A QUANTITATIVE CYTOCHEMICAL STUDY OF SOME HYPOTHALAMIC NERVE CELLS

JOHN A. RUSSELL

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To my family

SUMMARY

1. Quantitative cytochemical techniques have been used to measure changes in the synthetic activity of the nucleoli of anatomically defined hypothalamic neurones in response to various stimuli in the rat. Interference microscopy was used to measure the dry mass of nucleoli in isolated neuronal nuclei. In some experiments ultraviolet absorption microspectrography was used to measure nucleolar nucleic acid content and confirm, by a technique based on different optical principles, the trend of the nucleolar dry mass measurements.

Increased nucleolar dry mass or nucleic acid content was interpreted as a sign of increased ribosome and protein synthesis.

2. Nucleolar changes in paraventricular and supraoptic neurones were measured during lactation and pregnancy. Nucleolar dry mass of supraoptic and paraventricular neurones is increased on the 20th day of pregnancy and throughout lactation. Suckling causes a further increase in the nucleolar dry mass of paraventricular neurones within seven days post partum, but no further change occurs in supraoptic neurones. In the absence of suckling after parturition, the changes in nucleolar dry mass of both supraoptic and paraventricular neurones regress within seven days. The nucleolar changes in lactation are graded according to the size of the litter, but are maximal with a litter of 10 pups.

3. The quantitative relationship between nucleolar changes and the functional load in paraventricular and supraoptic neurones during lactation was investigated. Peripheral stimulus and secretory response parameters of the milk ejection reflex were measured. Nucleolar dry mass changes in supraoptic and paraventricular neurones are related quantitatively to the secretory activity of these neurones and not to the intensity of the suckling stimulus. A mechanism is proposed whereby secretory activity of the nerve endings of a neurone could regulate synthetic activity in the nucleolus.

4. The effects of other hormones regulated by suckling on the activity of the nucleoli of paraventricular and supraoptic neurones were studied. Experiments were performed on lactating rats with unilateral nipple line ablation to show that nucleolar dry mass changes in paraventricular neurones are partially independent of humoral factors, and that afferent fibres of the milk ejection reflex are distributed mainly to the contralateral paraventricular nucleus. Nucleolar changes in supraoptic neurones in these animals can not be so interpreted. The changes in the nucleoli of paraventricular and supraoptic neurones during lactation do not depend on the hormonal state of the preceding pregnancy because nucleolar dry mass increased when the suckling stimulus was intensified seven days post partum.

In virgin females, 1 mg of estradiol benzoate daily for five days increases the nucleolar dry mass of supraoptic and paraventricular neurones: estrogen may partly cause

the nucleolar changes in pregnancy and lactation. Ovariectomy or progesterone treatment do not alter the nucleoli of paraventricular or supraoptic neurones in virgin females, and no changes occur in these neurones during the estrous cycle in intact females.

5. The interaction of lactation and water deprivation was studied. Water deprivation alone for three days or lactation alone for eight days produce similar changes in the nucleolar dry mass of supraoptic and paraventricular neurones. When lactating animals are deprived of water for three days the nucleolar changes in supraoptic and paraventricular neurones are the sum of the nucleolar responses to each stimulus applied separately. The effects of a diuretic, frusemide, in dehydrated animals confirm that nucleolar dry mass changes are quantitatively related to the secretory activity of supraoptic and paraventricular neurones.

Histograms of individual nucleolar dry mass measurements in these experiments show that the changes in mean nucleolar dry mass which result from the combined stimuli of water deprivation and suckling may be due to summation of changes in separate populations of neurones.

The PV nucleus contains neurones responsive to suckling, some neurones responsive to hyperosmotic or hypovolemic stimuli and an unresponsive population of neurones.

The SO nucleus contains neurones mainly responsive to hyperosmotic or hypovolemic stimuli, and few neurones responsive only to suckling; most of the changes in SO

neurones during lactation could be due to hyperosmotic or hypovolemic stimulation resulting from milk secretion.

6. In male rats the effects of castration on several hypothalamic areas were investigated. Nucleolar dry mass increases only in suprachiasmatic neurones after castration, and decreases in ventromedial, arcuate, medial preoptic, preoptic and posterior hypothalamic neurones. These results support other evidence that the suprachiasmatic neurones may synthesise gonadotrophin releasing factors, and changes in the other neurones have been discusssed in relation to functions of these neurones dependent on testosterone. Nucleoli of paraventricular or anterior hypothalamic neurones did not change after castration.

7. In male rats the effects of adrenalectomy on several hypothalamic areas were investigated. Nucleolar dry mass of paraventricular, arcuate and ventromedial neurones increases after adrenalectomy. These changes can not be interpreted as indicating localisation of corticotrophin releasing factor synthesis but have been discussed in relation to the possible role of the adrenal cortex in regulating the activity of these neurones.

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ACTH	Adrenocorticotrophic hormone
ADH	Antidiuretic hormone
ATP	Adenosine triphosphate
ATP-ase	Adenosine triphosphatase
CRF	Corticotrophin releasing factor
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
FRF	Follicle cell stimulating hormone releasing factor
FSH	Follicle cell stimulating hormone
LH	Luteinising hormone
LHRH	Luteinising hormone releasing hormone
LRF	Luteinising hormone releasing factor
mU	milliunit*
mOsm	milliosmole
μU	microunit
μm	micron
N.A.	Numerical aperture
nm	nanometer
PV	Paraventricular
RNA	Ribonucleic acid
SO	Supraoptic

*Compared with the 3rd International Standard for Oxytocic, Vasopressor and Antidiuretic Substances (1957):-

One micromole of pure synthetic oxytocin contains 4.5×10^{5} mU of oxytocic activity or 1 mU of oxytocic activity is present in 2.23×10^{-9} g of pure synthetic oxytocin.

One micromole of pure synthetic arginine vasopressin contains 4.65×10^{5} mU of antidiuretic activity or 1 mU of antidiuretic activity is present in 2.33×10^{-9} g of pure synthetic arginine vasopressin.

SECTION I

GENERAL INTRODUCTION

1. Prologue

This thesis describes a study of changes in synthetic activity of hypothalamic neurones in response to defined changes in functional activity. Quantitative microchemical techniques have been used to measure responses of nucleoli of hypothalamic neurones.

The general introduction has several aims. The biological basis of the cytochemical methods is reviewed, with special reference to supraoptic and paraventricular neurones. The activation of neurosecretory mechanisms by peripheral stimuli is discussed, and the quantitative relationship between stimulus and secretory response is emphasised. The relationship between enhanced neurosecretory activity and ribosome and protein synthesis is established. Some of the changes in secretory activity of endocrine glands caused by stimuli which also cause oxytocin and ADH to be secreted are described, because these changes may modify synthetic activity in supraoptic or paraventricular neurones.

The introduction shows how these aspects of neurosecretion have been drawn together to form the basis of the present study.

2. General Introduction

2.1 Historical perspective

The similar cytochemical properties of vertebrate nerve cells and exocrine cells of the pancreas were described by Scott (1905) who suggested that nerve cells should be considered not as a mere system of conducting paths, but also as true 'proteid' secretory cells. He tested this suggestion, and found that at the endings of the frog sciatic nerve and dorsal roots of the spinal cord transmission ceased after a short time if these nerve fibres were disconnected from their cell bodies. He surmised that 'nerve cells secrete a substance the passage of which from the nerve endings is necessary to stimulation' (Scott, 1906). Scott did not distinguish neurosecretion and chemical transmission, although these two processes were later considered to be distinct (e.g. Dale, 1935; Bargmann, 1966). The site of delivery of the secreted product is the only known feature which distinguishes these two processes (Scharrer, 1969). A definition of neurosecretion based on the prolonged actions of the released substance (Picard, 1969) breaks down when other actions of the same substance at different sites are considered (e.g. Nicoll & Barker, 1971; Dyball & Cross, 1972).

Study of the neurosecretory process arose from the discovery of the affinity of neurosecretory material for certain stains. This material was found to be distributed along the supraoptico- and paraventriculo-hypophyseal tracts in vertebrates (Bargmann, 1949).

Investigation of the endocrine functions of the posterior pituitary developed separately from study of the

associated neurosecretory processes. Extracts of the posterior pituitary were shown to have vasopressor (Howell, 1898), antidiuretic (von den Velden, 1913), oxytocic (Dale, 1906) and milk ejecting (Ott & Scott, 1910) properties.

The supposed pituicyte origin of posterior pituitary hormones was universally rejected after studies of neurosecretory material in various functional states associated this material with the hormones, and established its synthesis within the SO and PV neurones (Leveque & Scharrer, 1953).

2.2 Neurosecretion: Synthesis and transport of secretory product

On the basis of comparative cytochemical studies, Scott (1905) proposed the nerve cell nucleus to be 'the centre of metabolism', which controls the cytoplasmic Nissl substance from which the secretory products, 'neurosomes', are derived. This view was reiterated by Hyden (1943) who used the technique of ultraviolet absorption microscopy, developed by Caspersson (Caspersson & Schultz, 1940) to study changes in protein and nucleic acid synthesis by neurones during embryonic development. Hyden found that enlargement of nucleoli preceded increased protein and RNA synthesis, and he emphasised the role of the nucleolus in the synthesis of cytoplasmic RNA and protein. This close functional relationship between the nucleolus and cytoplasmic RNA and protein content has been described for all nucleated cells studied (Caspersson & Schultz, 1940; Caspersson, 1950; Brachet, 1960).

2.2.1 Ribosome synthesis: role of the nucleolus

The evidence that the nucleolus is the site of ribosomal RNA synthesis in nucleated cells is overwhelming (Busch & Smetana, 1971).

The nucleolus has similar staining and light absorption properties to the Nissl substance of the cytoplasm, and these properties are removed by treatment with ribonuclease (Caspersson & Schultz, 1940; Brachet, 1960). The nucleotide composition of nucleolar and cytoplasmic RNA are similar (Edstrom, Grampp & Schor, 1961). Synthesis of cytoplasmic RNA is reduced by about 70% in HeLa cells if the nucleoli, but not other nuclear areas, of these cells are irradiated with a microbeam of ultraviolet light (Perry et al, 1961). Anucleolar Xenopus laevis mutants cannot synthesise ribosomal RNA or its precursors (Brown & Gurdon, 1964).

