STUDIES ON BRAIN PROTEIN SYNTHESIS IN RELATION TO SEXUAL DIFFERENTIATION AND MATURATION IN THE RAT

HELEN F. STANLEY

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I declare that the work presented in this Thesis is the result of my own independent investigation apart from the studies in Chapter 7 involving the H-POA analysis by high performance liquid chromatography with electrochemical detection which were carried out in collaboration with Dr. A.G. Watts (MRC Brain Metabolism Unit, Edinburgh).

The work has not been and is not being concurrently submitted for candidature for any other degree.

Dedicated to my Mother

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ABBREVIATIONS

A_{260}	Absorbance at 260 nanometers
ATP	Adenosine 5' triphosphate
cm	Centimetre
cpm	Counts per minute
Ci/mmol	Curies per millimole
CO_2	Carbon dioxide
$CaCl_2$	Calcium Chloride
$Cu\ SO_4$	Copper sulphate
°C	Degrees centigrade
dpm	Disintegrations per minute
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
EDTA	Ethylenediaminetetracetic acid
fmol	Fentomole
g	Gravitation force Gram
$HClO_4$	Perchloric acid
h	Hour
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate
mA	Milliamp
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
mg	Milligram
$Mg\ Cl_2$	Magnesium chloride
$Mg\ SO_4.7H_2O$	Magnesium sulphate
ng	Nanogram
NaOH	Sodium hydroxide
Na Cl	Sodium chloride
$Na_2\ CO_3$	Sodium carbonate

Abbreviations continued...

rpm	Revolutions per minute
r_{av}	Average radius
r_{max}	Maximum radius
RNA	Ribonucleic acid
RNAase	Ribonuclease
s	Seconds
TCA	Trichloracetic acid
Tris	Tris (hydroxymethyl) aminomethane
µM	Micromolar
µl	Microlitre
µg	Microgram
vol	Volumes
V	Volts
Vh	Volthours
v/v	Volume per volume
w/v	Weight per volume
W	Watts

ABSTRACT OF THESIS

The aim of this thesis was to investigate brain protein synthesis in male, female and androgenised female (4 day female injected with 1.25mg testosterone propionate) rats to determine whether sex differences in specific brain proteins occur during or as a result of sexual differentiation. Proteins from subcellular fractions of the hypothalamus-preoptic area (H-POA) were analysed by polyacrylamide gel electrophoresis (PAGE) and changes in brain protein synthesis were also investigated indirectly by measuring the content of 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in the H-POA and midbrain raphe (MR) by high performance liquid chromatography with electrochemical detection (LCED).

Developmental changes in brain proteins were detected by staining proteins in the soluble, nuclear, mitochondrial-lysosomal and microsomal fractions. Sex differences were found in H-POA soluble proteins at days 0, 25 and 80 and in soluble proteins from the 80 day pituitary gland. A 50,000 molecular weight protein was present in the 4 day male and androgenised female rat H-POA but not in the control female. The H-POA content of a protein with the electrophoretic characteristics of tubulin was higher in the male than the female at birth and the translation of H-POA mRNA *in vitro* showed that this sex difference was probably due to higher levels of tubulin mRNA in the male than the female. Analysis of the translation products of H-POA mRNA also showed developmental changes in proteins. Testosterone administration to 4 day females for 6h stimulated the synthesis of a 70,000 molecular weight protein and inhibited that of a 20,000 species. Synthesis of the latter protein was also less in the 4 day male than the control or androgenised female. Together with the findings of sex differences in two other H-POA proteins (37,000 and 21,000) these results suggest that the synthesis of specific proteins appears to be influenced by androgen at the mRNA level in the neonatal rat. By labelling proteins *in vivo* the synthesis of two low molecular weight proteins (approximately 14,000) was found to be influenced by the administration of testosterone to 4 day females. An incidental but important finding in the latter studies was the induction of a 77,600 molecular weight protein in the H-POA of a rat which had undergone a series of 'fit' episodes. Experiments carried out *in vitro* showed that both steroid and thyroid hormones could affect the synthesis of specific proteins in H-POA slices and in primary cultures of foetal rat hypothalamic neurons.

No major sex differences were found in the concentrations of 5-HT, 5-HIAA, DA, or DOPAC at any of the ages investigated, in either brain region. The 5-HIAA/5-HT molar ratio reached a peak at 4 days of age in both regions and a sex difference in the ratio occurred at day 80 in the H-POA (female greater than male) but not MR. The rate of 5HT synthesis, determined using an aromatic amino acid decarboxylase inhibitor, was greater in the 80 day female compared with the 80 day male and 5 times greater in the 80 day H-POA than the 4 day H-POA.

CONTENTS

CHAPTER 1

Introduction

1.1 Sexual Differentiation of the Brain: History

The adult patterns of gonadotrophin secretion and sexual behaviour in a number of species are known to be dependent not on genetic sex but on the endocrine status of the animal during a 'critical period' of development. Steinach (1912, 1913) was the first to report that male and female rats and guinea-pigs could be feminised and masculinised, respectively, by implanting gonads of the opposite sex at an early stage of development. In 1934, Goodman demonstrated that ovarian transplants in an adult, castrated male rat failed to show ovulation or corpora lutea formation, but if the same experiment was performed on an adult female, cyclical formation of corpora lutea was observed. Pfeiffer (1936) investigated more fully the effects of gonadectomy and transplantation in neonatal rats on the pattern of gonadotrophin output in the adult. His major findings were that male rats castrated at birth and transplanted with an ovary when adult showed corpora lutea formation in the transplant. The same results were obtained when the experiment was performed on female rats. However, neither male rats in which the testes were transplanted to the neck at birth and which received an ovarian graft when adult nor female rats transplanted with testes at birth showed corpora lutea formation in the ovary. The female rats were also shown to enter a state of constant vaginal oestrus after puberty. Pfeiffer therefore established that the sex difference in gonadotrophin output is dependent upon the nature of the differentiated gonad during the neonatal period and postulated that the anterior pituitary gland was undifferentiated at birth. That is the gonad, in his view, caused sexual differentiation by a direct effect on the anterior pituitary gland.

Testosterone was found to be as effective as a testicular graft in preventing the occurrence of oestrous cycles. A single injection of 1.25 mg testosterone propionate (TP) in the female rat ('androgenised female') from 0 to 10 days after birth (the 'critical period' of sexual differentiation) permanently abolished cyclical gonadotrophin release (Barraclough, 1961; Barraclough and Gorski, 1961) and changed the patterns of female sexual behaviour (Segal and Johnson, 1959; Barraclough and Gorski, 1962; Swanson and van der Werff ten Bosch, 1964, 1965). The sterilising action of testosterone was found to be dose- and age-dependent (Gorski, 1968; Barraclough, 1961; Brown-Grant, 1974) and although as little as 10μg TP were effective (Barraclough and Gorski, 1962; Gorski and Barraclough, 1963), animals injected with this low dose exhibited what was termed a 'delayed anovulatory syndrome', characterized by normal cyclic and ovulatory behaviour at puberty followed by anovulation from about 90 days of age (Gorski, 1966 and 1968; Swanson and van der Werff ten Bosch, 1964). Swanson and van der Werff ten Bosch (1964, 1965) showed that female rats could be masculinised prenatally by either injecting pregnant rats with large doses (10-25mg) of TP, or injecting the rats 'in utero' with 20-100μg TP 1 to 4 days before birth.

The site of action of testosterone in producing the masculinising effects in female rats was debatable at first, but clearly three sites of action were possible: the ovary, the pituitary gland and the central nervous system. To investigate an ovarian site of action the ovaries from testosterone-treated females were transplanted into a 'normal' adult female that had been ovariectomised; ovulation was found to occur cyclically in the ovarian grafts (Bradbury, 1941; Harris, 1964; Harris and Levine, 1965). The experiments of Segal and Johnson (1959) and Adams Smith and Peng (1966) showed that pituitary glands from androgenised females transplanted under the median eminence (ME) of normal females that had been hypophysectomised were capable of maintaining oestrous cycles, although these cycles were of longer duration and less regular than those in untreated adult female rats. Together with the earlier classic studies of Harris and Jacobsohn (1952) in which male pituitary glands were grafted under the ME of hypophysectomised females, the data of Segal and Johnson (1959) and Adams-Smith and Peng (1966) suggested that the brain rather than the pituitary gland was the main site of the masculinising action of the testicular hormones. A central action of testosterone was supported by the observations that central nervous depressant drugs (e.g., chlorpromazine, pentobarbital and phenobarbital) could block the effects of testosterone (Arai and Gorski, 1968a). Further evidence for a hypothalamic or preoptic area (POA) site of action of testosterone came from electrical stimulation and lesion studies (Barraclough, 1961; Barraclough and Gorski, 1961).

Therefore, animals of either sex which are exposed to testosterone during the 'critical period' of sexual differentiation become incapable of maintaining cyclic gonadotrophin output and normal patterns of sexual behaviour. There is evidence that similar mechanisms for sexual differentiation of the brain also operate in other non-primate species such as the mouse (Barraclough and Leathem, 1954), hamster (Swanson, 1966; Alleva, Alleva and Umberger, 1969), guinea-pig (Brown-Grant and Sherwood, 1971), and sheep (Short, 1974; Karsch and Foster, 1975). Despite a relative paucity of evidence, there are indications that similar principles of sexual differentiation also apply to primates. Testosterone administration to pregnant rhesus monkeys results in masculinisation of female offspring as assessed by certain behaviour patterns (Goy, 1970). However, at present there are insufficient data to extrapolate these principles of sexual differentiation to the human.

1.2 Characteristics of the Androgenised Female Rat

The androgenised female rat is characterised chiefly by the inability to show a preovulatory surge of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and a reduced ability to show lordosis. Other abnormalites are also seen. For example, androgenised female rats show precocious puberty. Vaginal opening is advanced by about 7-10 days compared with that in normal female rats (Segal and Johnson, 1959; Harris and Levine, 1962; Chiappa and Fink, 1977; Sarkar and Fink, 1979) and the animals are in a state of constant oestrus with persistent vaginal cornification. Structural and functional changes occur in the ovary (Peters, Sørensen, Byskov, Pedersen and Krarup, 1970; Stegner and Poelmann, 1970) and uterus (Tushimaa and Johannson, 1971; Lobl, Trotta and Brumberger, 1974). The ovaries are small and white and contain follicles but no corpora lutea. Neonatal administration of TP also appears to reduce gonadotrophin synthesis and release during the prepubertal period (Barraclough, 1966; Chiappa and Fink, 1977;

Sarkar and Fink, 1979). The ovary is far less responsive to LH (Mennin, Kubo and Gorski, 1973) and the changes reported in 3-β-hydroxysteroid dehydrogenase activity in the ovary of the pre-pubertal animal which might lead to altered steroid synthesis (Peters et al., 1970) could contribute to the abnormal synthesis of gonadotrophins seen in androgen-sterilised rats (Chiappa and Fink, 1977). Although the pituitary of the androgenised female can be induced to release an ovulatory quota of LH by administration of luteinising hormone-releasing hormone (LHRH) (Borvendeg, Hermann and Bajusz, 1972) and electrical stimulation of the POA (Gorski and Barraclough, 1963; Terasawa, Kawakami and Sawyer, 1969), the responsiveness of the pituitary gland to LHRH, which is known to change with the hormone environment (Debeljuk, Arimura and Schally, 1972; Aiyer and Fink, 1974) is reduced in the androgenised female rat (Barraclough and Turgeon, 1974; Fink and Henderson, 1977). This change in pituitary responsiveness could conceivably be due to an effect of TP on the hypothalamus leading to a change in LHRH output which thereby affects pituitary responsiveness. However, although testosterone appears to act primarily on the brain (Barraclough, 1966; Harris, 1964, 1970; Adams-Smith and Peng, 1966) an action on the pituitary gland or indeed the gonads cannot be entirely ruled out.

Prolactin secretion is elevated in androgenised female rats (Mallampati and Johnson, 1974). Oestrogens are known to stimulate prolactin synthesis and release (Ratner, Talwalker and Meites, 1963; Neill, 1980) and, therefore, the high prolactin levels are possibly due to the constant secretion of oestrogen from the polyfollicular ovary (Naftolin, Brown-Grant and Corker, 1972). Gunnet and Freeman (1982) have shown that the normal patterns of prolactin secretion in male (acyclic) and female (cyclic) rats can be partially reversed by neonatal administration of TP to female rats or by castrating neonatal males.

1.3 Normal Patterns of Gonadotrophin Output

Barraclough (1966) proposed the theory of dual hypothalamic control of gonadotrophin release on the basis of experiments which showed that after priming with progesterone, electrical stimulation of the ventromedial-arcuate nucleus but not the medial POA (mPOA) could cause ovulation in androgenised female rats. Barraclough suggested that the mPOA was the major centre involved in the control of cyclicity ('surge centre') and that on proestrus, activation of this centre caused the ventromedial-arcuate region, normally involved in 'tonic' gonadotrophin output, to signal an ovulatory discharge of gonadotrophins from the pituitary gland. The basic concept of the control of gonadotrophin secretion has not changed dramatically. However, it is now known that, apart from at the time of the preovulatory surge, gonadotrophins are released in a pulsatile, not a 'tonic', manner (Gallo, 1980), and that LHRH neurons originate chiefly in the septal and mPOA and suprachiasmatic (SCN) nuclei and project directly to the external layer of the ME (Merchenthaler, Kovācs, Lovāsz and Sētālo, 1980; Rethelyi, Vigh, Sētālo, Merchenthaler, Flerkō and Petrusz, 1981; Witkin, Paden and Silverman, 1982; Shivers, Harlan, Morrell and Pfaff, 1983). The latter studies suggest that a surge of LH could occur as a consequence of LHRH release from the LHRH neurons that project directly from the POA to the ME, and that the ventromedial-arcuate region is not necessarily involved in the surge. A few perikarya containing immunoreactive LHRH have been demonstrated in the medial-basal hypothalamus (MBH) by some workers (Kelly, Ronnekliev and Eskay, 1982) and these neurons could conceivably be involved in the control of 'pulsatile'/'basal' mode of LH release which occurs even when the MBH is separated surgically from the mPOA (Blake and Sawyer, 1974).

Throughout most of the oestrous cycle secretion of LH and FSH is controlled by negative feedback actions of oestradiol and progesterone, which appear to act both at the level of the hypothalamus by inhibiting LHRH release (Smith and Davidson, 1974; Sherwood and Fink, 1980) and at the anterior pituitary gland inhibiting gonadotrophin secretion (for review see Labrie et al., 1978). Progesterone potentiates the inhibitory effect of low plasma oestradiol concentrations on LH release (McCann, 1962; Goodman, 1978). During the afternoon of dioestrus, plasma concentrations of oestradiol-17β begin to rise, reaching a peak during the morning and early afternoon of proestrus. Oestradiol, by a positive feedback action on the mPOA (Goodman, 1978), stimulates a surge of LHRH and increases pituitary responsiveness to LHRH (Aiyer and Fink, 1974). These two events, acting by way of a 'cascade', result in the preovulatory LH surge (Fink, 1979a and b). At this stage of the cycle, the effects of oestrogen are enhanced by progesterone, secreted in response to LH secreted on the afternoon of proestrus (e.g., Everett, 1964; Aiyer and Fink, 1974; Tapper, Greig and Brown-Grant, 1974; Fink and Henderson, 1977).

Pulsatile gonadotrophin release seems to be dependent upon pulses of LHRH released into the portal vessels, as shown by studies of LHRH release into hypophysial portal blood (Sarkar and Fink, 1980) and the effects of intravenous injections of anti LHRH-serum in ovariectomised rats (Snabes and Kelch, 1979). In the rat, pulsatile release of LH is most easily seen after gonadectomy (Gay and Seth, 1972; Gallo, 1980). Noradrenaline (NA) and dopamine (DA) but not 5-hydroxytryptamine (5-HT) (Arendash and Gallo, 1979; Soper and Weick, 1980) have been implicated in the modulation of pulsatile release (for a review see Barraclough and Wise, 1982).

A diurnal release of LH can also be detected in long-term ovariectomised female rats treated with oestradiol (Caligaris, Astrada and Taleisnik, 1971; Legan, Coon and Karsch, 1975; Henderson, Baker and Fink, 1977) and this is due in part to a daily surge of LHRH (Sarkar and Fink, 1980). The diurnal rhythm of LH depends upon the integrity of the SCN (Coen and MacKinnon, 1980; Wiegand and Terasawa, 1982) and appears to be driven by a different neural mechanism to that involved in pulsatile LH release (Watts and Fink, 1981). However, the relevance of the circadian pattern of LH release to the control of spontaneous LH release in the intact animal has not been established.

In contrast to the inhibitory role of NA in the regulation of pulsatile LH release, data from experiments on NA turnover, α-methyl-p-tyrosine (αMPT) administration, intraventricular infusion of NA and from measurements of LHRH in portal blood from immature rats treated with para-chlorophenylalanine (PCPA) and αMPT, suggest a facilitatory role for NA in the preovulatory surge (for review, see Kalra and McCann, 1973; Fink and Geffen, 1978; Weiner and Ganong, 1978; Barraclough and Wise, 1982). Dopamine involvement in the LH surge control mechanism is still controversial but the majority of studies suggest an inhibitory influence (Barraclough and Wise, 1982).

1.4 Steroid Effects on Neurotransmitter Systems Involved in The Neural Control of Reproduction

Peptidergic and non-peptidergic neuronal pathways are known to be involved in the control of reproductive function. Gonadal steroids can influence the functional activity of these systems by acting at the level of transmitter synthesis, at transmitter degradation or by effects on receptor sites.

The effects of steroids on non-peptidergic transmitter synthesis have been well documented, principally by way of studies on the activity of rate limiting enzymes. There is little evidence in the central nervous system for a direct regulatory control of catecholamine synthesis at the level of the genome although ten days after castration, tyrosine hydroxylase (TOH) in the ME did show an elevated V_{max} but no change in K_m value suggesting an increase in enzyme content (Kizer, Humm, Nicholson, Greeley and Youngblood, 1978). As far as peptide and protein hormones are concerned, the effect of estradiol on prolactin (Maurer, 1982) and gonadotrophin synthesis (Godine, Chin and Habener, 1980), for example, does involve alterations in mRNA levels for these hormones.

The effects of steroids on TOH activity are largely inhibitory as shown, for example, by an elevation of the K_m value for pteridine cofactor by progesterone and testosterone (Beattie and

Soyka, 1973; Beattie and Martin, 1976). Tyrosine hydroxylase activity is elevated in the hypothalamus of ovariectomised rats (Beattie, Rodgers and Soyka, 1972). The changes in activity appear to be restricted to the ME and the fact that lesions of the ventral NA tracts which resulted in greater than an 85% decrease in dopamine-β-hydroxylase (DBH) activity had no effect on TOH, suggests that the feedback effects of gonadal steroids are mediated largely by the tubero-infundibular DA system (Kizer, Muth and Jacobowitz, 1976). Not only estradiol, but also 2-hydroxy-estradiol, a catechol derivative, can inhibit TOH activity both _in vivo_ and _in vitro_ (Lloyd, Boyd, Walega, Ebersole and Weisz, 1982). Androgens are also believed to have negative feedback effects on gonadotrophin output by way of DA pathways but the control mechanism is, as yet, unknown (Vermes. Varszegi, Toth and Talegdy, 1978; Simpkins, Kalra and Kalra, 1980). The functional integrity of central NA systems is required for the LH surge (for review see Barraclough and Wise, 1982) and hypothalamic DBH activity was shown to be highest during the evening of proestrus in cycling rats (Banerji, Parkening and Collins, 1982).

Tryptophan hydroxylase activity in the hypothalamus was not affected significantly by gonadectomy, with or without steroid replacement (Kizer, Palkovitz, Kopin, Saavedra and Brownstein, 1976b). However, gonadal steroids have been shown to affect both the 5-HT brain content and uptake mechanisms (Vogel, Janowsky and Davis, 1970; Ladowsky and Gaziri, 1970; Giulan, Pohorecky and McEwen, 1973; Kizer, Palkovits, Zivin, Brownstein, Saavedra and Kopin, 1974). Therefore, steroid modulation of 5-HT does occur but not necessarily, it seems, at the enzyme level.

The cholinergic neuronal system is affected by estradiol which increases both choline acetyltransferase (CAT) activity in the mPOA, cortico-medial amygdala and hippocampus, and the actual enzyme levels in the POA, as shown by immunocytochemistry (Luine, Khylchevskaya and McEwen, 1975; Luine, Park, Joh, Reis and McEwen, 1980). This increased enzyme synthesis is an example of a genomic action of estradiol, inducing new protein synthesis. One of the key markers of estradiol action in the pituitary and MBH, as in the uterus, is an elevation of glucose-6-phosphate dehydrogenase activity, giving rise to pentose sugars for RNA synthesis resulting from estradiol-activated transcription (Luine, Khylchevskaya and McEwen, 1974).

In addition to effects on synthesis, transmitter catabolism is also modulated by steroids. Removal of monoamines from the synaptic cleft by re-uptake mechanisms normally follows transmitter release. Oestrogen has been shown to inhibit DA uptake whereas 5-HT uptake is enhanced by progesterone (Wirz-Justice, Hackmann and Lichtsteiner, 1974), but how these effects occur is not known. The activity of monoamine oxidase (MAO) in the MHB and cortico-medial amygdala is inhibited by estradiol (Luine et al., 1975).

A number of studies have shown that the degradation of peptide hormones may be as important as their synthesis in regulating control of gonadotrophin output. The activity of both hypothalamic and pituitary peptidases varies throughout the oestrous cycle (Kuhl, Rosniatowski and Taubert, 1978; Griffiths and Hooper, 1973) with ovarian steroids elevating peptidase activity. The activity of these enzymes varies little in the male brain and androgenised female rat brain does not show cyclic variations in enzyme activity (Kuhl et al., 1978). Studies on extracts of hypothalamic cytosol suggest that changes in enzyme activity are not due to variations in actual synthesis of peptidases but perhaps an allosteric effect of the steroids on the enzymes (Swift and Crighton, 1979).

Steroid hormones can also influence neurotransmission by effects upon receptor sites. Thus for example, in addition to the modulatory role of steroids in the self-priming effect of LHRH (Aiyer and Fink, 1974; Aiyer, Chiappa and Fink, 1974) there is evidence that estradiol can modify the number of LHRH binding sites on pituitary cells (Clayton and Catt, 1981; Clayton, Solano, Garcia-Vela, Dufau and Catt, 1980; Meidan and Koch, 1981). Two sites of differing affinity have been described (Spona, 1974) and it has been shown that the high affinity site, reduced in chronically ovariectomized rats, is increased by oestradiol (Clayton and Catt, 1981). Oestrogen also induces muscarinic receptors in the ventromedial nucleus (VMN) (Rainbow, DeGroff, Luine and McEwen, 1980) and there are reports of effects of oestrogen on α and β adrenoreceptors in the hypothalamus and cerebral cortex (Wilkinson, Herdon, Pearce and Wilson, 1979; Wagner, Crutcher and Davis, 1979). The number of DA receptors in the amygdala and cerebral cortex is also influenced by the early steroid environment. Males and androgenised females have higher levels of DA receptors than female rats (Karakiulakis, Knight, Powell and Thomas, 1978; Jalilian-Tehrani, Karakiulakis, Le Bland, Powell and Thomas, 1982;). These results suggest a sexual dimorphism in projections from the amygdala to the hypothalamus, involving DA pathways. Steroids can therefore act postsynaptically to modify neural transmission. Neurotransmitter sensitive adenyl cyclase activity is higher in the female rat hypothalamus than the male and oestradiol can also inhibit this enzyme in vitro (Mohsen, Butterworth and Thomas, 1978; Ani, Butterworth and Thomas, 1980). Therefore, in addition to the mechanisms described, it appears that oestradiol can also affect neurotransmission by way of the second messenger.

1.5 Sexual Behaviour

The classical behavioural endpoint in the female rodent, during which a state of maximal receptivity is reached, is the lordosis response. Occurring around the time of ovulation in the cycling rat (0200-0400h of oestrus) or elicited by steroid priming in the long-term ovariectomised rat model, the lordosis reflex is the result of steroid effects on both the patterns of electrical activity and protein synthesis in target neurons (Pfaff, 1980, 1983).

The primary centres involved in the behavioural effects of oestradiol were discovered using a number of complementary techniques. Oestradiol target cells have been extensively mapped using ^{3}H-oestradiol autoradiography (Pfaff, 1968; Stumpf, 1968; Pfaff and Keiner, 1973), and subsequent experiments in which localised oestradiol implants facilitated reproductive behaviour (Lisk, 1962; Barfield and Chen, 1977; Dörner, Döcke and Moustafa, 1968; Yanase and Gorski, 1976; Davis, McEwen and Pfaff, 1979) highlighted the importance of the mPOA, anterior hypothalamus and VMN in the control of sexual behaviour. It is now apparent that the mPOA and the VMN of the hypothalamus fulfill opposite roles in the expression of oestradiol-mediated behaviours. Electrophysiological recordings in the VMN and anterior hypothalamus of ovariectomised rats given oestradiol showed increased neuronal firing rates in these areas compared to control rats (Bueno and Pfaff, 1976). Similar studies on the mPOA, however, show a reduction in cell firing after oestradiol. Results from such experiments suggest that the VMN is involved in facilitating, while the mPOA inhibits the lordosis response. Lesions of the VMN inhibit lordosis (Carrer, Ash and Aron, 1973; Dörner, Döcke and Gotz, 1975; Kennedy, 1964; La Vaque and Rodgers, 1974) while lesions of the mPOA were shown to facilitate lordosis (Powers and Valenstein, 1972). The net effect of the mPOA on lordosis is inhibitory, and so it appears that oestradiol, by inhibiting cell firing in the mPOA, may facilitate lordosis.

In addition to the electrophysiological effects on the cells which selectively bind it, oestradiol has marked effects on the biosynthetic and secretory mechanisms within the cells of the VMN (Cohen and Pfaff, 1981).

The oestrogenic control of lordosis is a slow process, consistent with genomic involvement. Oestradiol has to be elevated for at least 16h before any facilitatory effect on behaviour can be detected (Green, Luttge and Whalen, 1970). However, whilst maximal nuclear binding of ^{3}H-oestradiol is achieved by 4h, maximal sexual receptivity does not occur until approximately 20h (McEwen, Pfaff, Chaptal and Luine, 1975), a time when nuclear oestradiol is low. From the latter studies, and from data which have shown the abolition of lordosis by anisomycin (Rainbow, Davis and McEwen, 1980; Rainbow, McGinniss, Davis and McEwen, 1982) it appears that oestrogen facilitates sexual behaviour by an action on the genome. Part of the new protein synthesis may be stimulation of progesterone receptors, known to occur in the POA and MBH as a result of oestradiol action (MacLusky and McEwen, 1978; Blaustein and Wade, 1978; MacLusky and McEwen, 1980a). In a study comparing sexual receptivity with progesterone receptor levels in these sites (Parsons, MacLusky, Krey, Pfaff and McEwen, 1980) a 'threshold' level of 30% of maximal induction in the hypothalamus was associated with the ability to display lordosis behaviour. The role of progesterone receptor induction in the mediation of sexual receptivity is not clear, particularly when it has been shown that receptivity can be induced by oestradiol alone (Pfaff, 1970; Edwards, Whalen and Nadler, 1968; Davidson, Rodgers, Smith and Bloch, 1968). The enhancement of lordosis by progesterone may be due to an inhibition by the steroid of the activity of the raphe-septal pathway. Lesions of both the raphe nuclei and the septum facilitate lordosis (Beach, 1967; Komisurak, 1974) as do 5-HT receptor antagonists (Everitt, Fuxe, Hökfelt and Jonsson, 1975) and systemic administration of inhibitors of 5-HT synthesis, such as PCPA (Everitt et al., 1975; Meyerson and Lewander, 1979). Progesterone can reduce neuronal activity in the raphe-septal loop, thereby removing or reducing an inhibitory input to the hypothalamus (Kow, Malsbury and Pfaff, 1974). Raphe discharge is in fact lower in darkness than in light, and this may be associated with the increased sexual activity that occurs in the dark in the rat (Mosko and Jacobs, 1974). Dopamine and 5-HT seem to have primarily inhibitory effects on sexual behaviour (Dewsbury, 1975; Everitt et al., 1975; Meyerson, 1975).

Experiments carried out to test whether LHRH could facilitate lordosis have revealed that LHRH can enhance the lordosis response to low doses of oestradiol in hypophysectomised, ovariectomised rats (Pfaff, 1973; Moss and McCann, 1973). Blockade of the effect by LHRH antiserum infused into the midbrain central grey suggests that LHRH may play an important role in both the ovulatory LH surge and the lordosis response by way of pathways to the central grey (Sakuma and Pfaff, 1980, 1983) .

Separate neuronal systems are involved in the control of male and female reproductive behaviours. Androgen-sensitive pathways projecting from the mPOA by way of the medial forebrain bundle (MFB) are important in eliciting male mating behaviour (Caggiula, Antelman and Zigmond, 1973) while lesions of the MFB in the female have no effect on reproductive behaviour (Modianos, Flexman and Hitt, 1973). Also, whereas in the female the POA primarily inhibits lordosis, lesions of the POA decrease or abolish mating behaviour in male rats. Outputs from the MBH by way of the central grey to the lateral reticular formation are of major importance in the female, but are not required for male sexual behaviour (Pfaff, 1980).

1.6 Site of Action of Testosterone

Both anovulatory sterility and masculine patterns of sexual behaviour have been observed in adult female rats after the implantation of testosterone, just after birth, in brain areas which are known to regulate reproductive functions (Nadler, 1972, 1973;

Sutherland and Gorski, 1972; Hayashi and Gorski, 1974; Lobl and Gorski, 1974). Together with studies using tritiated steroid autoradiography (Pfaff, 1968; Sar and Stumpf, 1973) and morphological and electrophysiological techniques (Raisman and Field, 1973; Dyer, MacLeod and Ellendorf, 1976) these approaches have indicated that those brain regions which can specifically bind androgen and are involved in the control gonadotrophin output, exhibit a number of sexually dimorphic features.

A sexually dimorphic nucleus within the POA has been described (Gorski, Shryne and Southam, 1978; Gorski, Harlan, Jacobson, Shryne and Southam, 1980) together with reports of sex differences in the dendritic branching pattern (Greenough, Steerman and De Voogd, 1977), synaptic terminals (Raisman and Field, 1973) and nuclear (Dorner and Staudt, 1968; Pfaff, 1966) and nucleolar (Pfaff, 1966) size in the same or nearby regions. The volume of an 'intensely staining component' of the POA is greater in the male than the female rat and can be significantly influenced by the neonatal steroid environment (Gorski, et al. 1978). However, although the mouse brain undergoes sexual differentiation, in contrast to the rat, sex differences in the volume of this nucleus have not been detected (Young, 1982). Similar patterns of synaptic organisation seen in the POA of male and female rats found by Raisman and Field (1973) have also been described in the arcuate nucleus (Matsumoto and Arai, 1980,1981) and in the medial amygdala (Nishizuki and Arai, 1981a and b). Therefore, the amygdala, POA and arcuate nucleus, which are all immature at birth and therefore susceptible to the differentiating effects of gonadal steroids show sexually dimorphic anatomical features. The SCN which is required for the preovulatory LH surge (Coen and McKinnon, 1976), is also influenced structurally by the neonatal hormone environment (Le Blond, Morris and Karakiulakis, 1982).

Although the androgenised female rat does not ovulate under normal conditions, electrical stimulation of the POA/anterior hypothalamus, as in an adult male rat with a transplanted ovary, will elicit an LH surge (Quinn, 1966; Terasawa, Kawakami and Sawyer, 1969; Everitt, Holsinger, Zeilmaker, Redmond and Quinn, 1970). The results from these and a number of other studies have suggested that projections from extrahypothalamic sites could also be sexually differentiated. In their classic morphological study, Raisman and Field (1973) showed that the dorsal POA, which is innervated by fibres in the stria terminalis from the amygdala, was sexually dimorphic with respect to its non-strial synaptic connections. Twice as many spine synapses existed in this region in the male than the female and the synaptic patterns could be altered by endocrine manipulations of the neonate. Electrophysiological studies (Dyer et al., 1976) have shown that significantly more neurons connecting the POA with the MBH receive synaptic connections from the amygdala in the male than in the female rat.

1.7 Nature of The Hormone Which Causes Sexual Differentiation

Within the areas which exhibit sexually dimorphic features the conversion of testosterone to oestrogen metabolites has been reported to occur from as early as 21 days of gestation as a result of an aromatase enzyme system (Reddy, Naftolin and Ryan, 1974; Naftolin, Ryan, Davies, Reddy, Flores, Petro and Khun, 1975).

Oestrogens are equally as, or more, potent than testosterone in causing androgenisation (Gorski, 1963; Gorski and Barraclough, 1963; Doughty, Booth, McDonald and Parrot, 1975), and synthetic antioestrogens, for example MER-25 and CI-628, are effective inhibitors of the process (McDonald and Doughty, 1972; McEwen, Lieberburg and Krey, 1977). Dihydrotestosterone (DHT), a non-aromatizable androgen (McGuire, Hollis and Tompkins, 1960) is largely ineffective in causing androgenisation (McDonald and Doughty, 1972; Luttge and Whalen, 1970; Brown-Grant, Munck, Naftolin and Sherwood, 1971). For these reasons it has been proposed that oestrogen rather than testosterone itself renders the hypothalamus acyclic. The in vivo conversion of ^{3}H-testosterone to ^{3}H-oestrogen has been demonstrated (Weisz and Gibbs, 1974; Lieberburg and McEwen, 1975) and 1,4,6-androstatriene-3,17-dione (ATD), an inhibitor of the aromatase system (Lieberburg, Wallace and McEwen, 1977) can block the central effects of testosterone (Vreeburg, van der Vaart and van der Schoot, 1977; Booth, 1977; McEwen et al., 1977). Synthetic non-steroidal oestrogens, diethylstilbestrol (DES) and RU 2858, for example, are highly potent in causing sexual differentiation (Slaughter, Wilken, Ryan and Naftolin, 1977) and the effects can again be blocked by administration of anti-oestrogens (Doughty et al., 1975). It does appear, however, that oestrogen may not be the only mediator of the central actions of testosterone in the neonatal rat. Sexual differentiation can be regarded as being made up of defeminising and masculinising components (Beach, 1975). Masculinisation can be described as an enhanced readiness to show male mounting behaviour whereas defeminisation is characterised by a reduced ability to show lordosis and an oestrogen-induced gonadotrophin surge. Studies on the action of ATD have revealed that defeminisation can be blocked without affecting masculinisation (Vreeburg et al., 1977) and suggest that whereas defeminisation may be attributable to oestrogen, both androgen and oestrogen bring about masculinisation (Nadler, 1969; Ward and Renz, 1972; Clemens, Gladue and Coniglio, 1978). Experiments in which the antiandrogen, cyproterone acetate, was injected with TP in neonatal females also showed, by examination of the ovaries in adulthood which contained corpora lutea, that the masculinising effects of androgen could be abolished by antiandrogen (Neumann and Kramer, 1967). Support for a major role of aromatisation in defeminisation of the brain and a role for androgen in the expression of male behaviour has come from studies on the androgen-insensitive (Tfm) mutation (Beach and Buehler, 1977; Olsen, 1979; Shapiro, Levine and Adler, 1980). Although male Tfm rats are deficient in androgen receptors, they are defeminised and this defeminisation can be prevented by castration on day 2, the central aromatase and oestrogen receptor systems are not defective (Fox, 1975; Naess, Haug, Attramadal, Aakvaag, Hansson and French, 1976; Krey, Lieberburg, MacLusky and Davis, 1981).

The female rat may be protected from high maternal oestrogen levels in utero and the high plasma oestrogen levels in neonatal life by alpha fetoprotein (AFP), a plasma oestrogen-binding protein (Raynaud, Mercier-Bodard and Baulieu, 1971). Plasma concentrations of AFP are high during the first week of life and AFP has been identified within neurons in brain (Plapinger, McEwen and Clemens, 1973; Toran-Allerand, 1980a) although the protein is not synthesised in brain but in the liver. AFP has a high affinity for oestradiol (a K_d of 10^{-10}M at 4°C) but not testosterone and therefore testosterone is free to reach the brain where it is converted intracellularly to oestradiol. Synthetic oestrogens, which are 50-100 times more potent than oestradiol in inducing sexual differentition, have lower affinities for AFP (McEwen, Plapinger, Chaptal, Gerlach and Wallach, 1975) and the administration of antibodies to AFP can significantly interfere with the normal patterns of brain differentiation in neonatal rats (Mizejewski, Vonnegut and Simon, 1980).

In addition to its role in masculinising the brain in the male, it has been suggested that oestradiol may be essential for 'feminising' the female brain (Döhler and Hancke, 1978), challenging the basic concept that the developmental potential of the brain is inherently female. Post-natal treatment of female rats with tamoxifen, an oestrogen antagonist, results in permanent acyclicity and loss of female sexual receptivity (Hancke and Döhler, 1980). This defeminisation was not accompanied by a masculinisation of sexual behaviour and it could be counteracted by concomitant oestrogen administration. Döhler and Hancke (1978) have therefore proposed that the embryonic brain is neutral or 'asexual' and that low concentrations of oestrogen will induce a female type brain whereas higher oestrogen concentrations and possibly androgens masculinise the brain. This hypothesis also suggests that AFP, rather than sequestering oestrogen, may serve as a carrier or reservoir for the maternal oestrogens allowing uptake of oestrogen into neurons in late foetal and early neonatal life when the ovaries are still inactive. Support for this hypothesis comes from the finding that AFP is localised within neurons (Toran-Allerand, 1980a) and that embryonic neurons do not develop neuritic processes in the absence of oestrogens (Toran-Allerand, 1976) suggesting that development of the female phenotype also requires induction. However, tamoxifen is known to possess oestrogenic properties as shown by Koseki, Zava, Chamness and McGuire (1977), for example, who demonstrated induction of progesterone receptors in the uterus by tamoxifen. Therefore, in the experiments by Hancke and Döhler (1980), an oestrogenic effect of tamoxifen in causing defeminisation cannot be ruled out.

1.8 Steroid Receptors In Brain

The distribution of neurons that concentrate oestradiol and testosterone have been determined by autoradiography (Sar and Stumpf, 1973; Pfaffand Keiner, 1973). In general, neurons that take up both steroids are found predominantly in the mPOA, the basal hypothalamus and the amygdala.

The ontogeny of steroid receptors in brain could be responsible, in part, for the differences in response of the brain to hormones between the neonate and the adult. The general patterns of oestrogen and androgen receptor development seem to differ, with oestrogen receptors rising rapidly to adult levels during the 'critical period' of sexual differentiation (MacLusky, Lieberburg and McEwen, 1979; MacLusky, Chaptal and McEwen, 1979; Vito and Fox, 1981) whereas androgen receptors increase more slowly (Vito, Wieland and Fox, 1979; Lieberburg, MacLusky and McEwen, 1980).

McLusky et al. (1979) showed that oestrogen receptors started increasing at day 21 of gestation, reaching a plateau at approximately 6 days but the studies of Vito and Fox (1981) suggested that oestrogen receptors have almost reached adult levels by birth. The physicochemical characteristics of oestrogen receptors are reported not to change during development in brain, in contrast to their distribution (MacLusky, Chaptal, Lieberburg and McEwen, 1976). Oestrogen receptors are present in the cerebral cortex during the first 2-3 weeks of life but then disappear (MacLusky et al., 1976). The physiological significance of these oestrogen receptors is still unclear and no aromatase enzyme system has been detected in the cerebral cortex, suggesting that the local conversion of testosterone to oestradiol is not a source of hormone for binding to the receptor (Barley, Ginsburg, Greenstein and MacLusky, 1974; Lieberburg and McEwen, 1975).

Oestrogen receptors in the brain and pituitary gland are similar to cytoplasmic receptors in peripheral target tissues with respect to their affinity for labelled oestrogens and their binding specificity (MacLusky and Clark, 1980). However, differences in the biochemical responses to oestrogen are seen. In the uterus, oestrogen stimulates both RNA polymerase I and II whereas in brain only RNA polymerase I is stimulated by oestrogen (Peck, Miller and Kelner, 1978). The rate of 'transformation' of the oestrogen receptor is slower in the hypothalamus than the uterus (Fox, 1977; Linkie, 1977) and the depletion of cytoplasmic oestrogen receptors after oestrogen administration is less extensive in brain than in the pituitary or uterus (Lieberburg, MacLusky and McEwen, 1980;

Clark, MacLusky and Naftolin, 1982). These differences in oestrogen receptor transformation and nuclear translocation between the brain and peripheral target tissues may underlie the differences in responses to oestrogen.

A sex difference in the number of oestrogen and progesterone receptors within specific brain nuclei has recently been described (Rainbow, Parsons and McEwen, 1982). Although Flerkó, Mess and Illei-Donhoffer (1969), Vértes and King (1969), Maurer and Woolley (1971) and McEwen and Pfaff (1970) have demonstrated that the capacity of the brain to bind oestrogen is reduced in androgenised female rats, the results of Green, Luttge, and Whalen (1969) and Barley, Ginsburg, MacLusky, Morris and Thomas (1977) argue against a receptor defect in the androgenised female. However, the report of Rainbow et al. (1982) supports the hypothesis that the relative insensitivity of the male to gonadal steroids may be due to a lack of oestrogen receptors in the mPOA resulting in a loss of positive feedback.

1.9 Mechanism of Action of Steroid Hormones

1.9.1 General

A large body of evidence has been collected showing that steroid hormones exert their effects in target tissues primarily at the level of gene transcription (O'Malley and Means, 1974; Darnell, 1982; Spelsberg, Littlefield, Seelke, Martin, Toyoda, Boyd-Leinen, Thrall and Kon, 1983). The ultimate effect of the hormones appears to be the induction of specific proteins. Many studies have shown that the changes in protein synthesis are preceded by an increase in the activity of RNA polymerase I (Gorski, 1964), II (Glasser, Chytil and Spelsberg, 1972; Borthwick and Smellie, 1975) and III (Weil, Sidikaro, Stancel and Blatti, 1977) with a subsequent increase in the levels of ribosomal, messenger and transfer RNA. DNA synthesis is also stimulated in the uterus by oestrogen (Kaye, Sheratzky and Lindner, 1972) and an increase in DNA polymerase activity has been demonstrated (Harris and Gorski, 1976).

One of the most significant advances in the study of steroid hormone action was made by Jensen and Jacobson (1962) when they synthesised high specific activity ^{3}H-steroids in an attempt to analyse target tissue specificity and demonstrated the existence of a cytoplasmic protein which could selectively bind oestrogen and lead to the accumulation of the hormone within the tissue. Receptor proteins are believed to be the mediators of hormone action which bind to nuclear chromatin and regulate gene expression.

Steroid hormones are thought to enter the cell by passive diffusion although the presence of oestrogen receptors on the cell surface has been demonstrated (Pietras and Szego, 1977, 1979). Oestrogen was shown to bind to the cytoplasmic receptor, forming a non-covalent, high affinity complex (Maurer and Chalkley, 1967). Many workers have examined multiple forms of hormone receptors by analysing their sedimentation rate on sucrose gradients. Steroid receptors show a change in sedimentation constant with both temperature and ionic strength (Erdos, Best-Belpomme and Bessada, 1970; Jensen, Numata, Brecher and De Sombre, 1971). Oestrogen receptor monomers sediment at about 4S and associate with each other to form dimers or higher aggregates. Conversion of the 4S to 8S form does not require hormone binding (Jensen and De Sombre, 1973). In the rat uterus, interaction of oestrogen with the receptor protein causes a shift in the sedimentation constant from 4S to about 5.4S and corresponds to an increase in molecular weight from 70,000 to 130,000 (Notides, Hamilton and Auer, 1975). This process may be synonymous with the so-called 'transformation' process (Bailly, Le Fevre, Savouret and Milgrom, 1980) and is a prerequisite for 'translocation' of the hormone-receptor complex to the nucleus. Two oestrogen binding sites have been described in the cytoplasm and nucleus of uterine cells (Clark, Hardin, Upchurch and Eriksson, 1978; Eriksson, Upchurch, Hardin, Peck and Clark, 1978). The classical cytosolic oestrogen receptor (type I) has a high affinity for oestradiol (K_d = 0.8nM), a low binding capacity (0.6pmol/uterus) and is depleted from the cytosol after oestradiol administration. The type II sites have a lower affinity (K_d = 30nM), a higher capacity (2.0pmol/uterus), are not depleted after oestradiol treatment and may be involved in the effects of estradiol upon cell division. Schenborn and Karavolas (1983) have now provided evidence for two classes of high affinity progesterone binding sites in the hypothalamus.

The progesterone receptor is also composed of subunits. Studies on the progesterone receptor in the chick oviduct have shown that, in contrast to the oestrogen receptor, the dimer is made up of dissimilar subunits (A and B) which differ with respect to their size, charge and nuclear function (Schrader and O'Malley, 1972; Vedeckis, Schrader and O'Malley, 1980).

The suggestion that movement of the hormone-receptor complex from the cytoplasm to the nucleus occurred after hormone binding originated from the work of Jensen, Suzuki, Kawashima, Stumpf, Jungblut and De Sombre (1968) and Shyamala and Gorski (1969) who showed that with nuclear accumulation of bound oestradiol there was a concomitant depletion of the cytoplasmic receptor. The extent of translocation of oestrogen receptors to the nucleus is known to be related to the fluctuating plasma oestradiol concentration during the oestrous cycle in the uterus (Clark, Anderson and Peck, 1972; Ginsburg, MacLusky, Morris and Thomas, 1975), brain (Ginsburg et al., 1975) and pituitary gland (Ginsburg et al., 1975; Sen and Menon, 1978). Nuclear retention of the hormone-receptor complex is an important feature of the mechanism of hormone action and has been used as an index of agonist and antagonist properties of oestrogenic compounds (Clark, Pasko and Peck, 1977) since oestrogen antagonists appear to remain in the nucleus for longer than agonists.

Both DNA and chromosomal proteins, particularly the non-histone proteins, have been implicated in the binding of the hormone-receptor complex to chromatin. An intranuclear location of the 'acceptor' sites for the complex was demonstrated by fractionating nuclei previously exposed to ^{3}H-oestradiol-receptor and finding the radioactivity associated with the chromatin (O'Malley, Toft and Sherman, 1971; Spelsberg, Steggles and O'Malley, 1971; O'Malley, Spelsberg, Schrader, Chytil and Steggles, 1972). The experiments of Shyalama-Harris (1971) suggested that the hormone-receptor complex was bound to DNA itself, by demonstrating that DNAase treatment could release bound oestrogen from uterine nuclei. These results were confirmed by DNA-cellulose chromatography (Yamamoto and Alberts, 1972). Incubation of the

^{3}H-oestrogen-receptor complex (but not ^{3}H-oestrogen alone) with uterine chromatin resulted in retention of the complex on chromatin (Steggles, Spelsberg, Glasser and O'Malley, 1971) and removal of the histone proteins increased the number of binding sites (King and Gordon, 1972). The importance of the non-histone chromosomal proteins for nuclear binding of the progesterone receptor was demonstrated in experiments where chromatin was fractionated and reconstituted with histone and/or non-histone proteins from another tissue or species (Spelsberg et al., 1971; O'Malley et al., 1972;' Spelsberg, Steggles, Chytil and O'Malley, 1972). More recently, progesterone receptor recognition sites on the ovalbumin gene have been demonstrated (Mulvihill, Le Pennec and Chambon, 1982) lying 135-300 base pairs upstream from the transcription initiation site. It has been demonstrated that the A subunit preferentially binds to DNA whereas the B subunit does not (Schrader and O'Malley, 1972). The B subunit may bind to an acceptor site in chromatin, allowing the A subunit to interact with the regulatory regions of the DNA. It is not yet certain, however, whether the progesterone receptor, or other steroid hormone receptors, specifically recognises and binds to double stranded DNA (Mulvihill et al., 1982) or whether the receptor acts as an 'unwinding' protein and has a preference for single stranded DNA, supported by Schrader's group. Gronemeyer and Pongs (1980) have demonstrated, using photo-affinity labelling, that the metamorphic hormone β-ecdysterone, can be localised <u>in vivo</u> at the sites of hormone-induced puffs in the salivary gland chromosomes of Drosophila larvae. Schaltmann and Pongs (1982) also detected a protein in Drosophila larvae salivary glands and in cells in culture which exhibited characteristics of a steroid hormone receptor, including translocation from the cytoplasm to the nucleus. These results provide the first direct evidence for the *in vivo* localisation of a hormone-receptor complex at the site of hormone-activated genes.

1.9.2 Steroid Hormone Action In Brain

The effects of hormones on the brain depend to a large extent upon the age of the animal. In the newborn rat, sex steroids exert permanent effects upon the functioning of the hypothalamo-hypophysial axis whereas in the adult, the effects are of a reversible nature and serve to modulate the activity of fully differentiated neuroendocrine circuits. Such actions have been described as 'organisational' and 'activational' (Phoenix, Goy, Gerall and Young, 1959), respectively, and are presumably due to the state of differentiation of the target neurons at the time the steroid reaches them. For example, activational effects of gonadal steroid hormones in the adult rat include the fine control of gonadotrophin output throughout the oestrous cycle, the induction of progesterone receptors by oestradiol and the control of sexual behaviour in both male and female rats. All of these characteristics do not become operational until after the 'critical period' of sexual differentiation. Female rats start to show receptive and proceptive responses to manual stimulation after oestrogen and progesterone administration around postnatal day 15 (Södersten, 1975) and the oestrogen induction of progesterone receptors cannot be observed until after the 'critical period' (MacLusky and McEwen, 1980b). In addition, although the limiting factor for the onset of regular oestrous cycles is maturation of the ovary, the hypothalamo-hypophysial system is capable of responding to gonadal steroids in an adult manner from as early as 22 days of age (Caligaris, Astrada and Taleisnik, 1972, 1973). Therefore it seems that the emergence of these characteristics after the end of the 'critical period' of sexual differentiation may indicate that the target cells are changing in their dominant mode of response to steroids. However, despite the major difference in the final effects of gonadal steroids on the brain between the neonate and the adult, an activation of the genome is probably partly responsible for the actions of the hormones at both stages of development. Protein synthesis is involved in the activation of lordosis by oestrogen and progesterone and there is evidence that some of the actions of oestrogen on enzyme activity in brain are the result of genomic effects (see sections 1.4 and 1.5). Steroids may, however, also act by more rapid and direct effects on the membrane itself as assessed by electrophysiological techniques (Yagi, 1973; Kelly, Moss and Dudley, 1977) and by measuring the plasma concentration of LH after an intravenous injection of oestradiol-17β in long-term ovariectomised rats (Sarkar and Fink, 1980). There is evidence that such an action may be involved in sexual differentiation of the brain (Arai and Gorski, 1968a and b).

The precise mechanism by which testosterone, after conversion to oestradiol, causes sex differences in synaptic pattern and nuclear volume, for example, in the neonatal rat hypothalamus-preoptic area is not understood although in view of the main mode of steroid hormone action, it would seem likely that testosterone affects protein synthesis in developing brain and thereby leads to oestrogen-dependent changes in neurons. Studies on mouse brain explants in vitro have demonstrated a marked enhancement of dendritic outgrowth after exposure of the explants to oestrogen (Toran-Allerand, 1976,1980b). Brain regions which showed a growth response to oestrogen contained oestrogen receptors and the effect could be abolished by antibodies to oestrogen or by oestrogen antagonists. The involvement of DNA, RNA and protein synthesis in the actions of testosterone on sexual differentiation of the brain have been demonstrated (Kobayashi and Gorski, 1970; Gorski and Shryne, 1972; Salaman and Birkett, 1974) suggesting that hormonal regulation of gene expression is a critical factor in the androgenisation process.

1.10 Aims of Thesis

The aim of this thesis was to investigate the effects of gonadal steroid hormones on brain protein synthesis in the male, female and androgenised female rat in order to determine whether a change in one or more proteins was associated with sexual differentiation. A limited study was also carried out on the effects of thyroid hormones on brain protein synthesis since although the importance of thyroid hormones in differentiation of the cerebellum has been investigated, little is known about the effects of these hormones on development of the hypothalamus-preoptic area. A variety of approaches, both in vivo and in vitro, were used with analysis of proteins by polyacrylamide gel electrophoresis. Proteins were also analysed by isolating and translating messenger RNA in vitro. One possible mode in which changes in protein synthesis may be expressed is through the synthesis of neurotransmitters and, therefore, studies were also carried out to investigate sex differences in catecholamines and indoleamines in the hypothalamus-preoptic area and midbrain.

CHAPTER 2

Materials and Methods

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals

Wistar Cob (Caesarian Originated Barrier Sustained) rats used in all experiments were obtained from either the departmental breeding colony or Charles River (Margate, Kent). Animals were maintained under controlled conditions of lighting (lights on 14 h, off 10 h) and temperature (22°C) with free access to food (Diet 41B or Oxoid breeding diet; Oxoid, Basingstoke, Hampshire) and tap water. The day of birth was taken as day 0 and littermates were used whenever possible.

2.1.2 Hormones

Testosterone, 17β-oestradiol (E_2), L-thyroxine (T_4) and 3, 3', 5-Triiodo-L-thyronine (T_3) in crystalline form and for use in *in vitro* experiments (Chapter 6), were obtained from Sigma (Poole, Dorset). Stock solutions (1 mM) were prepared in ethanol and kept at 4°C.

Testosterone propionate (TP), supplied in ethyl oleate from Paines and Byrne (Greenford, Middlesex) was diluted to a working concentration with arachis oil (Hopkin and Williams, Essex) on the day of the experiment and administered by subcutaneous (s.c.) or intraperitoneal (i.p.) injection. Throughout this thesis, crystalline testosterone will be called testosterone and testosterone propionate will be abbreviated to TP.

2.1.3 Radiochemicals

The following radiochemicals were obtained from Amersham International (Bucks. U.K.):

1. L-[^{35}S] methionine specific activity > 800 Ci/mmol.
2. L-[4,5-^{3}H] leucine specific activity 187 Ci/mmol.
3. L-[U-^{14}C] leucine specific activity 330 mCi/mmol.
4. High specific activity [^{3}H]-amino acid mixture containing:

	Specific Activity (Ci/mmol)
L-[4,5-^{3}H] leucine	130
L-[4,5-^{3}H] lysine monohydrochloride	76
L-[2,4,6-^{3}H] phenylalanine	84
L-[2,3,4,5-^{3}H] proline	100
L-[2,3,5,6-^{3}H] tyrosine	50

5. [^{14}C] methylated protein mixture (10-50μCi/mg protein) containing:

	Molecular Weight
[^{14}C] methylated myosin	200,000
[^{14}C] phosphorylase b	92,500
[^{14}C] bovine serum albumin	69,000
[^{14}C] ovalbumin	46,000
[^{14}C] carbonic anhydrase	30,000
[^{14}C] lysozyme	14,300

2.1.4 Electrophoresis Calibration Kits

Protein mixtures used to calibrate gels for the estimation of either molecular weight (Mol.Wt.) or isoelectric point (pI) were obtained from Pharmacia (Great Britain) Ltd. (Hounslow, Middlesex) and are described in Appendix I.

All other chemicals were 'Analar' grade and obtained from British Drug Houses (Poole, Dorset) or Sigma (Poole, Dorset) unless stated otherwise.

2.2 METHODS

2.2.1 Androgenisation of Neonatal Female Rats

Four day old female rats were injected with 1.25 mg TP in 0.1 ml arachis oil either s.c. (for chronic experiments) into the back of the neck, or i.p. (for acute experiments).

2.2.2 Dissection of the Hypothalamus and Preoptic Area (see Fig. 2.A)

Rats were killed by decapitation and the brain quickly removed and placed, ventral surface upwards, on a glass slide on ice. The cerebellum was removed by cutting at the level of the pons. The hypothalamus-preoptic area (H-POA) was dissected out by way of an incision which extended from the rostral edge of the optic chiasm to the caudal edge of the mammillary body, laterally to the hypothalamic sulci and about as deep as the anterior commisure. In a number of experiments the preoptic area (POA) alone was removed as a block of tissue; in the 4 day animal this was approximately 1 mm wide, and as long as the breadth of the optic chiasm. In these animals the hypothalamus was dissected out separately. The remaining tissue was termed 'rest of brain'.

2.2.3 Subcellular Fractionation

Brain tissue was homogenised in 5-10 vol ice-cold sucrose-TKM buffer (250mM sucrose, 500 mM Tris-HCl, pH 7.4, 25mM KCl, 5mM $MgCl_2$) and centrifuged at 4°C according to the experimental

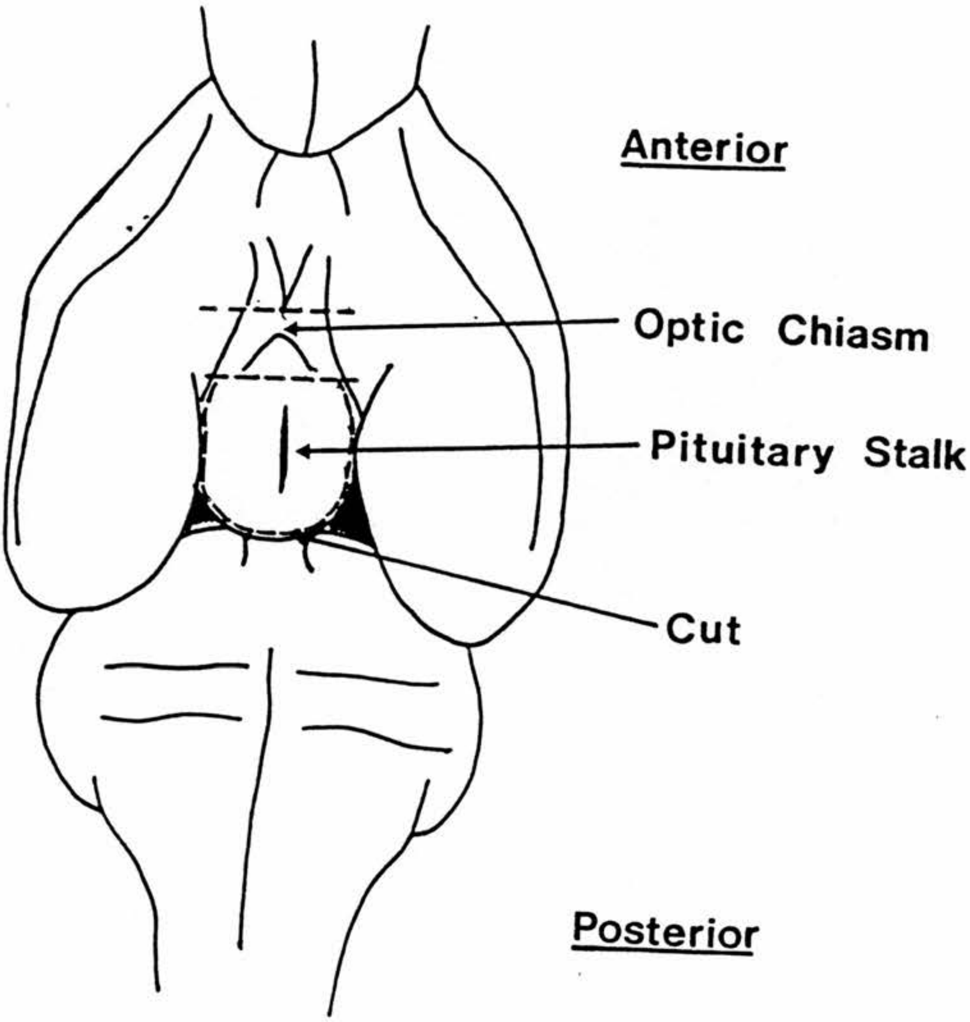


FIGURE 2.A: Ventral Surface of Rat Brain Showing Positions of Cuts For Dissection of The POA and Hypothalamus.

protocol, in either 5mm x 41mm or 13mm x 51mm Ultra-clear TM centrifuge tubes (Beckman Ltd., Bucks) in a Sorvall AH 650 swinging-bucket rotor (r_{av} = 8.33cm) in a Sorvall OTD-65B ultracentrifuge. The protocol used in Chapter 3 for a complete subcellular fractionation of brain tissue is illustrated in Fig. 2.B. In all other experiments homogenates were centrifuged at either 15,000g for 20 min or at 120,000g for 1h at 4°C. Membrane-associated proteins were extracted from pellets by the method of Mahadik, Korenovsky and Rapport, (1976). To remove lipid from the pellets they were allowed to stand overnight in 20 vol of chloroform:methanol (2:1, v/v) at 4°C. One third volume of methanol was then added, the mixture was centrifuged for 15 min at 500g and the pellet was solubilised by heating at 80-100°C for 10 min in a modified Laemmli (1970) sample buffer (125mM Tris-HCl, pH 6.8, 1.25% SDS (w/v), 1% 2-mercaptoethanol (v/v), 12.5% glycerol (v/v), 2mM Na_2 EDTA). Any particulate material remaining after this process was precipitated by centrifugation for 10 min at 1,000g.

