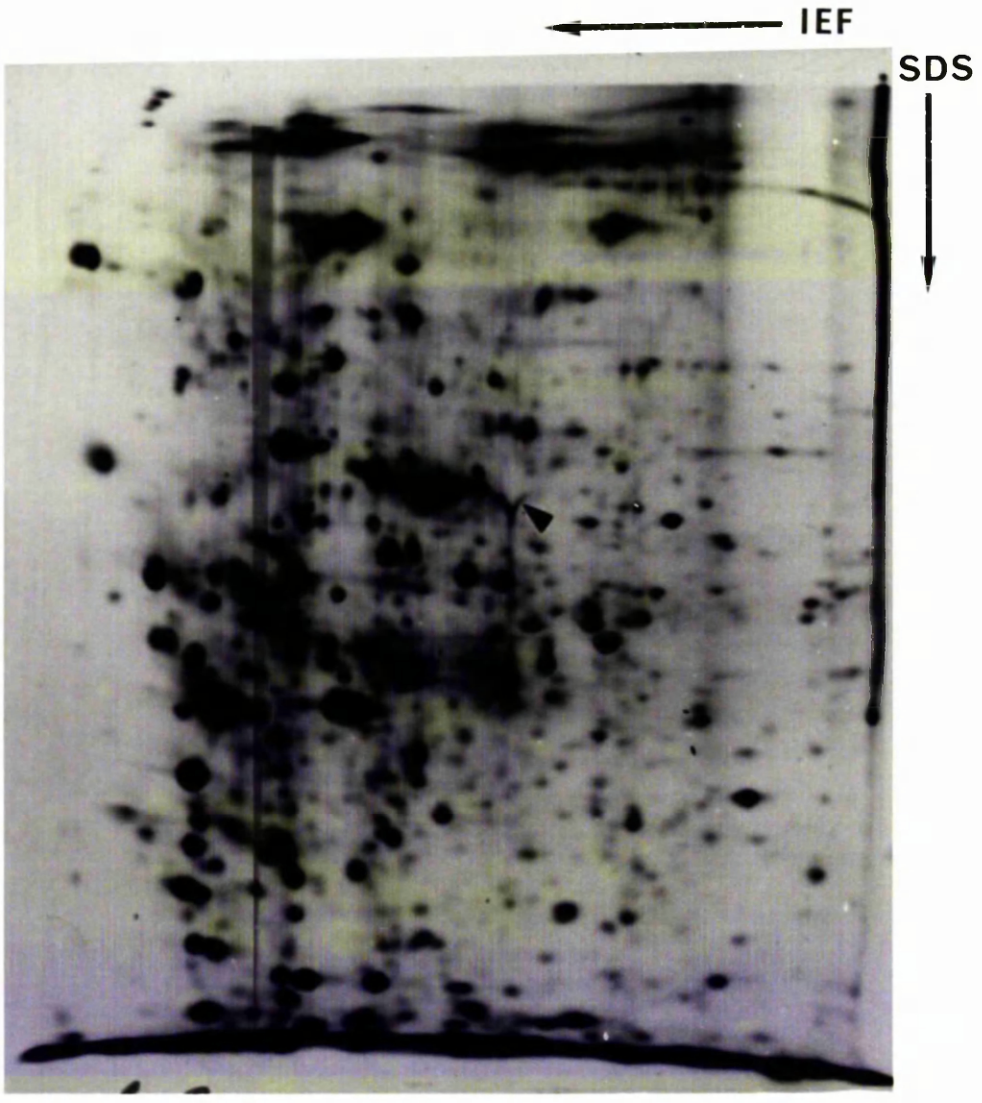
Electrophoresis of L-[U-^{14}C]-labelled uterine proteins on two-dimensional polyacrylamide gels

The uteri from 6 immature rats were incubated in 1ml of Eagles medium containing one-thousandth the usual concentration of amino acids, and 20 μCi of a high specific activity L-[U-^{14}C]-labelled amino acid mix, as described in the Methods section (2.5.1). A total protein sample was prepared and the specific activity determined. The proteins were subjected to two-dimensional gel electrophoresis by the method of O'Farrell (1975), which uses isoelectric focussing in the first dimension. The sample was loaded at the basic end of the gel and run at 400V for 6000-8000 volt-hours. The second dimension was an 8.75% polyacrylamide slab gel containing 0.4% SDS. The gel was stained in 0.25% (w/v) Coomassie Brilliant Blue R250 in 50% methanol, 10% acetic acid, and destained in the solvent mix. The wet gel was photographed, prepared for fluorography according to the method of Bonner and Laskey (1974) and exposed to X-ray film for 12 days. The gel was loaded with 250μg protein from L-[U-^{14}C] labelled uteri, containing 10^5 cpm. The direction of the first dimension was from right (basic) to left (acidic), and the slab gel was run from top to bottom.

a) Coomassie Brilliant Blue staining pattern

b) Fluorogram

a



b

FIGURE 1.

focussing in the first dimension and SDS-polyacrylamide gel electrophoresis in the second dimension. Fig. 1a shows the Coomassie Blue staining pattern achieved, and Fig. 1b the fluorograph of the same gel. This gel was overloaded (250µg protein, $2x10^5$ cpm) to show the staining pattern. The major protein species lie in the acidic part of the gel and form a complex, distinct pattern. Many minor components are present, and these are difficult to compare between gels due to their faintness, even after long exposures. O'Farrell (1975) suggests a maximum loading of 100µg protein per gel, to avoid artifacts in the separation. He indicates that the main problem is in the change of major spot shape and the displacement of minor spots as larger neighbours increase in size. However in a complex mixture of proteins, only a small number of species are present as major components, and gels can be severely overloaded with respect to these with only a small decrease in overall resolution of the majority of species. The arrowed area of Fig. 1 illustrates the distortion of a labelled component by a major stainable spot. This particular distortion occurs on all gels of total and cytoplasmic protein.

O'Farrell further suggests that for true comparison, the same loading of protein must be used on each gel. This is not always possible using the uterine samples, due to variation in the specific activities between different preparations. Most gels were run with a loading of 10^5 cpm , but the possible effects of variation in protein loading on the results obtained must be borne in mind in all subsequent gel analyses.

Although many of the stained proteins in Fig. 1a also appear as labelled species in Fig. 1b there are many more spots visualised by fluorography and the pattern is very complex. To try to simplify this

picture and allow easier detection of hormone-induced changes, the labelled uterus was fractionated into cytoplasmic and nuclear preparations before analysis on gels. Such fractionations had the added advantage that oestrogen-induced changes might be identified with specific cell compartments.

Specific activities for cytoplasmic protein samples varied from 500 to 2,500 cpm/µg protein, and for nuclear samples from 600 to 2800 cpm/µg protein. The degree of labelling varied with hormone treatment, and preparations from control animals generally had lower specific activities. Nuclear samples always had higher specific activities than their corresponding cytoplasmic fractions.

Fig. 2 shows the spot patterns of ^{14}C-labelled cytoplasmic uterine proteins. The fluorograms shown are typical of the protein populations found in immature rats responding to 0, 2 and 4 hours of oestrogen stimulation. The overall resemblance of these fluorograms to each other and to the spot pattern in Fig. 1 is obvious. However, once the general pattern is recognised, it is possible to detect some differences between the preparations from control and hormone-treated animals. Spots arrowed and numbered 1 to 6 appear after hormone treatment or increase in response to it. Spots 7 and 8 appear between 2 and 4 hours of treatment and the spots marked 9 progressively decrease and are absent from the population after 4 hours of oestrogen treatment. However, these differences are not consistent for every set of gels. For example, the three spots marked 1 are absent from the control gel of Fig. 2a, but some or all of them regularly appear on other control gels (e.g. Fig. 3a, following page 69). The two spots marked 3 are a similar case. These increase in intensity after the longer hormone treatment and cannot be seen in the control sample (Fig. 2a), only the reference spot 'A' being present.

Figure 2.

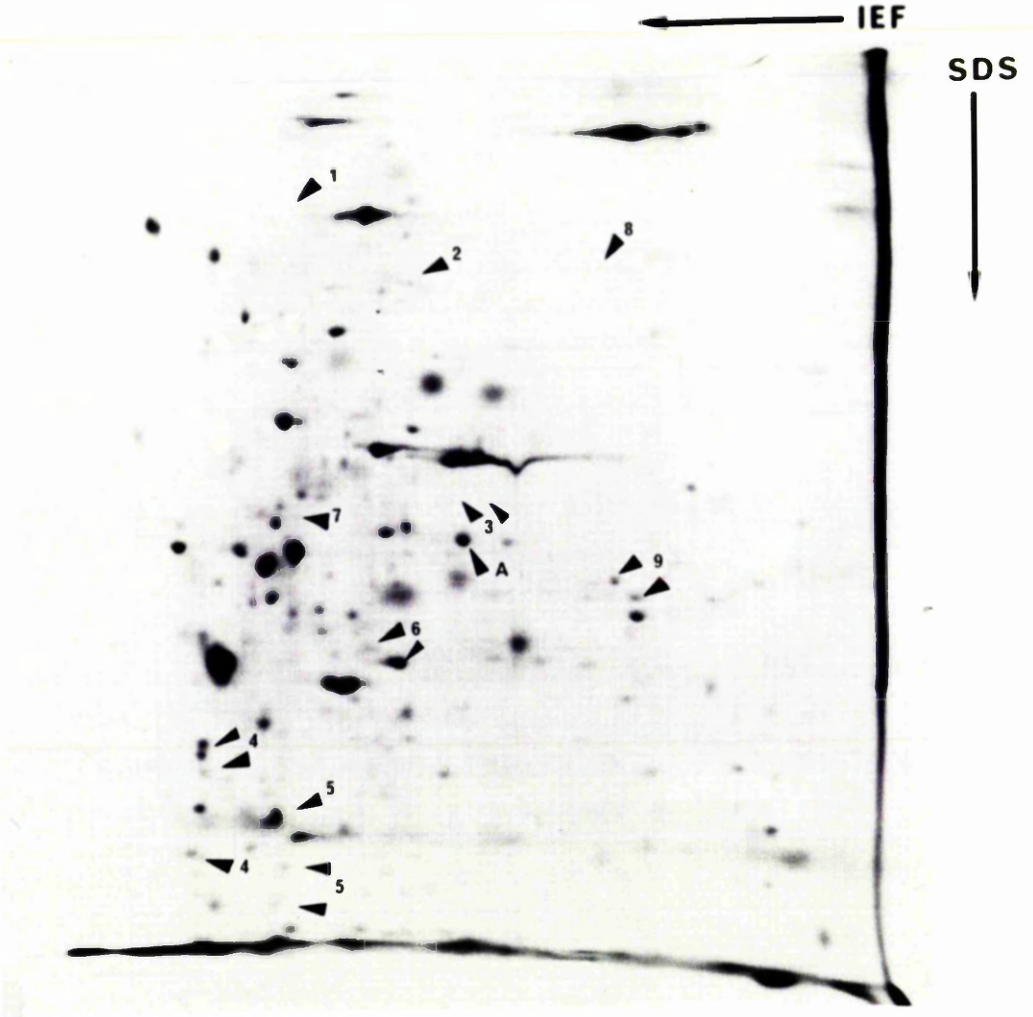
Electrophoresis of L-[U-^{14}C]-labelled uterine cytoplasmic proteins from rats responding to short periods of oestrogen stimulation.

Groups of six immature female rats received 1µg oestradiol-17β in saline by intraperitoneal injection, 2 or 4 hours before death. Control animals received saline only, 4 hours before death. Uteri were labelled as described in the legend to Figure 1. After incubation, the uteri were homogenised and nuclei were isolated using the method of Knowler (1976). Protein samples were prepared from the post-nuclear supernatant and the nuclear pellet, as described in the Methods section (2.5.1). The samples were subjected to two-dimensional gel electrophoresis as described in the legend to Figure 1. Gels were fluorographed and films were exposed for 12 days. The fluorograms shown are as follows:-

a) Control uterine cytoplasmic protein. The first dimension gel was loaded with 130µg protein containing 10^5 cpm.

b) Uterine cytoplasmic proteins from rats receiving 1µg 17β-oestradiol 2 hours before death. Loading: 40µg, 10^5 cpm.

c) Uterine cytoplasmic proteins from rats receiving 1µg 17β-oestradiol 4 hours before death. Loading: 95µg, 10^5 cpm.



a

FIGURE 2.

FIGURE 2.

However, it is exceedingly difficult to locate these two spots on any other gel, for example the 12 hour sample in Fig. 3. Furthermore, they may even be present in the control sample in Fig. 1 just below and to the left of the arrowed distortion. The two spots marked 6 correspond to the expected position of IP. These are exclusively cytoplasmic, as is IP (See Fig. 5, following page 70) but occur in some control gels (Fig. 3a, following page 69). Spot 7 seems to be a more reliable difference. It appears between 2 and 4 hours after oestrogen and cannot be seen on any control gel. It is also absent from the 12 hour sample and would therefore be a good candidate as a key intermediary protein. However, further experiments have shown that this spot is also very difficult to identify consistently.

Other examples of minor differences can be seen but the validity of these is even more difficult to assess, because of the variation between gels and samples of cytoplasmic protein from uteri receiving the same hormone treatment. The basic end of a gel always shows the largest variation. This is where the most interference from any nucleic acids in a sample may be expected, and where the pH gradient is most variable between first dimension gels that have not been run together (O'Farrell, 1975).

Fig. 3 shows a direct comparison between samples of the same specific activity, from control animals and those receiving oestrogen stimulation 12 hours before death. The most obvious result of this comparison is the high degree of similarity between the newly made proteins in the uterine cytoplasms of control and hormone-stimulated animals. No major changes can be identified in the population, and many of the recognisable 'constellations' of minor proteins agree closely between the two fluorograms. This is surprising in view of the

Figure 3.

Electrophoresis of L-[U-^{14}C]-labelled uterine cytoplasmic proteins from rats responding to 0 & 12 hours of oestrogen stimulation.

Groups of 6 immature female rats received either 1μg of oestradiol-17β or saline only, 12 hours before death. Experimental details are the same as those for Figure 2. Films were exposed for 11 days and the fluorograms are shown. The first dimension gels were each loaded with 150μg of proteins containing 10^5 cpm.

a) Control uterine cytoplasmic proteins.

b) Uterine cytoplasmic protein from rats responding to 12 hours of oestradiol-17β.

a

FIGURE 3.

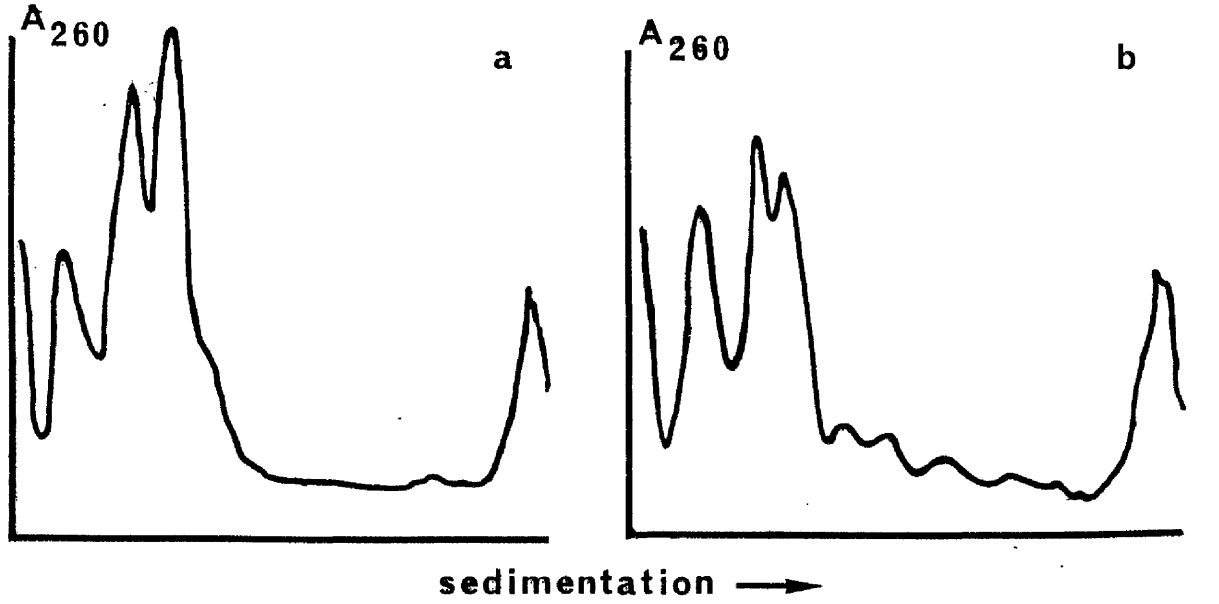
b

Figure 4.

The effect of oestradiol-17β on polysome profiles of immature rat uteri.

Immature female rats were given a single injection of 1μg oestradiol-17β in saline, at various times before death. Control animals received saline only. Polysomes were prepared from the uteri and analysed on 15-45% (w/v) sucrose density gradients as described in the Methods section (2.5.7). The profiles shown are examples of polysomes from uteri responding to the following times of oestrogen treatment:

a) 0 hrs, b) 2 hrs, c) 4 hrs, d) 12 hrs.



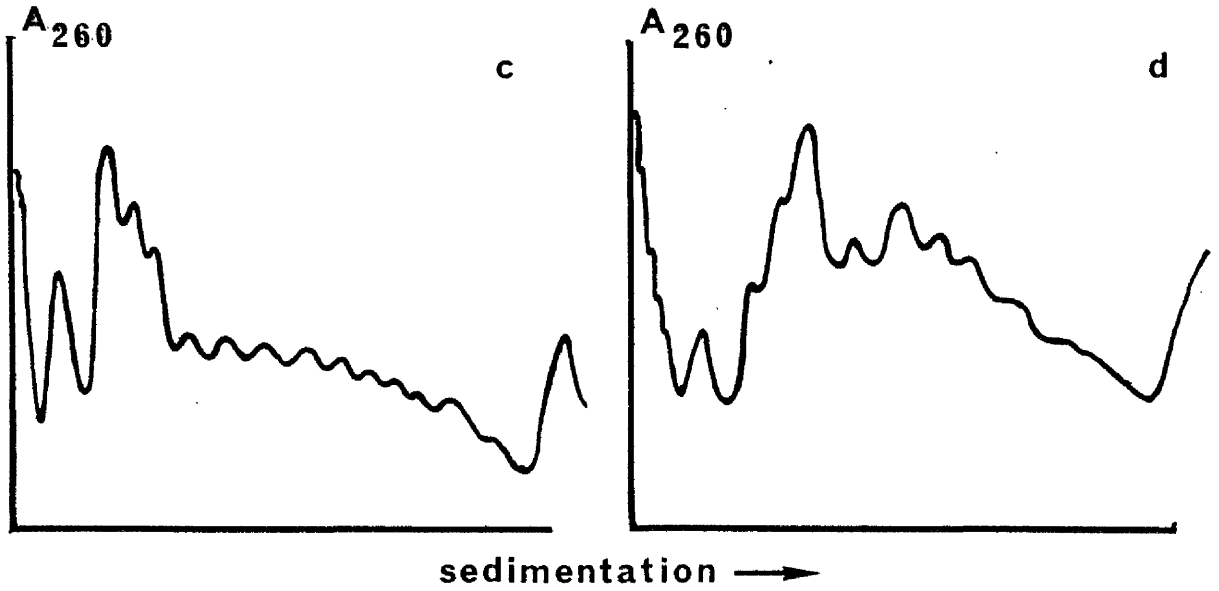


FIGURE 4

difference in protein synthetic activity in vivo, between 0 and 12 hours of oestrogen stimulation. The profiles of sucrose density gradient separations of polysomes in various hormonal states can be seen in Fig. 4. These show that the majority of ribosomes in the untreated immature rat uterus exist as free monosomes or in small aggregates of two or three ribosomes. As oestrogen treatment progresses the ribosomes are isolated in increasing amounts at the heavy end of the gradient as large polysomes. This indicates that the unstimulated uterus is a quiescent tissue not very active in protein synthesis, whereas after 12 hours of oestrogen treatment, the tissue has entered the phase of vastly increased protein synthesis characteristic of the hypertrophic response. In view of this, and in view of the known changes in uterine mRNA populations in the oestrogen -treated rat (Aziz et al., 1979b), one might have expected to see considerable differences between the control and 12 hour protein populations shown in Fig. 3.

The comparison of nuclear samples at first seemed more informative. The protein populations seen at 0, 4 and 12 hours are shown in Fig. 5. The spot patterns share some of the proteins seen in the cytoplasmic samples, but there are fewer components resolved. Greater differences can be seen between the control and hormone-treated samples on the gels shown than for the cytoplasmic samples and these are arrowed in yellow. These differences are not reproducible, with the exception that the synthesis of the protein arrowed in white always decreases after hormone treatment. A recent analysis of mouse uterine proteins shows a very similar spot pattern (Korach et al., 1981). The authors and a number of workers with other cell systems, have identified a protein migrating in the same position, as a subunit of tubulin (Schultz et al., 1979; Bravo & Celis, 1980). There is also reason to

Figure 5.

Electrophoresis of L-[U-^{14}C]-labelled uterine nuclear proteins from rats in various hormonal states.

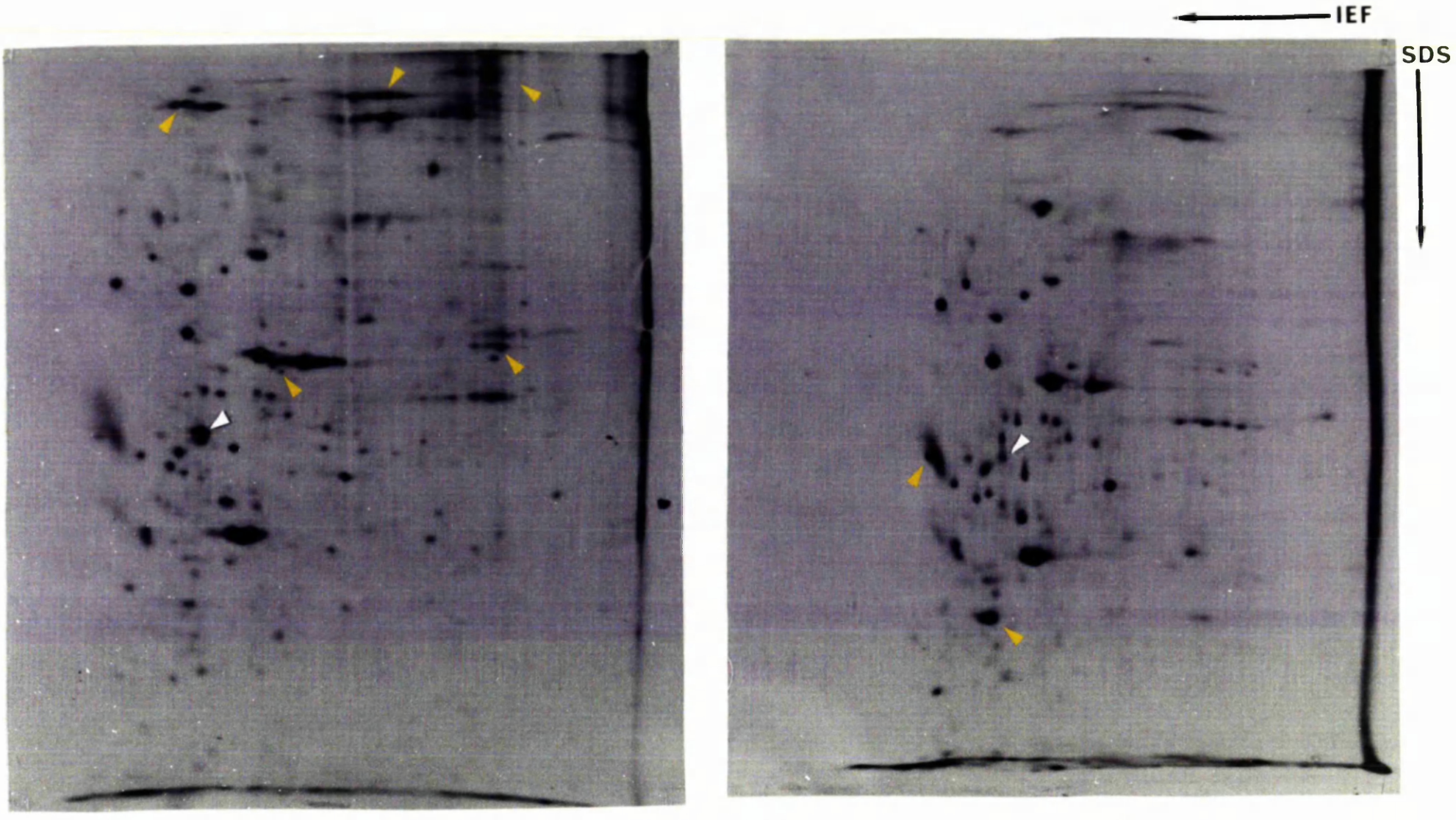
Nuclear protein samples were prepared from labelled uteri by the method of Knowler (1976). Details of the labelling in vitro, and electrophoresis are given in the legend to Figure 1. Fluorograms were exposed for 11 days in a) and b), and for 21 days in c) and d). The rats were treated with oestradiol-17β for the following times:

a) 0 hrs. Loading: 80μg protein, 10^5 cpm.

b) 4 hrs. Loading: 100μg protein, 10^5 cpm.

c) 0 hrs. Loading: 80μg protein, $5x10^4$ cpm.