Autoradiographic or fractionation studies after exposure of cells to isotopically labelled RNA precursors provide further support for the nucleolar origin of ribosomes. A few minutes after ^{3}H-cytidine is added to a culture of human amnion cells the whole nucleus is labelled (Goldstein, 1959). After a further short period of time labelled RNA is localised to the nucleolus but the cytoplasm is only labelled after the nucleolus; this lag between labelling of the nucleolus and the cytoplasm varies in duration between tissues, and is present in neurones (Leblond & Amano, 1962; Watson, 1965a, b; Bondy, 1966). The pattern of uptake by nerve cells of isotopically labelled RNA precursors demonstrates that RNA is synthesised continuously; the half life of ribosomal RNA of the whole brain is about six days (Dawson, 1967).

The sedimentation properties of nucleolar RNA and ribosomal RNA are similar (Birnstiel et al, 1963). Detailed sedimentation analyses coupled with studies of isotopically labelled precursor incorporation have shown for several cell types, including neurones, that the 18S and 28S components of ribosomal RNA are formed by successive cleavages of a precursor RNA within the nucleolus (Perry, 1962; Murumatsu et al, 1966; Jarlstedt & Hamberger, 1971).

Actinomycin D inhibits ribosomal RNA synthesis predominantly (Perry, 1962; Ro et al, 1966). Isotopically labelled actinomycin D is localised to the perinucleolar chromatin (Ro et al, 1966; Ebstein, 1967), although such apparent localisation may be artefactual and depend on the method of fixation (Watson, 1968a, pers. obs.). About 0.04% of the total nuclear DNA of rat tissues, including brain, is complementary to ribosomal RNA (Mohan et al, 1969), although there is evidence for amplification of ribosomal DNA in rat brain (Stevenin et al, 1968). Nucleolar DNA, however, has more than five times the number of sequences complementary to ribosomal RNA than has whole nuclear DNA (McConkey & Hopkins, 1964). Nucleolar DNA hybridises with the high molecular weight precursors of ribosomal RNA (Birnstiel et al, 1968; Quagliarotti et al, 1970). The anucleolar Xenopus laevis mutant has been useful in the study of ribosome synthesis, since nucleolar, or satellite, DNA is absent in this mutant. Together with the inability of this mutant to synthesise ribosomes, this supports other evidence that ribosomal RNA precursors are transcribed from nucleolar DNA (Birnstiel et al, 1968).

Active RNA synthesis in neurones is indicated by the higher activity of RNA polymerase in brain compared with liver (Bondy & Waelsch, 1965), although there are regional variations within the brain (McEwen et al, 1972). Nucleolar, or ribosomal, RNA polymerase and DNA-like RNA polymerase can be distinguished by their different ionic dependencies and inhibitor sensitivities (Roeder & Rutter, 1970; Jacob et al, 1970).

About 85% of the total neuronal nucleolar dry mass is protein (Watson, 1968a, 1969). The assembly of ribosomes depends on the simultaneous supply of ribosomal RNA and protein, and ribosomal RNA precursors mature only if ribosomal protein is supplied (Craig & Perry, 1970, 1971). Isolated nucleoli can synthesise protein in vitro, but it is not ribosomal protein (Zimmerman et al, 1969) which is synthesised by cytoplasmic ribosomes and transferred to the nucleolus (Ogata et al, 1967; Tsurugi et al, 1972).

The stage in their production at which ribosomes combine with messenger RNA is not known. Messenger RNA has been extracted from brain, but messenger RNA coding for a specific protein has not been isolated (Zomzely et al, 1970).

In summary, ribosomal RNA is transcribed as a large precursor molecule from nucleolar DNA and is subsequently fragmented and combined within the nucleolus with ribosomal protein, synthesised in the cytoplasm.

2.2.2 Cytoplasmic ribosomes

Within normal neurones ribosomes are either free in the cytoplasm or bound to endoplasmic reticulum, which may be

more concentrated in some areas of the cytoplasm and is then identified as Nissl substance (Palade, 1955; Palay & Palade, 1955). Brain ribosomes, complexed with messenger RNA, are most active in protein synthesis when aggregated as polyribosomes, although this form is unstable and infrequently seen in electron micrographs (Zomzely et al, 1968).

The types of complex which ribosomes form in the cytoplasm have been considered to indicate the classes of proteins synthesised as well as the rate of synthesis. It was believed that ribosomes attached to the endoplasmic reticulum synthesise only export proteins, and those ribosomes free in the cytoplasm synthesise cytoplasmic proteins. This distinction does not hold under vectorial analysis of protein synthesis in vitro. Membrane attached ribosomes of brain or liver release protein both into the cytoplasm and into the cisternae of the endoplasmic reticulum (Andrews & Tata, 1971), and free ribosomes synthesise export and cytoplasmic proteins (Siekevitz & Palade, 1960). However, export proteins are released into the cisternae of the endoplasmic reticulum (Redman et al, 1966; Palade, 1966). In stimulated supraoptic neurones, filamentous material which may be newly synthesised export protein can be seen in the cisternae of the endoplasmic reticulum and attached to its ribosomes (Zambrano & De Robertis, 1966). In electron micrographs of neurones ribosomes have not been seen distal to the axon hillock region, apart from a few ribosome-like particles at nodes of Ranvier (Zelena, 1970); although mammalian axons contain a low concentration of RNA (Koenig, 1965b). Transport of isotopically labelled RNA along axons has been claimed

(Bondy, 1971b; Jarlstedt & Karlsson, 1973), and whole nerve fibres, including the sheath, synthesise acetylcholinesterase and other proteins (Koenig, 1965a, 1967). Synaptosome preparations, which also contain a post synaptic component, can synthesise protein although the product is not defined (Cotman & Taylor, 1971) and the synthetic mechanism is resistant to ribonuclease (Ramirez et al, 1972). Most ribosome dependent protein synthesis can be expected to occur in the perikarya of nerve cells (Ochs et al, 1967; Watson, 1968a; Satake, 1972). There is no evidence that neurosecretory products of PV or SO neurones are synthesised in the axons or nerve endings.

2.2.3 Synthesis and storage of neurosecretory material

Neurosecretory material, defined by its staining properties, is present in small amounts in the perikarya of PV and SO neurones and in larger amounts along the axons of these neurones and in the posterior pituitary (Bargmann, 1949).

Membrane limited, dense cored vesicles are seen in electron micrographs of nerve endings in the posterior pituitary (Palay, 1955). There are similar granules, about 0.1 μmdiameter, in the perikarya and axons of PV and SO neurones (Rinne, 1966; Enestrom, 1967; Kalimo, 1971; Flament-Durand, 1971). The number of these granules in the posterior pituitary decreases when it is depleted of hormone (Morris & Cannata, 1973). Similar granules are seen in electron micrographs of homogenates of posterior pituitary prepared by differential or density gradient centrifugation: ADH, oxytocin and neurophysin are distributed in the

centrifugate with the granules (Barer et al, 1963; Norstrom & Hansson, 1972). The general hypothesis that chemical transmitters are stored within vesicles at nerve endings embraces also neurosecretory products. These vesicles are equivalent to the 'neurosomes' postulated by Scott (1905).

The stages in the synthesis of neurosecretory material have been defined with autoradiography and fractionation studies after exposure to isotopically labelled amino acids. Isotopically labelled precursors of ADH, oxytocin and associated proteins, usually ^{35}S labelled cysteine or methionine, are initially incorporated into protein in the perikarya of SO and PV neurones and appear in the posterior pituitary later (Flament-Durand, 1961; Sloper & King, 1963; Leonardelli, 1964; Talanti et al, 1972).

In electron micrographs, neurosecretory granules appear to form from the Golgi apparatus (Enestrom, 1967). In electron microscopic autoradiographs isotopically labelled arginine or cysteine is initially incorporated into protein in the endoplasmic reticulum and is later transferred to the Golgi apparatus (Nishioka et al, 1970), as in other neurones (Droz, 1965). The Golgi apparatus functions in PV and SO neurones as in other secretory cells as an organelle in which secretory products are packaged (Palade, 1966; Whaley et al, 1972). Acid phosphatase and thiamine pyrophosphatase are present in the Golgi apparatus of PV and SO neurones (Osinchak, 1964). Some consider these enzymes tobe specifically involved in the packaging of neurosecretory granules (Jongkind & Swaab, 1967). However these enzymes may be

related to other functions of the Golgi apparatus, although the membranes of neurosecretory granules close to the Golgi apparatus do contain small amounts of acid phosphatase (Osinchak, 1964). After injection of ^{35}S labelled amino acids either intracisternally or close to the cell bodies of the SO nucleus via an indwelling cannula, the label subsequently appears in the posterior pituitary as ADH, oxytocin or neurophysin (Takabatake & Sachs, 1964; Norstrom & Sjostrand, 1971a; Norstrom, 1972; Jones & Pickering, 1972). In the first 90 minutes after ^{35}S-cysteine has been injected into the IIIrd ventricle of dogs, ADH is not labelled (Takabatake & Sachs, 1964). This lag is interpreted as a period in which a hormonally inactive precursor protein is synthesised, which is subsequently modified to form active hormones. Puromycin does not inhibit formation of labelled hormone if injected after the labelled amino acid, but does if given before. This is direct evidence for synthesis of hormonally inactive precursor (Sachs & Takabatake, 1964). There is a similar lag period before neurophysin is labelled (Norstrom & Sjostrand, 1971c). The lag may represent the time for the precursor to be processed by the Golgi apparatus.

Because labelled amino acid is initially incorporated into protein within the SO and PV perikarya anddoes not appear in the posterior pituitary in any form until several hours have elapsed, it is clear that synthesis of the precursor of neurophysin, oxytocin and ADH occurs in the perikarya and is ribosome-dependent. The precursor may not all be cleaved before it leaves the perikarya, although

oxytocin and ADH can be demonstrated in the PV and SO nuclei (Hild & Zetler, 1951). The ratio of ADH to oxytocin is lower in the posterior pituitary than in the SO and PV nuclei; this suggests that oxytocin is formed from the precursor as it is transported along the axons (Lederis, 1962). The neurosecretory granules in the perikarya are smaller and their contents more electron-dense than those in the posterior pituitary; the population in the axons is heterogeneous. These morphological changes may reflect breakdown of a precursor into more osmotically active products, with consequent swelling of the granules (Cannata & Morris, 1973).