2.2.4 Protein Determination

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as a standard. See Appendix I for experimental details. In some experiments the Bio-Rad protein assay kit was used, based on the method of Bradford (1976).

2.2.5 Incorporation of Radioactivity into Protein

The incorporation of radioactive amino-acid into protein was determined by adding 10µl sample to 1 ml ice-cold 5% TCA (w/v) and

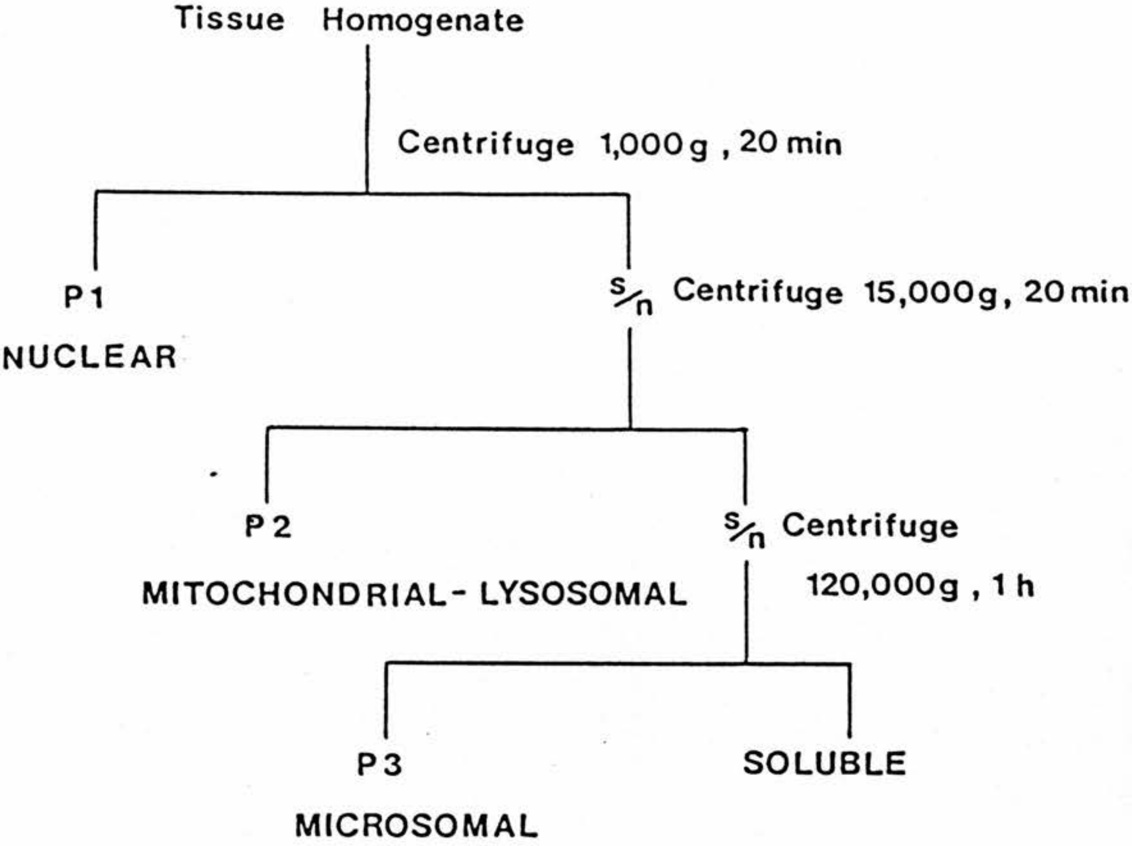


FIGURE 2.B: Schematic Representation of Procedures Used for The Subcellular Fractionation of Rat H-POA.

All procedures were carried out at 4°C.

allowing precipitation to occur at 4°C over 30 min. Precipitates were then poured onto pre-soaked GFC filter discs (Whatman, Maidstone, Kent) in a filter manifold (Millipore (U.K.) Ltd., Harrow, Middlesex), tubes were rinsed out with 2ml 5% TCA (w/v) and filters washed twice with 20ml 5% TCA and finally with 20ml 95% ethanol. Air-dried filters were put into miniature polyethylene scintillation vials (Packard Instrument Ltd., Berks), to which was added 4ml scintillant (Aqualuma Plus, Lumac, Holland). The vials were counted in an LKB-Wallac Rack Beta II liquid scintillation spectrometer (LKB Instruments Ltd., Surrey) at counting efficiencies of 35-40% for ^{3}H and 70-75% for ^{35}S.

2.2.6 Polyacrylamide Gel Electrophoresis (PAGE)

Separation of proteins by molecular weight was carried out using the SDS-discontinuous system originally described by Laemmli (1970). The discontinuous buffer system of Ornstein (1964) and Davis (1964) in the presence of SDS was used. SDS, an ionic detergent, dissociates proteins into their individual polypeptide subunits. When SDS is present in excess, proteins bind approximately equivalent amounts of SDS and the intrinsic charge of the polypeptide becomes insignificant compared to the negative charge of the detergent. The SDS-polypeptide complexes have almost identical charge densities and therefore migrate in polyacrylamide gels strictly according to polypeptide size. Estimation of the molecular weight of unknown proteins was carried out as described by Weber and Osborne (1969) after calculating the relative mobilities (R_f) of the marker proteins (by dividing the migration distance of each by that of the dye front). By plotting the logarithm of the molecular weight of each of the markers against Rf value (Fig. 2.C), the molecular weight of an unknown protein was estimated from its R_f value.

Linear gradient gels (8.5–20% acrylamide (w/v)) were always used allowing the analysis of proteins over a wide molecular weight range (14,000 - 200,000).

High resolution was obtained by the sharpening of protein bands during migration due to the gradient in pore size which is continually decreasing. Slab gels (180mm x 200mm x 0.7mm) were poured using a GM–1 Gradient Mixer and GSC–2 Gel Slab Casting Apparatus (Pharmacia (Great Britain) Ltd.). Solutions used in the preparation of all gels are listed in Appendix I. After polymerisation was complete (approximately 30 min at room temperature) the casting apparatus was dismantled and a 4.75% stacking gel applied, using well formers which allowed 14 samples (up to 50µl each) to be loaded.

Samples containing either equal protein (20–100µg) or acid-insoluble radioactivity were heated at 80–100°C for 2–3 min after the addition of an equal volume of sample buffer (125mM Tris–HCl, pH 6.8, 20% glycerol (v/v), 10% 2-mercaptoethanol (v/v), 4.6% SDS (w/v)). Electrophoresis was carried out for 16–20h at 15mA (constant current) per slab gel using the GE–2/4 LS vertical electrophoresis apparatus and ECPS 2000/300 power supply (Pharmacia (Great Britain) Ltd.). Running buffer (25mM Tris base, 192mM glycine, 0.1% SDS (w/v), pH 8.3) was continuously circulated and cooled.

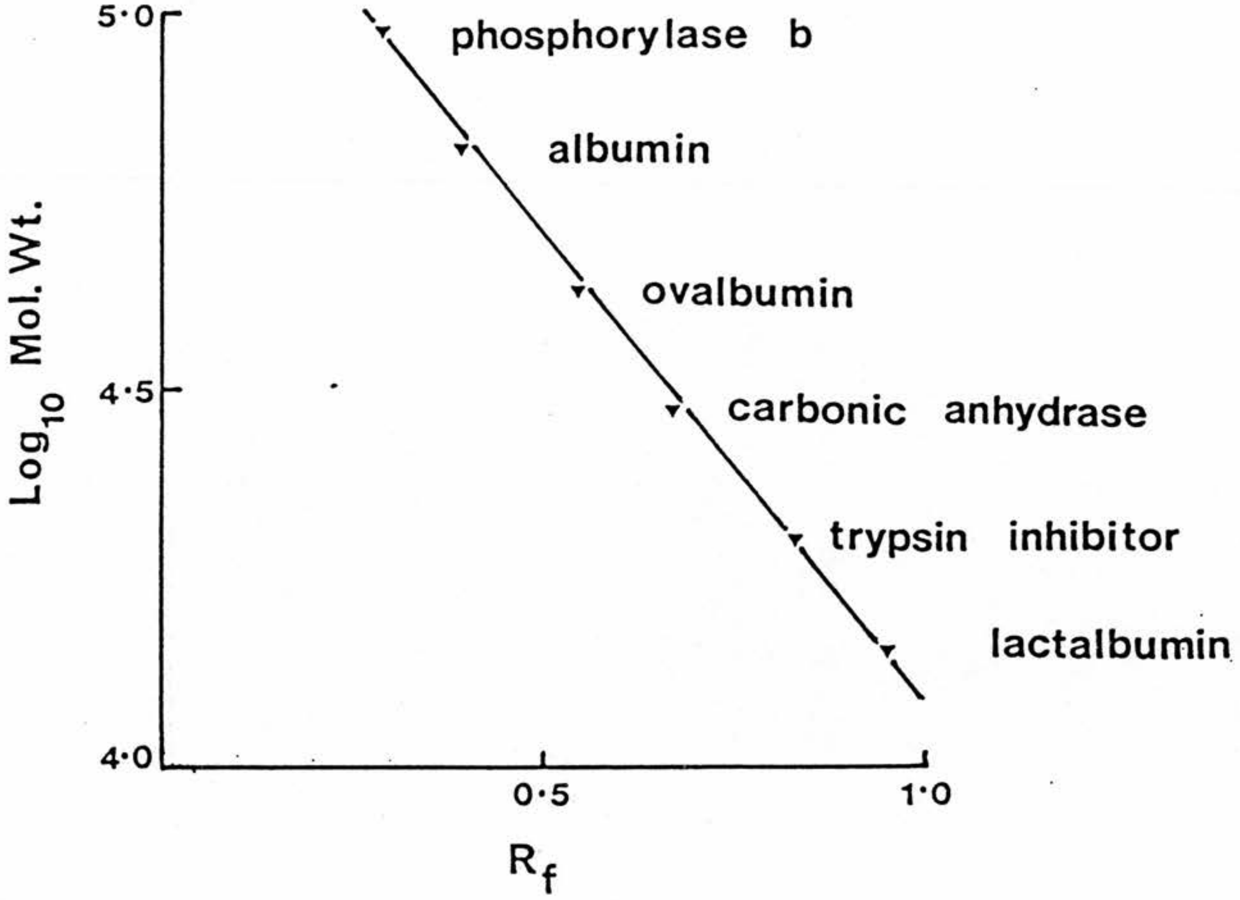


FIGURE 2.C: Calibration Curve For The Estimation of The Molecular Weight of An Unknown Protein.

2.2.7 Isoelectric Focussing (IEF)

Isoelectric focussing of proteins was carried out under denaturing conditions (9 M urea, 'Aristar', British Drug Houses) on a pH gradient established between the electrodes and stabilized by carrier ampholytes (Ampholines pH 3.5-9.5 and pH 4.0-6.5, LKB Instruments Ltd.). By measuring the distance of marker proteins (see Appendix I) from one electrode and plotting pI against distance, the pI of unknown proteins was estimated.

Two systems were used for the electrophoretic separation of proteins according to pI.

(a) Flat Bed

To allow easy comparison of large numbers of samples, a flat bed system (LKB Instruments Ltd.) utilising 230mm x 115mm x 1mm rectangular gels was the method of choice. Gels were prepared as in Appendix I. An equal volume of lysis buffer (9.5 M urea, 2% NP-40 (w/v), 5% 2-mercaptoethanol (v/v), 2% Ampholines) was added to all samples containing equal amounts of protein (20-100µg). A good contact between the gel support (thin glass or plastic sheet) and cooling plate was achieved by spreading a few drops of liquid paraffin onto the cooling plate before resting the gel on top. Electrode strips soaked in buffer were laid along the entire gel length at the cathode (1 M NaOH) and anode (1 M H_3PO_4). Samples and marker proteins (see Appendix I) were applied to the centre of the gel by pipetting onto sample applicators laid on the gel surface. Focussing was carried out at 20W (constant power) for 2h. Sample applicators were removed after 30 min. Once focussing was complete, the gel was fixed in 20% TCA (w/v) for 30 min, stained in 0.15% Coomassie Blue (w/v) (see Appendix I for details) for 30 min and destained in several changes of 25% methanol, 10% acetic acid.

(b) Rod Gels

Rod gels were cast in 180mm x 2.7mm (internal diameter) glass tubes, 5 at a time. The glass tubes were placed in a flat-bottomed, 50ml pyrex measuring cylinder and 5ml gel solution (see Appendix I) poured in. By overlaying with distilled water and slowly filling the cylinder to the brim, gels of equal length were obtained. Polymerisation was generally complete by 30 min at room temperature. Rods were removed from the cylinder and the gel surface rinsed with cathode buffer (20mM NaOH) before covering the top of the glass rod with perforated parafilm (Gallenkamp, Sussex) to prevent gels slipping out of tubes during focussing. Rods were inserted into the GE-2/4 LS apparatus using the appropriate rubber gasket and up to 20 rod gels could be run simultaneously. The lower buffer reservoir was filled with 4.3l anode buffer (10mM H_3PO_4) and sufficient cathode buffer added to the upper reservoir to cover the glass rods (300-400ml). Anode and cathode buffer reservoirs remained entirely separate during IEF. Rod gels were prefocussed at 250V (constant voltage) for 1h and the gel surface then rinsed with fresh cathode buffer. Samples containing equal protein or acid-insoluble radioactivity were prepared as in 2.2.7 (a) and carefully applied to the rod gel using a 20µl Gilson pipette (Anachem, Luton). Focussing was carried out for 7,000 -10,000 Vh at 480 V (constant voltage). Once focussing was complete, gels were forced out of the tubes into a flat, glass dish by pressure from a 60ml syringe connected with rubber tubing. Electrode orientation was marked by a pin inserted into the foot of the gel and rods were either processed for staining (2.2.9) or frozen at -20°C in 5ml buffer (62.5mM Tris-HCl pH 6.8, 10% glycerol (v/v), 5% 2-mercaptoethanol (v/v), 2.3% SDS (w/v)) until run in the second dimension (2.2.8). A 'blank' rod gel was run with each set of samples to determine the pH gradient established during IEF. The gel was sliced (5mm slices), individual slices shaken in 5ml distilled water for 2-3h and the pH values read on a PW 9409 digital pH meter. A typical pH gradient is shown in Fig. 2.D.

2.2.8 Two-dimensional PAGE

Isoelectric focussing in a rod gel (2.2.7 (b)) followed by gradient gel electrophoresis (2.2.6) was the basic methodology employed for separation of proteins in two dimensions (O'Farrell, 1975). The first dimension separated proteins according to net charge while the second dimension separated by molecular weight. As denaturants were used in both dimensions, the system resolved oligomeric proteins in terms of their polypeptide subunits.

Rod gels were thawed at room temperature and loaded directly onto a gradient gel by embedding in molten agarose (1% (w/v)) in sample buffer containing 0.1% (w/v) bromophenol blue), ensuring that no air bubbles were trapped below the rod. The acidic end of the rod was routinely placed at the left of the slab gel to standardise the procedure. Electrophoresis was carried out for 16-20h at 15 mA (constant current) per gel.

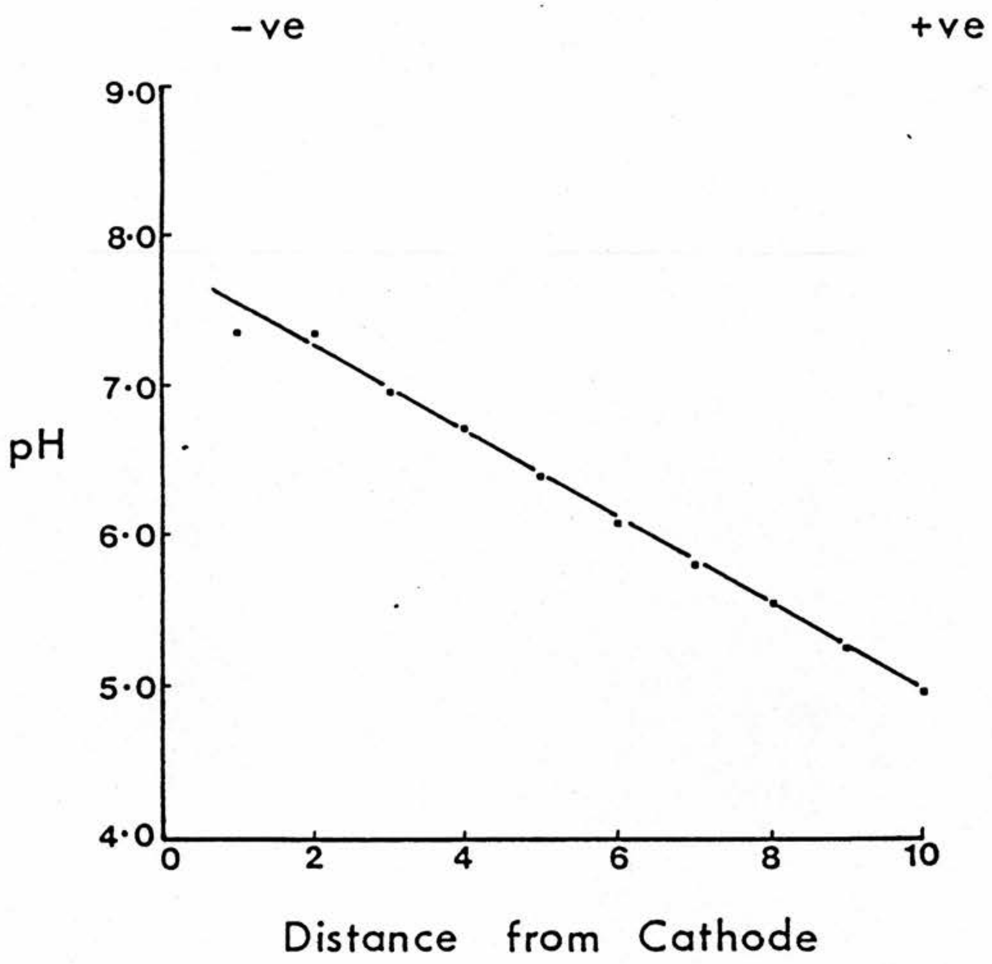


FIGURE 2.D: Typical pH Gradient In An IEF Rod Gel After Focussing For 8,000-10,000Vh.

2.2.9 Detection of Proteins

(a) Staining Techniques

Coomassie Brilliant Blue: Single dimension gradient gels were soaked overnight in 40% methanol (v/v), 7% acetic acid (v/v) to remove SDS. A stock solution of 0.25% Coomassie Brilliant Blue R250 (w/v) (Sigma) in 95% methanol (v/v) was prepared and 5ml stock solution mixed with 95ml 12% TCA (w/v). Individual gels were stained overnight at room temperature in 400-500ml staining solution and destained in several changes of 25% methanol, 10% acetic acid.

IEF rod gels were stained for 1h in 0.2% Coomassie Brilliant Blue R250 (w/v) in 45% ethanol, 10% acetic acid and then destained in several changes of 25% methanol, 10% acetic acid.

Silver Stain: Silver staining is a highly sensitive method of detecting proteins in polyacrylamide gels. All sources of protein contamination must therefore be minimised. Clean glassware was rinsed thoroughly with distilled water before use and gloves worn for handling gels.

Gels were stained using the Bio-Rad Silver Stain Kit (Bio-Rad Laboratories Ltd., Herts) based on the method of Merril, Danau and Goldmann, (1981) or by the method of Sammons, Adams and Nishizawa, (1981) as follows. Staining was carried out in shallow, pyrex dishes and solutions were agitated gently throughout. Gels were soaked overnight in 50% ethanol, 10% acetic acid and then given three 1h distilled water washes. Equilibration in silver nitrate solution (0.3g per 160 ml distilled water per gel) for 2h was followed by a 15s wash in distilled water and gels were then developed in 750mM NaOH containing formaldehyde (2.1 ml per 280 ml) for approximately 10 min, or until colour development seemed complete. Gels were soaked for 1h in Na_2CO_3 (7.5 g per litre distilled water) and stored in more of the same solution.

(b) Fluorography

The technique of fluorography, first described by Bonner and Laskey (1974) allows detection in polyacrylamide gels of proteins labelled with a tritiated amino-acid and increases the sensitivity to ^{35}S-labelled proteins. After the removal of water, gels are impregnated with a scintillator. When β-particles interact with the scintillator within the gel, visible light is emitted and an image is formed on blue-sensitive x-ray film.

Gels were soaked in 300-400ml dimethylsulphoxide (DMSO) for 30 min. This was repeated using fresh DMSO and then gels were soaked for 3h in 300-400ml 22.2% 2,5-diphenyloxazole (w/v) (PPO) in DMSO. After soaking for at least 1h in a few changes of distilled water, gels were dried down under vacuum, placed in a Kodak X-Omatic Cassette (18cm x 24cm with regular intensifying screen) with a sheet of X-Omat RP film and exposed at -70°C for 2 days to 2 months. Films were developed using Kodak DX-80 developer and FX-40 X-ray liquid fixer (Kodak Ltd., Glasgow).

(c) Slicing Gels

The incorporation of radioactive amino-acid into proteins separated by PAGE was also analysed by slicing the gel and counting radioactivity within slices. Following electrophoresis, gels were stained with Coomassie Blue (2.2.9 (a)) and then individual tracks sliced into 1mm sections using a hand-held slicer made in the departmental workshop. Slices were placed into scintillation vials containing 0.2ml of 0.1% SDS (w/v) and shaken overnight. Next day 4ml scintillant was added and samples counted. Samples labelled with both ^3H and ^{14}C-leucine (Chapter 6) were counted with efficiencies of 35-40% and 75-80% respectively.

Gels and autoradiograms were photographed on a light box and scanned using a Quick Scan R ə D Electrophoresis Densitometer (Helena Laboratories, M.I. Scientific Ltd., Newcastle).

2.2.10 Isolation of RNA

The isolation of RNA can be affected by ribonuclease contamination. All procedures were therefore carried out under sterile conditions. All glassware was siliconised (Sigmacote, Sigma) and dissecting instruments were sterilised by dry heat (120°C overnight) or autoclaving and solutions were either autoclaved or filtered using a Millex-GS sterilising filter unit (Millipore (U.K.) Ltd.) before use. Gloves were worn throughout all the experimental procedures.

Total RNA was extracted from the H-POA (see 2.2.2) of male, female and androgenised female (2.2.1) rats of various ages. Two extraction procedures were used although the SDS-phenol/chloroform method (Spradling, Penman and Saveria Campo, 1974) was superceded by the guanidine thiocyanate method (Chirgwin, Przybyla, McDonald and Rutter, 1979), shown by other workers in this laboratory to give higher yields of RNA from rat hypothalamus.

Immediately after dissection, the tissue was weighed, frozen in liquid nitrogen and stored until sufficient material was collected for RNA extraction.

SDS-Phenol/chloroform

Tissue was allowed to thaw and homogenised at room temperature in a glass homogeniser (Jencons Scientific Ltd., Bedfordshire) in 5 vol (w/v) homogenisation buffer (0.5% SDS (w/v), 25mM Na_2 EDTA, 75mM NaCl, pH 8.0, containing heparin at 1 mg/ml). Homogenates were transferred to conical flasks, extracted with an equal volume of water-saturated phenol containing hydroxyquinoline (1 mg/ml) by stirring gently for 15 min at room temperature and centrifuged for 15 min at 16,000g in an MSE High Speed 18 centrifuge. The upper aqueous phase plus the interface, then the phenol layer were removed and re-extracted separately with buffer-saturated phenol containing chloroform (1:1) for 15 min at room temperature. After centrifugation at 16,000g for 15 min the aqueous phases were pooled, proteinase K (Sigma) added at 20µg/ml and incubated at 37°C for 15 min. A final phenol-chloroform extraction was performed and the aqueous phase made 200mM in NaCl. RNA was precipitated by adding two volumes of ice-cold ethanol, collected by centrifugation at 16,000g for 1h and DNA removed by three successive precipitations with 3M sodium acetate, pH 6.0 as follows. Nucleic acids were dissolved in sterile distilled water at approximately 1mg/ml and 3 vol of 4M sodium acetate, pH 6.0 were added. After standing at 4°C for 30 min, the RNA was recovered by centrifugation at 16,000g for 30 min. The pellet was dissolved in distilled water and the procedure repeated twice more. Finally, RNA was made 200mM in NaCl and precipitated overnight in 2 vol ethanol at -20°C. RNA was collected by centrifugation, dissolved in a known volume of distilled water and the concentration determined from spectrophotometric absorbance at 260nm. One A_{260} unit was taken to represent a nucleic acid concentration of 40µg/ml. RNA was reprecipitated overnight, collected by centrifugation and dissolved in distilled water at a final concentration of 1mg/ml.

Guanidine Thiocyanate (GTC)

The following solutions were prepared the day before use and stored at 4°C:

(i) 4M guanidine thiocyanate/1 M 2-mercaptoethanol, pH 5.0 (GTC solution)

23.6g guanidine thiocyanate (Eastman Kodak Ltd., Liverpool) in 30ml distilled water was stirred for 2h at 60°C then allowed to stand at room temperature for 2h. 3.49ml 2-mercaptoethanol was added and the solution adjusted to pH 5.0 with glacial acetic acid. After making up to 50ml with distilled water the solution was filtered through Whatman No 1 filter paper and sterilised by passage through a 0.22µm Millex-GS filter unit.

(ii) Caesium Chloride (Cs Cl solution)

CsCl (9.59g) was dissolved in 4ml 250mM EDTA, pH 6.5 and made up to 10ml with distilled water. On the morning of the experiment 4.19µl 2-mercaptoethanol were added.

(iii) 100mM Tris-Acetate/100mM Sodium Acetate, pH 9.0

Tris base (1.21g) and sodium acetate (0.82g) were dissolved in 50ml distilled water, adjusted to pH 9.0 with glacial acetic acid, made up to 100ml, filtered (0.22µm) and autoclaved.

(iv) 2M Potassium Acetate, pH 5.5 (0.22µm filtered and autoclaved)

Tissue was allowed to thaw, homogenised in 5-10 vol GTC solution in an all glass homogeniser and left to stand at room temperature for 2h (for example, 30 H-POA weighing approximately 1.5g were homogenised in 8ml GTC solution). The homogenate was then loaded onto a 2.5ml cushion of caesium chloride in sterile, polyallomer centrifuge tubes (14mm x 95mm, Beckman r_{max} = 15.87cm) and centrifuged in a SW 40 swing out rotor (Beckman) at 193,000g for 20h at 25°C. Once centrifugation was complete, the surface fat layer was removed using a spatula, the aqueous layer sucked off and the highly viscous DNA layer removed. Finally the caesium chloride was carefully removed, leaving the clear, gelatinous RNA pellet at the base of the centrifuge tube. The pellet was dissolved in 500µl 100mM Tris-acetate/sodium acetate and transferred to a Corex centrifuge tube (Corning Ltd., Staffordshire). The polyallomer tube was rinsed out with a further 500µl buffer and after pooling the rinses, an A_{260} reading was taken. 100µl 2 M potassium acetate, pH 5.5 and 10ml ice-cold ethanol were then added and the RNA precipitated at -20°C for 2-3 days. After centrifugation for 1h at 6,000g, the RNA was washed by dissolving in 2ml 100mM sodium acetate, pH 5.5 and re-precipitated overnight at -20°C.

2.2.11 Isolation of Poly (A)$^+$ RNA

Total cellular RNA was enriched for Poly (A)$^+$ containing messenger RNA (mRNA) by oligo (dT)-cellulose chromatography as described by Aviv and Leder (1972).

Total RNA, dissolved at 1mg/ml in 1 mM Na_2 EDTA, pH 7.0, was heat-denatured at 70°C for 45s and then rapidly chilled on ice. RNA was added to an equal volume of twice concentrated binding buffer (800mM NaCl, 2 mM Na_2 EDTA, 0.2% SDS (w/v), 20 mM Tris-HCl, pH 7.5) at room temperature and applied to the oligo (dT)-cellulose (Sigma or Collaborative Research (Type II), Cambridge) column, pre-equilibrated in binding buffer (400mM NaCl, 1 mM Na_2 EDTA, 0.1% SDS (w/v), 10 mM Tris-HCl, pH 7.5). The first eluate of unbound RNA was reapplied to the column and the process repeated for the second eluate. The column was then washed with binding buffer until no U.V.-absorbing material eluted from the column (generally 1 h). Poly $(A)^+$-enriched mRNA was eluted from the column with elution buffer (1 mM Na_2 EDTA, 0.1% SDS (w/v), 10 mM Tris-HCl, pH 7.5). Significant poly $(A)^+$ mRNA containing fractions were located by A_{260} readings, pooled, and precipitated by making 200mM in NaCl and adding 2 vol ice-cold ethanol. After standing overnight at -20°C, mRNA was collected by centrifugation at 16,000g for 30 min and dissolved at 1μg/μl in distilled water.

The normal yield of poly $(A)^+$ RNA was 1-5% of the total RNA applied to the column.

All RNA samples were stored in sterile distilled water at -20°C.

2.2.12 Translation of mRNA In Vitro

mRNA was translated in vitro in either a cell-free, rabbit reticulocyte lysate system (Amersham International) or wheat germ extract (Bethesda Research Laboratories Ltd., Cambridge) supplemented with ^{35}S-methionine, or ^{3}H-amino acid mixture (see section 2.1.3).

Rabbit Reticulocyte Lysate

mRNA was translated in a nuclease-treated, mRNA-dependent lysate prepared by a modification of the method described by Pelham and Jackson (1976). A number of translations were carried out using a lysate (N90) manufactured with concentrations of potassium and magnesium ions suitable for the translation of a wide range of mRNA species. The final reaction mix (total volume 22µl) contained 16µl lysate mix, 4µl ^{35}S-methionine at 5µCi/ul, and 2µl mRNA or distilled water. Incubations were for 90 min at 30°C.

In the majority of experiments, mRNA was translated in an amino-acid depleted lysate (N 150) which required the addition of optimal concentrations of potassium and magnesium ions, a mixture of cold amino-acids (excluding those being used as the radiolabel) and the radioactive amino-acid(s). The final reaction mixture (20µl) contained 14µl lysate, cold amino-acids at 50µM (1µl), radioactive amino-acid at 1µCi/µl (2µl), potassium acetate at 170 mM (2 µl), and 0.5-1.0µl mRNA or distilled water. No additional magnesium was added. The mixture was incubated at 30° for 60 min.

Wheat Germ

Translations were carried out in a 30µl final assay volume, at 25°C for 90 min. The reaction mixture contained 10µl nuclease-treated wheat germ extract, 3µl protein biosynthesis reaction mix, potassium acetate (96mM), magnesium acetate (2.37 mM), radioactive amino acid at 1µCi/µl and 1µl mRNA or distilled water. The reaction volume was made to 30µl with distilled water. The manufacturer's recommended concentrations of potassium and magnesium ions for globin mRNA were found to result in efficient incorporation of radiolabel into protein using hypothalamic mRNA and no further trials were performed.

2.2.13 Estimation of Protein Synthesis Directed by mRNA

At fixed time intervals during the incubation, 1µl samples were removed from the reaction mix and added to 0.5ml 1M NaOH containing 5% hydrogen peroxide (100 vol (v/v)). After heating at 37°C for 10 min, tubes were placed on ice and 3ml ice-cold 25% TCA (w/v) containing 2% casein hydrolysate (w/v) were added and left for 30 min. The mixture was then filtered through pre-soaked GFC filter discs, washed twice with 20 ml 5% TCA (w/v), once with 95% ethanol (v/v) and the air-dried filters counted by liquid scintillation spectroscopy as in section 2.2.5.

2.2.14 Primary Culture of Foetal Rat Hypothalamic Neurons

Primary cultures of 18 day foetal rat hypothalamic neurons were prepared according to the method of Vaccaro and Messer (1977). Sterile technique was employed throughout these experiments.

Time-mated female Wistar Cob rats (from departmental breeding colony) were decapitated on day 18 of pregnancy (day of conception = day 1) and after swabbing the abdomen with 70% ethanol (v/v) the uterus was removed and placed in a sterile dish on ice. Three embryos were removed from the uterus at a time and transferred to another sterile Petri dish under a dissecting microscope. Holding an embryo at the neck with one pair of forceps, layers of overlying epithelium were peeled away with another pair of forceps to reveal the brain. By making major incisions with micro-scissors rostrally at the olfactory bulb and caudally at the spinal cord and cutting other fine attachements, the brain was carefully dissected free of the head. Keeping the brain moist with a few drops of Spinners minimum essential medium (Spinners MEM, calcium and magnesium free, Gibco-Biocult Ltd., Paisley), the brain was placed ventral surface upwards and the H-POA was dissected out with micro-scissors. Four to six H-POA were placed in a sterile, plastic tube containing Spinners MEM (1 ml) and kept on ice until all dissection work was complete. Dissections were carried out as quickly as possible and all subsequent manipulations were performed in a Class II Microbiological Safety Cabinet (Envair (U.K.) Ltd., Lancashire). The Spinners MEM was removed from all tubes and replaced with 1ml Spinners MEM containing 130mg/100ml $MgSO_4.7H_2O$, 0.1% DNAase (w/v, Sigma) and 0.1% papain type III (w/v, Sigma). Trituration was performed three times with a sterile, plugged pasteur pipette, previously fire-polished to give approximately a 1mm tip. Cells were incubated for 30 min at room temperature, triturated three times and centrifuged at 150g for 5 min. The supernatant was removed and replaced with Eagles MEM (Flow Laboratories Ltd., Irvine) containing 0.1% DNAase (w/v), trituration carried out ten times with a fresh fire-polished pipette and cells incubated at room temperature for 5 min. After triturating again twenty times, centrifuging as before and replacing the supernatant with plating medium (Dulbeccos modified Eagles medium containing 5% foetal calf serum (v/v) and 2mM glutamine), cells were gently resuspended and after pooling, counted in a haemocytometer. The volume of medium was then adjusted to give approximately 8×10^5 cells/1.5ml medium and cytosine arabinoside (Ara-C, Sigma) was added at 2μM. Cells were then plated out at 8×10^5 cells per 35mm Petri dish previously coated with polylysine (Polylysine B VII, Sigma, prepared at 100µg/ml) and maintained at 37°C in an atmosphere of 5% CO_2/95% air in a Humidity Cabinet Incubator (Gallenkamp, Crawley, Sussex). Every third day the medium was changed by removing 1ml medium from each dish and replacing with 1ml fresh medium. Ara-C was omitted after the first three days.

Cells in culture prepared in this way begin to attach to the culture dish within a day, start sending out processes shortly afterwards and have been shown to exhibit electrophysiological characteristics of nerve cells (Vaccaro, Messer and Leeman, 1976b). Non-neuronal cell growth is effectively controlled by the initial addition of Ara-C to the medium and only form a thin layer of cells upon which the neurons grow out.

2.2.15 High Performance Liquid Chromatography with Electrochemical Detection

The concentrations of indoleamine and catecholamine neurotransmitters and their metabolites were measured in brain tissue by high performance liquid chromatography with electrochemical detection (LCED) (Mefford and Barchas, 1980; Mefford, 1981).

Dopamine (DA), 3,4-dihydroxyphenyl acetic acid (DOPAC), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were measured simultaneously in the tissue extract with an assay sensitivity comparable to radioenzymatic assays previously described for these compounds (Coyle and Henry, 1973; Saavedra, Brownstein and Axelrod, 1973; Cuello, 1978).

The electrochemical detection method relies on the ability of a molecule to oxidise when subjected to a fixed oxidative potential (Adams, 1969; Weber and Purdy, 1978). Catecholamines and indoleamines generally require an oxidation potential of between +0.5 and +0.7V, with reference to a silver-silver chloride reference electrode, to yield two electrons (Fig. 2.E) and the total current generated from the oxidation reaction is proportional to the molar concentration of the compound.

All apparatus was obtained from Scotlab Instrument Sales Ltd., Carluke.

Tissue samples were weighed, homogenised in 200mM $HClO_4$ in 0.1mM cysteine solution containing 1ng/20µl n-acetyl dopamine (internal standard) and centrifuged at 2,000g for 20 min to pellet precipitated protein. The supernatant was filtered using a 0.2µm regenerated cellulose filter (BAS Inc., West Lafayette Ind.) and 20µl final extract introduced into the LCED system using an SGE injection syringe and a Rheodyne 7125 injection valve fitted with a 20µl sample loop. The mobile phase (100mM sodium acetate, 100mM citric acid in 10% methanol (v/v), pH 4.5) was pumped at a constant flow rate of 1ml/min using an Altex 110A pump.

The chromatographic system (Fig. 2.F) consisted of a 10 cm x 0.40cm Ultrasphere ODS pre-column and a 25cm x 0.46cm Ultrasphere ODS analytical column (5µm particles, reverse phase). Both columns were obtained pre-packed. DA, DOPAC, 5-HT and 5-HIAA were detected electrochemically using an LC-5A glassy carbon transducer cell with a working potential set at +0.7V and a BAS LC-4A Amperometric detector. Generated current was amplified and converted to a voltage for display on a Tarkan 600 chart recorder, set at 1V full

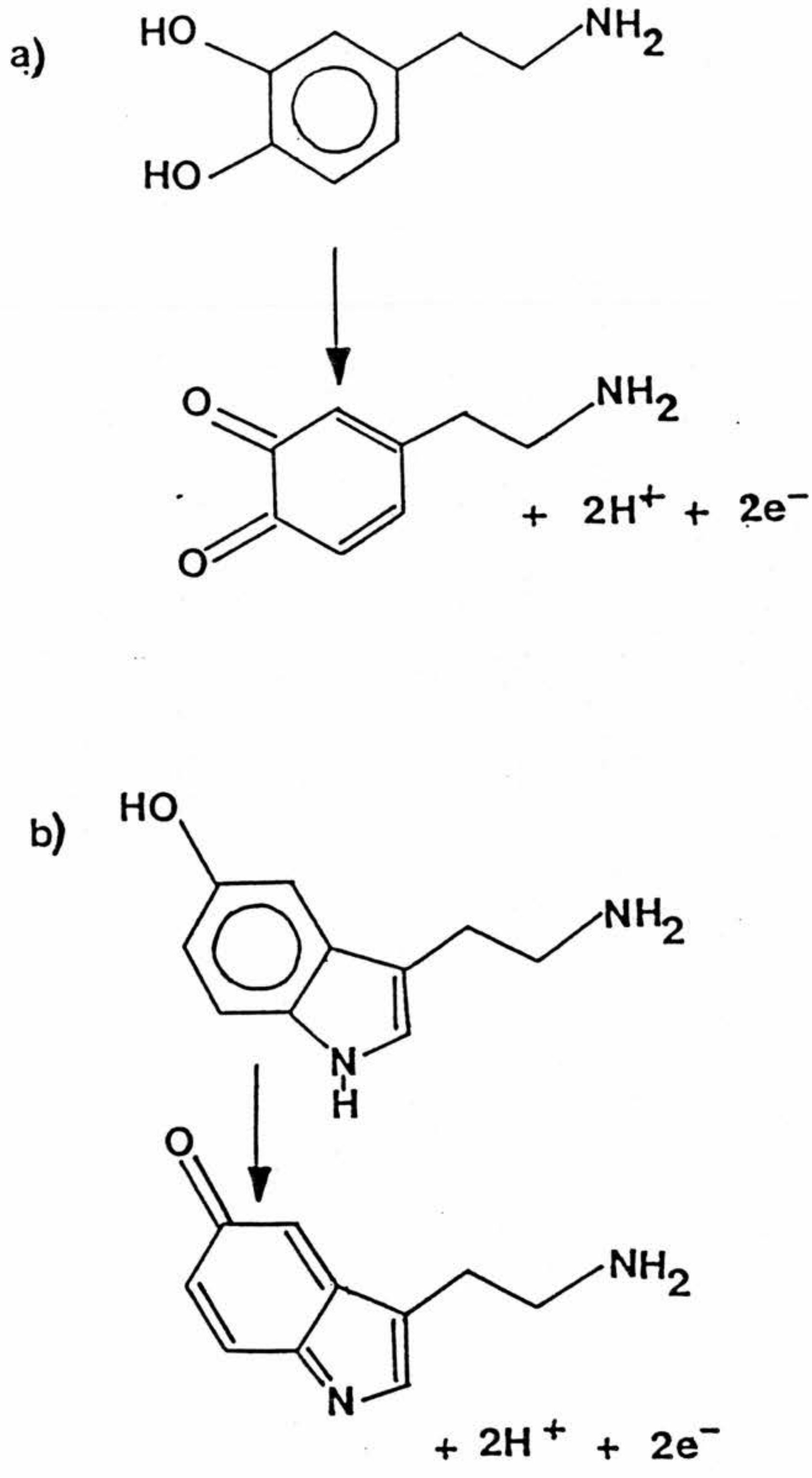


FIGURE 2.E: Diagramatic Representation Of The Oxidative Mechanism For a) Catecholamines b) Indoleamines.

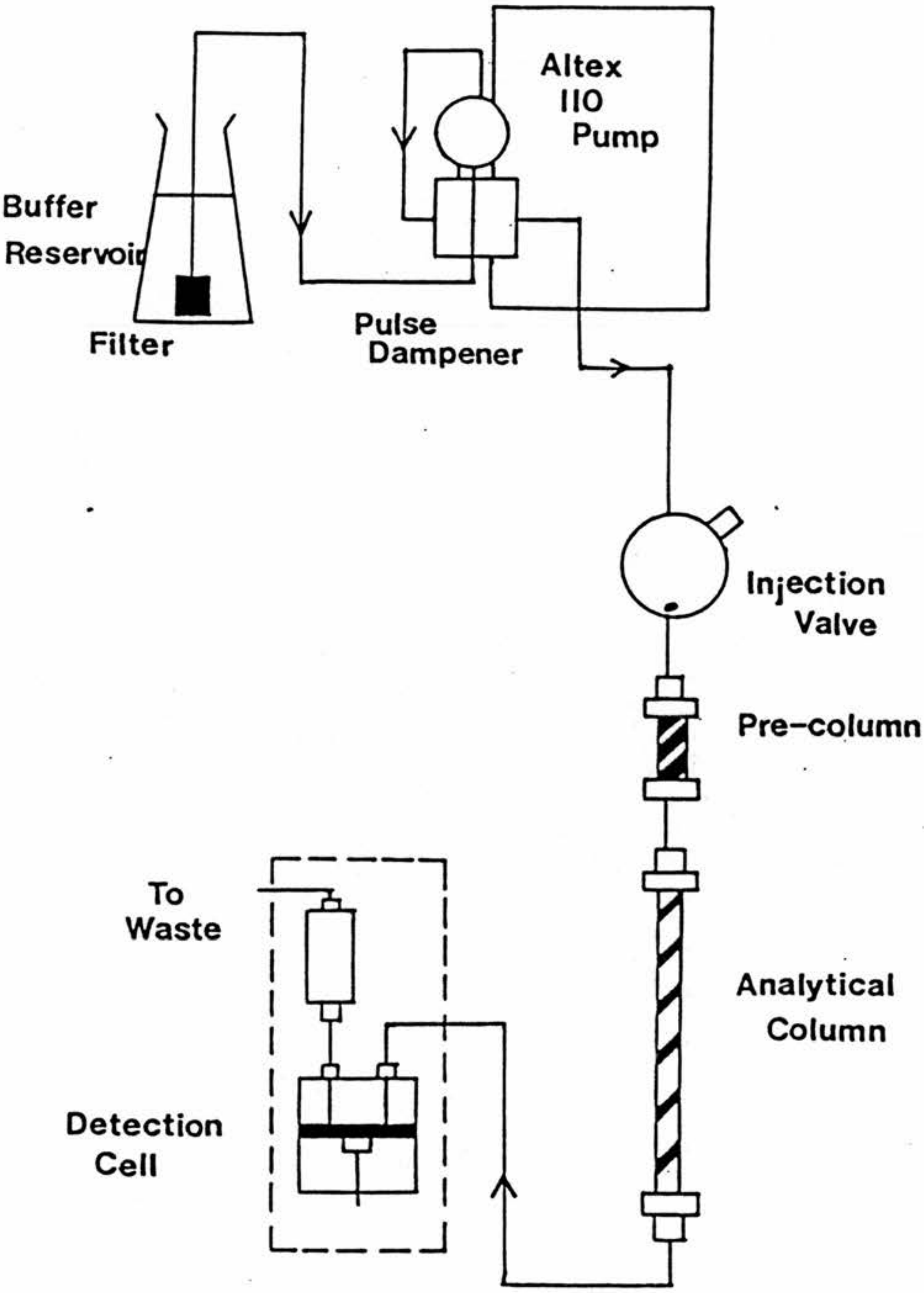


FIGURE 2.F: Diagramatic Representation Of The High Performance Liquid Chromatography Apparatus (LCED).

scale deflection.

Stock solutions of standards (DA, DOPAC, 5-HT and 5-HIAA) and internal standard (n-acetyl dopamine) were prepared at 0.1 - 0.2 mg/ml in 0.1 mM cysteine and stored at 4°C, in the dark. For each experiment, standards were diluted with 200mM $HClO_4$ to give 2,000pg, 200pg, 100pg and 50pg per 20µl working standard solutions and column retention times were noted for each compound. Standard curves (Fig. 2.G) were linear between 50-2,000pg per 20µl and the lower limit of sensitivty was between 15-25pg per 20µl for all compounds. A typical chromatogram for a mixture of standards, including internal standard, is shown in Fig. 2.H and a chromatogram for 20µl of H-POA extract is shown in Fig. 2.I.

Calculation of Results

Peak heights were measured using a perpendicular drawn from the curve peak to the baseline (Fig. 2.I). For each concentration of standard, the ratio of peak height, relative to the internal standard (1ng/20µl in standards and sample) was calculated i.e.

$$\frac{\text{Peak Height of Standard}}{\text{Peak Height of Internal Standard}}$$

to give a standard curve of peak height ratio versus concentration. The mass of a compound in the sample was then calculated by linear regression from a standard curve, using the peak height ratio of that compound relative to the internal standard in the sample. The use of peak height ratios relative to an internal standard allowed for slight variations in injection volume and recovery.

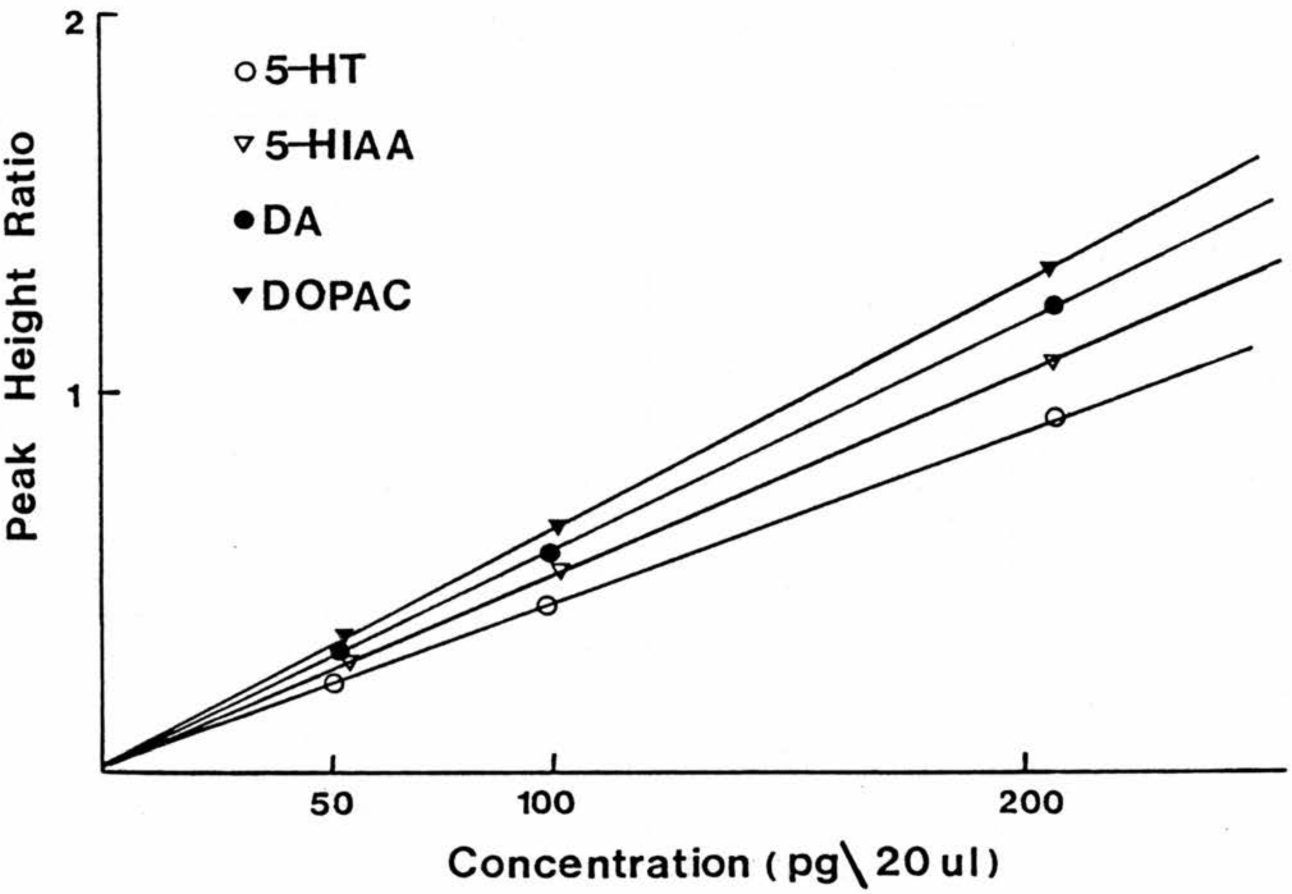


FIGURE 2.G: Standard Curves For 5-hydroxytryptamine (5-HT), 5-hydroxyindole acetic acid (5-HIAA), dopamine (DA) and 3,4-dihydroxyphenyl-acetic acid (DOPAC).

All standard curves linear to 2ng.

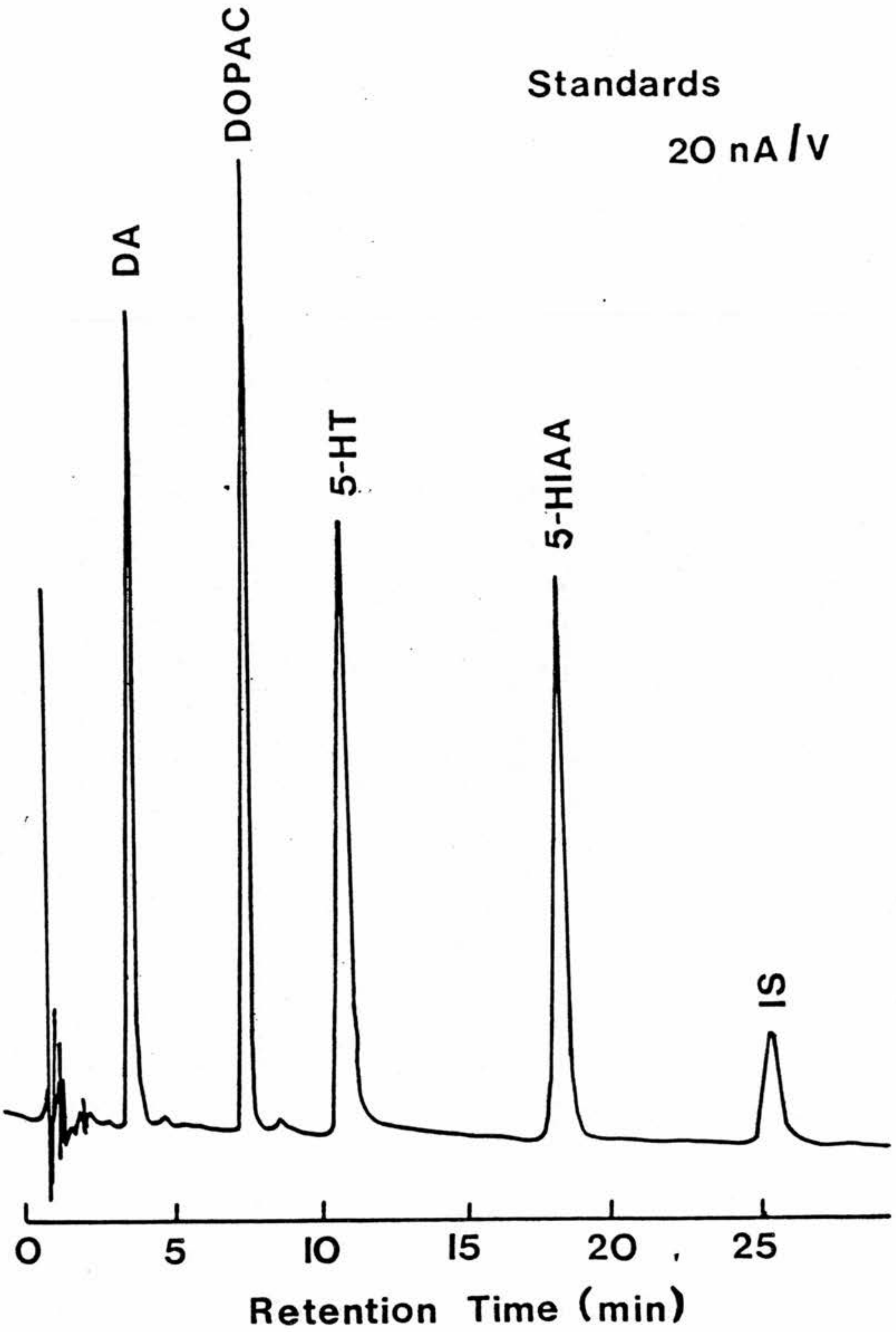


FIGURE 2.H: Standard Chromatogram for 2ng/20μl of 5-hydroxytryptamine (5-HT), 5-hydroxyindole acetic acid (5-HIAA), dopamine (DA) and 3,4-dihydroxyphenyl-acetic acid (DOPAC) with 1ng/20μl n-acetyl dopamine internal standard (IS).

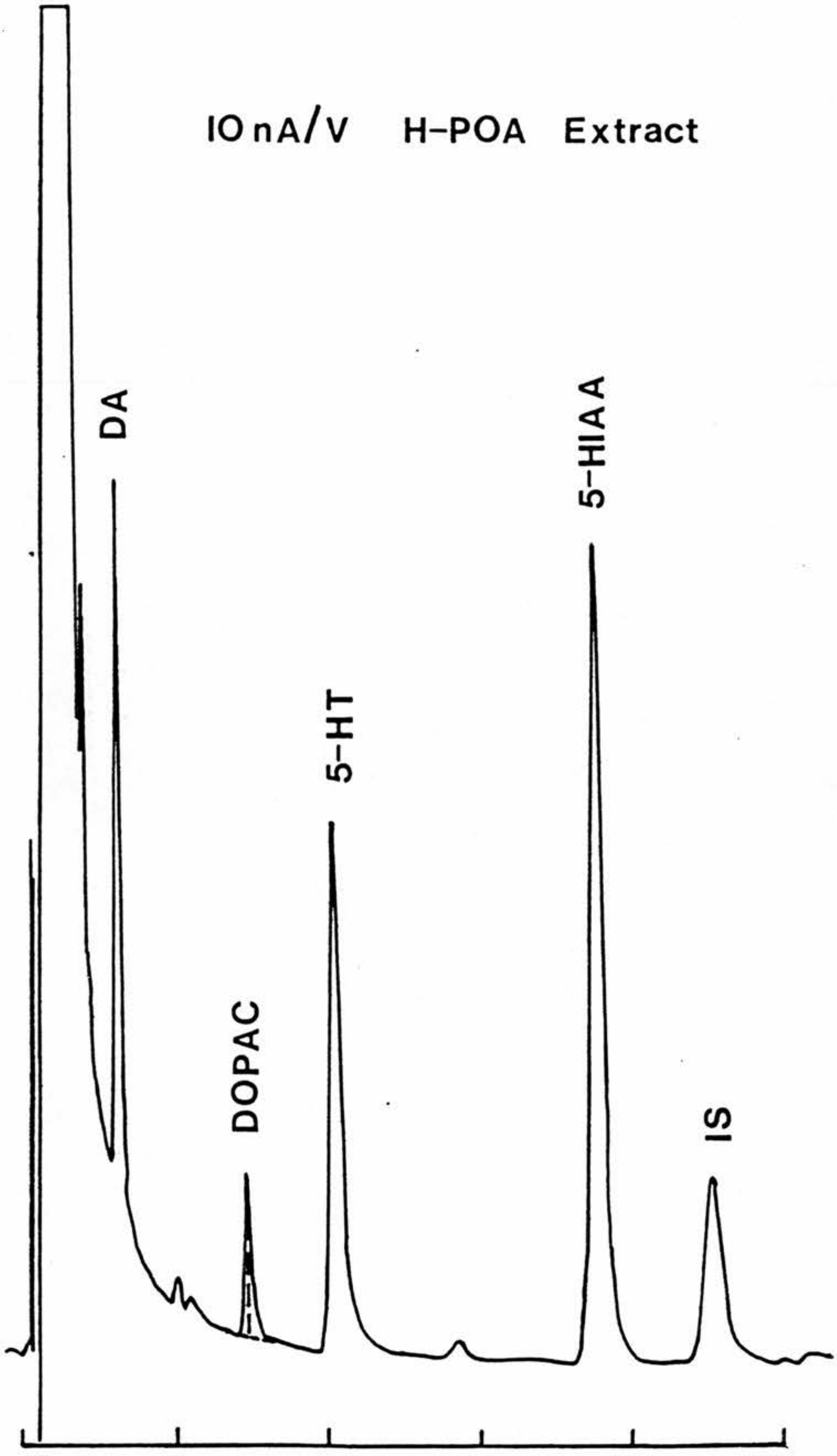


FIGURE 2.I: Typical Chromatogram For 20µl H-POA Extract.

Abbreviations as in Figure 2.H. Peak height shown by dotted line.

CHAPTER 3

Brain Protein Changes During Development
In The Male and Female Rat

3.1. INTRODUCTION

Sexual differentiation of the brain has been shown to be associated with several sexually dimorphic morphological features in the rostral diencephalon (Raisman and Field, 1973; Jacobson and Gorski, 1981; Matsumoto and Arai, 1981); however, so far no biochemical markers for the process of sexual differentiation have been identified. The involvement of DNA, RNA and protein synthesis in the actions of oestrogen during the period of sexual differentiation have been reported (Gorski and Shryne, 1972; Salaman and Birkett, 1974) indicating that oestrogen regulation of gene expression may be an important factor in the differentiation process. Although the high rates of DNA, RNA and protein synthesis in developing rat brain (Matthieu, Widmer and Herschkowitz, 1973) are probably associated largely with the processes of glial cell proliferation and myelination (Vanier, Holm, Öhman and Svennerholm, 1971), they may also be involved in an organising effect of gonadal steroids upon neurons, perhaps including an oestrogen-dependent growth of nerve processes (Toran-Allerand, 1980) or oestrogen effects upon neurotransmitter systems (Luine et al., 1980; Rainbow et al., 1980; Biegon and McEwen, 1982). A considerable number of data have been obtained on the changes in brain proteins during development (Grossfield and Shooter, 1971; Waehneldt and Neuhoff, 1974; Kelly and Luttges, 1976; Lau, Ryan and Beesley, 1982); however, relatively little is known about patterns of protein synthesis in the male and female rat brain either during the period of sexual differentiation and/or at later stages of development. The purpose of this study was to investigate whether sexual differentiation involves a change in the brain proteins associated with various subcellular fractions at different stages of postnatal development.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Male and female Wistar Cob rats from the departmental breeding colony or Charles River (Margate, Kent) were used at day 0, 8, 25, 72 and 80 (day 0 = day of birth). Littermates were used whevever possible. Animals were killed by decapitation, the brain was quickly dissected out (without the cerebellum, which was removed at the level of the pons) and placed ventral surface upwards on a glass slide on ice. The optic nerves and chiasm were carefully removed and the H-POA dissected out as described in section 2.2.2. The remaining tissue was termed 'rest of brain'. Four or 5 H-POA from both male and female rats and the 'rest of brain' from 3 or 4 rats were taken from animals aged 8, 25 and 72 days. The number of animals used at day 0 was increased to 10 to provide sufficient tissue for subcellular fractionation of the H-POA.

In a separate experiment, the H-POA and pituitary were removed from 80 day control female, androgenised female (section 2.2.1) and male rats. H-POA or pituitary gland tissue from 3 animals was pooled for each group.