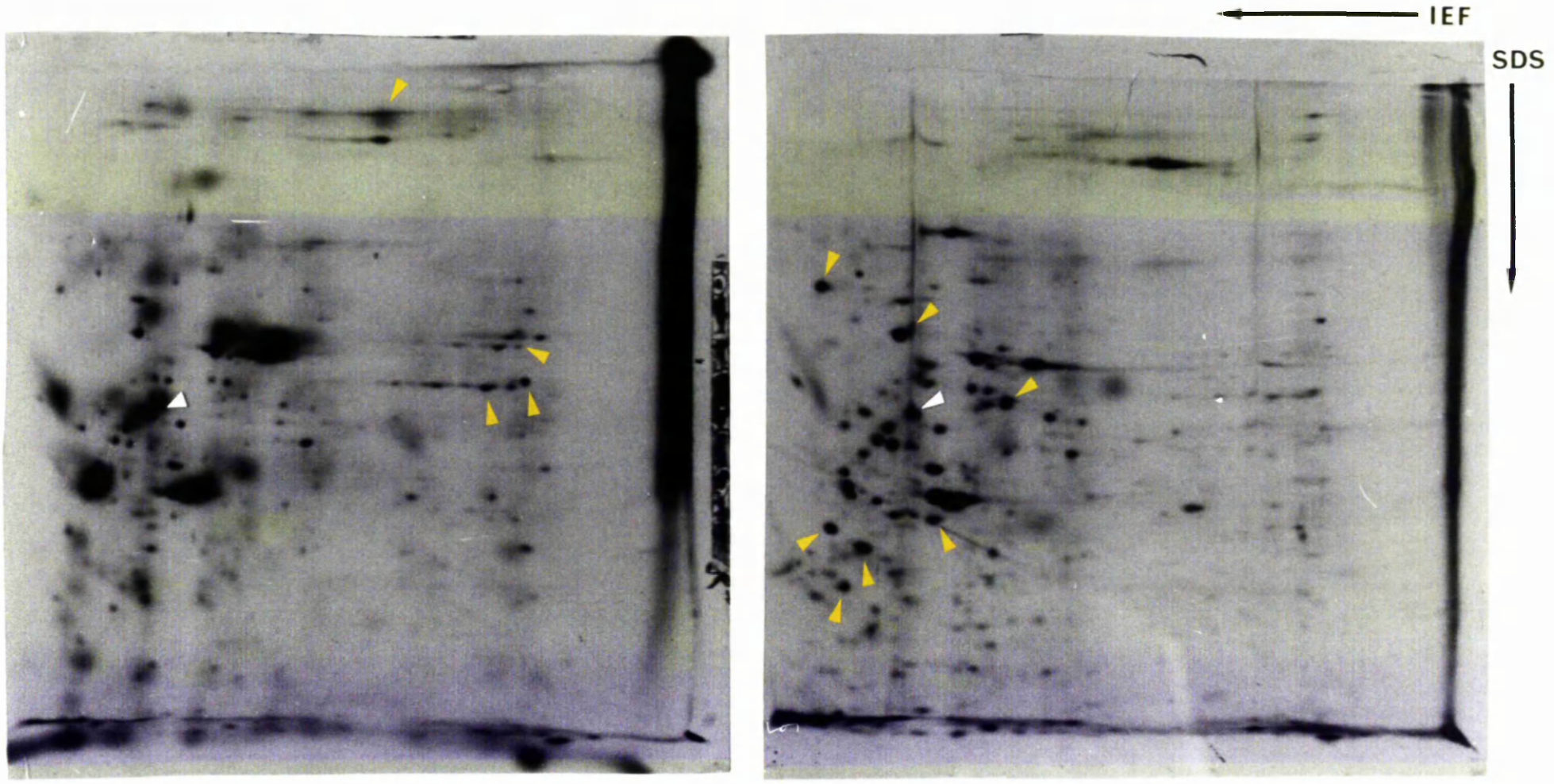
d) 12 hrs. Loading: 50μg protein, $5x10^4$ cpm.



a

FIGURE 5.

b



c

FIGURE 5.

d

suppose that the major triple spot below the arrowed spot is actin. This is discussed later.

3.3 The investigation of the protein population of isolated cell types.

Light microscopy was used to look for damage to cells during the fractionation procedure, and samples of each cell type were also examined by transmission electron microscopy (TEM). This allowed a check on the integrity of cells after preparation, and an assessment of the degree of contamination between cell types. These methods were used by McCormack & Glasser who developed the fractionation procedure (1980), and the micrographs compare well with their published results.

Epithelial cells were isolated as large plaques of cells in which many lipid droplets could be seen under both light and transmission electron microscopy. They also had many microvilli on their surfaces (Fig. 6a). There were very few cells of any other kind in epithelial fractions.

The stromal cells did contain a few small epithelial plaques, but these did not exceed 5% of the preparation. The cells appeared rounded and had large nuclei, but could not easily be distinguished from myometrial cells under the light microscope. TEM revealed separated stromal cells to be engaged in the production of collagen fibrils and they showed small irregular blebs and protrusions at their surfaces (Fig. 6b). This agreed with the appearance of these cells in the scanning electron micrographs published by McCormack & Glasser. TEM also showed the presence of intact glandular epithelial structures in this fraction (Fig. 6c).

Figure 6.

Transmission electron microscopy of fractionated uterine cell types.

Uterine cell types were isolated using an enzymatic digestion technique developed by McCormack & Glasser (1980). Full experimental details are given in the Methods section (2.5.4). Samples were sent for electron microscopy immediately after isolation. This analysis was performed by Mr. I. Montgomery of the Physiology Department, University of Glasgow. The figure shows typical micrographs of the cell types isolated from control uteri.

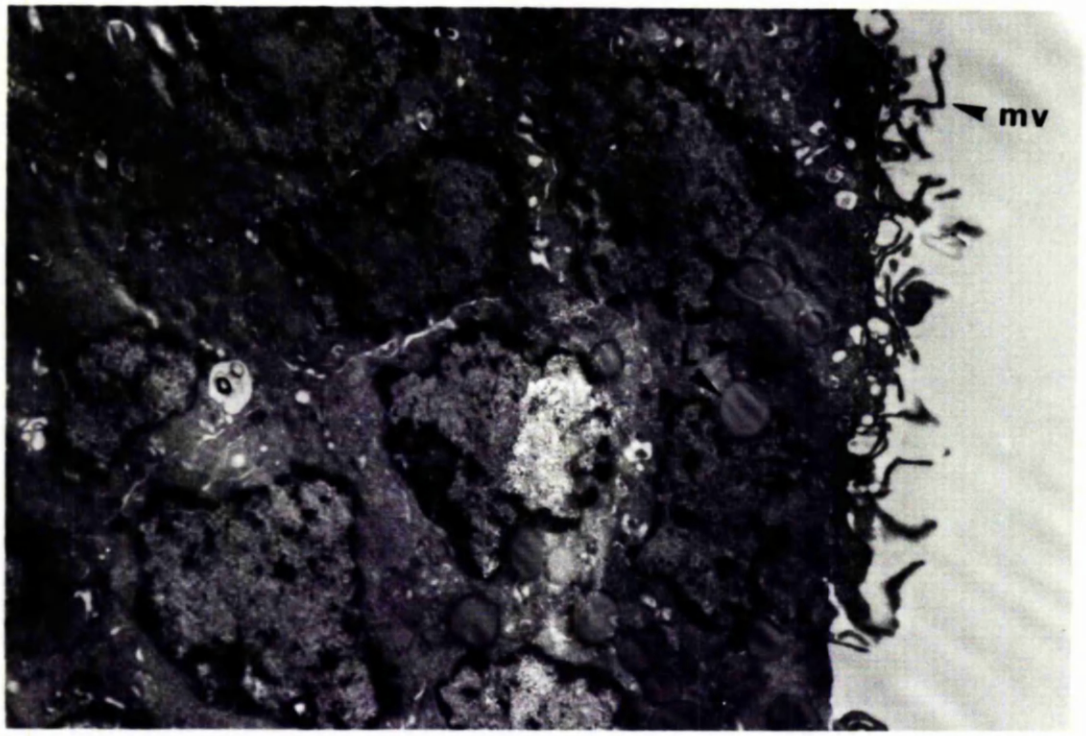
a) Luminal epithelial cells x3,900.

b) Stromal cells x3,900.

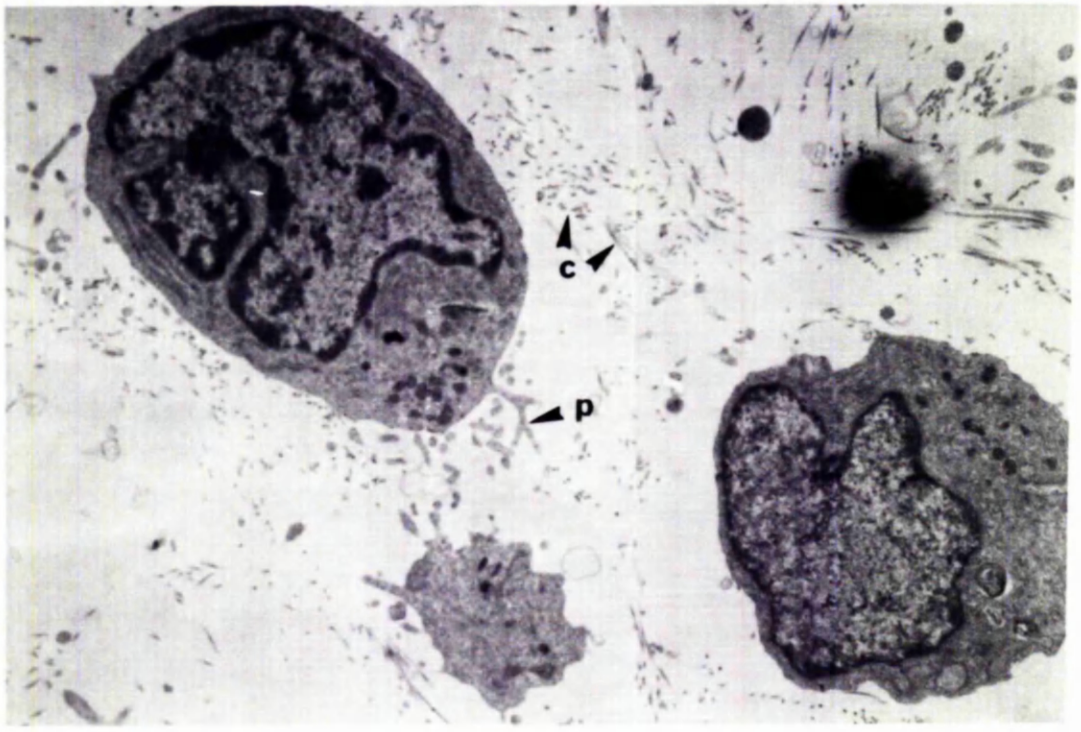
c) Glandular epithelial structure in the stromal fraction x2,350.

d) Myometrial cells x7,800.

mv, microvilli; L, lipid droplets; c, collagen fibrils;
p, protrusions; gl, glycogen granules; t, membrane thickening;
sf, stress fibres.

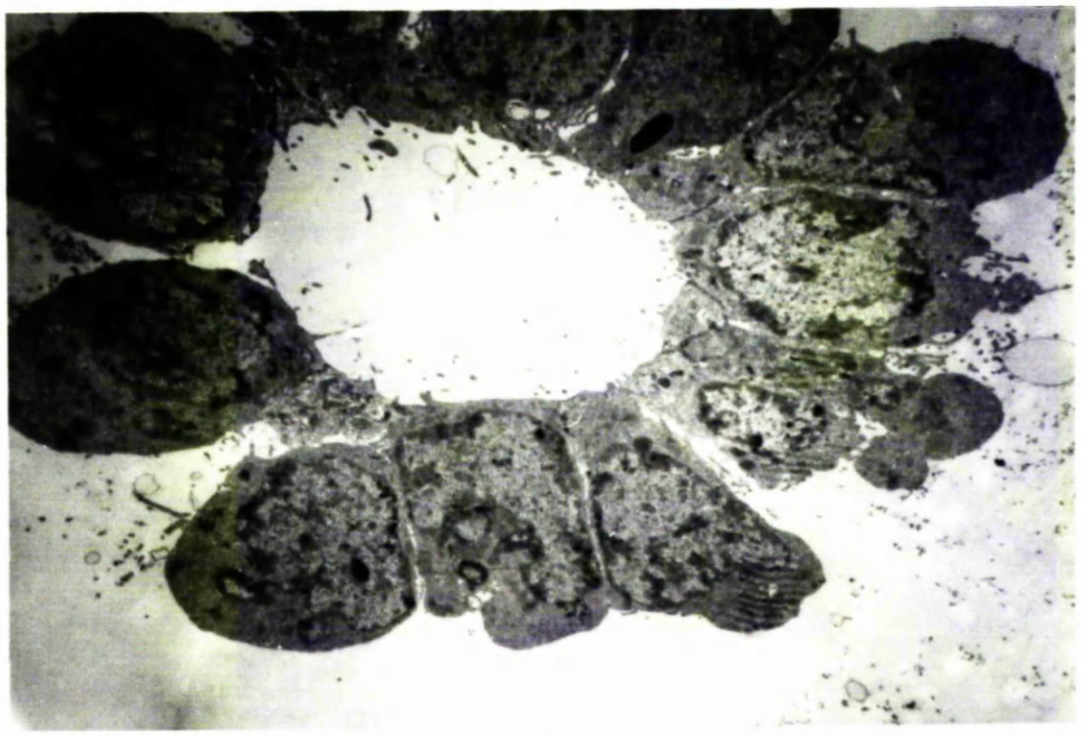


a

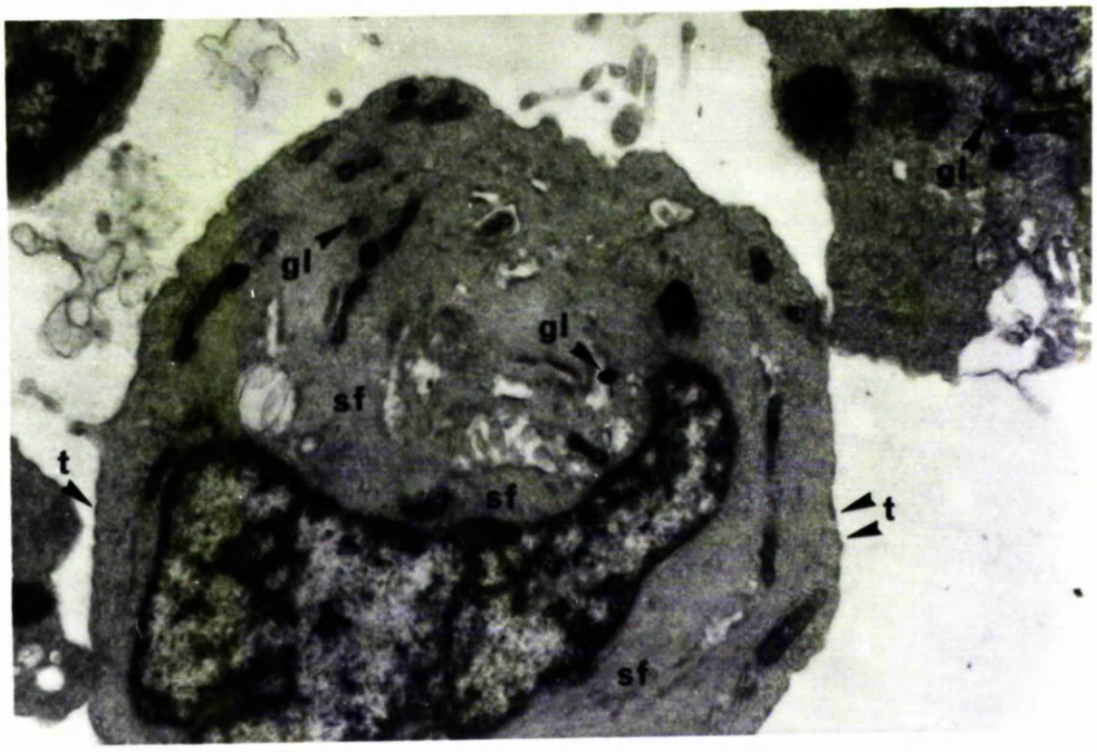


b

FIGURE 6.



c



d

FIGURE 6.

The myometrial cell fraction contained no epithelial cells, but did contain some stromal contamination. This was difficult to assess except where stromal cells could be seen in micrographs but appeared to be at a low level. The myometrial cells were irregular and often elongated with nuclei occupying a much smaller proportion of the cell than in the stromal cells. The cells possessed membrane thickenings indicative of subsarcolemmal filament attachment areas, and also contained glycogen granules and stress fibres (Fig. 6d).

After isolation, each cell type was labelled in vitro with ^{35}S-methionine and total protein samples were prepared and subjected to two-dimensional gel electrophoresis. Specific activities for these samples varied from 500 to 800 cpm/µg protein for epithelial and stromal cell types but were always below 500 cpm/µg, and were usually about 200 cpm/ug for myometrial cells.

Fig. 7 shows the patterns of radioactive proteins detected in epithelial, stromal and myometrial cells from the uteri of control animals. The patterns are not as complex as those seen for total or cytoplasmic protein samples from whole uteri, and do not at first appear very similar. The staining patterns (not shown) showed a few faint spots, and one large spot which was identical in position to the major component arrowed in Fig. 1a (following page 66). This polypeptide was not labelled in vitro but is severely overloaded on these gels and causes a serious distortion spreading over a larger area of the gel than in Figs. 1-3. It completely displaces the more basic of the spots which migrate at the same molecular weight position and is particularly prominent in the myometrial fraction. This distortion is useful as an internal marker for comparing the fluorograms of cell-type proteins with those of whole uteri. Although

Figure 7.

Electrophoresis of proteins from L-[^{35}S]-labelled fractionated cell types.

The uteri of 24 immature rats were used to prepare epithelial, stromal and myometrial cell fractions, as described in the Methods section (2.5.4). Following isolation, each cell type was rested for 4 hours at 37^{o}C in an aerated tube containing 5 mls of complete Eagles medium. After this incubation each cell type was washed twice in phosphate buffered saline, and then incubated in 1ml Eagles medium lacking methionine, and containing 5% charcoal-stripped, heat-inactivated foetal calf serum, and 70µCi of L-[^{35}S]-methionine. Protein samples were prepared from each cell type as described in the Methods section. Two-dimensional gel electrophoresis and fluorography were performed for each sample as described in the legend to Figure 1, except that second dimension slab gels were 10% polyacrylamide. All subsequent figures of SDS gels show 10% polyacrylamide slabs. Films were exposed for 28 days.

a) Control epithelial cell-type proteins. Loading: 190µg protein, $9.6x10^4$ cpm.
b) Control stromal cell-type proteins. Loading: 100µg protein, $7x10^4$ cpm.
c) Control myometrial cell-type proteins. Loading: 150µg protein, $3.6x10^4$ cpm.

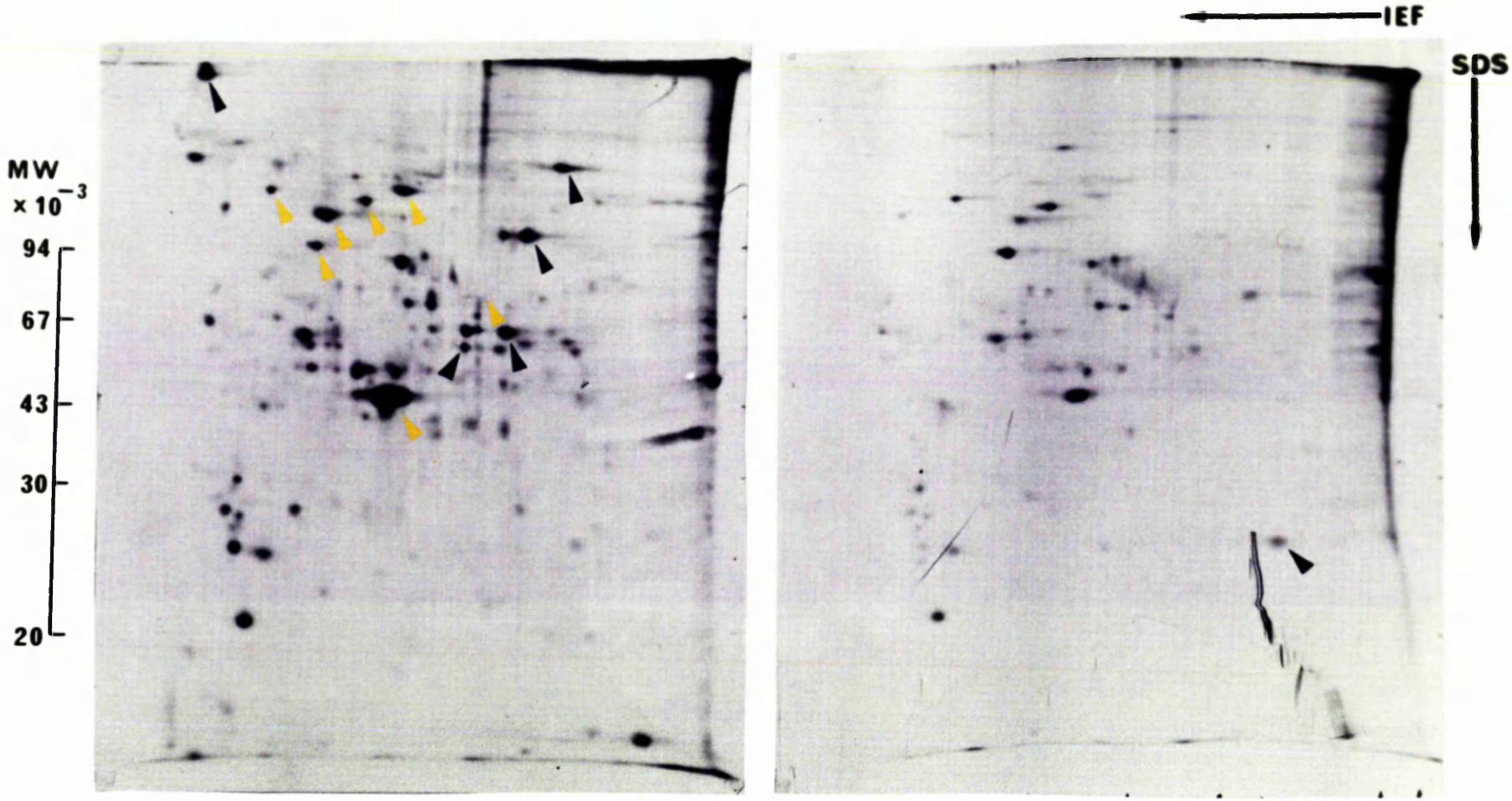


FIGURE 7.

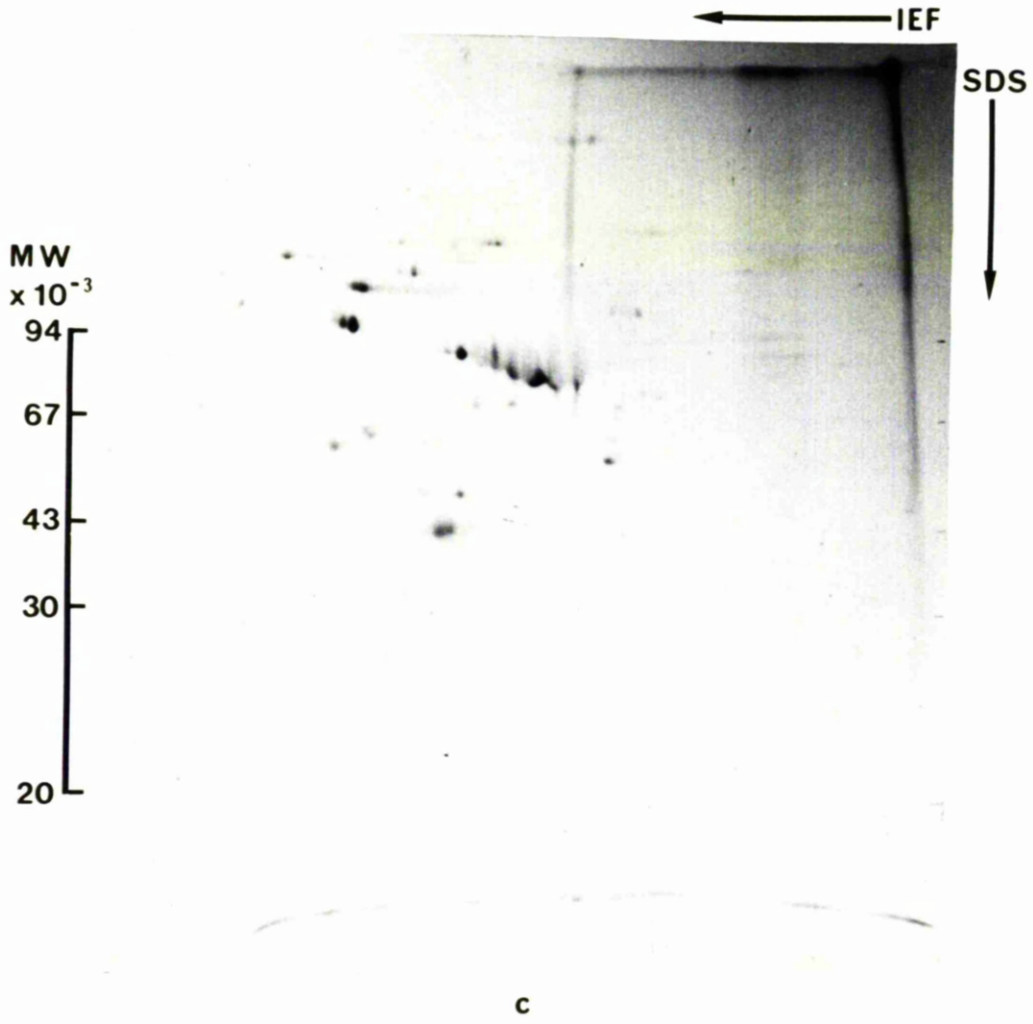


FIGURE 7.

c

they have not run so far, the gels in Fig. 7 may be compared most easily with those in Fig. 3 (following page 69). The putative actin spot, and a distinctive constellation of higher molecular weight proteins in the acidic region are arrowed in yellow in Fig. 7a. All of these are easy to identify in Fig. 3 and in other gels. They can be seen in gels published by Walker & Kaye (1981). Many other spots are less easy to identify particularly without the molecular weight markers which were not used on the early gels.