2.2.4 Axonal transport of neurosecretory material

Because hormone, neurophysin and the precursor are synthesised in the perikarya then the neurosecretory granules must be transferred to the nerve endings in the posterior pituitary.

Scott (1906) demonstrated that synaptic transmission depends on the perikarya of the presynaptic neurones. To explain this dependence he proposed 'the passage of a portion of this substance /secreted at the ending/ down the nerve fibre to the nerve ending'. Weiss (1967) further developed the concept of axoplasmic flow from the observation that nerve fibres swell on the proximal side of a ligation; this axoplasmic flow rate is about 1 mm per day (Weiss & Hiscoe, 1948).

The observations of Scott have been confirmed for the rat phrenic nerve-diaphragm preparation in vitro (Miledi & Slater, 1970). Neuromuscular transmission, in response to phrenic nerve stimulation, fails after a period of time which depends on the length of the distal segment of the cut phrenic nerve. The transmission failure is accompanied by a decrease in the frequency of miniature end-plate potentials. These changes are consistent with the failure of arrival at the ending of axonally transported materials essential for transmission: the transport rate is about 360 mm per day. Bidirectional movements of particles, at rates of 80-260 mm per day, occur in cultured neurones (Burdwood, 1965). After axotomy of the rat sciatic nerve there is a ten-fold increase within 48 hours in the organelle content of the axonal segment immediately proximal to the lesion (Martinez & Friede, 1970). The additional concept of axonal transport has been introduced to account for the movement along axons of organelles and transmission associated materials at rates faster than are possible by axoplasmic flow.

Two main approaches have been used to study axonal transport. In the first the rates of accumulation or disappearance of endogenous materials are measured on either side of an obstruction to flow: this may be mechanical, for instance a ligation or axotomy, or chemical, produced by colchicine. In the second approach the fate is followed of isotopically labelled moieties, usually amino acids, to which one end of the neurone is exposed.

The ligation method has been used on the rat or cat sciatic nerve to show the proximo-distal transport of noradrenaline, chromogranin and dopamine β-hydroxylase at a rate of about 120 mm per day (Dahlstrom & Haggendal, 1966a; Dahlstrom, 1967, 1971). Acetylcholinesterase accumulates proximal to the site of section of peripheral axons; the rate of accumulation is consistent with a proximo-distal transport rate of 260 mm per day (Lubinska, 1971). Redistribution of transmitters and associated materials in isolated nerve segments without a change in total content confirms that the material transported is synthesised in the perikarya and not in the axons (Dahlstrom & Haggendal, 1966b; Lubinska & Niemierko, 1971). ^{3}H-glycine injected by microiontophoresis into a spinal motoneurone is incorporated into protein and transported throughout the perikaryon, dendrites and along the axon at a rate of at least 10 mm per day (Globus et al, 1968). Spinal cord dorsal root ganglion cells of the cat incorporate ^{3}H-L-leucine injected into the ganglion. The labelled protein is transported along both the central and peripheral processes; two peaks of radioactivity are found in each process, corresponding to transport rates of 1.3 and 500 mm per day (Lasek, 1968). In a variety of mammalian species the average rate of fast axonal transport in the sciatic nerve is 410 mm per day; the rate of fast transport in ascending fibres in the spinal cord is similar (Ochs, 1972a). Inhibition of protein synthesis, with inhibitors such as acetoxycycloheximide, before or after administration of isotopically labelled amino acids, shows that most amino acids are incorporated into

protein before transportation (McEwen & Grafstein, 1968; Ochs, 1972b). The only free amino acid which is transported is γ amino butyric acid. ^{14}C-proline and ^{3}H-fucose are incorporated into microtubular protein and glycoprotein respectively, which are then transported (Bondy, 1971a). The transport of glycoprotein indicates that products of the Golgi apparatus are carried by the fast transport system (Ochs, 1972b).

Axonal transport in the central nervous system is shown by the gradual appearance, after exposure to isotopically labelled amino acids, of labelled protein in the synaptosome fraction: this isnot the result of protein synthesis in synaptosomes (Barondes, 1968).

Studies on PV and SO neurones have also shown fast rates of transport for the neurosecretory materials, although early calculations suggested a transport rate of only 0.19 mm per day (Sloper, 1958). In the acutely stimulated preoptic neurones of the goldfish, neurosecretory granules are transferred along the axons at a rate equivalent to 2880 mm per day (Jasinski et al, 1966). Synthesis of ADH in vitro by hypothalami containing SO perikarya, but not by isolated posterior pituitaries (Takabatake & Sachs, 1964; Pickering & Jones, 1971) is a priori evidence for the transport of neurosecretory products from the cell bodies to the endings in the posterior pituitary. Neurophysin, identified by immunofluorescence, accumulates on the proximal side of a crush injury of the dog pituitary stalk (Alvarez-Buylla et al, 1970). After ^{35}S-cysteine or methionine are injected

intracisternally in the rat the delay between the appearance, shown autoradiographically, of labelled protein in the SO and PV nuclei and its appearance in the posterior pituitary is evidence for proximo-distal transport of neurosecretory protein. This delay has been variously estimated as 5.5 hours (Talanti et al, 1972), 6 hours (Nishioka et al, 1970), 10 hours (Flament-Durand, 1961, 1967) and 18 hours (Sloper & King, 1963). If the SO-hypophyseal tract is 2 mm long the respective transport rates are between 8 and 2.7 mm per day, if the initial lag for precursor synthesis is not considered, or between 12 and 3 mm per day if this lag is taken into account.

After intracisternal injection of ^{3}H-tyrosine, in the rat, ^{3}H-ADH appears in the posterior pituitary within two hours; the equivalent transport rate is 24-48 mm per day, or 48-96 mm per day if the synthesis lag is considered (Pickering & Jones, 1971; Jones & Pickering, 1972). A detailed analysis of transport in the hypothalamo-neurohypophyseal system has been made in rats which were injected with ^{35}S-cysteine via cannulae chronically implanted in the SO nuclei. The labelled proteins were identified by electrophoretic and immunological techniques. The changes in the pattern of labelled proteins at different times in various parts of the system are consistent with three rates of transport, 0.5, 1.5 and 60 mm per day. Neurophysin is transported at the fastest rate (Norstrom & Sjostrand, 1971b,c). If allowance is made for the lag between synthesis of labelled precursor and formation of active hormone and carrier (Takabatake & Sachs, 1964), the fast transport rate may be 200 mm per day(Norstrom & Sjostrand,

1971c). This is comparable to the rate of transport of transmitters and associated proteins in other axons.

2.2.5 Mechanism of axonal transport

It has been proposed that neurotubules are responsible for the fast transport from the perikarya to the nerve endings of materials essential for the maintenance of transmission (Schmitt, 1968; Ochs et al, 1969). In electron micrographs of axons of the lamprey spinal cord structures similar to synaptic vesicles are arranged alongside microtubules, to which they are connected by crossbridges (Smith,D.S., 1971). Microtubules may interact with vesicles by a mechanism similar to that of the proposed sliding filament model of muscle contraction (Schmitt, 1968; Ochs, 1972b). Circumstantial evidence for this suggestion is that axonal transport of isotopically labelled protein depends upon a local energy supply in the form of ATP; and local axonal anoxia blocks axonal transport in the anoxic segment (Ochs, 1972b).

Synaptic transmission in the optic tectum of the pigeon is blocked within three days by intraocular injection of colchicine, which binds with microtubules (Perisic & Cuenod, 1972). Local application of colchicine to post-ganglionic axons of the chick ciliary ganglion begins to block transmission at the post-ganglionic fibre terminals within 24 hours (Pilar & Landmesser, 1972). Local application of colchicine to the sciatic nerve inhibits transport of amine storage granules (Dahlstrom, 1971). After subarachnoid or intracisternal injection of colchicine, in the rat, transport

of ^{35}S-cysteine labelled protein and neurosecretory material from PV perikarya stops (Flament-Durand & Dustin, 1972) and labelled neurophysin does not appear in the posterior pituitary (Norstrom et al, 1971). Similarly, colchicine given intracisternally to dehydrated rats prevents re-accumulation of neurosecretory material or neurosecretory granules in the posterior pituitary when these animals are rehydrated, while neurosecretory material and granules accumulate in the perikarya of SO neurones (Boudier et al, 1972).

2.2.6 Retrograde or disto-proximal axonal transport

There is substantial evidence that in many types of neurone material may be transported from the nerve endings along the axon to the perikaryon. If ^{3}H-leucine is injected into the tongue, it is incorporated into protein in axons of the hypoglossal nerve, and transported within the axons toward the perikarya (Watson, 1968b). Horse radish peroxidase or Evans blue with bovine albumin is similarly transported by rabbit hypoglossal axons, at a rate of 120 mm per day (Kristensson et al, 1971). Disto-proximal transport of ^{14}C-glutamate, without incorporation into protein, occurs in a frog nerve muscle preparation in vitro (Kerkut et al, 1967).

Six days after injection of ^{3}H-leucine into a spinal dorsal root ganglion the sciatic nerve contains isotopically labelled protein; if a segment of this nerve is isolated labelled protein accumulates at both ends of the isolated segment. Bidirectional transport is implied (Lasek, 1967).

Acetylcholinesterase and noradrenaline accumulate on both sides of a ligation or axotomy of a peripheral nerve, but the rates of accumulation and maximum contents are less on the distal side of the lesion (Dahlstrom, 1967; Lubinska & Niemierko, 1971). The disto-proximal transport rate for acetylcholinesterase is 134 mm per day (Lubinska & Niemierko, 1971). Disto-proximal axonal transport occurs in the central nervous system, and has been shown for dopamine and horse radish peroxidase (Ungerstedt et al, 1969; Lavail & Lavail, 1972).

Retrograde axonal transport has not been demonstrated in SO or PV neurones, although the nerve terminals in the posterior pituitary incorporate horse radish peroxidase in vitro (Nagasawa et al, 1971).

The mechanism of retrograde transport is not clear, but it may be similar to that proposed for proximo-distal transport because colchicine prevents disto-proximal transport of Herpes simplex virus (Kristensson et al, 1970).

2.3 Secretion of hormones from the posterior pituitary

2.3.1 Electrical activity of neurosecretory neurones

The electrical properties of SO and PV neurones are similar to those of other neurones; the posterior pituitary hormones are released from the PV and SO nerve endings after the endings have been depolarised.