3.2.2 Subcellular Fractionation

Fresh tissue from the 0, 8, 25 and 72 day rats was homogenised in 5-10 vol ice-cold sucrose-TKM buffer and subcellular fractions were prepared as described in section 2.2.3 and Fig. 2.B using a Sorvall OTD-65B Ultracentrifuge, at 4°C. The 80 day samples were homogenised as described and then centrifuged at 120,000g for 1h at 4°C.

All pellets were rinsed twice with homogenisation buffer. Membrane-associated proteins were extracted from the nuclear (1,000g), mitochondrial-lysosomal (15,000g), microsomal (120,000g) and the 120,000g pellets from the 80 day samples, according to the method of Mahadik et al. (1976), as described in section 2.2.3.

Protein concentrations were determined by the method of Lowry et al., (1951) using bovine serum albumin (Sigma, Dorset) as standard (Appendix 1). Samples containing equal amounts of protein were prepared for PAGE as described in section 2.2.6.

3.2.3 Electrophoresis

The separation of proteins by molecular weight was carried out using linear gradient SDS-polyacrylamide gels as described in section 2.2.6. Four gels were cast and subjected to electrophoresis simultaneously to aid comparison of samples. Low molecular weight protein standards (Appendix I) were run on each gel. Proteins were visualised by staining with Coomassie Brilliant Blue R250 and the gels were scanned, as described in section 2.2.9. The molecular weight of unknown proteins was determined by the method of Weber and Osborn (1969), as described in section 2.2.6.

Isoelectric focussing of proteins in rod gels was carried out as described in section 2.2.7(b). Gels were prefocussed at 250V (constant voltage) for 1h and the samples were then focussed for 8-10,000Vh. The gels were removed from the glass rods and stained as described in section 2.2.9(a). To simplify the comparison of a large number of samples, flat bed isoelectric focussing was also carried out using the procedures described in section 2.2.7(a). Protein markers of known pI (Appendix I) were routinely focussed at the same time as the samples and the pI values of unknown proteins were calculated as described in section 2.2.7.

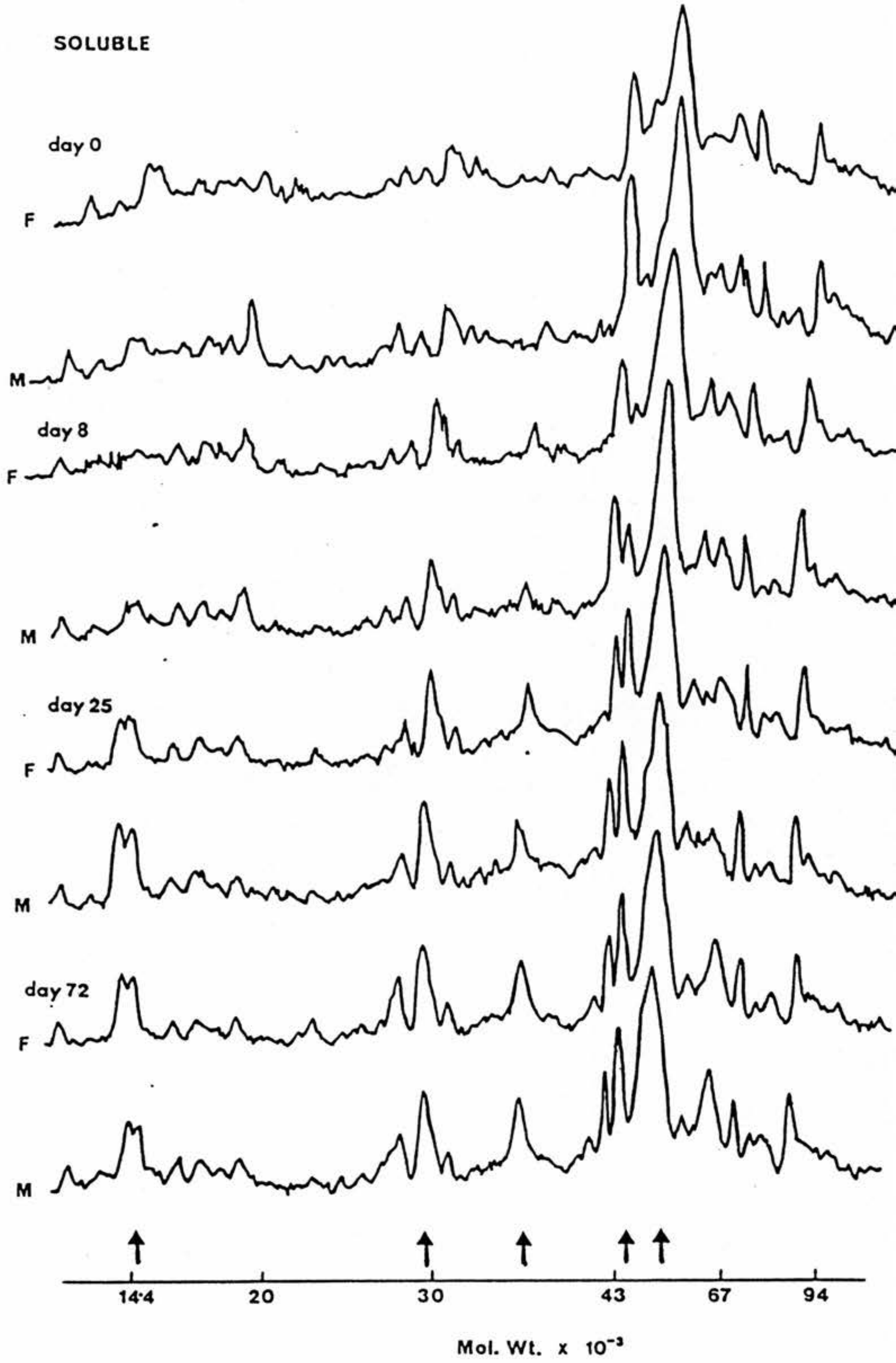
3.3 RESULTS

The fractionation procedure used in these experiments, including two thorough washes and delipidation of the pellets, resulted in quite characteristic electrophoretic profiles for each subcellular fraction.

No major differences were found between the electrophoretic profiles of proteins prepared from H-POA or the 'rest of brain'. Therefore only proteins from the H-POA from male and female rats (and in the case of 80 day animals, androgenised female rats) at different stages of development are described.

3.3.1 Soluble Fraction

A number of quantitative changes in proteins in the soluble fraction were found to occur during brain maturation. Gradual developmental increases in polypeptides with apparent molecular weights of 29,900 and 38,900 were detected, with a more abrupt increase of a 49,000 molecular weight species occurring at 25 days (Figs. 3.A(a) and 3.A.(b)). Two closely associated low molecular weight proteins (14,600 and 15,000), which were at their lowest concentration at 8 days, increased toward adulthood. A sex difference was detected in the density of the latter two proteins at 25 days, the density in the male being about twice that in the female. The 53,100 to 56,200 molecular weight bands were denser in the male than in the female at day 0 (by approximately 30%).



b) Densitometric scans of the soluble proteins.

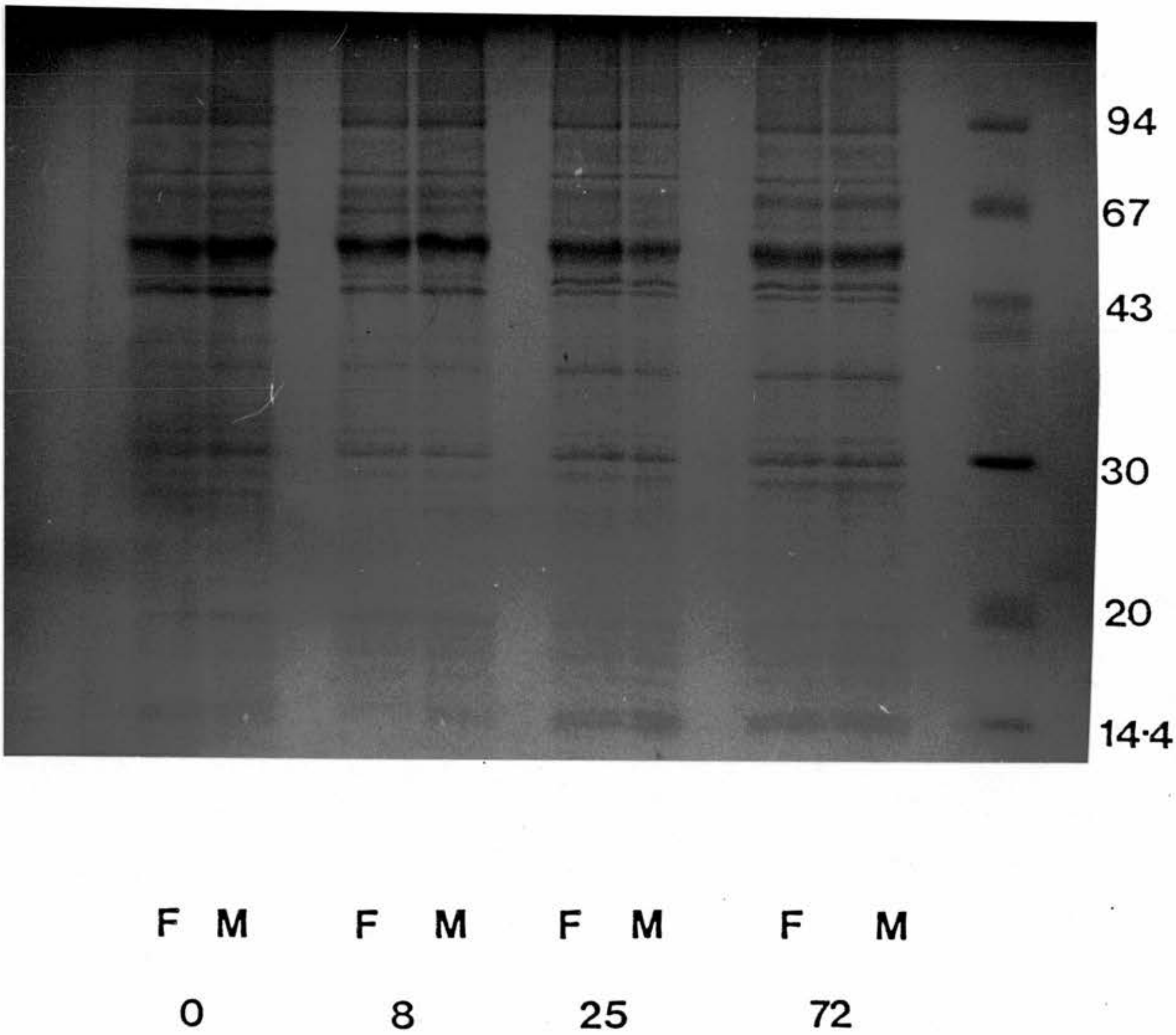


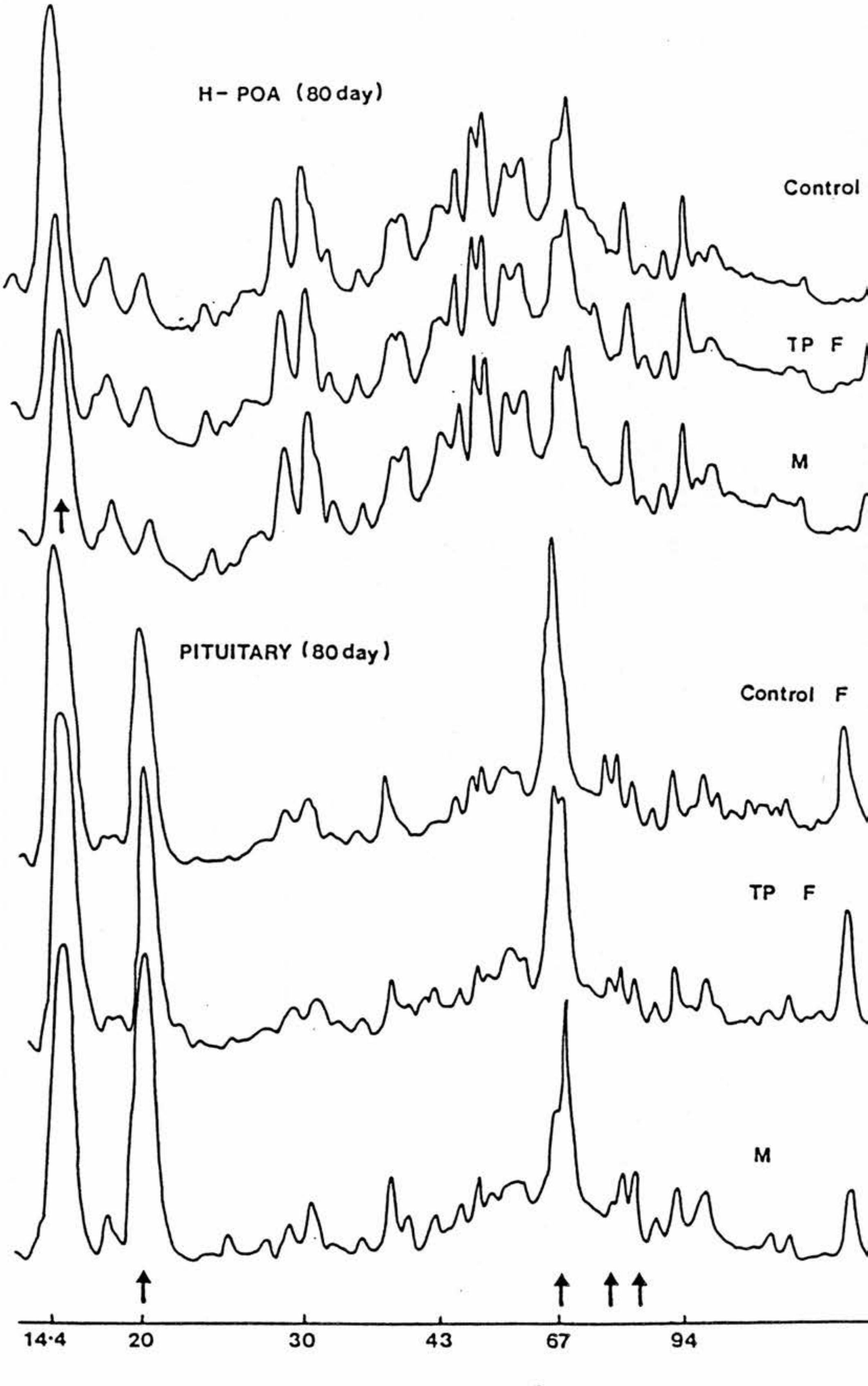
FIGURE 3.A: Developmental Changes In Soluble Proteins From Rat H-POA

a) Electrophoretic profiles of proteins from the 120,000g supernatant, analysed by SDS-PAGE. The gel was loaded with 48µg protein and stained with Coomassie Brilliant Blue R250. Numbers at the foot of the gel represent age (days) and numbers at the side of the gel represent marker protein molecular weights X 10^{-3}.
F = Female, M = Male

In contrast to the soluble proteins isolated from 72 day animals, in which there was no difference between male and female H-POA, in the 80 day H-POA a quantitative sex difference was found in a very broad band with an approximate molecular weight of 14,800. (Figs. 3.B.(a) and (b)). This protein(s) was reduced by approximately 30% in both the male and androgenised female H-POA compared with the control female. Soluble proteins from the pituitary glands of 80 day rats showed a number of differences between groups (Figs. 3.B.(a) and (b)). A dense, low molecular weight band of approximately 18,600 daltons was present in greater quantities (approximately 20%) in the androgenised female than in the control female and in even greater quantities in the male (25%) than in the control female. An increase in the density of a 64,600 molecular weight protein was found in the androgenised female compared with both the control female and the male. In addition, a protein with an apparent molecular weight of 75,000 daltons was reduced in the androgenised female (by approximately 50%) and further reduced in the male (by approximately 90%) compared with the control female, and an 82,200 molecular weight species was increased in the male compared with both the control and androgenised female.

3.3.2 Nuclear Fraction

Major quantitative changes in nuclear proteins occurred throughout development, particularly in the 40,000-50,000 molecular weight range and in the low molecular weight proteins between 13,800 and 16,000 daltons (Figs. 3.C.(a) and 3.C.(b)). The 42,000, 46,800 and 48,400 molecular weight proteins increased in density from day 0 to day 8 and then gradually decreased with age. Developmental



b) Densitometric scans of the 80 day rat soluble proteins from the H-POA and pituitary gland.

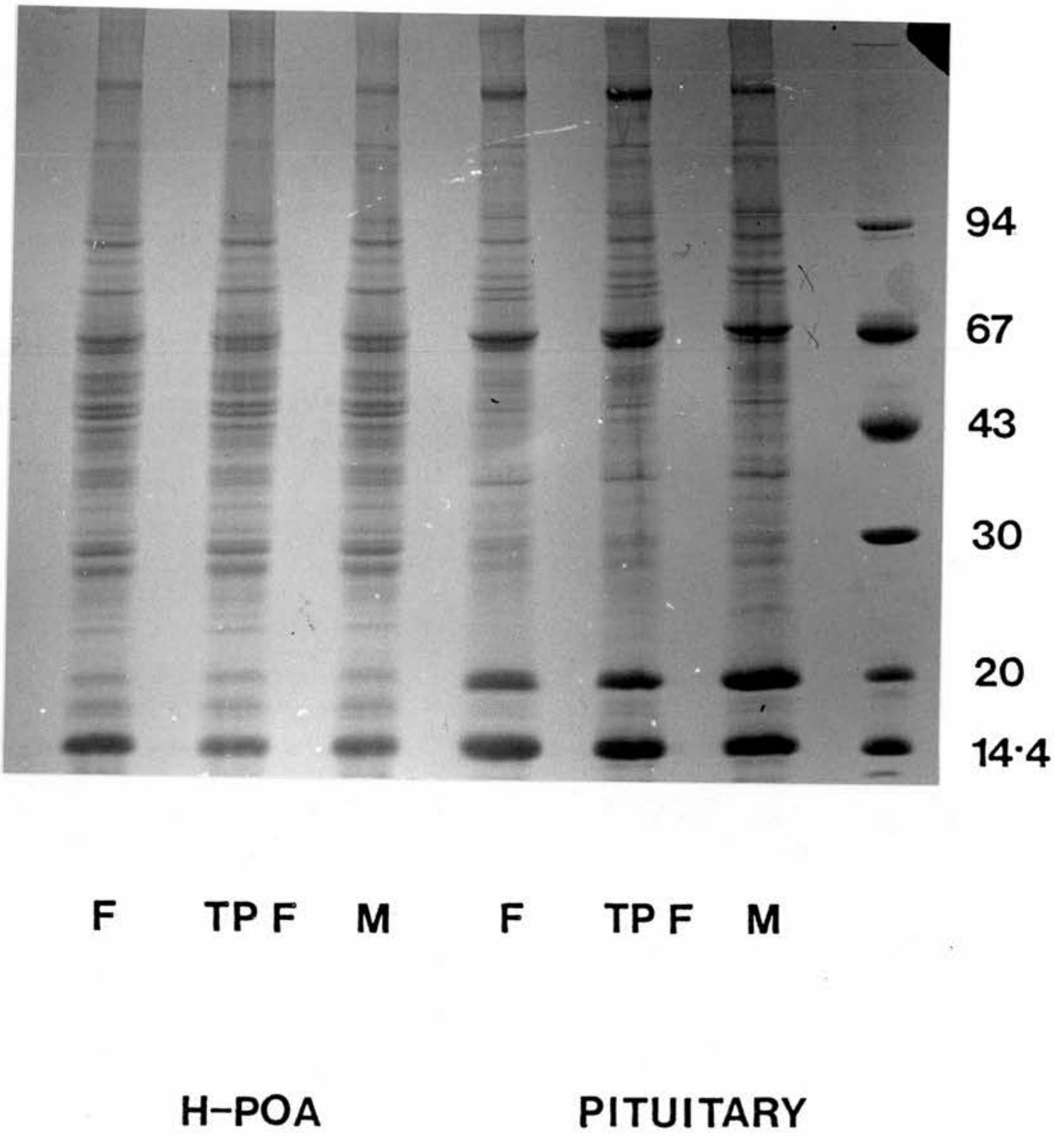
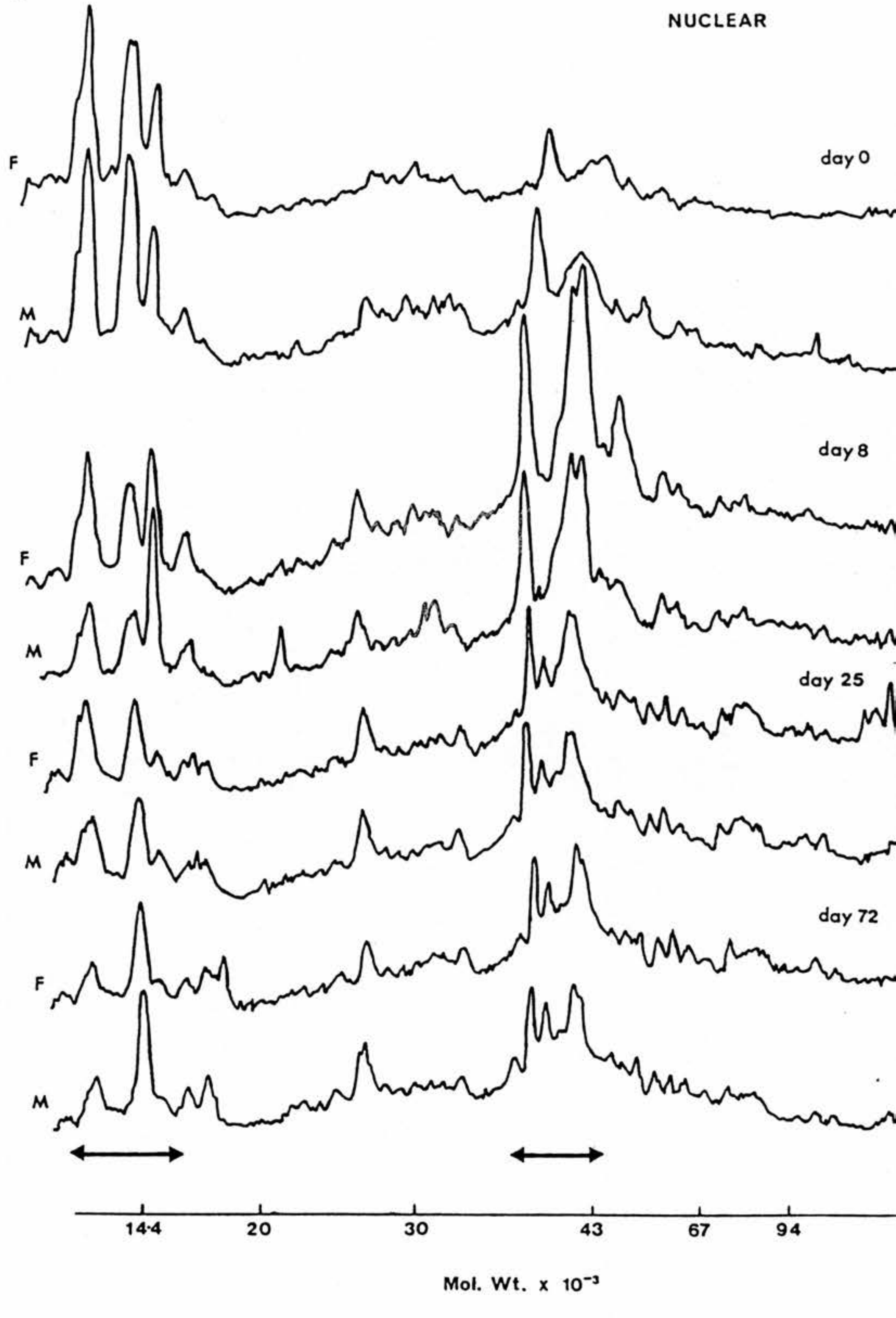


FIGURE 3.B: Soluble Proteins From The 80 day Rat H–POA And Pituitary Gland.

a) Proteins from the 120,000g supernatant were analysed by SDS–PAGE. The gel was loaded with 100µg protein and stained with Coomassie Brilliant Blue R250.
Numbers at the side of the gel represent marker protein molecular weights X 10^{-3}.
F = Female, TP F = Androgenised Female and M = Male.



b) Densitometric scans of the nuclear proteins.

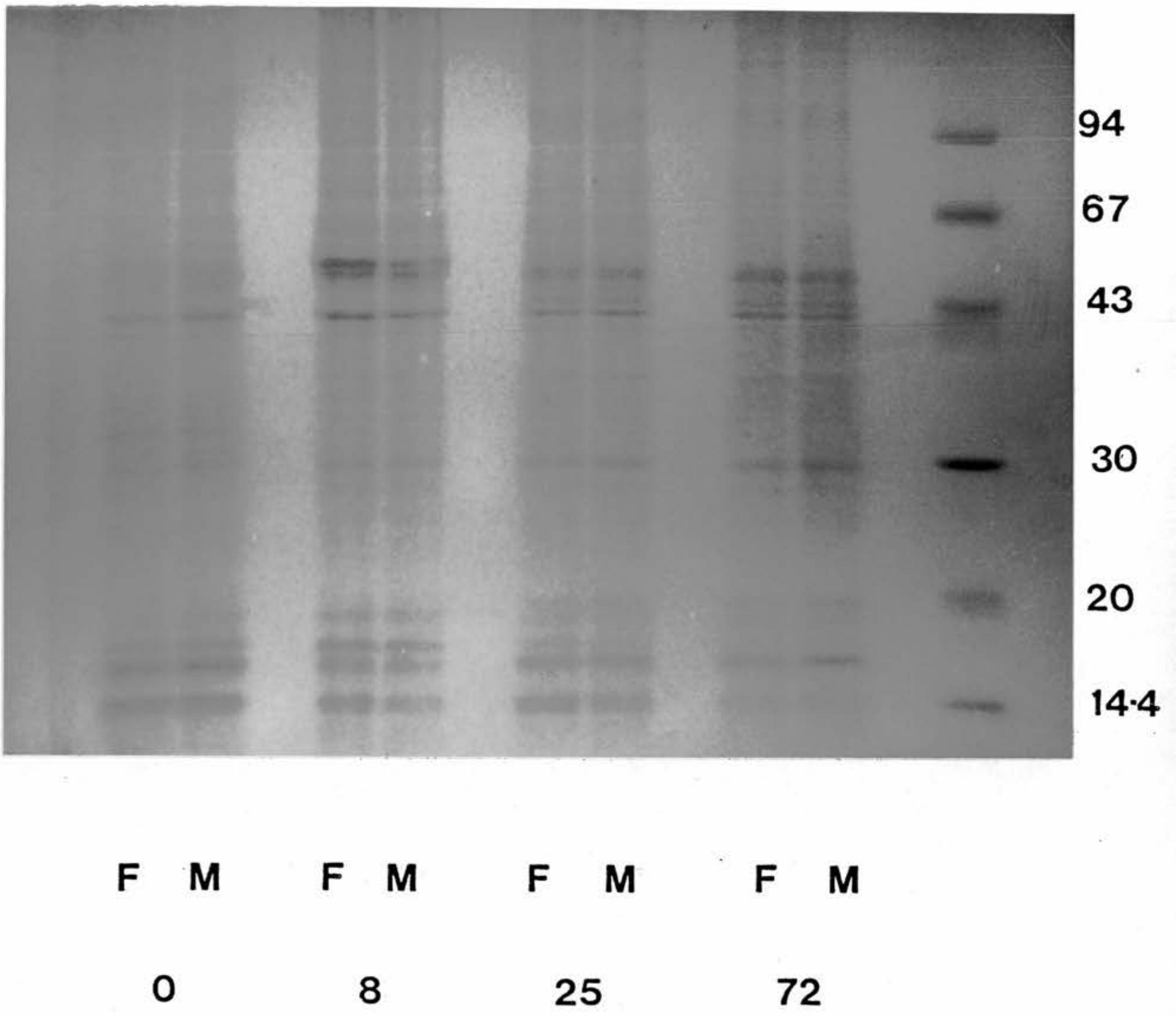


FIGURE 3.C: Developmental Changes In Nuclear Proteins From Rat H-POA

a) Electrophoretic profiles of SDS extract from the 1,000g pellet. Proteins (100µg) were analysed by SDS-PAGE and stained with Coomassie Brilliant Blue R250.
Numbers at the foot of the gel represent age (days) and numbers at the side represent marker protein molecular weights X 10^{-3}.
F = Female, M = Male

decreases occurred in the 13,800 to 16,000 molecular weight range of proteins. A protein with an apparent molecular weight of 43,200 appeared between days 8 and 25 and increased with age.

No consistent sex differences were detected in proteins associated with the nuclear fraction.

3.3.3 Mitochondrial-Lysosomal Fraction

Developmental decreases in proteins with apparent molecular weights of 49,000 (by approximately 80%) and 52,000 (by approximately 60%) occurred in the mitochondrial-lysosomal fraction (Figs. 3.D. (a) and 3.D. (b)).

No consistent sex differences were detected.

3.3.4 Microsomal Fraction

The most striking developmental change in proteins from the microsomes occurred between days 8 and 25, with a huge reduction in protein density at 50,100 and 56,900 daltons (Figs. 3.E. (a) and 3.E. (b)). An increase in a high molecular weight protein (approximately 130,000 daltons) occurred with age. Low molecular proteins between 14,400 and 20,000 also increased with age.

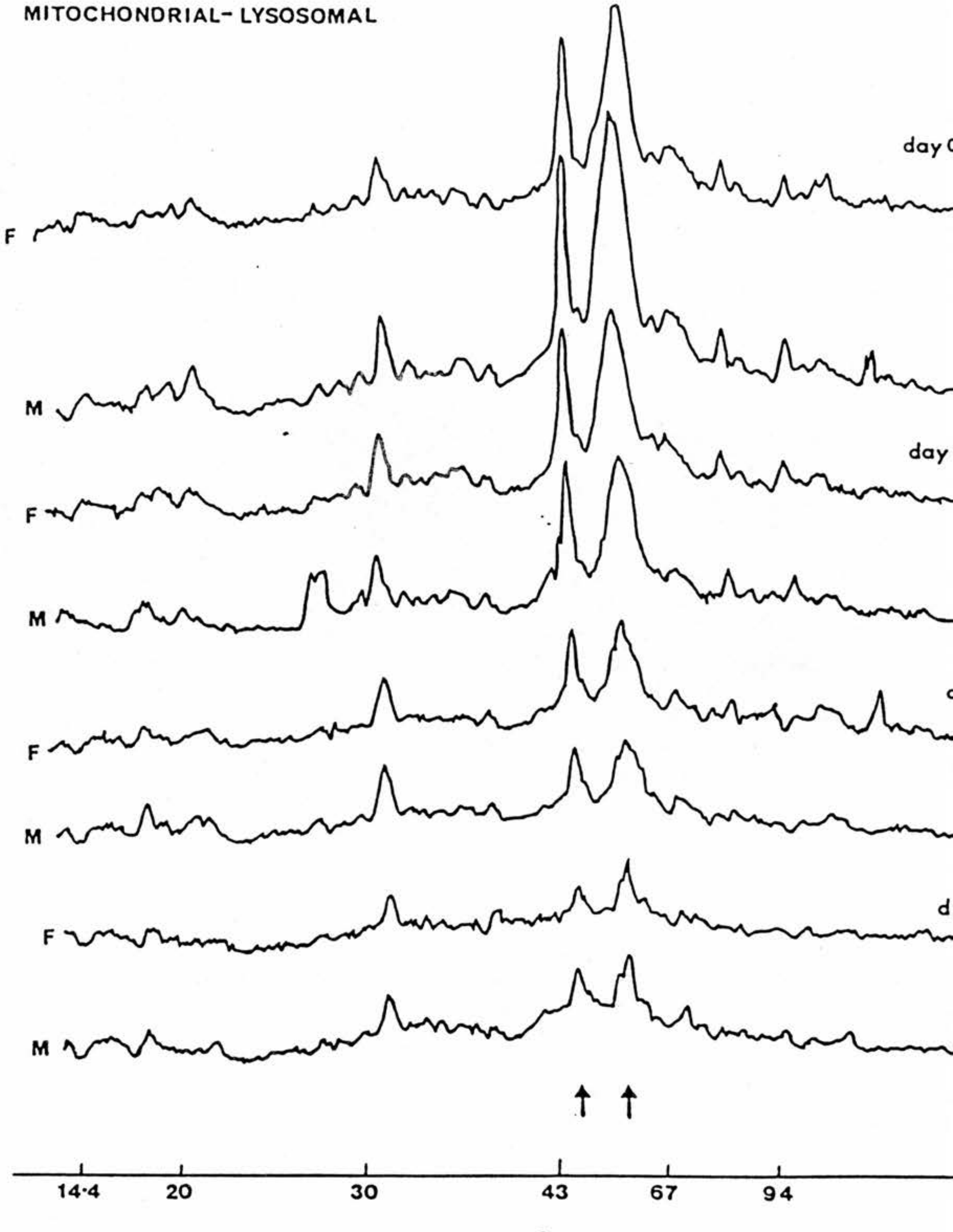
Male and female protein patterns were similar at all ages.

3.3.5 120,000g Pellet from 80d H-POA and Pituitary

No consistent differences between groups were detected in the proteins extracted from the 120,000g pellet from either the H-POA or pituitary gland (Fig.3.F.).

3.3.6 Isoelectric Focussing

Developmental changes in proteins associated with all subcellular fractions were found when proteins were separated according to pI in rod gels (data not shown). No consistent sex



b) Densitometric scans of the mitochondrial-lysosomal proteins.

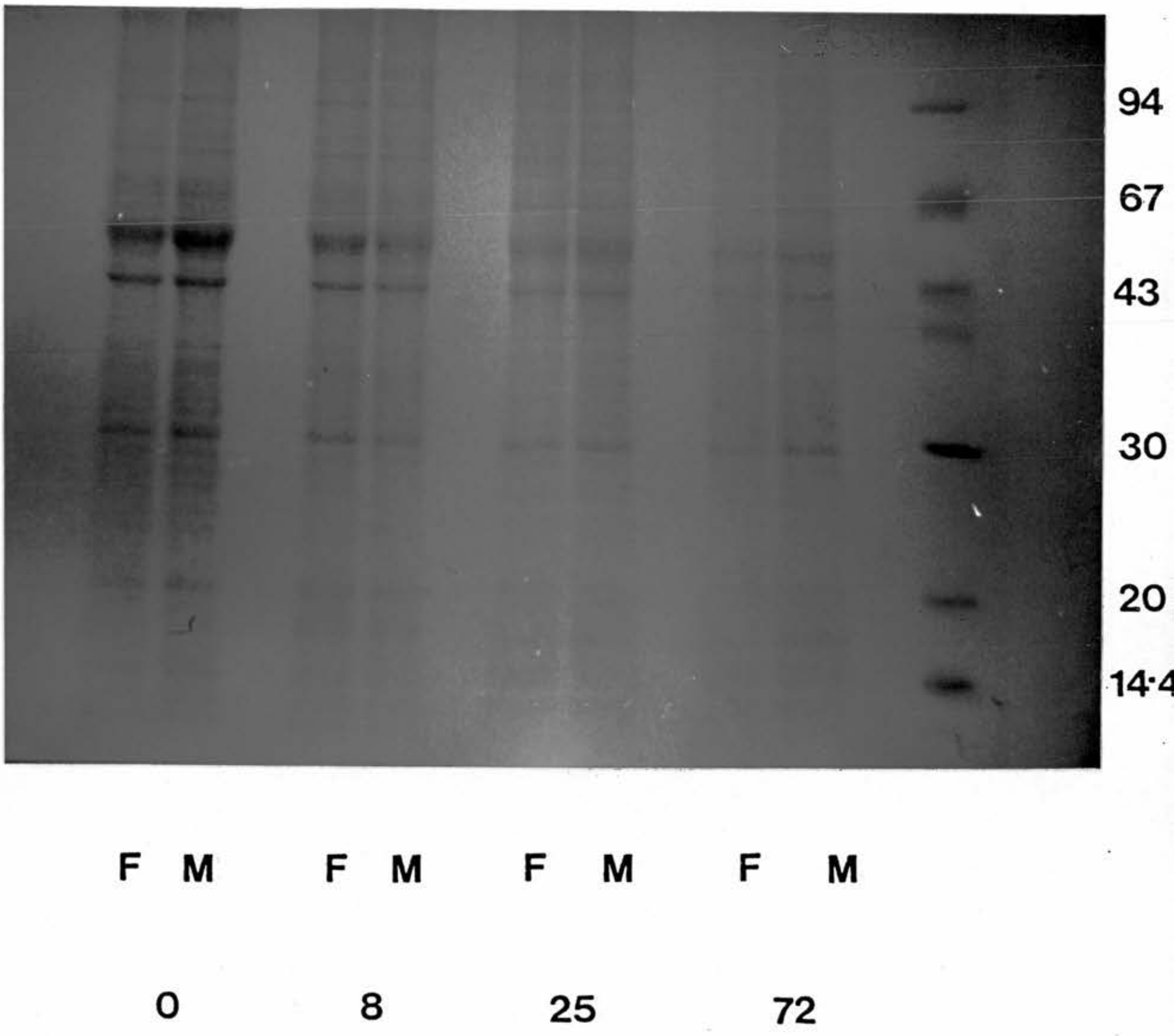
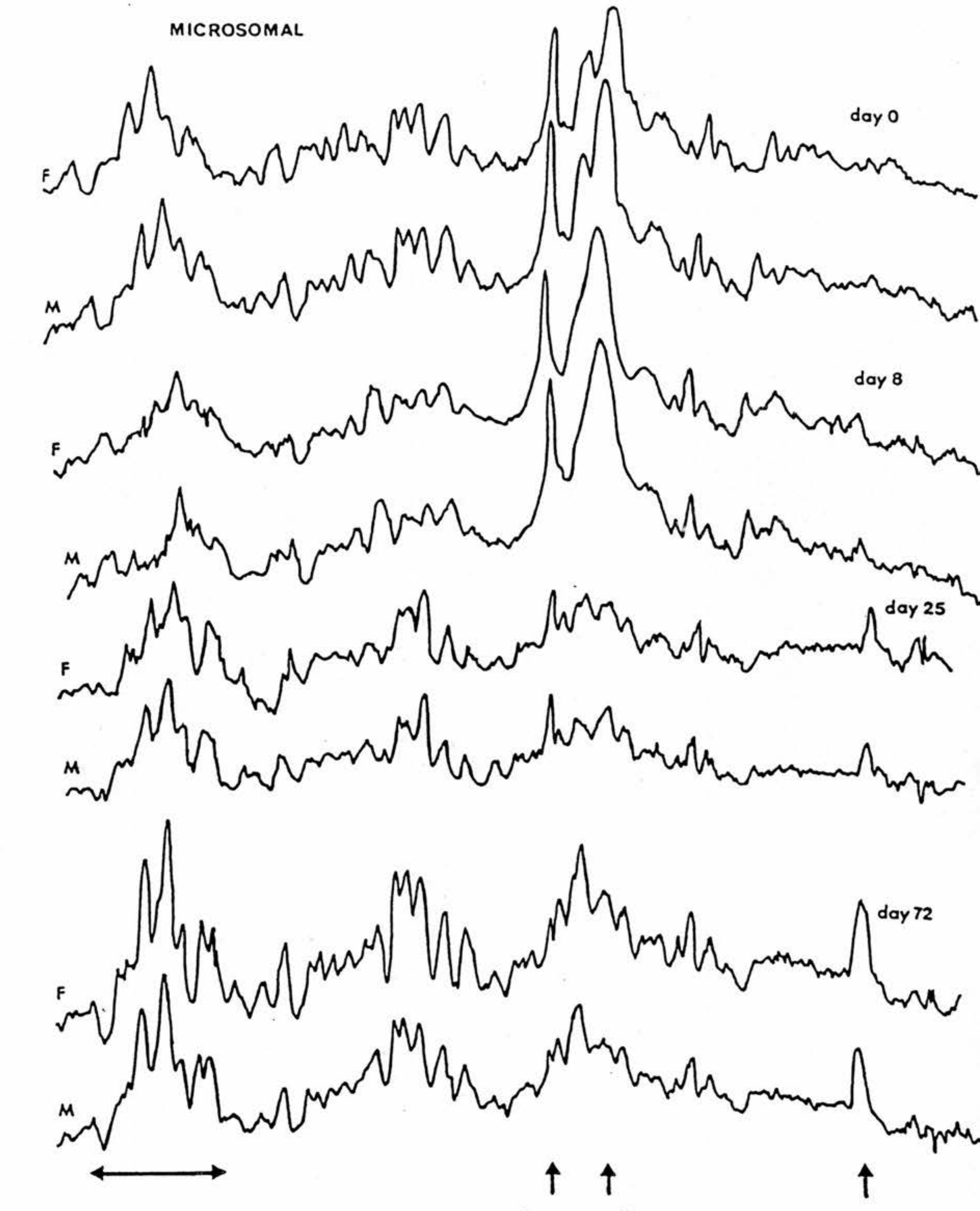


FIGURE 3.D: Developmental Changes in Mitochondrial-Lysosomal Proteins From Rat H-POA.

a) Proteins (100µg) from the SDS extract of the 15,000g pellet were analysed by SDS-PAGE and stained with Coomassie Brilliant Blue R250.
Numbers at the foot of the gel represent age (days) and numbers at the side represent marker protein molecular weights X 10^{-3}.
F = Female, M = Male.



b) Densitometric scans of microsomal proteins.

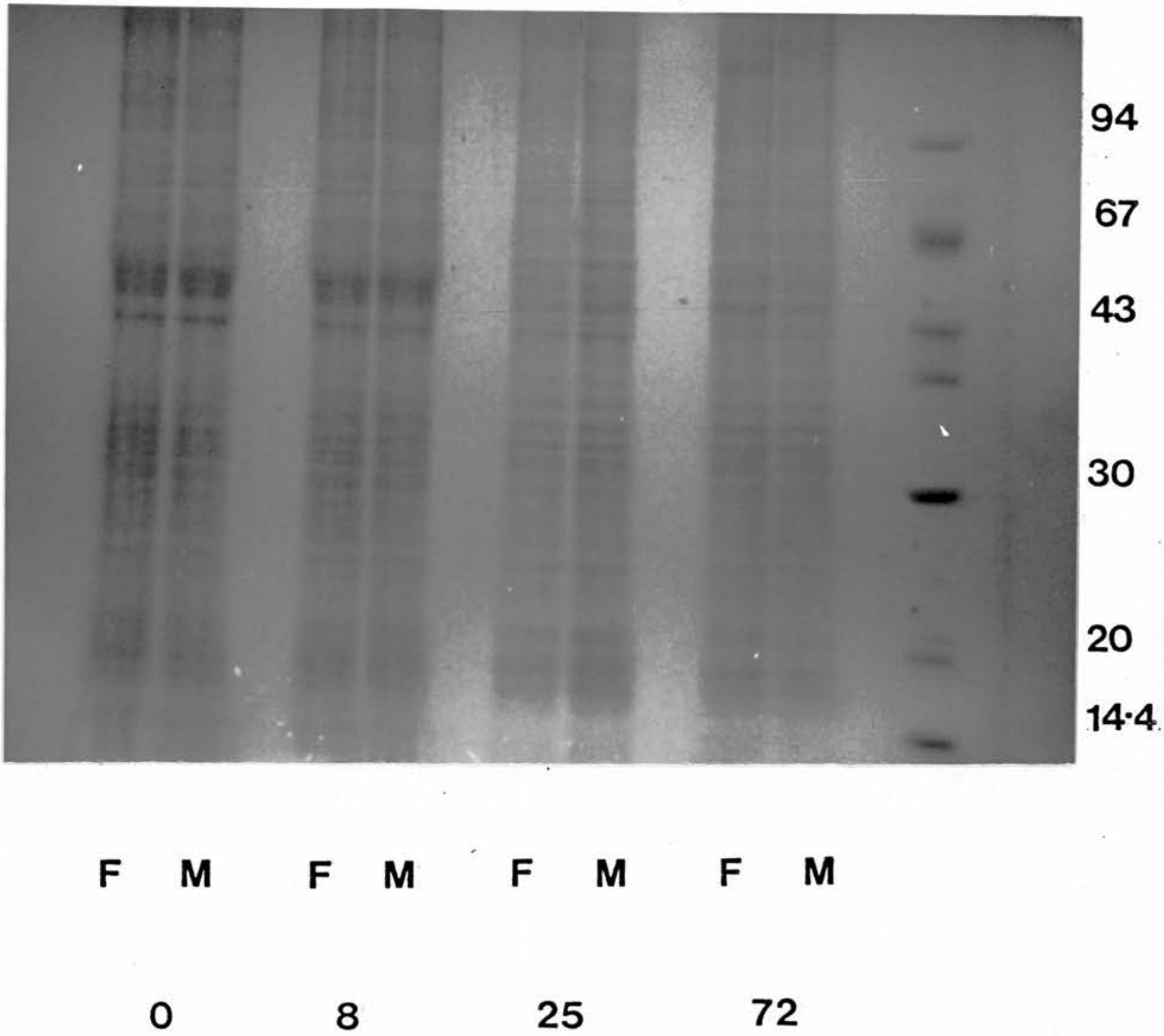


FIGURE 3.E: Developmental Changes In Microsomal Proteins From Rat H–POA.

a) Proteins (100μg) from the SDS extract of the 120,000g pellet were analysed by SDS–PAGE and stained with Coomassie Brilliant Blue R250.
Numbers at the foot of the gel represent age (days) and numbers at the side represent marker protein molecular weights X 10^{-3}.
F = Female, M = Male

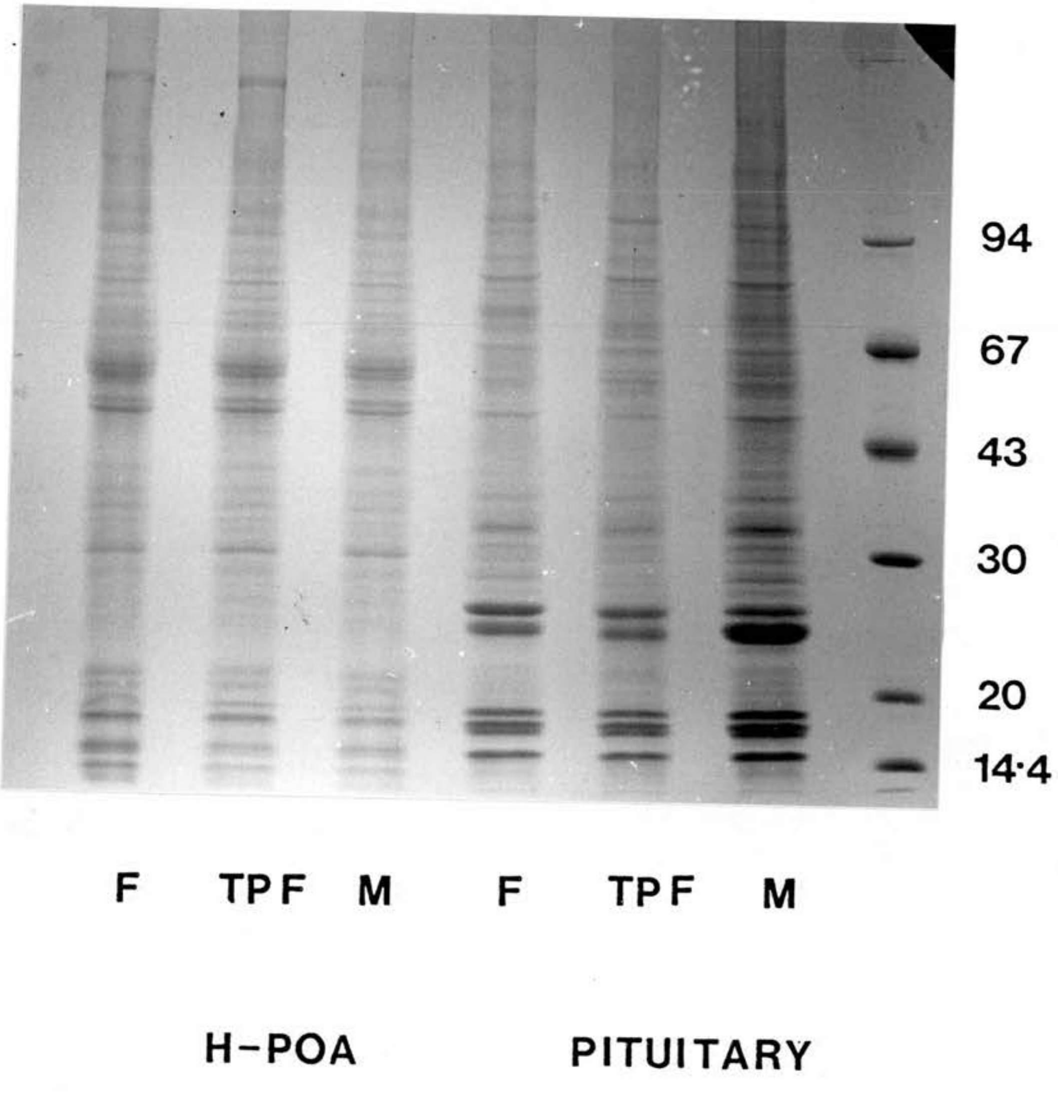


FIGURE 3.F: Pellet Proteins From The 80 day Rat H-POA And Pituitary Gland.

a) Electrophoretic profiles of the SDS extract from the 120,000g pellet.
F = Female, TP F = Androgenised Female, M = Male.
Numbers at the side of the gel represent marker protein molecular weights X 10^{-3}.
100µg protein was analysed by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue R250.

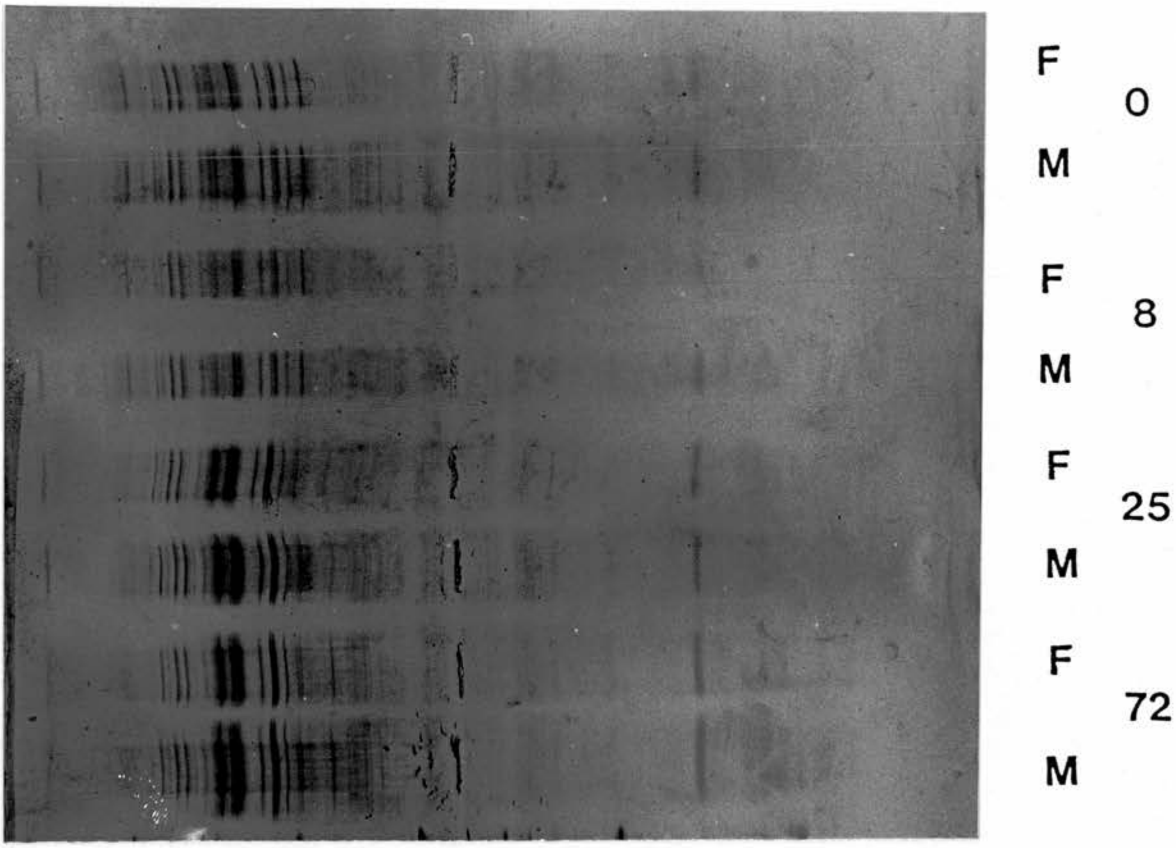


FIGURE 3.G: Developmental Changes In Soluble Proteins: Analysis By Flat Bed IEF.

Proteins from the soluble fraction of male (M) and female (F) rat H–POA throughout development were analysed by flat bed IEF as described in section 2.2.7. The gel was loaded with 30µg each sample and focussed at 20W (constant power) for 2h. Proteins were detected by staining with Coomassie Brilliant Blue R250. Numbers at the foot of the gel represent the age (days).

differences were detected. Developmental changes in the soluble protein pattern are seen in Fig. 3.G. where the flat bed IEF gel allows an easier comparison of many samples to be made. The major developmental changes were in proteins with PI values of 5.95, 5.85, 5.35 and 4.45.

3.4 DISCUSSION

The results show that marked quantitative changes occur in a number of the most abundant cell proteins in both male and female rat H-POA during development (Figs. 3.A, 3.C, 3.D, 3.E and 3.G). Although it is difficult to directly correlate the major protein changes found with specific physiological events, a number of functional changes in brain associated with development and aging might be expected to cause changes in patterns of protein synthesis within various subcellular fractions. Data are available on developmental changes in the activities of a number of enzymes which serve as markers for various subcellular fractions (Banik and Davison, 1969; James and Kanungo, 1978). For example, during development, glycolysis in the newborn brain is largely replaced by respiratory activity and the increase found in succinic dehydrogenase activity is directly related to this fundamental change in glucose metabolism (McIlwain, 1966; Davison and Dobbing, 1968; Gregson and Williams, 1969). Therefore, in view of the undifferentiated nature of neonatal rat brain, both structurally and functionally, a variety of structural proteins and enzymes which have been shown to be typically associated with certain subcellular fractions might have been expected to increase or decrease in quantity with development of the brain.

Sex differences occurred in the soluble fraction in proteins with apparent molecular weights of 14,600 and 15,000 and in proteins in the molecular weight range 53,100 to 56,200 (Figs. 3.A(a) and 3.A(b)). The major sex difference was in the latter group of proteins which were present in greater quantities in the male than in the female at birth. The 53,100 to 56,200 molecular weight region of the gel will have included the subunits of tubulin but whether this sex difference represents a difference in microtubule content, possibly related to enhanced neuritic growth in the male can only be speculative. No age-related decrease in tubulin content was found as reported by others using immunochemical (Gozes, Littauer, Geiger and Fuchs, 1977) and electrophoretic (Schmitt, Gozes and Littauer, 1977) techniques: in this study this may have been due to the relatively poor resolution obtained in the 53,000 to 56,000 region of the gel, possibly due to the heterogeneity of tubulin itself (Fiet, Slusarek and Shelanski, 1971; Gozes and Littauer, 1978; Marotta, Harris and Gilbert, 1978).

The finding of a marked developmental increase of a 49,000 molecular weight protein occurring in the soluble fraction between days 8 and 25 (Figs. 3.A(a) and 3.A(b)) may relate to (i) the report of Grossfield and Shooter (1971) that a soluble protein, found midway in the gel, but of no specified molecular weight, increased in mouse brain during the first month of life and (ii) the report by Kelly and Luttges (1976) that a marked increase in a 50-55,000 molecular weight species occurred in the soluble fraction at 20 days of age in the mouse. Assuming the identity of these proteins to be the same, the difference in molecular weight values between the data of Kelly and Luttges (1976) and those reported here could perhaps be accounted for by differences in gel gradients and therefore estimates of molecular weight. Although the 49,000 molecular weight protein migrated with the same Rf value (0.44) as creatine kinase, the major component of oestrogen-induced protein in brain (Reiss and Kaye, 1981), further studies would be required for positive identification. Oestrogen-induced protein has been detected in unfractionated rat brain cytosol (Walker, Negreanu, Gozes and Kaye, 1979) and found to increase during the first 4 weeks after birth (Kaye, Reiss and Walker, 1980).

In the soluble fraction of H-POA, a sex difference was found in a broad band with an approximate molecular weight of 14,800 at 80 days (Figs. 3.B(a) and 3.B(b))but not at 72 days (Figs. 3.A(a) and 3.A(b)). The fact that the 30% reduction of this band in the 80 day male compared to the female was also found in the 80 day androgenised female suggests that the sex difference at this age may be due to the exposure of the brain to high levels of testosterone during the perinatal period. The reason for the late expression of this characteristic is not clear, but the gene for the protein would appear to be switched on between days 72 and 80. The electrophoretic profiles of the 120,000g supernatants seen in Figs. 3.A and 3.B are not identical and either the difference in sample preparation (section 3.2.2) or age-related changes in protein patterns may account for this.

Pituitary proteins from 80 day rats also showed sex differences in the soluble fraction (section 3.3.1, Figs. 3.B(a) and 3.B(b)) and the changes seen in the 18,600 and 75,000 dalton proteins in the male compared with the control female could be produced by TP administration to neonatal female rats. The results therefore show that exposure of the hypothalamus and pituitary gland to testosterone during the 'critical period' caused long-lasting changes in the electrophoretic pattern of soluble proteins. The experiments of Harris and Jacobsohn (1952) and Adams-Smith and Peng (1966) showed that pituitary tissue grafted under the median eminence of the female rat brain would initiate, or reinitiate, the pattern of female reproductive cycles irrespective of whether the graft tissue was derived from male, female or androgenised female rats. This suggested that the masculinising action of the testes or testosterone in the neonatal female was due to an action predominantly on the brain rather than the pituitary gland. However, the responsiveness of the anterior pituitary gland to LHRH was found to be markedly less in the male and androgenised female than in the normal female (Barraclough and Turgeon, 1974; Fink and Henderson, 1977) and, in contrast to the normal female, the manipulation of steroids had relatively little effect in the male and virtually no effect in the androgenised female (Fink and Henderson, 1977). The decreased pituitary responsiveness occurred within 24 hours after TP administration and persisted into adult life (Barraclough and Turgeon, 1974). By virtue of the priming effect of LHRH (Aiyer, Chiappa and Fink, 1974; Fink, Chiappa and Aiyer, 1976; Speight, Popkin, Watts and Fink, 1981), the responsiveness of the anterior pituitary gland to LHRH can be significantly influenced by LHRH itself, and therefore the studies by Barraclough and Turgeon (1974) and Fink and Henderson (1977) do not negate the conclusions drawn from the experiments of Harris and Jacobsohn (1952) and Adams-Smith and Peng (1966). However, the data on the sex difference in the responsiveness of the pituitary to LHRH are at least compatible with the possibility that as well as exerting an effect on the brain, TP administered neonatally could exert a long lasting effect on the pituitary gland. In the mouse, TP had a striking effect on ovarian structure which was attributed to a direct action on the gonad as well as an action mediated by the hypothalamic- pituitary system (Peters et al., 1970). This shows further that the peripheral effects of TP should not be dismissed, and that, therefore, the sex differences in the pituitary proteins (Fig. 3.A(a) and (b)) could be due to a peripheral as well as a central action of testosterone.

Brain protein content increases rapidly up to postnatal day 12 with the steepest increase occurring between days 8 and 12 (Matthieu et al., 1973) during which there is also an exponential increase in the number of transmitter vesicles in the median eminence (Fink and Smith, 1971) and in the hypothalamic content of non-peptide (e.g. Smith and Simpson, 1970) and peptide (e.g. Araki, Toran-Allerand, Ferin and Vande Wiele, 1975; Chiappa and Fink, 1977) transmitters. After day 12, protein synthesis begins to decline and DNA and RNA concentrations in brain reach adult values (Matthieu et al., 1973). The rate of DNA synthesis increases from day 1 to day 8 and this is associated with a high rate of synthesis of the nuclear proteins, particularly the histones (Burdman, 1972). In the present study, a reduction in low molecular weight nuclear proteins (13,800 to 17,000) was found with increasing age (Figs. 3.C(a) and

3.C(b)). These low molecular weight proteins have the characteristics of histones as assessed by their electrophoretic pattern on SDS gels (Mahadik et al., 1976; Greenwood, Silver and Brown, 1981), but positive identification is still required. Although the rate of histone synthesis is higher on day 8 than day 1 (Szijan and Burdman, 1973), the concentration of histones has been reported not to change throughout development (Szijan and Burdman, 1973; Biessman and Rajewsky, 1975; Kelly and Luttges, 1976). Waehneldt and Neuhoff (1974) did however report a developmental decrease in histones, starting between day 5 and 15, a finding similar to the present results.