The combination of the three cell-type spot patterns does not add up to the total spectrum of uterine proteins seen in the earlier gels. A few proteins are exclusive to the epithelial cell types (black arrows), but there are no obvious stromal or myometrial markers. The spot arrowed in Fig. 7b is discussed in the section on Fig. 8. Most of the proteins are common to the three cell types.

Fig. 8 shows the epithelial and stromal cell patterns from uteri responding to 4 hours of oestrogen. A satisfactory gel of myometrial proteins was never obtained. The greatest difference found, was in fact, in the specific activities of the stromal fractions. These were consistently stimulated following 4 hours of oestrogen treatment from about 500 to 2000 cpm/μg protein. The specific activities of epithelium (500 cpm/μg) and myometrium (200 cpm/μg) were unaffected.

There is virtually no change in the proteins labelled in the epithelium after 4 hours of oestrogen treatment, except that three of the proteins exclusive to the epithelium increase in response to the hormone (black arrows, compare Figs. 7a & 8a). When the stromal proteins of untreated (Fig. 7b) and 4 hour oestrogen-treated rat uterus (Fig. 8b) were compared, however, hormone-induced differences

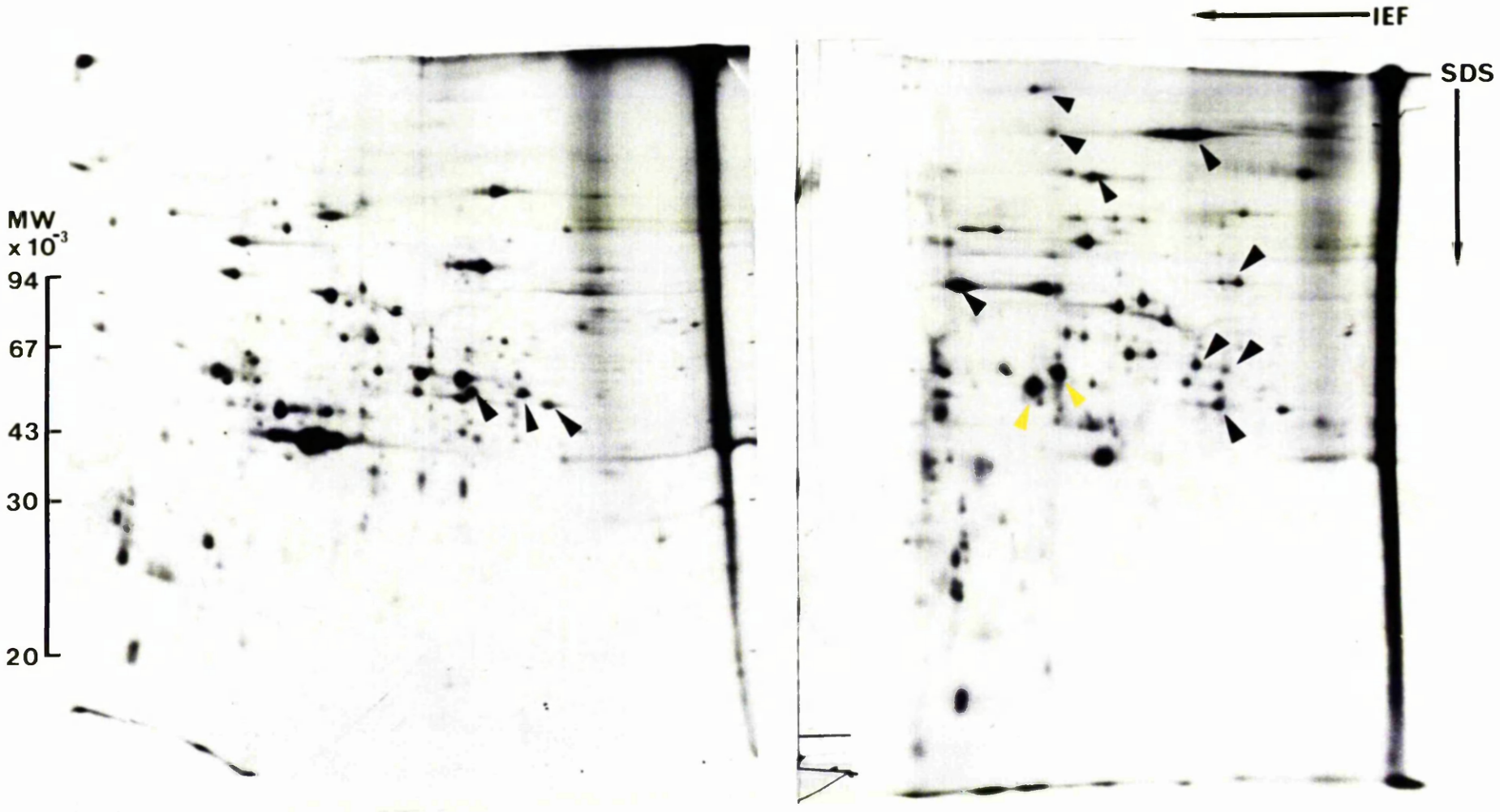
Figure 8.

Electrophoresis of L-[^{35}S]-labelled uterine cell-type proteins from immature rats responding to 4 hours of oestrogen treatment.

24 immature female rats received 1ug oestradiol-17β 4 hours before death. All other experimental details are the same as described in the legend to Figure 7. Films were exposed for 28 days.

a) 4hr oestrogen-treated epithelial cell proteins. Loading: 200μg protein, 1.2×10^5 cpm.

b) 4hr oestrogen-treated stromal cell proteins. Loading: 65μg protein, 1.45×10^5 cpm.



a FIGURE 8. b

were detected. Firstly, as expected from the higher specific activity of the protein from oestrogen-treated animals, most proteins were more heavily labelled. In addition some new spots were detected after hormone treatment and those which proved reproducible are indicated by black arrows in Fig. 8b. Most of these are exclusive to the stroma. The labelling of a single stromal polypeptide decreased after hormone treatment; this is arrowed in Fig. 7b.

Some further differences were detectable between the two cell types, particularly in the labelling of the putative actin spot relative to the other components. In epithelium, this is a major triple spot, with a smaller streak to the acidic side of the gel. In stroma the spot is considerably smaller and may consist of only a single polypeptide. Actin is known to occur in multiple forms which have peptide differences. Garrels and Gibson (1976) have identified three major actins of which two occur in all eukaryotic cells (beta, gamma) and the other (alpha) is exclusive to muscle cells. Although there are clearly three components to the actin spot in fluorograms of protein samples from whole uteri (e.g. Fig. 1, following page 66), it is difficult to resolve the exact number of components in the cell-type protein samples. However, there is clearly some difference between the epithelial and stromal cell types. Another noticeable difference is in the spots in the same positions as those identified as tubulins by Korach et al. (1981) (yellow arrows, Fig. 8b). These are easily recognisable in gels of whole uteri and are heavily labelled in hormonally treated stromal fractions. In contrast, the epithelium has a number of smaller spots which migrate at this position. These are probably different forms of the α and β tubulins which are known to exhibit microheterogeneities between different tissues, and during development, in many species (Raff et al., 1982). It is odd that, in

these stromal preparations the synthesis of this putative tubulin was strongly stimulated (compare Figs. 7b & 8b), while the investigations of isolated nuclei showed what appeared to be the same protein synthesised in smaller quantities after hormone treatment (Fig. 5, white arrow, following page 70). Korach et al. (1981) have described hormone-induced movement of specific proteins into different cell compartments and this may explain the anomaly.

The efficient labelling of the separated cell types was only possible after they had been allowed to recover from the trauma of isolation, by resting in a complete, aerated medium for 4 hours. Thus it is possible that the differences between the labelled protein patterns of cell types and whole uteri, stemmed from the labelling technique, rather than the source of material. To test this possibility, cell types were prepared from uteri which had been labelled in vitro as in the earlier experiments. To avoid any artifacts due to storage, cell protein samples were always prepared on the same day as the uteri were isolated, immediately following the labelling incubation. The specific activities of cell types were higher using this method; about 2500 cpm/μg for epithelium and stroma, and 1500 cpm/μg for myometrium. Fig. 9 shows the pattern of ^{35}S-methionine labelled proteins derived in this way from the uteri of immature rats responding to 4 hours of oestrogen treatment. The films, although over-exposed, show almost all of the components seen in the comparable experiment in which the cells were labelled after fractionation (Fig. 8). The spot patterns of the epithelial and stromal preparations (Figs. 9a & 9b) are very complex, but there are still relatively few differences between the two fluorograms. However, these preparations are enriched in low molecular weight and basic proteins relative to those of Fig. 8. Most of the exclusive and hormone-stimulated stromal proteins seen in Fig. 8b can

Figure 9.

Electrophoresis of L-[^{35}S]-labelled uterine cell-type proteins, fractionated from pre-labelled uteri.

24 immature female rats received oestradiol-17β , 4 hours before death. The uteri were incubated in 1ml Eagles medium lacking methionine, but containing 150μCi L-[^{35}S]-methionine. The labelling incubation and preparation of fractionated cell-type protein samples was conducted as described in the Methods section. Fluorograms of the two-dimensional separations of the 3 cell types are shown. A separation of total, L-[^{35}S]-labelled uterine proteins is included for comparative purposes. This sample was prepared from the uteri of 6 rats responding to 4 hours of oestrogen-treatment. These were labelled in 1ml Eagles medium lacking methionine but containing 50μCi L-[^{35}S]-methionine. Protein samples were prepared as described in the Methods section.

a) 4 hour epithelial cell-type proteins. Loading:80μg protein, 1.9×10^5 cpm.

b) 4 hour stromal cell-type proteins. Loading: 75μg protein, 2.25×10^5 cpm.

c) 4 hour myometrial cell-type proteins. Loading: 105μg protein, 1.54×10^5 cpm.

d) 4 hour oestrogen treated total uterine proteins. Loading: 120μg, 2×10^5 cpm.

Films a,b & c were exposed for 40 days and film d for 27 days.

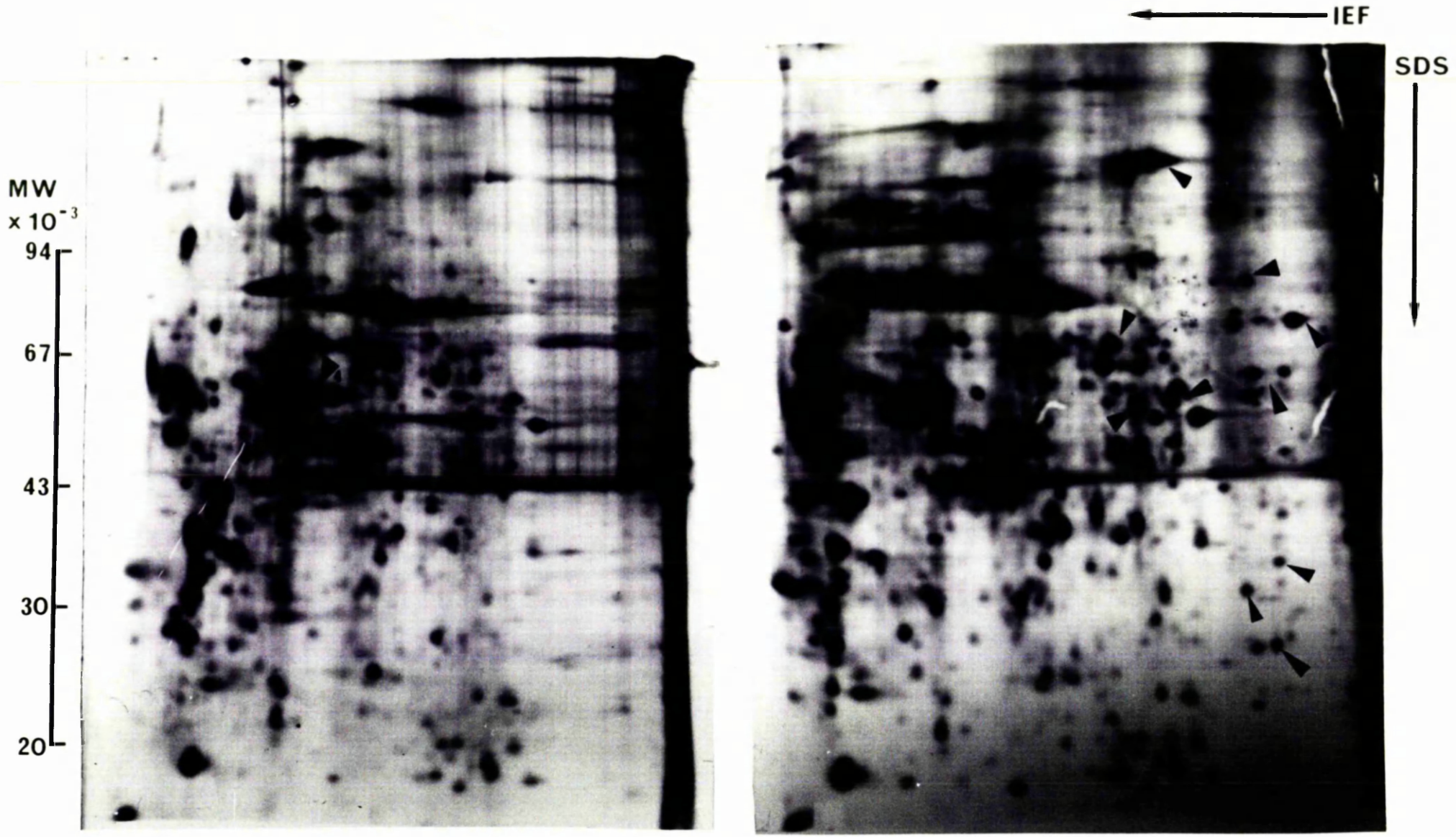
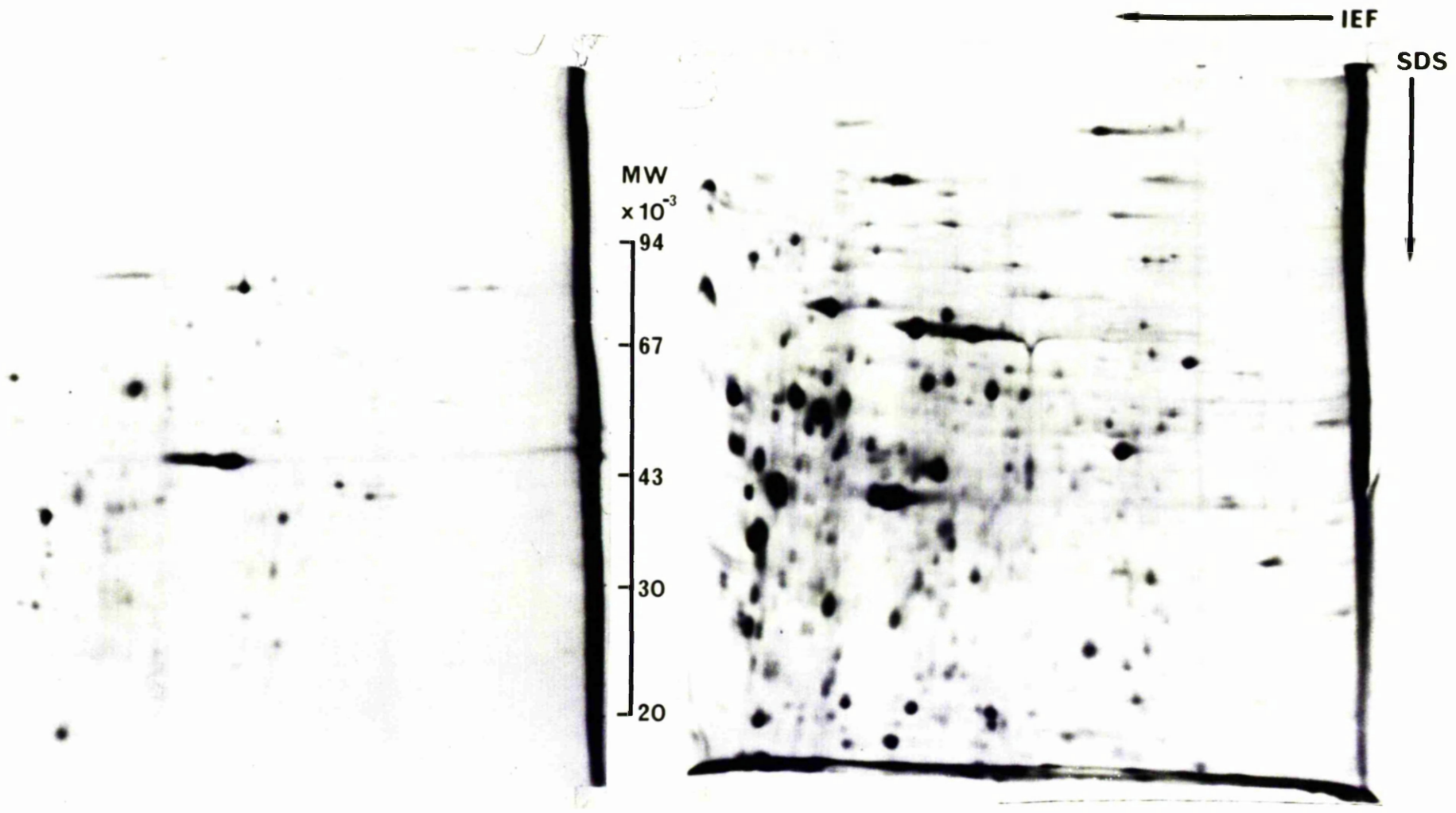


FIGURE 9.



c

FIGURE 9.

d

be picked out in Fig. 9b (black arrows) and some additional differences between the epithelium and stroma are also arrowed. The differences between the epithelial and stromal 'actin' spots in Figs. 7 & 8 are not detectable in the samples shown in Fig. 9. The spot labelled '4' in Fig. 9a is for reference in discussing Fig. 12, and Fig. 9d is a total uterine protein population included for comparative purposes.

The myometrial sample (Fig. 9c) shows few spots, and none are exclusive to the myometrial fraction. This is in agreement with all the gel separations performed with material from this fraction. The spots in this prelabelled 4 hour treated myometrium are, however, difficult to identify with those of control myometrium labelled after separation from whole uteri (Fig. 7c, following page 72). As previously described, attempts to label isolated myometrium from hormone-treated uteri were unsuccessful and the control preparation of Fig. 7c was labelled very inefficiently and therefore may not represent proteins made in vivo. The higher specific activity of the prelabelled myometrial sample enabled protein overloading to be avoided for this gel and the distortion in Fig. 7c was thus abolished. The prelabelled sample shows the 'actin' spot to be a major component whereas it is very faint in Fig. 7c.

Protein samples from whole uteri (Figs. 1-3 & 5) were labelled with a mixture of high specific activity ^{14}C amino acids, whilst the protein preparations from isolated cell types shown in Figs. 7-9 were from ^{35}S-methionine labelled tissue, or cells. To ensure that any differences observed were not due to the use of different radioactive precursor, one of the prelabelled cell-type samples was prepared again, using ^{14}C amino acid mixture in the labelling incubation. The

sample prepared was from unstimulated rat uteri and thus could be compared with ^{35}S-labelled control cell samples labelled post-fractionation (Fig. 7); and with the ^{14}C-labelled total uterine protein population from control rats (Figs. 1b & 13d, following pages 66 & 68).

Fluorograms of the ^{14}C prelabelled cell-type samples are shown in Fig. 10. This figure shows a much greater difference between one cell type and another than was detected after ^{35}S-methionine labelling. Spots exclusive to, or particularly heavily labelled in, each cell type are arrowed. Most of these are seen in the epithelial fraction (Fig. 10a) but there are four proteins which are very heavily labelled in the stroma only (Fig. 10b). The myometrium (Fig. 10c) shows no exclusive components. Some of the arrowed proteins in Fig. 10a correspond to exclusively epithelial proteins in Fig. 7a. Most of the labelled species correspond to stainable proteins (Fig. 11) although not all stainable proteins are labelled (see arrows, Fig. 11a).

The 'actin' spot differs between the cell types. The epithelium shows only two components whereas there are clearly three components in the stroma and myometrium. From Garrels & Gibson's observations (1976) one would expect the most acidic spot, alpha actin, to be absent from epithelial and probably stromal fractions since they have shown it to be exclusive to muscle cells. It is clearly absent from the epithelial fraction but is present in the stroma. This is in contrast to Figs. 7 & 8 which show the stroma to have less ^{35}S-labelled actin-like components than epithelium. As in the other cell protein samples the preparations of Fig. 10 are enriched in basic species.

When comparing Figs. 9 & 10, it is seen that most proteins labelled

Figure 10.

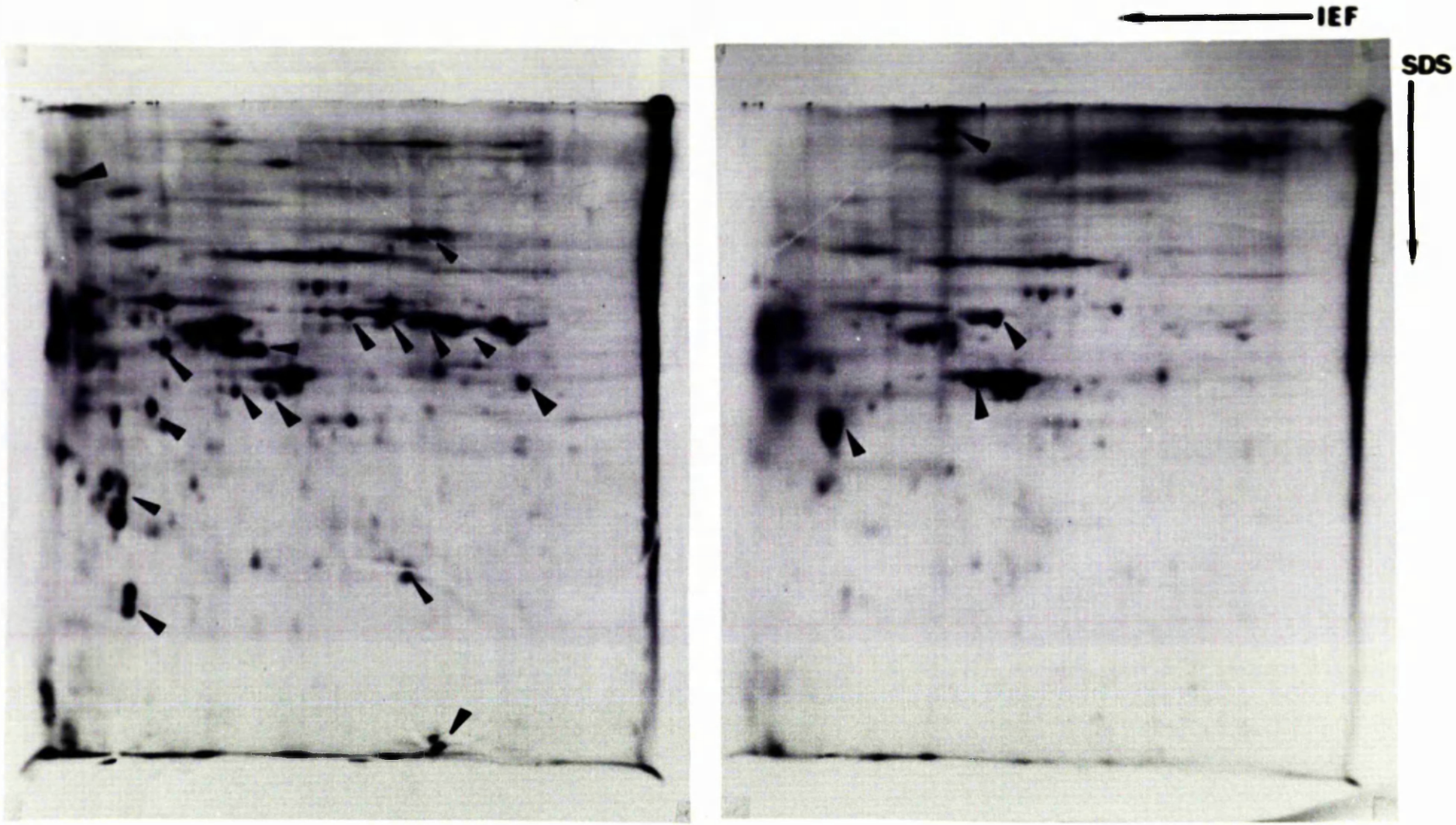
Electrophoresis of L-[U-^{14}C]-labelled uterine cell-type proteins, fractionated from pre-labelled uteri.

24 immature rat uteri from control animals were labelled as described in the legend to Figure 1. All other experimental details are the same as those for Figure 9. Fluorograms of the two-dimensional separations of the three cell types are shown. Films were exposed for 30 days.

a) Control epithelial proteins. Loading: 130µg protein, 9.1×10^4 cpm.

b) Control stromal proteins. Loading: 200µg protein, 9.9×10^4 cpm.