Harris (1947, 1948a,b,c) used rabbits with implanted electrodes, stimulated remotely, to show that electrical stimulation of the supraoptico-hypophyseal tract causes oxytocin and ADH to be secreted. Direct electrical stimulation

of the PV nucleus in male rats releases oxytocin (Hawker et al, 1959). Electrical stimulation in the region of the PV and SO nuclei in the lactating goat causes milk ejection and antidiuresis respectively (Andersson & McCann, 1955).

The conduction properties of neurosecretory axons were first described in the goosefish, Lophius, and found to be similar to those of other unmyelinated axons (Potter & Loewenstein, 1955), and the electrical properties of preoptic neurosecretory neurones of the goldfish are in all respects similar to those of other neurones (Kandel, 1964). Action potentials can be recorded in the perikarya of SO and PV neurones of the rat when the posterior pituitary is stimulated electrically (Yagi et al, 1966). These antidromically propagated action potentials are similar to action potentials in other neurones (Novin et al, 1970). Action potentials can be recorded from isolated posterior pituitary glands stimulated electrically in vitro, and are abolished if the potassium ion concentration of the medium is raised (Yagi et al, 1966). Direct electrical stimulation of the posterior pituitary causes oxytocin and ADH to be secreted in vivo (Harris, 1947; Wakerley & Lincoln, 1973a) and in vitro (Douglas, 1967).

If posterior pituitary glands are incubated in media which contain depolarising concentrations of ions, usually high potassium, then oxytocin, ADH and neurophysin are released (Douglas, 1963, 1967; Dicker, 1966; Cheng et al, 1972). Acetylcholine or a hyperosmotic medium are not effective stimuli for the release of ADH and oxytocin from posterior pituitaries in vitro (Dicker, 1966).

There is a general relationship between electrical

activity of SO and PV neurones and the secretion of oxytocin and ADH. In rats with diencephalic islands the firing rate of PV neurones and the blood concentration of oxytocin are both decreased (Dyball & Dyer, 1971). During suckling, in lactating rats, a pulse of oxytocin is secreted immediately after each burst of electrical activity in PV neurones (Wakerley & Lincoln, 1973a). Acute hyperosmotic stimulation in cats increases the firing rate of PV neurones and the secretion of ADH and oxytocin (Brooks et al, 1966); and in rats, after acute hyperosmotic stimulation the firing rates of both PV and SO neurones and the secretion of ADH and oxytocin increase. However, ADH secretion does not fall immediately after removal of the osmotic stimulus, so the relationship between electrical activity and ADH secretion may not be simple (Dyball, 1971). Chronic hyperosmotic stimulation, induced by drinking 2% sodium chloride solution instead of water, increases the firing rates of PV and SO neurosecretory neurones (Dyball & Pountney, 1973).

2.3.2 Secretory process

The mechanism by which depolarisation of the PV and SO nerve endings causes the secretion of ADH and oxytocin is of interest because it is during this process of release and its immediate sequelae that the state of the ending is changed. This change may generate a signal which could regulate synthetic activity in the perikarya. There are two mechanisms by which the neurohormones could be discharged from the nerve ending into the perivascular space. Either the contents of the neurosecretory granules are released into the cytoplasm

of the nerve endings and diffuse to and through the terminal membrane (Barer et al, 1963), or the neurosecretory granules fuse with the terminal membrane and discharge their contents by exocytosis (Douglas, 1967, 1968). The weight of evidence favours the latter mechanism.

Whichever mechanism is correct it requires an interaction between some component of the terminal and the neurosecretory granules because, in vitro, ionic conditions which release ADH and oxytocin from intact posterior pituitaries do not release hormone from isolated neurosecretory granules (Barer et al, 1963). ATP stimulates the release of hormone from isolated neurosecretory granules but this action is probably not important in vivo (Douglas, 1968; Poisner & Douglas, 1968; Norstrom, 1972).

Evidence in favour of release of hormone within the endings is partly based on electron micrographs which show decreased opacity of neurosecretory granules when hormone release is stimulated (Monroe & Scott, 1966). It is claimed that changes in electron density of neurosecretory granules can be artefacts caused by the preparative technique (Morris & Cannata, 1973).

The posterior pituitary stores of hormone are heterogeneous; 10 to 20% of the stored hormone is more readily released than the remainder (Sachs et al, 1967). In response to haemorrhage less than 20% of the posterior pituitary stores of ADH are released, and no further ADH is released if the stimulus is repeated within one hour. ADH is not released from the posterior pituitaries of animals so treated when the

posterior pituitaries are incubated in vitro in a medium with a high concentration of potassium ions (Sachs et al, 1967). Posterior pituitaries from dehydrated rats have a similar limited capacity to release ADH: this is not related to the total ADH content (Vilhardt, 1970). In vitro, ATP has a greater effect on the release of ^{35}S-cysteine labelled neurophysin from old neurosecretory granules, labelled 14 days previously, than from young neurosecretory granules, labelled four hours previously. A medium with a high concentration of potassium ions is less effective in causing the release of ^{35}S-cysteine labelled neurophysin from posterior pituitaries labelled 30 days previously than from recently labelled posterior pituitaries. These findings indicate that as they age neurosecretory granules leak neurophysin into an extragranular pool (Norstrom, 1972; Norstrom & Hansson, 1972). The readily releasable pool is intragranular, but may be replenished from the extragranular pool (Norstrom, 1972). The high specific activity of ^{35}S labelled hormone indicates that this readily releasable pool may be newly synthesised (Sachs, 1971; Norstrom, 1972). In contrast, under resting conditions the kinetics of ^{3}H-tyrosine labelled ADH and oxytocin turnover are not consistent with a readily releasable pool (Jones & Pickering, 1972).

The consequences of the exocytosis hypothesis are: that hormone and neurophysin should be released together, that exocytotic profiles should be apparent in electron micrographs of actively secreting posterior pituitaries, and that as the result of fusion of the neurosecretory granule

membrane with the terminal membrane either the membrane expands or there is a mechanism to recapture neurosecretory granule membranes (Bennett, 1956).

Electron micrographs have been published of exocytotic profiles in nerve endings of the posterior pituitary stimulated in vitro by incubation in a high potassium medium (Douglas et al, 1971a). The frequency of exocytotic profiles is increased in the nerve endings of posterior pituitaries taken from rats subjected to haemorrhage (Santolaya et al, 1972). Exocytotic profiles have also been demonstrated in the chromaffin cells of the adrenal medulla (Grynszpan-Winograd, 1971), and at the terminals of rat phrenic axons (Hubbard & Kwanbunbumpen, 1968). In electron micrographs of cat cervical sympathetic ganglia fixed during electrical stimulation the surface area but not the volume of presynaptic terminals is increased and the number of vesicles per ending is decreased (Pysh & Wiley, 1972).

Early observations showed depletion of neurosecretory material and hormones to occur together (Ortman, 1951). When dogs, rabbits or rats are dehydrated one of the proteins disappears which is normally present in the electrophoretic pattern of posterior pituitary extracts (Rennels, 1966; Friesen & Astwood, 1967). This protein is probably neurophysin (Norstrom & Sjostrand, 1972a; Watkins & Evans, 1972). The concentration of neurophysin in blood increases when the secretion of ADH and oxytocin increase (Cheng & Friesen, 1970; Cheng et al, 1972). Neurophysin, oxytocin and ADH are released together from posterior pituitaries incubated in vitro, and the

effects of varying ionic concentrations are similar for all three materials (Uttenthal et al, 1971; Cheng et al, 1972). Neurophysin is released in proportion to oxytocin and ADH in vivo (Forsling et al, 1973) and in vitro (Uttenthal et al, 1971).

The two major predictions of the exocytosis hypothesis are fulfilled: profiles consistent with exocytosis are seen in electron micrographs, and neurophysin and the associated hormones are secreted in proportion to each other.

The incorporation of synaptic vesicle membrane into presynaptic terminal membrane could increase the area of the presynaptic membranes of a stimulated motor neurone by 0.2 to 2 mm^2 per hour (Bittner & Kennedy, 1970). Although during transmission the presynaptic terminal membrane may temporarily increase in area (Pysh & Wiley, 1972), it seems likely that there is a mechanism to recapture neurosecretory granule membrane (Douglas et al, 1971a; Holtzmann et al, 1973). Micropinocytosis occurs in a variety of nerve endings including those of PV and SO neurones. The nerve endings of rat posterior pituitaries incorporate horse radish peroxidase into smooth and coated vesicles in vivo, after intravenous injection, or in vitro (Nagasawa et al, 1971). The formation of coated vesicles (Gray, 1961; Kanaseki & Kadota, 1969) may be the process by which neurosecretory granule membrane is recaptured from the preterminal membrane (Smith, 1970; Douglas et al, 1971b), and may also enable the neurone to sample the synaptic environment (Zachs & Saito, 1969; Smith, A. D., 1971). The failure of neurosecretory granules

to incorporate ferritin indicates that these granules are not reformed after exocytosis and that the small vesicles, which do incorporate ferritin, are formed from the preterminal membrane and not directly from neurosecretory granules within the cytoplasm (Smith, U., 1971). Coated vesicles can be seen apparently forming from the bases of exocytotic profiles (Douglas et al, 1971a). It is possible that coated vesicles shed their coats soon after formation and then have the appearance of 'synaptic vesicles' (Gray & Willis, 1970; Douglas et al, 1971b). Several studies suggest increased formation of small vesicles within nerve endings when transmitter release is stimulated. In foetal mouse spinal cord, horse radish peroxidase incorporation increases with electrical activity (Holtzmann et al, 1973). There are large numbers of small vesicles in electron micrographs of nerve endings of the stimulated posterior pituitary (Douglas et al, 1971a).

It is possible that recaptured neurosecretory granule membrane is used in some way to regulate synthetic activity within the neurone according to secretory activity.

2.3.3 Mechanism of exocytosis

The release of neurophysin, ADH and oxytocin from posterior pituitary glands in vitro depends on calcium ions and is inhibited by magnesium ions (Douglas, 1963; Dicker, 1966; Uttenthal et al, 1971); these ionic dependencies are similar to those of secretory mechanisms in other glands (Douglas, 1968). It is not clear whether depolarisation of PV and SO nerve terminals increases entry of calcium ions into the terminal or releases calcium ions from the terminal

membrane (Douglas, 1968; Rasmussen, 1970). Although neurosecretory granules contain ATP and ATP-ase (Poisner & Douglas, 1968), these may not play a direct part in exocytosis (Douglas, 1968).