The group of 40,000 - 50,000 molecular weight nuclear proteins belong to the non-histone group of nuclear proteins (Mahadik et al., 1976; Greenwood et al., 1981). The non-histone proteins, which probably include structural proteins and enzymes of nucleic acid metabolism (Patel, Howk and Wang, 1967; Wang, 1968; O'Conner, 1969), may be involved in the control of gene expression (Spelsberg, Wilhelm and Hnilica, 1972) and their synthesis in the uterus appears to be affected by oestrogen administration (Teng and Hamilton, 1969; Cohen and Hamilton, 1975). No sex differences were detected in the present study in the non histone nuclear proteins indicating that sexual differentiation did not significantly alter the concentration of any of those proteins which are perhaps closely associated with the regulation of gene expression. However, as found by Bondy and Roberts (1969) and Szijan and Burdman (1973), developmental changes in the non-histone nuclear proteins were detected, with high concentrations of these proteins occurring in the 8 day H-POA (Figs.

3.C(a) and 3.C(b)).

During brain development the number of mitochondria and the biochemical properties of mitochondria change. With increasing age, there is a 4-6 fold increase in enzyme activity (Gregson and Williams, 1969) and a decrease in mitochondrial protein synthesis (Roodyn, Reis and Work, 1961; Roodyn, 1965). In addition to the enzymes of the tricarboxylic acid cycle (e.g. succinic dehydrogenase) and electron transport chain (e.g. cytochrome c oxidase), the mitochondrial fraction usually also contains CAT and MAO enzymes which, respectively, synthesise and degrade neurotransmitters and which are affected by gonadal steroids (Luine and McEwen, 1977; Luine et al., 1980). CAT activity, for example, decreases with age (James and Kanungo, 1978) reflecting a loss of cholinergic nerve function. Lysosomes, which are also present in the 15,000g pellet, contain a number of degradative enzymes including RNase, deoxyribonuclease and both acid and alkaline phosphatases. The phosphatase enzymes have been shown to be more active in embryonic brain than at later stages of development (Cohn and Richter, 1956), but whether the decreases found with age in the mitochondrial-lysosomal proteins in this study (Figs. 3.D(a) and 3.D(b)) relate to this finding is not so clear. Various structural and functional properties of the mitochondria and lysosomes in proximal tubule cells of the kidney are known to be sexually dimorphic (Koenig, Goldstone, Blume and Lu, 1980). In addition to a generalised increase in RNA and protein synthesis related to hypertrophy of proximal kidney tubules, testosterone also causes specific increases in both the tubular content of acid hydrolases, β-glucuronidase, for example, and the activity of cytochrome c oxidase. In contrast to these data on the kidney, no sex differences were detected in proteins extracted from the mitochondrial-lysosomal fraction of brain and, therefore, no extrapolation can be made to the brain from the responses of the kidney to testosterone.

The microsomal fraction comprises a heterogeneous mixture of membrane fragments from, for example, the endoplasmic reticulum, and Golgi apparatus, and plasma membranes from neurons and glial cells and also contains the ribosomes (approximately 30% RNA, 70% protein) and various enzymes such as 5'nucleotidase, Na$^+$,K$^+$-ATPase and adenyl cyclase. Developmental changes in the properties of brain polysomes have been reported (Yamagami and Mori, 1970) including a decrease in the proportion of heavy polysomes with age, lower rates of polysomal protein synthesis in the adult than in the neonate and a reduction with age in the activity of enzymes associated with the polysomes (e.g., RNAase, phosphodiesterase and 5'-nucleotidase). Samson and Quinn (1967) have also described changes in Na$^+$-K$^+$-ATPase activity with brain maturation. Any of these developmental changes could change the electrophoretic profile of proteins extracted from the microsomes. Although the major developmental changes in proteins associated with the microsomal fraction are described in section 3.3.5, Figures 3.E(a) and 3.E(b) also show numerous less obvious changes in the protein pattern, which probably reflect the variety of subcellular fragments found in this fraction and the changes in proteins associated with them.

In summary, the key results in this chapter are that sex differences were found in soluble proteins in the H-POA at day 0 (53,000-56,000), day 25 (14,600 and 15,000) and day 80 (14,800) and in the pituitary gland at day 80 (18,600 and 75,000), and that major developmental changes also occurred in H-POA proteins in the soluble (14,600 and 15,000, 29,900, 38,900 and 49,000), nuclear (40,000-50,000 and 13,800-16,000), mitochondrial-lysosomal (49,000 and 52,000) and microsomal (14,400-20,000, 50,100, 56,900 and 130,000) fractions. Whether any of the changes in proteins described, either during development or as a result of neonatal exposure to testosterone, relate to functional changes in the hypothalamic-pituitary system, can only be speculative.

CHAPTER 4

Short-Term Effects of Testosterone Propionate On Brain Protein Synthesis In The Neonatal Rat: Investigations *In Vivo*

4.1 INTRODUCTION

Inhibitors of DNA, RNA and protein synthesis can block or partially protect the neonatal rat brain from the masculinising effects of testosterone. Cyproterone acetate, a synthetic antiandrogen can also prevent normal masculinisation in the male H-POA (Neumann, Hahn and Kramer, 1967; Neumann and Kramer, 1967) and inhibit the effects of TP in the neonatal female (Arai and Gorski, 1968c; Wollman and Hamilton, 1967). Salaman and Birkett (1974) found that androgen-induced sterilization in the neonatal female could be prevented by administering either hydroxyurea or α-amanitin in divided doses together with, and 6h after TP. In the same study, inhibition of protein synthesis by puromycin was found to be less effective than hydroxyurea or α-amanitin in blocking the central effects of TP, but the authors maintained that the results were still compatible with an obligatory role of protein synthesis in the process of sexual differentiation. Kobayashi and Gorski (1970) also found that puromycin, injected 4h after TP, could significantly inhibit the masculinising effects of TP as assessed by ovarian morphology at 45 days of age.

The available data therefore suggest that TP exerts some of its most important effects on brain protein synthesis, perhaps by way of mRNA synthesis, within 12h of administration or less. The aim of the present experiments was to investigate the short-term effects of TP on protein synthesis in the neonatal rat H-POA by labelling proteins *in vivo* with ^{35}S-methionine and analysing them by one and two-dimensional PAGE.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Male and female Wistar Cob rats (littermates) aged 4 days (day 0 = day of birth) from the departmental breeding colony were used in all experiments. Animals were taken from the mother and kept in cages in the laboratory under a lamp for warmth throughout the experiment. At time zero (10.00h) female rats were injected (i.p.) with either 0.1ml oil or 1.25mg TP in 0.1ml oil. Four day females which had been injected with TP were then kept in the same cage as the males, separate from the oil-treated females.

4.2.2 Intraventricular Injection of ^{35}S-methionine

At various time intervals (see RESULTS) after the injection of oil or TP, approximately 20-30μCi ^{35}S-methionine (specific activity > 800Ci/mmol) in a volume of 2 or 3μl were injected into the third ventricle of the rat using a 5μl Hamilton syringe (S.G.E. Ltd., Melbourne, Australia). The animal was anaesthetised with ether and a small incision (approximately 5mm long) was made in the scalp in a rostral-caudal direction. The needle of the syringe was aligned with the sagittal suture and then carefully pushed through lambda at 90° in the coronal plane and about 45° in the sagittal plane. The needle was pushed to a depth of approximately 9mm, which, as assessed in preliminary studies, placed the tip in the third ventricle just above the optic chiasm. In some animals the tip touched the floor of the skull, and in these cases it was withdrawn about 1mm. The ^{35}S-methionine was then expelled over a period of about 30 sec. The needle was carefully withdrawn, the wound closed with a small piece of tape, and the animal returned to the appropriate cage. One hour later the rat was killed by decapitation and the H-POA and 'rest of brain' were dissected out as described in section 2.2.2. In view of the possibility that changes in protein synthesis induced by TP, in the POA in particular, might be masked by analysing the whole H-POA, experiments were also carried out in which the POA and hypothalamus were dissected out (section 2.2.2) and analysed separately.

For a straight comparison of proteins synthesised in male and female H-POA, in some experiments 4 day male and female rats which had received no i.p. injection of either oil or TP were injected with ^{35}S-methionine as described.

4.2.3 Subcellular Fractionation and PAGE

Tissue samples were homogenised (immediately after dissection) in 5-10 vol sucrose-TKM buffer and centrifuged at 15,000g for 20 min or 120,000g for 1h at 4°C as described in section 2.2.3. The supernatants were collected and stored at -70°C until further analysis. Proteins associated with the pellets were extracted according to the method described in section 2.2.3. The incorporation of radioactivity into protein and the protein concentrations were determined as in sections 2.2.4 and 2.2.5. Samples containing either equal amounts of acid-insoluble radioactivity or equal amounts of protein were analysed by one and two-dimensional PAGE (sections 2.2.6 and 2.2.8). Gels were stained or fluorographed as described in section 2.2.9. Fluorographed gels were dried and exposed to film for 5 days to 6 weeks.

4.2.4 Spread of ^{35}S-methionine in Brain after Intraventricular Injection

To investigate the spread of ^{35}S-methionine in brain during the 1h period after the intraventricular injection, 4 animals were injected with ^{35}S-methionine as described in section 4.2.2. and decapitated 1h later. The whole brain was dissected out and frozen at -30°C in isopentane. Serial coronal sections (25μm) were cut on a cryostat (Bright O.T.F., Cambridge) at -22°C. The sections were exposed to Kodak X-Omat RP film for approximately 20h and the films were then developed as described in section 2.2.9.

4.3 RESULTS

4.3.1 Spread of ^{35}S-methionine in Brain after Injection into the 3rd Ventricle

Figure 4.A shows that 1h after the injection of ^{35}S-methionine into the 3rd ventricle of 4 day rats the radioactive amino acid had reached all regions of the brain since low density radioactive labelling was found in all areas of all brain sections. As expected, the greatest concentration of ^{35}S-methionine was around the 3rd ventricle itself but spreading out ventrally and laterally in the brain.

4.3.2 Analysis of Soluble Proteins from the H-POA which had Incorporated ^{35}S-methionine, By PAGE and Fluorography

The following data are the results obtained from individual experiments.

Proteins in the 120,000g supernatant which were labelled with ^{35}S-methionine in the whole H-POA of oil-treated (C) and

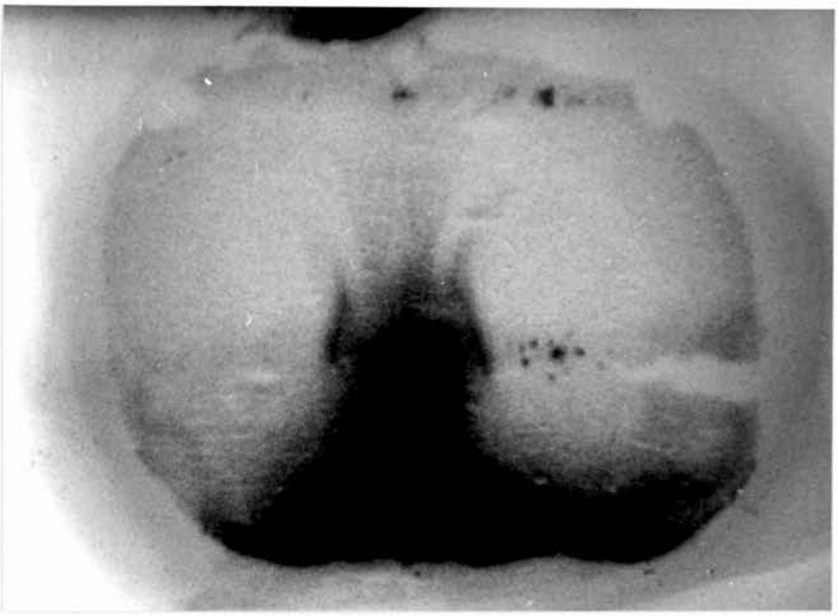
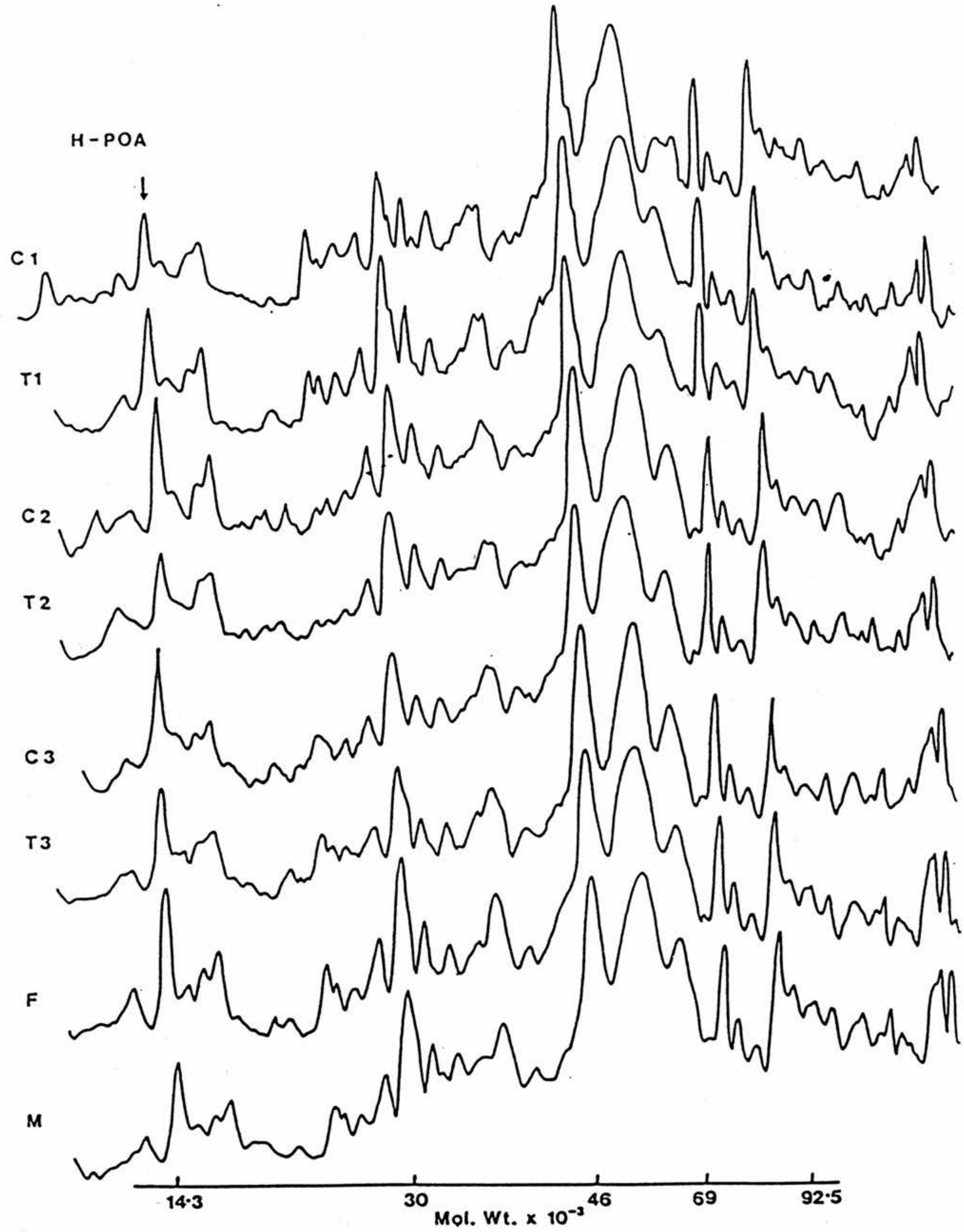


FIGURE 4.A: Coronal Section Of 4 Day Rat Brain After An Intraventricular Injection of ^{35}S-methionine.

Approximately 25µCi ^{35}S-methionine (specific activity > 800Ci/mmol) in a volume of 2µl were injected into the third ventricle of a 4 day female rat under ether anaesthesia, as described in section 4.2.2. One hour later the rat was killed, the brain removed and processed as described in section 4.2.4. The section was exposed to film for 20h at -70°C.

TP-treated (T) rats during the first hour (C1,T1), the third hour (C2,T2) and the sixth hour (C3,T3) after injection of oil or TP are shown in Figure 4.B(a) with corresponding scans in Figure 4.B(b). No major qualitative differences were detected in proteins synthesised in the H-POA of oil-treated or TP-treated rats at any of the times investigated. However, quantitative changes were detected in a protein with an approximate molecular weight of 14,000. After an initial stimulation in synthesis of this protein (by approximately 30%) in the TP-treated female H-POA (Figure 4.B(b), C1 and T1) a reduction in synthesis by approximately 50% and 20% occurred in the TP-treated H-POA compared to the oil-treated H-POA in the third (C2 and T2) and sixth (C3 and T3) hours respectively after TP injection. Visual inspection of two-dimensional gels of the same samples revealed no other effects of TP on protein synthesis in the H-POA during the first (Figure 4.C(a) and 4.C(b)) or sixth hour (data not shown) after TP administration. A comparison of the proteins synthesised in the 4 day female H-POA with the 4 day male H-POA, where neither animal had received an injection of either oil or TP, showed a reduction in synthesis (by approximately 30%) of the same 14,000 molecular weight protein in the male H-POA compared with the female.

Figures 4.D(a) and 4.D(b) show that in an experiment where the POA and hypothalamus were analysed separately, the synthesis of two closely associated low molecular weight proteins found in the 15,000g supernatant (approximately 13,300 and 13,600) was reduced by at least 50%, 2-3h after TP administration. No other changes in protein synthesis were detected.



b) Densitometric scans of the soluble proteins.

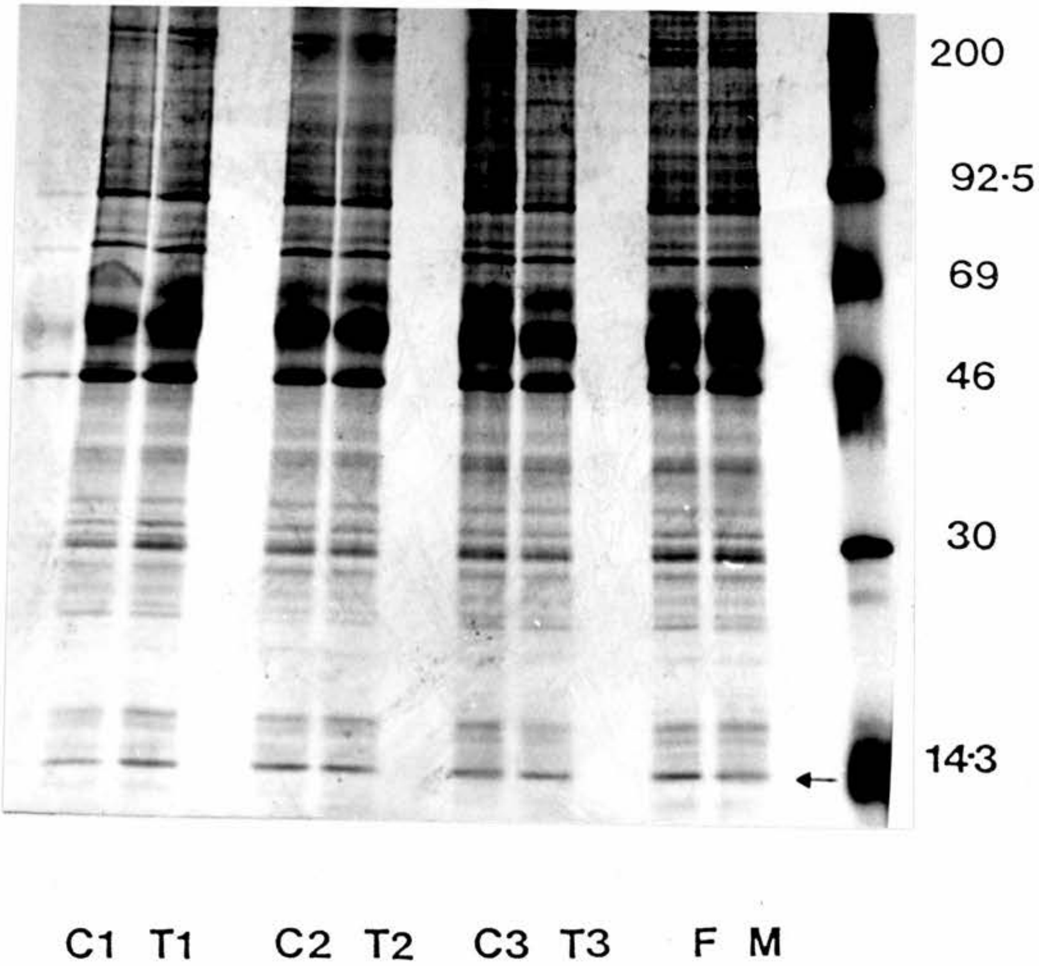
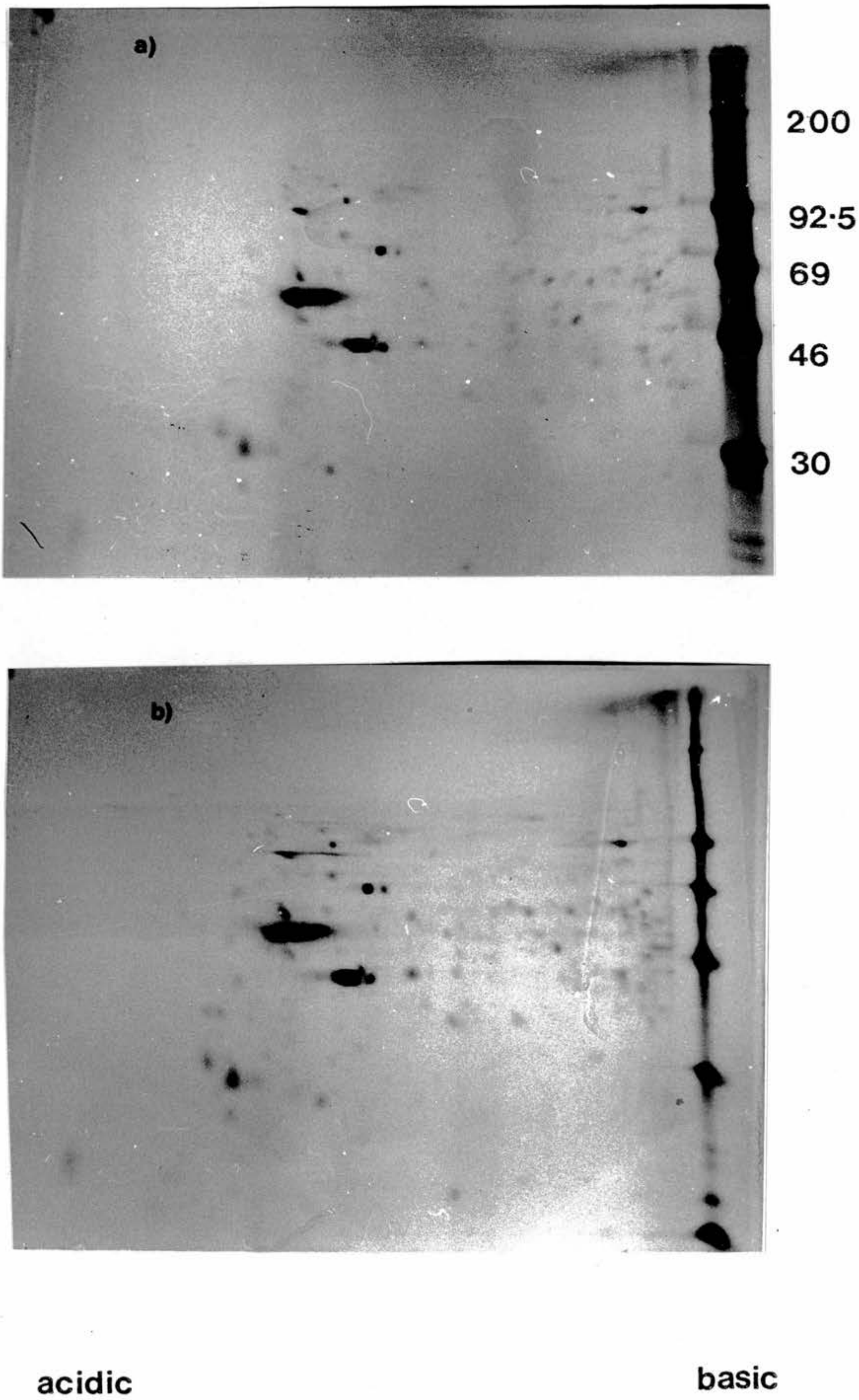


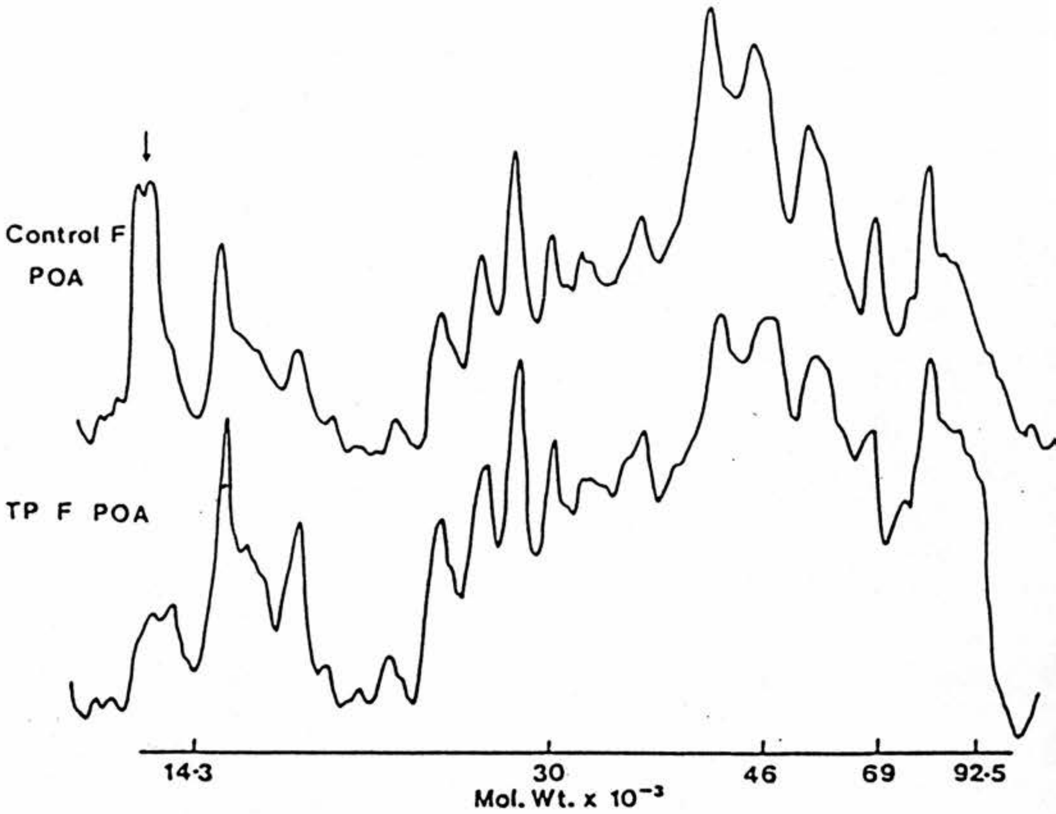
FIGURE 4.B: Soluble Proteins From The H-POA Of 4 Day Rats Labelled In Vivo With ^{35}S-methionine.

a) Electrophoretic profiles of soluble proteins from the H-POA of 4 day oil-treated female (C), TP-treated female (T) and untreated male (M) and female (F) rats. At time zero rats were injected (i.p.) with either 0.1ml arachis oil or 1.25mg TP in 0.1ml arachis oil. Immediately (C1,T1), 2 (C2,T2) or 5h (C3,T3) later, animals were given an intraventricular injection of ^{35}S-methionine (approximately 20μCi in 2μl) and killed 1h later. Soluble proteins were prepared from the H-POA and the incorporation of ^{35}S-methionine into protein was estimated. Samples containing equal amounts of acid-insoluble radioactivity (approximately 20,000 cpm) were analysed by SDS-PAGE. The gel was fluorographed, dried and exposed to film for 5 days. Numbers at the side of the gel represent marker protein molecular weights x 10^{-3},

FIGURE 4.C: Two-Dimensional Gel Analysis Of Soluble Proteins From The H-POA Of 4 Day Rats.

Soluble proteins labelled with ^{35}S-methionine from the H-POA of an a) oil-treated and b) TP-treated 4 day female rat were analysed by PAGE in two dimensions. The proteins analysed were those labelled during the first hour after the i.p. injection of oil or TP and correspond to the C1 and T1 samples of Fig. 4.B. Equal amounts (approximately 20,000 cpm) of acid insoluble radioactivity were loaded onto each gel. After two dimensional PAGE the fluorographed gels were dried and exposed to film for 20 days at -70°C. The acidic and basic ends of the IEF gel are indicated. Numbers at the side of the gel represent marker protein molecular weights x 10^{-3}.


a)
200
92·5
69
46
30
b)
acidic
basic



b) Densitometric scans of C2 and T2 samples.

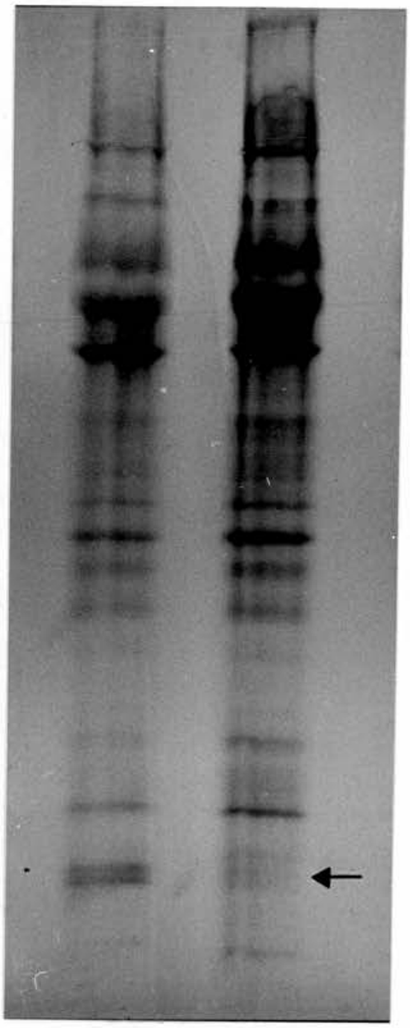
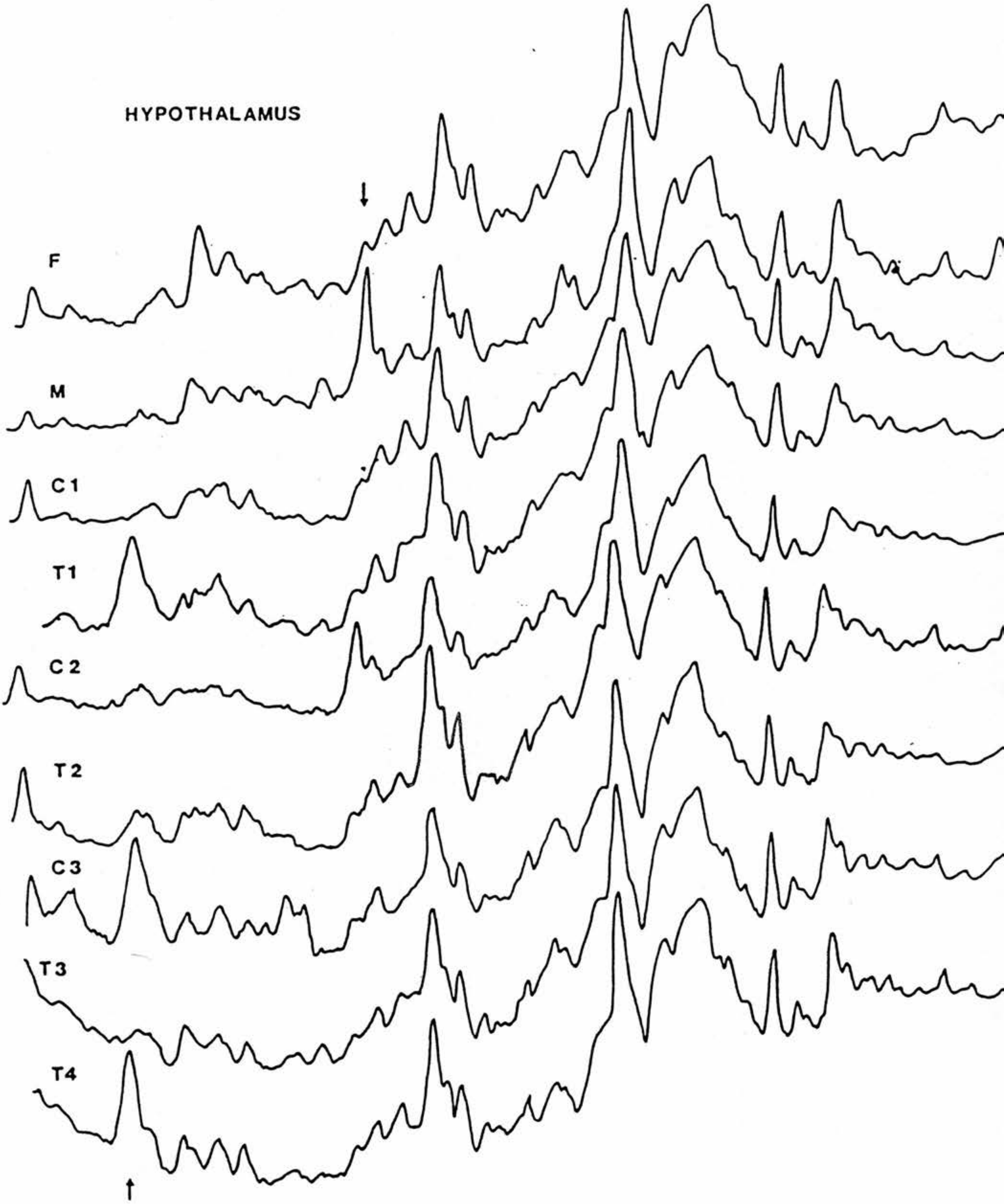


FIGURE 4.D: Proteins Labelled With ^{35}S–methionine From The POA of 4 Day Rats.

a) Electrophoretic profile of proteins in the 15,000g supernatant labelled in vivo 2-3h after the injection of either oil (C2) or TP (T2) in 4 day female rats. For each sample, 8,000 cpm were loaded onto the gel. After electrophoresis the gel was fluorographed, dried and exposed to film for 14 days at -70°C.

In a further experiment, the synthesis of two low molecular weight proteins in the 15,000g supernatant (approximately 13,500 and 13,800) was again found to vary with time after the i.p. injection of oil or TP. Figures 4.E(a) and (b) and Figures 4.F(a) and (b) show the autoradiograms of this experiment and the corresponding scans. In 4 day male and female rats given no injection of oil or TP the synthesis of the low molecular proteins (13,500 and 13,800) was very low (Figures 4.E). As in the previous experiments, after an initial stimulation by approximately 95%) in the synthesis of these proteins in the TP-treated female hypothalamus (Figures 4.E,C1 and T1), a reduction in their synthesis occurred 2-3h after injection of TP (Figs. 4.E and 4.F, C2 and T2) to the levels seen in the oil-treated animal. By 5-6h after the injection of oil or TP, synthesis of the 13,500 and 13,800 molecular weight proteins was increased in the oil-treated female hypothalamus and POA but was still very low in the TP-treated hypothalamus and POA (Figs. 4.E and 4.F, C3 and T3). However, by 7.5h-8.5h after the injection of TP the synthesis of the low molecular weight proteins was as high as that seen at T1 (Figs. 4.E and 4.F, T4). No data for an oil-treated female at 7.5-8.5h was obtained since no more littermate females were available.

In the animals which had no i.p. injection of oil or TP, the synthesis of a 31,600 molecular weight protein was higher in the male than in the female (Figs. 4.E). The same protein was synthesised in greater quantities in the C2 female hypothalamus than in any other animal injected with either oil or TP (Fig. 4.E and 4.F, C2).



b) Densitometric scans of the hypothalamic proteins.

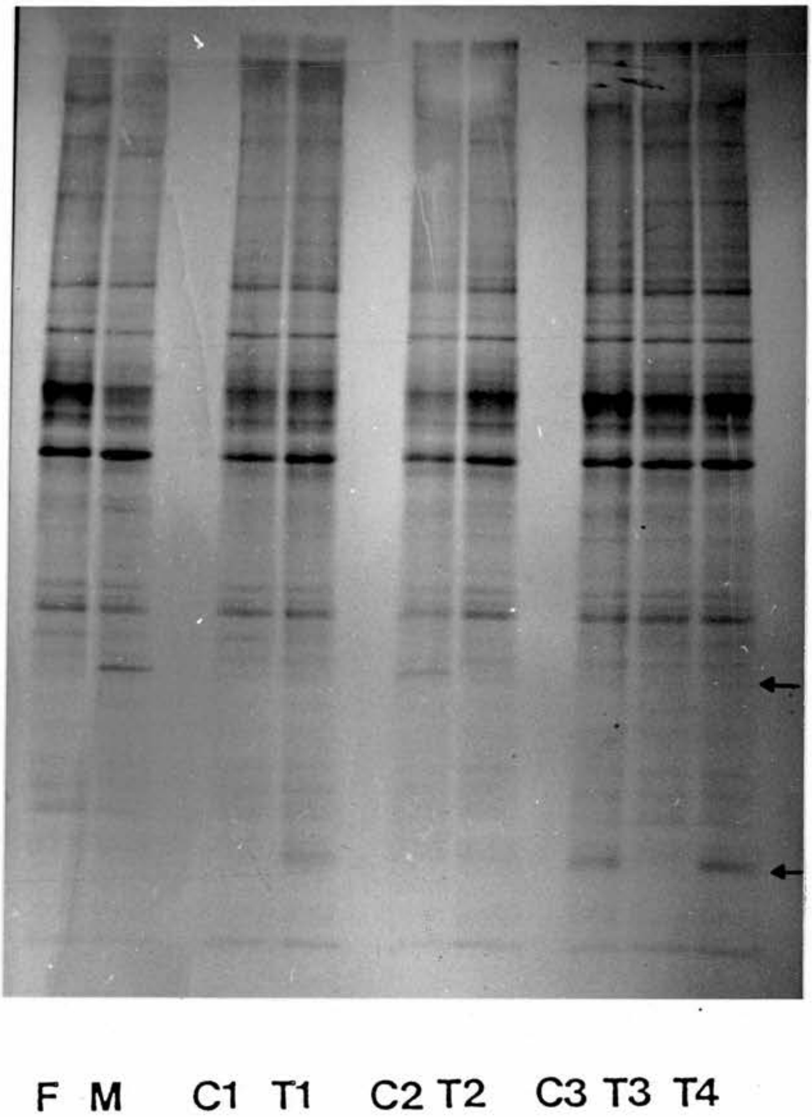
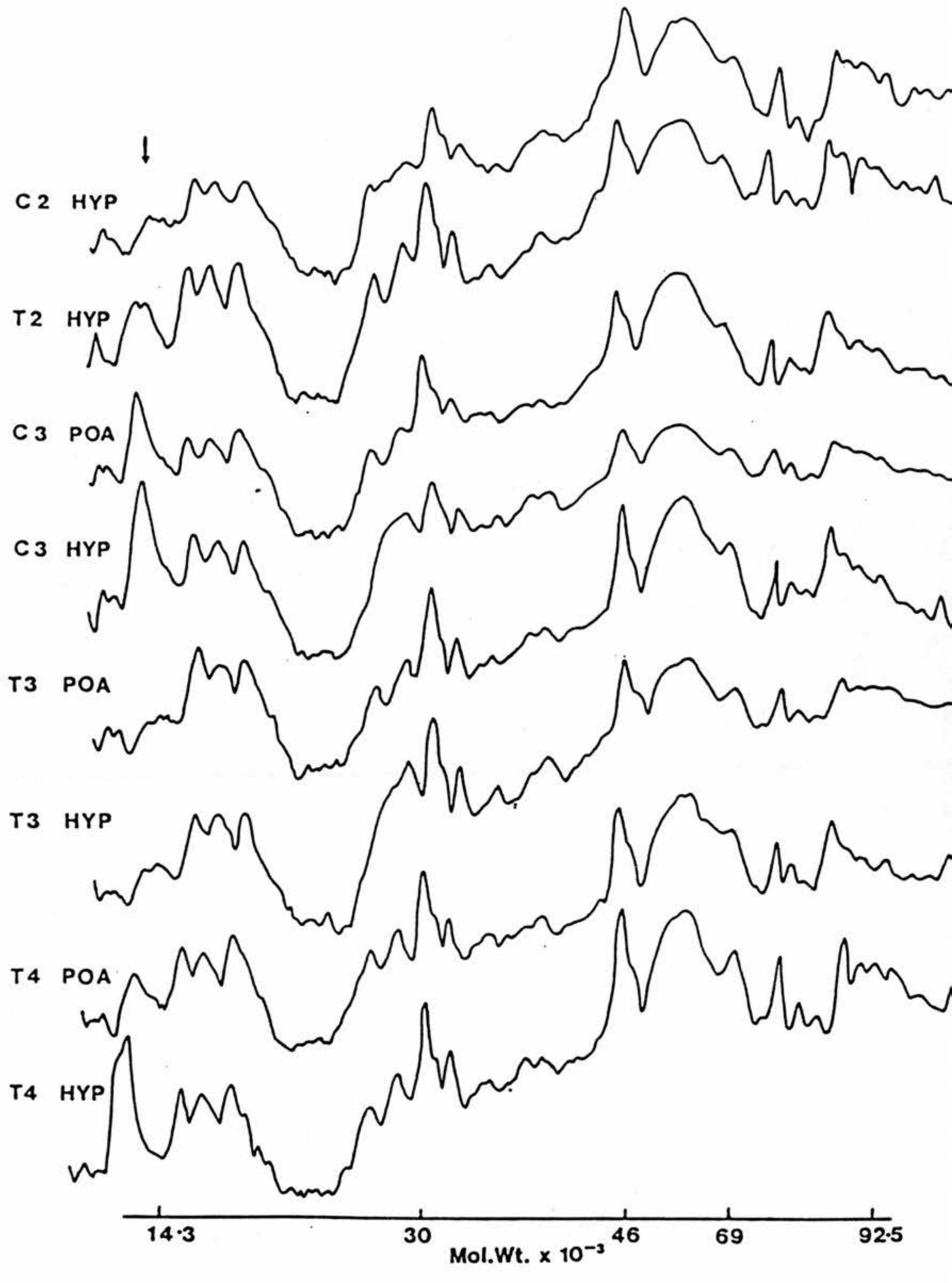


FIGURE 4.E: Proteins From The Hypothalamus Of 4 Day Rats Labelled In Vivo With ^{35}S-methionine.

a) Electrophoretic profiles of proteins in the 15,000g supernatant from the hypothalamus (HYP) of oil-treated female (C), TP-treated female (T) and untreated male (M) and female (F) rats. Proteins were labelled in vivo with ^{35}S-methionine during the first (C1,T1), the third (C2,T2), the sixth (C3,T3) and the seventh to eighth hours (T4) after the i.p. injection of oil or TP. Equal amounts of acid-insoluble radioactivity (15,000 cpm) for each sample were loaded onto the gel. After SDS-PAGE the gel was fluorographed, dried and exposed to film at -70°C for 6 days.



b) Densitometric scans of the POA and hypothalamus proteins.

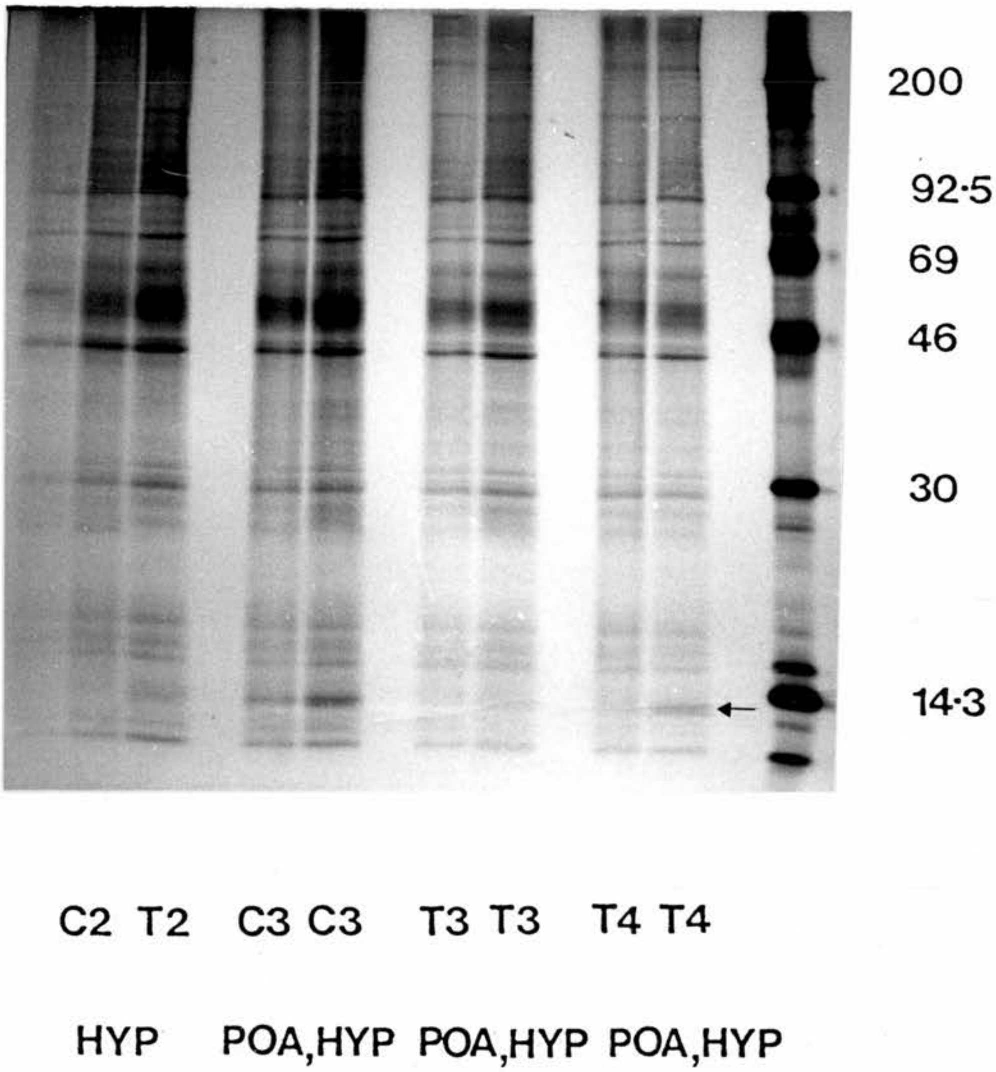


FIGURE 4.F: Proteins From The POA And Hypothalamus Of 4 Day Rats

a) Electrophoretic profiles of proteins in the 15,000g supernatant from the POA and hypothalamus (HYP) of oil-treated (C) and TP-treated (T) female rats from the same experiment as illustrated in Figure 4.E. The autoradiogram shows proteins labelled with ^{35}S-methionine during the third (C2,T2), the sixth (C3,T3) and the seventh to eighth hours (T4) after the injection of oil or TP. 15,000 cpm were loaded onto each lane and after electrophoresis the gel was fluorographed, dried and exposed to film for 6 days at -70°C. Numbers at the side of the gel represent marker protein molecular weights x 10^{-3}.

Although the results of a further experiment (Fig. 4.G) show that the incorporation of ^{35}S-methionine into soluble protein was poor and the autoradiogram, particularly in the low molecular weight region, was faint, induction of a 77,600 molecular weight protein occurred only in the POA and hypothalamus of the C2 rat. This animal went through a period of 'fitting' during the hour following the intraventricular injection of ^{35}S-methionine. The 77,600 molecular weight protein was not seen in any of the other experiments.

In summary, the results of these PAGE studies show that as assessed by the incorporation of ^{35}S-methionine into H-POA protein of 4 day rats during a 1h period in vivo, the synthesis of two low molecular weight proteins (approximate molecular weight 13,000 - 14,000), was not constant in either oil-treated or TP-treated animals during the 8.5h after the i.p. injection of oil or TP. In one experiment, the synthesis of a 31,600 molecular weight protein was higher in the 4 day male than the female and in another experiment, a 77,600 molecular weight protein was induced in the H-POA of an oil-treated female rat which had experienced 'fits' during the 1h labelling period.

4.3.3 Analysis Of Proteins Labelled with ^{35}S-methionine from 'Rest of Brain' and Proteins Isolated from the Pellet Fractions by PAGE and Fluorography

No major quantitative or qualitative changes in protein synthesis were detected in soluble proteins from the 'rest of brain' or from any of the pellets examined (data not shown).

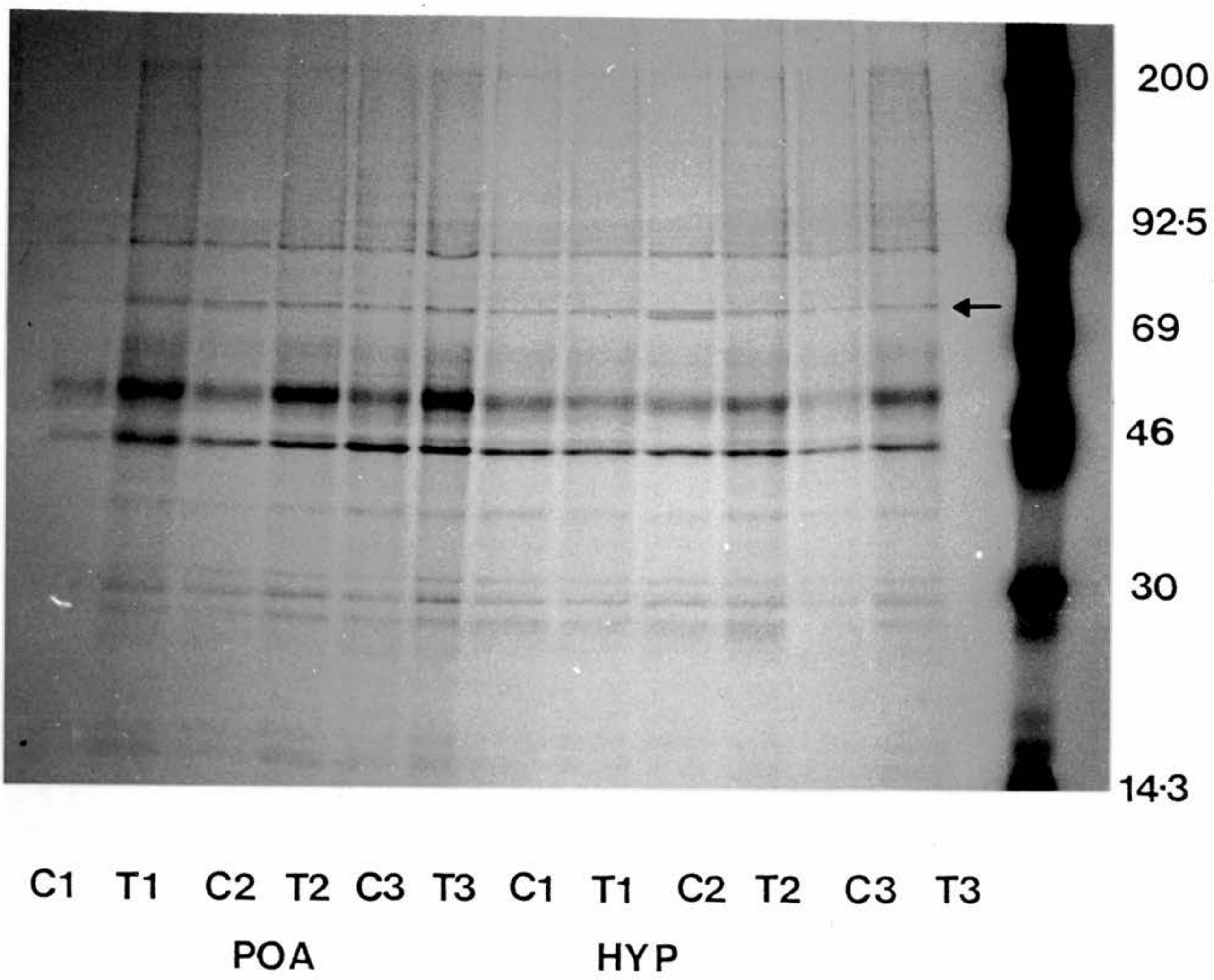


FIGURE 4.G: Autoradiogram Of Soluble Proteins Labelled With ^{35}S-methionine From The POA And Hypothalamus Of 4 Day Rats.

Proteins synthesised in the POA and hypothalamus (HYP) of oil-treated (C) and TP-treated (T) female rats were labelled in vivo either immediately (C1,T1) or 2 (C2,T2) or 5h (C3,T3) after the i.p. injection of oil or TP. The animals were killed 1h later and soluble proteins (120,000g supernatant) were prepared from the POA and hypothalamus. Samples containing equal amounts of acid-insoluble radioactivity (4,000 cpm) were analysed by SDS-PAGE and the gel was fluorographed, dried and exposed to film for 10 days at -70°C.
Numbers at the right of the gel represent marker protein molecular weights x 10^{-3}.

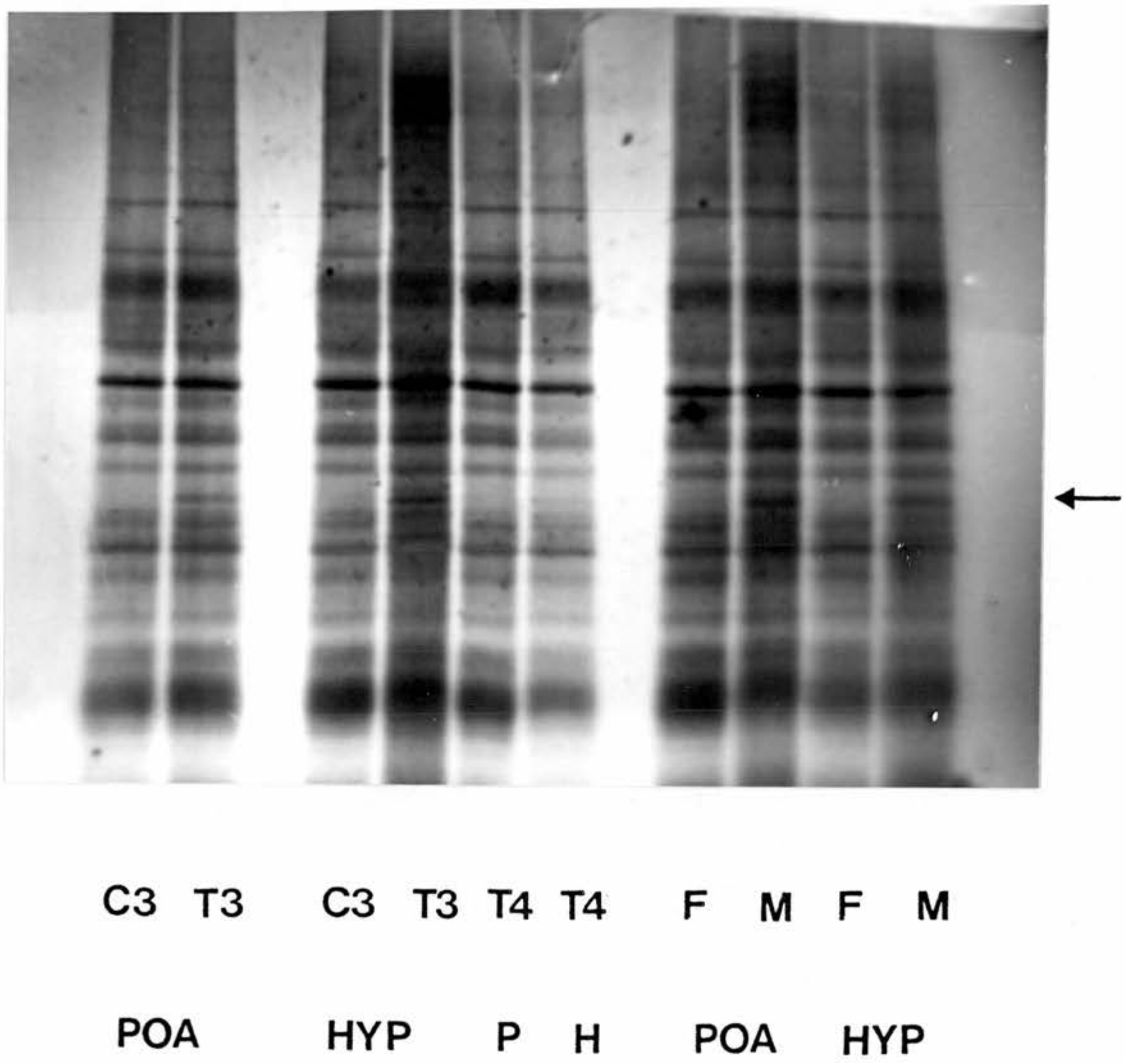


FIGURE 4.H: Electrophoretic Profile Of Proteins In The 15,000g Supernatant From The POA And Hypothalamus Of 4 Day Rats.

4 day female rats were injected (i.p.) with either oil (C) or 1.25mg TP in oil (T) at time zero. Proteins were labelled for 1h in vivo by intraventricular injection of ^{35}S-methionine six (C3,T3) and eight (T4) hours later. Male (M) and female (F) rats which received no i.p. injection of oil or TP but were given an intraventricular injection at time zero were also included in this experiment. Animals were killed at the appropriate time and the POA and hypothalamus (HYP) were dissected out. A 15,000g supernatant was prepared and approximately 20µg protein from each sample were analysed by SDS-PAGE. Proteins were detected by silver staining as described in section 2.2.9 using the method of Sammons et al., (1981).

4.3.4 Analysis of 15,000g Supernatant Proteins by PAGE and Silver Staining

Figure 4.H shows proteins found in the 15,000g supernatant from the POA and hypothalamus of oil and TP-treated male and female 4 day rats, detected by the silver stain method of Sammons et al., (1981). A protein with an approximate molecular weight of 50,000 which was induced 5-6h after TP injection in the female POA and hypothalamus was also present in the 4 day male POA and hypothalamus. This protein was not detected by fluorography (data not shown).

4.4 DISCUSSION

The data presented in this chapter show that changes in the synthesis of specific proteins occur in the H-POA of neonatal female rats within 6-8h of TP administration and support the data of Arai and Gorski (1968), Kobayashi and Gorski (1970) and Salaman and Birkett (1974) which suggested that some of the most important central effects of TP occur within 12h or less. The key results were (i) the finding that the synthesis of two closely associated low molecular weight proteins (approximately 13,000-14,000) in the 4 day H-POA showed inter-animal variation and could be modified by TP (Figs. 4.B, 4.D, 4.E, 4.F) (ii) the detection by silver staining of a 50,000 molecular weight protein which was induced in the POA and hypothalamus of 4 day female rats 5-6h after TP administration and was also present in the 4 day male (Fig. 4.H) and (iii) an incidental finding of the induction of a 77,600 molecular weight protein in the POA and hypothalamus of a 4 day control female rat which had undergone a series of fits throughout the experiment (Fig. 4.G).

Although the initial results from the autoradiograms suggested that the synthesis of two closely associated low molecular weight proteins was inhibited at 2-3h after TP administration (Figs. 4.D), further experiments showed changes in the synthesis of these proteins in the H-POA of both oil-treated and TP-treated animals during the 1h labelling period (Figs. 4.E and 4.F). The results could be interpreted as a stimulation in synthesis of the proteins 5-6h after an i.p. injection of oil or TP and a modification of their synthesis by TP. Whatever the mechanisms involved in the control of synthesis of these proteins, the H-POA content changed markedly in the 4 day rat H-POA during these experiments and their synthesis may be sensitive to stress. The molecular weights of the proteins suggest that they could be large polypeptide hormones or precursor molecules for smaller species. Slight variation in the estimates of the molecular weights of these proteins (which also appeared on some gels as one broader band) occurred between experiments, probably a result of slight differences in the gradient within the gels and the sharpness of the marker protein bands which might have caused variation in the calculation of R_f values. Two important variables in these experiments were litter size and precise age of the animals. Depending upon when the litters were born and actually recorded, differences in the actual age of litters could be as much as 12h, a time interval which could be critical when investigating a system such as the sexually differentiating brain where previous exposure to endogenous steroids may have some bearing on the effects of exogenous hormone on protein synthesis. In addition, litters were not reduced to a constant size just after birth in these experiments and differences in nutrient intake may affect brain protein synthesis in the neonate. However, the results of Dunlop, van Elden and Lajtha, (1977) showed that changes in the rates of total protein synthesis in brain did not occur when litter size was reduced.