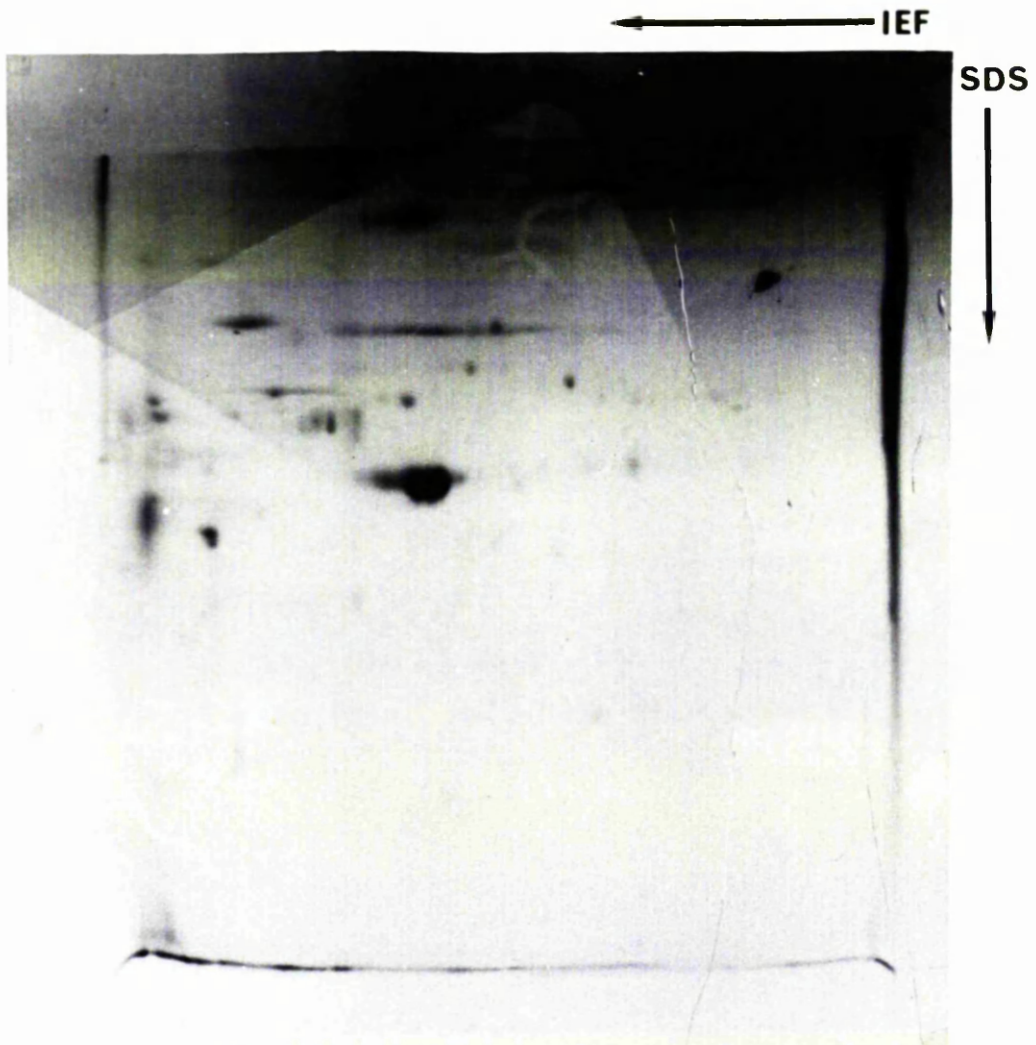
c) Control myometrial proteins. Loading: 130µg protein, 6×10^4 cpm.



a

FIGURE 10.

b



c

FIGURE 10.

Figure 11.

Staining patterns of control cell-type proteins.

These photographs show the Coomassie Brilliant Blue staining patterns of the three gels whose fluorograms appear in Figure 10 a-c.

a) Control epithelial proteins. Loading: 130µg protein.

b) Control stromal proteins. Loading: 200µg protein.

c) Control myometrial proteins. Loading: 130µg protein.

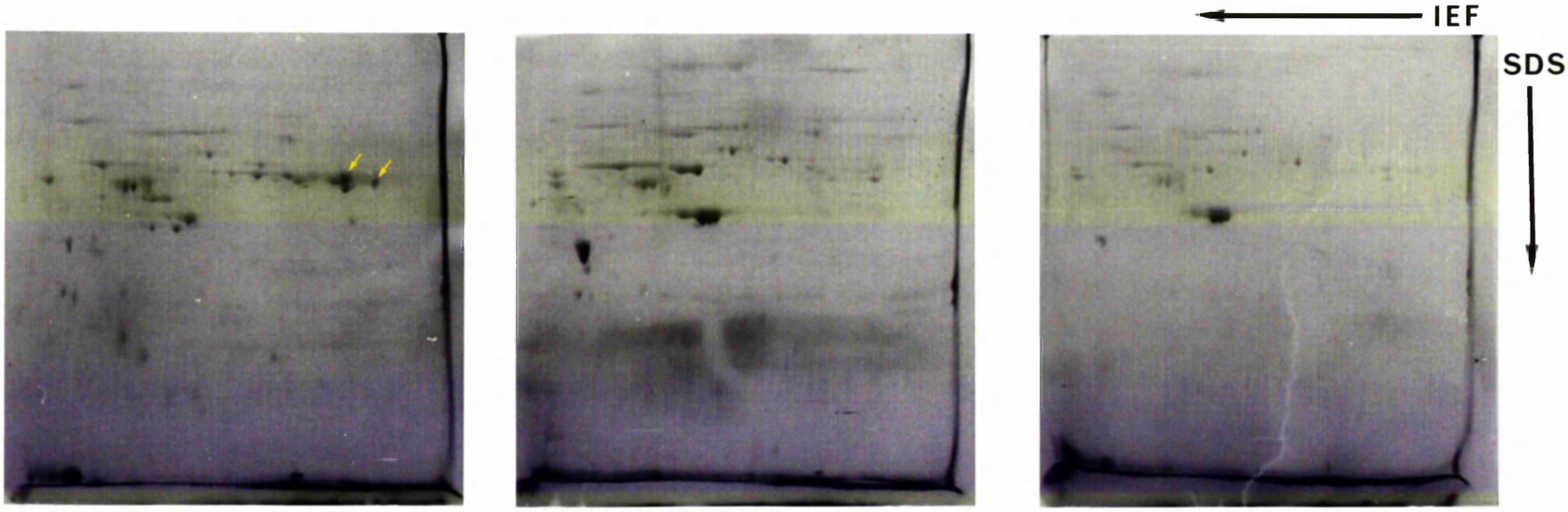


FIGURE 11.

with ^{14}C-amino acids are also labelled with ^{35}S-methionine. The multiple spots in the region of the polypeptides identified by others as tubulins, are not seen in the ^{35}S-labelled samples. This may be either a difference in the labelling of the proteins, or an artifact due to sample preparation of the ^{14}C-labelled fractions. This area of the gel is also difficult to compare with that in Fig. 7 (following page 72), which comprises protein species labelled in isolated cell types labelled after fractionation from control uteri. The constellation of spots arrowed for reference (black) in Fig. 7a is not obvious in Fig. 10a and may either be absent or have been disguised by streaking. Most of the major spots, however, are consistently reproduced.

The comparison between ^{14}C-labelled samples from total uterus (Fig. 3a) and cell types (Fig. 10) shows little similarity. The gel of total protein is better resolved and has run further. If the 'actin' spot is used as a reference point, some of the other spot groupings can be recognised. However, many of the minor components are not present in the cell fractions.

3.4 The investigation of uterine proteins synthesised in vitro by cell-free translation systems.

3.4.1 The choice of translation system.

The third method of examining any possible changes in the uterine protein population in response to oestrogen, was to use cell-free protein synthesising systems to label samples.

Initially, several approaches were used. Firstly, uterine polysomal messenger RNA was prepared, and polyadenylated messengers were

purified by oligo-dT cellulose column chromatography. These samples were translated in wheat germ extracts prepared by the method of Roberts and Paterson (1973). The results from this system were not always consistent and the method required that several additions be made to the translation cocktail immediately prior to the incubation. This, and the fact that wheat germ extracts varied considerably with source, as to endogenous ribonuclease activities, probably accounted for the unreliable results. A messenger-dependent translation system (MDL) was also prepared, from rabbit reticulocyte lysates, according to the method of Pelham & Jackson (1976). This proved to give better results and was easier to use. Another approach was to use polysomes rather than purified mRNA and this was tried in both wheat germ and MDL assays. Preparation of polysomes was faster and gave a better yield for the number of animals used. The use of polysomes also avoided the loss from the sample of any non-polyadenylated messenger species being translated in vivo at the time of death. Furthermore, it has the advantage that nascent polypeptides being translated at the time of death potentially from hormone-induced mRNA, are completed and made radioactive. The use of polysomes in MDL assays became the system of choice.

For a period of 18 months I used MDL prepared by myself and optimised for salt concentration, pH, and messenger levels, but this was subsequently dropped in favour of a commercially produced lysate, for a number of reasons. The preparation of the lysate was laborious and involved trauma to the rabbits which sometimes died before their treatment was complete. The waste of animals and effort was not justified for the small-scale usage of the lysate. In addition the results from the home-made lysate were not as good as those produced by a commercial lysate because the samples often caused a 'fogging' of

the fluorograms which obscured the labelled spot patterns. A number of methods were tried to alleviate this, but all failed. The best results were obtained when Amersham MDL was used and this was adopted for all subsequent experiments.

3.4.2. Cell-free translation directed by total uterine polysomes.

Incorporation of label into acid-precipitable material varied with the preparation of polysomes used, and with the number of hours of oestrogen treatment given to the rats in vivo. Levels of incorporation varied from 2 x background to 15 x background incorporation, but an average was 6 x background.

Fig. 12 shows the protein translation products using the uterine polysomes from rats in various hormonal states. Fig. 12a shows the proteins translated by the polysomes of unstimulated rat uteri. The pattern is somewhat different to the proteins visualised by labelling the whole organ in vitro (for example, see Fig. 9d, following page 75) but a number of common, major proteins are seen. The putative actin spot is obvious (see Fig. 12a, yellow arrow, 1) as are the spots identified as tubulins by Korach (2,3). However, some other protein spots do not appear in the patterns of total protein obtained from labelling the whole organ (eg. Fig. 9d). An example is the spot 4. Since this appears consistently in fluorograms of cell free translation products and of heavily loaded gels of isolated cell proteins (e.g. Fig. 9a, following page 75) it may represent a relatively minor protein with a high rate of turnover, thus it is enriched in the sample obtained by cell-free translation.

Translation products of polysomes from uteri responding to 2, 4 and 12 hours of oestrogen treatment are shown in Fig. 12b,c & d. The most

Figure 12.

Electrophoresis of MDL translation assays directed by total uterine polysomes from rats in various hormonal states.

A translation cocktail containing MDL (Amersham) and 1mCi/ml L-[^{35}S]-methionine was mixed freshly before each experiment. Polysomes in distilled water were added in a volume not exceeding 10-15% of the final volume, and an equal volume of distilled water was added to the control assay. The incubation was performed in a sealed 1.5ml Eppendorf tube for 90 minutes in a gently shaking water bath at 30^{o}C, and the assay was stopped by rapid cooling to 0^{o}C. 1µl duplicates from the 10µl or 25µl incubation mix were assayed for counts incorporated into acid precipitable material, as described in the Methods section (2.5.9).

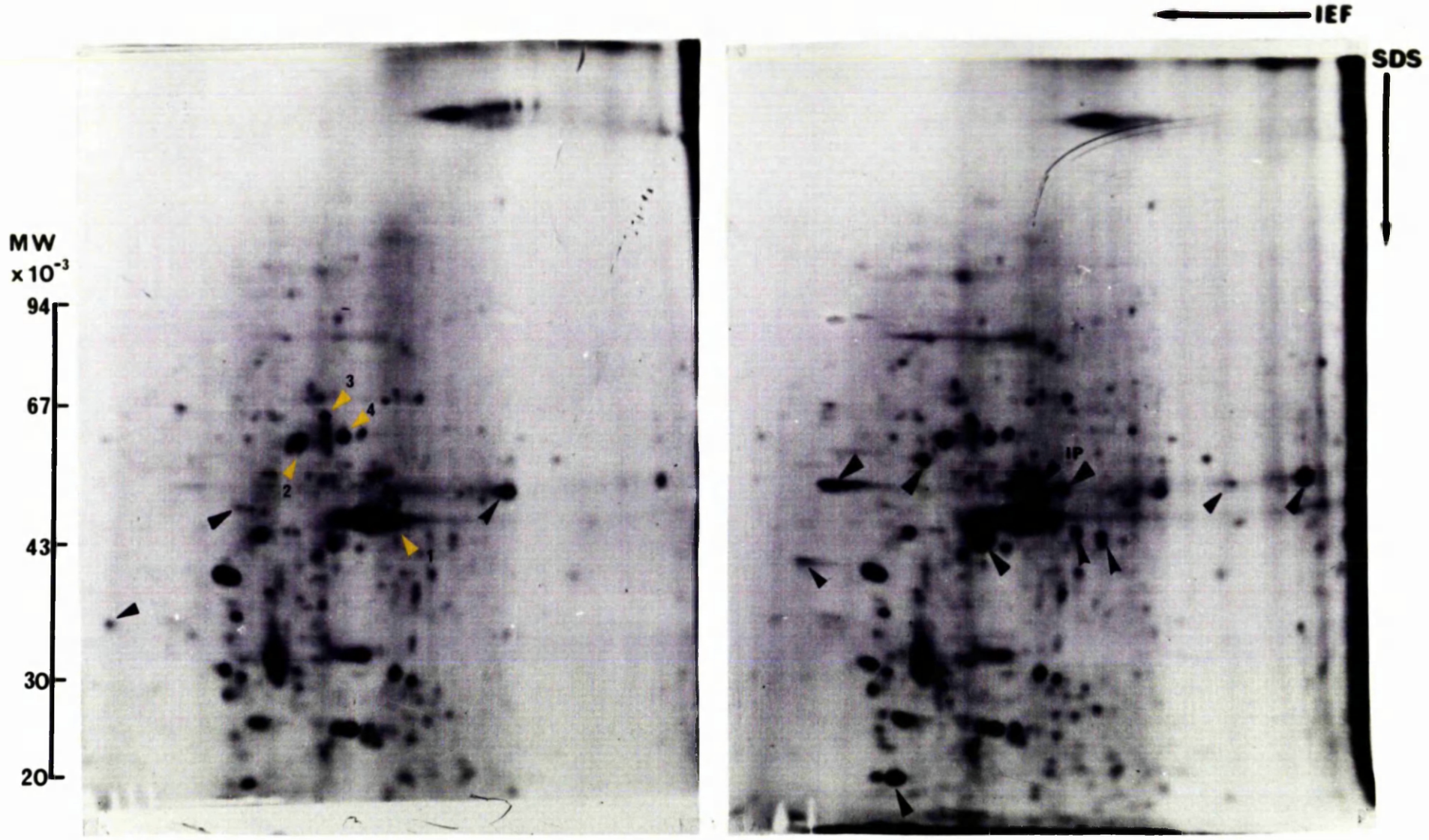
For electrophoresis, equal volumes of lysis buffer were added to the MDL samples and gels were run in the usual way. 10% SDS slab gels were used for all experiments. Fluorography was performed on the slabs and films shown were exposed for 42 days. Sample loadings given below have been adjusted for control assay incorporation. Fluorograms of MDL translation products of polysomes from uteri responding to the following periods of oestradiol treatment are shown:

a) 0 hrs, 1.47×10^5 cpm.

b) 2 hrs, 2.05×10^5 cpm.

c) 4 hrs, 2.5×10^5 cpm.

d) 12 hrs, 1.71×10^5 cpm.



a

FIGURE 12.

b

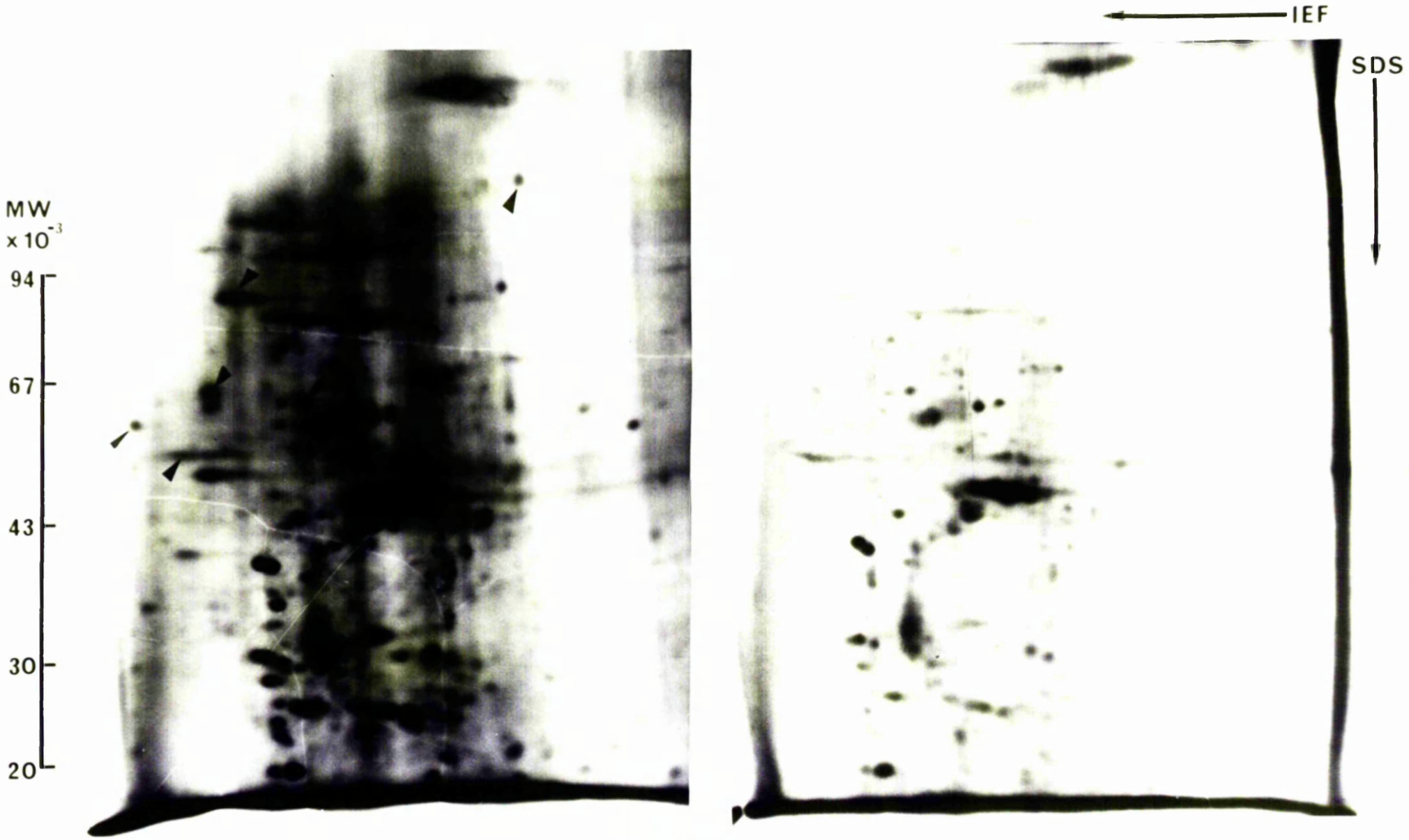


FIGURE 12.

striking observation is the exceptionally high degree of correspondence between the fluorograms, even in the most minor spots. Some of the most marked differences are arrowed in black. Spots which are new or strongly stimulated in the 2 hour sample are arrowed in Fig. 12b whereas those decreasing after oestrogen treatment are arrowed in the control sample (Fig. 12a). These changes are maintained in the 4 hour sample (Fig. 12c), except that the two spots of molecular weight about 49,000 which have increased at two hours are no longer stimulated. These are marked IP on Figs. 12b & 13b and correspond to the expected position of the induced protein creatine kinase. Some further increases and new spots are arrowed in black. The two 'tubulin' spots also decrease in intensity at four hours (yellow arrows) which was also observed in the nuclear fractions from whole labelled uteri (Fig. 5, following page 70). The labelled translation products of 12 hour polysomes (Fig. 12d) show virtually no difference to those of the 4 hour pattern. An exception is the tubulin spot marked 3 in Fig. 12a which has decreased markedly (see also Fig. 5d). The decreases noted between 2 and 4 hours of oestrogen treatment are maintained, as are some of the increases.

A large number of comparisons of the cell-free translation products of polysomes from rats in various hormonal states have been performed. The patterns obtained show a high degree of correspondence of most of the spots when examined closely. However, many of the apparent hormone-induced differences observed are not conserved between different preparations of polysomes. Fig. 13 illustrates this point. The figure shows the protein population produced from control (Fig. 13a) and 2-hour (Fig. 13b) polysome-directed translation. Spots decreasing after oestrogen treatment are arrowed in (a) and those increasing are arrowed in (b). Only those increases arrowed in yellow

Figure 13.

Comparison of MDL translation products of polysomes from uteri responding to short periods of oestrogen treatment with total L-[U-^{14}C]-labelled uterine protein.

Details of experimental procedure are given in the legend to Figure 12. The L-[U-^{14}C]-labelled total uterine protein sample was prepared as described in the legend to Figure 1 and a gel (d) is included for comparative purposes. Fluorograms shown are of MDL translation assays, directed by polysomes from uteri responding to the following periods of oestrogen treatment:

a) 0 hrs, 1.39×10^5 cpm.

b) 2 hrs, 1.61×10^5 cpm.

c) 4 hrs, 2.3×10^5 cpm.

d) Total L-[U-^{14}C]-labelled uterine protein from immature rats responding to 4 hours oestrogen stimulation. Loading: 50µg protein, 10^5 cpm.

Films a-c were exposed for 36 days; Film d for 27 days.

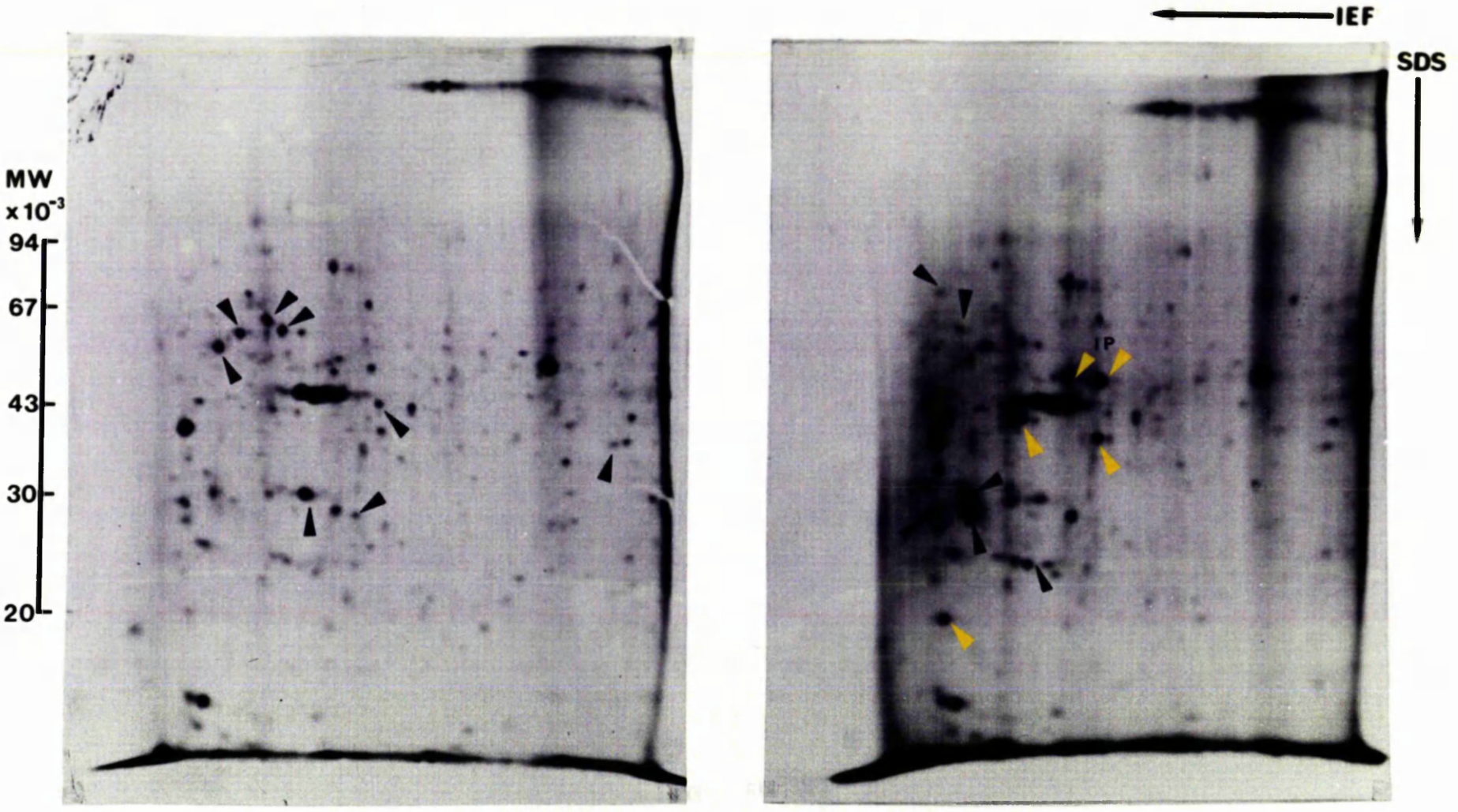
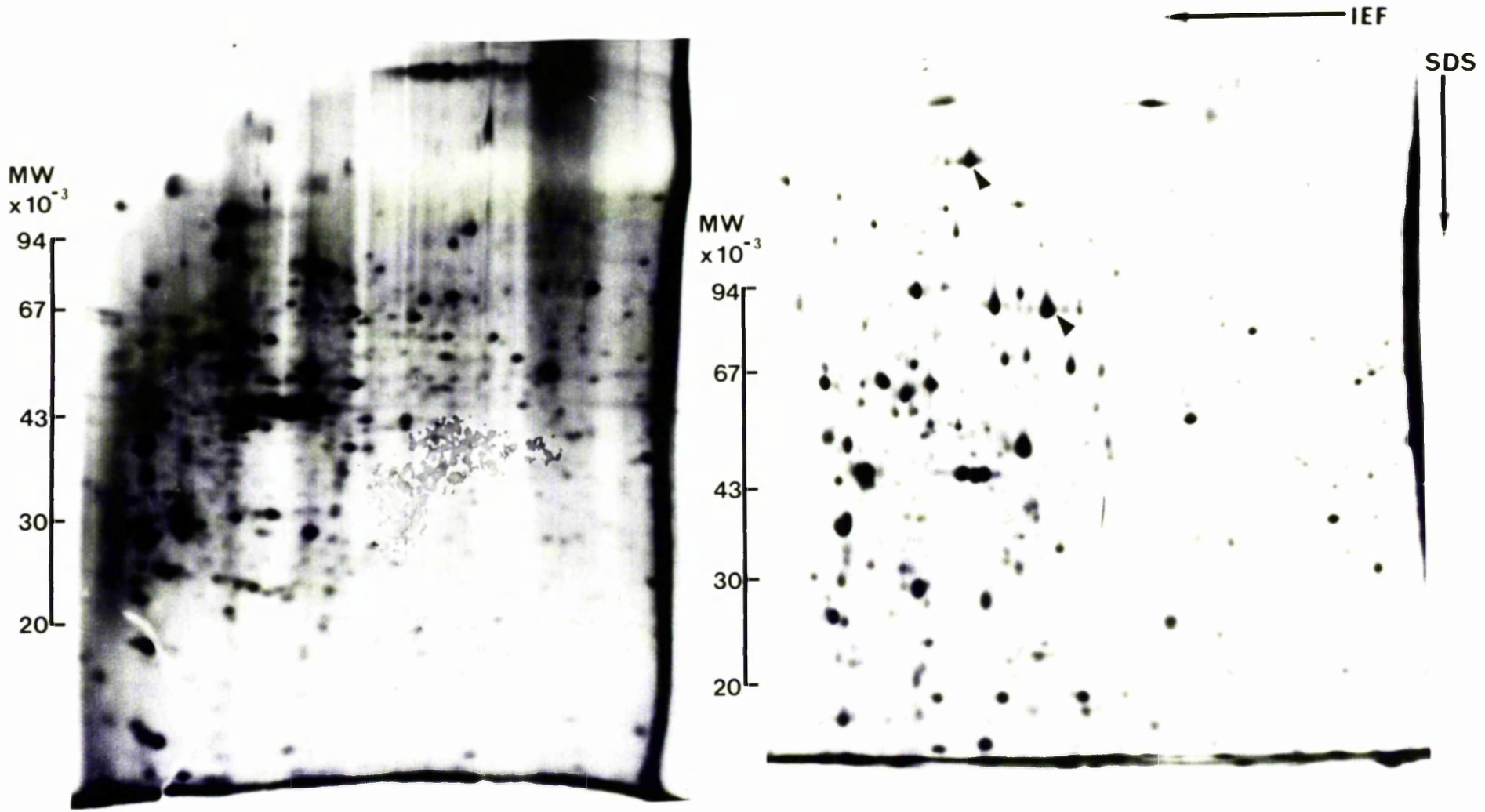


FIGURE 13.



c FIGURE 13. d

correspond to those seen in Fig. 12b. These increases are consistently observed.