The effects of ions on exocytosis have been compared with their effects on muscle contraction (Douglas, 1968). This theme has been developed by Rasmussen (1970), who suggested a role in exocytosis for microtubules. It is of interest in this context that neurosecretory granules are arranged in an orderly fashion in PV and SO nerve terminals (Sachs, 1971), as are synaptic vesicles in other nerve terminals (Hubbard & Kwanbunbumpen, 1968). Colchicine prevents the release of neurosecretory material from the posterior pituitary during dehydration (De Groot, 1957), and causes accumulation of neurosecretory granules in the posterior pituitary (Norstrom et al, 1971), although arrest of axonal transport may be the explanation for these effects (Norstrom et al, 1971; Flament-Durand & Dustin, 1972; Boudier et al, 1972).

If, in the PV and SO nerve terminals, there is a contractile mechanism to move neurosecretory granules into contact with the terminal membrane, the neurosecretory granule membrane and the terminal membrane may then fuse; perhaps as a result of a high lysolecithin content in the former, by analogy with chromaffin granules (Winkler, 1971). The readily releasable pool of hormone is in neurosecretory granules which have recently arrived in the terminal (Norstrom, 1972) and may still be attached to microtubules along which they have been transported.

The fate of ageing neurosecretory granules is not established. PV and SO nerve endings contain lysosomes (Whitaker et al, 1970) which may modify recaptured neurosecretory granule membrane, or may degrade old neurosecretory granules and so regulate the amount of hormone in the posterior pituitary (Shin et al, 1970; Whitaker et al, 1970) in a manner analogous to that in anterior pituitary prolactin cells (Farquhar, 1971). The posterior pituitary contains cathepsins, although the cellular origin of these enzymes is not known (Pickup & Hope, 1971). ^{3}H-L-vasopressin is taken up and broken down in vitro by posterior pituitaries, but not by cerebral cortex (Pliska et al, 1971).

2.4 Electrical activation of neurosecretory neurones

2.4.1 Osmosensitivity

The classical studies of Verney (1947) established that there are 'osmoreceptors within the vascular bed of the internal carotid artery', which, when stimulated by intracarotid injection of hypertonic saline, cause the release of ADH from the posterior pituitary. Subsequent work has demonstrated neurones in the PV and SO nuclei which alter their firing rates afterintracarotid hyperosmotic stimulation: but it is not known whether neurosecretory neurones are also osmoreceptors.

The activity of single units, recorded with microelectrodes, in the SO and PV nuclei may be increased or decreased by injection of hypertonic saline into a carotid artery (Cross & Green, 1959; Koizumi et al, 1964; Brooks et al, 1966). Neurones

outwith these nuclei also respond (Cross & Green, 1959; Brooks et al, 1966). The PV and SO responses still occur in hypothalamic islands; this indicates that the osmoreceptors are in the hypothalamus (Koizumi et al, 1964).

The responses of neurones in and around the SO nucleus have been studied in conscious, restrained rhesus monkeys with indwelling electrodes. Intracarotid injection of hypertonic saline elicits a biphasic response, excitatory then inhibitory, in neurones within the SO nucleus and a monophasic, excitatory response in neurones around the SO nucleus. The biphasic responses may occur in neurosecretory neurones and the monophasic responses may occur in separate osmoreceptors (Vincent & Hayward, 1970). However, in these studies active units were not identified as neurosecretory neurones.

Neurosecretory neurones in the PV and SO nuclei can be identified as those neurones from which antidromically propagated action potentials are recorded when the posterior pituitary, which contains their endings, is electrically stimulated (Kandel, 1964; Yamashita et al,1970; Sundsten et al, 1970).

Antidromically identified PV and SO neurones respond to hyperosmotic stimulation. After intracarotid injection of hypertonic saline excitatory responses occur in slow firing units, and inhibitory responses in fast firing units (Dyball, 1971). Increased activity of SO neurones continues for an hour after a single intracarotid injection of hypertonic saline (Dreifuss & Kelly, 1972). In rats given 2% sodium chloride solution to drink for three days there are more fast

firing antidromically identified neurosecretory neurones in the SO and PV nuclei than in controls. The firing rates are unaltered in units identified as non neurosecretory (Dyball & Pountney, 1973).

Although osmosensitivity of neurosecretory neurones has been established it is still not clear whether they are also osmoreceptors; other neurones may have this function.

2.4.2 Synaptic transmission

Intracarotid injections of acetylcholine or adrenaline excite or inhibit respectively the secretion of ADH (Abrahams & Pickford, 1956). The perikarya of SO and PV neurones have synapses (Peterson, 1965; Kalimo, 1971). Large numbers of noradrenergic terminals in the SO and PV nuclei have been demonstrated by fluorescence microscopy (Fuxe & Hokfelt, 1967). The number of granulated vesicles in presynaptic terminals within the PV and SO nuclei are increased by treatment with nialamide and L-dihydroxyphenylalanine; this effect confirms that noradrenergic terminals are present in the PV and SO nuclei (Zambrano, 1968). Acetylcholinesterase is present inSO and PV perikarya (Rinne, 1966).

The actions of various drugs implicate both cholinergic and monoaminergic synaptic mechanisms in the control of the release of ADH and oxytocin. Peripherally administered reserpine inhibits the milk ejection reflex (Moon & Turner, 1959); so do 5-hydroxytryptamine and iproniazid (Mizuno et al, 1967). Carbachol, injected into the IIIrd ventricle of lactating rats, causes milk ejection and antidiuresis (Kuhn & McCann, 1971). The effects of intraventricular injection

of various drugs suggest that acetylcholine acts on muscarinic receptors to stimulate, and through adrenergic β receptors to inhibit ADH secretion, while noradrenaline acts on adrenergic α receptors to stimulate ADH secretion (Bhargava et al, 1972). Acetylcholine releases oxytocin from isolated rat hypothalamo-posterior pituitary units in vitro (Nordmann et al, 1971). Such studies have not established the properties of synapses upon the PV and SO perikarya.

The properties of these synapses have been defined by microiontophoretic application of various transmitters and inhibitors to the perikarya of antidromically identified PV and SO neurones. Noradrenaline acts on β receptors to inhibit 83-90% of antidromically identified neurosecretory neurones in the SO or PV nucleus, and excites none (Barker et al, 1971b, d; Moss, Urban & Cross, 1972). There is less consistency in reports on the effects of microiontophoretic application of acetylcholine. In the cat acetylcholine inhibits 84% and excites 16% of SO neurosecretory neurones by acting on muscarinic and nicotinic receptors respectively (Barker et al, 1971b,d). In the rat and rabbit acetylcholine excites more SO or PV neurones than it inhibits, although most of the excitatory responses of SO or PV neurones are mediated by nicotinic receptors (Moss et al, 1971; Dreifuss & Kelly, 1972; Moss, Urban & Cross, 1972). Acetylcholine may have variable effects because there are nicotinic and muscarinic receptors on the same neurone (Barker et al, 1971b,d; Moss, Urban & Cross, 1972). Excitatory responses may be mediated by nicotinic or muscarinic receptors (Moss, Urban & Cross,

1972), although inhibitory responses of SO neurones are all mediated by muscarinic receptors (Barker et al, 1971), and so are most inhibitory responses of PV neurones (Moss, Urban & Cross, 1972). The response to acetylcholine will presumably depend on the relative numbers of available excitatory and inhibitory receptors at any time.

The organisation of synapses on SO and PV neurones is complicated by recurrent axon collaterals, which may be inhibitory or excitatory. Inhibitory post synaptic potentials can be recorded from the perikarya of cat SO or PV or goldfish preoptic neurones when these neurones are stimulated antidromically (Kandel, 1964; Barker et al, 1971c). Spontaneous activity of the neurosecretory neurones in either the PV or SO nucleus is inhibited when the SO or PV nucleus, respectively, is stimulated (Yamashita et al, 1970); although Laqueur (1954) did not find PV neuronal terminals in the SO nucleus. Inhibitory recurrent collaterals, operating via interneurones, have been postulated to explain biphasic responses to a hyperosmotic stimulus in presumed neurosecretory neurones and monophasic responses in presumed osmoreceptors (Vincent & Hayward, 1970). A similar mechanism was proposed to explain phasic responses of antidromically identified neurosecretory neurones to iontophoretically applied acetylcholine and inhibitory responses to an acute hyperosmotic stimulus (Dyball, 1971; Dreifuss & Kelly, 1972).

Pharmacological blockade of synaptic transmission does not prevent recurrent inhibition in the SO nucleus; lysine vasopressin applied iontophoretically inhibits 50% of

antidromically identified SO neurones, but excites cerebral cortical neurones. It is probable that ADH acts as the inhibitory transmitter, without the intervention of an interneurone (Nicoll & Barker, 1971). This recurrent inhibitory system may restrict secretion of ADH in response to a persistent stimulus. In contrast, oxytocin applied iontophoretically stimulates 90% of antidromically identified neurosecretory PV neurones; too few SO neurones have been studied to allow any conclusions to be drawn (Moss, Dyball & Cross, 1972). Intravenous oxytocin is not effective (Dyball & Dyer, 1971). Conversely, a large dose of oxytocin injected into the IIIrd ventricle of lactating rats inhibits milk ejection, although the site of action is not known (Kuhn & McCann, 1970).

Periodically during suckling the firing rate of antidromically identified PV neurones accelerates (Wakerley & Lincoln, 1973); recurrent excitatory collaterals which release oxytocin may synchronise these bursts of activity. Such a mechanism could ensure that a pulse of oxytocin is secreted which is adequate for milk ejection. Recurrent inhibitory collaterals of neurones secreting ADH may inhibit simultaneous release of oxytocin, if oxytocin and ADH are secreted by separate neurones. Recurrent inhibitory collaterals end not only on the neurone of origin but also on adjacent neurones (Barker et al, 1971c).

It is not known what proportion of the neurosecretory neurone's content of ADH or oxytocin is liberated at the endings of recurrent collaterals, but it is presumably small compared with the amount secreted by the posterior pituitary.

2.4.3 Peripheral stimulation: milk ejection reflex

The milk ejection reflex was formulated by Turner & Slaughter (1930): 'nerve paths would lead to the pituitary gland causing the discharge of the hormone which would in turn bring about the well known change in the /mammary/ gland'. They showed that injections of posterior pituitary extracts into lactating cows at the time of milking increased the milk yield. The distinction between milk secretion and ejection was later emphasised by Ely & Peterson (1941). They found that the effects of injected posterior extracts on milk yield did not depend on an intact nerve supply to the mammary gland.