The results cannot easily be compared with those of any of the published studies on protein synthesis in relation to sexual differentiation, because most of these studies measured the incorporation of radioactive amino acid into total brain protein determined by either tissue analysis without PAGE (Mogiulevsky, Scacchi and Christot, 1971; Ter Haar, MacKinnon and Bulmer, 1974) or by amino acid autoradiography of brain sections (Darrah, MacKinnon and Rogers, 1971; Litteria, 1973). Administration of TP to neonatal female rats was found to increase incorporation of ^{3}H-lysine into the ventral nucleus of thalamus 12h later (Darrah et al., 1971) and to decrease the incorporation of ^{3}H-lysine into protein in the arcuate, paraventricular, periventricular and supraoptic nuclei of the adult rat (Litteria, 1973). The studies of Mogiulevsky et al., (1971) and Litteria (1973), for example, which examined the effects of TP on total protein synthesis and showed long-lasting effects of TP upon this, provide data on the general increases or decreases in protein synthesis occurring within specific brain nuclei. Although the masculinising effects of TP in neonatal rat brain may involve only a generalised change in synthesis of all proteins or an abundant class of proteins within certain nuclei the classic mode of action of steroids on peripheral target tissues suggests that selective changes in the synthesis of specific proteins may also be involved in the central effects of TP and these selective changes can be detected using PAGE.

Nottebohm (1980) showed that testosterone stimulates the growth of certain nuclei in avian brain which are involved in the control of song production. In an interesting example of a similar type of study, the effects of testosterone on protein synthesis in avian brain were investigated (Konishi and Akutagawa, 1981) and androgen-induced changes in protein synthesis in sexually dimorphic brain nuclei were found to occur in the castrated male and the normally mute female song bird. Konishi and Akutagawa (1981) showed that an increase in protein synthesis within the vocal control centres in avian brain preceded the induction of song by androgen, as assessed by amino acid autoradiography 5-6 days after androgen administration.

One of the most interesting results of the present experiments was the detection of a 50,000 molecular weight protein, presumably methionine-free, in the 15,000g supernatant of POA and hypothalamus from 4 day androgenised females which was also present in the 4 day male (Fig. 4.H). The identity of this protein remains to be established but the fact that it was only detected by silver staining suggests that it was present in very low quantities and, therefore, might prove difficult to isolate for further study. Although the silver based stains are extremely sensitive and may, in fact, be comparable to the sensitivity obtained using PAGE and fluorography, they are not as useful as Coomassie Blue or autoradiography for the quantitative analysis of proteins (Poehling and Neuhoff, 1981). However, in investigations of this type the sensitivity of the method is the great advantage. A lack of reproducibility of the silver stain is one disadvantage and although the change in synthesis of the 50,000 molecular weight protein by TP was only detected in one out of four experiments, factors other than the lack of reproducibility of the stain, for example age differences, could explain this finding. The results of this experiment in particular highlight what are perhaps the chief problems of investigations of this type into hormone effects on protein synthesis in brain and those are the questions of timing and sensitivity. Although the effects of androgen on the neuroendocrine function of the neonatal rat brain appear to be permanent, any changes in the synthesis of specific proteins may only be transient, perhaps involving a 'switch' in the pattern of neuronal growth, differentiation and/or metabolism and such changes, which could represent only a small percentage of the total protein, may not easily be detected.

An incidental but important finding in these experiments was the induction of a 77,600 molecular weight protein in rat POA and hypothalamus, apparently the result of a 'fit' episode(s) and probably related to a period of intense neuronal activity in brain (Fig. 4.G); this may be the first report of such an effect. Studies have been carried out investigating the effects of a variety of functional loads on protein synthesis in brain, for example light (Haywood, Hambley and Rose, 1974) and electrical stimulation and convulsants (Dunn, Giuditta and Pagliuca, 1971). However, changes in the synthesis of specific proteins have not been demonstrated. The 77,600 molecular weight protein was not seen by incorporation of ^{35}S-methionine into newly-synthesised protein in any of the other experiments which suggests that the induction was specific and a direct result of the massive change in electrical discharge in brain probably occurring during the seizures in the rat. Recent studies have shown, however, that the synthesis of a 71,000 molecular weight stress protein (SP 71) can be induced in rat brain *in vivo* by hyperthermic shock (Currie and White, 1981) and in cell cultures of rat cerebellum in response to heat shock (Pearce, Dutton and White, 1983). Synthesis of the SP 71 protein is also induced in brain slices *in vitro* but not in brain slices prepared from rats less than 3 weeks old (White, 1981). It seems that SP 71 is one of a group of proteins which are now known to be synthesised in a variety of tissues in response to stress and it is possible that the 77,600 molecular weight protein detected in these experiments could belong to such a group of stress-induced proteins.

CHAPTER 5

The Isolation And Translation *In Vitro* Of RNA From Male, Female And Androgenised Female Rat Hypothalamus-Preoptic Area

5.1 INTRODUCTION

Evidence from a number of experiments indicates that steroid hormones bring about their regulatory effects on protein synthesis in target tissues by increasing the concentration of mRNA for these proteins. For example, transcriptional regulation of gene expression by steroids has been demonstrated in the rat uterus (Walker and Kaye, 1981), rat ventral prostate (Hiremath, Mpanias and Wang, 1981), mouse kidney (Toole, Hastie and Held, 1979), chick oviduct (Chan, Means and O'Malley, 1973), and rabbit endometrium (Loosfelt, Fridlansky, Savouret, Atger and Milgrom, 1981). Glucocorticoid and thyroid hormones have been shown to alter transcription of the gene for growth hormone in rat pituitary gland (Evans, Birnberg and Rosenfeld, 1982) and oestrogen is known to regulate mRNA levels for the pituitary gonadotrophins (Godine et al., 1980). Although oestrogen is known to affect the activity of a number of enzymes in both the rat pituitary gland and brain (Luine et al., 1974) and to change the content of CAT in the rat POA (Luine et al., 1980) it is not known whether these effects of oestrogen are the result of transcriptional and/or translational regulation.

Androgen-induced sexual differentiation is blocked by injecting α-amanitin, an inhibitor of nucleoplasmic RNA polymerase II, at the same time as and 6h after TP administration to neonatal female rats (Salaman and Birkett, 1974). Long-lasting changes in brain RNA populations as a result of neonatal exposure to androgen have been reported (Shamida and Gorbman, 1970; Namiki and Gorbman, 1973). Changes in brain protein synthesis during development have been studied by translating mRNA *in vitro* and analysing the products by

PAGE (Schmitt et al., 1977; Hall and Lim, 1981; Morrison, Pardue and Griffin, 1981; Masuda, Sakimura, Yoshida, Kuwano, Isobe, Tsuneo and Takahashi, 1983) but no such data are available regarding sex differences in gene transcription in the H-POA during brain maturation.

The aim of these experiments was to try to detect differences in the synthesis of specific proteins in the male, female and androgenised female rat H-POA by the isolation and translation of H-POA mRNA in vitro.

5.2 MATERIALS AND METHODS.

5.2.1 Animals

Wistar Cob rats used in this study were from the departmental breeding colony apart from the 72 day male and 72 day control female rats which were obtained from Charles River (Margate, Kent).

Male, control female and androgenised female (section 2.2.1) rats were used at 4, 12 and 72 days of age (day 0 = day of birth). For the group of rats aged 4 days, littermate females were injected (i.p.) with either 0.1ml arachis oil or 1.25mg TP in 0.1ml arachis oil. The oil-treated and TP-treated animals were then kept apart and killed by decapitation 6h later. For the experiment on 12 day rats, all of the females from a litter were injected (s.c.) with either 0.1ml arachis oil or 1.25mg TP in 0.1ml arachis oil at 4 days and killed by decapitation at 12 days. 72 day control female rats received no injection of oil at 4 days. Androgenised female rats for use at 72 days were injected with TP (s.c.) at 4 days. All 72 day animals were weighed before decapitation.

To confirm that the 72 day androgenised female rats were in a state of persistent oestrus, vaginal smears were recorded once daily for a week before decapitation and the histology of the ovaries from a few animals was examined in paraffin sections at the ovaries stained with haematoxylin and eosin (see Appendix II for details).

5.2.2 Isolation of RNA

For each group of rats at the appropriate age, the animals were killed by decapitation, the H-POA was dissected out (section 2.2.2) under sterile conditions and the tissue was immediately frozen in liquid nitrogen. It was necessary to pool tissue for these experiments and so all samples were left at -70°C until approximately 30 H-POA from each group had been collected.

Total RNA was extracted from the H-POA using either the SDS-phenol/chloroform or GTC method, as described in section 2.2.10. Two experiments were carried out using H-POA from 4 day rats. In the first experiment RNA was extracted from the H-POA of oil-treated and TP-treated rats using the SDS-phenol/chloroform extraction method. In the second experiment, in addition to oil-treated and TP-treated female rats, RNA was also isolated from the H-POA of a group of 4 day male rats using the GTC extraction method. The RNA from 12 day and 72 day H-POA samples was extracted using the GTC procedure.

The RNA preparations were enriched for Poly(A)$^+$ containing mRNA by oligo (dT)-cellulose chromatography as described in section 2.2.11.

5.2.3 Translation of RNA In Vitro

RNA was translated in a cell-free system <u>in vitro</u> using either the N-90 or N-150 rabbit reticulocyte lysate (RRL, Amersham

International) or wheat germ extract (Bethesda Research Laboratories Ltd., Cambridge) supplemented with ^{35}S-methionine or ^{3}H-amino acid mixture (section 2.1.3).

Optimum concentrations of K^+ and Mg^{2+} ions (present as acetates) for use in the N-150 lysate were determined by testing a range of ion concentrations in the standard translation mix. N-150, as supplied, contains K^+ at 20mM and Mg^{2+} at 0.7mM. Final K^+ concentrations of 95-220mM and Mg^{2+} concentrations of between 0.7 and 1.95mM were tested.

All *in vitro* translations were set up as described in section 2.2.12. The optimum mRNA concentration was determined for each mRNA preparation. All translation mixes contained approximately 0.5-1.0µl mRNA. The no-message blank contained 1µl sterile distilled water.

Protein synthesis directed by RNA was estimated by measuring the incorporation of radioactive amino acid into acid-insoluble material at various times (see RESULTS) during the incubation, using the method described in section 2.2.13. The reaction was terminated by placing the tubes on ice.

5.2.4 Electrophoresis

The translation products were analysed by PAGE in one and two dimensions (sections 2.2.6 and 2.2.8) and the gels were fluorographed, dried and exposed to film at -70°C for between 2 days and 2 months, as described in section 2.2.9. Gels were calibrated using ^{14}C-methylated protein markers (section 2.1.3). The protein patterns on two-dimensional gels were compared by visual inspection of the autoradiograms. Single dimension gels were scanned but the high background on these autoradiograms made analysis of some scans very difficult.

5.3 RESULTS

All 72 day androgenised female rats showed a majority of cornified epithelial cells in the vagina and the ovaries contained follicles but no corpora lutea (Fig. 5.A). Androgenised females at 72 days were significantly heavier than control female rats ($p < 0.001$). The average weights of the 72 day control female, androgenised female and male rats were 224 ± 11, 246 ± 15 and 301 ± 21 g (± S.D.) respectively, although these were not littermates.

5.3.1 Yield of Poly (A)$^+$ mRNA from Total RNA Preparations

The average yield of Poly (A)$^+$ mRNA obtained from total RNA by oligo (dT)-cellulose chromatography (section 2.2.11) was 1-5% of the total RNA applied to the column (Table 5.1). Some of the nucleic acids in the RNA preparations could have been DNA since no DNAase treatment was carried out.

5.3.2 Optimum Concentrations of K$^+$ and Mg^{2+} Ions for the Translation of mRNA In N-150 RRL

Figures 5.B.(a) and (b) show that the optimum concentrations of K$^+$ and Mg^{2+} ions (including the endogenous lysate cations) were 170mM and 0.7mM respectively. Therefore no additional Mg^{2+} ions were added to the lysate and the K$^+$ ion concentration was raised to 170mM by adding an appropriate amount of 1.5M potassium acetate.

5.3.3 Time Course of Incorporation of ^{35}S-methionine into Protein Directed by RNA In Vitro

All RNA preparations enhanced the incorporation of ^{35}S-methionine into protein when added to a cell-free extract.

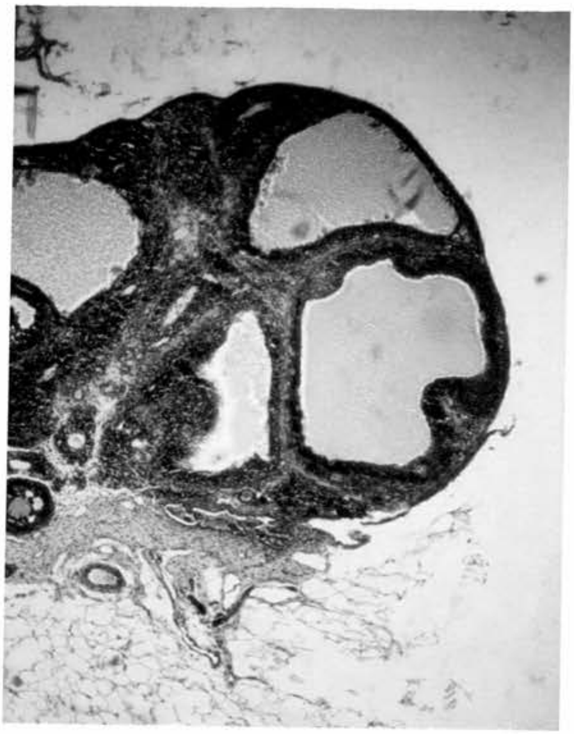


FIGURE 5.A: 72 Day Androgenised Female Rat Ovary

The ovaries were removed from 72 day androgenised female rats, fixed, mounted in a paraffin wax block, sectioned (7μm) and stained with haematoxylin/eosin as described in Appendix II. Note the presence of normal large follicles and the absence of corpora lutea.

Table 5.1

Estimated Yields of Total RNA and Poly $(A)^+$ mRNA From Rat H-POA (Absorbance Read at 260nm)

Extraction Method	Source of Tissue	Wet Weight (g)	Total RNA (μg)	Poly $(A)^+$ mRNA (μg)	% yield Poly $(A)^+$mRNA
Phenol/Chloroform	4 day C F	0.6	985	–	–
	TP F	0.6	625	–	–
GTC	4 day C F	1.0	1200	20	1.6
	TP F	1.0	1400	29	2.1
	M	1.0	1950	48	2.5
GTC	12day C F	1.6	1960	56	2.8
	TP F	1.3	1980	60	3.0
	M	1.8	2500	51	2.0
GTC	72day C F	1.1	1900	50	2.6
	TP F	1.3	1400	68	4.8
	M	1.2	1200	52	4.3

FIGURE 5.B: Effect Of Changes In Potassium And Magnesium Ion Concentration On The Translation Of mRNA In N-150 RRL.

Concentrations of a) 95–220mM potassium acetate and

b) 0.7–2.0mM magnesium acetate were used in N-150 RRL to test the efficiency of mRNA translation. Incorporation of ^{35}S-methionine into acid-insoluble material was used as the index of translation efficiency.

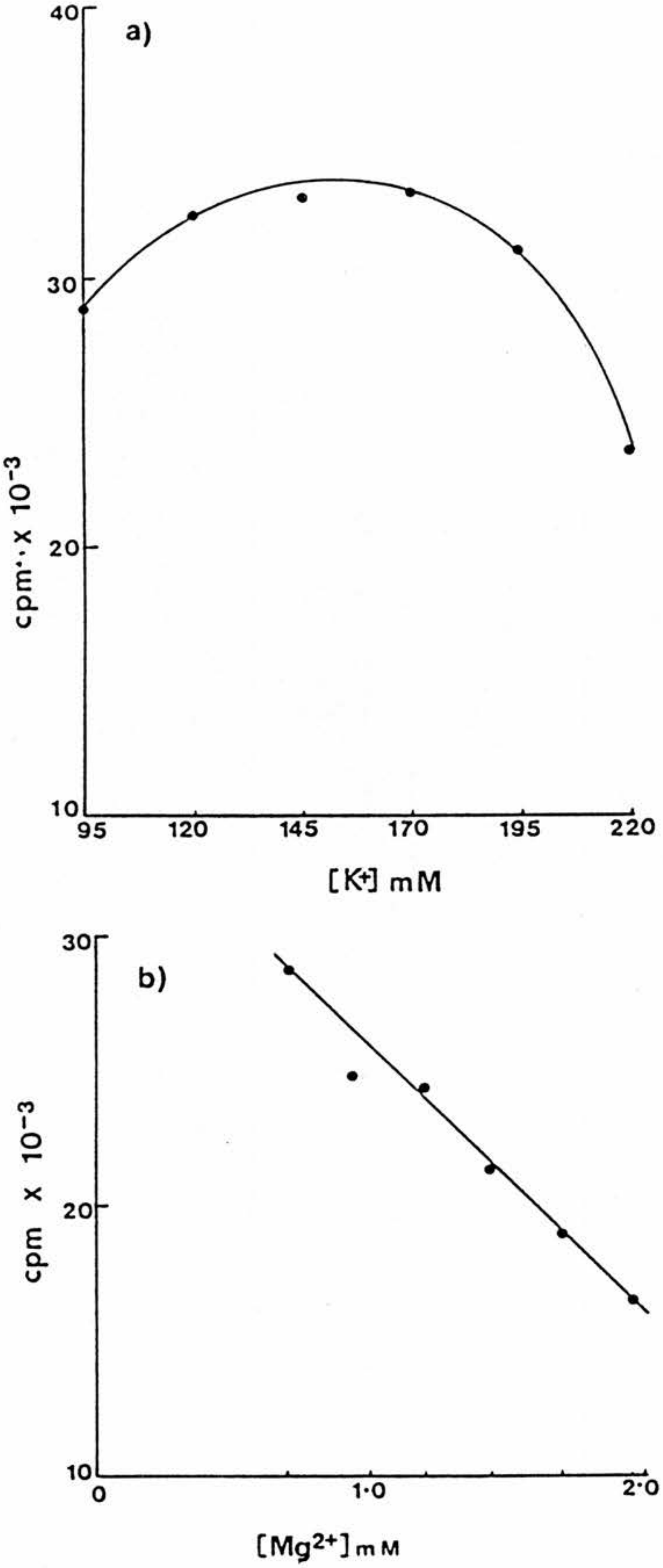

a)
40
30
20
10
cpm · x 10⁻³
95
120
145
170
195
220
[K+] mM
b)
30
20
10
cpm x 10⁻³
0
1·0
2·0
[Mg2+] mM

Figure 5.C.(a) shows that RNA from 4 day control female and androgenised female rat H-POA, extracted using SDS-phenol/chloroform, caused a 3.5 fold stimulation in incorporation of ^{35}S-methionine into protein in N-90 lysate compared with the blank. The incorporation data for the translation (in N-150 lysate) of mRNA, extracted using GTC from control female, androgenised female and male rat H-POA at 4, 12 and 72 days of age, are shown in Figs. 5.C.(b), 5.C.(c), and 5.C.(d) respectively. A two to six fold stimulation in incorporation of ^{35}S-methionine into protein occurred in the various preparations compared with the blank. The lowest incorporation was found using the 72 day control female and androgenised female mRNA (Fig. 5.C.(d)).

The results obtained using the wheat germ extract are shown in Figs. 5.D. The incorporation of ^{35}S-methionine into protein when added to a wheat-germ cell-free extract, compared with the blank, was approximately 8-10 fold.

5.3.4 Analysis Of Translation Products By Two-Dimensional PAGE

5.3.4.1 Total RNA Isolated from 4 day Control Female and Androgenised Female Rat H-POA using SDS-Phenol/Chloroform and Translated in N-90 RRL

Two-dimensional PAGE analysis of the proteins synthesised *in vitro* by total H-POA RNA showed that administration of TP to a 4 day female rat for 6h caused a quantitative change in the synthesis of two proteins (Figs. 5.E.(a) and 5.E.(b)). The synthesis of a 70,000 molecular weight protein was stimulated by TP whereas the synthesis of a 20,000 molecular weight protein was inhibited.

FIGURE 5.C: Time Course Of Incorporation Of ^{35}S-methionine Into Protein Directed By H-POA RNA In RRL.

RNA was added to either N-90 (a) or N-150 (b, c and d) RRL and the incorporation of ^{35}S-methionine into acid-insoluble material was estimated at fixed time intervals during the incubation. All procedures were carried out as described in sections 2.2.12 and 2.2.13.

Key
○———○ Control Female
●— — —● Androgenised Female
▲———▲ Male
■--------■ No-message blank.

a) 4 day RNA Isolated Using SDS-Phenol/Chloroform

b) 4 day RNA Isolated Using GTC

c) 12 day RNA Isolated Using GTC

d) 72 day RNA Isolated Using GTC.

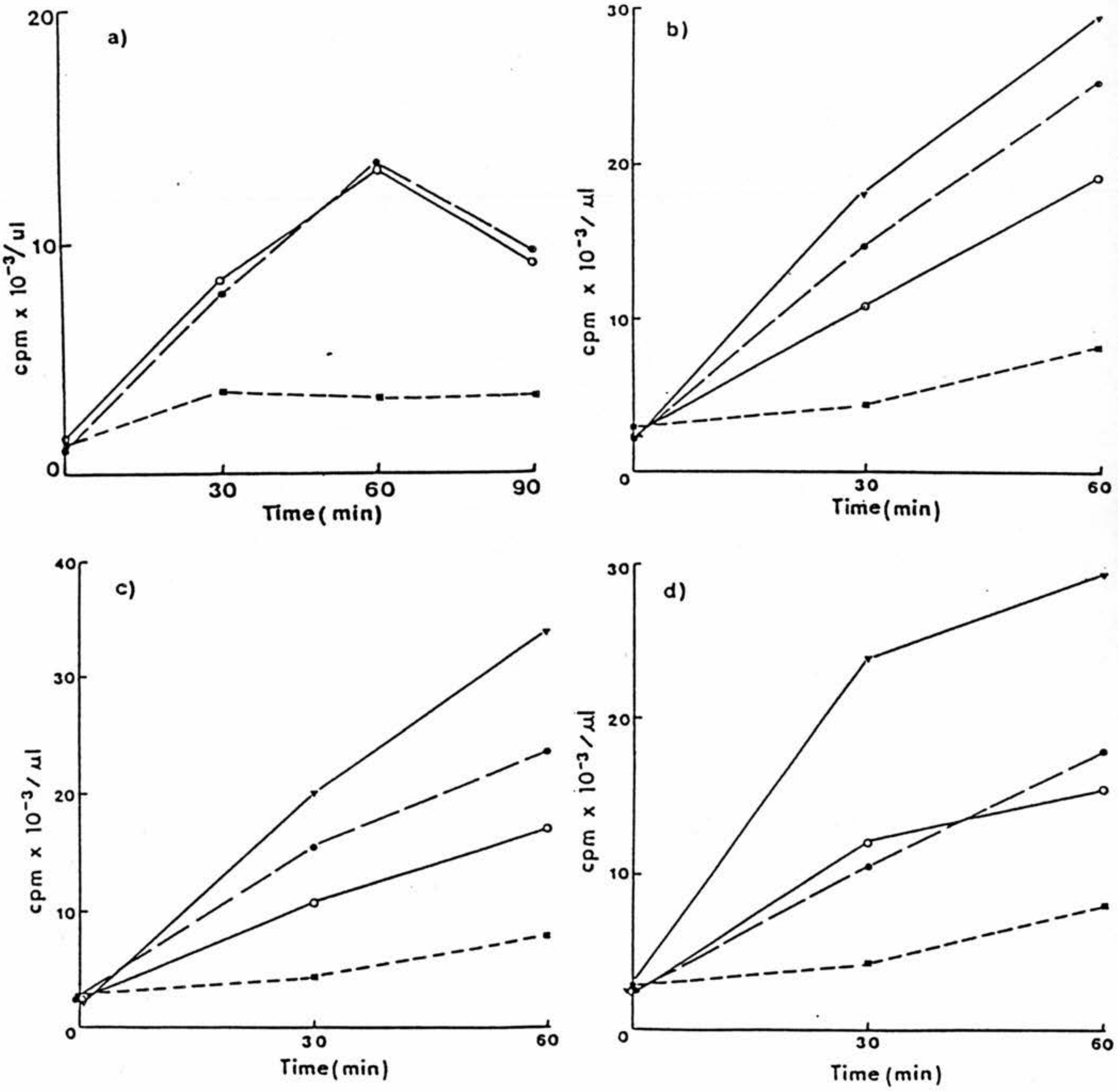

a)
20
10
0
cpm x 10⁻³/ul
30
60
90
Time(min)
b)
30
20
10
0
cpm x 10⁻³/ µl
30
60
Time(min)
c)
40
30
20
10
0
cpm x 10⁻³/ µl
30
60
Time(min)
d)
30
20
10
0
cpm x 10⁻³/ µl
30
60
Time (min)

FIGURE 5.D: Time Course Of Incorporation Of ^{35}S-methionine Into Protein Directed By H-POA mRNA In A Wheat Germ Cell-Free Extract.

Wheat germ translation mixes were prepared as described in section 2.2.12. The incorporation of ^{35}S-methionine into acid-insoluble material, directed by mRNA, was estimated at fixed time intervals during the incubation. All RNA was isolated using the GTC procedure and mRNA was purified by oligo (dT)-cellulose chromatography.

Key ○————○ Control Female

●— — —● Androgenised Female

▲————▲ Male

■- - - - -■ No-message blank

a) 4 day RNA

b) 12 day RNA

c) 72 day RNA

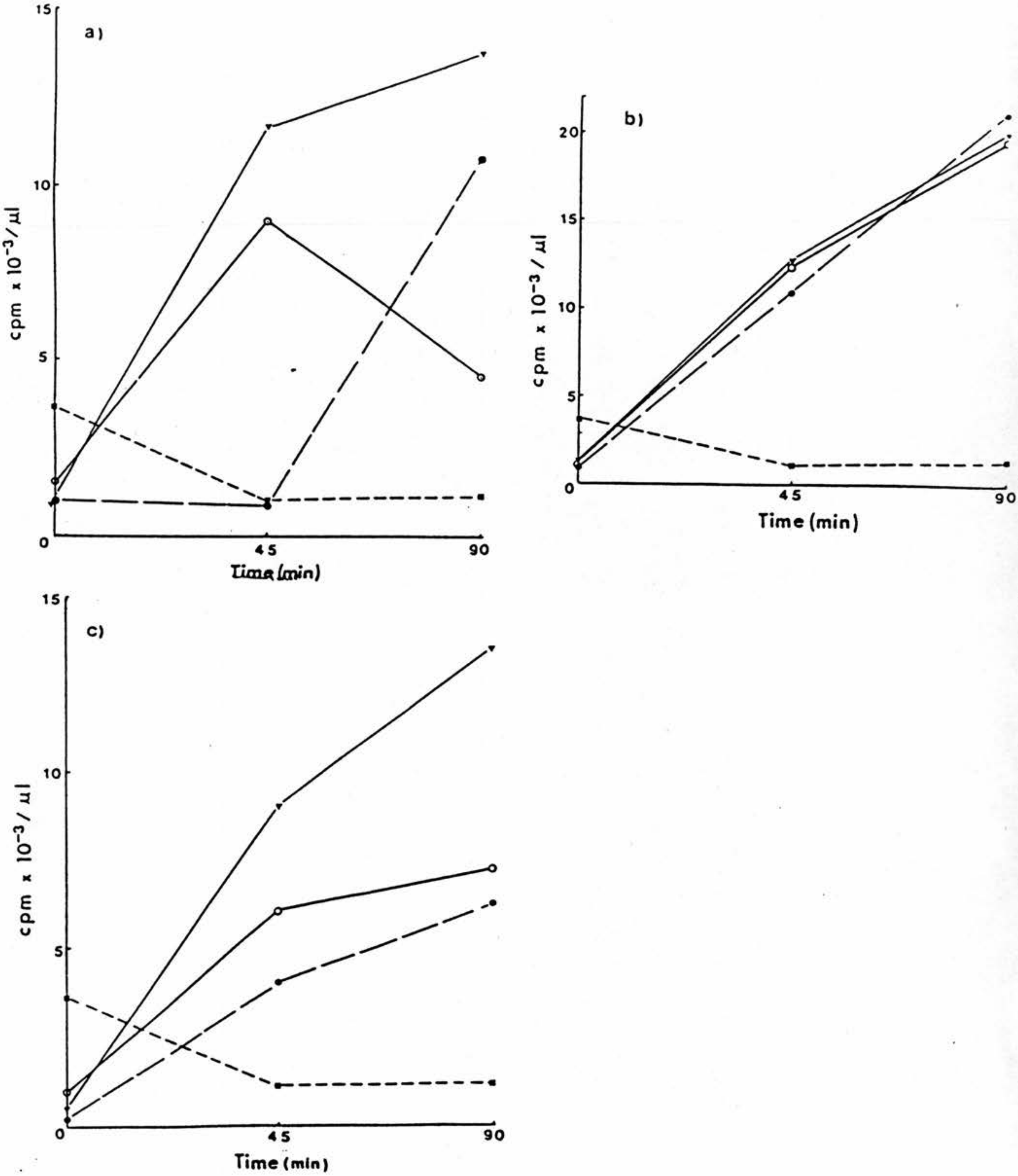
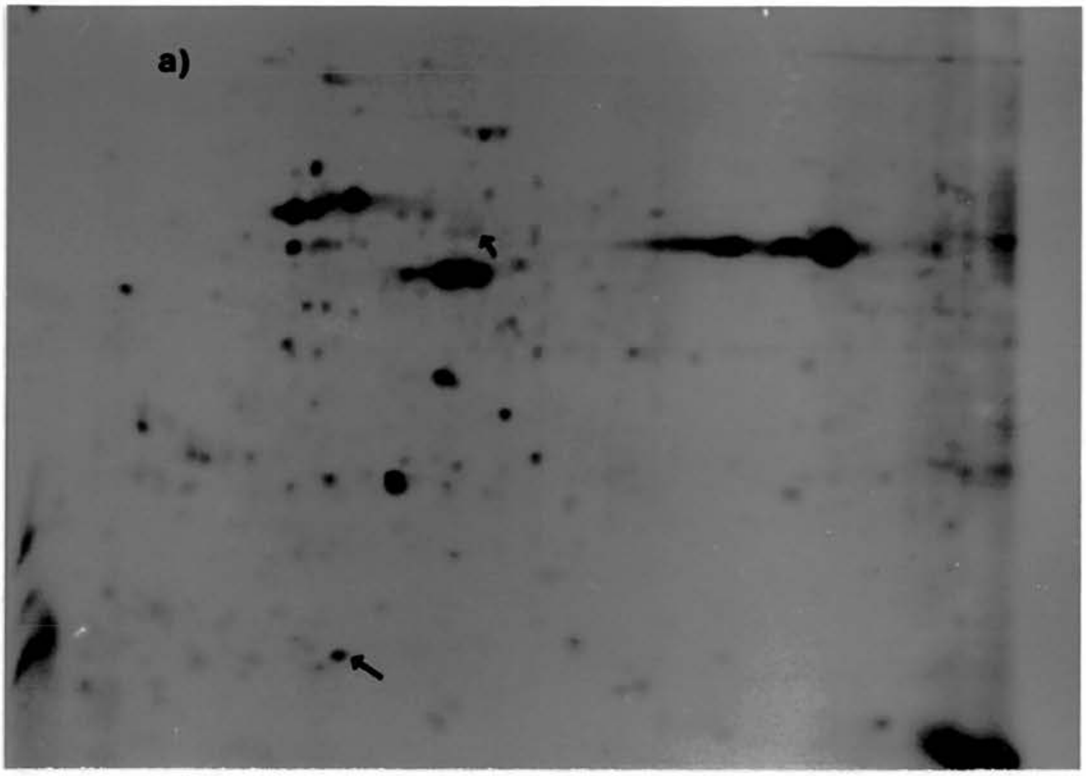
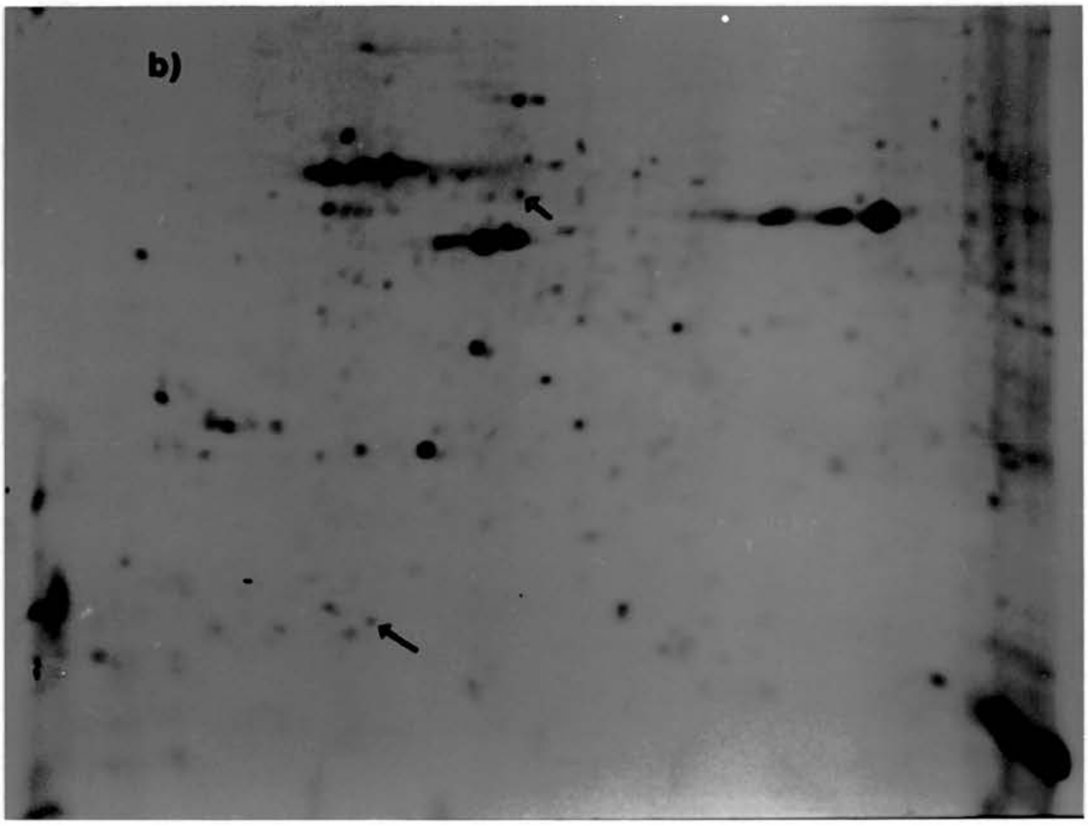

a)
b)
c)
cpm x 10⁻³/µl
0
5
10
15
20
45
90
Time (min)

FIGURE 5.E: Two-Dimensional Gel Analysis Of Proteins Synthesised In Vitro In N-90 RRL By RNA Isolated From 4 Day H-POA Using SDS-Phenol/Chloroform.

Total RNA was isolated from the H-POA of 4 day a) oil-treated and b) TP-treated female rats using the SDS-phenol/chloroform procedure and translated in N-90 RRL containing ^{35}S-methionine. Each gel was loaded with approximately 10,000 cpm. After two-dimensional PAGE the gels were fluorographed, dried and exposed to film for 21 days at -70°C.


a)


b)

5.3.4.2 mRNA Isolated from 4 day Control Female, Androgenised Female And Male Rat H-POA using GTC and Translated in N-150 RRL

The translation products are shown in Figs. 5.F.(a),(b) and (c). As shown in section 5.3.4.1, the synthesis of a low molecular weight protein (approximately 20,000) was reduced by the administration of TP to a 4 day female for 6h. Figure 5.F.(c) shows that synthesis of the same protein was further reduced in the 4 day male H-POA. Figure 5.F.(d) shows the translation products of the no-message blank.

5.3.4.3 mRNA Isolated from 12 day Rat H-POA using GTC and Translated in N-150 RRL

Two-dimensional gels of the proteins synthesised *in vitro* from mRNA from the H-POA of 12 day rats are shown in Figs. 5.G.(a),(b) and (c). No major differences between oil-treated females, TP-treated females and males were detected.

5.3.4.4 mRNA Isolated from 72 day Rat H-POA using GTC and Translated in N-150 RRL

Apart from the 72 day male mRNA preparation, the stimulation of protein synthesis in RRL was poor (Fig. 5.C.(d)) and a comparative analysis of the 72 day samples by two-dimensional PAGE was not possible.

5.3.5. Analysis of Translation Products By One-Dimensional PAGE

5.3.5.1 mRNA Isolated from Rat H-POA using GTC and Translated in N-150 RRL

Figure 5.H.(a) shows an autoradiogram of the ^{35}S-methionine-

FIGURE 5.F: Two-Dimensional Gel Analysis Of Proteins Synthesised In Vitro In N-150 RRL By mRNA Isolated From 4 Day Rat H-POA Using The GTC Procedure.

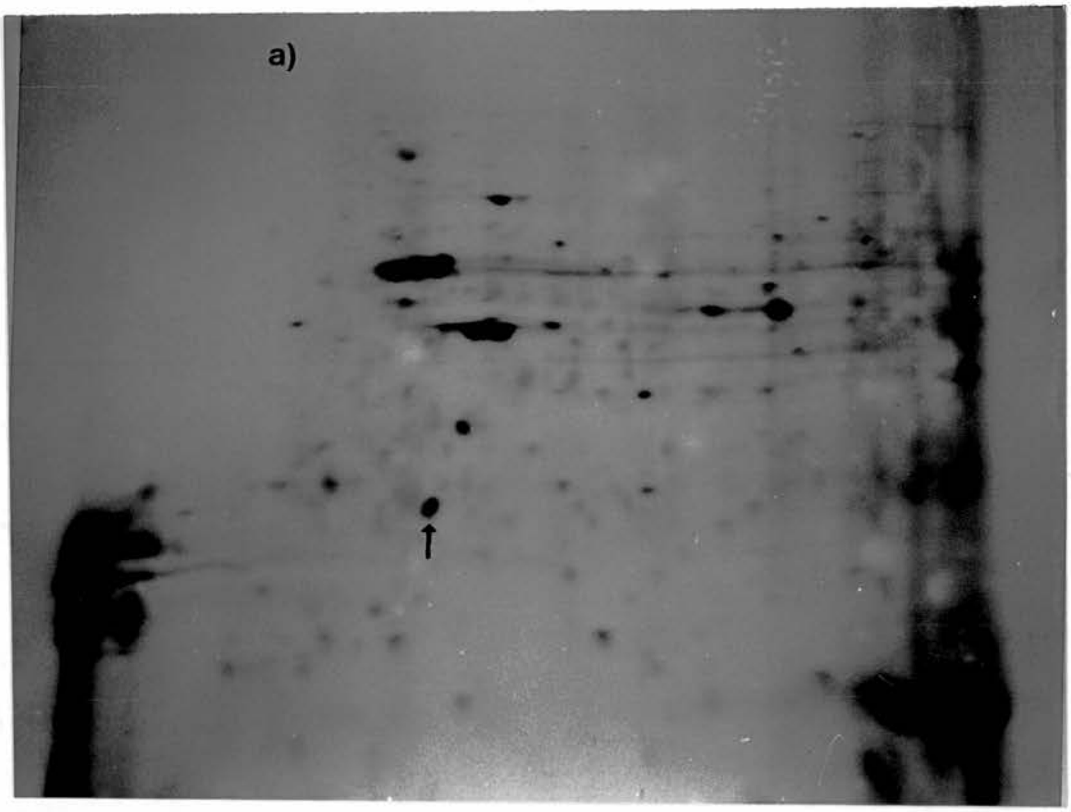
RNA was extracted from 4 day oil-treated, TP-treated and male rat H-POA using GTC and enriched for mRNA by oligo (dT)-cellulose chromatography. mRNA was translated in N-150 RRL containing ^{35}S-methionine, as described in section 2.2.12. Translation products (40,000 cpm) were analysed by two-dimensional PAGE, the gels were fluorographed, dried and exposed to film for 20 days at -70°C.

a) Control Female

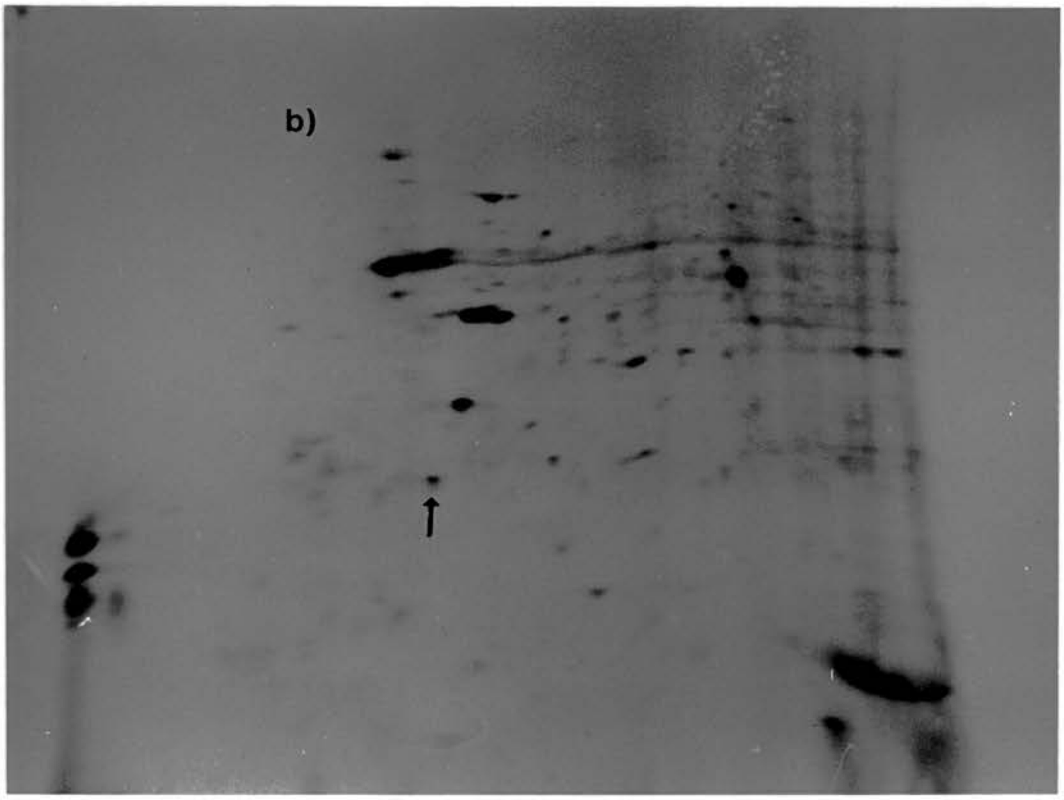
b) Androgenised Female

c) Male

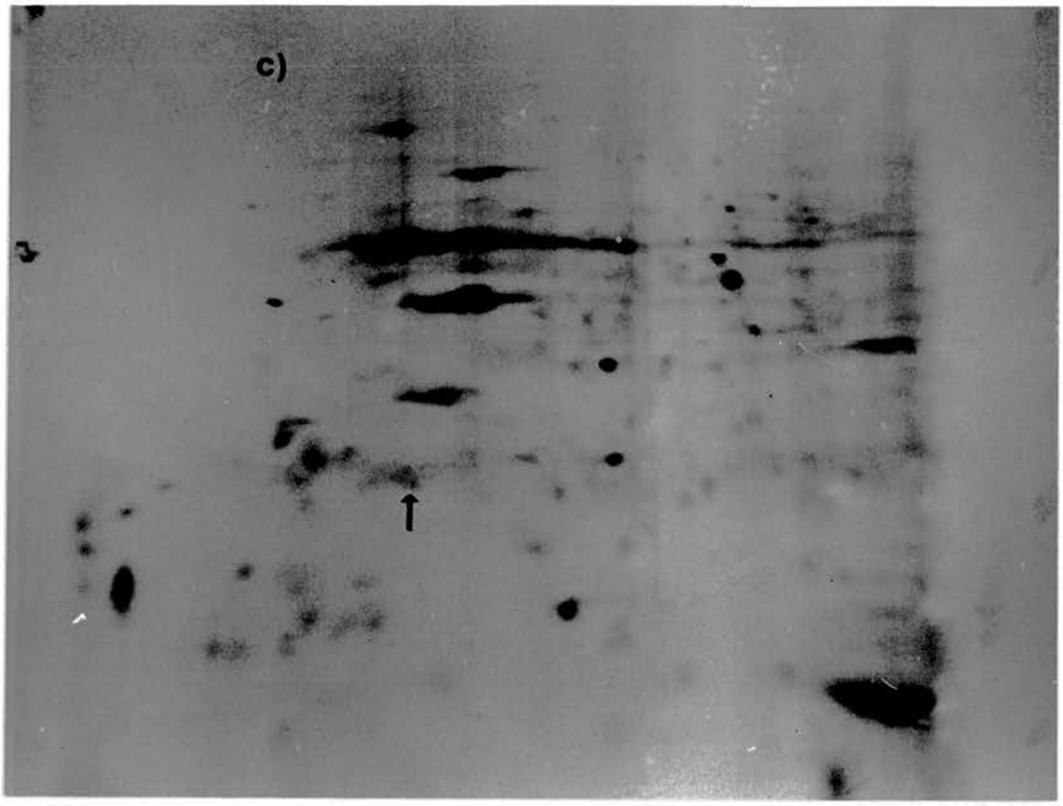
d) No-message blank



a)



b)


c)

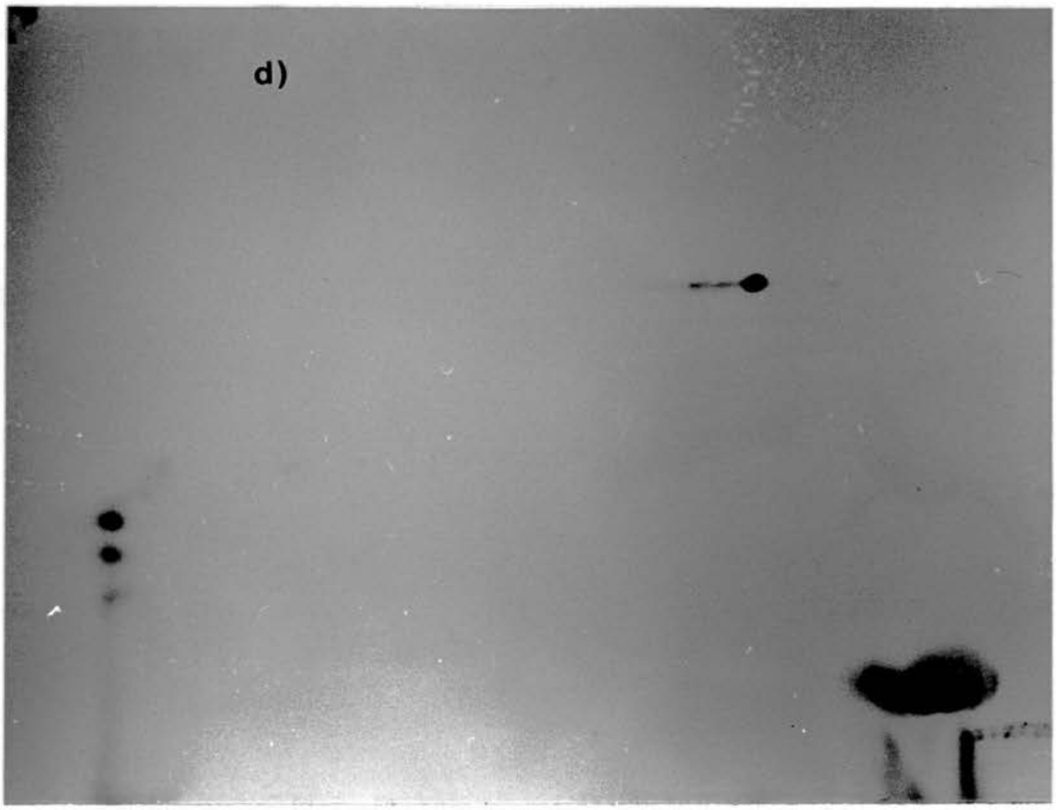

d)

FIGURE 5.G: Two-Dimensional Gel Analysis Of Proteins Synthesised In Vitro In N-150 RRL By mRNA Isolated From 12 Day Rat H-POA Using The GTC Procedure.

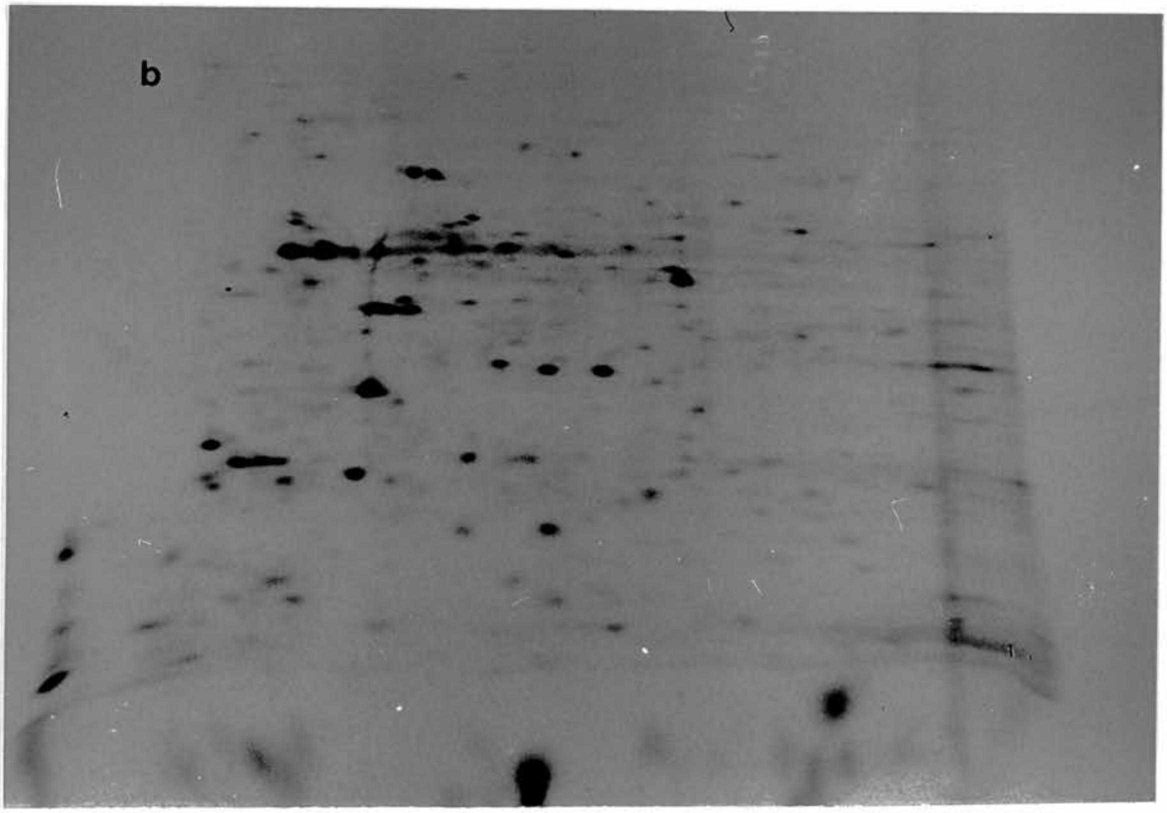
RNA was extracted from 12 day oil-treated, TP-treated and male rat H-POA using GTC and enriched for mRNA by oligo (dT)-cellulose chromatography. mRNA was translated in N-150 RRL containing ^{35}S-methionine, as described in section 2.2.12. Translation products (40,000 cpm) were analysed by two-dimensional PAGE, the gels were fluorographed, dried and exposed to film for 21 days at -70°C.

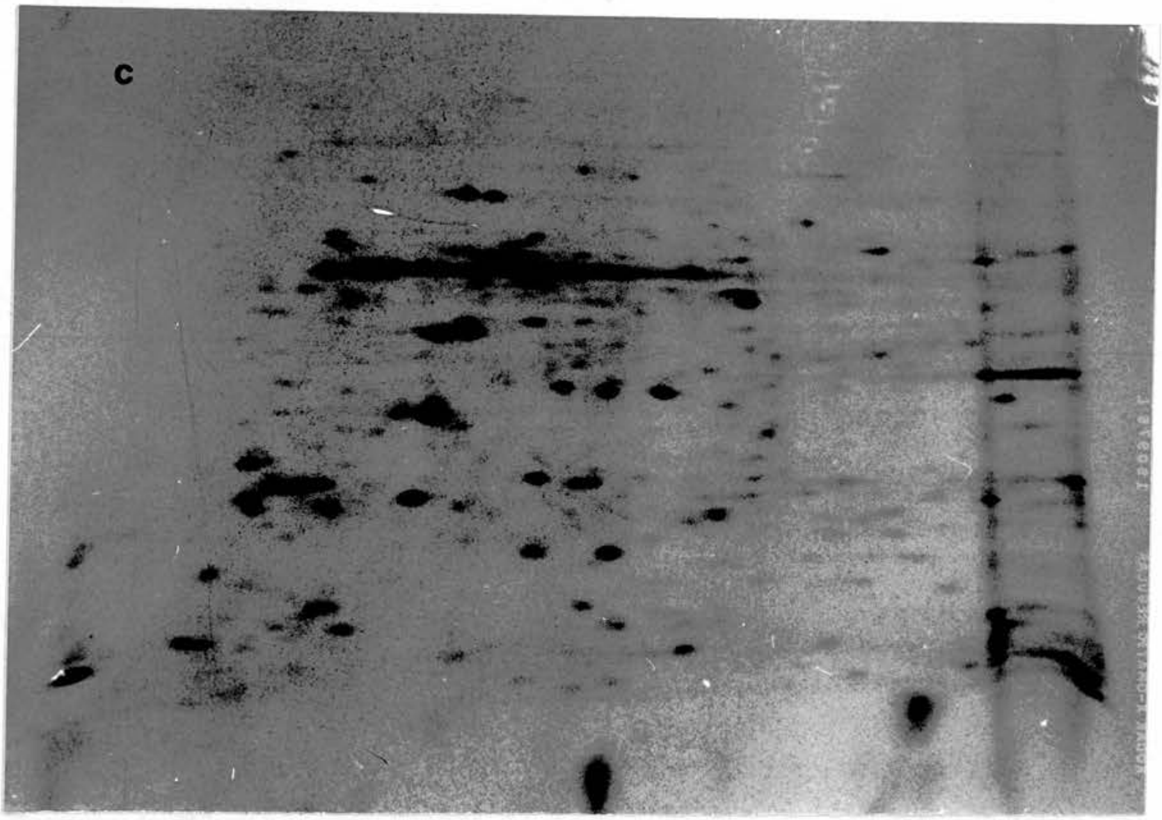
a) Control Female

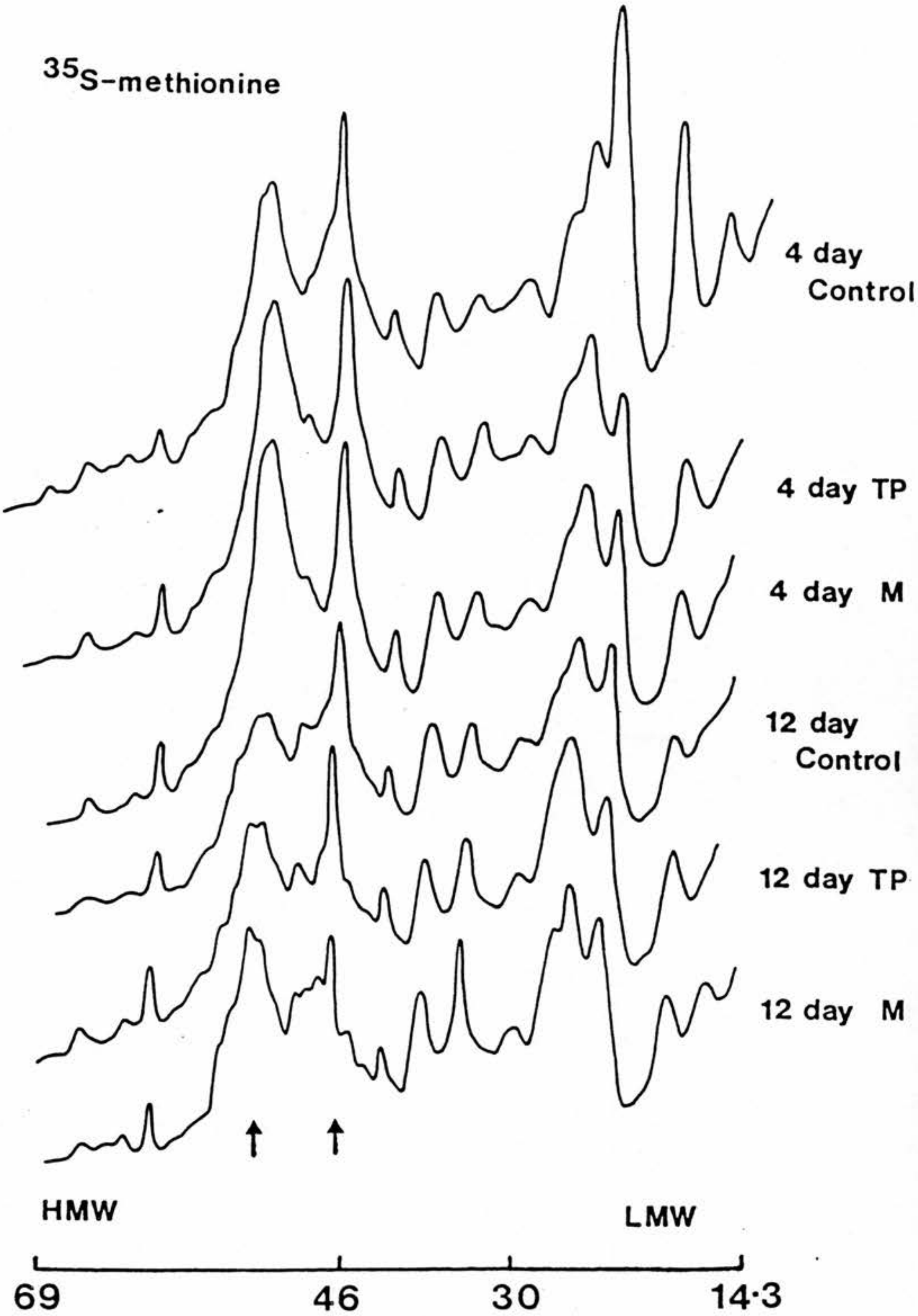
b) Androgenised Female

c) Male


a


b


c



b) Densitometric scans of the ^{35}S-methionine labelled translation products.