The 4 hour sample is shown in Fig. 13c. The first dimension gel was loaded with 100,000 cpm more than the control sample, and strictly, therefore, cannot be compared to the control or 2 hour samples. However, it illustrates the large number of components that can be resolved using this method of labelling uterine proteins. The species present in this separation may be compared to a typical gel of *in vitro* ^{14}C-labelled total uterine protein from 4 hour oestrogen-treated rats (Fig. 13d). Although the gel has run further, most of the major spots can be identified; the polysome directed translation products are enriched in high molecular weight basic proteins and minor species. Some major spots are missing however, such as the more basic of the two spots of molecular weight 87,000 and the 150,000 molecular weight protein. These are arrowed in Fig. 13d. There are also differences in the 'actin' spots which appear to have four components in Fig. 13c, but only three in Fig. 13d.

3.4.3. Cell-free translation directed by polysomes isolated from fractionated cell types.

This was the last method used to detect proteins synthesised early in the oestrogenic response of immature rat uterus. The experiment was very difficult in that the time required to prepare the isolated cells and their polysomes was 4 to 6 hours, which made the mRNA and the protein synthesising enzymes very vulnerable to ribonuclease and proteolytic digestion. These problems were made even worse by the time involved in the excision of the uteri from the large numbers of animals used.

Two experiments of this type were performed and polysomes were prepared from the uterine epithelial cells of 12 hour oestrogen-treated rats and from the epithelial, stromal and myometrial cells of 4 hour stimulated rats. Although sufficient polysomes were recovered for the cell-free translations presented in Figs. 14 & 15, the yields were very low and did not justify further experimentation. The polysome yields from 132 rats treated with oestradiol for 4 hours were: 3.99, 2.73 and 0.39 Optical Density Units (260nm) from the epithelial, stromal and myometrial fractions respectively. Far too many rats would have been required for the preparation of polysomes from the fractionated cells of unstimulated rats.

The cell-free translation assays incorporated about 5 x background counts into trichloroacetic acid-precipitable material using epithelial polysomes from 12 hour treated uteri, 2 x background for epithelial polysomes from 4 hour treated uteri, and 3.5 x background for stromal polysomes. Myometrial polysomes only incorporated 1.3 x background counts. These activities were considerably less than those achieved using polysomes from whole uteri (average 6 x background) but gave sufficient counts to attempt two-dimensional gels. Fluorograms of these are shown in Figs. 14 & 15. There were insufficient myometrial polysomes at too low a concentration to repeat the translation assays and the fluorogram was too faint to reproduce. However, the very few spots detected, were also present in the epithelial and stromal patterns, which are shown in Fig. 14. These epithelial and stromal translation products resemble each other closely. Furthermore, all of the spots can be identified with total polysome translation products from 4 hour stimulated uteri (Fig. 12c, 13c, following pages 80 & 81). It must be stressed that these gels, although duplicated, emanate from

Figure 14.

Fluorograms of MDL translation products of polysomes isolated from fractionated uterine cell types.

132 rats received 1μg oestradiol-17β 4 hours before death. The uteri were fractionated into epithelial, stromal and myometrial cell types, each of which was used to prepare polysomes. Full details of these techniques are given in the Methods section (2.5.8). MDL translation assays were conducted as described in the legend to Figure 12, and the samples subjected to two-dimensional gel electrophoresis, and the gels to fluorography. Films were exposed for 46 days. Loadings have been adjusted for control assay incorporation.

a) Translation products of 4 hr oestrogen-treated epithelial cell polysomes. Loading: 1.44×10^5 cpm.

b) Translation products of 4 hr oestrogen-treated stromal cell polysomes. Loading: 2.4×10^5 cpm.

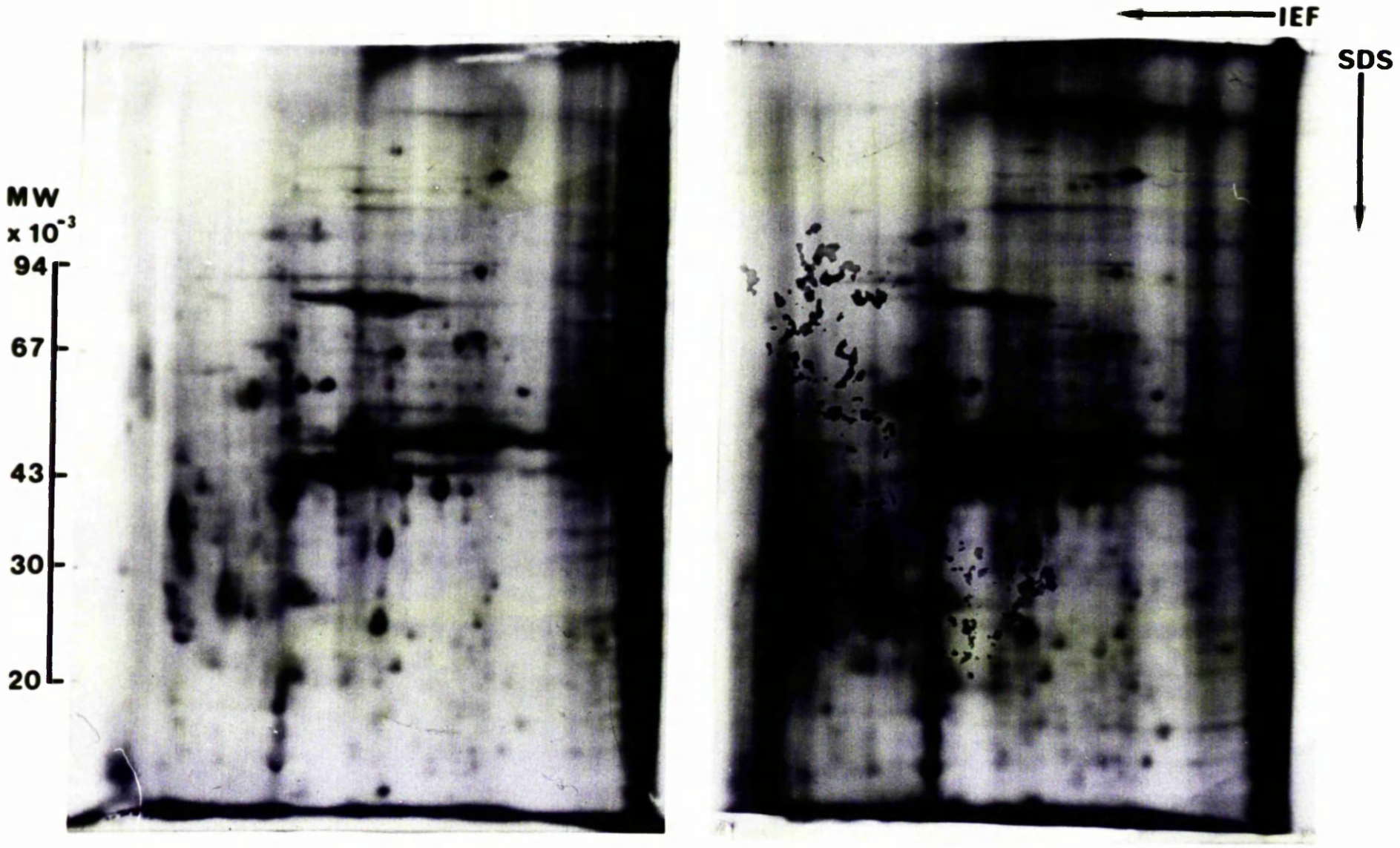


FIGURE 14.

Figure 15.

Translation products of epithelial cell-type polysomes from uteri responding to 12 hours oestrogen treatment.

44 immature female rats received 1μg oestradiol-17β 12 hours before death. The uteri were used to prepare epithelial cells and from these a polysome fraction was isolated. Translation assays and analysis of the products was performed as described in the legend to Figure 12. The film was exposed for 30 days. The loading, adjusted for control assay incorporation, was 3×10^5 cpm.

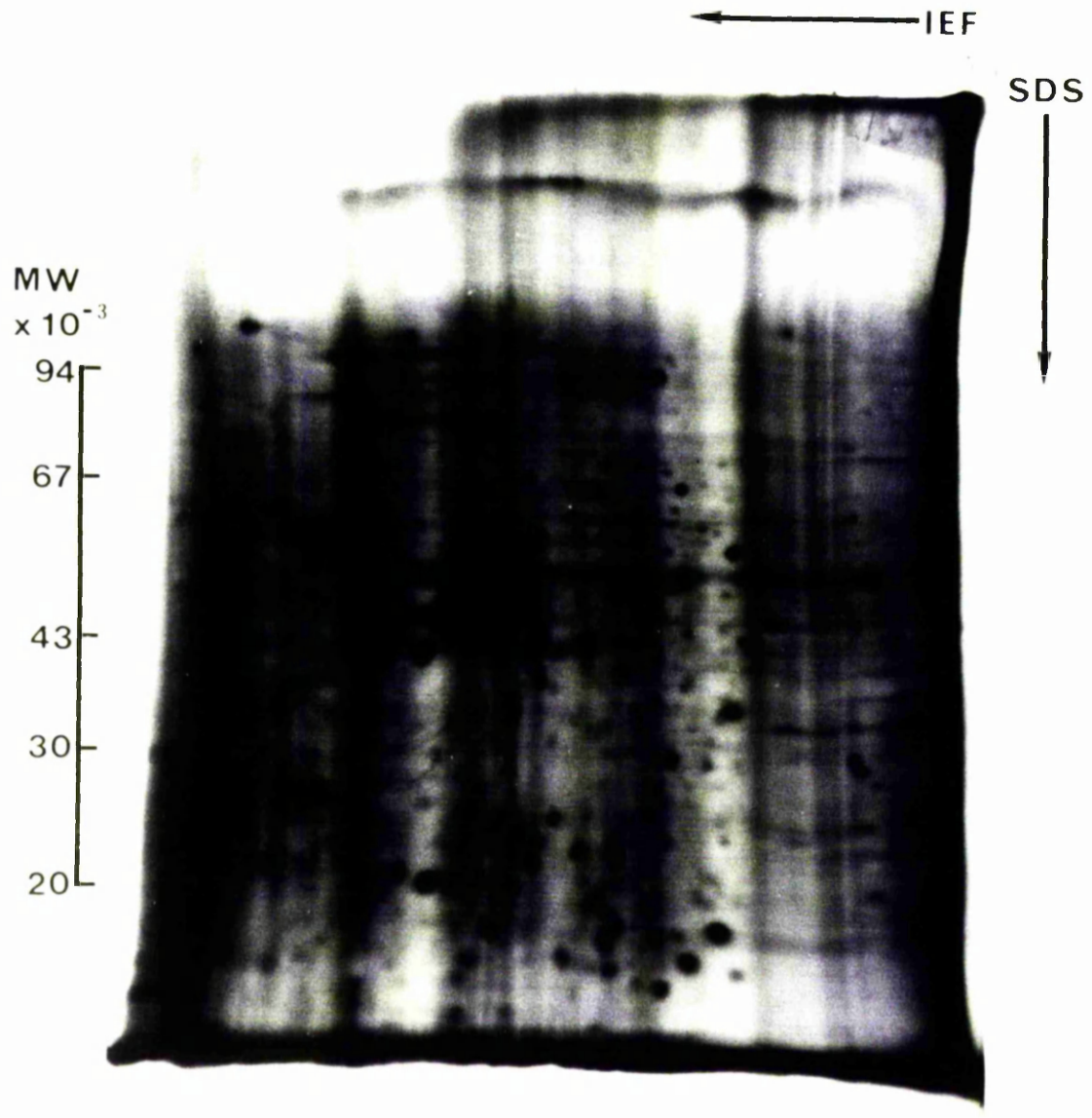


FIGURE 15.

single preparations and are therefore only preliminary results.

Fig. 15 illustrates the proteins labelled in cell-free translation assays directed by epithelial polysomes isolated from the uteri of rats receiving oestrogen 12 hours before death. Again, a large number of spots are resolved in a highly reproducible manner. Considerable differences can be seen between the translation products of these 12 hour epithelial cell polysomes and those of the 4 hour epithelial preparation, but thesedata werederived from a single preparation of polysomes and, as discussed with respect to Figs. 12 & 13, different preparations appear to give slightly different results. All that can be said with confidence is that many of the polypeptides detected in Figs. 14 & 15 are also seen in Figs. 12 & 13 and in other figures, but that the significance of the differences between the different preparations remains uncertain.

3.5 Cofractionation of known proteins with labelled uterine samples.

3.5.1 The contribution of the cofractionation technique to the identification of uterine proteins.

In order to identify some of the proteins synthesised in the immature rat uterus a number of approaches are possible. Firstly it is possible to raise antibodies to purified proteins and use them to identify proteins labelled *in vitro* in the uterus or in cell-free translation assays. Secondly, uteri labelled *in vitro* could be used to prepare specific proteins and see if oestrogen treatment stimulates isotope incorporation into these fractions. Examples of proteins which could be used are ribosomal proteins, hnRNP proteins, mRNP proteins, and the nucleolar proteins. However, a number of technical problems with either method have made them difficult or impractical to develop in the time available.

A third approach is to cofractionate isolated proteins with radioactive uterine protein samples. Comigration of a protein with another of known identity can help to confirm their possible unity, and may also be useful as a negative assay, although it is not a sufficient test to identify a protein unambiguously. This last method was however, the only one feasible in the time available.

3.5.2 Cofractionation of uterine protein samples with actin.

Because the most prominent spot in labelled uterine protein gels ran at a position expected for actin on two-dimensional gels, and its multiple components resembled published spot shapes of the protein (Garrels and Gibson, 1976; Korach et al., 1981) this was the first purified protein to be analysed. Actin was obtained from two sources. Firstly, it was extracted from a rabbit skeletal muscle acetone powder kindly donated by Dr. J. Dow of this Department. Secondly, a sample of pure rat leg muscle actin was obtained from Dr. J. Elce of the Department of Biochemistry, Queen's University, Kingston, Canada.

Lyophilised actin was dissolved in lysis buffer and a sample was subjected to two-dimensional gel electrophoresis. The gel was stained and compared with the staining patterns of actin coelectrophoresed with labelled uterine samples. Fig. 16a shows the position of a sample of rabbit muscle f-actin on the two-dimensional gels used. Fig. 16b shows the staining pattern obtained when the rabbit protein was cofractionated with ^{14}C-labelled total uterine protein, and Fig. 16c shows the fluorogram of this gel. The centre of the major triple spot in the fluorogram is displaced by the unlabelled rabbit muscle actin, the position of which is arrowed.

Figure 16.

Cofractionation of rabbit muscle actin with L-[U-^{14}C]-labelled uterine proteins on two dimensional gels.

Actin was extracted from rabbit skeletal muscle acetone powder as described in the Methods section (2.5.10,a). This was subjected to two-dimensional gel electrophoresis alone, and mixed with total L-[U-^{14}C]-labelled uterine protein from 4 hour oestrogen-treated rats. Slab gels were stained and photographed. Those with labelled samples were then fluorographed. The film was exposed for 20 days.

a) 20µg unlabelled rabbit muscle f-actin, stained with Coomassie Brilliant Blue.

b) Coomassie Brilliant Blue staining pattern of 20µg unlabelled f-actin coelectrophoresed with L-[U-^{14}C]-labelled uterine protein from oestrogen-treated rats. The uterine sample contained 110µg protein and 10^5 cpm.

c) Fluorogram of b). The arrow marks the position of the stained actin spot.

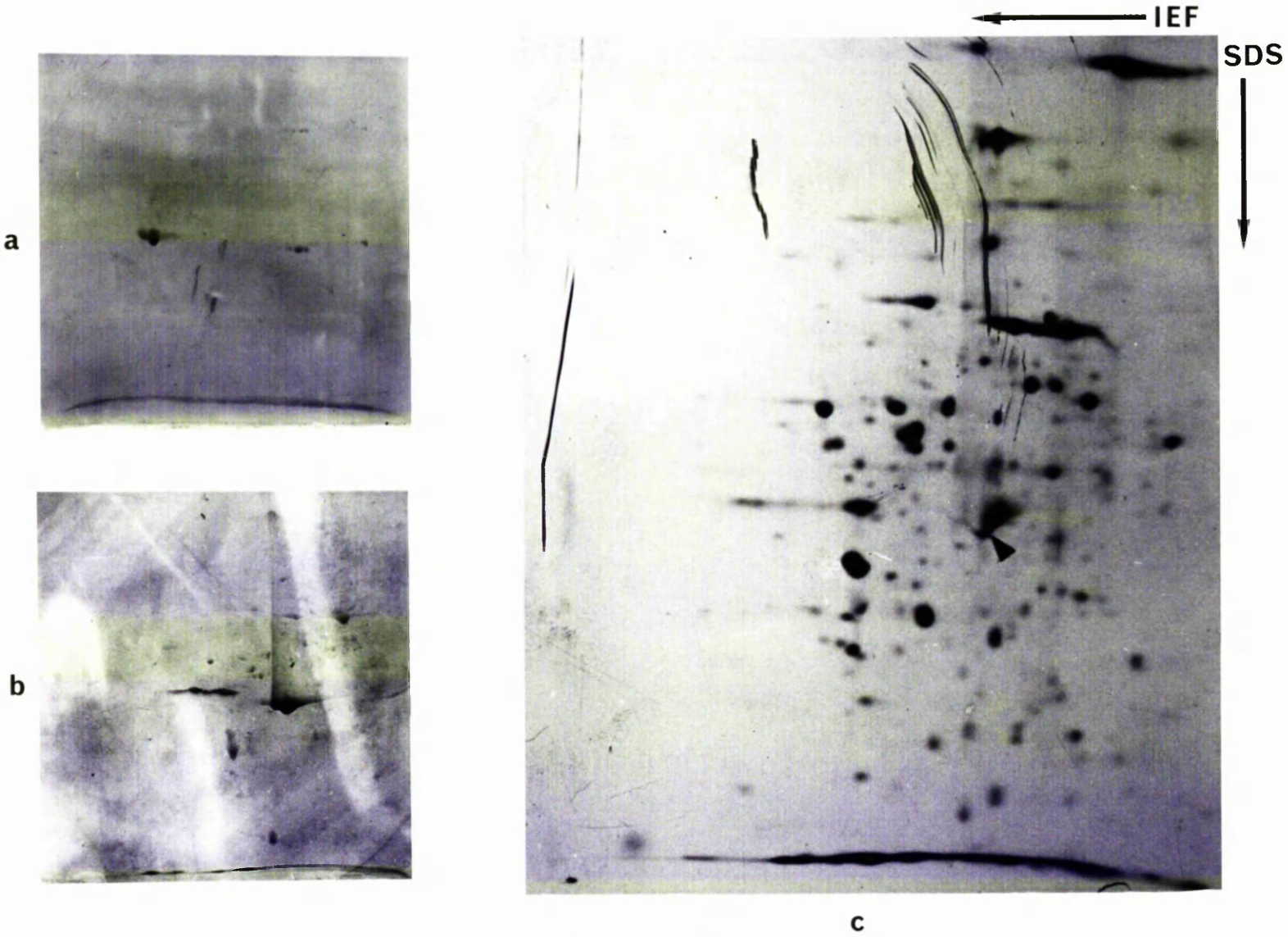


FIGURE 16.

Figure 17.

Cofractionation of pure rat leg muscle actin with L-[U-^{14}C]-labelled uterine proteins.

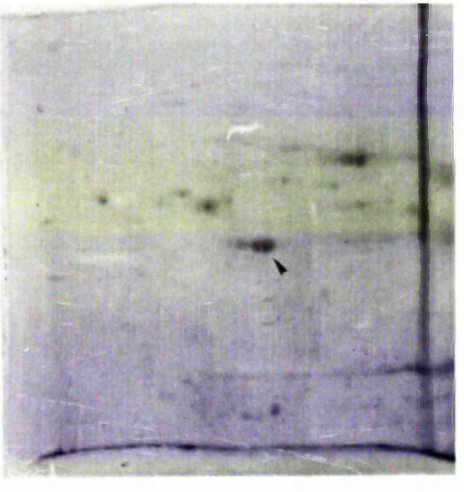
A purified sample of rat leg actin was obtained from Dr. J. Elce of the Department of Biochemistry, Queen's University, Kingston, Canada. Two-dimensional gel electrophoresis was performed with samples of pure actin and samples of actin mixed with L-[U-^{14}C]-labelled total uterine protein from rats responding to 4 hours of oestrogen-treatment. Gels were stained and photographed and those with labelled samples were fluorographed. The film was exposed for 28 days.

a) 10µg pure rat leg actin stained with Coomassie Brilliant Blue.

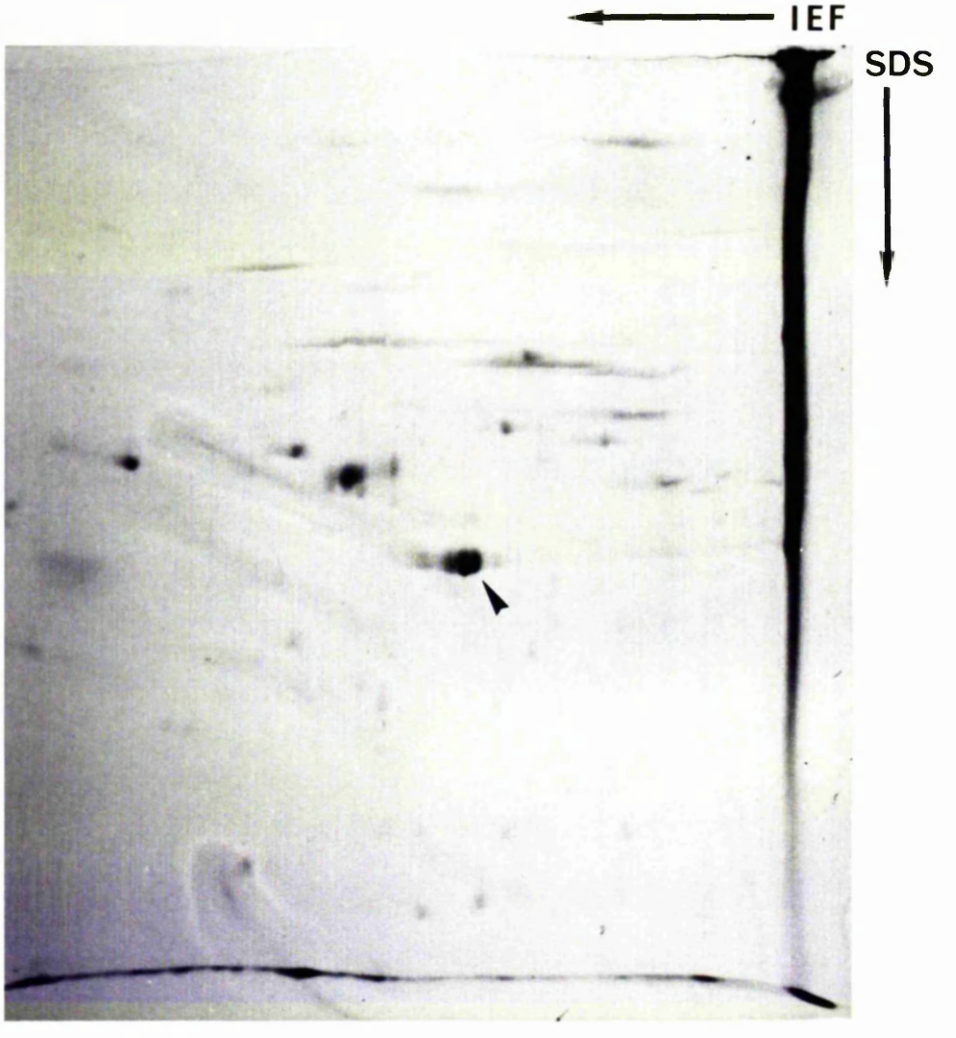
b) Staining pattern of 10µg pure rat leg actin cofractionated with a L-[U-^{14}C]-labelled uterine protein sample from 4 hr oestrogen treated rats. The uterine sample contained 100µg protein and 10^4 cpm. The arrow marks the position of actin.

c) Fluorogram of b). The arrow marks the position of actin in b).

a



b



c

FIGURE 17.