Suckling rabbits obtain only small quantities of milk if their mothers are anaesthetised or have lesions of the SO-hypophyseal tract: the milk yield can be increased by oxytocin injection. Electrical stimulation of the intact SO-hypophyseal tract or intravenous injections of oxytocin increase intramammary pressure and milk flows copiously (Cross & Harris, 1952).

Subsequent studies in many mammals have confirmed the importance of the milk ejection reflex in lactation (e.g. Harris, 1958; Cross, 1961; Denamur, 1965).

Removal of the posterior pituitary glands of lactating rats prevents litter growth, which can be partly restored by oxytocin injections twice daily (Benson & Cowie, 1956). A specific antibody to oxytocin injected into rats in late pregnancy reduces litter growth for the first four days of lactation (Kumaresa et al, 1971). Changes in the posterior

pituitary content of oxytocin during lactation are not marked, although the increased ADH to oxytocin ratio during lactation is consistent with increased oxytocin secretion (Dicker & Tyler, 1953; Acher et al, 1956; Heller, 1959; Denamur, 1965). The concentration of oxytocin in venous blood of the lactating rabbit is increased by suckling (Bisset et al, 1970). The quantitative characteristics of the mammary response to posterior pituitary stimulation or exogenous oxytocin are identical, and is further proof that oxytocin is released in response to suckling (Cross & Harris, 1952). The turnover of neurophysin in the posterior pituitary is increased in lactating animals (Norstrom & Sjostrand, 1972b), but only a slight and insignificant increase is found in the neurophysin concentration in venous blood of lactating rats removed from their litters for ½ to 2 days and killed after then nursing their litters for 30 minutes (Cheng et al, 1970, 1972). Because oxytocin is released intermittently during suckling (vide infra) a similar intermittent release of neurophysin is expected, which may explain the failure of Cheng et al to detect increased neurophysin secretion during suckling. In lactating rats, anaesthetised with Nembutal, the weight gain of a litter suckling for 30 minutes is a function of the dose of injected oxytocin (Grosvenor & Turner, 1957a). Similarly in the rabbit milk yield varies with the amount of oxytocin secreted, assayed endogenously, in response to suckling (Fuchs & Wagner, 1963).

The major stimulus which releases oxytocin during lactation is suckling. Suction applied to the nipples of lactating cats increases electrical activity of units in the PV nucleus and causes the secretion of oxytocin (Brooks et al, 1966). Intramammary pressure rises in the anaesthetised or conscious lactating rat only after suckling has commenced (Wakerley & Lincoln, 1971; Lincoln, Hill & Wakerley, 1973). Milk ejection does not occur in response to suckling in lactating rabbits if the nipples are anaesthetised (Findlay, 1968) and lactation fails in rats if the nipples are denervated by spinal dorsal root section or by spinal cord transection (Eayrs & Baddeley, 1956; Edwardson & Eayrs, 1967; Beyer & Mena, 1970). A stimulus adequate to maintain lactation in the rat can be applied if only two nipples are innervated, but if the innervation to these nipples is progressively reduced milk yield falls progressively (Edwardson & Eayrs, 1967). However, these studies did not distinguish milk ejection from milk secretion.

A detailed quantitative analysis of the milk ejection reflex has recently been made, based on four key observations:

1. Rat litters display a synchronised and periodic behaviour, the 'stretch reaction', during suckling. This reaction can be evoked by oxytocin, ADH or acetylcholine, but not other milk ejecting agents, injected into the anaesthetised mother (Vorherr et al, 1967).
2. The stretch reaction occurs within 1 second after each and every milk ejection. The threshold of the

stretch reaction is higher than the threshold of milk ejection in response to oxytocin, but milk ejections equivalent to the release of oxytocin in amounts subthreshold for the stretch reaction are rarely (Lincoln, 1973) or never (Lincoln et al, 1973) observed. The frequency of the stretch reaction can be used to measure the frequency of milk ejections (Wakerley & Lincoln, 1971).

3. The rise in intramammary pressure and the stretch reaction associated with each milk ejection can be mimicked by the intravenous injection of oxytocin (Wakerley & Lincoln, 1971; Wakerley et al, 1973).
4. The milk ejection reflex in the rat is not abolished by surgical anaesthesia (Lincoln, 1973; Lincoln et al, 1973).

The description of the milk ejection reflex which has been obtained by using these observations is as follows:

Milk ejection in the rat is the result of secretion of a pulse of between 0.4 and 1.5, but on average 1, mU of oxytocin from the posterior pituitary gland. The secretion of each pulse of oxytocin is preceded by a synchronised, explosive increase in the firing rate of PV neurones (Wakerley & Lincoln, 1973). Milk ejection starts after a latent period of about 12 minutes from the onset of suckling in conscious animals continuously with their litters, and the mean interval between milk ejections thereafter is 10.5 minutes. Milk ejections are more frequent if the suckling stimulus is continuous; removal of pups from the nipples

for more than 1 minute increases the next milk ejection interval (Lincoln et al, 1973). The frequency of milk ejections is increased if the mammary glands are distended with milk; milk ejection is inhibited by pain, but not by surgical anaesthesia. Milk ejections only occur when pups are attached to the nipples, and are abolished if the nipples are anaesthetised, or the posterior pituitary is removed (Wakerley & Lincoln, 1971). During a normal lactation 50-80 mU of oxytocin may be secreted each day in response to suckling (Lincoln et al, 1973).

In the rabbit, there is a quantitative relationship between the number of suckling young and the amount of oxytocin secreted during each milk ejection, measured as the rise in intrauterine pressure which accompanies milk ejection (Fuchs & Wagner, 1963). In the studies on lactating rats, described above, litters of 10 pups were used; if the number of pups suckling is reduced to 5, the lag to the first milk ejection increases and the subsequent intervals may also increase; if the number of pups is reduced further then milk ejections are 'rare', although each is equivalent to an amount of oxytocin similar to that secreted with 10 pups suckling (Lincoln et al, 1973).

Suckling young release stimuli other than suckling which may reflexly cause secretion of oxytocin (Tindal & Knaggs, 1970). If litters are only permitted to suckle denervated nipples litter weight gain is reduced to zero; continued secretion, and ejection, of some milk is implied (Grosvenor, 1964). After the seventh day post partum the milk ejection reflex operates with a shorter latency in lactating

rats if they are exposed only to auditory signals from the pups for 15 minutes prior to suckling. This effect can be mimicked by 20 mU of oxytocin per 100 g body weight injected before the pups are permitted to suckle (Deis, 1968).

In summary, the pattern of oxytocin secretion in lactation is determined by temporal and spatial summation of the suckling stimulus, modulated by information from mammary gland volume receptors and other stimuli.

ADH is probably also secreted during suckling (Cross, 1951; Abrahams & Pickford, 1954; Pickford, 1960), although this is disputed (Bisset et al, 1970).

2.5 Other effects of suckling

Selye (1934) demonstrated the importance of suckling in the maintenance of milk secretion. He proposed that suckling reflexly causes the secretion of prolactin. The existence of this neuroendocrine reflex has since been confirmed.

The amount of prolactin measured by bio- or radioimmunoassay in the anterior pituitaries of lactating rats decreases during a 30 minute suckling period after the litters and mothers have been separated for 10-12 hours (Grosvenor & Turner, 1957b; Amenomori, Chen & Meites, 1970). In similar experiments the number of exocytotic profiles in prolactin cells (Shiino et al, 1972) and the serum prolactin concentration, measured by radioimmunoassay, are increased by suckling (Amenomori et al, 1970; Shiino et al, 1972; Blake & Sawyer, 1972).

Lactation ceases when afferent fibres from the mammary glands are cut and is not maintained by exogenous oxytocin treatment (Grosvenor, 1964). This is clear evidence that milk secretion is suckling dependent. However, pups older than 14 days can stimulate the release of prolactin when they are in close proximity to their mothers but prevented from suckling (Grosvenor, 1965); indeed adult female rats may exert a similar effect (Mena & Grosvenor, 1972).

ACTH and growth hormone are essential for normal lactation (Cowie, 1957; Cowie et al, 1960) and are secreted in response to the suckling stimulus (Grosvenor et al, 1968; Voogt et al, 1969).

Milk yield is related to the demands of the litter (Bateman, 1957; Blaxter, 1961). Milk production, measured indirectly as litter weight gain, increases with the age and size of the litter (Cowie & Folley, 1947; Schultze, 1954; Reddy & Donker, 1964; Tucker et al, 1967; Edwardson & Eayrs, 1967; Ota & Yokoyama, 1967b). Maximum daily litter weight gain may be reached with litters of 12 pups (Schultze, 1954; Edwardson & Eayrs, 1967) and with litters of less than three pups lactation is shortened (Bruce, 1961).

Spatial summation of the suckling stimulus occurs with respect to milk yield (Edwardson & Eayrs, 1967) and other parameters related to lactation.

After the post partum ovulation further ovulation is inhibited only if the litter is kept with the mother (Stotsenburg, 1923; Slonaker, 1925). The duration of lactation diestrus is related to litter size (Weichert &

Schurgast, 1942; Rothchild, 1960; Edwardson & Eayrs, 1967).

Plasma corticosterone concentration rises more with six pups than with two pups suckling (Smith & Convey, 1970), and the secretion of growth hormone is similarly related to the number of pups suckling (Grosvenor et al, 1968).

Suckling can stimulate the secretion of the lactogenic complex of hormones, and restart lactation, in non-lactating multiparous rats (Uyldert, 1943; Bruce, 1958; Ota & Yokoyama, 1965). Suckling prolongs normal lactation if suitable litters are used (Nicoll & Meites, 1959; Bruce, 1961) and can induce pseudopregnancy in non-lactating multiparous rats (Selye & McKeown, 1934; Bruce, 1958; Ota & Yokoyama, 1965).

In summary, suckling plays an essential role in lactation by regulating the secretion of anterior and posterior pituitary hormones. Milk yield reflects temporal and spatial summation of the suckling stimulus. The interaction between the hormones secreted in response to suckling must be considered when any one of them is studied.