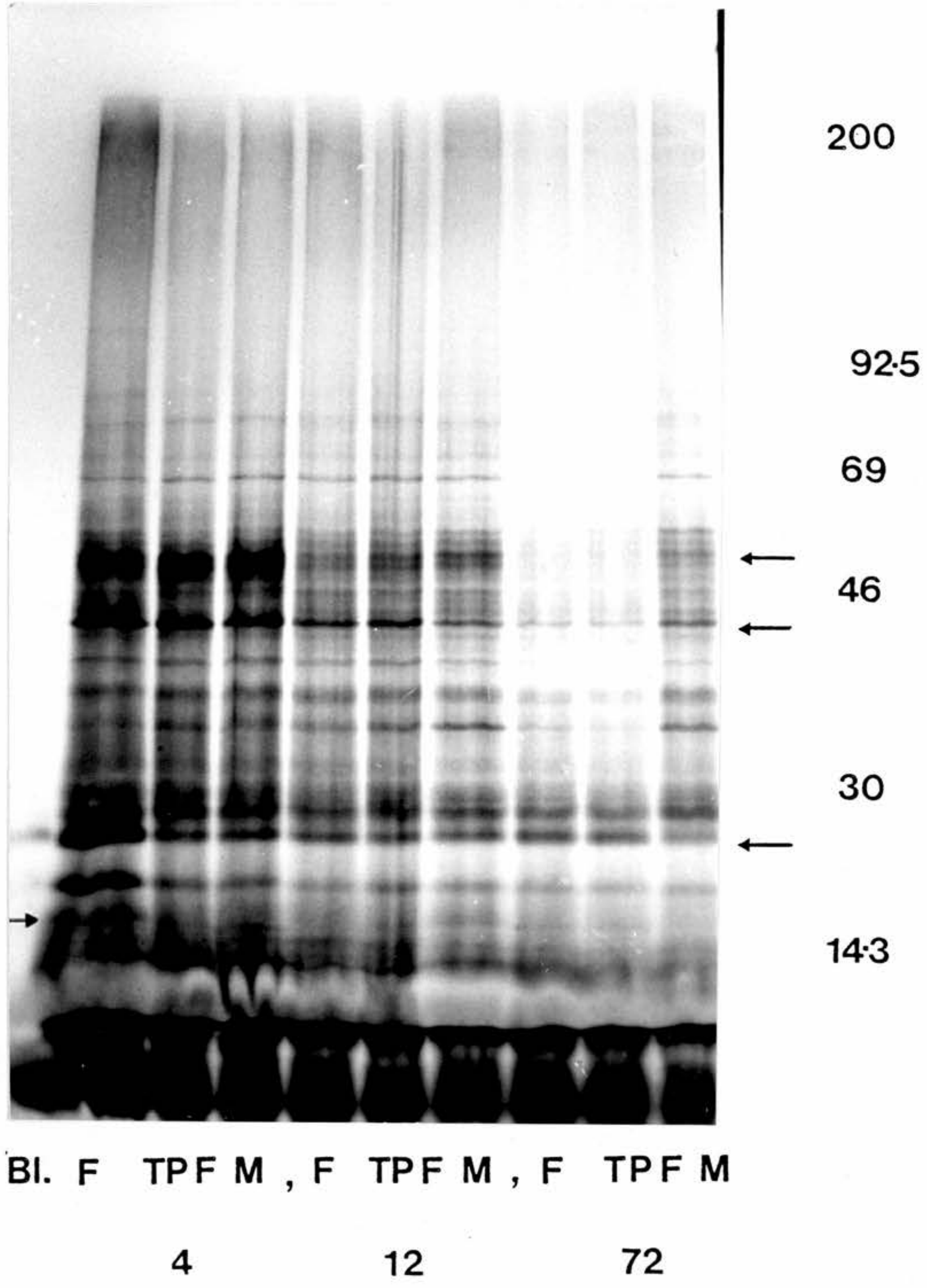


FIGURE 5.H: Developmental Changes In The Translation Products Of mRNA Isolated From Rat H-POA Using GTC And Translated In N-150 RRL Containing ^{35}S-methionine.

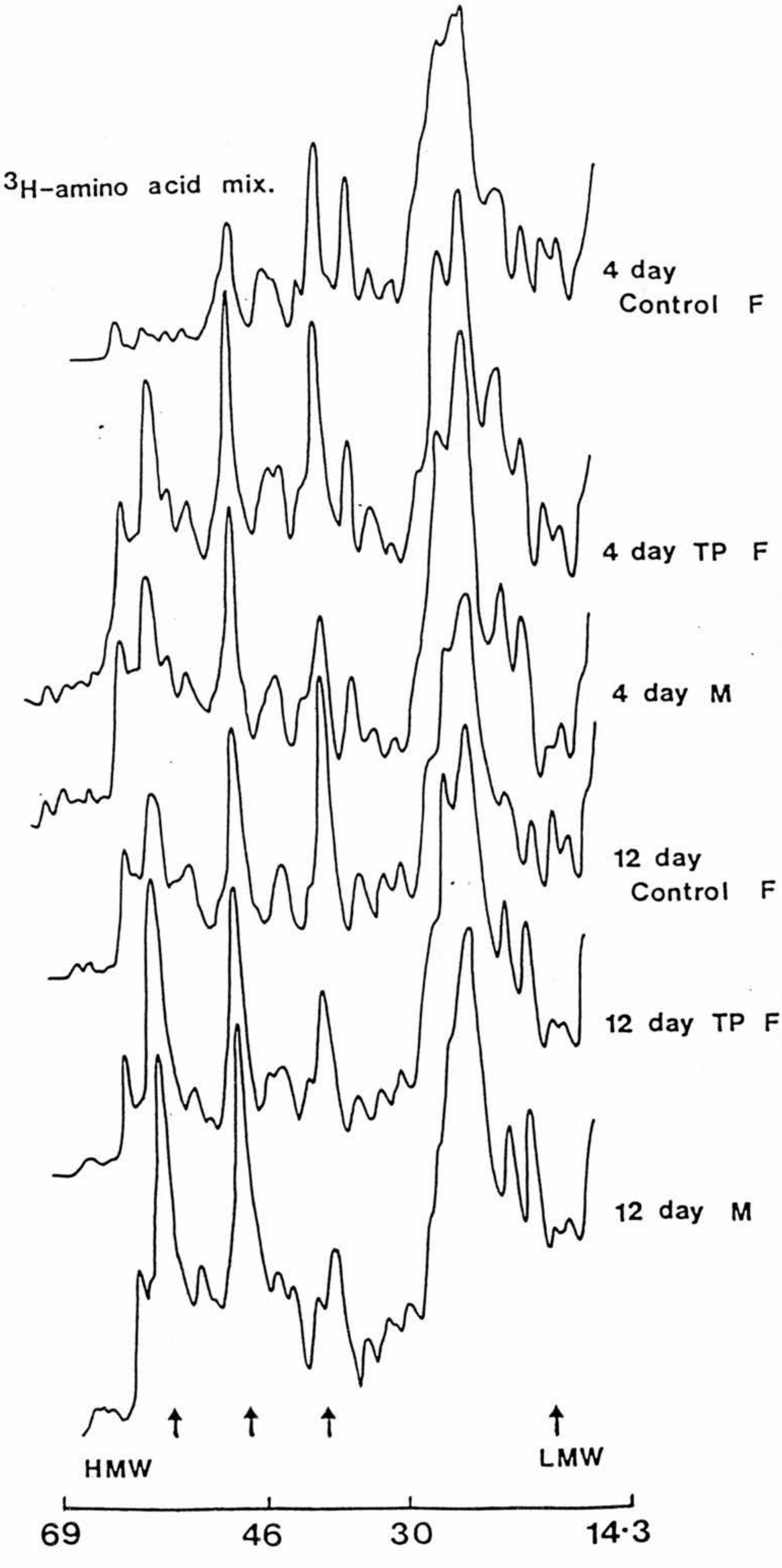
mRNA from 4, 12 and 72 day rat H-POA was translated in N-150 RRL containing ^{35}S-methionine.

a) Translation products (75,000 cpm) were anlysed by SDS-PAGE and the gel was fluorographed, dried and exposed to film for 4 days at -70°C. Numbers at the foot of the gel represent age (days) and numbers at the side of the gel represent marker protein molecular weights x 10^{-3}.

F = oil-treated female, TP F = TP-treated female,
M = Male, Bl. = Blank.

labelled proteins which were synthesised in vitro in the presence of mRNA extracted from 4, 12 and 72 day rat H-POA. Two major differences between the translation products from control female, androgenised female and male rat mRNA at 4 days were detected. A low molecular weight band (approximately 24,500) was much denser in the control female than in the androgenised female or male and a 58,000 molecular weight protein(s) was synthesised in greater amounts in the 4 day androgenised female and male than in the control female (Fig. 5.H.(b)). The latter difference between groups was also detected at 12 days. A decrease in synthesis of the 58,000 and a 46,000 molecular weight protein was found to occur between days 4 and 12 (Fig. 5.H.(b)). In addition, a less obvious change occurred in a polypeptide with an approximate molecular weight of 20,000 (Fig. 5.H.(a)) which was present at 4 days but its' synthesis was greatly reduced after this time.

Figure 5.I.(a) shows the translation products of mRNA from 4 and 12 day rat H-POA only which had incorporated ^{3}H-amino acid mixture. At 4 days the synthesis of a 37,000 molecular weight protein was lower in the male than in the control or androgenised female and at 12 days the mRNA from both the androgenised female and the male rat H-POA directed the synthesis of reduced amounts of the same protein, compared with the 12 day control female (Fig. 5.I.(b)). Synthesis of a 35,000 molecular weight protein (Fig. 5.I.(b)) decreased between days 4 and 12. Proteins with approximate molecular weights of 57,000 and 47,000 were synthesised in greater quantities in the androgenised female and male than in the control female at 4 and 12 days (Fig. 5.I.(b)). The synthesis



b) Densitometric scans of the ^{3}H-labelled translation products.

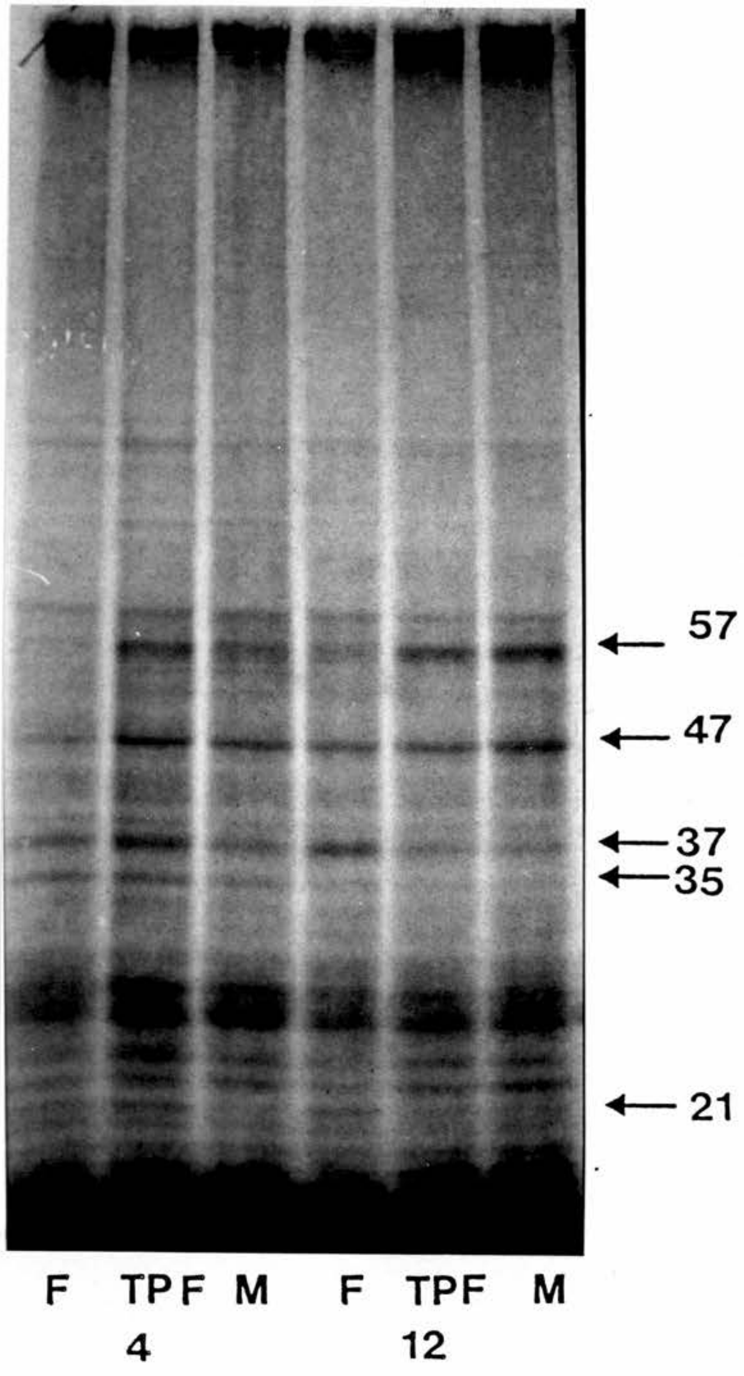


FIGURE 5.I: Developmental Changes In The Translation Products Of mRNA Isolated From Rat H-POA Using GTC And Translated In N-150 RRL Containing ^{3}H-amino acid mixture.

mRNA from 4 and 12 day rat H-POA was translated in N-150 RRL containing ^{3}H-amino acid mixture.

a) Translation products (40,000 cpm) were analysed by SDS-PAGE and the gel was fluorographed, dried and exposed to film for 30 days at -70°C. Numbers at the foot of the gel represent age (days) and numbers at the side represent marker protein molecular weights x 10^{-3}.

F = oil-treated female, TP F = TP-treated female, M = Male

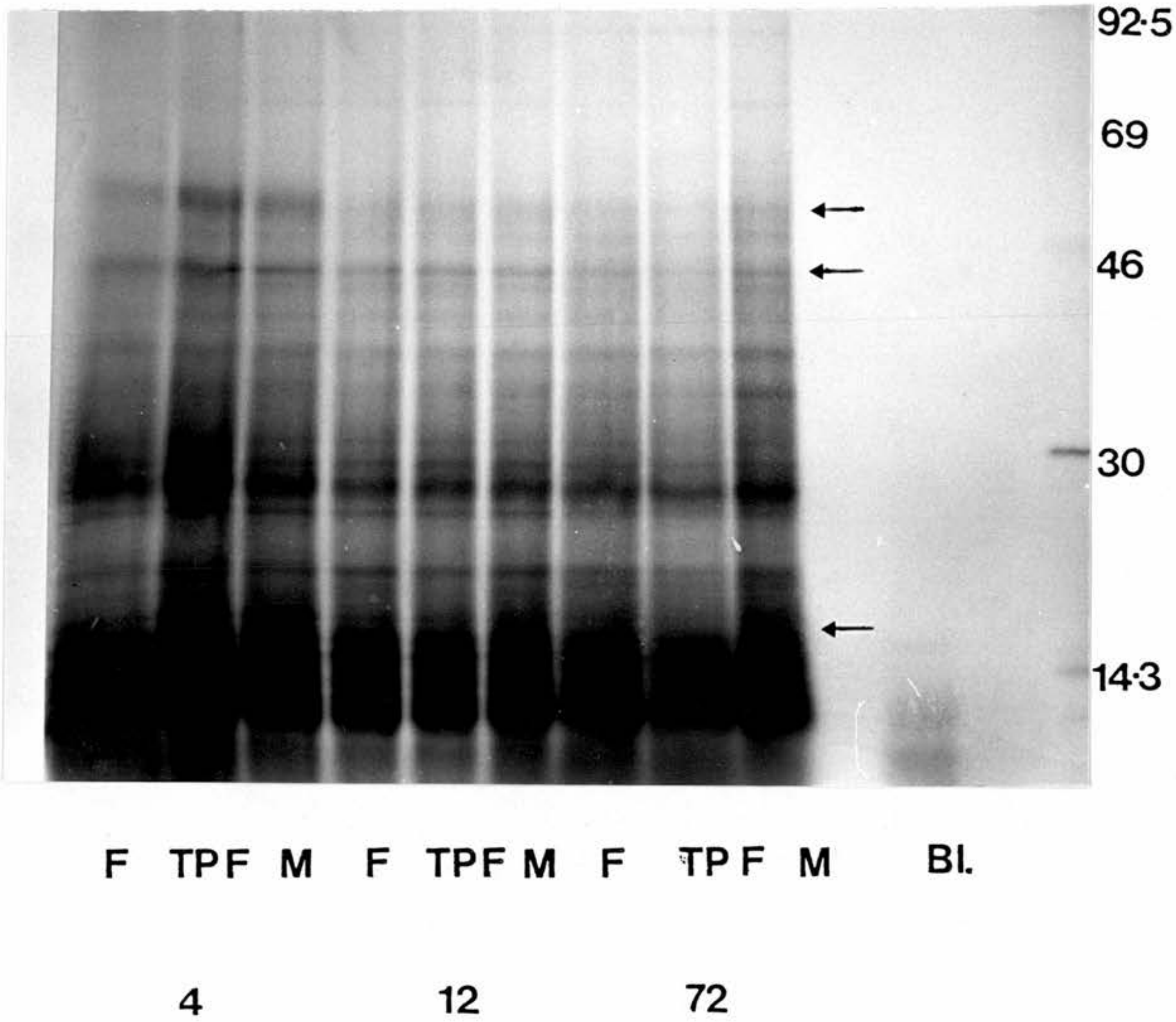


FIGURE 5.J: Developmental Changes In The Translation Products Of mRNA Isolated From Rat H-POA Using GTC And Translated In Wheat Germ Extract Containing ^{35}S-methionine.

mRNA from 4,12 and 72 day rat H-POA was translated in wheat germ extract containing ^{35}S-methionine. Translation products (50,000 cpm) were analysed by SDS-PAGE and the gel was fluorographed, dried and exposed to film for 8 days at -70°C. Numbers at the foot of the gel represent age (days) and numbers at the side represent marker protein molecular weights x 10^{-3}.

F = oil-treated female, TP F = TP-treated female, M = Male.

Bl. = Blank

of a low molecular weight protein (approximately 21,000) was lower in the 4 day male and 12 day androgenised female and male compared with the 4 day control female, androgenised female and 12 day control female (Fig. 5.I.(b)).

5.3.5.2 mRNA Isolated from Rat H-POA using GTC and Translated in a Wheat Germ Cell-Free Extract

Figure 5.J shows an autoradiogram of the proteins which were synthesised in a wheat germ extract, supplemented with ^{35}S-methionine, in the presence of mRNA from 4, 12 and 72 day rat H-POA. No scan is included for this gel as the background was too high, particularly in the low molecular weight region. As was found using N-150 RRL (section 5.3.5.1), mRNA translated in a wheat germ extract directed the synthesis of reduced amounts of 60,000 and 50,000 molecular weight proteins at 12 and 72 days of age compared with mRNA extracted at 4 days. Synthesis of a protein with an approximate molecular weight of 20,000 decreased dramatically after 4 days.

5.4 DISCUSSION

The data presented in this chapter show that developmental changes in the synthesis of proteins in the male, female and androgenised female rat H-POA can be detected by translating H-POA mRNA *in vitro* in two heterologous cell-free systems and analysing the translation products by one and two-dimensional PAGE. The results also show that the synthesis of specific proteins appears to be influenced by androgen at the mRNA level.

Figures 5.H and 5.J show that analysis of the translation products of the RRL and wheat germ systems, supplemented with

^{35}S-methionine, showed developmental decreases in proteins with approximate molecular weights of 58,000-60,000 and 47,000-50,000. When the R_f values of these proteins were compared with standard preparations of tubulin and actin run on gradient gels under the same conditions, the proteins were found to correspond to tubulin and actin, respectively. A decrease in synthesis of these proteins with age was also reported by Schmitt et al., (1977) and Hall and Lim (1981). Four and 12 day androgenised female and male rat H-POA have the capacity to synthesise more tubulin than the respective control female H-POA (Figs. 5.H.(b) and 5.I.(b)). In chapter 3 (section 3.3.1) it was shown that, at birth (day 0), the male H-POA contained more tubulin than the female H-POA as assessed by Coommassie Blue staining of soluble proteins. The results presented here suggest that this sex difference is probably accompanied by changes in mRNA concentrations for tubulin. In the same set of experiments in chapter 3, the sex difference in H-POA tubulin content seen at day 0 was no longer apparent at day 8. Therefore, in contrast to the data obtained on tubulin content by staining protein from soluble brain extracts in gels, the translation of mRNA in vitro shows that the effect of androgen on tubulin synthesis is still detectable at 12 days. However, due to the relatively poor translatability of the 72 day control and androgenised female mRNA preparations in RRL, no results are available for a further comparison of tubulin synthesis in the male and female 72 day H-POA with that found in the 4 and 12 day H-POA. In chapter 3, the measurements of H-POA tubulin content after day 0 by staining soluble protein and scanning gels were probably not sensitive enough to detect the sex difference in tubulin synthesis described here. Since tubulin comprises approximately 30% of soluble brain protein in the neonate and approximately 15% of translation products (Schmitt et al., 1977) it is likely that only large changes in the H-POA content of tubulin, such as that seen at day 0, could be detected by staining soluble brain proteins.

The lower translatability of the 72 day control and androgenised female mRNA in RRL compared with the 4, 12 and 72 day male mRNA preparations is probably largely the result of a poor extraction procedure. However, a decrease in synthesis of poly A^+ mRNA is known to occur with age in brain (De Larco, Abramowitz, Bromwell and Guroff, 1975; Hall and Lim, 1978; Guroff and Brodsky, 1971) and a reduction in chromatin template activity has been demonstrated (Bondy and Roberts, 1969).

An increase in synthesis by RRL of a 24,500 (approximately) molecular weight species was seen in the 4 day control female H-POA compared with the 4 day androgenised female and male H-POA using ^{35}S-methionine to label proteins (Figs. 5.H). Since this was not detected when the mRNA was translated in wheat germ in the presence of ^{35}S-methionine and the molecular weight of the polypeptide is very close to that of one of the endogenous lysate mRNA products, the physiological importance of this finding is questionable.

The synthesis of a polypeptide labelled with ^{35}S-methionine with an approximate molecular weight of 21,000 was greatly reduced between days 4 and 12 in all groups. This reduction in synthesis was most obvious in the translation products from the wheat germ extract (Fig. 5.J) but as can be seen in Fig. 5.H this change was also detected by translating the mRNA in RRL. On both gels, the polypeptide had an R_f value of 0.89. The identity of the 21,000 molecular weight polypeptide is not known but it could represent a high molecular weight precursor form of another polypeptide(s) since the translation systems used in these studies are generally considered not to carry out any post-translational modifications. However, reports by Peeters, Mous, Van Bellegem and Rombauts (1979) and Pascall, Boulton, Parker, Hall and Craig (1981) suggest that both RRL and wheat germ may in fact be capable of a limited degree of post-translational processing.

The translation of mRNA in vitro has helped to clarify the hormonal regulation of gene expression in a number of tissues by allowing a distinction to be made between transcriptional and translational control mechanisms. Two translation systems were used in the present study as a means of cross-checking results. However, the RRL and wheat germ systems possess a number of different characteristics. For example, RRL can translate larger mRNA species than wheat germ but it has a higher endogenous translation activity which, as can be seen in Fig. 5.H.(a), may interfere with the analysis of translation products on single-dimension gels. One disadvantage of both systems is that they maintain a high rate of protein synthesis for a relatively short time - perhaps only 90 min at the most.

The advantage of using two-dimensional PAGE for a more detailed analysis of the translation products is illustrated in Figs. 5E which, by visual inspection, show androgen-induced changes in the synthesis of two less abundant proteins in the 4 day H-POA after exposure to androgen for 6h. Although sex differences in the synthesis of tubulin (Figs. 5.H.(b) and 5.I.(b)) and actin (Fig. 5.I.(b)) could be detected by scanning single dimension gels, these proteins are major cell components and the detection of changes in synthesis of proteins present in much lower concentrations in the H-POA does require PAGE in two dimensions. The results also suggest that despite the disadvantage of a long film exposure, the ^{3}H-amino acid mixture is a useful method of labelling the translation products since a number of proteins not labelled by ^{35}S-methionine might be detected and sex differences are actually apparent on single dimension gels (Fig. 5.I.(a)).

A number of differences were detected between the 4 and 12 day mRNA preparations when a ^{3}H-amino acid mixture was used to label the proteins synthesised in RRL (Figs. 5.I). The synthesis of tubulin was higher in both the androgenised female and male than in the control female at 4 and 12 days as was found using ^{35}S-methionine (Fig. 5.H.(b)). The results show that hormone-induced changes in actin synthesis also occur (Fig. 5.I.(b)). In addition, the synthesis of proteins with approximate molecular weights of 37,000 and 21,000 was lower in the male than in the control and androgenised female at 4 days and their synthesis was also lower in the 12 day androgenised female and male compared with the 12 day control female (Figs. 5.I). The latter results suggest that neonatal exposure to androgen can influence the levels of mRNA for these two proteins but that the inhibitory effect of TP administration to a neonatal female upon their synthesis does not become apparent until day 12.

The results from the in vitro translation of mRNA coding for actin (and possibly other proteins) may not be entirely representative of synthesis of the protein in vivo. Messenger RNA coding for actin is known to exist in both poly(A)$^+$ and poly(A)$^-$ forms (Hunter and Garrels, 1977; Kaufmann, Milcarek, Berissi and Penman, 1977) and the Poly (A)$^-$ form would be lost at the oligo (dT)- cellulose chromatography step in these experiments. Although poly (A)$^-$ mRNA was originally thought to code for only a few specific proteins, for example, the histones (Adesnik and Darnell, 1972), the lack of a poly (A)$^+$ tail, or a very short length of Poly(A)$^+$at the 3' end of the mRNA molecule is becoming recognised as a more general phenomenon and complex populations of poly (A)$^+$ and poly (A)$^-$ mRNA are now being investigated in brain (Chikaraishi, 1979; Van Ness, Maxwell and Hahn, 1979).

There are other reports of changes in brain RNA after neonatal androgen administration, detected by measuring the incorporation of radioactive precursors into RNA and therefore reflecting changes in the gross synthesis of RNA. Clayton, Kogura and Kraemer (1979), using autoradiography of brain sections 3h after an injection of TP in neonatal female rats, showed a generalised decrease in incorporation of ^{3}H-uridine into RNA in all brain areas except the anterior hypothalamus and amygdala and Shamida and Gorbman (1970) demonstrated a slight decrease in ^{3}H-uridine incorporation into RNA in androgenised female rat brain at 47 days. Salaman (1970) also showed a consistently lower incorporation of ^{3}H-uridine into RNA in the androgenised female rat hypothalamus than in the normal cycling female rat hypothalamus. However, the data of Vértes,

Vértes and Kovács (1978) which showed a significantly greater incorporation of ^{14}C-orotic acid into RNA in the 7 and 14 day hypothalamus of neonatally androgenised females compared with control females seems to contradict the latter reports, although in each of these experiments, a different age was examined. The results of the work in this chapter cannot be compared with the latter studies since different methods were used. However, together with the data of Hall and Lim (1981) which showed developmental changes in the synthesis of 33,000 and 21,000 molecular weight proteins in female rat forebrain, the data presented in this chapter not only confirms that marked changes in the levels of mRNA coding for specific proteins occur during the 'critical period' but also suggests that androgen regulation of gene expression in brain does occur in the neonatal rat.

In conclusion, the data obtained in these studies using two cell-free translation systems and two radioactive amino acid preparations to label mRNA translation products in vitro show that both developmental and androgen-induced changes in protein synthesis occur in the rat H-POA. The synthesis of tubulin, actin and unidentified 20,000 and 35,000 molecular weight proteins was found to decrease during development of the H-POA. Sex differences in the synthesis of proteins with approximate molecular weights of 70,000, 37,000, 24,500, 21,000, and 20,000 and in tubulin and actin synthesis were also detected. It is not known, however, whether the changes described are restricted to the H-POA and therefore possibly related to the complex structural and functional changes occurring in this region during neonatal life.

CHAPTER 6

Steroid And Thyroid Hormone Effects On Brain Protein Synthesis: Investigations *In Vitro*

6.1 INTRODUCTION

Several methods have been successfully used in vitro to demonstrate steroid- induced changes in protein synthesis in a number of target tissues (Notides and Gorski, 1966; Chan, Means and O'Malley 1973; McKnight, 1978; Higgins, Colman, Fuller and Jackson, 1981). Many studies in vitro have been carried out either by (i) labelling proteins with a radioactive amino-acid in vitro after the administration of hormone in vivo (Notides and Gorski, 1966; Walker et al., 1979; Korach, Harris and Carter, 1981) or (ii) by the addition of both hormone and radioactive amino-acid to the isolated tissue (Katzenellenbogen and Gorski, 1972; Hall and Jenkins, 1982). However, few such studies have been reported for hormone effects on developing rat brain.

The aim of the following series of experiments was to use two types of in vitro techniques to investigate the effects of gonadal steroid and thyroid hormones on protein synthesis in the brain of foetal and neonatal rats. The first approach (section 6.2) involved the incorporation in vitro of radioactive amino-acids into proteins in the H-POA (and rest of brain in section 6.2.1.3) of 4 day rats. Hormones were administered either in vivo for various time intervals before incubation with radioactive amino-acids in vitro or added directly to the incubation medium containing tissue slices. The second approach (section 6.3) was to establish primary cultures of foetal rat hypothalamic neurons as described by Vaccaro and Messer (1977) and determine whether such cultures would be a useful in vitro system for studies on protein synthesis in the undifferentiated neurons from 18 day foetal rat H-POA. Although trophic effects of oestrogen and aromatizable androgen have been investigated in mouse brain explants (Toran- Allerand, 1976, 1980b) no electrophoretic studies investigating hormone effects on protein synthesis in either explants or dispersed cell systems have been reported.

6.2 PROTEIN SYNTHESIS STUDIES IN VITRO USING H-POA SLICES

Two separate types of experiment were carried out. In the first experiment, H-POA slices (or in one experiment slices of POA, hypothalamus and rest of brain; 6.2.1.3 and 6.2.2.3) from 4 day old rats were incubated with ^{35}S- methionine in order to label proteins to a high specific activity for analysis by one and two dimensional PAGE and fluorography. In the second experiment, double isotope labelling (originally used to detect oestrogen-induced proteins in the uterus by Notides and Gorski, 1966) was applied to experiments on neonatal rat brain.

6.2.1 MATERIALS AND METHODS

Incubation of H-POA In Vitro, Subcellular Fractionation and PAGE.

Four day old male and female Wistar Cob rats (day 0 = day of birth) from the departmental breeding colony were used in all experiments. Testosterone propionate (1.25mg in 0.1ml arachis oil) or arachis oil (0.1ml) were injected intraperitoneally at various times (see RESULTS) before the animals were killed. Animals were decapitated and the complete H-POA dissected out as described in section 2.2.2. The H-POA was then carefully sliced in the sagittal plane at room temperature, and the 0.5mm (approximately) thick sections were incubated in a 10ml conical flask containing 1 or 2ml

Krebs Ringer Phosphate medium, pH 7.4 (Appendix I) at 37°C, for 2h in a shaking water bath under an atmosphere of 95% air/5% CO_2. Hormones to be added to the incubates were prepared from stock solutions in ethanol (section 2.1.2), diluted with 0.5% (w/v) BSA in 0.9% (w/v) saline, and added to the incubation medium to give final hormone concentrations of 1µM (E_2 and testosterone) or 0.1µM (T_3 and T_4). The final ethanol concentration was 0.01% . Control slices were exposed to the same concentration of ethanol in BSA/saline solution. The radioactive amino acids (section 2.1.3) used were ^{35}S-methionine (50µCi/ml), ^{3}H-leucine (20µCi/ml) and ^{14}C-leucine (5 uCi/ml). At the end of the incubation period flasks were placed on ice for 5 min before collecting the tissue by centrifugation at 1,000g for 5 min at 4°C and rinsing the tissue once with incubation medium to remove excess free radioactive amino acid.

Tissue samples were homogenised in 5-10 vol sucrose-TKM buffer, centrifuged at 120,000g for 1h at 4°C, the supernatant collected and proteins extracted from the pellets, as described in section 2.2.3.

Protein concentration was determined as described in section 2.2.4 and the incorporation of radioactivity estimated as in section 2.2.5. PAGE was carried out as described in section 2.2.6 and gels were stained (section 2.2.9 (a)), fluorographed (section 2.2.9 (b)) or sliced (section 2.2.9 (c)) to detect proteins.

6.2.1.1 Time Course of Incorporation of Radioactive Labelled Amino Acids into Protein

The incorporation of radioactive amino acid into soluble protein in vitro was investigated by incubating H-POA slices with

^{35}S-methionine (15-25µCi/ml) for 1-6h and analysing the 120,000g supernatant as described in section 2.2.5.

6.2.1.2 Incubation of H-POA with Steroid or Thyroid Hormones.

The complete H-POA from 4 day male and female rats (litter mates) was incubated as described in section 6.2.1 in 1 ml medium in the presence of steroid or thyroid hormones and ^{35}S-methionine for 2h.

6.2.1.3 The Effect of TP injected In Vivo on Brain Protein Synthesis In Vitro

Female rats (litter mates) aged 4 days were injected i.p. with either oil or TP at approximately 10.00h (3 animals/group). Six hours later, the animals were decapitated and the brains were quickly dissected into POA, hypothalamus and rest of brain as described in section 2.2.2. For both control and experimental groups the tissue from each brain region from the 3 rats was pooled and incubated for 2h in vitro with ^{35}S-methionine as described in 6.2.1. The tissues were prepared for PAGE as described in 6.2.1.

6.2.1.4 Double Label Experiments

The principle of the double isotope labelling technique (Notides and Gorski, 1966) is the electrophoretic separation of a mixture of proteins from control and hormone-treated tissues (labelled with either ^{3}H-leucine or ^{14}C-leucine respectively in vitro) so that labelling of the individual proteins from each sample can be compared. Hormone-induced changes in protein synthesis can be detected by comparing the relative incorporation of the ^{14}C or ^{3}H-labelled amino acid into proteins. It is a particularly useful method for the detection of proteins which represent only a minor percentage of the total protein and which contribute little to the overall incorporation of radiolabelled amino acid.

The effect of TP administration to 4 day female rats on H-POA protein synthesis was investigated using the double isotope label technique. Animals were killed at 45 min, 6h and 26h after the injection of oil or TP (1.25mg in 0.1ml oil). The H-POA (prepared as in 6.2.1) from control animals was incubated in the presence of ^{3}H-leucine at 20µCi/ml and the H-POA from TP- treated animals incubated with ^{14}C-leucine at 5µCi/ml. After a 2h incubation, tissue from the control and experimental animals was pooled, homogenised and centrifuged as described in 6.2.1. The soluble fraction was concentrated by freeze-drying to approximately half the original volume. Soluble and pellet-associated proteins were prepared for PAGE as described in section 2.2.3. Gels were stained with Coomassie Blue and then sliced to estimate the incorporation of radioactive amino acid into protein by liquid scintillation counting (sections 2.2.9 (a) and 2.2.9 (c)).

6.2.2 RESULTS

6.2.2.1 Time Course Of ^{35}S-Methionine Incorporation into Protein In Vitro

Fig. 6.2.A ((a) and (b)) shows the time course of incorporation of ^{35}S-methionine into soluble protein *in vitro*. Incorporation was linear for the duration of the experiment indicating that the protein synthesising mechanisms were still fully functional in the H-POA slices after 6h *in vitro*. On the basis of these results, in all subsequent experiments a 2h incubation period was used since at this time point sufficient incorporation of ^{35}S-methionine into

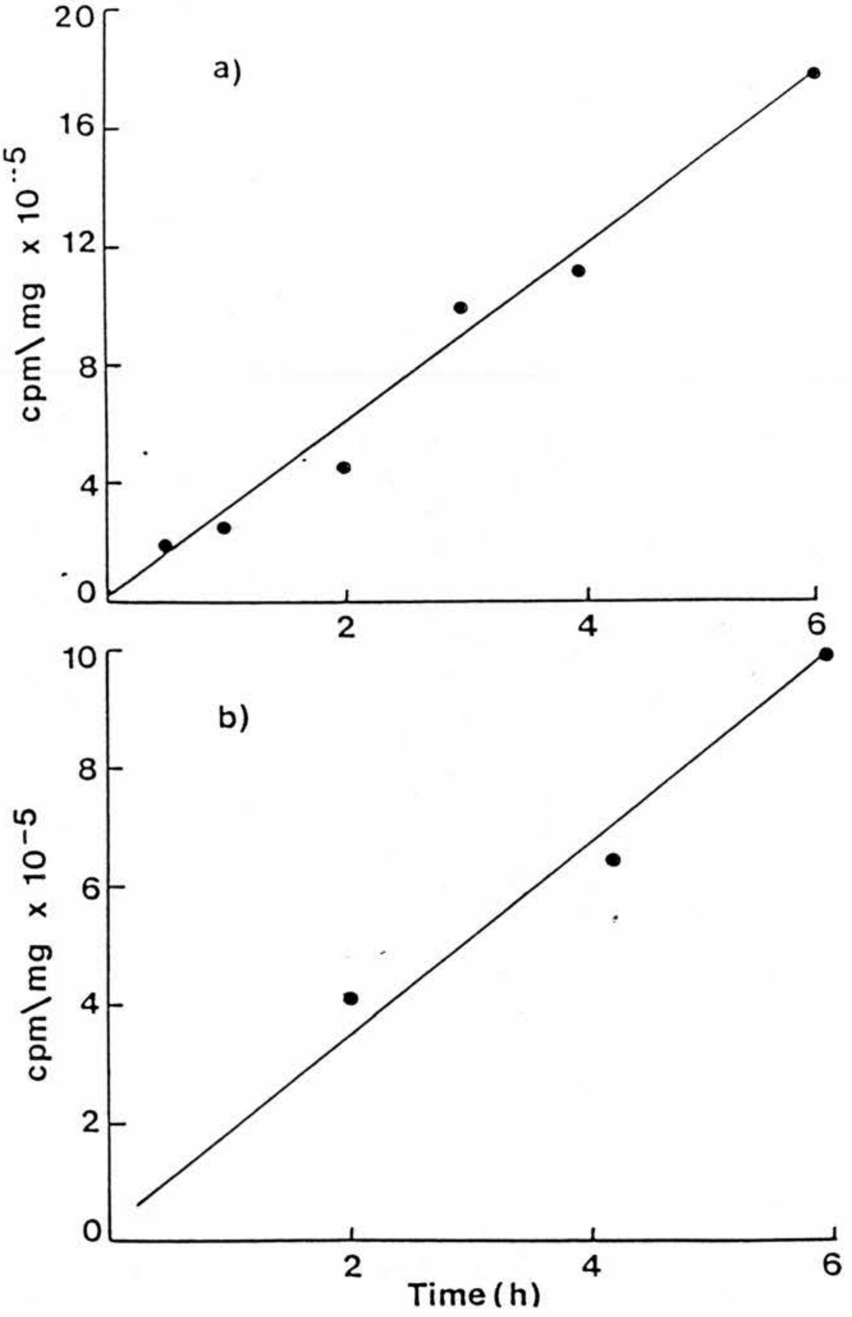


FIGURE 6.2.A: Incorporation Of ^{35}S-methionine Into Protein In H-POA Slices <u>In Vitro</u>

H-POA slices from 4 day rats were incubated in the presence of a) 25µCi/ml and b) 14µCi/ml ^{35}S-methionine in Krebs Ringer Phosphate buffer for up to 6h. The incorporation of ^{35}S-methionine into acid insoluble material in the soluble fraction was then estimated. Protein was measured by the method of Lowry et al., (1951).

protein had occurred to allow analysis of proteins by PAGE.

6.2.2.2 The Effects of Exposure to Hormones In Vitro on H-POA Protein Synthesis

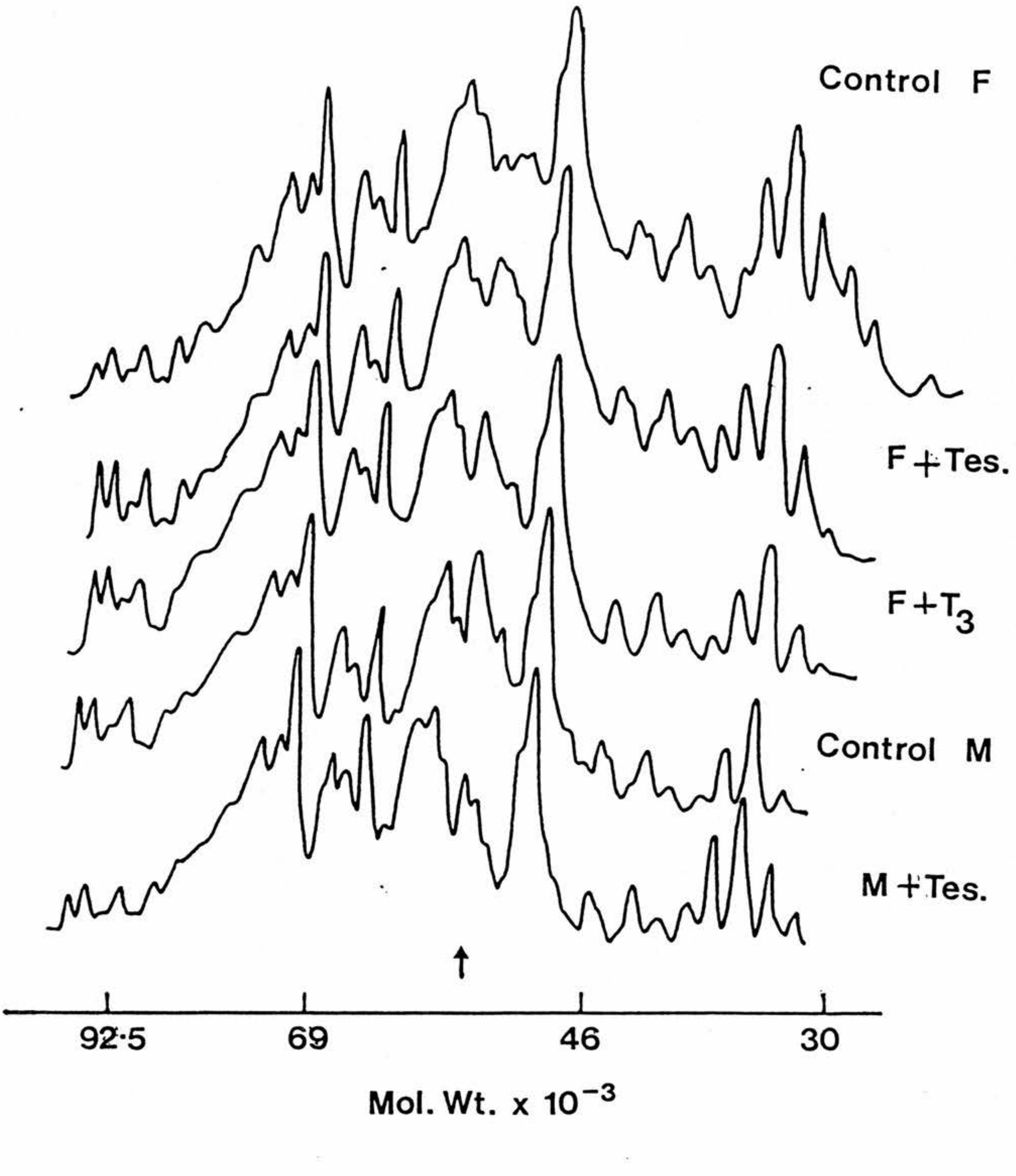
Steroid and thyroid hormones both stimulated and inhibited the synthesis of specific proteins in the isolated H-POA after only 2h in vitro (Figs 6.2.B(a) and 6.2.C.(a)).

In control H-POA, the synthesis of a soluble protein, approximately 57,500 daltons, was significantly higher (approximately 500%) in the male than in the female. Testosterone and T_3 (but not T_4) increased synthesis of this protein in the female H-POA by 300 and 400% respectively, whereas testosterone reduced its synthesis by approximately 60% in the male (Fig 6.2.B.(b)).

As in the soluble fraction, the synthesis of only one protein in the pellet fraction was significantly influenced by hormone administration. In female H-POA, the synthesis of a 58,900 molecular weight protein was reduced by at least 35% by testosterone, E_2 and T_3. A 60% reduction in synthesis of this protein occurred after the addition of T4 to the medium. In contrast to the female H-POA, only testosterone and T3 were effective in reducing synthesis of the 58,900 molecular weight protein in the male H-POA by 35% (Fig. 6.2.C.(b))

6.2.2.3 The Effect of TP Administration In Vivo on the Incorporation of ^{35}S-Methionine into Brain Protein In Vitro

There was no apparent effect of TP on the synthesis of soluble proteins in any brain region incubated in vitro 6h after the



b) Densitometric scans of proteins from the soluble fraction.

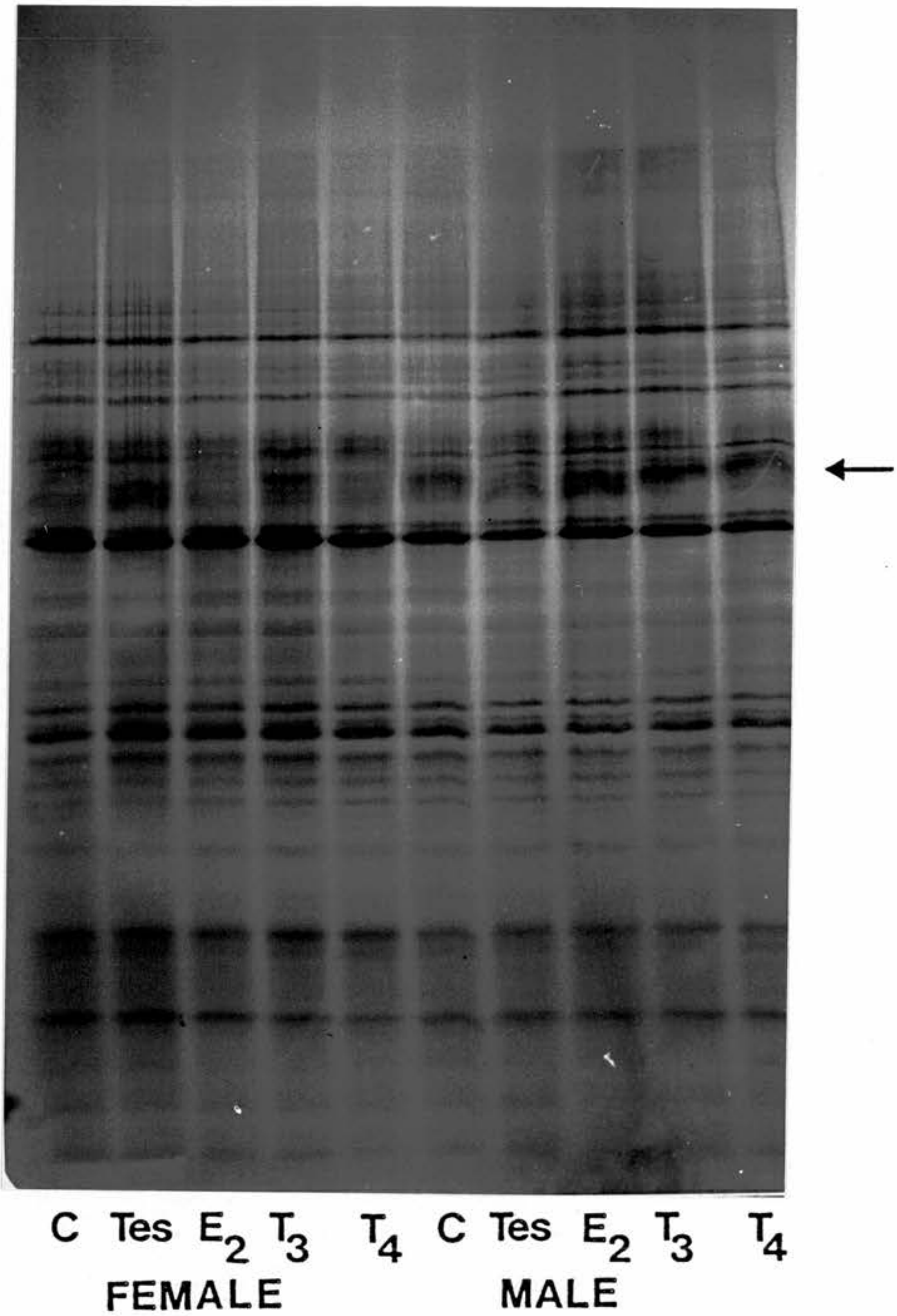
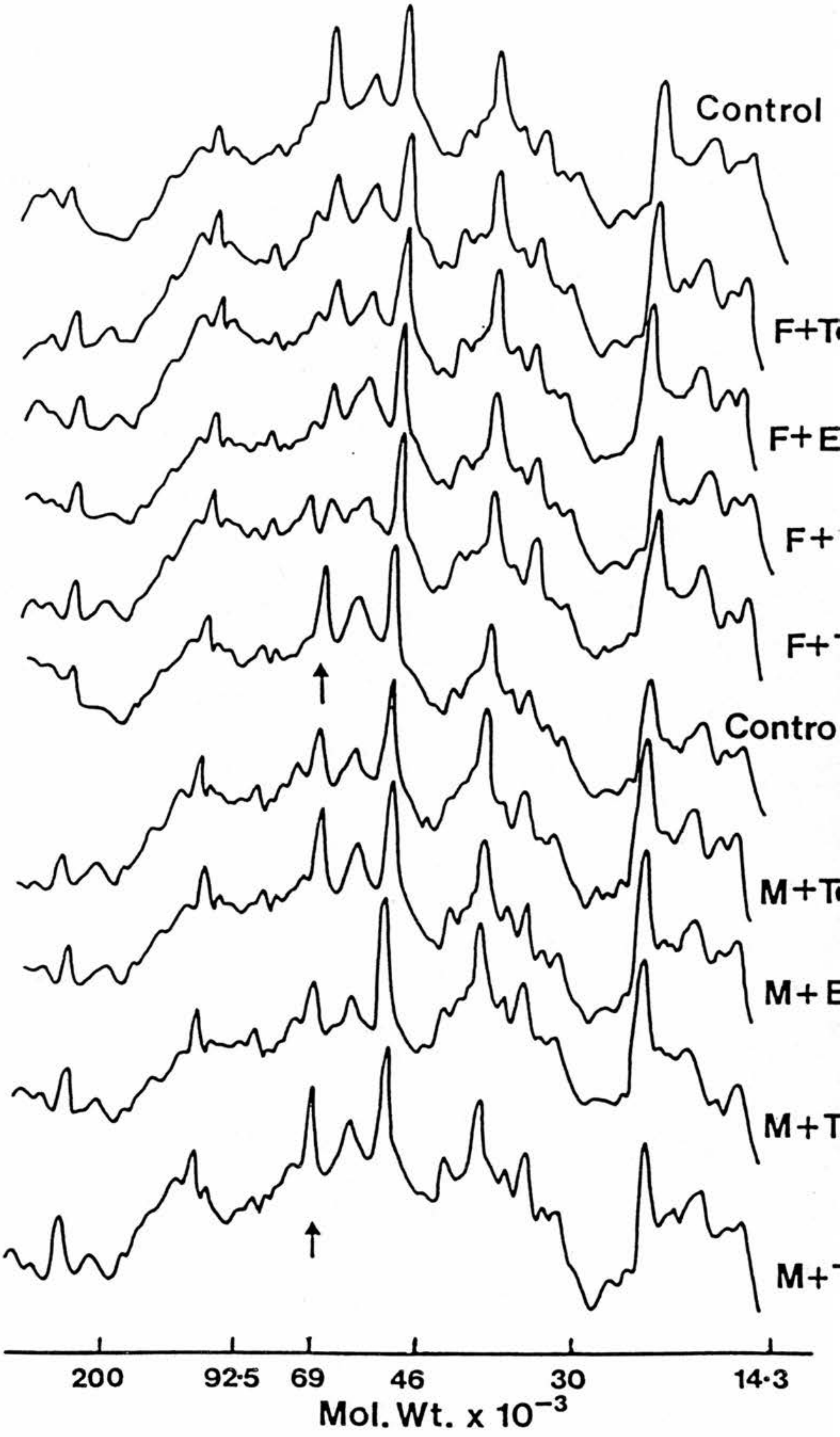


FIGURE 6.2.B: Electrophoretic Analysis Of Soluble Proteins Prepared From H-POA Slices Incubated In Vitro In The Presence of Steroid And Thyroid Hormones With ^{35}S-methionine.

a) H-POA slices from 4 day male (M) and female (F) littermates were incubated in Krebs Ringer Phosphate buffer at 37°C for 2h *in vitro* in the presence of ^{35}S-methionine (50µCi/ml) and either testosterone (T) or E_2 at 1µM or T_3 or T_4 at 0.1µM. Control (C) slices were exposed to the same concentration of ethanol (0.01%) in BSA/saline solution. Tissue was homogenised and a soluble fraction prepared. Incorporation of radioactivity into acid insoluble material was estimated and 200,000 cpm from each sample were analysed by SDS-PAGE. The gel was fluorographed, dried and exposed to film at -70°C for 2 days.



b) Densitometric scans of proteins extracted from the pellets.

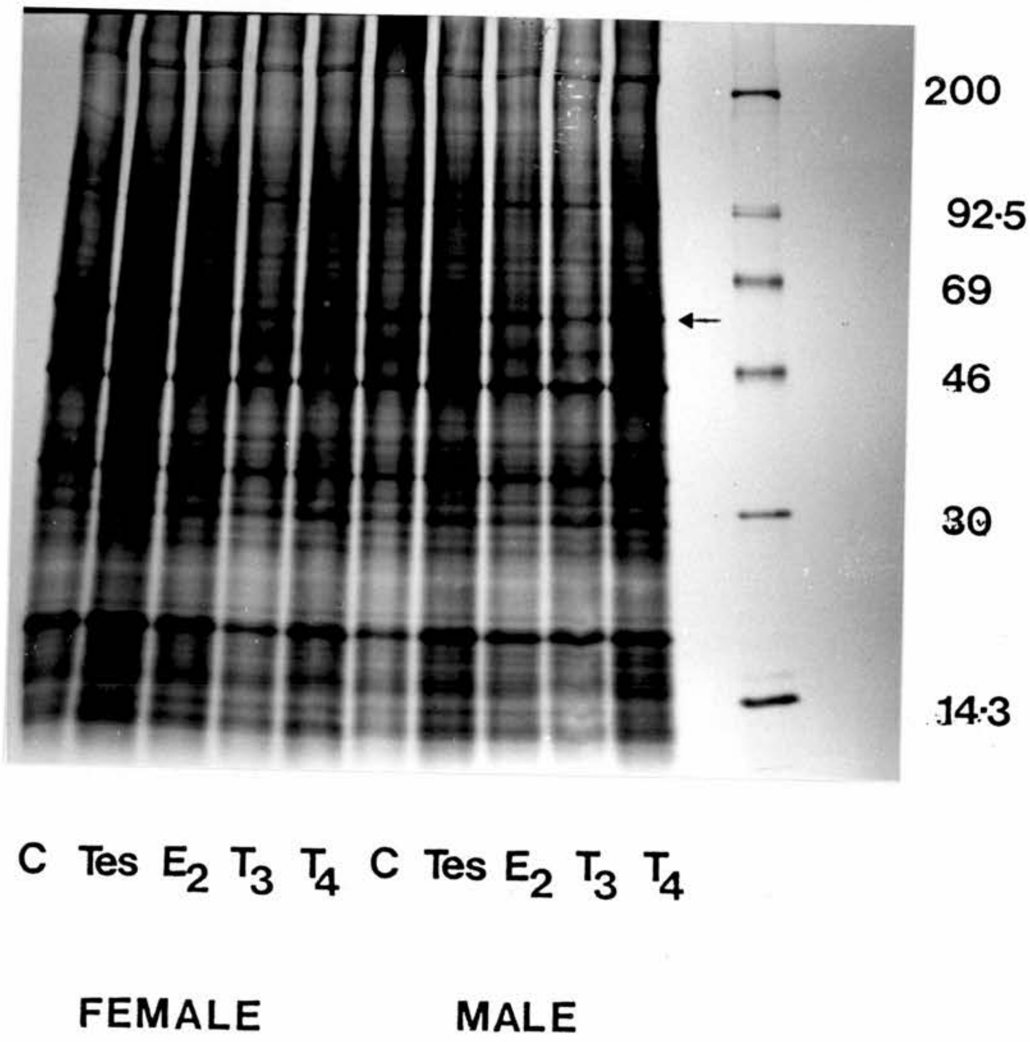


FIGURE 6.2.C: Electrophoretic Analysis Of SDS Extracts From The 120,000g Pellet From The H-POA Slices.

a) Proteins were extracted from the pellets as described in section 2.2.3 and 100,000 cpm from each sample were analysed by SDS-PAGE. The gel was fluorographed, dried and exposed to film at -70°C for 4 days. Numbers at the side of the gel represent marker protein molecular weights x 10^{-3}.

administration of TP in vivo as assessed by either single (Fig 6.2.D) or two-dimensional gel analysis of proteins (Figs 6.2.E (a) and 6.2.E (b)).

6.2.2.4 Double Label Experiments

As assessed by the incorporation of ^{3}H-leucine and ^{14}C-leucine, the H-POA soluble and pellet-associated proteins showed no evidence for a major induction or inhibition of synthesis of any proteins at 45 min (Fig 6.2.F (a)), 6h (Fig 6.2.F (b)) or 26h (Fig 6.2.F (c)) after TP administration. However, the stained pattern of soluble proteins (Fig. 6.2.G) from the pooled H-POA at 26h differed from the patterns seen at either 45 min or 6h, which were identical to each other. A protein of approximately 27,000 daltons was present in greatly reduced amounts in the 26h soluble sample.

The ^{3}H/^{14}C ratio in both soluble and pellet proteins at 45 min (Fig 6.2.F (a)) and 26h (Fig 6.2.F (c)) also showed that the labelling of proteins in H-POA from both the oil-treated and TP-treated rats was proportional only to the concentration of isotope in the medium. No effect of TP on protein synthesis was detected. However, at 6h after TP administration the ^{3}H/^{14}C ratio in both soluble and pellet proteins (Fig 6.2.F (b)) showed a generalised increase. Average values of the ^{3}H/^{14}C ratio were between 4 and 6 at 45 min and 26h, and 6 to 8 at 6h after TP injection.

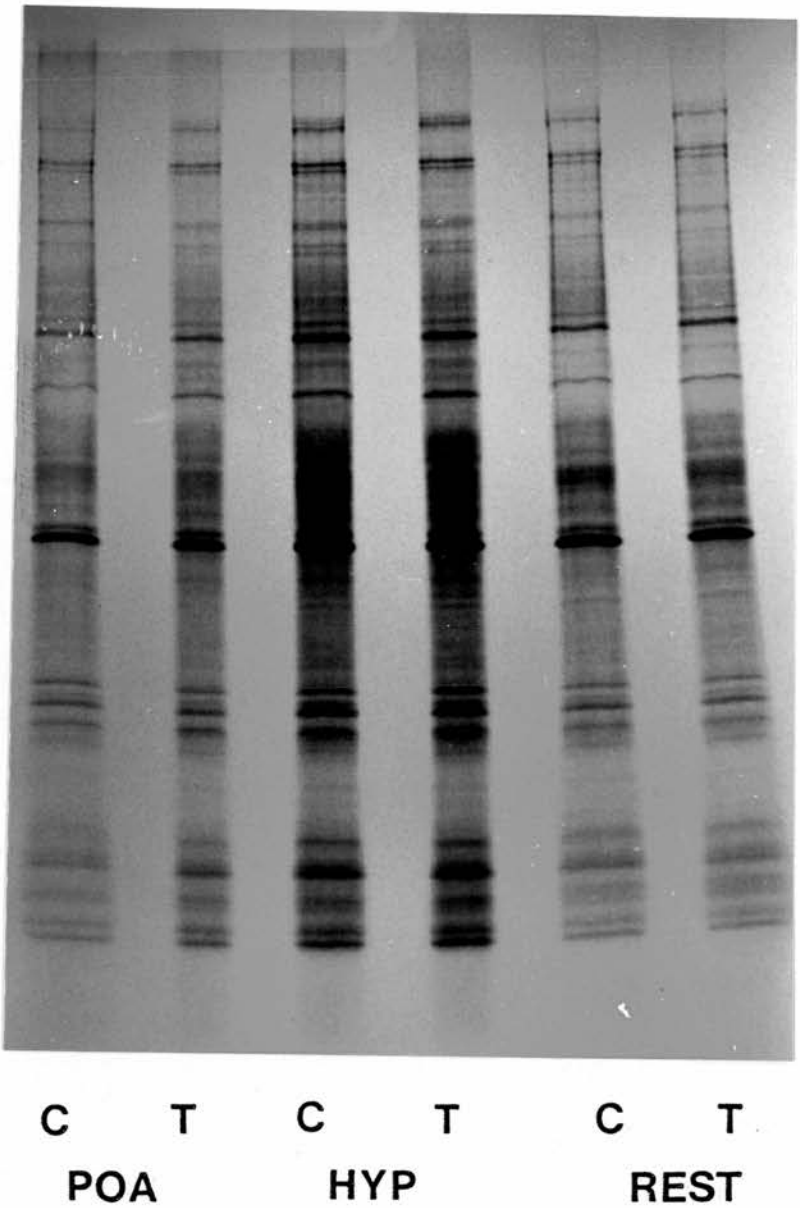
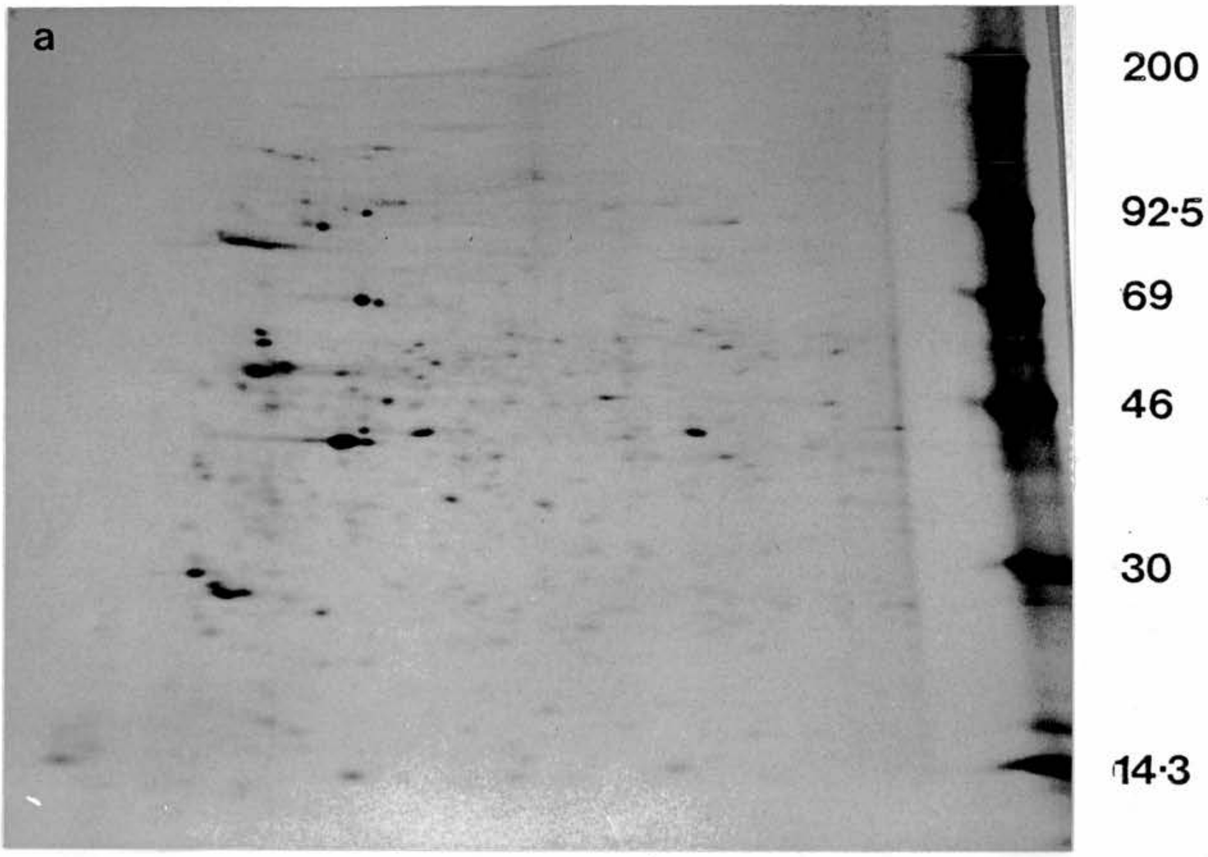


FIGURE 6.2.D: Autoradiogram Of Soluble Proteins From The POA, Hypothalamus And Rest Of Brain Labelled With ^{35}S-methionine In Vitro After TP Administration In Vivo.