Pure rat leg muscle actin was also cofractionated with ^{14}C-labelled uterine protein. Fig. 17a shows the two-dimensional gel of a sample, stained with Coomassie Brilliant Blue. Fig. 17b shows the staining pattern when mixed with total ^{14}C-labelled uterine protein prior to electrophoresis, and Fig. 17c shows the fluorogram of this gel. The staining pattern and the fluorogram show that this actin sample exactly comigrates with the multiple spot in the labelled uterine protein population. This would seem to confirm that the major multiple spot in all the fluorograms is indeed actin. The displacement of the spot by rabbit actin in Fig. 16c could be explained by slight differences in the actins of different species, such that the migration characteristics were slightly different.

3.5.3 Cofractionation of labelled uterine proteins with IP (creatine kinase).

The oestrogen induced protein known as IP, first described by Notides & Gorski (1966), has recently been shown to be mainly composed of creatine kinase (Reiss & Kaye, 1981). These workers have further shown that the enzyme is identical to rat brain creatine kinase which they have purified, and the brain enzyme used here was the generous gift of Dr. A.M. Kaye of the Weizmann Institute, Rehovot, Israel.

The results obtained with creatine kinase cofractionation were very disappointing. A stained two-dimensional gel of 10µg of the protein is shown in Fig. 18a. The compact spot was, however, distorted and spread into a streaked shape when mixed with uterine samples (Fig. 18b,d, yellow arrows) but the position of migration relative to actin agrees closely with published patterns (Manak et al., 1980; Walker & Kaye, 1981). The fluorograms of labelled uterine samples cofractionated with IP samples show only very diffuse and slight darkening of the film at

Figure 18.

Cofractionation of purified rat brain IP with labelled uterine proteins.

A sample of the oestrogen-induced protein IP was obtained from Dr. A. Kaye of the Weizmann Institute, Rehovot, Israel. Aliquots were subjected to two-dimensional gel electrophoresis alone, or mixed with uterine protein populations labelled in vitro in the whole organ or translation assays. Summaries of these techniques are given in the legends to Figures 1 & 2. Gels were stained, photographed and fluorographed as described in the Methods section (2.6). The films were exposed for 37 days.

a) Coomassie Brilliant Blue staining pattern of 10µg IP.

b) Coomassie Brilliant Blue staining pattern of 10µg IP, coelectrophoresed with L-[U-^{14}C]-labelled uterine protein from 4 hr oestrogen-treated rats. The uterine sample contained 100µg protein and 10^4 cpm.

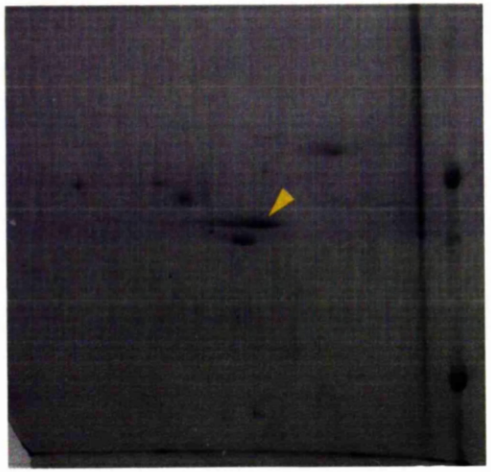
c) Fluorogram of b).

d) Coomassie Brilliant Blue staining pattern of 10µg IP, coelectrophoresed with L-[^{35}S]-methionine labelled MDL translation products of 4 hr polysomes. $2x10^5$ cpm (discounting background) were loaded.

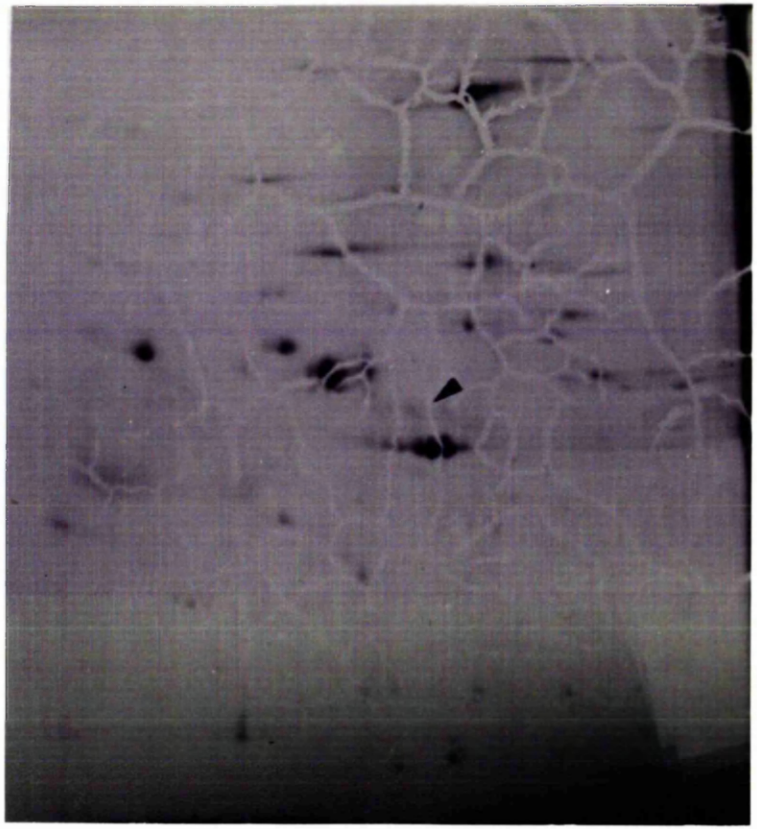
e) Fluorogram of d).

Arrows mark the position of IP.

a

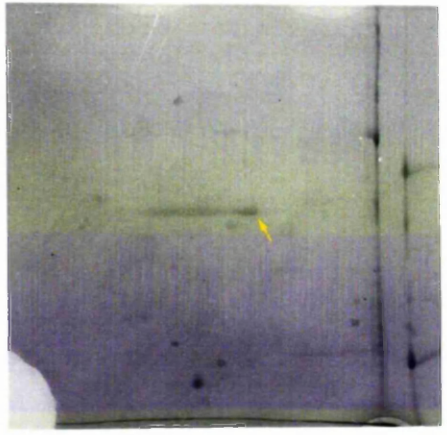


b



c

FIGURE 18.



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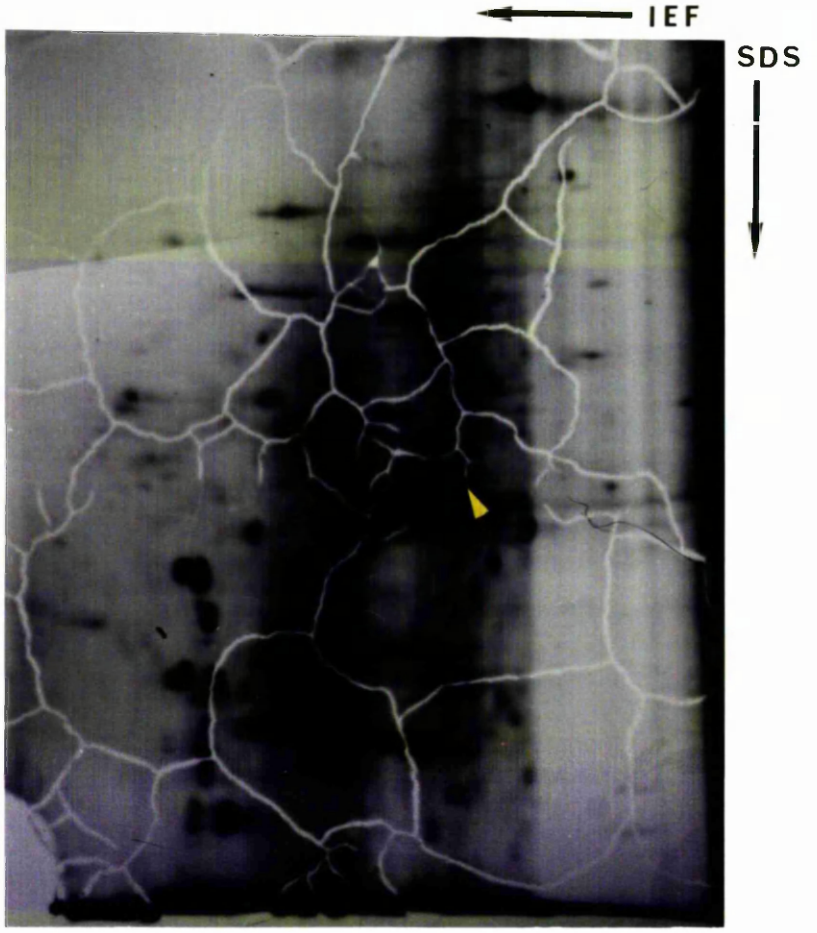


e

FIGURE 18.

this spot (Fig. 18c,e, see arrows).

It cannot be said with certainty whether any spot in either of the labelled samples corresponds to the position of IP. Two proteins which may be IP are found in cytoplasmic uterine proteins (Fig. 2, spots 6, following page 68) and these increase rapidly in response to oestrogen. These spots are not seen in nuclear samples which is consistent with their possible identity. The same proteins can be seen in the fluorograms of translation products of total polysomes. Both Figs. 12 & 13 show that the proteins are strongly stimulated early in the response; their labelling has greatly increased at 2 hours (see IP, Fig. 12a,b; Fig. 13a,b, following pages 80 & 81).

3.5.4 Cofractionation of MDL translation products with non-histone chromatin proteins.

Chromatin proteins were isolated from uterine nuclei. Non-histone proteins were obtained in small yield from the chromatin pellet, and were very difficult to dissolve in sample buffer; but sufficient protein was recovered after DNA digestion and histone removal to produce the staining pattern shown in Fig. 19b. Total chromatin proteins were used for cofractionation, because the yields from the preparation were greater, and they were more easily dissolved for electrophoresis. Fig. 19d shows the staining pattern of total chromatin proteins. There is some streaking due to DNA in the sample, and there is a small amount of actin in the sample. This may or may not be a contaminant (see discussion), but the major components are the acidic chromatin proteins present in Fig. 19b. The staining patterns of the MDL cell-free translation system and of chromatin cofractionated with MDL are shown in Fig. 19a & c respectively. The fluorograms of a & c are shown in e & f. MDL was used in this

Figure 19.

Cofractionation of purified rat uterine chromatin proteins with labelled uterine proteins.

Uteri from 24 rats responding to 4 hours of oestrogen-treatment were used to prepare uterine nuclei. From these, chromatin pellets were prepared using a modification of the method of Spelsberg (2.5.10,d). Non-histone proteins were prepared from the chromatin by dehistonisation and DNA digestion. MDL translation assays directed by uterine polysomes from 4 hour oestrogen-stimulated rats were prepared and samples analysed on the two-dimensional gels listed below. Gels were stained and photographed and those with labelled samples were fluorographed. Films were exposed for 27 days.

Staining patterns:-

a) 10µl MDL translation assay, loading: 2.75×10^5 cpm.

b) Non-histone protein sample, loading: 70µg.

c) Chromatin proteins cofractionated with MDL translation assay. 10µl of MDL containing 2.75×10^5 cpm, and 135µg chromatin protein were loaded.

d) Chromatin proteins, loading: 135µg.

Fluorograms:-

e) Fluorogram of a). MDL translation products of 4hr oestrogen treated uterine polysomes.

f) Fluorogram of c). MDL translation products of 4hr oestrogen treated uterine polysomes cofractionated with chromatin. The arrows mark the position of chromatin proteins stained in c) and d).

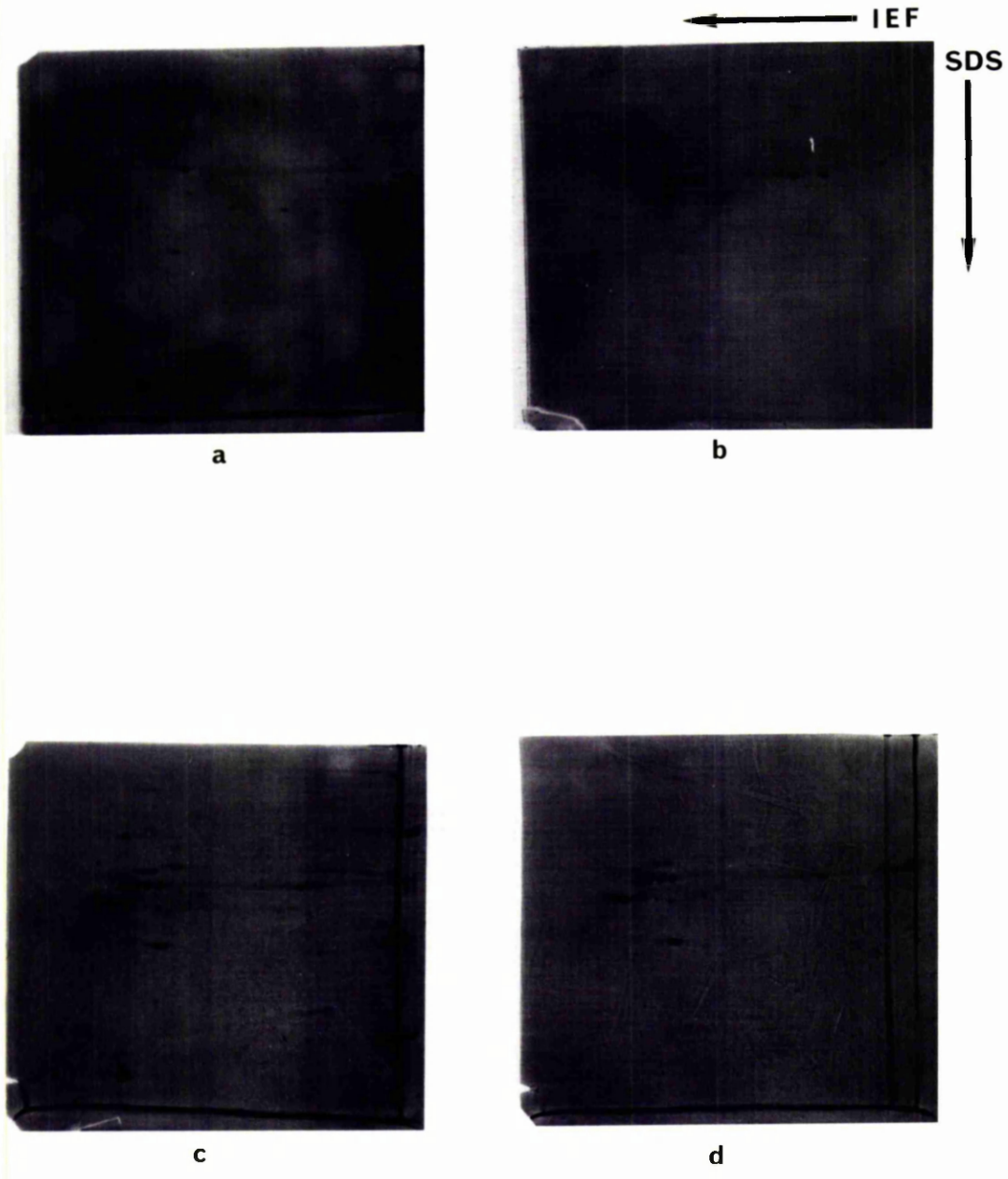
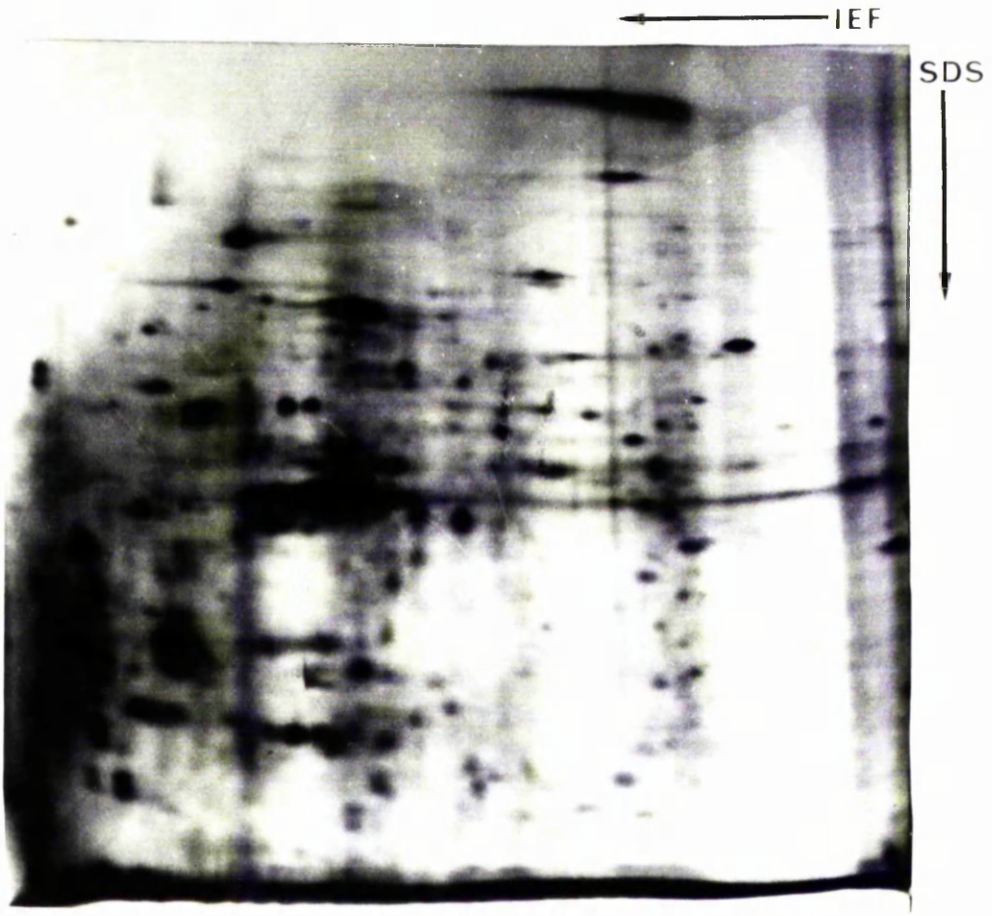
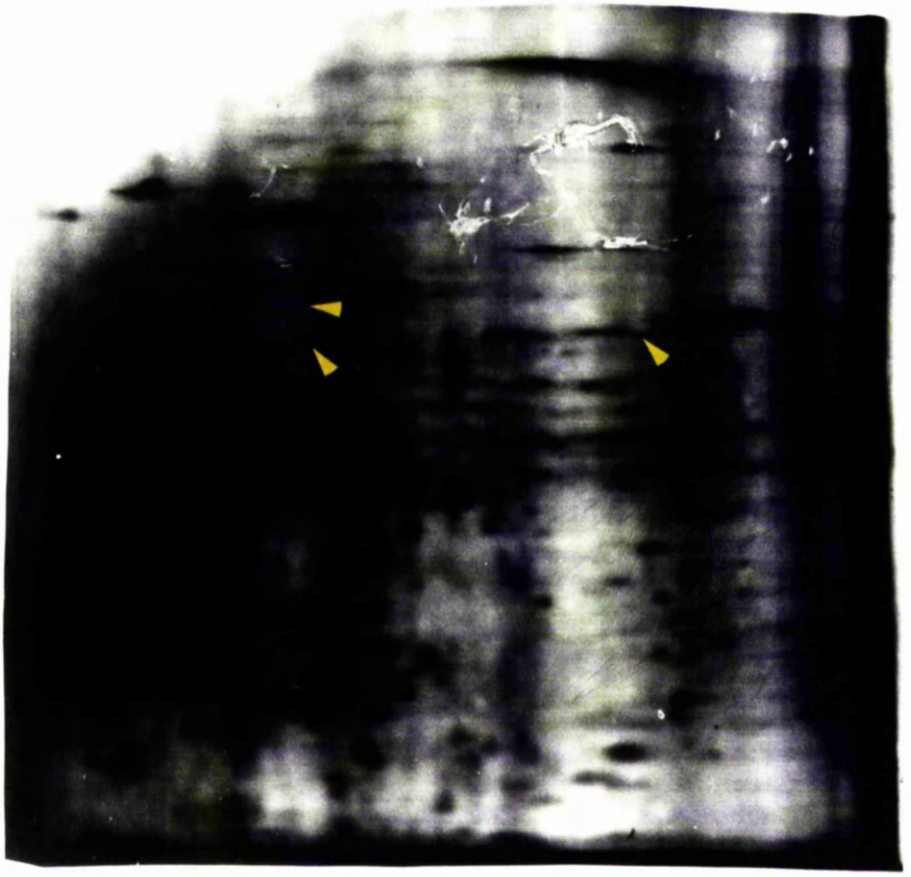


FIGURE 19.



e



f

FIGURE 19.

cofractionation because it was more likely to show minor components and could be used in the small volumes required for the first dimension gels.

The labelled proteins are the translation products of polysomes isolated from uteri responding to 4 hours of oestrogen treatment. The pattern in Fig. 19f shows that there are pale distortions in the background fogging of the film, which denote the position of some of the stained non-histone proteins (arrowed). That there are no labelled proteins present in this area of the gel can be seen in the fluorogram e. None of the non-histone proteins present in stainable quantities in b are labelled in in vitro translation assays.

3.5.5 Cofractionation of MDL translation products with hnRNP particle proteins.

Since these are basic proteins they cannot be resolved using isoelectric focussing in the first dimension, because the equilibrium pH gradient is too short at the basic end. However, basic proteins can be separated using basic ampholines in the first dimension gel, which is not run to equilibrium. This technique is known as Non-equilibrated pH gradient gel electrophoresis or NEpHGE (O'Farrell et al., 1977).

hnRNP particles were isolated from rat liver and the staining pattern of the proteins is shown in Fig. 20a. This compares favourably with published protein profiles of hnRNP particles (Wilks & Knowler, 1980). Labelled translation products of 4 hour polysomes, separated by NEpHGE in the first dimension are shown in Fig. 20b. The fluorogram pattern characteristic of samples separated by isoelectric focussing in the first dimension can be recognised but is contracted because the acidic

Figure 20.

Cofractionation of rat liver hnRNP particles with MDL translation products using NEpHGE.

Adult male rat livers were used to prepare nuclei by the method of Chauveau et al. (1956) and hnRNP particles were extracted from them by the method of Samarina et al. (1968). Experimental details are given in the Methods section (2.5.10,e). Samples of hnRNP particles and MDL translation assays were separated alone, or mixed, on two-dimensional gels. Non-equilibrated pH gradient gel electrophoresis (NEpHGE) (O'Farrell et al. 1977) was used in the first dimension (section 2.6.3) and 10% SDS polyacrylamide slabs in the second dimension. Gels were stained and photographed, and those with labelled samples were fluorographed. Films were exposed for 28 days.

a) Coomassie Brilliant Blue staining pattern of 150µg rat liver hnRNP particle proteins, showing nomenclature of Wilks and Knowler (1981).
b) Fluorogram of MDL translation products of uterine polysomes from rats responding to 4hrs of oestrogen treatment (10^5 cpm.)
c) Coomassie Brilliant Blue staining pattern of 150µg rat liver hnRNP particle proteins, cofractionated with MDL translation products of uterine polysomes from 4hr oestrogen-treated rats (10^5 cpm.).
d) Fluorogram of c). The arrows show the positions of labelled species comigrating with hnRNP particle proteins.