2.6 Metabolic changes in neurones

The cytological and cytochemical features of resting neurones indicate continuous ribosome and protein synthesis. This is confirmed by kinetic studies with isotopically labelled precursors (Section I - 2.2.1, 2.2.3). Under conditions in which increased amounts of materials associated with transmission or neurosecretion are used or in which the neurone is growing or regenerating its axon the activity of all or a part of the synthetic machinery will increase.

2.6.1 Axonal injury

For a long time chromatolytic changes in the perikarya of axotomised neurones were interpreted as signs of decreased protein and RNA synthesis (Lieberman, 1971).

The correct interpretation of chromatolysis arose from the early studies of absorption spectra of nerve cells after axotomy (Hyden, 1943). However, this pioneering work did not include measurements integrated with respect to area, and Hyden interpreted the delay of three weeks before perikaryal RNA and protein concentration rose as a period in which the axotomised neurone lay fallow. Total perikaryal nucleic acid and protein content decrease only for the first three or four days after axotomy; thereafter these parameters increase; because cell volume also increases the concentrations of protein and RNA decrease for about 12 days. After this period increased cell volume is matched by increased RNA and protein content. The cells return to normal about 70days after axotomy (Brattgard et al, 1957).

Watson (1968a, 1969) measured similar changes in the total content and concentration of RNA and protein in the isolated perikarya of axotomised rat hypoglossal neurones. In addition he found that the latency before perikaryal RNA and protein content increase is shorter when axotomy is performed closer to the perikarya (Watson, 1968a). RNA and protein synthesis, measured autoradiographically, increase within 48 hours of axotomy (Watson, 1965a), whilst after actinomycin D the perikaryal nucleic acid content decreases more rapidly in axotomised neurones (Torvik & Heding, 1967;

Watson, 1968a). Similarly after puromycin the rate of decrease of perikaryal dry mass is greater in axotomised than in intact neurones (Watson, 1969). These changes are consistent with increased synthesis and turnover of cytoplasmic RNA and protein within three to seven days after nerve injury. It can be inferred from electron micrographs that these changes are partly concerned with restoration of axoplasmic constituents (Takano, 1964).

During the phase of axonal regeneration synthesis of export proteins may be repressed (Watson, 1966, 1968a, 1973a). Interruption of functional contact between the nerve endings and tongue muscle with Botulinum toxin causes similar changes to axotomy, with the exception that perikaryal dry mass increases much earlier; probably because increased outflow of protein from the cell body does not occur (Watson, 1969).

Functional stimulation or axotomy has long been recognised to increase the nucleolar volume of the affected neurones (e.g. Guerrini, 1902; Hyden, 1943; Cragg, 1970). Since the cytoplasmic changes of injured neurones have been reinterpreted the temporal relationship of these changes to the nucleolar responses of axotomised neurones have been clarified. In general, nucleolar dry mass and RNA content increase about 24 hours before perikaryal RNA and protein content increase, while the maximum rate of transfer of RNA from nucleus to cytoplasm coincides with the peak nucleolar RNA content and dry mass (Watson, 1968a, 1969). Chromatolysis is inhibited by actinomycin D given at the time axotomy

is performed (Torvik & Heding, 1967) and more ^{3}H-actinomycin D is bound to isolated nuclei from axotomised than from normal hypoglossal neurones (Watson, 1973b).

The metabolic responses of the neurone to injury can be viewed as the result of derepression of ribosomal DNA, with increased synthesis and transport to the cytoplasm of ribosomes, and subsequent increased protein synthesis(Watson, 1968a, 1969).

2.6.2 Functional stimulation: relationship between secretory activity and metabolic changes

There is less complete evidence that the synthesis of ribosomes and protein within neurones increase after, or during, a period of stimulation. Much of this evidence is derived from studies of neurosecretory neurones.

If changes in protein and RNA synthesis are to be interpreted in relation to functional demand and to the classes of protein synthesised then the stimuli applied to the neurones should be controlled and the output of transmitter and associated material measured. Studies other than of functionally identifiable neurones are of limited value in this context.

It is possible that a neurone normally synthesises neurohormone at a rate which exceeds any possible demand; the surplus being broken down without release at the endings. In such a system increased rate of synthesis of neurohormone need not follow increased secretion. Clearly this system would be uneconomic, but it cannot be dismissed. However, the responses of neurosecretory neurones to increased

functional demand show that the rate of secretion in such circumstances exceeds the resting rate of supply.

This is implied by the loss of most of the neurosecretory material and hormone from the posterior pituitaries of dehydrated rats (Ortmann, 1951, and confirmed by many others). Increased secretion of ADH during chronic hyperosmotic stimulation is confirmed by measurement of the concentration of ADH in blood, which increases five-fold within 72 hours of water deprivation (Little & Radford, 1964a).

In lactation changes in the amount of neurohormone in the posterior pituitary are not consistently observed (Stutinsky, 1953; Dicker & Tyler, 1953; Malandra, 1955; Racadot, 1957; Heller, 1959). From a total pool of 350 mU in the posterior pituitary of the normal, non-lactating, rat endogenous oxytocin labelled with ^{3}H-tyrosine disappears at a rate of 19 mU per day, measured seven days after exposure to isotope; the resting synthesis rate may be similar (Jones & Pickering, 1972). From kinetic studies of the clearance of oxytocin a secretion rate for oxytocin an order of magnitude greater, 50 μU/100 g/minute or 180 mU/250 g rat/24 hours, has been calculated (Fabian et al, 1969). The lower estimate is consistent with the half life of neurophysin, isotopically labelled in vivo, measured by others (Norstrom & Sjostrand, 1971c).

In lactation 80 mU of oxytocin may be secreted daily in response to suckling (Lincoln et al, 1973); this is about four times the estimated resting synthesis rate of 19 mU per day, which may include breakdown without secretion. Alternatively the high

estimate of resting daily secretion rate, 180 mU per day, is increased by about 50% during lactation. If the content of oxytocin in the posterior pituitary does not decrease during lactation (Heller, 1959), then because 99% of hypothalamic oxytocin is in the posterior pituitary (Van Dyke et al, 1957; Lederis, 1962), oxytocin synthesis must increase during lactation.

The posterior pituitary stores of ADH and neurosecretory material are rapidly replaced when dehydrated rats are rehydrated (Bachrach, 1957; Moses & Miller, 1970). ADH synthesis greater than the resting rate is implied.

Increased production of neurohormone could result from an increased rate of maturation of a large pool of precursor, but the evidence indicates that synthesis of precursor is increased. The total radioactivity of ^{35}S ADH synthesised by dog hypothalami in vitro is greater if the hypothalami are from dehydrated dogs (Takabatake & Sachs, 1964). The lag period before ^{35}S ADH can be detected is unaltered by dehydration. If rats are given 1% or 2% sodium chloride solution to drink for several days and ^{35}S-cysteine is then injected into the SO nucleus the total radioactivity of neurophysin in the posterior pituitary is initially higher, but more rapidly depleted, than in normal rats. The total content of neurophysin in the posterior pituitary is less. Conversely the turnover rate of endogenously labelled ^{35}S-neurophysin is decreased by water loading (Norstrom & Sjostrand, 1972a). After haemorrhage the bulk transport of previously synthesised neurophysin

increases, and subsequently more neurophysin is synthesised (Norstrom & Sjostrand, 1971d).

The specific activity of neurophysin, labelled by injection of ^{35}S-cysteine into the SO and PV nuclei, reaches a maximum in the posterior pituitaries of lactating rats sooner than in controls. The specific activity then declines more rapidly in lactating rats (Norstrom & Sjostrand, 1972b).

These studies indicate that when secretion of neurohormone increases neither the time taken for the synthesis of each molecule of neurohormone precursor nor the time required for transport of each molecule to the posterior pituitary is altered: but the bulk of precursor synthesised in unit time and the bulk of neurophysin arriving in the posterior pituitary are increased.

The newly synthesised neurophysin is secreted preferentially after haemorrhage or chronic osmotic stress, but not in lactation (Norstrom & Sjostrand, 1971d, 1972a,b). The neurosecretory granules formed in stimulated neurones may not be identical to those formed in resting neurones; oxytocin is more readily released from isolated neurosecretory granules from the posterior pituitaries of lactating rats than from those of non-lactating females (Barer et al, 1963).

Autoradiography has been used to show that during chronic osmotic stress or lactation PV and SO neurones synthesise more protein and the turnover of labelled proteins in the posterior pituitary increases (Wells, 1961; Sloper & King, 1963; Flament-Durand, 1966; Talanti, 1971). More sedentary protein is also synthesised during chronic osmotic stress (Murray, 1967).

Increased synthesis of neurohormone could result from increased ribosome activity or from increased ribosome synthesis. At rest, ribosomes are continually synthesised (Section I - 2.2.1). The evidence supports the concept that increased ribosome synthesis is the mechanism by which synthesis of neurohormone is increased.

PV and SO neurones of water-deprived or salt loaded rats or mice incorporate more ^{3}H-uridine and ^{3}H-cytidine into RNA compared with other neurones in the same animals or PV and SO neurones in normal animals (Watson, 1965b; Norstrom, 1971).

Qualitative studies of PV and SO neurones of lactating or dehydrated rats suggest perikaryal enlargement and chromatolytic changes in the Nissl substance (Hillarp, 1949; Cavallero & Malandra, 1958; Enestrom, 1967). These observations are confirmed by quantitative studies. Perikaryal volume and cytoplasmic basophilia of SO and PV neurones increase in salt loaded rats (Campanacci, 1960; Sloper & King, 1963). The RNA content of SO neurones increases during dehydration but not lactation; PV neurones have not been studied (Edstrom, Eichner & Schor, 1961). The nucleic acid content and dry mass of isolated SO perikarya from dehydrated rats increase after the third day of water deprivation (Watt, 1970).

Qualitative assessment of electron micrographs of SO and PV neurones from dehydrated rats supports the conclusion that ribosome and protein synthesis are increased in these neurones. Filamentous material appears in the dilated endoplasmic

reticulum cisternae in the SO perikarya of dehydrated rats while the number of ribosomes appears to increase, particularly those free in the cytoplasm (Zambrano & DeRobertis, 1966a,b; Enestrom, 1967; Rechardt, 1969).