4 day females were injected (i.p.) with oil (C) or 1.25mg TP (T) and 6h later were killed and the brain dissected into POA, hypothalamus (HYP) and rest of brain (REST). Tissue slices were incubated in vitro for 2h in the presence of ^{35}S-methionine (15μCi/ml). The incorporation of radioactivity into acid insoluble material in the soluble fraction prepared from each area was estimated and 15-50,000 cpm loaded onto the gel. After SDS-PAGE the gel was fluorographed, dried and exposed to film for 20 days at -70°C. Numbers at the right of the gel represent marker protein molecular weights x 10^{-3}.

FIGURE 6.2.E: Two-Dimensional Gel Analysis Of Soluble Proteins From Hypothalamus, Labelled with ^{35}S-methionine _In Vitro_ After TP Administration _In Vivo_

Soluble proteins from the hypothalamus (HYP in Figure 6.2.D) of oil-treated (a) and TP-treated (b) 4 day female rats were analysed by PAGE in two dimensions. For each sample 50,000 cpm were loaded onto the gel. The track on the left of gel b) is the sample run only in the second dimension as a reference. After electrophoresis the gels were fluorographed, dried and exposed to film for 28 days at -70°C. The numbers on the right of the gel represent marker protein molecular weights x 10^{-3}.


a
200
92·5
69
46
30
14·3

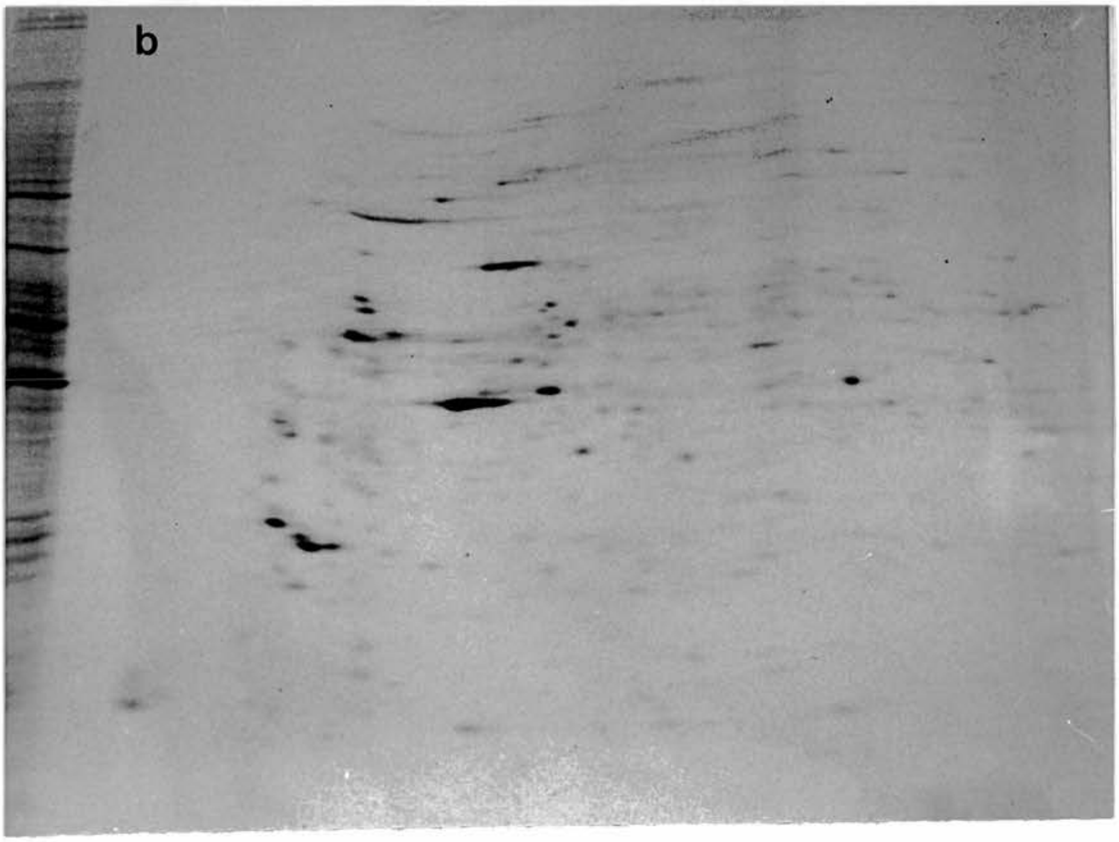
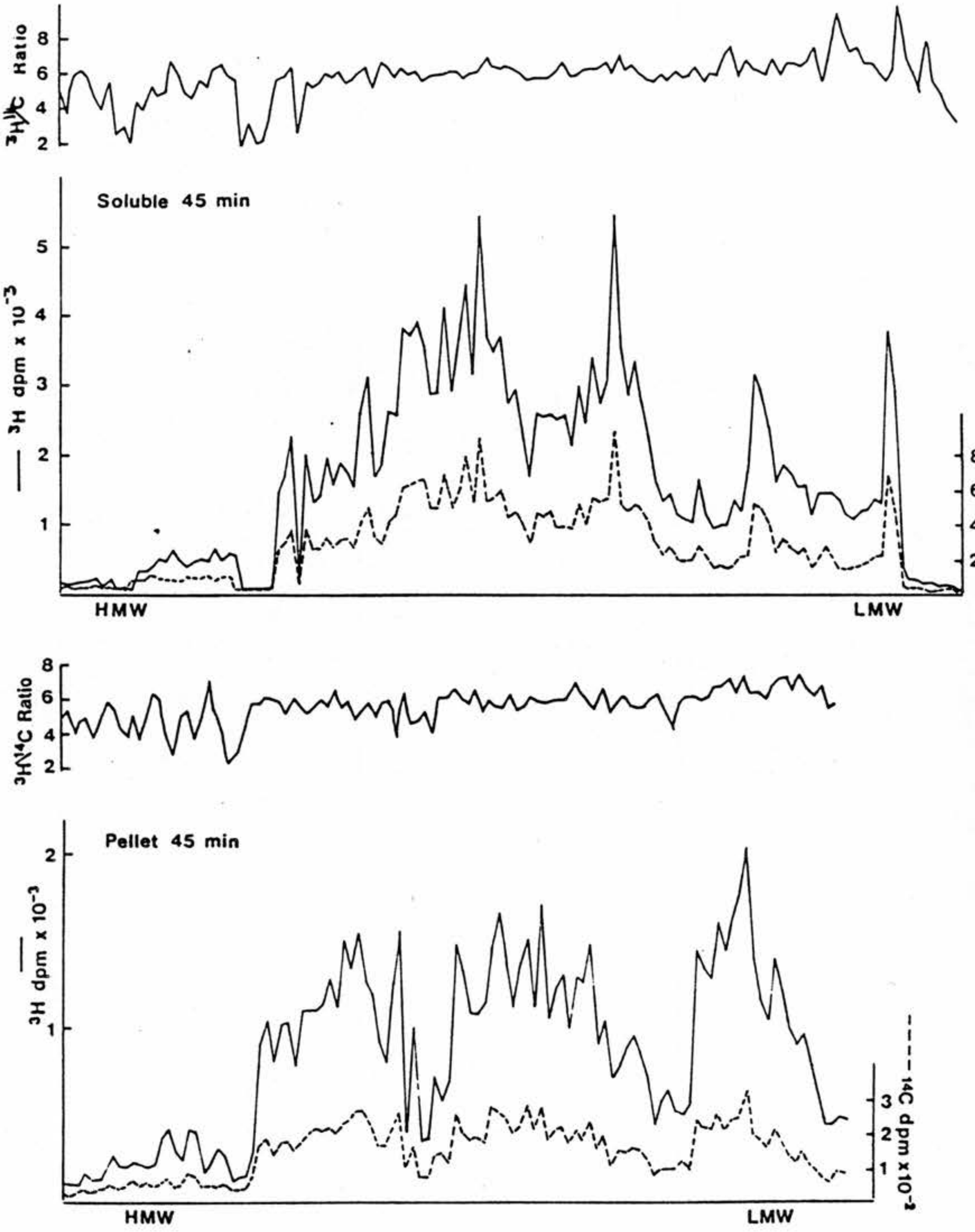

b

FIGURE 6.2.F: Double Label Experiments

Electrophoretic distribution on polyacrylamide gels of 4 day H-POA soluble and pellet proteins synthesised in vitro after oil or TP administration in vivo for a) 45 min
b) 6h
c) 26h

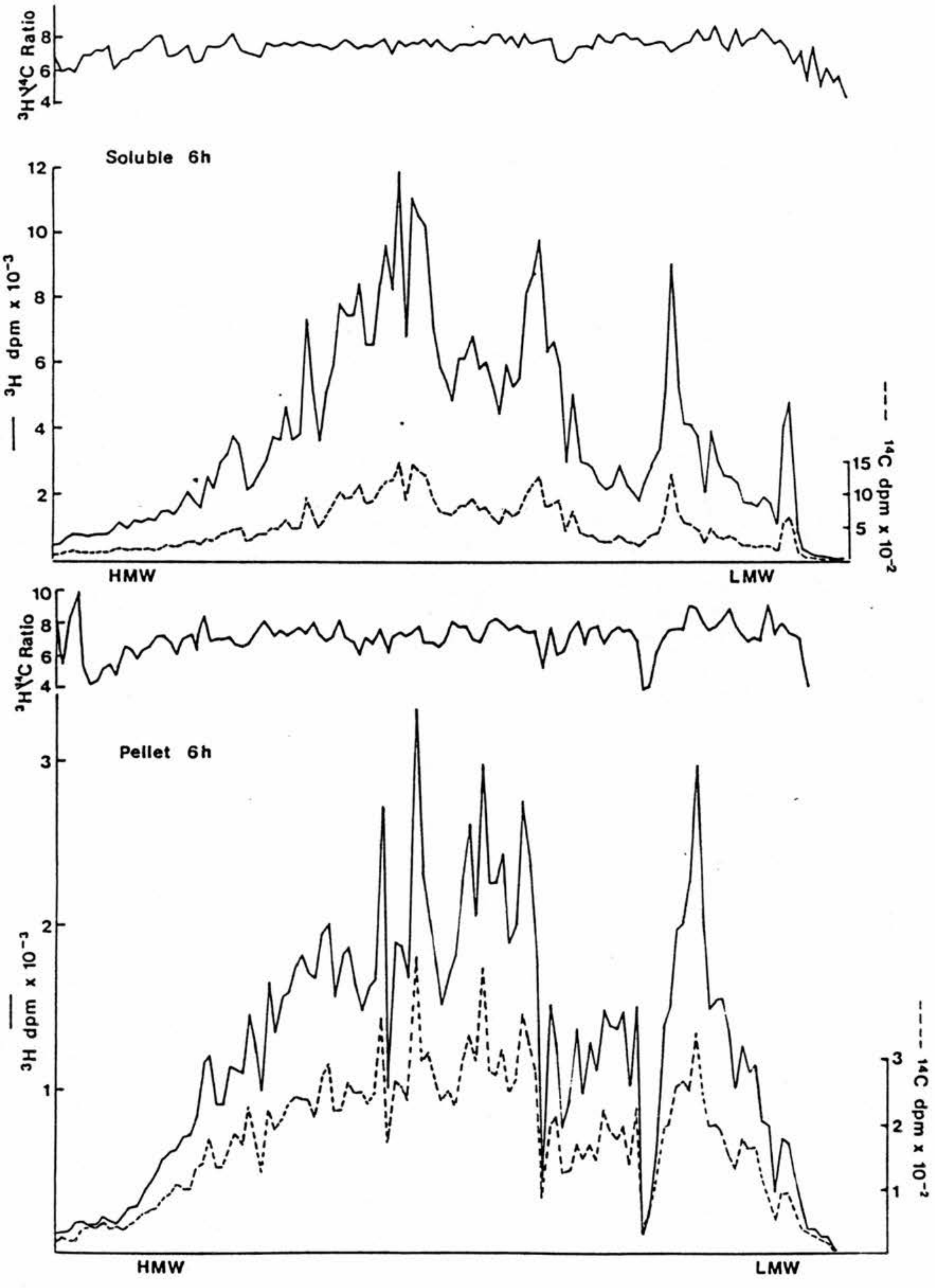
H-POA from oil-treated animals were incubated in vitro for 2h at 37°C in the presence of 20µCi/ml ^{3}H-leucine. The H-POA from TP-treated animals were incubated with 5µCi/ml ^{14}C-leucine.

The H-POA from oil-treated and TP-treated rats were then homogenised together, centrifuged at 120,000g for 1h at 4°C and the proteins in the pellet were extracted. Soluble and pellet-associated proteins were analysed by SDS-PAGE. The radioactivity and ^{3}H/^{14}C ratio in gel slices were then determined by liquid scintillation counting.



3H/14C Ratio
8
6
4
2
Soluble 45 min
— 3H dpm x 10-3
5
4
3
2
1
8
6
4
2
HMW
LMW
3H/14C Ratio
8
6
4
2
Pellet 45 min
— 3H dpm x 10-3
2
1
---- 14C dpm x10-2
3
2
1
HMW
LMW

FIGURE 6.2.F (a)



$^3H/^{14}C$ Ratio
8
6
4
Soluble 6h
12
10
8
6
4
2
— ^3H dpm x 10^{-3}
--- ^{14}C dpm x 10^{-2}
15
10
5
HMW
LMW
$^3H/^{14}C$ Ratio
10
8
6
4
Pellet 6h
3
2
1
— ^3H dpm x 10^{-3}
--- ^{14}C dpm x 10^{-2}
3
2
1
HMW
LMW

FIGURE 6.2.F (b)

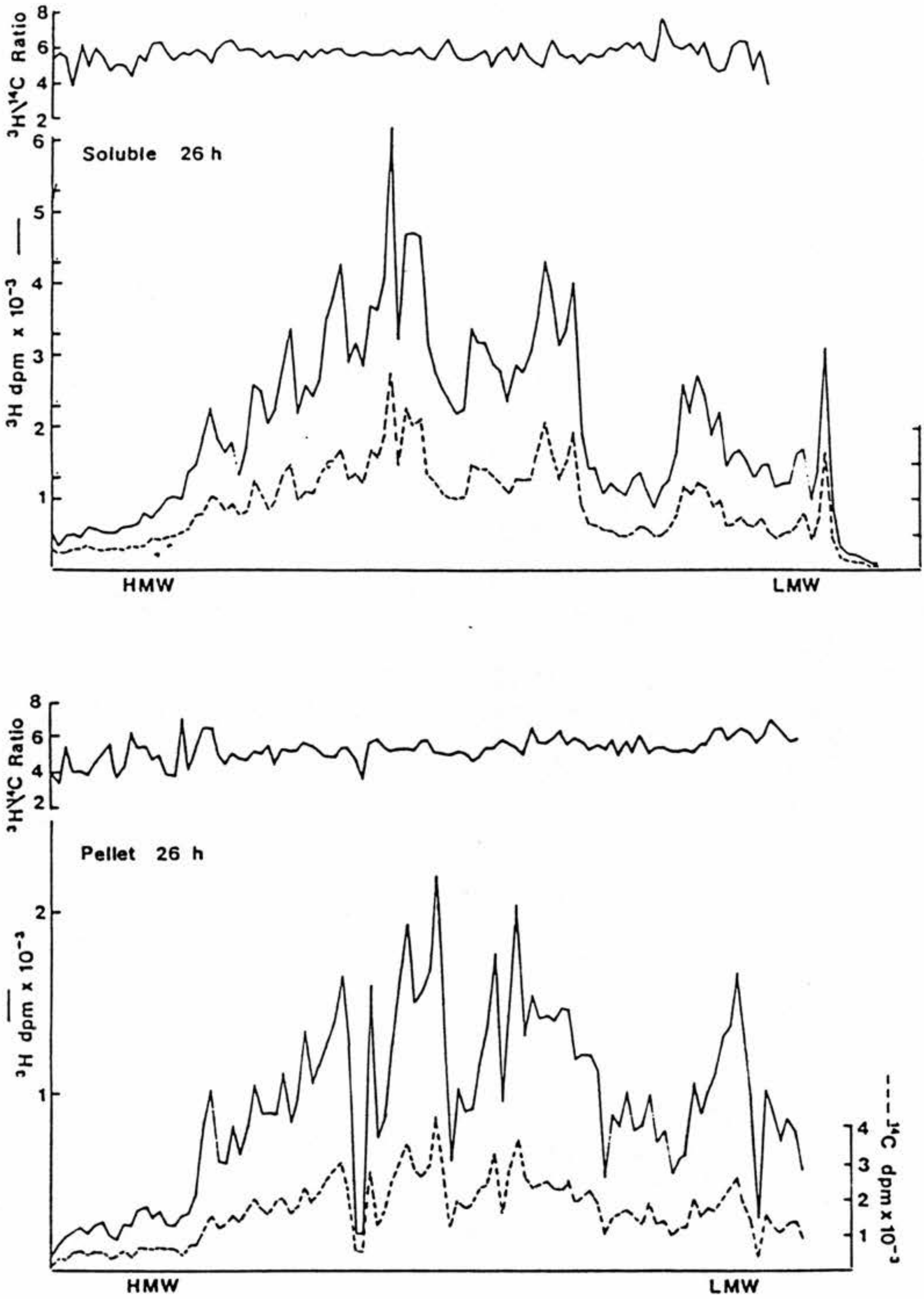

$^3H/^{14}C$ Ratio
8
6
4
2
Soluble 26 h
^3H dpm x 10^{-3} —
6
5
4
3
2
1
--- ^{14}C dpm x 10^{-2}
8
6
4
2
HMW
LMW
$^3H/^{14}C$ Ratio
8
6
4
2
Pellet 26 h
^3H dpm x 10^{-3} —
2
1
--- ^{14}C dpm x 10^{-3}
4
3
2
1
HMW
LMW

FIGURE 6.2.F (c)

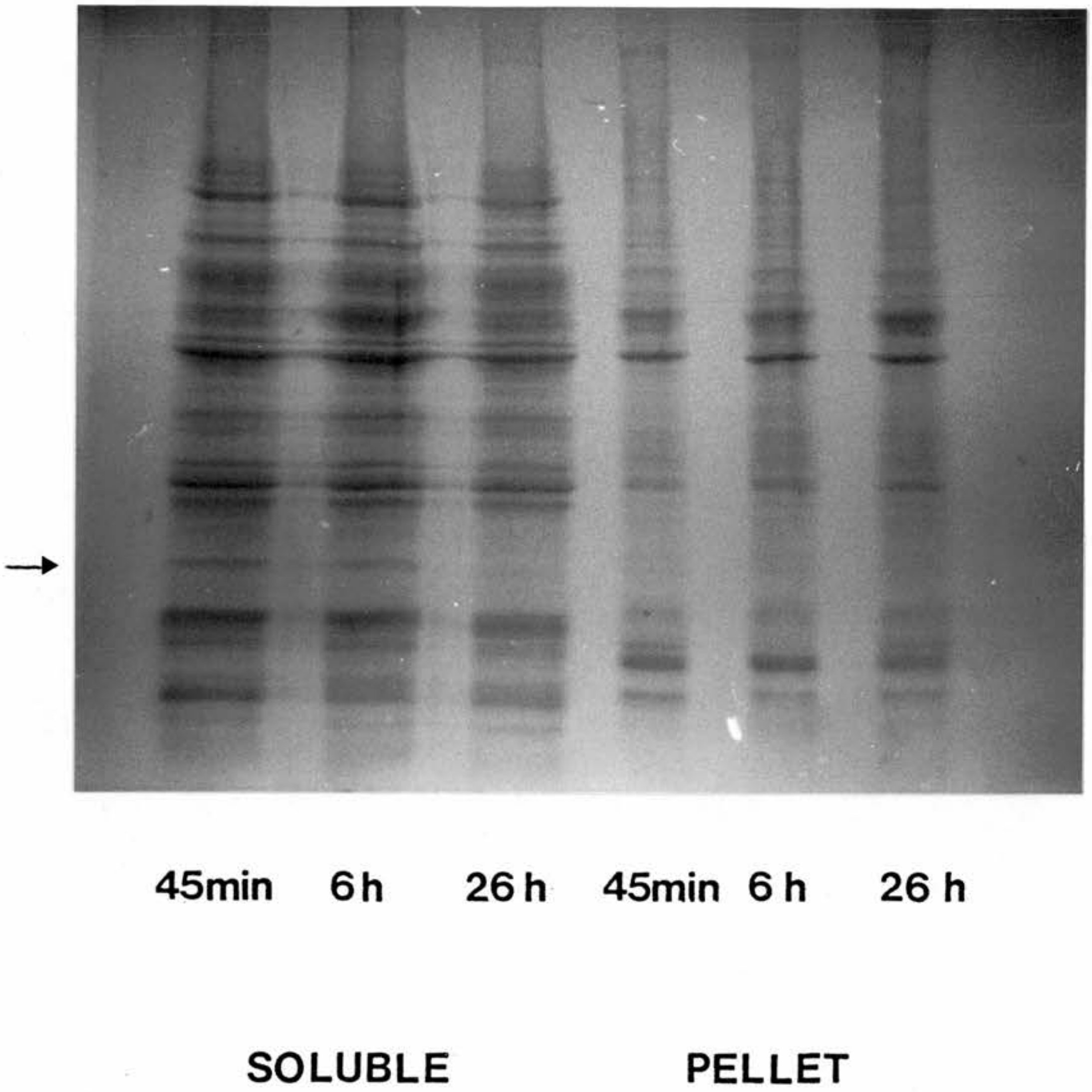


FIGURE 6.2.G: Double Label Experiments: SDS–PAGE Of Soluble And Pellet Proteins.

After the incubation *in vitro*, the H-POA from oil-treated and TP-treated rats were homogenised together, centrifuged at 120,000g for 1h at 4°C and proteins were extracted from the pellet. Approximately equal loads of protein (100μg) from the soluble and pellet fractions at 45 min, 6h and 26h were analysed by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue R250.

6.3 HORMONE EFFECTS ON PROTEIN SYNTHESIS IN NEURONS IN CULTURE

6.3.1 MATERIALS AND METHODS

Primary Culture of Foetal Rat Hypothalamic Neurons, Subcellular Fractionation and PAGE.

Primary cultures of foetal rat hypothalamic neurons were prepared (section 2.2.14) from male and female embryos at day 18 of gestation (day of conception = day 1) when the final cell divisions in hypothalamic nuclei have taken place (Ifft, 1972) and when foetal rat plasma testosterone concentrations in both sexes are at a peak (Weisz and Ward, 1980; Gogan, Slama, Bizzini-Koutznetzova, Dray and Kordon, 1981). Antibiotics were never used. The cultures were examined daily under an inverted microscope and were used for experimentation one week after their preparation at a time when a network of fine neuronal processes was established.

Hormones were prepared and added to the cells as described in section 6.2.1. Both hormone and ^{35}S-methionine were added to the cells at the time at which the medium was changed and the cultures returned to the incubator for a further 24 or 48h. ^{35}S-methionine was added to the medium at 15-25μCi/ml and two culture dishes were used per hormone treatment.

At the end of the incubation period, cells were scraped from the dish, collected by centrifugation at 1,000g for 5 min and homogenised in either 5-600μl sucrose-TKM buffer or lysis buffer (9.5 M urea, 2% (w/v) NP 40, 2% Ampholines, 5% (v/v) β-mercaptoethanol).

One and two-dimensional PAGE was carried out as described in sections 2.2.6 and 2.2.8 and proteins were detected either by staining (section 2.2.9 (a)) or fluorography (section 2.2.9 (b)).

6.3.1.2 Aromatisation of Testosterone by Primary Cultures

Seven day old cultures of foetal rat hypothalamic cells were tested for their capacity to aromatise testosterone.

20μCi [7-^{3}H(N)]-Testosterone (New England Nuclear, Boston) were added to each culture dish which was then incubated for 2h at 37°C. Cells were then scraped from the dishes and collected by centrifugation at 1000 g for 5 min. The pellet was resuspended in 0.8-1.0 ml plating medium and homogenised. Steroids were extracted from 0.7-0.9 ml homogenate with 3.5-4.5 ml chloroform/methanol (2:1 v/v) and the protein content determined in the remaining 0.1 ml homogenate as described in Appendix 1. The chloroform/methanol layer was removed, blown dry under nitrogen and the extract resuspended in 10-100μl ethanol. 10μl aliquots were then spotted onto a thin layer chromatography plate (E. Merck, Darmschadt) together with marker steroids and the plate run for 1-2h in hexane/ethyl acetate (60:40 v/v). Spots were visualised under ultra violet light at 245nm, scraped off the plate and counted as described in section 2.2.5.

6.3.2 RESULTS

Approximately 1.5 x 10^6 cells per H-POA were obtained on average from 18 day foetal rats. The dispersed cells began to attach to the culture dish and cell processes developed within the first 2 days after plating. Non-neuronal cells formed a thin layer upon which the neurons grew. The neurons sent out long processes making contact with other cells in the dish. Cells with single, two or multiple processes were all seen (Fig 6.3.A).

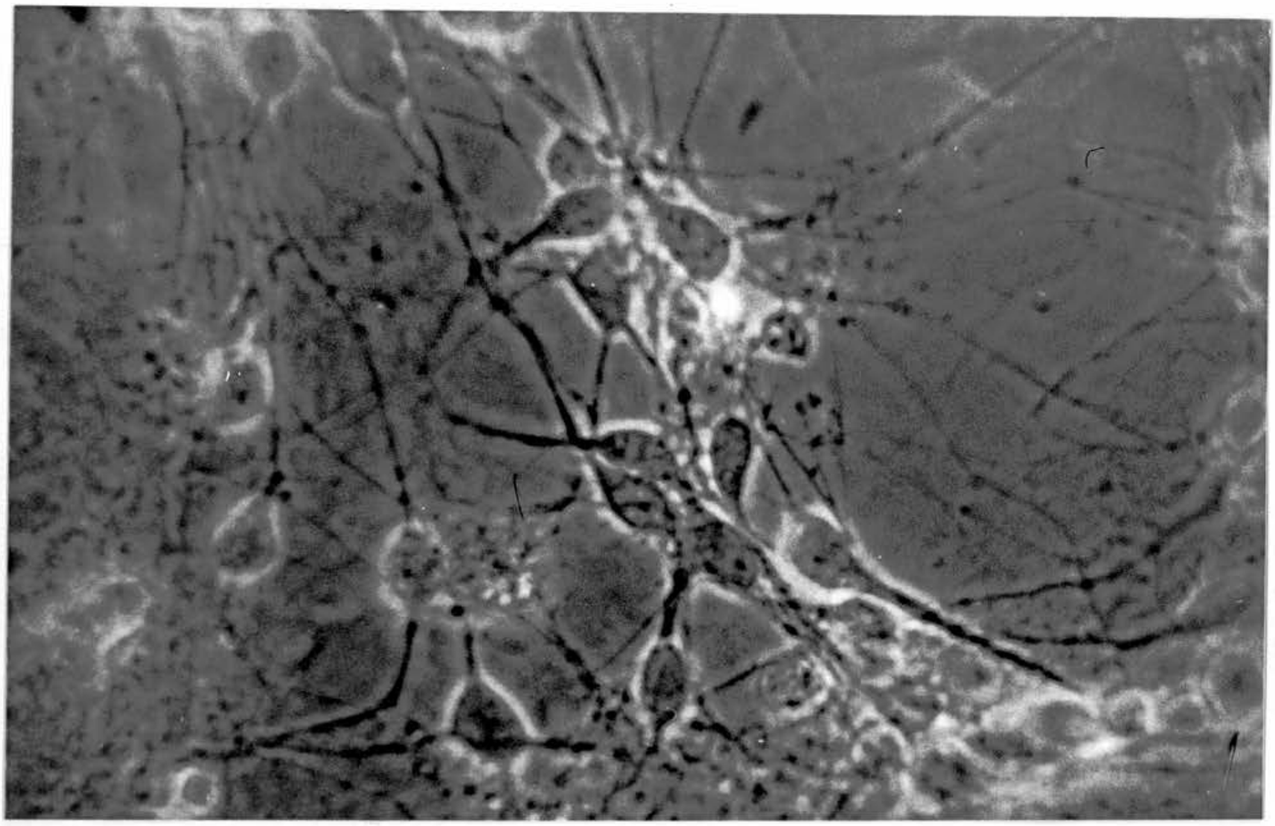


FIGURE 6.3.A: A Primary Culture Of Foetal Rat Hypothalamic Neurons.

Cells in culture were photographed 7 days after being prepared. (oil immersion, 40 x magnification)

6.3.2.1 Conversion of Testosterone to Oestrogen Metabolites in Primary Cultures

Table 6.3.1 shows that the primary cultures of foetal rat hypothalamus were capable of converting testosterone to both E_2 and oestrone (E_1). Of the total steroids recovered from the plate, 5-6% were in the form of oestrogen metabolites of testosterone. The conversion could be partially blocked by 1μM 1,4,6-androstatriene-3, 17-dione (ATD) an inhibitor of the aromatase system.

6.3.2.2 Changes in Synthesis of Specific Proteins in Cell Cultures as a Result of Exposure To Hormones

Both steroid and thyroid hormones affected the synthesis of certain proteins in 7 day old primary cultures of foetal rat hypothalamic neurons. The presence of testosterone in the culture medium for 24h induced the synthesis of a 93,000 molecular weight acidic protein (Figs 6.3.B (a) and 6.3.B (b).). The presence of either T_3 or T_4 in the culture medium for 24h reduced the synthesis of a 45,200 dalton protein (Fig. 6.3.C (a)) while T_4 but not T_3 increased the synthesis of a 60,000 molecular weight species by 100% (Fig.6.3.C (b)).

Figs 6.3.D (a) and 6.3.D (b) show that the presence in the culture medium for 48h of testosterone and T_3 had marked effects on the synthesis of a number of proteins. Silver staining a duplicate gel gave similar results (Fig 6.3.D (c)). The major changes which occurred in protein synthesis were as follows: testosterone and T_3 increased synthesis of a 14,800 dalton protein by approximately 30% and reduced synthesis of proteins with apparent

Table 6.3.1

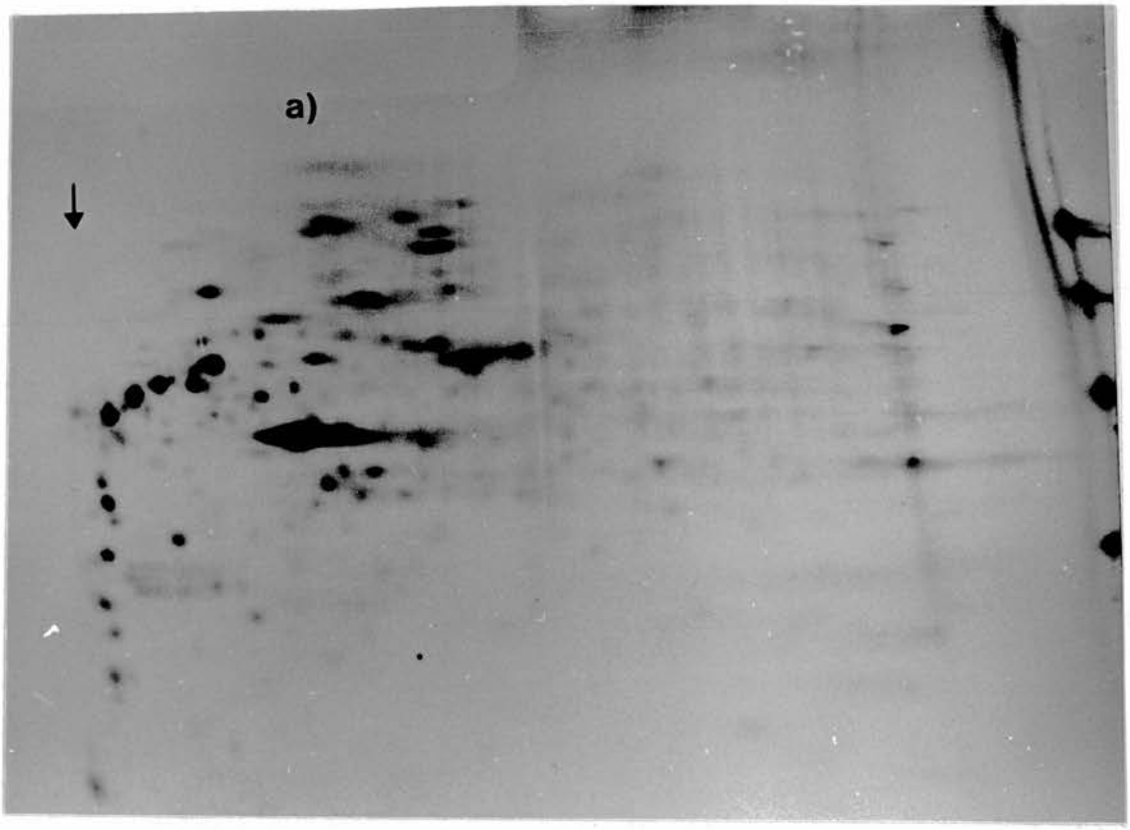
Conversion Of Testosterone To Oestrogen Metabolites In Primary Cultures of 18 Day Foetal Rat Hypothalamic Neurons

All data refers to steroid recovered from the TLC plate. The experiment was carried out as described in section 6.3.1.2

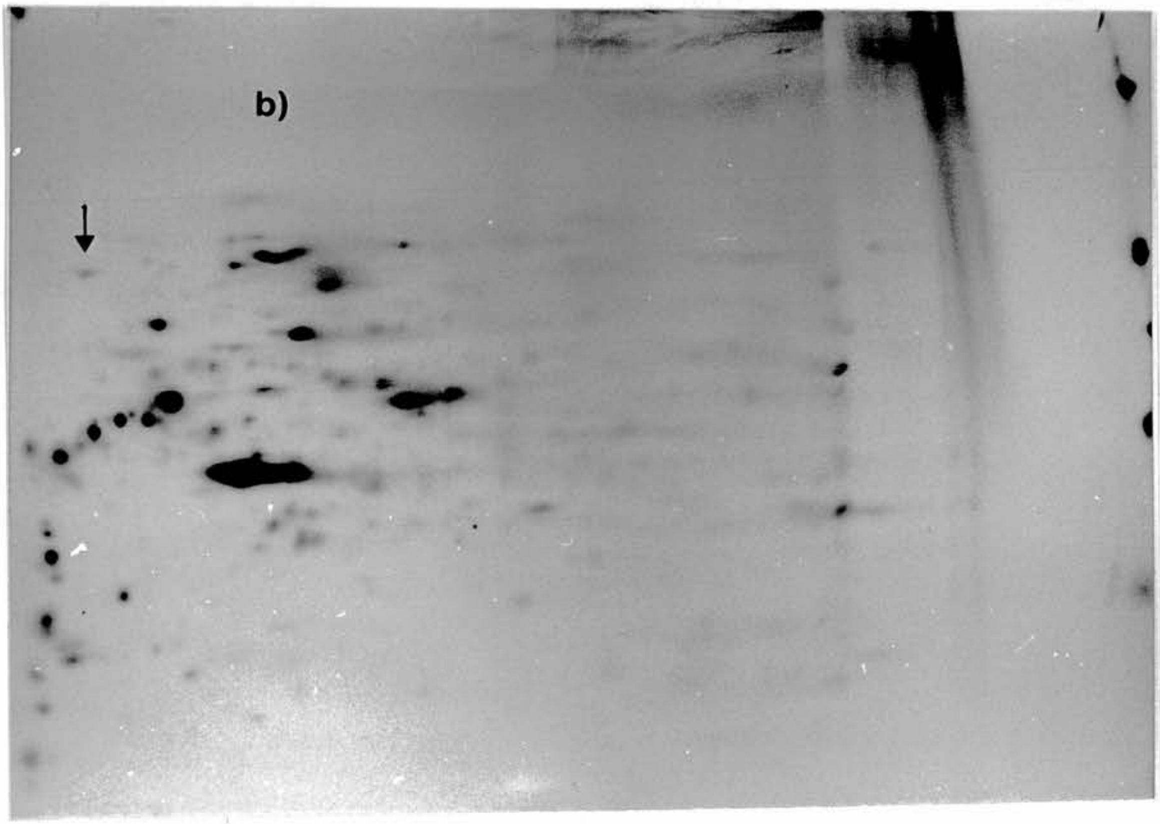
	- ATD, n = 3		+ ATD, n = 1	
Steroid	Steroid Recovered fmol/mg/h ± S.E.M.	% Conversion Of Testosterone	Steroid Recovered fmol/mg/h	% Conversion Of Testosterone
Testosterone	1024.4 ± 90.3	-	1104.2	-
E_2	58.7 ± 6.1	5.3 ± 0.3	30.9	2.8
E_1	11.9 ± 2.0	1.1 ± 0.2	6.4	0.5

FIGURE 6.3.B: Two-Dimensional Gel Analysis Of Proteins Synthesised In Foetal Rat Hypothalamic Neurons In Culture, Labelled With ^{35}S-methionine.

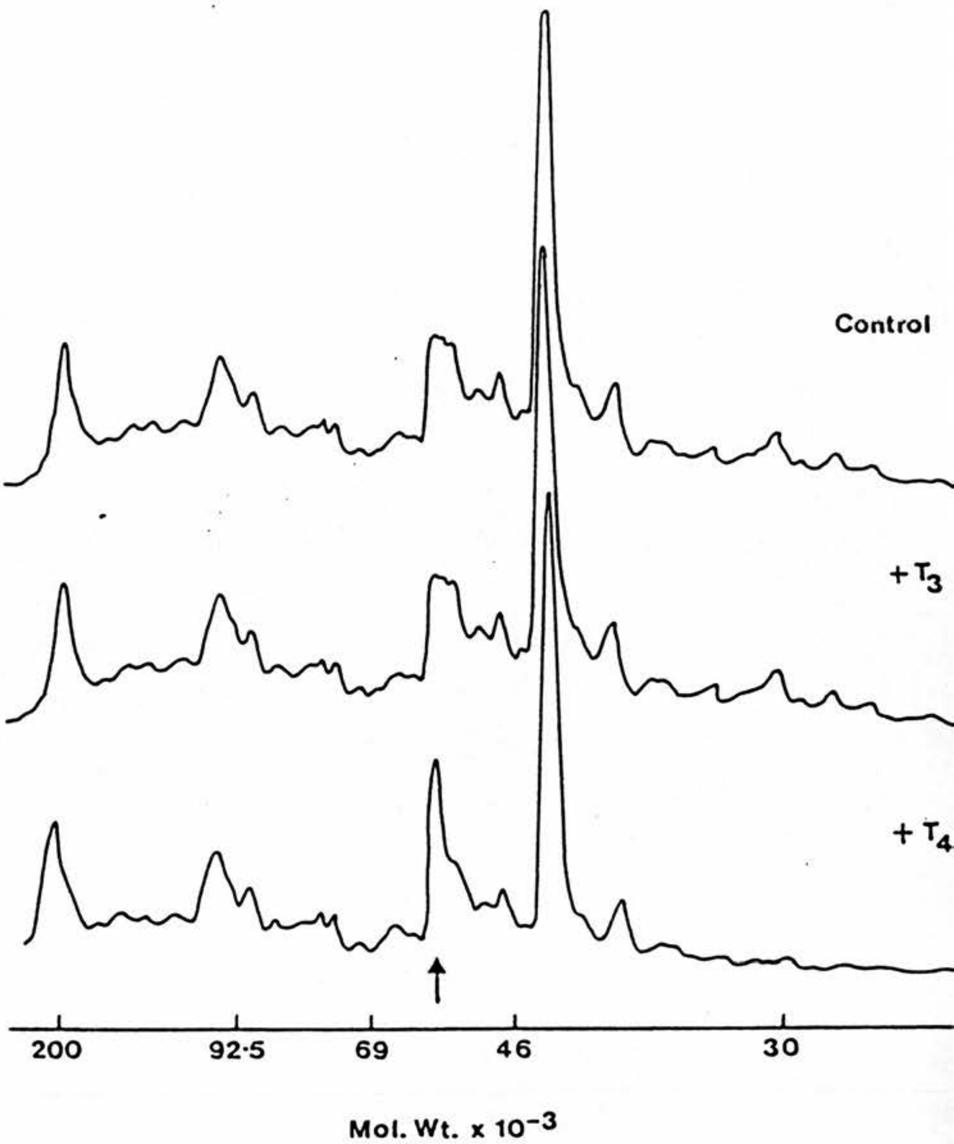
Primary cultures (7 days old) were incubated in the presence of either a) BSA/saline or b) 1μM testosterone and ^{35}S-methionine (20μCi/ml) for 24h. Cells were scraped from the dish, homogenised in lysis buffer and the incorporation of radioactivity into acid insoluble material was estimated. Two-dimensional gels were loaded with 80,000 cpm and after electrophoresis gels were fluorographed, dried and exposed to film for 10 days at -70°C.



a)



b)



b) Densitometric scans of proteins from the cells in culture.

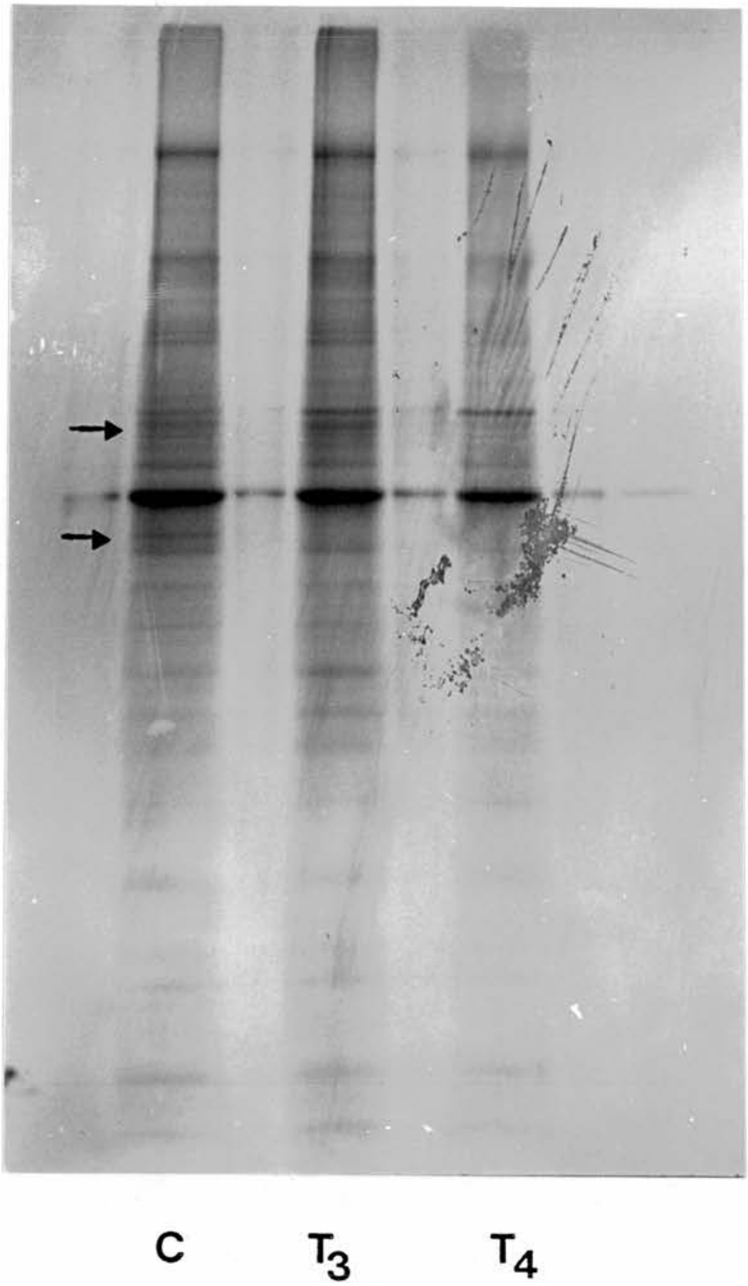
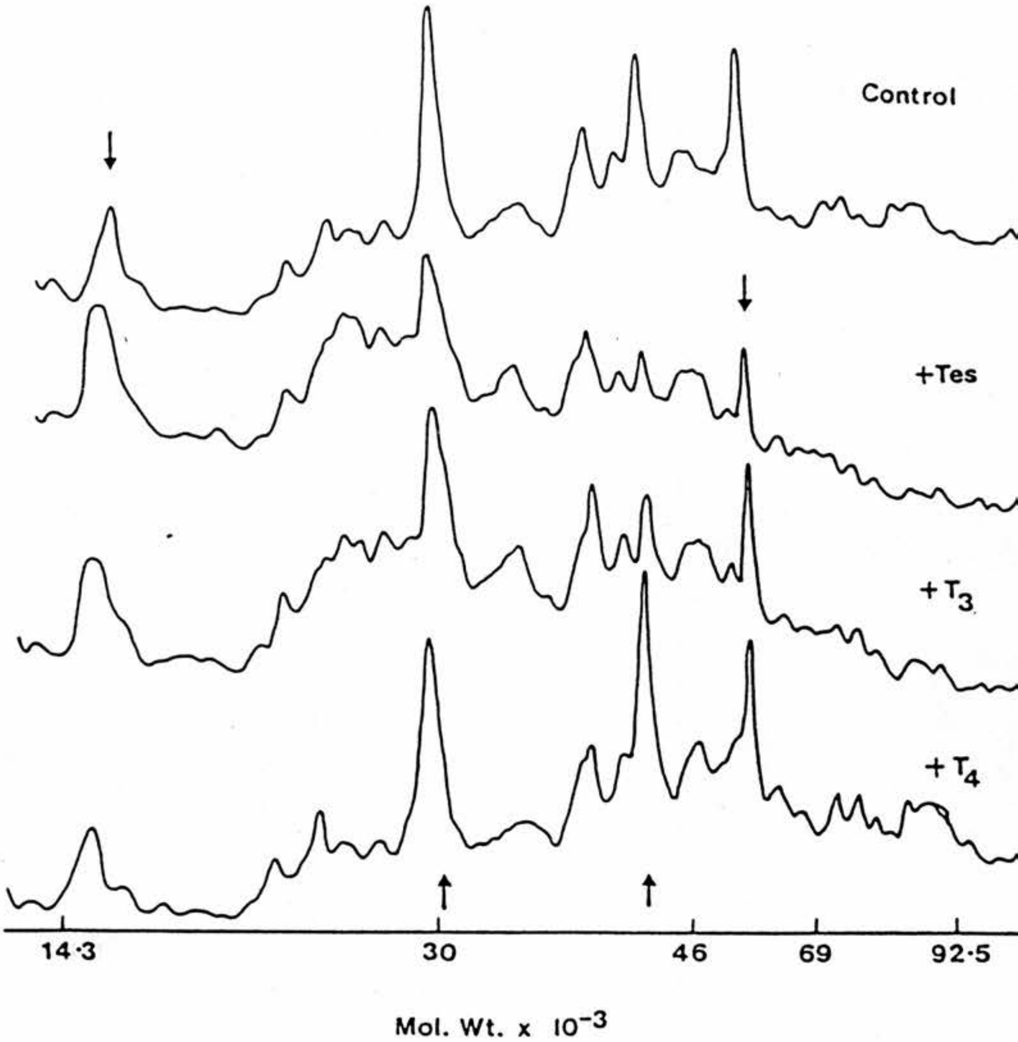


FIGURE 6.3.C: Effects Of Exposure To T_3 Or T_4 For 24h On Protein Synthesis In Primary Cultures Of Foetal Rat Hypothalamic Neurons.

a) Seven day old cultures were incubated in the presence of BSA/saline (C), 0.1μM T_3 or 0.1μM T_4 and ^{35}S-methionine (10μCi/ml) for 24h. Cells were scraped from the dish, homogenised in sucrose-TKM buffer and the incorporation of radioactivity into acid insoluble material was estimated. Samples containing 15,000 cpm were then analysed by SDS-PAGE. The gel was fluorographed, dried and exposed to film for 7 days at -70°C.



b) Densitometric scans of proteins from the cells in culture

c) see over

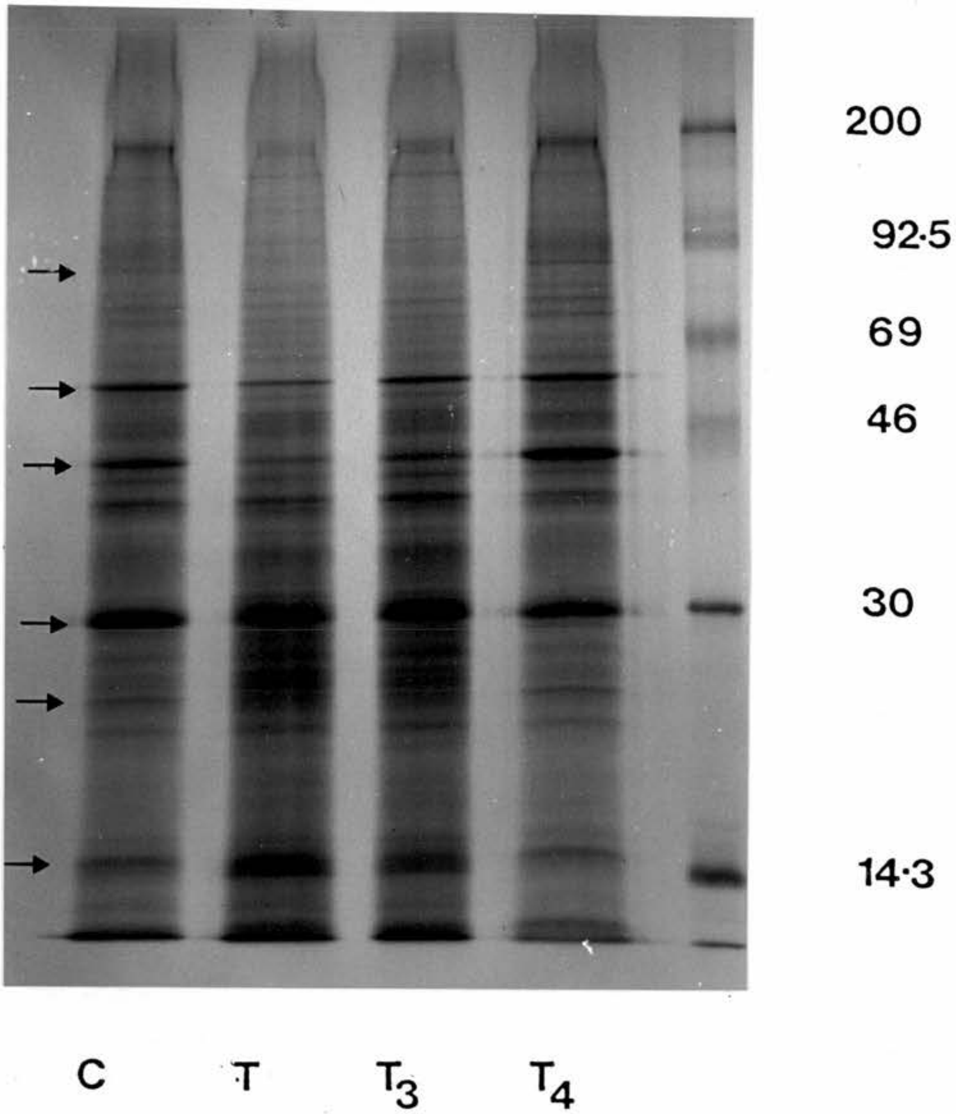


FIGURE 6.3.D: Effects Of Exposure To T_3, T_4 Or Testosterone For 48h On Protein Synthesis In Primary Cultures Of Foetal Rat Hypothalamic Neurons.

a) Seven day old cultures were incubated in the presence of BSA/saline (C) , 1μM testosterone (T), 0.1μM T_3 or 0.1μM T_4 and ^{35}S-methionine (20μCi/ml) for 48h. Cells were homogenised in sucrose-TKM buffer and the incorporation of ^{35}S-methionine into acid insoluble material was estimated. Samples containing 20,000 cpm were analysed by SDS-PAGE and the gel was fluorographed, dried and exposed to film for 5 days at -70°C.

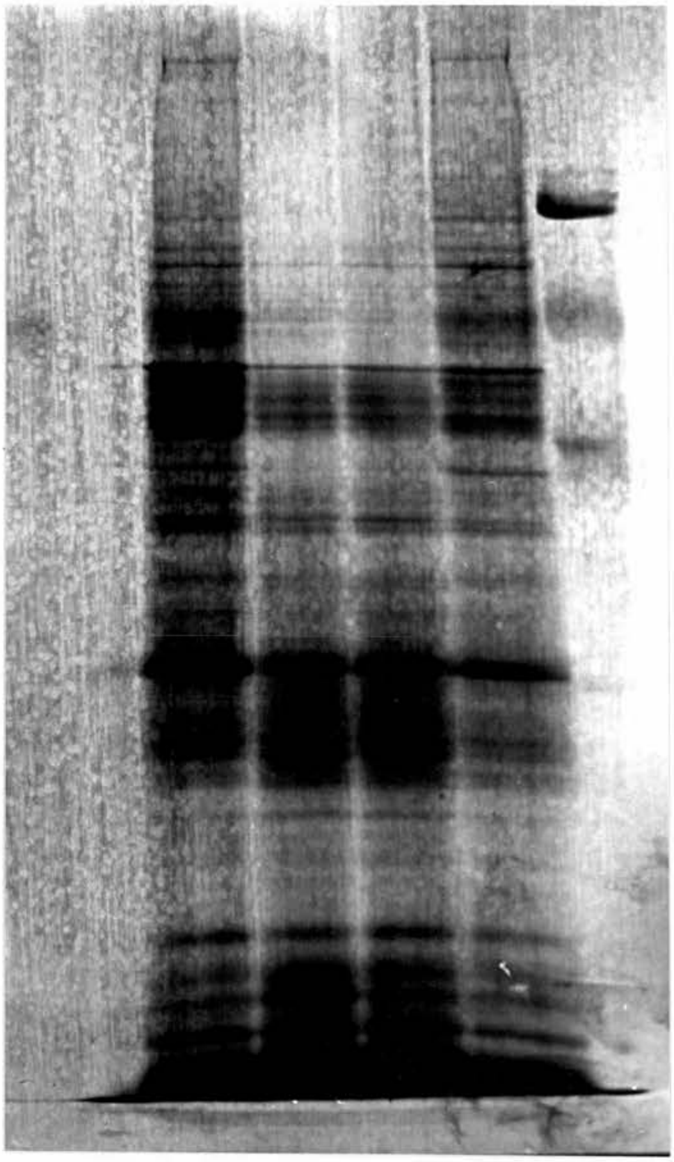


FIGURE 6.3.D c):

Approximately 20μg protein from each sample were analysed by SDS-PAGE and the gel was stained with silver using the Bio-Rad staining kit (see section 2.2.9).

molecular weights of a 90,000, 42,700 (by 60 and 30%respectively), 30,900 (by 60 and 50% respectively) and 26,400. Testosterone reduced the synthesis of a 56,900 molecular weight protein by approximately 50% .

Due to the low density of some protein bands (e.g. 90,000 and 26,400 in Fig. 6.3.D (a) and the 45,200 band in Fig.6.3.C (a)) some changes in proteins were seen by eye on the autoradiogram but not on the scans.

Oestradiol-17β had no obvious effect on protein synthesis after 24h in culture as shown by two-dimensional gel analysis (data not shown).

6.4 DISCUSSION

Steroid and thyroid hormones, in physiological concentrations, directly stimulated and inhibited the synthesis of specific proteins in both the isolated H-POA (Figs 6.2.B and 6.2.C) and in primary cultures of 18 day foetal rat hypothalamic neurons (Figs 6.3.B, 6.3.C and 6.3.D). Although the techniques used in these studies to investigate hormone effects upon protein synthesis *in vitro* were designed to investigate the same questions (section 6.1), the results from the H-POA slice experiments and from the cell culture work cannot be directly compared. This is largely due to the fact that the cultures were prepared from both male and female H-POA and therefore the results represent a combination of hormone effects on protein synthesis within cells obtained from the H-POA from both sexes. However, the data from the experiments described in sections 6.2 and 6.3 show that significant changes in the synthesis of specific proteins within H-POA cells were detected after 2h, 24h and 48h exposure to steroid and thyroid hormones. After incubating 4 day male and female H-POA slices with hormone for 2h in vitro the synthesis of two proteins, one in the soluble fraction (57,500 daltons) and one in the pellet (58,900 daltons), were altered (Figs. 6.2.F and 6.2.G). Exposure of the cells in culture to testosterone, T_3 or T_4 for 24h resulted in changes in the synthesis of 3 different proteins (45,200, 60,000 and 93,000 daltons, Figs. 6.3.B and 6.3.C) while after incubation of the cells with hormone for 48h the synthesis of at least 6 different proteins (14,800, 26,400, 30,900, 42,700, 56,900 and 90,000 daltons) was changed compared to those in control cultures (Fig. 6.3.D). These results show that increasing the duration of exposure of the tissue or cells to either testosterone or T_3 or T_4 seemed to result in changes in an increasing number of proteins, perhaps involving a cascade of events within the cells. Although the apparent molecular weight values of those proteins which were influenced by hormone administration in vitro did not correspond to any of the key structural or metabolic proteins, or subunits of proteins, listed in the literature, the changes found could still be related to the effects of hormones on the enzymes that synthesise non-peptide neurotransmitters, and the polypeptide precursor molecules for peptide neurotransmitters (or neurohormones) within the H-POA. Thus, for example, low levels of immunoreactive LHRH have been detected in foetal hypothalamus (Chiappa and Fink, 1977) and brain (Araki et al., 1975) at 17 days gestation. Studies on mouse brain explants (Toran-Allerand, 1978) have shown that endogenous steroids in the culture medium are important both for the survival of LHRH cells and the regulation of LHRH synthesis within these cells. It is therefore possible that the hormone-induced changes in protein synthesis described here could be linked to regulatory effects of the hormones upon the biochemical or electrical properties of LHRH cells. However, the identity of these proteins is still not known.