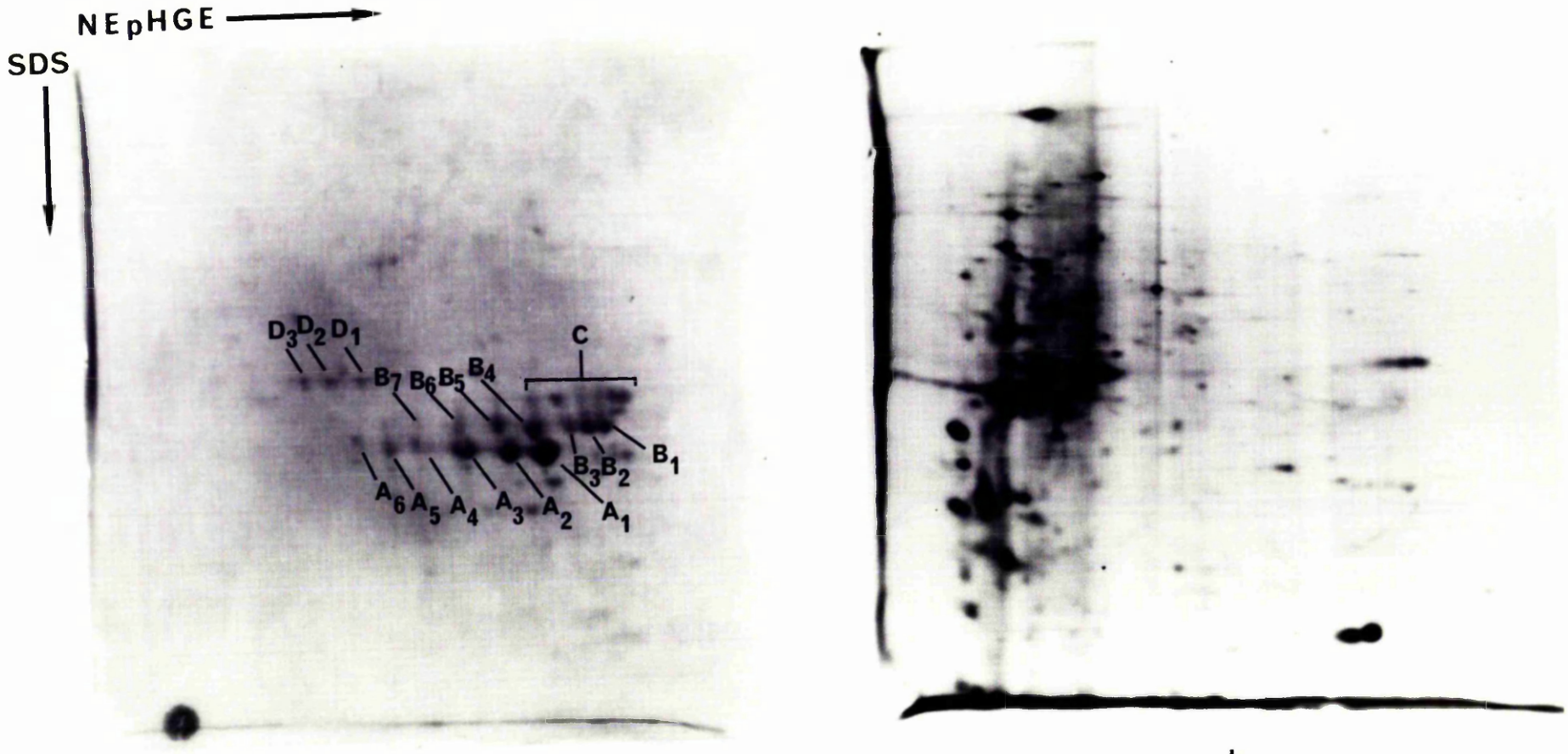


FIGURE 20.

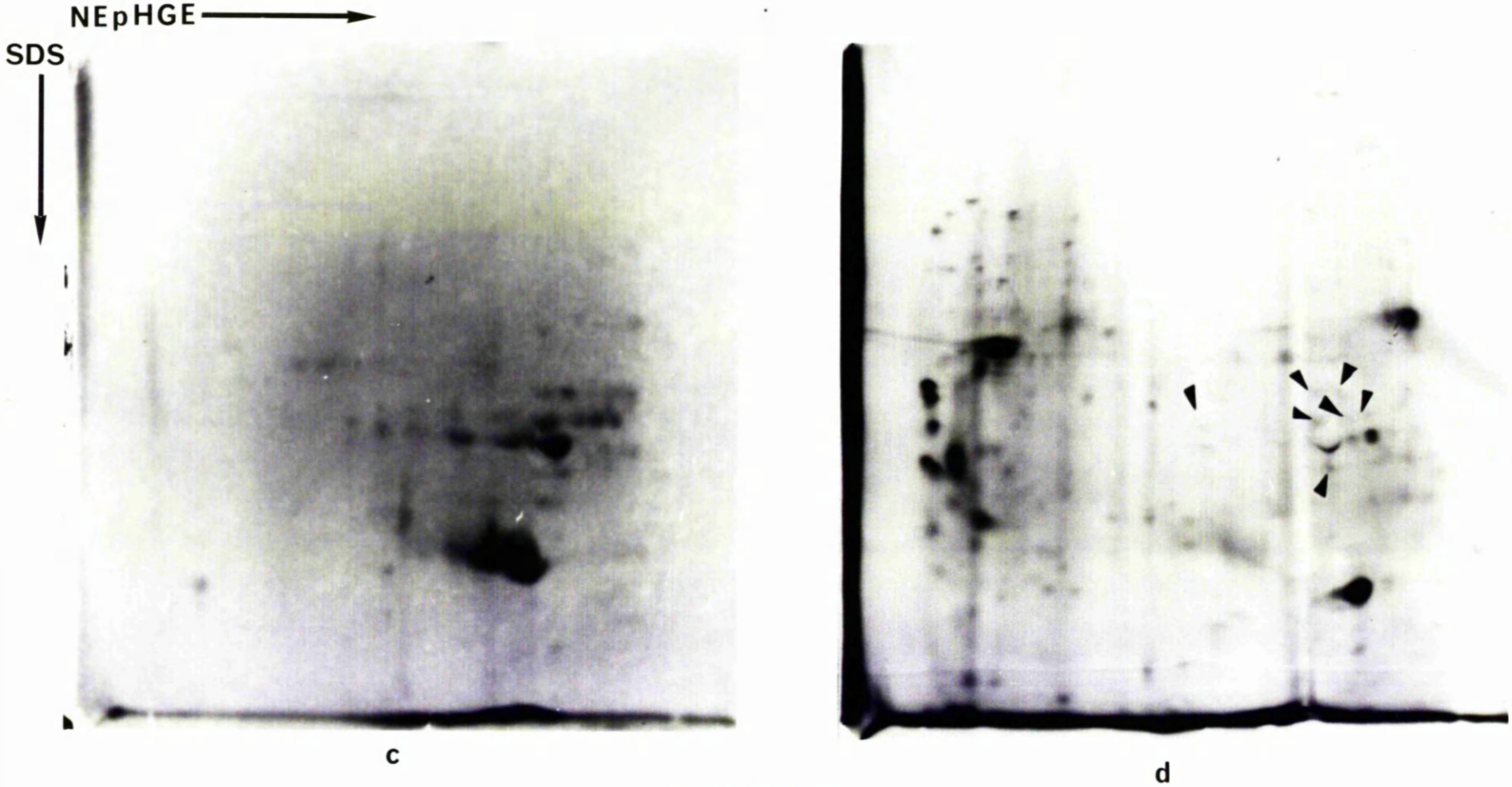


FIGURE 20.

end of the pH gradient is much shorter in these gels.

The stained gel of the cofractionated samples, Fig. 20c, shows a heavily staining, low molecular weight spot beneath the hnRNP proteins, which does not enter the first dimension when isoelectric focussing is used. This is a component of the MDL system.

The fluorogram, Fig. 20d, shows a few very faint spots which comigrate with hnRNP proteins. Using the nomenclature of Wilks & Knowler (1981) these are B2, B3, B4, A4 & two of the C-group proteins. Also, the spot A1 displaces a labelled protein, and the unnamed spot below A1 also comigrates with a labelled species.

3.5.6 Cofractionation of MDL translation products with ribosomal subunit proteins.

Ribosomal proteins cannot be satisfactorily separated in two-dimensions by either of the gel systems used. In order to determine whether ribosomal proteins were being synthesised in my *in vitro* systems, the established two-dimensional gel system of Kaltschmidt & Wittmann (1970) modified by Lastick & McConkey (1976) was employed.

Rat ascites tumour ribosomal subunit proteins were donated by Mr. M. McGarvey of this Department. They were coelectrophoresed with small samples of MDL translation products from uterine polysomes by Mr. I. Kennedy, also of this Department. The staining patterns and diagrammatic representations of the 40S ribosomal subunit proteins showing the accepted mammalian ribosome nomenclature (McConkey et al., 1979) are shown in Fig. 21a-d. MDL translation assay proteins do not contribute to the staining pattern because far too little assay sample was used. As can be seen from the fluorograms (Fig. 21e & f), many of

Figure 21.

Cofractionation of small ribosomal subunit proteins with MDL translation products of uterine polysomes.

40S ribosomal subunit protein samples were prepared from rat ascites tumour cells by Mr. M. McGarvey. Samples were mixed with small volumes of MDL translation assays and subjected to two-dimensional gel electrophoresis by the method of Kaltschmidt and Wittmann (1970) with modifications (Lastick & McConkey 1976). The electrophoresis was carried out by Mr. I. Kennedy as described in the Methods section (2.5.10,f). The direction of migration was from left to right in the first dimension and from top to bottom in the second dimension. Gels were stained, photographed and fluorographed in the same way as for O'Farrell gels and films were exposed for 51 days.

Coomassie Brilliant Blue staining patterns:

a) 100µg 40S ribosomal subunit proteins cofractionated with MDL translation products of control uterine polysomes ($1.5x10^5$ cpm.).

b) 100µg 40S ribosomal subunit proteins cofractionated with MDL translation products of 4hr oestrogen-treated uterine polysomes ($2.5x10^5$ cpm.).

c) Schematic representation of a).

d) Schematic representation of b).

e) Fluorogram of a) (Control polysome translation products).

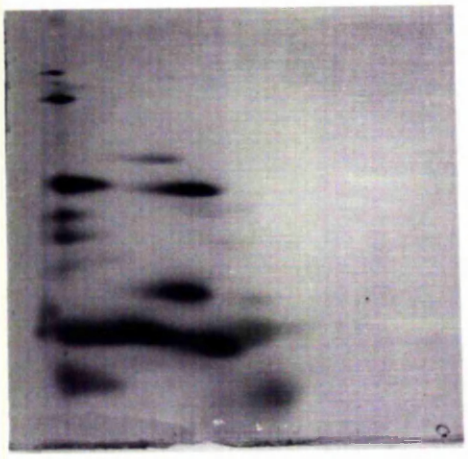
f) Fluorogram of b) (4hr polysome translation products).

g) Schematic representation of e).

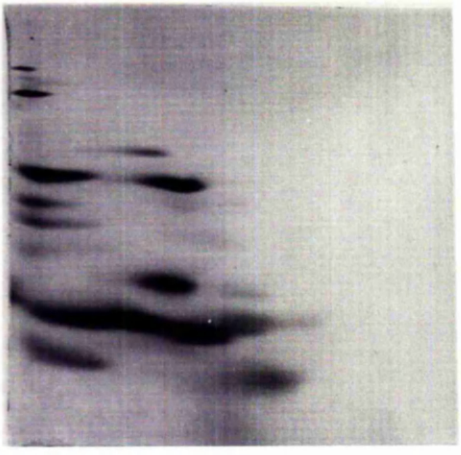
h) Schematic representation of f).

The schematic representations of the subunit proteins use the nomenclature of McConkey et al. (1979).

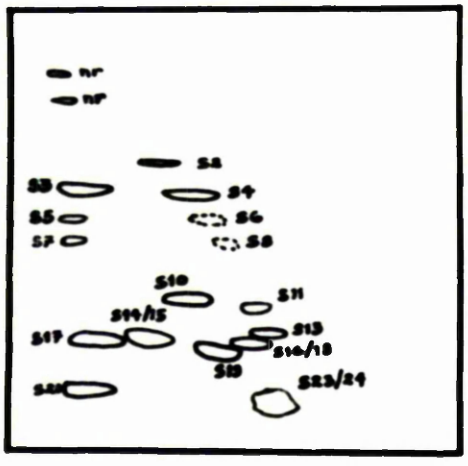
H1 = histone H1, PS6 = phosphorylated S6, nr = non-ribosomal



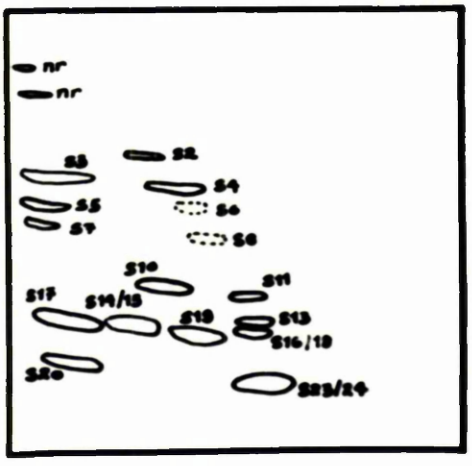
a



b

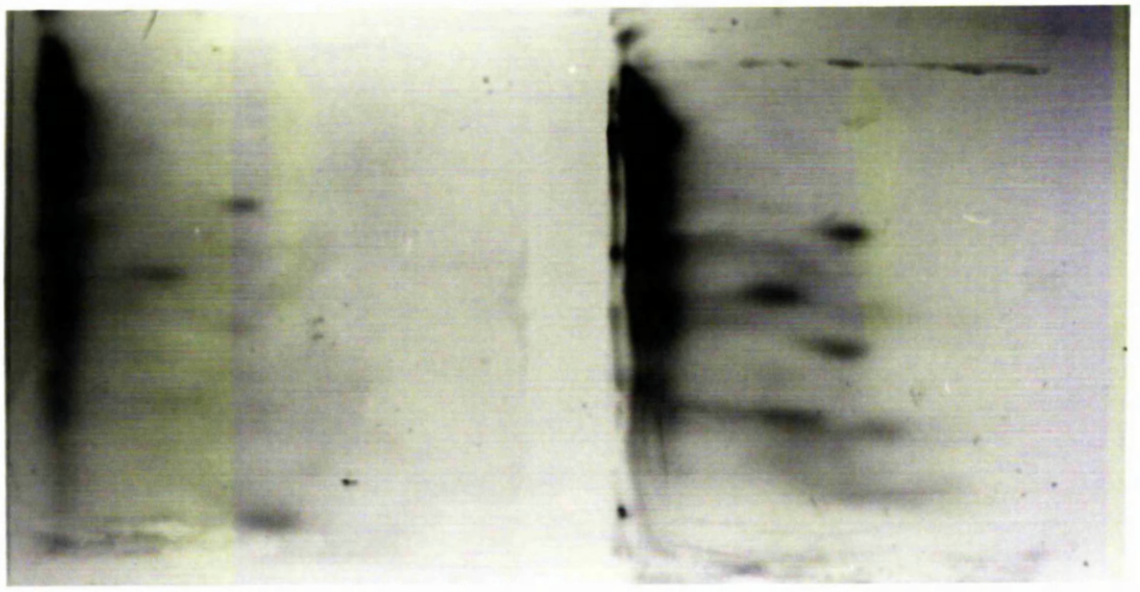


c

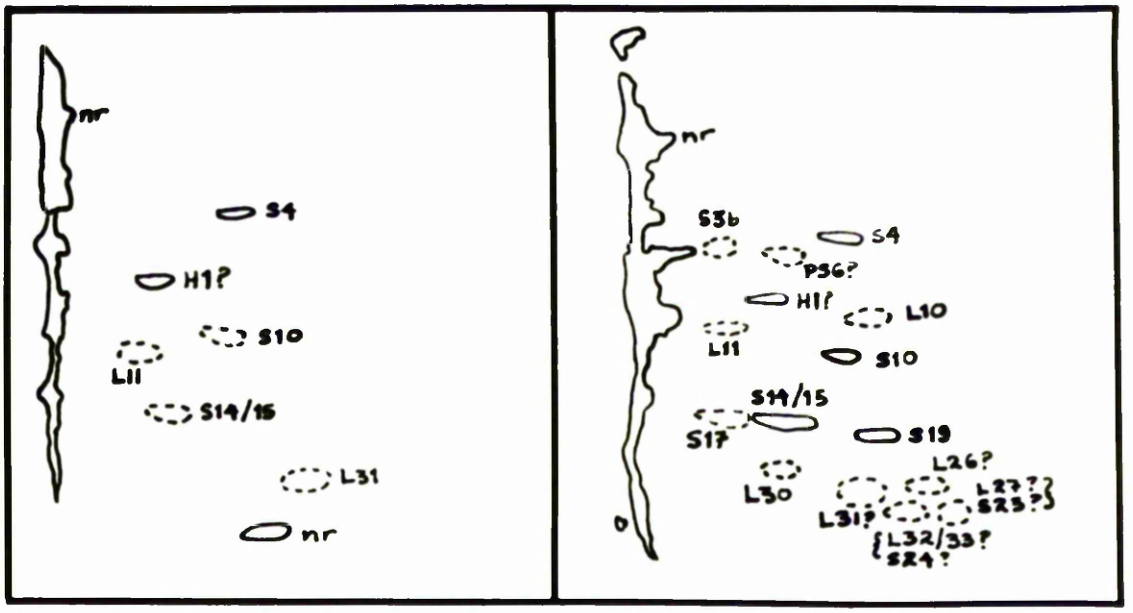


d

FIGURE 21.



e f



g h

FIGURE 21.

the proteins translated in vitro from control and 4 hour polysomes, comigrate with ribosomal proteins of the 40S subunit. These are shown in the diagrammatic representation of the fluorograms (Fig. 21g & h). Most prominent are those comigrating with S4, S10, S14/15 & S19. A dark spot lying between S4 and S10 does not coincide with any ribosomal protein and is found at a position where the histone H1 migrates in this gel system. Some low molecular weight protein and some material which does not enter the first dimension are also non-ribosomal proteins (nr).

Large subunit (60S) proteins cofractionated with MDL assays are shown in Fig. 22. This sample shows some contamination by 40S proteins and these are marked in the diagrams of the staining patterns (Fig. 22c,d). The fluorograms (e,f) show more detail than those in Fig. 21 although the same MDL samples were used. In addition to the labelled 40S ribosomal proteins in Fig. 21e & f, some large subunit proteins can be seen. The most heavily labelled of these are L10, L13/15, L26 & L32/33. The composition of the sample buffer and loading volume for these gels was critical and therefore the volume of MDL added to the ribosomal sample was kept to a minimum. Because MDL has a very high incorporation of counts in small volumes, small variations in the actual volume could quite easily account for the variation in the actual number of counts loaded and hence the variation between the films. The diagrams (g,h) show the spots identified in the fluorograms. Although some differences are seen between 0 and 4 hour translation products in Fig. 21, these are minimal in Fig. 22 and, due to the loading problem, no significance should be attached to them.

Figure 22.

Cofractionation of large ribosomal subunit proteins with MDL translation products of uterine polysomes.

60S ribosomal subunit protein samples were prepared from rat ascites tumour cells by Mr. M. McGarvey. All other methodological details are the same as those given in the legend to Figure 21.

Coomassie Brilliant Blue staining patterns:-

a) 100µg 60S ribosomal subunit proteins mixed with MDL translation assays using control uterine polysomes ($1.5x10^5$ cpm).

b) 100µg 60S ribosomal subunit proteins mixed with MDL translation assays using 4hr oestrogen-treated uterine polysomes ($2.5x10^5$ cpm).

c) Schematic representation of a).

d) Schematic representation of b).

e) Fluorogram of a).

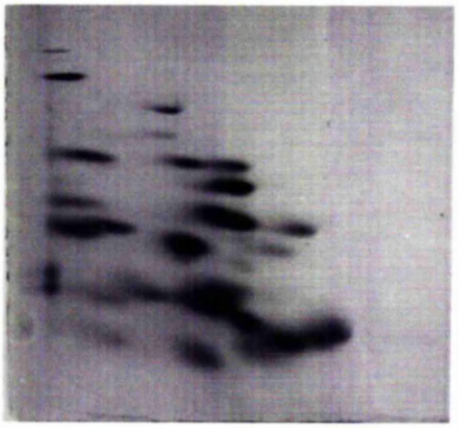
f) Fluorogram of b).

g) Schematic representation of e).

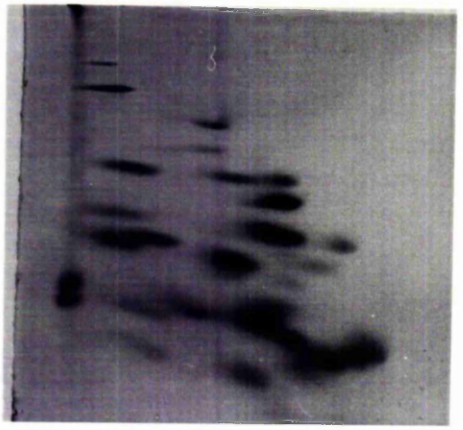
h) Schematic representation of f).

The schematic representations of the subunit proteins use the nomenclature of McConkey et al. (1979).

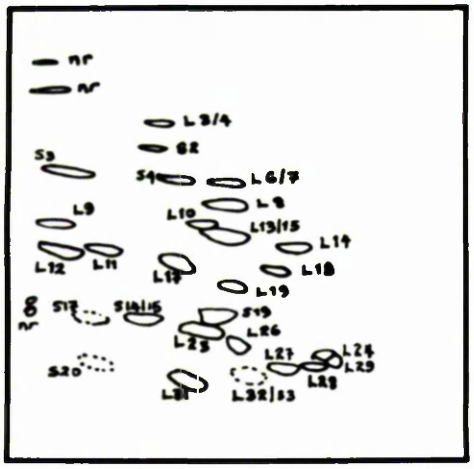
H1 = Histone H1, PS6 = phosphorylated S6, nr = non-ribosomal.



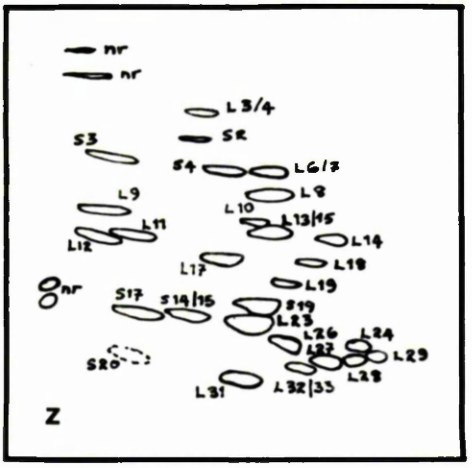
a



b

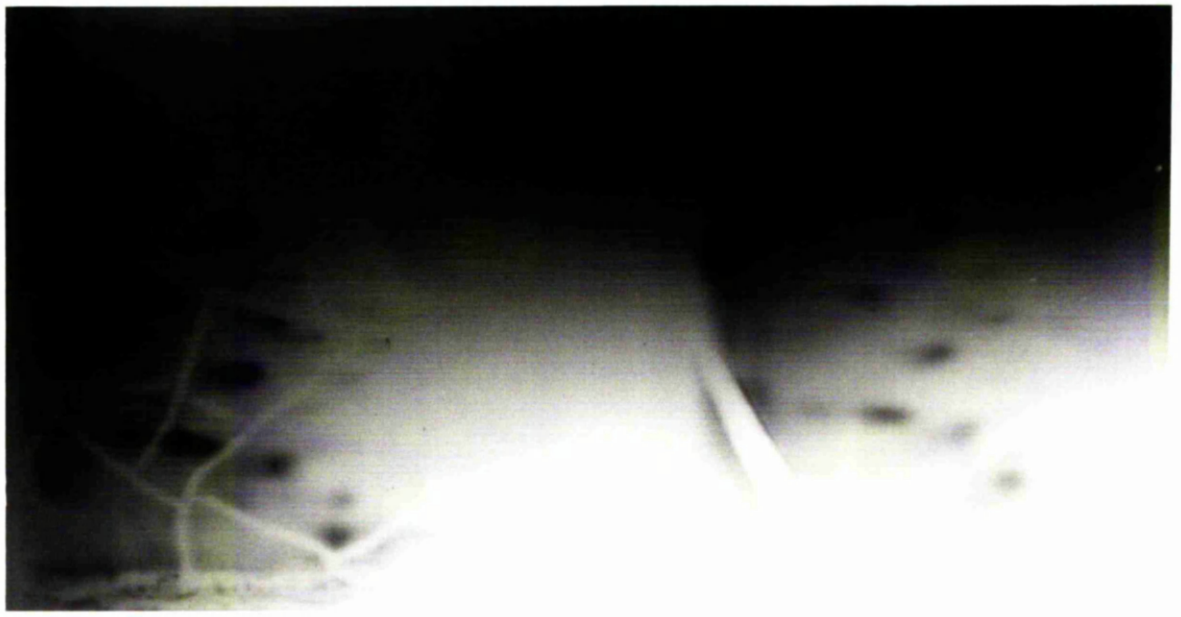


c

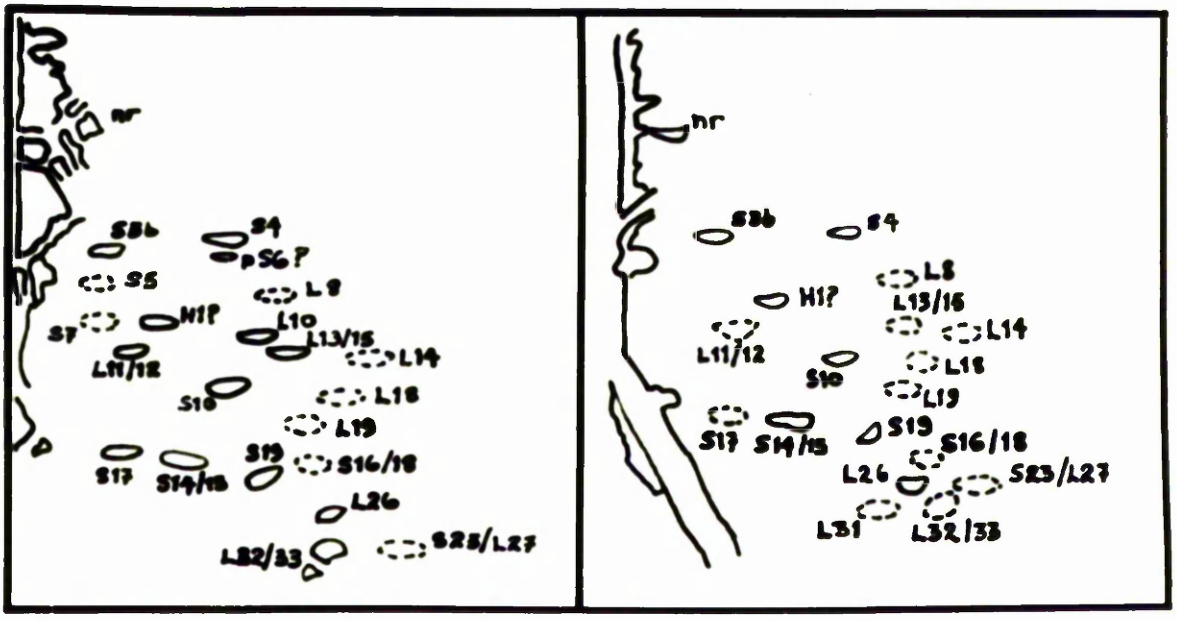


d

FIGURE 22.



e f



g h

FIGURE 22.