Although a recurring criticism of in vitro techniques in general is the extent to which observations can be related to the situtation in vivo, the data obtained in these studies suggest that changes in the synthesis of specific proteins in the H-POA from foetal and neonatal rats can be induced by hormone administration in vitro. In addition, H-POA neurons in culture still retain many of the physiological (Benda, De Vitry, Picart and Tixier-Vidal, 1975; Vaccaro, Messer, Lam and Leeman, 1976(a)) and biochemical (Vaccaro, Messer and Leeman, 1976(b); Canick, Vaccaro, Ryan and Leeman, 1977) characteristics of intact neuronal networks and in the present study the cells were capable of converting testosterone to oestrogen metabolites (Table 6.3.1). However, the results presented in Fig 6.2.D do not show the inhibition of synthesis of two low molecular weight proteins by TP, found by in vivo work in Chapter 4. Although the general patterns of ^{35}S-methione-labelled proteins obtained by in vivo and in vitro labelling are highly comparable, the reason for the lack of effect of TP administration in vivo on synthesis of the low molecular weight proteins in vitro is unclear.

The double label experiments showed that the concentration of a soluble 27,000 molecular weight protein was reduced at 26h after TP administration (Fig 6.2.G). This was a late response to the hormone as no inhibition was detected at 45 min or 6h after TP administration. However, this finding is complicated by the fact that the electrophoretic protein pattern represents proteins isolated from both oil and TP-treated female H-POA. Since no major change in the $^3H/^{14}C$ ratio was detected at 26h after TP administration.(Fig 6.2.B (c)) this reduction in the 27,000 molecular weight protein can possibly only be explained as an age-related change in protein synthesis.

Uterine IP, the major component of which is creatine kinase ββ (Reiss and Kaye, 1981), can be induced in vivo (Notides and Gorski, 1966) or in vitro (Katzenellenbogen and Gorski, 1972) and is detectable 40 min after oestrogen administration (Barnea and Gorski, 1970). IP has since been detected in rat brain and pituitary in higher concentrations than in the uterus (Walker et al., 1979; Kaye and Reiss, 1980) and found to increase in brain during the first 4 weeks after birth (Kaye et al., 1980). However, whereas induction of IP by oestrogen in rat uterus has been repeatedly demonstrated, from as early as 5 days (Walker, Gozes, Kaye, Reiss and Littauer, 1976), the question of IP inducibility in brain remains controversial. The data presented in Figs. 6.2.F. (a), (b), (c); 6.2.D.; and 6.2.B. provide no evidence for the induction of IP in 4 day female rat H-POA. Similar negative results were reported for 7 day H-POA (Vērtes, Kőrnyei, Nagy, Vērtes and Kovācs, 1981) and 25 day hypothalamus and pituitary (Walker et al., 1979), although Thomas and Knight (1978) have reported the induction of a protein in 3 day hypothalamus following exposure to testosterone for 3h in vivo. Differences in the age of rats or duration of exposure to testosterone could account for the differences in results. The elevated $^{3}H/^{14}C$ ratio seen in both the soluble and pellet fractions at 6h after TP administration (Fig 6.2.F (b)) indicates that TP had a general inhibitory effect on protein synthesis in the H-POA 6h after administration. However, by 26h, the inhibitory effect of TP was no longer apparent. Further experimentation is required, however, to investigate the effects of gonadal steroids on IP synthesis in brain, particularly in the neonate where the capacity of IP, to provide energy stores for functions such as macromolecular synthesis and axonal transport, may be important in the growth of neurons still undergoing the final stages of differentiation and synapse formation.

The relative refractory nature of brain to induction of IP may be one example of differential activation of target tissues by steroid hormones. The uterus, pituitary and hypothalamus of the adult rat vary in their responsiveness to oestrogen. The peripheral target tissues are more sensitive, with nuclear translocation of oestrogen receptors occurring at lower concentrations of oestrogen than those present in the hypothalamus (Kelner, Kirchick and Peck, 1982). Tissue differences in oestrogen receptor affinities are not responsible for this effect (Peck and Clark, 1979; Kelner, Miller and Peck, 1980). Goodman (1978) has demonstrated that whereas oestrogen concentrations in pituitary and uterus are comparable, the hypothalamic concentration of oestrogen is only 1/15th that found in the peripheral target tissues. However, limited availability of oestrogen, particularly in the neonatal rat, seems an unlikely explanation for the lack of IP induction since intracellular aromatisation of testosterone is occurring at this time and at approximately twice the rate as that found in the adult (Reddy et al., 1974). In addition, although the affinities of the 'classical' oestrogen receptor are identical in uterus and brain, a type II receptor has been described in the uterus (Clark et al., 1978). Type II receptors seem to be found only in tissues which respond to oestrogen by cell growth and, perhaps more importantly, cell division. IP induction is thought to be closely linked to these responses. Type II receptors have not yet been identified in rat brain (Kelner, Panko and Peck, 1980), a tissue which does not respond to oestrogen by hyperplasia and in which IP induction is still not firmly established. The lack of type II receptors in brain may therefore be relevant to the difference in responses to oestrogen of brain and uterus.

Eighteen day foetal rat H-POA cultures are reported to synthesise γ amino- butyric amino acid (GABA) and acetylcholine (ACh) (Vaccaro et al., 1976(a)) and secrete LHRH, adrenocorticotrophic hormone, vasopressin and β-endorphin (Denizeau, Dubē, Antakly, Lemay, Parent, Pelletier and Labrie, 1981). Neurons containing thyrotropin-releasing hormone have also been immunochemically identified in primary cultures of mouse hypothalamus (Faivrē-Bauman, Nemesker, Tougard and Vidal, 1980). Whether any of the hormone-induced changes in protein synthesis found in the present studies are related to changes in the synthesis and/or release of these peptide and non-peptide transmitters can only be speculative. T_3 and T_4 affected the synthesis of a number of proteins both in H-POA slices and in foetal rat H-POA cultures and the effects of one hormone did not always parallel those of the other (see Fig 6.3.C (a) and 6.3.D (a) and (b)). The actions of thyroid hormone are believed to be mediated largely through T_3 which binds to a nuclear receptor (Tata, Ernstero, Lindberg, Arrhenius and Pederson, 1963; Tata and Widnell, 1966; Oppenheimer, Koerner, Schwartz and Surks, 1972(a)) and alters gene transcription in both a generalised (Tata and Widnell, 1966; Dillmann, Mendecki, Koerner, Schwartz and Oppenheimer, 1978;) and more specific manner (Kurtz, Sippel and Feigelson, 1976; Seo, Vassart, Bocas and Refetoff, 1977; Martial, Baxter, Goodman and Seeburg, 1977). The nuclear receptor shows a higher affinity for T_3 than T_4 (Oppenheimer, Schwartz and Surks 1972(b)) and only 15% of thyroid hormone bound in cells is T_4, the latter deriving most of its biological potency from peripheral conversion to T_3 (Schwartz, Surks and Oppenheimer, 1971; Oppenheimer et al., 1972(b)). Neonatal and adult brain do not respond to thyroid hormone administration in the 'classical' way either by increasing oxygen consumption (Schwartz and Oppenheimer, 1978) or stimulating specific dehydrogenase enzymes (Hemon, 1968; Lee and Lardy, 1965) although the concentration of T_3 receptors in the brain at birth is comparable to that in liver (Oppenheimer, Schwartz and Surks, 1974; Schwartz and Oppemheimer, 1978). High concentrations of T_3 receptor are therefore present during the period when the plasma T_3 concentration rises rapidly (Schwartz and Oppenheimer, 1978), hypothalamic neurons are still undergoing differentiation and when the well established T_3-induced changes in cerebellum (Hamburgh, Mendoza, Burkart and Weil, 1971) and cortex (Eayrs, 1953) begin. The early work of Eayrs (1953) on thyroid hormone deficiency highlighted the effects of thyroid hormone on neurogenesis, nerve fibre outgrowth and dendritic arborization in brain. However, the biochemical basis for this hormonal influence is still poorly understood and although thyroid hormone receptors have been demonstrated within hypothalamus (Stumpf and Sar, 1975) the role of thyroid hormones, if any, in hypothalamic maturation is even less clear.

The contribution made by glial cells to the pattern of protein synthesis by cells in culture is not known. However, the addition of Ara-C to the cultures largely prevents growth of non-neuronal cells which form a thin layer underneath the growing neurons. In mature brain, neurons and glia can be distinguished by their isoenzyme content of the glycolytic enzyme enolase: neuron specific enolase (NSE) and non-neuronal enolase (NNE). Brain NSE levels are low at birth and an increase in levels correlate with neuronal differentiation when an increase in the NSE/NNE ratio occurs (Marangos, Schmechel, Parma and Goodwin, 1980). NSE has been identified in 17 day foetal rat brain cultures (Secchi, Lecaque, Cousin, Lando, Legault-Demare and Raynaud, 1980), localized in cells with the morphological characteristics of neurons. In view of the fact that NNE and NSE levels can not only provide information on the glia/neuron ratio but may also indicate the extent of neuronal differentiation, immunohistochemical studies in these cultures would be extremely valuable, particularly with regard to the effects of steroid and thyroid hormones on cell growth and maturation.

CHAPTER 7

Neurotransmitters In The Hypothalamus-Preoptic Area And Midbrain Of Male And Female Rats Throughout Development

7.1 INTRODUCTION

Neurotransmitter systems in brain are not fully developed at birth and are therefore susceptible to the differentiating effects of gonadal steroids during the 'critical period'. Although the effects of neurotransmitter interactions with steroids in the control of ovulation (Weiner and Ganong, 1978; Fink et al., 1983) and sexual behaviour have been extensively studied in adult rats, the effects of gonadal steroids on indoleamine systems both during and after the process of sexual differentiation are less well understood.

Serotonergic mechanisms are implicated in two sexually differentiated brain functions; the control of LH release (Héry, Laplante and Kordon, 1978; Coen and MacKinnon, 1979) and feminine sexual behaviour (Meyerson, 1964; Meyerson, Correr and Eliasson, 1974; Everitt, Fuxe, Hökfelt and Johnsson, 1975; Ward, Crowley, Zemlan and Margules, 1975; Foreman and Moss, 1979). A daily surge of LH release occurs in adult female rats which have been gonadectomised and treated with oestradiol benzoate (Caligaris, Astrada and Taleisnik, 1971; Legan, Coon and Karsch, 1975; Henderson, Baker and Fink, 1977) but not in similarly treated male rats or in androgenised female rats (Henderson et al., 1977; Gogan, Beattie, Héry, Laplante and Kordon, 1980). Control of the daily neural signal for LH release is believed to involve 5-HT neurons (Héry, Laplante and Kordon, 1976, Héry et al., 1978; Coen and MacKinnon, 1979; Héry, Faudon, Dusticier and Héry, 1982) which originate in the midbrain raphe nuclei and project to the H-POA (Dahlström and Fuxe, 1964; Ungerstedt, 1971; Parent, Descarries and

Beaudet, 1981; Steinbusch, 1981; Consolazione and Cuello, 1982) where they may interact with LHRH neurons (Coen and Mackinnon, 1980; Kawakami, Arita and Yoshioka, 1980; Héry et al., 1982; Wiegand and Terasawa, 1982). Sexual behaviour in the male and female rat appears to be controlled by different neuronal pathways (Caggiula, Antelman and Zigmond, 1973; Modianos, Flexman and Hitt, 1973) and 5-HT may not be involved in the control of male sexual behaviour (Bradshaw, Erskine and Baum, 1982).

Gonadal steroid hormones affect the activity of non-peptidergic neurotransmitter systems in a number of ways. For example, they regulate the activity of enzymes involved in neurotransmitter synthesis (Beattie et al., 1972; Kizer et al., 1974; Luine et al., 1977; Kizer et al., 1978; Luine et al., 1980) and degradation (Luine and McEwen, 1977; Luine and Rhodes, 1983) and they can modify neurotransmission by increasing or decreasing receptor numbers (Biegon et al., 1980; Hruska et al., 1980; Rainbow et al., 1980; Biegon and McEwen, 1982). Some of the effects of oestrogen on neurotransmission are known to involve a direct action on the genome (Kizer et al., 1978; Luine et al., 1980). Therefore, to investigate the possibility that exposure of the neonatal rat brain to testosterone during the 'critical period' may influence the expression of a particular neurotransmitter phenotype, the concentrations of 5-HT, and 5-HIAA were measured in the H-POA and MR of male and female rats throughout development using LCED (see 2.2.15). In addition, NSD 1015 was used to block aromatic amino acid decarboxylase and the accumulation rate of 5-hydroxytryptophan (5-HTP) was measured in the H-POA of 4 and 80 day old rats of both sexes. The accumulation rate of 5-HTP is believed to be an index of the rate of synthesis of 5-HT (Carlsson, 1974; Curzon, 1981) since 5-HTP is the immediate precursor of 5-HT and it is rapidly converted to 5-HT.

Since LCED allows the simultaneous detection of DA and DOPAC, the concentrations of these compounds in the H-POA and MR of male and female rats throughout development were also measured.

7.2 MATERIALS AND METHODS

7.2.1 Animals

Male and female Wistar Cob rats from the departmental breeding colony or Charles River (Margate, Kent) were used at 36h and 4, 12, 20, 40 and 80 days of age. Vaginal opening in rats in the departmental breeding colony occurred on about day 37-38. Littermates were used whenever possible. Animals were killed by decapitation between 1000h and 1100h, the brains quickly dissected out and placed ventral surface upwards on a glass slide on ice. After removing the optic nerves and chiasm the H-POA was dissected out as described in section 2.2.2 and Fig. 2.A. In a separate series of experiments, the MR was removed from a 1mm (approximately) thick coronal brain section cut immediately caudal to the interpeduncular fossa (Figure 7.A). The section was trimmed using 2 cuts each 1mm either side of the midline and a third cut to remove the most ventral 1mm of tissue.

7.2.2 Analysis of Tissue Samples by LCED

As described in section 2.2.15, all tissue samples were weighed, homogenised in 80-300μl 200 mM $HClO_4$ in 0.1mM cysteine solution containing 1ng/20μl n-acetyldopamine (Sigma, Dorset) as internal

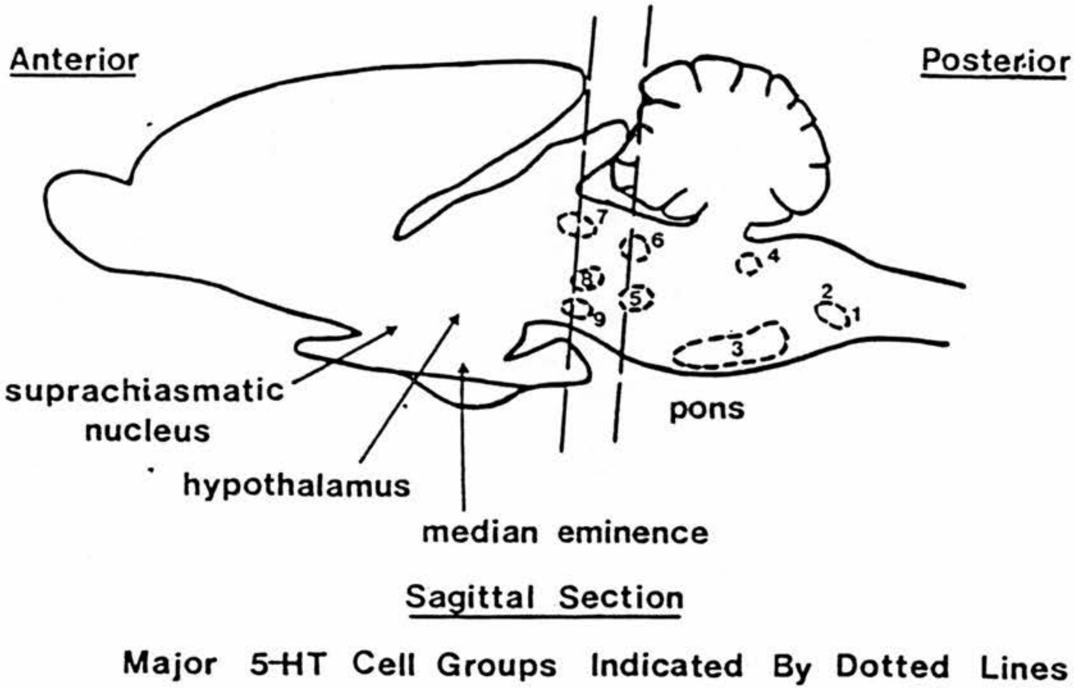


FIGURE 7.A: Diagramatic Representation Of The Section of Brain Removed In The First Step Of The Dissection Of The Midbrain Raphe Region. (MR)

Dotted areas represent approximate locations of the main raphe cell groups.

standard, and centrifuged for 30 min at 2,000g at 4°C. The supernatant was filtered (0.2μm filter) and 20μl of the tissue extract was then analysed by LCED as described in section 2.2.15.

Standard solutions of 5-HT, 5-HIAA, 5-HTP, DA and DOPAC were prepared (section 2.2.15) and standard curves were plotted for each experiment (Figure 2.G). Standard curves were linear over a range of 20pg to 2ng/20μl injection. The concentrations of compounds in samples were calculated by linear regression from a standard curve using the peak height ratio of the compound with respect to 1ng/20μl internal standard, added to all standards and samples.

7.2.3 Measurement of the Rate of Accumulation of 5-Hydroxytryptophan

The aromatic amino acid decarboxylase inhibitor NSD-1015 (m-hydroxybenzylhydrazine, Sigma, Dorset) was dissolved in 0.9% saline and injected (i.p.) at 100mg/kg (body weight) into male and female rats at 4 and 80 days of age.

The first experiment was designed to establish whether, as in the adult (Carlsson et al., 1972; Carlsson, 1974) the accumulation of 5-HTP was linear for 60 min after inhibition of aromatic amino acid decarboxylase in the neonate. The concentration of 5-HTP was measured by LCED in the 4 day H-POA at 10, 20, 30, 40 and 60 min after the injection of NSD-1015 (Fig.7.C).

In the second experiment, NSD-1015 was injected into 4 and 80 day male and female rats and 20 or 30 min later the animals were decapitated, the H-POA was dissected out and the 5-HTP concentration was determined by LCED. The accumulation rates of 5-HTP in 4 and 80 day H-POA were calculated from the mean of values obtained 20 and 30 min after the NSD-1015 injection.

7.2.4 Statistics

The significance of the difference between the mean values for males and females at any age was determined by the unpaired Students *t*-test. The significance of differences throughout development was determined by one way analysis of variance and the multiple range test of Duncan (1957) using the tables of Harter (1960). Where no significant sex difference occurred, data were pooled at each age apart from DA and DOPAC in the MR at 36h. The 5-HIAA/5-HT ratio in the H-POA was analysed separately for each sex.

7.3 RESULTS

7.3.1 Serotonin

No sex differences were found in the concentration of 5-HT in either the H-POA or MR at any of the ages studied. Table 7.1 shows that in the H-POA there was a small increase in the 5-HT concentration between 4 and 12 days ($P < 0.05$) and then a massive increase between days 12 and 20 ($P < 0.001$) to a value which was not significantly different from that in the adult . 5-HT concentrations fell significantly ($P < 0.001$) in the H-POA between days 20 and 40, but then increased significantly ($P < 0.001$) between days 40 and 80.

5-HT concentrations in the MR at 36h were approximately 5 times higher than in the H-POA at the same age. However, in contrast to the H-POA, 5-HT concentrations remained steady throughout development with only a small but significant increase ($P < 0.05$) occurring between days 20 and 40. 5-HT concentrations at 36h, 12 and 20 days were not significantly different from adult the concentrations in the adult.

Table 7.1

The mean (± S.E.M.) concentrations (ng/mg tissue wet weight) of serotonin (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) in the hypothalamic-preoptic area and midbrain raphe region of male and female rats at various ages

		Age (days)					
Hypothalamus-Preoptic Area		36h	4	12	20	40	80
5-HT	Female	0.25 ± 0.03	0.31 ± 0.02	0.40 ± 0.03	1.16 ± 0.03	0.81 ± 0.07	1.14 ± 0.08
	Male	0.26 ± 0.02	0.30 ± 0.02	0.39 ± 0.01	1.15 ± 0.02	0.77 ± 0.12	1.22 ± 0.02
5-HIAA	Female	0.28 ± 0.04	0.40 ± 0.05	0.32 ± 0.02	0.79 ± 0.02	0.47 ± 0.04	0.49 ± 0.03
	Male	0.25 ± 0.02	0.37 ± 0.07	0.31 ± 0.01	0.77 ± 0.04	0.49 ± 0.08	0.46 ± 0.01

n = 5 (except at 4 days where n = 10).

Midbrain Raphe							
5-HT	Female	1.27 ± 0.10	1.01 ± 0.09	1.40 ± 0.06	1.52 ± 0.12	1.61 ± 0.08	1.46 ± 0.06
	Male	1.18 ± 0.13	1.12 ± 0.09	1.22 ± 0.04	1.46 ± 0.14	1.97 ± 0.23	1.36 ± 0.17
5-HIAA	Female	1.68 ± 0.06	1.47 ± 0.10	1.26 ± 0.06	1.66 ± 0.12	1.15 ± 0.05	1.13 ± 0.04
	Male	1.59 ± 0.10	1.63 ± 0.12	1.02 ± 0.07	1.55 ± 0.16	1.16 ± 0.06	0.97 ± 0.11

n = 5 (except 36h where n = 4).

7.3.2 5-Hydroxyindoleacetic acid

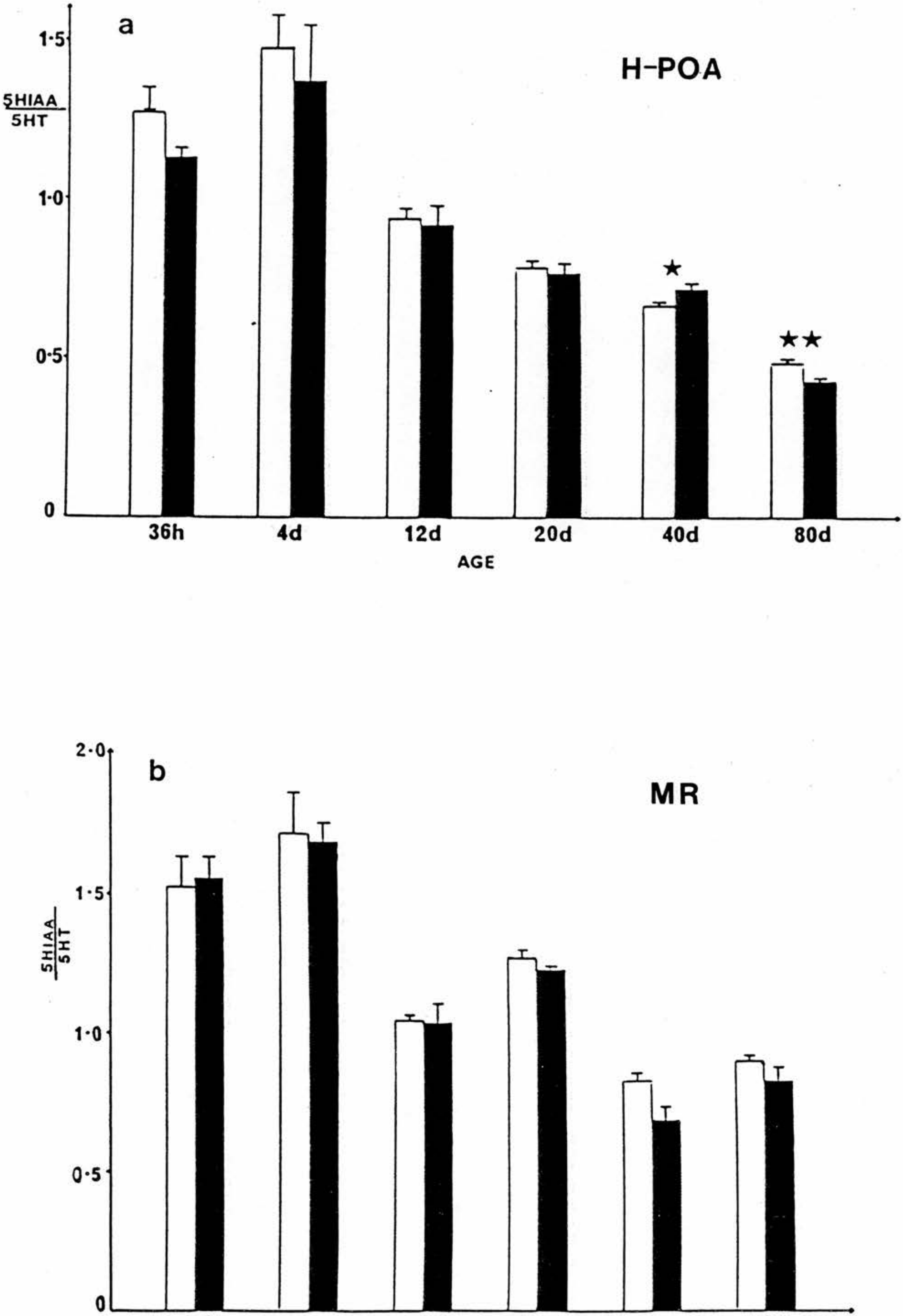
Table 7.1 shows that there was no sex difference in the concentration of 5-HIAA in either the H-POA or MR throughout development. However, unlike the concentrations of 5-HT, the concentrations of 5-HIAA in the H-POA did not increase markedly during development. Concentrations at day 4, 12 and 40 were not significantly different from concentrations on day 80. Between days 12 and 20 there was a significant increase ($P < 0.001$) in the concentration of 5-HIAA in the H-POA to a value which was significantly greater ($P < 0.001$) than that at any other time.

In contrast to the H-POA, the concentrations of 5-HIAA in the MR were significantly greater at 36h and 4 days (both $P < 0.001$) than at 80 days. Between days 4 and 12 concentrations fell significantly ($P < 0.001$) to values which were not significantly different from 40 or 80 day old animals. Between days 12 and 20 there was a significant increase ($P < 0.001$) in the concentration of 5-HIAA in the MR.

7.3.3 The Molar Ratio of 5-HIAA/5-HT

Figure 7.B.(a) shows that in the H-POA the 5-HIAA/5-HT ratio was slightly greater in males than females ($P < 0.05$) at 40 days, but at 80 days this pattern was reversed so that the ratio was significantly higher in females than in males ($P < 0.001$). The maximum ratio occurred at day 4 when the value was approximately 3 times that found at day 80 (both $P < 0.001$) and a gradual decline from 4 days to 80 days occurred in the 5-HIAA/5-HT ratio.

Figure 7.B.(b) shows that in contrast to the H-POA, no sex differences occurred in the 5-HIAA/5-HT ratio in the MR. However,


a
H–POA
5HIAA / 5HT
0
0·5
1·0
1·5
★
★★
36h
4d
12d
20d
40d
80d
AGE
b
MR
5HIAA / 5HT
0
0·5
1·0
1·5
2·0
36h
4d
12d
20d
40 d
80d
AGE

as in the H-POA, the highest ratio occurred at day 4 but the value was only about twice that found at day 80 ($P < 0.001$). Between days 4 and 80 there was a gradual decline in the 5-HIAA/5-HT ratio, with a small increase between days 12 and 20 ($P < 0.05$).

7.3.4 The Rate of 5-HTP Accumulation after Treatment with NSD 1015

Figure 7.C shows that the accumulation of 5-HTP in the 4 day H-POA was linear ($r = 0.87$, $P < 0.001$) during the 60 min after injection of NSD 1015. Figure 7.D shows that there was no sex difference in the rate of 5-HTP accumulation at day 4 but at day 80 the rate of accumulation of 5-HTP was significantly greater ($P < 0.025$) in females than males. The rate of accumulation was significantly greater in both sexes at 80 days than at 4 days (both $P < 0.001$).

7.3.5 Dopamine

Table 7.2 shows that there was no sex difference in the concentration of DA in the H-POA throughout development. Between 36h and 12 days the concentration of DA in the H-POA was fairly constant. Significant increases in DA concentration occurred between days 12 and 20 ($P < 0.001$) and days 20 and 40 ($P < 0.001$). A smaller increase also occurred between days 40 and 80 ($P < 0.01$). The highest concentration of DA was found in the 80 day H-POA.

In contrast to the H-POA, a small but significant ($P < 0.05$) sex difference occurred in DA concentration in the MR at 36h with higher values in the female than the male (Table 7.2). The concentration of DA in the MR did not show the same increase throughout development as the H-POA; rather, concentrations remained relatively

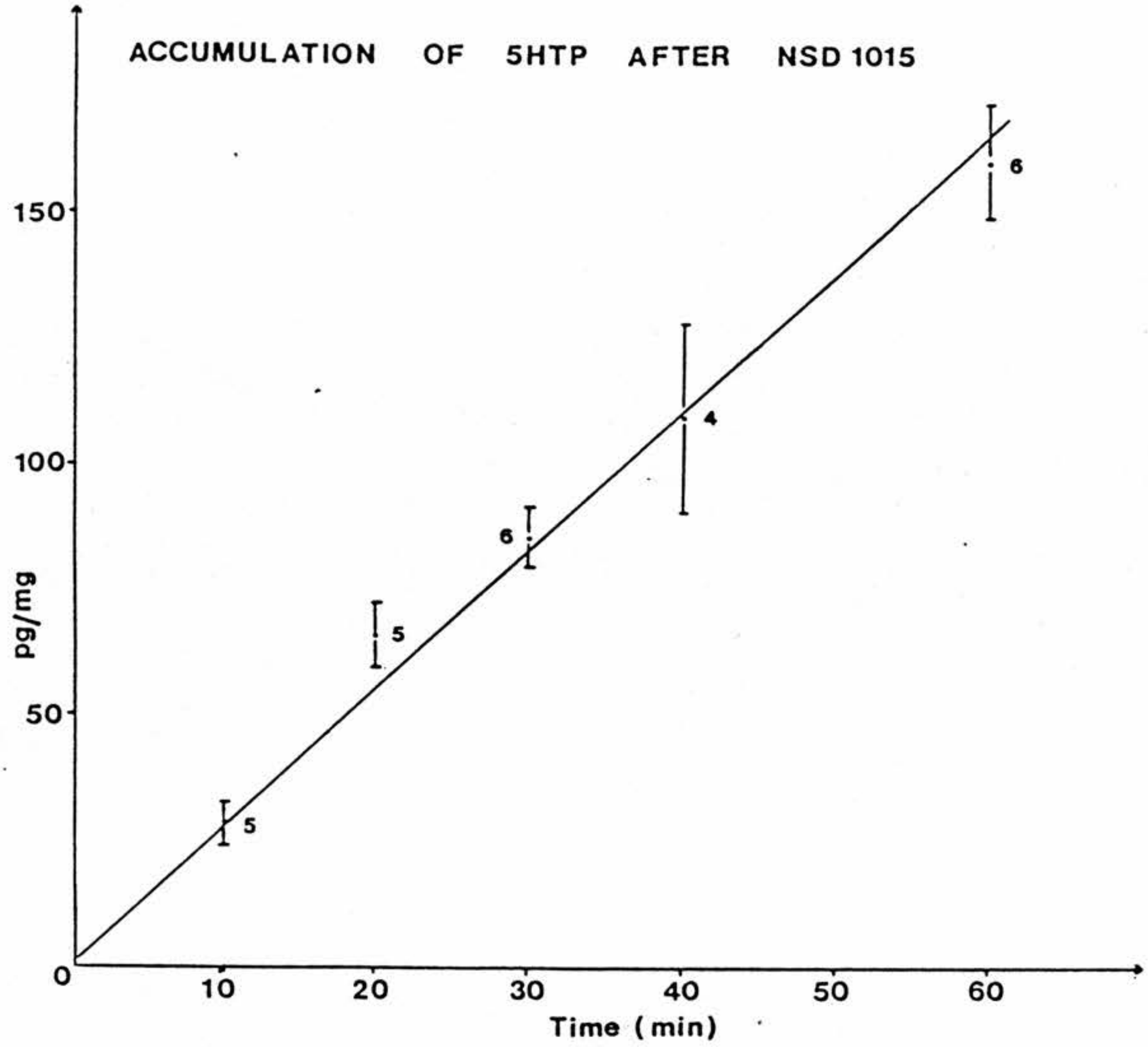


FIGURE 7.C:

The mean (± S.E.M.) concentration of 5-HTP (pg/mg) in the hypothalamus-preoptic area (HPOA) of 4 day old rats of both sexes at various times after injection (i.p.) of NSD 1015. Figures at each time represent number of samples at that time.

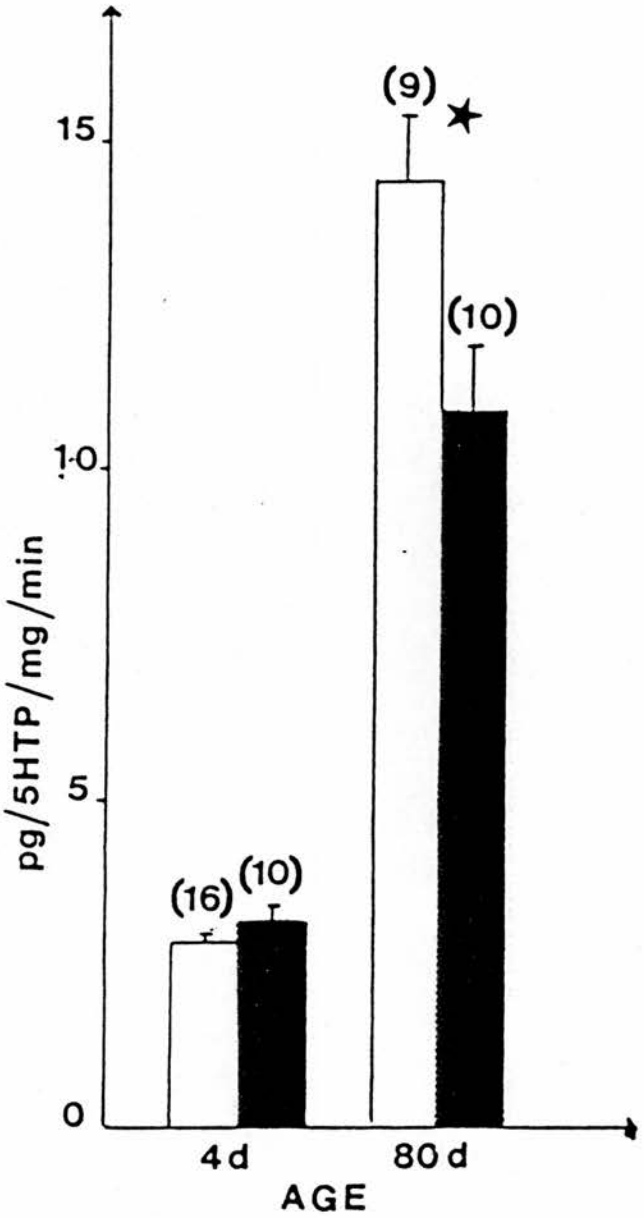


FIGURE 7.D:

The mean (± S.E.M.) rate of accumulation of 5-hydroxytryptophan in the hypothalamus-preoptic area (HPOA) of female (open bars) and male (solid bars) rats killed at 4 or 80 days of age. Animals were decapitated 20 or 30 min after injection with 100mg/kg of NSD 1015. Figures in parenthesis are the number of animals/group. *$p < 0.025$ when comparing males to females at 80 days of age.

Table 7.2

The mean (± S.E.M.) concentrations (ng/mg tissue wet weight) of dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in the hypothalamus-preoptic area and midbrain raphe region of male and female rats at various ages

Hypothalamus-Preoptic Area	36h	4d	12d	20d	40d	80d
DA female	0.10 ± 0.01	0.12 ± 0.02	0.16 ± 0.01	0.36 ± 0.04	0.61 ± 0.02	0.73 ± 0.03
male	0.11 ± 0.02	0.10 ± 0.01	0.17 ± 0.02	0.38 ± 0.03	0.59 ± 0.02	0.66 ± 0.03
DOPAC female	0.037 ± 0.005	0.042 ± 0.005	0.048 ± 0.005	0.074 ± 0.006	0.074 ± 0.003	0.074 ± 0.005
male	0.042 ± 0.004	0.040 ± 0.002	0.047 ± 0.003	0.078 ± 0.009	0.077 ± 0.003	0.074 ± 0.005

n = 5 (except at 4 days where n = 10)

Midbrain Raphe	36h	4d	12d	20d	40d	80d
DA female	0.48 ± 0.1	0.64 ± 0.09	0.75 ± 0.05	0.89 ± 0.03	0.67 ± 0.03	0.59 ± 0.03
male	*0.25 ± 0.05	0.73 ± 0.05	0.74 ± 0.04	0.89 ± 0.09	0.73 ± 0.08	0.56 ± 0.09
DOPAC female	0.081 ± 0.01	0.17 ± 0.01	0.19 ± 0.004	0.17 ± 0.02	0.050 ± 0.004	0.13 ± 0.02
male	**0.042 ± 0.005	0.16 ± 0.01	0.19 ± 0.008	0.16 ± 0.01	0.043 ± 0.02	0.10 ± 0.008

n = 5 (except at 36h where n = 4)

* $p < 0.05$
** $p < 0.025$

constant after day 4. At 36h, the concentration of DA was significantly lower than at 4, 12, 20 and 40 days ($P < 0.001$) and at 80 days ($P < 0.05$). Peak concentrations of DA in the MR were found at 20 days. A small decrease occurred between days 20 and 40 ($P < 0.05$).

7.3.6 3,4-Dihydroxyphenylacetic Acid

Table 7.2 shows that no sex difference occurred in DOPAC concentrations in the H-POA at any age. A significant increase in DOPAC concentration occurred between days 12 and 20 ($P < 0.001$). Between 36h and 12 days and also 20 days and 80 days, no changes in the concentration of DOPAC occurred.

In contrast to the H-POA, Table 7.2 shows that a sex difference in DOPAC concentration occurred in the MR at 36h (females greater than males, $P < 0.025$). The concentration of DOPAC increased significantly between 36h and 4 days ($P < 0.001$) to a value which was not significantly different from those found at 4, 12 and 20 days. Peak DOPAC concentrations were found at 12 days. A massive drop in DOPAC concentrations occurred in the MR between days 20 and 40 ($P < 0.001$) to a value which was not significantly different to that found at 36 h. Between days 40 and 80, DOPAC concentrations increased ($P < 0.001$) to a value which was significantly greater than that found at 36h ($P < 0.001$) and less than that found at 12 days ($P < 0.001$) and 4 and 20 days ($P < 0.005$).

7.4 DISCUSSION

In the present study no sex differences were detected in the content of 5-HT or 5-HIAA in the H-POA or MR at any of the ages investigated (Table 7.1). Therefore any sex difference in central serotonergic neuronal systems which may occur during or as a result of sexual differentiation are not reflected in the indoleamine content of these regions. A high 5-HIAA/5-HT ratio, with a peak at 4 days, was found to occur in neonatal rat brain in both the H-POA and MR (Figs. 7.B.(a) and (b)) and a major sex difference in the 5-HIAA/5-HT ratio was found in the 80 day H-POA (females greater than males). Using the accumulation rate of 5-HTP after aromatic amino acid decarboxylase inhibition as an index of the rate of 5-HT synthesis, the high 5-HIAA/5-HT ratio in the 80 day female H-POA was found to be due, in part at least, to a higher rate of 5-HT synthesis in the female than in the male. Accumulation of 5-HTP in the 4 day H-POA was linear for 60 min after the injection of NSD 1015 (Fig. 7.C) and a comparison of results from the 4 day with the 80 day H-POA (Fig. 7.D) showed that the accumulation of 5-HTP in the 80 day H-POA was approximately 5 times that in the neonate. In addition to these data, the content of 5-HT, DA and their metabolites in both the male and female H-POA and MR throughout development were established.

The data on 5-HT concentrations in the H-POA and MR (Table 7.1) shows that, as found by others (Lidov and Molliver, 1982(a)) the 5-HT cell groups in the MR are relatively well developed at birth. The MR of neonatal rats was found to contain as much 5-HT as the 80 day MR. 5-HT synthesis has been detected in the brainstem as early as the thirteenth day of gestation (Olson and Seiger, 1972, Seiger and Olson, 1973). Serotonergic fibres do not, however, invade their major terminal fields within the hypothalamus until approximately postnatal day 10 and terminal formation extends throughout the first 3 post natal weeks (Lidov and Molliver, 1982(b)). This continuing postnatal development of the 5-HT neuronal network within the H-POA probably contributes to the increase in 5-HT concentration in the H-POA during the first 3 weeks after birth although the dramatic increase in tryptophan hydroxylase activity which is known to occur during this period (Schmidt and Sanders-Bush, 1971; Deguchi and Barchas, 1972) is perhaps more important. Other workers have also shown that, as shown here in the H-POA, brain 5-HT concentrations at birth are only approximately 25% that found in the adult (Kato, 1959).

The pattern of changes in 5-HIAA content in the H-POA, with a maximum around day 20, agrees with previously reported patterns for forebrain and whole brain (Bourgoin, Artand, Adrien, Héry, Glowinski and Hamon, 1977; Ziesel, Mauron, Watkins and Wurtman, 1981; Sarna, Tricklebank, Kantameneni, Hunt, Patel and Curzon, 1982) but the abrupt increase in the content of 5-HT in the H-POA between days 12 and 20 compared to the more steady pattern in the MR was not reported in forebrain by Bourgoin et al., (1977) or in whole brain by Sarna et al., (1982). A number of factors influence brain concentrations of 5-HT and 5-HIAA including plasma tryptophan metabolism, brain tryptophan levels, tyrosine hydroxylase and MAO A activities and changes in any or all of these parameters during development will affect brain 5-HT and 5-HIAA concentrations. A comparison of the data for the H-POA and MR in Table 7.1 does show, however, that the concentrations of each compound do not change to the same degree in each area, suggesting that changes in precursor availability, or perhaps more importantly, enzyme activities, affect each area to different extents.

Ladowsky and Gaziri (1970) measured the 5-HT concentration in whole brain (without cerebellum) and showed a greater concentration of 5-HT in the female than the male at 12 days. The present results suggest that the sex difference in 5-HT content reported by Ladowsky and Gaziri (1970) was probably due to changes in brain areas outside the H-POA and MR although the concentrations of 5-HT in cerebral cortex, for example, are very much lower than those in the H-POA or MR (Palkovits, Brownstein and Saavedra, 1974(a); Saavedra, Palkovits, Brownstein and Axelrod, 1974). The reports of sex differences in 5-HT concentrations in the whole brain of adult (Kato, 1959) and 2 day old rats (Hardin, 1973) were not confirmed in the regions examined in the present study.

Since the 5-HIAA/5-HT ratio appears to be a reliable indicator of 5-HT turnover and neuronal activity (Tozer, Neff and Brodie, 1966; Aghajanian, Rosecrans and Sheard, 1967; Smythe, Bradshaw, Cai and Symons, 1982) the data in Figs. 7.B, and 7.D suggest that during the first 3 weeks of postnatal development in the H-POA and at all ages studied in the MR, there were no significant sex differences in the activity of serotonergic neurons. The sex differences found in the H-POA at 40 and 80 days suggest different neuronal activities at these times but the basis for these differences is difficult to explain. Figure 7.C confirmed that in 4 day rats, as in the adult (Carlsson, Davis, Kehr, Lindquist and Atack, 1972; Carlsson, 1974), the administration of NSD 1015 results in a linear accumulation of 5-HTP during the 60 min after injection of NSD 1015. Figure 7.D shows that the sex difference in 5-HIAA/5HT ratio in the H-POA at 80 days (Fig. 7.B, females greater than males) is accompanied by an increase in the accumulation rate of 5-HTP in the female H-POA, suggesting that an increased rate of 5-HT synthesis accompanies the increased neuronal activity in the H-POA of 80 day female rats. Figure 7.D also shows that the high 5-HIAA/5-HT ratio found in neonatal HPOA compared with 80 day H-POA is not accompanied by a higher rate of 5-HT synthesis. Therefore, the high 5-HIAA/5-HT ratio found in the neonatal H-POA is probably largely the result of an increased MAO A activity similar to that reported in the forebrain and brainstem by Nelson, Herbet, Glowinski and Hamon, (1979) and in the brainstem by Bourgoin et al., (1977). Bourgoin et al., (1977) have also shown that reduced clearance of 5-HIAA or formation of 5-HIAA from peripheral 5-HTP do not contribute to the increased ratio.

The DA neurons of the central nervous system have been implicated in the control of gonadotrophin (Weiner and Ganong, 1978; Sarkar and Fink, 1981; Barraclough and Wise, 1982) and prolactin release (Schally, Arimura and Kastin, 1973; Vale, Rivier and Brown, 1980) and in the greatly enhanced motor activity which occurs in female rats on the evening of proestrus (Finger, 1969; Steiner, Katz, Baldrighi and Carroll, 1981). Manipulations of the dopaminergic systems also affect the lordosis reflex (Ahlenius, Engel, Eriksson, Modigh and Södersten, 1972; Ahlenius, Engel, Eriksson, Modigh and Södersten, 1975; Everitt, Fuxe and Hökfelt, 1974; Caggiula, Herndon and Scanlon, 1979a; Caggiula, Antelman and Chiodo, 1979b.

Oestrogen can influence the functioning of the nigro-striatal and tuberoinfundibular (TIF) DA systems either directly, probably by an effect on the genome as a result of the uptake and binding of oestrogen in neurons of the TIF system (Grant and Stumpf, 1973; Heritage, Stumpf, Sar and Grant, 1980; Sar and Stumpf, 1981) or indirectly through the release of anterior pituitary hormones, for example prolactin (Eikenburg, Ravitz, Gudelsky and Moore, 1977; Demarest and Moore, 1980; Gudelsky, Nansel and Porter, 1981). The structure of the arcuate nucleus itself is known to be dependent upon the gonadal steroid hormone environment of the neonate (Matsumoto and Arai, 1981).

Cyclic changes in DA turnover are known to occur in the median eminence during the rat oestrous cycle and a persistently high DA turnover in the median eminence of sterile, adult female rats has been reported (Fuxe, Hökfelt and Nilsson, 1972) probably a result of elevated concentrations of oestrogen in the plasma. The present results show that other than a small but significant sex difference at 36h in the MR, no permanent change in the actual concentration of DA occurred as a result of sexual differentiation of the brain. The patterns of development of the concentrations of DA and DOPAC were different in the H-POA and MR (Table 7.2), probably a reflection of different functional requirements and, in agreement with other studies, the results demonstrate the relative immaturity of the monoaminergic system at birth (Hyyppä 1969, 1971; Loizou and Salt, 1970; Loizou, 1972) particularly in the H-POA.

Studies using fluorescence histochemistry have shown a good correlation between the appearance of primary catecholamines and structural and functional events in the hypothalamo-hypophysial axis. Catecholamines are visible in the midbrain at day 18 of gestation, and, by postnatal day 1, when midbrain concentrations are already increasing some fluorescence is also visible in the hypothalamus (Smith and Simpson, 1970). The median eminence, which contains the highest concentration of DA in the adult brain (Palkovits, Brownstein, Saavedra and Axelrod, 1974b), shows a rapid increase in catecholamine content at approximately postnatal day 4 (Smith and Simpson, 1970) when the primary capillary plexus of the portal system develops and starts to function (Rinne and Kivalo, 1965; Campbell, 1966; Glydon, 1975). Although studies using fluorescent techniques have shown that catecholamine fluorescence reaches adult intensities by approximately 3 to 6 weeks (Bjorklund, Enemar and Falck, 1968; Hyyppä, 1969, 1971), the present data and the results of Agrawal, Glisson and Himwich, (1966) and Coyle and Henry (1973) show that DA concentrations continue to rise beyond that time. The difference in the data obtained by the two techniques is probably due to the fact that beyond a certain tissue concentration of catecholamines, fluorescence intensity is not directly proportional to catecholamine concentration.

CHAPTER 8

Summary and Hypothesis

8.1 SUMMARY

In order to investigate the effects of steroid and thyroid hormones on protein synthesis in the developing rat brain, the following experiments were carried out:

(i) Subcellular fractions were prepared from the brains of intact male and female rats at days 0, 8, 25, 72 and from the H-POA and pituitary gland of male, female and androgenised female rats at day 80. Proteins associated with each subcellular fraction were analysed by PAGE.

(ii) The synthesis of brain proteins *in vivo* in 4 day male and female rats was investigated by injecting ^{35}S-methionine into the 3rd ventricle and 1h later, extracting brain proteins and analysing them by PAGE. The short-term effects of TP administration to 4 day female rats were also investigated using this method.

(iii) Protein synthesis was studied by the isolation and translation *in vitro* of H-POA mRNA from male, female and androgenised female rats at 4, 12 and 72 days of age.

(iv) In a series of *in vitro* experiments, the direct effects of testosterone, E_2, T_3 and T_4 on protein synthesis in 4 day H-POA slices and in primary cultures of foetal rat hypothalamic neurons were examined. In addition, the effects of TP administration *in vivo* to 4 day female rats on H-POA protein synthesis *in vitro* was investigated using the double label isotope technique.

(v) Changes in brain protein synthesis were investigated indirectly by measuring the concentrations of 5-HT, 5-HIAA, DA and DOPAC in the H-POA and MR of male and female rats throughout development by LCED.

In the study of proteins in the H-POA from intact male and female rats throughout development, changes in proteins associated with the soluble (14,600, 15,000, 29,900, 38,900 and 49,000), nuclear (40,000-50,000 and 13,800-16,000), mitochondrial-lysosomal (49,000 and 52,000) and microsomal (14,400, 20,000, 50,100, 56,900 and 130,000) fractions were detected with increasing age, as assessed by staining proteins with Coomassie Blue. Sex differences in H-POA proteins in the soluble fraction were detected at days 0 (53,000-56,000: males greater than females), 25 (14,600 and 15,000: males greater than females) and 80 (14,800: females greater than males) and in soluble proteins (18,600: greater in males than females and 75,000: greater in females than males) extracted from the pituitary gland of 80 day rats.

By labelling proteins in vivo with ^{35}S-methionine changes in the synthesis of two 13,000 - 14,000 molecular weight proteins in the 4 day H-POA were detected. There was variation in the synthesis of these low molecular weight proteins between animals and their synthesis could be modified, at least in the short-term, by the administration of TP to 4 day female rats. An incidental but important finding of the in vivo labelling experiments, was the induction of a 77,600 molecular weight protein in the H-POA of one animal which had undergone a series of fit episodes during the experiment. Synthesis of this protein could have been a direct result of a period of intense neuronal activity in the brain. Silver-staining proteins from these experiments revealed a 50,000 molecular weight protein which was present in the male and androgenised female but not the control female H-POA and although this sex difference was detected in only one out of four such experiments, this unidentified protein may be involved in mediating the central effects of testosterone in the neonatal rat.

Studies using two cell-free translation systems and two radioactive amino acid preparations to label mRNA translation products in vitro showed both developmental and androgen-induced changes in protein synthesis in the H-POA. A greater synthesis was detected in proteins with the electrophoretic characteristics of tubulin and actin in the H-POA of 4 and 12 day male and androgenised female rats compared with that in control female rats. These results suggest that the sex difference in content of a tubulin-like protein in the H-POA detected at day 0 by staining soluble proteins was probably also due to higher levels of 'tubulin' mRNA in the male than in the female.

Steroid and thyroid hormones stimulated and inhibited the synthesis of specific proteins, varying in molecular weight from 14,800 to 93,000, in H-POA slices in vitro and in primary cultures of foetal rat hypothalamic neurones. There appear to be no reports in the literature of thyroid hormone effects upon protein synthesis in neonatal rat brain using PAGE and although the physiological significance of the changes demonstrated in these experiments can only be speculative, it may be that thyroid hormones are involved in the differentiation of H-POA as well as the cerebellum. In H-POA slices from 4 day rats, the synthesis of a 57,500 molecular weight soluble protein was significantly greater in the male than the female and could be increased in the female H-POA by the addition of testosterone or T_3, but not T_4, to the medium. Despite the slight differences in molecular weight estimates in the experiments in this thesis, the latter 57,500 molecular weight protein and proteins with similar molecular weights in chapters 3 and 5 had the electrophoretic characteristics of tubulin. The in vitro H-POA slice experiments support the findings seen in chapters 3 and 5, of a higher synthesis of a tubulin-like protein in the neonatal male H-POA than in the female. When the effects of TP administration in vivo on H-POA protein synthesis in vitro were studied using the double label isotope technique, no evidence for the induction or inhibition of any specific proteins was obtained.

In conclusion, steroid and thyroid hormones were found to influence the synthesis of specific proteins in the foetal and neonatal rat H-POA. In addition, neonatal exposure to high levels of testosterone was found to have long-lasting effects on proteins in the H-POA and pituitary gland.

The concentrations of 5-HT and 5-HIAA did not differ significantly in the male and female H-POA or MR at any of the ages investigated and other than a small but significant sex difference at 36h in the MR, no permanent change in the concentrations of DA or DOPAC occurred in either brain region as a result of sexual differentiation. The 5-HIAA/5-HT molar ratio, believed to be an index of 5-HT turnover and neuronal activity, was highest at 4 days of age in both the H-POA and MR of male and female rats. A small but significant sex difference in the 5-HIAA/5-HT ratio was detected in the H-POA but not MR at 40 days of age (males greater than females) and at 80 days a highly significant sex difference (females greater than males) was also detected in the H-POA. In support of the latter data, studies using NSD-1015 to allow measurement of the accumulation of 5-HTP as an index of 5-HT synthesis, showed that the 80 day female rat H-POA synthesised significantly more 5-HT than the 80 day male. In addition, the rate of 5-HT synthesis was found to be 5 times higher in the 80 day than in the 4 day H-POA.

8.2 HYPOTHESIS

The results presented in this thesis support the hypothesis that exposure of neonatal rats to testosterone brings about hormone-induced alterations in protein synthesis. Whether any of the changes detected in specific proteins relate to events which normally occur during or as a result of sexual differentiation or are the result of non-specific effects of high doses of testosterone, is not known. However, androgen-induced alterations in gene expression were demonstrated and by comparing the results obtained using different experimental approaches, it appears that certain changes may reflect the situation in vivo. In particular, the discovery of a sex difference in actin and tubulin synthesis in the immature rat H-POA has important implications, in that, such a change may reflect structural alterations in the developing H-POA. Such changes have been reviewed in Chapter 1 of this thesis but it has been shown that sexual differentiation of the brain is associated with changes in synapse formation and possibly the patterns of neuritic outgrowth in specific brain areas. The results presented here may support the theory that the structural changes which occur during sexual differentiation of the brain could involve enhanced neuronal outgrowth in the male.

Sexual differentiation may also involve very subtle biochemical alterations in specific nuclei within the H-POA and although both steroid and thyroid hormones were found to influence specific proteins within this region, the identities of the proteins are not known and the precise mechanism of the process of sexual differentiation remains obscure.

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Appendices

APPENDIX I

Protein Determination

The following stock solutions were prepared:

A. 100g Na_2CO_3 per 1000ml 0.5 M NaOH

B. 1g $CuSO_4.7H_2O$ per 100ml distilled water

C. 2g potassium tartrate per 100ml distilled water

D. Bovine Serum Albumin (BSA) 1mg/ml in distilled water

Standards containing 10–300μg BSA and samples (5–20μl) were prepared in triplicate in a final volume of 1 ml with distilled water.

15ml solution A, 0.75ml solution B and 0.75ml solution C were mixed and 1ml added to all assay tubes. After standing at room temperature for 15 min, 3ml Folin's reagent (prepared by adding 5 ml commercial stock solution to 50ml distilled water) were added, mixed immediately and left at room temperature for 45 min before determining the absorbance at 750nm.

Solutions for Polyacrylamide Gel Electrophoresis

1. Gradient Gels

Stock Solutions

Acrylamide 154g acrylamide

4g N,N'-Methylene-bis-acrylamide (bis-acrylamide)

to 500ml distilled water

Filter through Whatman No. 1 filter paper

Buffer L 200g Tris base to 1 litre

pH 8.5–8.6 with HCl

L–10 3 parts buffer L

5 parts distilled water

L–20 3 parts buffer L

1 part glycerol

10% SDS (w/v)

10% ammonium persulphate (w/v) (APS)

The preparation of 4 gradient gels required the mixing of light and heavy gel solutions as follows:

	Light (ml)	Heavy (ml)
Stock acrylamide	19.2	46.4
L–10	50.4	–
L–20	–	23.2

To each of these solutions the following solutions were added just before pouring the gels:

10% SDS	740µl
10% APS	440µl
N,N,N',N'-Tetramethylethylenediamine (TEMED)	20µl

To pour gels: 120ml 20% methanol (v/v) were first run into the gel slab casting apparatus to allow formation of a flat surface on the top of the gradient gels (poured from the foot of the casting apparatus). The complete light and heavy solutions were then quickly poured into the appropriate chambers of the gradient mixer (light solution nearest outlet) and the gradient gels poured slowly. When the gradient mixer was almost empty, 90ml 50% glycerol (v/v) containing a little bromophenol blue were poured into the mixer and allowed to fill the gel casting apparatus up to the foot of the gel plates. Gels were left undisturbed until polymerisation was complete.

2. <u>Stacking Gel</u> (4.75% acrylamide)

Stock buffer 0.5M Tris-HCl, pH 6.8
0.4% SDS (w/v)

For 30ml gel solution mix:

4.5ml stock acrylamide (as in part 1)
7.5ml buffer

18.0ml distilled water

90µl 10% APS

30µl TEMED

3. <u>Flat Bed Isoelectric Focussing Gel</u> (4% acrylamide, 9M urea)

Stock solutions

Acrylamide 22.2g acrylamide

1.4g bis-acrylamide to 100ml distilled water

40% APS (w/v)

For 40ml gel solution mix:

7.2ml stock acrylamide

21.6g urea

2.0ml Ampholines (pH 3.5–9.5) and make to 40ml with distilled water

Add 40µl 40% APS

Staining solutions

A 10g copper sulphate per litre 20% acetic acid (v/v)

B 3g Coomassie Brilliant Blue per litre 90% methanol (v/v)

Mix A and B in a 1:1 ratio

4. <u>Rod Gels for Isoelectric Focussing</u> (4.75% acrylamide, 9.2 M urea)

Stock Solutions

Acrylamide 28.38 g acrylamide

1.62g bis-acrylamide to 100ml distilled water

10% Nonidet P-40 (w/v)

10% APS (w/v)

For 10ml gel solution mix:

5.5g urea

1.33ml acrylamide

2.00ml Nonidet P 40

1.97ml distilled water

0.4ml Ampholines, pH 4.0–6.5

0.1ml Ampholines pH 3.5–9.5

Add 10µl 10 APS

7µl TEMED

MARKERS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

High Molecular Weight

			Mol. Wt.
(HMW)	Thyroglobin	(hog thyroid)	330,000
(subunits)	Ferritin	(horse spleen)	60,000
	Catalase	(bovine liver)	18,500
	Lactate Dehydrogenase	(bovine heart)	36,000
	Albumin	(bovine serum)	67,000

Low Molecular Weight

(LMW)	Phosphorylase b	(rabbit muscle)	94,000
	Albumin	(bovine serum)	67,000
	Ovalbumin	(egg white)	43,000
	Carbonic Anhydrase	(bovine erythrocyte)	30,000
	Trypsin Inhibitor	(soy bean)	20,000
	α-lactalbumin	(bovine milk)	14,400

pI Calibration Kit

pH 3-10		pI (24°C)
	amyloglucosidase	3.50
	soybean trypsin inhibitor	4.55
	β-lactoglobulin A	5.20
	bovine carbonic anhydrase B	5.85
	human carbonic anhydrase B	6.55
	horse myoglobin -acidic band	6.85

horse myoglobin - basic band	7.35
lentil lectin - acidic band	8.15
lentil lectin - middle band	8.45
lentil lectin - basic band	8.64
trypsinogen	9.30

Krebs Ringer Phosphate Medium

Stock Solutions (all w/v)	Parts
0.9% NaCl	100
1.15% KCl	4
1.22% $CaCl_2$	3
3.82% $MgSO_4\ 7H_2O$	1
2.11% KH_2PO_4	1
100mM Phosphate buffer, pH 7.4	21
(1.42g Na_2HPO_4 - add HCl until reach pH 7.4, make to 100ml).	

Add 1mg/ml glucose

APPENDIX II

Preparation of Ovaries for Staining

The ovaries were left overnight in 10% formaldehyde-saline solution and then soaked in a series of graded alcohols (70% (v/v) - absolute) followed by 2 changes of chloroform. Tissue was then submerged in a molten wax bath (at 54°C), vacuum-embedded in paraffin wax and mounted on wooden blocks for sectioning (7μm).

Haematoxylin And Eosin Staining Procedure

Sections were placed in xylene to remove the wax and were then hydrated by soaking in a series of graded alcohols (absolute - 70%) and finally water. After staining for 5 min in Harris' haematoxylin, sections were left under running tap water until a blue colour developed. After placing in aqueous eosin (1%) for 30s, the sections were dehydrated and finally soaked in xylene before mounting in D.P.X. (Bio-Rad) and viewed under a low power microscope.