DISCUSSION

4. DISCUSSION

The aim of this project was to investigate the nature of any oestrogen-induced changes in the protein population of immature rat uterus responding to the hormone. In particular, it was hoped to detect individual proteins whose synthesis was induced by oestrogen very early in the uterotropic response. Before discussing the results it is pertinent to assess the experimental approaches used.

It is not possible to label rat uterine protein *in vivo* to a specific activity adequate for the analysis of the individual components of the population. This is because of the dilution and metabolism of labelled precursor before it reaches the organ. Therefore a number of different methods were used to investigate the effects of hormone treatment *in vivo* by monitoring the *in vitro* incorporation of precursor into the uterine protein of oestrogen-treated rats.

The success of this approach depended on several factors. Firstly, it is necessary that the response initiated *in vivo* should be continued *in vitro* without alteration, and without any additional response caused by the post mortem manipulation of the tissue. This is virtually impossible to establish before the study is conducted, particularly since the protein population of the uterus is complex and ill-characterised. With a clear-cut response, such as the induction of a specific protein detected *in vitro*, it would be possible to confirm retrospectively that the response occurs *in vivo*. This would employ specific probes such as antibodies or, in the case of proteins such as ovalbumin, and vitellogenin, by cDNA hybridisation to detect the messenger RNAs. If the protein is abundant enough, staining of gel separations may suffice. In the uterine system, gel staining did not reveal any clear cut difference between control and hormone-treated

animals, so the assay depended on the faithful labelling *in vitro* of proteins whose synthesis had been induced *in vivo*. This made the physiological state of the tissue of prime importance, so that as far as possible, labile substances and processes were preserved.

Three methods of labelling proteins *in vitro* were tried. Firstly, the whole organ was labelled in a nutritive medium containing a high specific activity amino acid mix. Labelled uteri could then be fractionated into cell types or subcellular fractions for analysis. This method should entail the least trauma to the tissue, with the least interruption in the time course of the response.

Secondly uteri were fractionated into epithelial, stromal and myometrial cell types before the labelling incubation, which should overcome the possibility of over- or under-representing the proteins of a particular cell type in the labelled population. This could occur if diffusion or uptake of label was unequal in the different cell layers of the uterus. The possible disadvantages of the method are the greater length of time between death, or the initiation of the response, and the time at which the resulting products were labelled. This gives rise to the possibility that very early, transient products may not be detected, and that the interruption of the response *in vivo* may have altered the chain of events.

Thirdly, polyribosomes were isolated from uteri immediately after death. These were added to cell-free translation systems containing radioactive amino acids, to label the uterine proteins synthesised *in vitro*. This method had several advantages: the oestrogenic response was effectively frozen at the time of death which prevented any spurious results due to trauma. The isolation of polyribosomes meant

that only the products of messengers actually being translated at the time of death would be labelled, and, having immediate access to the radioactive precursors, these should be labelled more efficiently. The use of polysomes to direct the cell-free translation system, rather than polyadenylated messenger RNA also avoided the omission of any proteins from the analysis that were encoded by nonadenylated messengers.

Other factors influencing the success of the experimental approach were the methods of separating and detecting the labelled products. Two-dimensional gel electrophoresis was used because of the large number of components it is capable of resolving. A sample of labelled uterine protein shows up to 10 times more components when separated on two-dimensional rather than one-dimensional polyacrylamide gels. The reproducibility of shape and juxtaposition of individual spots are also of use in comparing separations. Indeed, some workers are currently trying to identify and catalogue protein populations by defining their positions on two-dimensional gels run under controlled, standardised conditions (Bravo & Celis, 1980; Celis & Bravo, 1981; Clark, 1981). The use of labelling increases the detection of uterine proteins many-fold as can be seen by comparing Figs. 1a and 1b (following page 66). Labelling depends on the turnover rate of the proteins and hence it is also a method for screening out components which are abundant in the tissue, but are not turning over, for example the protein arrowed in Fig. 1a. As described in the Methods section, the use of fluorography increases the efficiency of detection ten-fold over that achieved by autoradiography for carbon- and sulphur-labelled compounds.

The three labelling methods yielded different results. The labelling of the whole organ in vitro revealed a very complex population of proteins in the uteri of both control and oestrogen-treated rats (Fig.2, following page 68). This was not expected because the immature rat uterus is a quiescent tissue before hormone stimulation, having its ribosomes largely in the form of monosomes, and making very much less RNA and protein than in stimulated animals (Merryweather & Knowler, 1980). The lack of difference between the two samples was also apparent when a double label technique was employed (Choo et al., 1980). In this system, control uteri were labelled with ^{14}C amino acids and protein samples mixed with ^{3}H-labelled samples from hormone treated uteri. Two-dimensional gel separations of this mixture were subjected to fluorography which detects both isotopes, and then exposed autoradiographically through carbon paper. This detects only ^{14}C, the low energy beta-particles of tritium having too short a path length to reach the film and PPO fluorescence being blocked by the carbon paper. This technique failed to show any hormonal effects on the protein population that were not detectable using separate gels.

There is some precedent for believing that the incorporation of precursors in incubations in vitro may distort results. Knowler (1976) demonstrated that oestrogen-stimulated hnRNA synthesis can be observed by this method, although stimulated rRNA synthesis is apparently abolished. Nevertheless, stimulated synthesis of ribosomal proteins (Merryweather & Knowler, 1980) as well as IP (Notides & Gorski, 1966) can be detected by this method. It may be that oestrogen-stimulated protein synthesis is obscured because the incubation conditions also induce protein synthesis. The other possible explanation is that the gel system is detecting only abundant proteins. If protein abundance mirrors messenger abundance, this would agree with the results of Aziz

et al. (1979b) that there are few changes in the abundant class of messenger RNA in the first 4 hours of the oestrogenic response.

The patterns of uterine protein separations described in this thesis are representative of similar fractionations by others. Thus, Korach et al. (1981) have fractionated the proteins of mature mouse uteri and have published polyacrylamide separations which are very similar to those shown in Figs. 1 & 2 (following pages 66 & 68). In their study, the uteri of mice treated in vivo with oestrogen were labelled in vitro with radioactive methionine. In order to test their hypothesis that oestrogen caused movement of proteins between intracellular compartments, they also added the hormone to the in vitro incubation. They then went on to prepare and fractionate the 30% ammonium sulphate precipitable cytoplasmic proteins, the supernatant proteins from this fraction and salt extractable nuclear proteins. Their results, which revealed only very minor oestrogen-induced differences in the cytoplasmic fractions, hardly substantiate their claim that some proteins move from the cytoplasm to the nucleus. Nevertheless, the hypothesis that oestrogen causes movement of proteins between cellular compartments is interesting, and could explain the apparent loss of some of my proteins after hormone treatment. Indeed, LeStourgeon et al. (1974) propose a flux of contractile proteins in and out of the nucleus during changes of chromatin structure.

The differences between my own cytoplasmic fractions are rather more clearcut than in the mouse study, but their lack of reproducibility argues that the system is responding in a more random manner than the hormone should dictate.

This problem may also apply to the studies on fractionated cells, but these experiments did help to answer some of the questions concerning the differential responses of the cell types. Firstly, the specific activities of samples from cells isolated after the labelling of whole uteri show that all cell types are represented in the labelled protein population, although the myometrium contributes least. Secondly, the labelling of uteri in vitro does not favour the epithelium because the serosal surface has more immediate access to the labelled precursors in the medium. Qualitatively, there is great similarity in the protein patterns of the epithelium and stroma but the two cell types are strikingly different in their responses to the hormone. The synthesis of many of the stromal proteins is consistently stimulated, although this is less detectable in prelabelled samples. It is difficult to determine whether some of the stimulated proteins are completely absent in control samples as the gels are faint, but many stromal species did appear to be present only after hormone stimulation and to be exclusive to that cell type (Fig. 8, following page 73). This could not be predicted from the available evidence that we have on the response of individual cell types in the rat (see 1.6), although in the mouse, hypertrophy is a property of the stroma and myometrium, whilst hyperplasia occurs in the luminal epithelium. Hypertrophy is known to be the earlier event and it may be the manifestation of the synthesis of proteins vital to the epithelial response, as suggested by Korach & Lamb (1981).

The results obtained with prelabelled cell proteins are slightly different to those obtained with cells labelled after fractionation. The spot patterns achieved using the former correlate closely with the gels of whole uteri but are slightly more complex than those of cell types labelled after fractionation (compare Figs. 8 & 9, following

pages 73 & 75). This may be due to a greater loading on the gels seen in Fig. 9, but it may also reflect the different method of labelling. Indeed, if there is stimulation of the whole organ by incubation in vitro, this would show in Fig. 9, whereas cells labelled in isolated fractions may not respond in the same way. For example, an isolated cell type cannot be influenced by another to react to the incubation conditions. Alternatively, the cells may be disturbed by the trauma of isolation and may not be responding fully to the stimulation by oestrogen in vivo. This is possible because the cells will not incorporate label into proteins immediately after isolation, but require the four hour rest described in the Methods section. This suggests that there could be some perturbation to normal protein synthesis. Cells are capable of quite marked responses to trauma. An example is the shutdown of current protein synthesis and rapid induction of the 'heat-shock' proteins in HeLa and Drosophila after short periods of temperature shock. A more likely explanation is that the cell membrane and transport systems require some reorganisation after the enzymic damage during isolation. The appearance of the cells under the electron microscope, and the fact that they exhibit the same stimulated RNA synthesis as the whole organ (McCormack & Glasser, 1980), argue against the cells being damaged by the isolation procedure.

There is some precedent for expecting a difference in the response of fractionated cells to that of the whole tissue. Higgins et al. (1981) showed that castration markedly decreases the incorporation of labelled methionine into proteins by seminal vesicle tissue, but not by isolated cells. Higgins also discounts the possibility of cell damage, and finds that isolated cells are more active in protein synthesis than tissue pieces. He noted that the activities of the

cells are modified by epithelial-stromal interactions in the tissue, and that epithelial cells are subject to vectorial constraints, which are removed when they are isolated. It is not entirely clear how these factors could account for my results. If isolation released the cells from some in vivo operative repression of protein synthesis then all the cell types should have exhibited a similar derepression. In the seminal vesicle study described above, the tissue was not fractionated into cell types.

Perhaps the technique of choice for a study of this sort is the translation of uterine messengers by cell-free systems. With this method, one can be sure that there is no change in the actively translating polysome population present at death, before the proteins being translated are labelled. It is also possible to check the integrity of the polysomes immediately prior to the translation assay (see Fig. 4, following page 69). The system yields samples of high specific activity which enable the ideal loadings to be used on isoelectric focussing gels, thus reducing the problems of streaking and spot displacement which can result from overloading. It also means that the likelihood of detecting minor species is increased.

The system has a high translational efficiency ranging from five to fifty rounds of chain reinitiation during the assay (Pelham and Jackson, 1976). Reinitiation can be demonstrated indirectly by incubating the assay with sparsomycin, which increases the average size of polysomes seen on subsequent sucrose density centrifugation. This also demonstrates the virtual absence of ribonuclease activity. In these respects the messenger-dependent reticulocyte lysate system (MDL) is far preferable to the wheat germ system which has much lower levels of reinitiation and has variable and often high ribonuclease

activities (Doel & Carey, 1976; Pelham and Jackson, 1976). Both systems do have some aminopeptidase activity which may account for the background fogging on films, but this is 1-3000 times lower in rabbit reticulocyte lysates than wheat germ extracts, which also have a trypsin-like activity (Mumford et al., 1981). For ovalbumin, it has been demonstrated that reticulocyte lysate acetylates the protein during its synthesis in vitro. Although it is not glycosylated, the product is indistinguishable from the naturally secreted ovalbumin if this is synthesised in the presence of the glycosylation inhibitor tunicamycin (Palmiter et al., 1978). Thus the protease activity may not be a problem and apart from large modifications such as glycosylation the lysate produces an identical product to the tissue in vivo.

If protease action is minimal then the differences between the products of cell-free translation and the labelling of the whole organ in vitro must have some other basis. One possible explanation is that the tissue is derepressed by removal from the animal and that the normally quiescent, inactive uterus of the immature animal is stimulated by incubation in vitro.

Another suggestion is that, because the bulk of tissue in immature uteri is myometrium, purified total polysome samples consist predominantly of myometrial ribosomes and messengers. Three lines of evidence refute this. Firstly, the specific activities of myometrial samples prepared from labelled uteri are lower than those of epithelium and stroma, suggesting this cell type to be less active in protein synthesis. Secondly the isolated myometrial cells also give samples of lower specific activity. The lower levels of incorporation and the consistently small number of proteins visualised in the

myometrial samples probably indicates that the myometrium is comprised of relatively few types of proteins which are mainly structural and have low rates of turnover in the cell. Lastly, the isolation of polysomes from fractionated cell types yields ten times more polysomes from epithelium and seven times more polysomes from stroma than from the myometrium of the same group of oestrogen-stimulated uteri. At all times it was difficult to obtain satisfactory gels of myometrial samples, thus it is extremely unlikely that the translation products of total uterine polysomes are significantly enriched in myometrial proteins.

The differences obtained between the translation products of oestrogen-stimulated and control polysomes labelled in MDL are fairly slight. Although several species decrease or increase with oestrogen, only five protein spots are consistently stimulated in the first two hours of the hormone stimulation (Fig. 13b, following page 81). Of these, two correspond to the expected position of the induced protein, IP. This also appears as two spots on the two-dimensional patterns published by Manak et al. (1980) and has been shown to contain a major component, creatine kinase, and a minor component, enolase, of very similar molecular weight (Reiss & Kaye, 1981). Skipper et al. (1980) report that it has a third minor component, but this has not been identified. Of the other three proteins, the one just below actin (molecular weight 42,000) is an exclusively nuclear component as can be seen by comparing Figs 2 & 5 (following pages 68 & 70). In nuclei isolated from labelled whole uteri, the synthesis of this protein is not seen to be stimulated until 12 hours after hormone administration. This may be because it is at too low a level to be significant in such a complex protein mixture, or because its increase is masked by the derepression in vitro of other species. Its translation by polysomes

is greatly stimulated between 0 and 2 hours as seen in Figs. 12 & 13 (following pages 80 & 81). It cannot be identified in any of the protein groups studied by cofractionation, but it may correspond to a non-histone protein described by King et al. (1974). This can be induced by administration of oestrogen to uteri in vitro for one hour. The fourth protein, of molecular weight 39,000 is stimulated by oestrogen in preparations from whole labelled uteri. This small stimulation occurs in both nuclear and cytoplasmic compartments and is therefore not likely to be a component specifically regulating gene expression. The last protein, of molecular weight 20,000 could be the 20,700 molecular weight oestrogen-induced non-histone protein described by Cohen & Hamilton (1975b), but it is not readily identified on gels of uterine nuclear proteins. It may be the smallest of the hormone-enhanced stromal proteins seen in Fig. 8 (following page 73). These results are internally consistent and corroborate the earlier work by other authors. However, the study has not revealed as much information as could have been expected. Neither has it been possible to unequivocally identify any of the induced proteins with the key intermediary proteins considered likely to be involved in oestrogen-induced uterine growth.

Estimates of the maximum theoretical resolving power of the O'Farrell gel system range from 2,500 to 5,000 different components (O'Farrell, 1975; Chrambach, 1980). The total sequence complexity of the polysomal mRNA in immature rat uterus shows that there are 8 to 12,000 distinct sequences (Aziz et al., 1979a). Even if only 50% of the protein types do not enter the isoelectric focussing gel, this heterogeneity is sufficient to overwhelm the theoretical resolving power of the system. In animal cells, relatively few specific proteins (50-60) may comprise greater than 50% of the total cellular protein mass (Peterson &

McConkey, 1976). These two factors taken together with the limitations of total protein load, restrict the absolute amounts of minor constituents in the sample applied to the gel to low levels. Thus it is more than possible that the gel system is only detecting the abundant protein species of the uterus. This explanation would account for the results, although it is likely that there is an additional incubation effect on whole uteri.

A recent study by Higgins and Fuller (1981) has also used two-dimensional gel electrophoresis to detect differences in rat seminal vesicle proteins before and after castration. This is the only other system where hybridisation studies show that the major effect of a steroid hormone is on mRNA sequences of moderate abundance (Higgins et al., 1979). The 100-200 intermediate abundant mRNA species decrease ten-fold after castration, but of the 20 or so protein species which are depleted, about half seem to be secretory products, previously shown to be encoded by highly abundant messengers. Examination of the fluorograms of these authors reveals that many of the depleted proteins are very minor components of the total population. These results support the idea that changes in moderately abundant proteins may not be detectable using two-dimensional gels. However, Higgins et al. (1981) propose that the action of testosterone in this system is the stabilisation of the messengers or proteins, rather than the control of transcription, so that one cannot draw too close a parallel between the uterine and seminal vesicle systems.

There is further support for our explanation, in that the proteins we expected to identify by cofractionation, namely, hnRNP particle proteins, non-histone chromatin proteins, and to a lesser extent ribosomal proteins, were very difficult to detect in labelled uterine

samples. If these proteins are representative of moderately abundant proteins then our inability to detect them supports the contention that we were only detecting abundant uterine species efficiently. Thus it is clear that efficient analysis of oestrogen-induced changes in the synthesis of uterine proteins must depend on a more sensitive assay. This could employ more efficient fractionation, further subfractionation of proteins of higher specific activity, or cell-free translation by fractionated cell-type polysomes. Preliminary results using the latter approach did reveal a more marked difference between 4 hour- and 12 hour-stimulated epithelia (Figs. 14 & 15, following page 83).

A method which has been used to increase the detection of minor components (particularly IP) is three-dimensional gel electrophoresis (Skipper et al., 1980). This incorporates non-denaturing gels as the first separation and the stacking of several corresponding gel segments onto a single isoelectric focussing gel, followed by the final SDS slab gel. This is a time-consuming procedure and does not simultaneously analyse all the components in a single protein map. The non-denaturing gel also allows protein-protein interactions which may cause the distribution of a single component between several segments of the gel. This could confuse the final analysis especially if minor components participate in such interactions.

The cofractionation studies reported in the results section were only partially successful. Whilst confirming the migration positions of proteins thought to be actin and IP, the labelled species comigrating with IP and hnRNP particle proteins were so faint in samples from hormone-treated animals that comparison with control samples would not have been convincing.

Ribosomal proteins were shown to be synthesised in cell-free translation assays using messengers from both control and 4 hour hormone treated rats. The small differences between these two samples and the faintness of the spots after long exposure times, supports the observation that ribosomal protein synthesis is not stimulated until 8 to 12 hours after treatment with oestradiol (Merryweather & Knowler, 1980).

No labelled proteins could be seen comigrating with non-histone proteins. A possible exception is actin which was present on the gel of chromatin proteins and is a major uterine species. There is controversy as to whether actin is, or is not, a nuclear protein species (Comings & Harris, 1976), but it has been reported to be a constituent of non-histone chromosomal proteins in a wide variety of organisms. These include the myxomycetes Physarum & Dictyostelium, HeLa cells and rat liver (Douvas et al., 1975; Peterson & McConkey, 1976; Fukui & Katsumaru, 1980). It has been suggested that actin and other contractile proteins found in the nucleus may function in the condensation of chromatin and chromosomes (LeStourgeon et al., 1974, 1975). Fukui & Katsumaru (1980) demonstrated that DMSO induced the formation of huge bundles of actin filaments from the nuclear matrix in Dictyostelium nuclei and suggested their involvement in changes of nuclear shape and possibly of chromatin structure. It has also been suggested that microfilaments as well as microtubules are involved in chromosome movement during mitosis (reviewed, Fukui & Katsumaru, 1980).

Actin shows no increase in response to oestrogen in the uterus, but this does not preclude its involvement in early nuclear events

initiated by the hormone. The nuclear matrix is a residual structural element, remaining after nuclease digestion, salt extraction and detergent treatment of nuclei. Actin is most probably a constituent of this, which suggests a number of ways in which it might be involved in the oestrogenic response. Firstly it may participate in specifying the salt-resistant oestrogen binding sites, reported by Barrack's group to reside in the nuclear matrix (Barrack et al., 1977; Barrack & Coffey, 1980). Secondly there is growing evidence that the nuclear matrix plays an important role in transcription and replication. DNA is arranged in the cell nucleus in supercoiled loops which are attached by their ends to this matrix (Cook & Brazell, 1976).

A number of actively transcribing gene sequences and virtually all of the rapidly labelled RNA have been isolated in association with this skeleton and a similarly isolated structure known as the nuclear cage (Herman et al., 1978; Miller et al., 1978; Jackson et al., 1981). Ultra-violet light-induced crosslinking studies in HeLa cells have shown that hnRNA sequences are specifically associated to the matrix via two of the particle proteins. These proteins are those tightly associated to the RNA in hnRNP particles (van Eekelen & van Venrooij, 1981). The genes for ribosomal RNA in rat liver, SV40 in transformed mouse cells, beta-globin in duck erythrocytes and Alu family sequences in human tissue culture cells have all been isolated in association with the nuclear matrix (Pardoll & Vogelstein, 1980; Nelkin et al., 1980; Maundrell et al., 1981; Small et al., 1982).

The best evidence, however, for the specific requirement for matrix attachment during transcription, comes from a recent study on the ovalbumin gene (Robinson et al., 1982). Ovalbumin gene sequences are preferentially associated to the nuclear skeleton in the target

tissue; chick oviduct, but are not, in a non-target tissue; liver. A control probe complementary to beta-globin gene sequences shows that non-transcribed sequences are not associated to the matrix in oviduct nuclei.

Thus all of the proteins which are candidates as key intermediary proteins; non-histone chromosomal and matrix proteins, hnRNP particle proteins, contractile proteins, and also the different types of receptor, may all interact in the process of gene transcription.

Actin and the other contractile proteins are also found in large amounts in the cytoplasm, in a variety of skeletal filaments and matrices. These extend throughout the cell and have many functions including maintenance of structure and shape, cell mobility and surface attachment. If actin does interact with oestrogen or its receptors, this could help to explain the existence of type II cytoplasmic binding sites, and the numerous reports of oestrogen-specific sites in the membranes and cytoskeletons of various cells (Pietras & Szego, 1979; Puca et al., 1981; Puca & Sica, 1981). There is some variation in the forms of actin found in the different cell types which may result from these forms having different functions or locations in the different cells.

Although it turns over rapidly in the uterus, actin is not however, one of the small number of uterine proteins specifically induced between 2 and 4 hours of oestrogen stimulation. The attempts in this study to identify these have succeeded only in showing that two proteins of MW about 40,000 and 20,000 are consistently induced early after oestrogen administration. There is no evidence that these <u>are</u> the key intermediary proteins we set out to investigate. The latter

may be at too low a level to detect by the methods used.

It seems likely that many of the problems of the control of gene expression in hormone-responsive tissues can only be resolved using the elegant and sensitive tools of recombinant DNA technology. Cloned cDNAs and genomic sequences have so far been used to show that increased levels of mRNA mirror increases in the cellular content of many hormone-induced proteins (Palmiter, 1973; Parker & Scrace, 1978). They have been used to show that the protein induction can result from increased transcription of the specific genes (Palmiter, 1973; Ryffel et al., 1977; Swaneck et al., 1979a), and other studies have indicated that hormones can influence the stability and turnover of specific mRNA species (Guyette et al., 1979). More recently, cloned genes have been used to show that hormones produce their effects by interacting with the DNA close to the activated genes. Two systems have been particularly informative in this respect. Both these systems involve glucocorticoid-inducible genes - rat liver α_2 μ-globulin and the mouse mammary tumour virus (MMTV) DNA. Genomic DNA coding for α_2 μ-globulin and cDNA to MMTV RNA were each cloned by different research groups into vector plasmids carrying marker enzyme genes, and were used to transfect host cells lacking these enzymes. Selection of transformed cells led to the isolation of clones carrying the α_2 μ-globulin gene and MMTV cDNA (Kurtz, 1981; Hynes et al., 1981; Buetti & Diggelman, 1981). The host cells used in both these sytems contained glucocorticoid receptors and these hormones were shown to induce the expression of the cloned DNAs. This showed that the DNA specifying their response was included in the cloned sequences.

The MMTV study has progressed further using chimaeric plasmids constructed from the terminal repeat sequences of the MMTV gene and a

cloned marker gene specifying dihydrofolate reductase which is not normally under glucocorticoid control. Cells transformed with such a plasmid acquire dexamethasone-inducible expression of dihydrofolate reductase (Lee et al., 1981), thus showing that in this system, hormonal responsiveness is associated with the long terminal repeat of the viral DNA.

The system of the oestrogen-inducible uterine proteins is more complex since there are no known products. This makes the cloning of individual DNAs complementary to single specific messengers impossible, and the making of specific probes difficult. Some recent progress has been made in this laboratory in cloning some of the DNA complementary to the polyadenylated messengers appearing in the polysome population between 2 and 4 hours of oestrogen administration. The isolation of these was described at the end of section 1.5.3, and these should include the sequences coding for at least some key intermediary proteins. With such cloned sequences, it should be possible to quantify and study oestrogen-induced transcription and to trace more precisely the kinetics of their appearance and processing in the nuclear and cytoplasmic RNA populations.

Notwithstanding the contribution of genetic manipulation to the study of hormone action, it cannot at the moment answer all problems. Thus although one might identify, through cloned sequences, many species of uterine mRNA induced by oestrogen it is technically very difficult to use an mRNA or a cloned derivative of it to identify a protein. It is the proteins however, on which the hormonal response of a cell depends, and it is clearly important to identify them. Also, it is unlikely that all the responses of a cell to a steroid hormone will be at a transcriptional level and it would be of tremendous advantage in

studying the effects of oestrogen on possible post-transcriptional and translational control of gene expression in the uterus, if induced proteins and antibodies to them were available as probes. For these reasons it is unfortunate that the work described in this thesis has not provided a more usable indicator of oestrogen-induced growth.

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