Some microscopists attach significance to the greater number of dark, or strongly basophilic, neurones in the SO nucleus during dehydration (Enestrom, 1967), and consider these neurones to be at a stage of increased protein synthesis in a cycle of synthesis and secretion (Zambrano & De Robertis, 1966b). Others regard 'dark' neurones as fixation artefacts (Kalimo, 1971). However, in the mink SO perikarya which contain few neurosecretory granules incorporate more ^{3}H-uridine than do those with large numbers of neurosecretory granules (Golubitsa & Korotchkin, 1971).

Prolonged functional stimulation of SO and PV neurones has been frequently reported to result in enlargement of nucleoli. There is a linear relationship between nucleolar volume and perikaryal RNA content (Edstrom & Eichner, 1958).

After chronic stimulation by water deprivation for about five days or salt loading for about seven days the nucleolar volumes, measured in fixed histological sections, are increased in both SO and PV neurones (Hillarp, 1949; Ortmann, 1951; Wolter, 1956; Bachrach & Koszegi, 1957; Rinne, 1960; Campanacci, 1960; Watson, 1965b; Enestrom, 1967; Murray, 1967). Nucleolar dry mass and nucleic acid content of rat SO neurones increase within 48 hours of water deprivation, and nucleolar changes precede changes in perikaryal total nucleic acid content or dry mass (Watt, 1970).

Reports of measurements on SO and PV neuronal nucleolar volume during lactation conflict. Nucleolar volumes of SO and PV neurones either increase (Flament-Durand, 1966; Swaab et al, 1971) or do not alter significantly (Rinne, 1960).

In general, the reported changes in nucleolar volume, dry mass or nucleic acid content of PV and SO neurones are consistent with other evidence that ribosome and protein synthesis increase during chronic stimulation.

During dehydration two new species of RNA appear in SO neurones; these may be newly synthesised messenger RNA (Norstrom, 1971). The earliest measured response of SO neurones during water deprivation is increased nuclear volume (Watt, 1970), without a change in nuclear dry mass (Watt, personal communication). Nuclear swelling is an essential step in DNA derepression (Leake et al, 1972) whereby ribosome synthesis is increased.

The Golgi apparatus of SO and PV neurones contains more thiamine pyrophosphatase during dehydration or lactation. This change may reflect a greater rate of packaging of neurosecretory material by the Golgi apparatus (Jongkind & Swaab, 1967; Jongkind, 1969; Swaab & Jongkind, 1970).

The rate of transport, as distance per unit time, of export protein along neurosecretory axons and in spinal cord motor neurones is not increased upon stimulation (Lux et al, 1970; Norstrom & Sjostrand, 1971d, 1972a,b), although the bulk of material transported in unit time does increase (Norstrom & Sjostrand, 1971d, 1972a,b).

In summary, in PV and SO neurones, when the rate at which secretory products are released from the endings is increased, the synthesis of proteins, including and associated with these secretory products, is increased. These synthetic changes involve increased activity of nucleoli. There is little evidence on the quantitative relationship between these changes and the functional load. Changes in SO perikaryal and nucleolar dry mass and nucleic acid content are greater when rats are dehydrated at a higher environmental temperature (Watt, 1970). SO neurones synthesise RNA at a greater rate with more severe salt loading (Watson, 1965b).

Apart from the orthodox stimuli which control the secretion of oxytocin and ADH from SO and PV neurones, other hormonal changes which result from these stimuli (Section I - 2.5) may modify SO or PV neuronal secretory or synthetic activity, or both.

2.7 General aims of investigation

1. To measure changes in SO and PV neuronal nucleoli during lactation (Section III).

2. To investigate the dependency of such changes on the suckling stimulus, and the quantitative relationship between nucleolar changes and secretory activity (Sections III, IV).

3. To explore the role of other hormones in producing these changes (Section V).

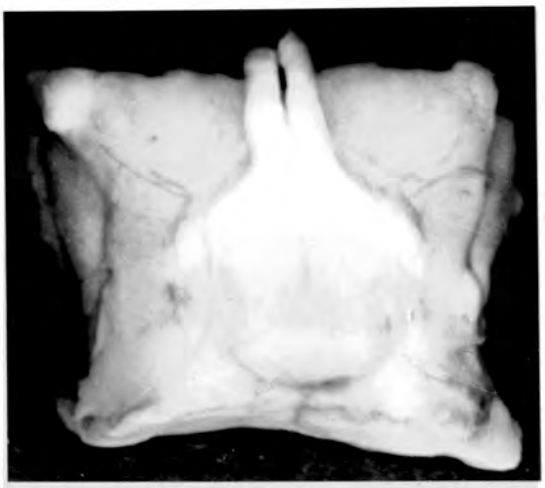
4. To see if functional activity of PV and SO neurones can be distinguished by applying qualitatively different stimuli simultaneously and separately (Section VI).

5. To measure nucleolar changes in other hypothalamic neurones after castration or adrenalectomy (Section VII).

Nucleolar dry mass was selected as the parameter to indicate changes in nucleolar activity: in some experiments nucleolar nucleic acid content was also measured.

SECTION II

GENERAL DESCRIPTION OF METHODS



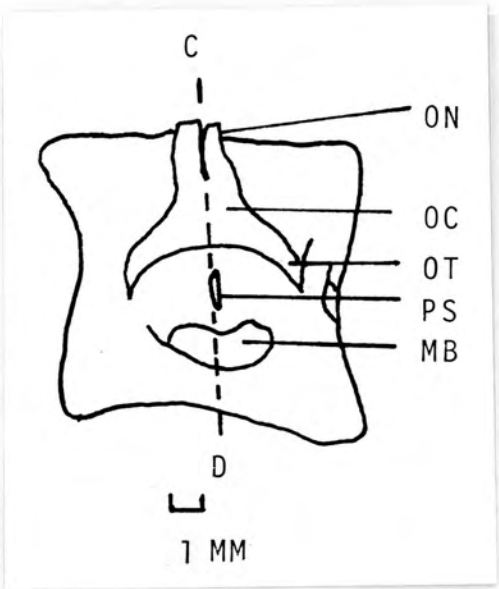


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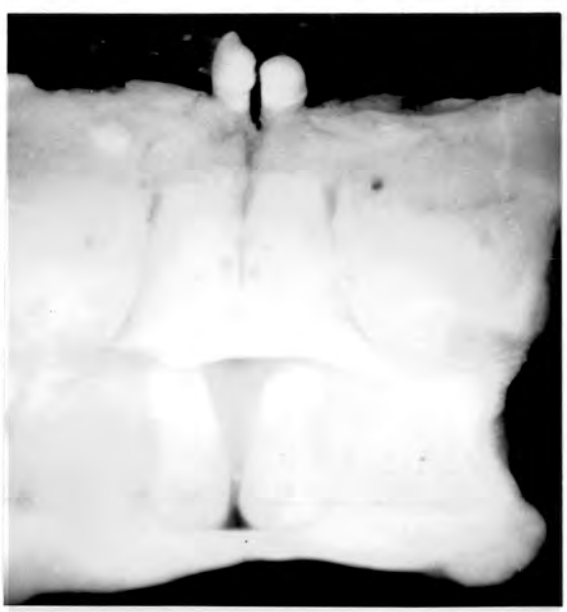
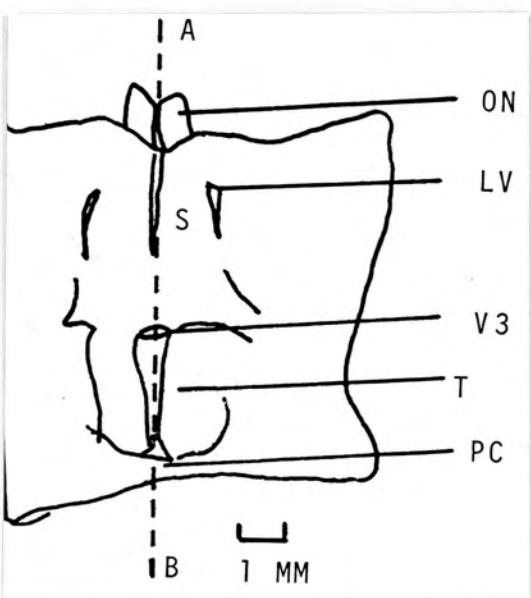
Figure 1 Block of tissue containing hypothalamus

a. Ventral aspect

b. Dorsal aspect. The block was bisected along line A-B.

b





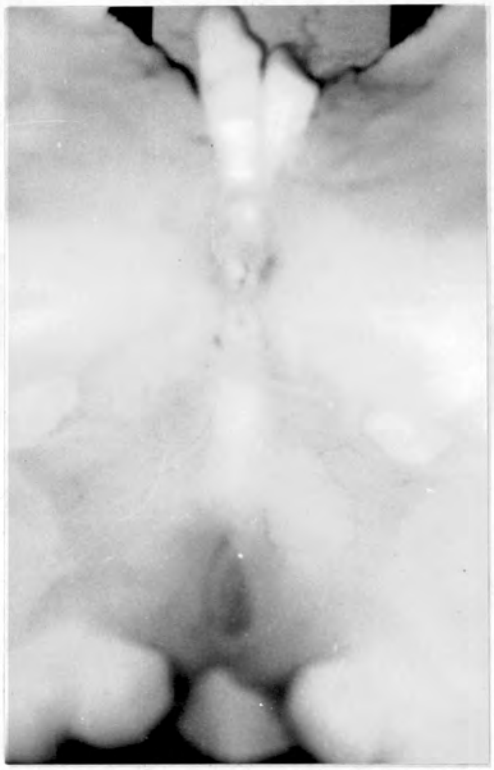
List of abbreviations used in Figs. 1-15

Hypothalamic nuclei

AH	Anterior hypothalamic
ARC	Arcuate
DM	Dorsomedial
MPO	Medial preoptic
PO	Preoptic, pars suprachiasmatica
PH	Posterior hypothalamic
PV	Paraventricular
SCH	Suprachiasmatic
SO	Supraoptic
VM	Ventromedial
	C: pars centralis
	M: pars medialis
	L: pars lateralis

Other structures

AC	Anterior commissure
BV	Blood vessel
F	Fornix
IR	Infundibular recess of IIIrd ventricle
LV	Lateral ventricle
LT	Lamina terminalis
MB	Mamillary body
ME	Median eminence
MT	Mamillothalamic tract
OC	Optic chiasm
ON	Optic nerve
OT	Optic tract
PC	Posterior commissure
PME	Posterior part of median eminence
PS	Pituitary stalk
S	Septum
T	Thalamus
V3 or III	IIIrd ventricle



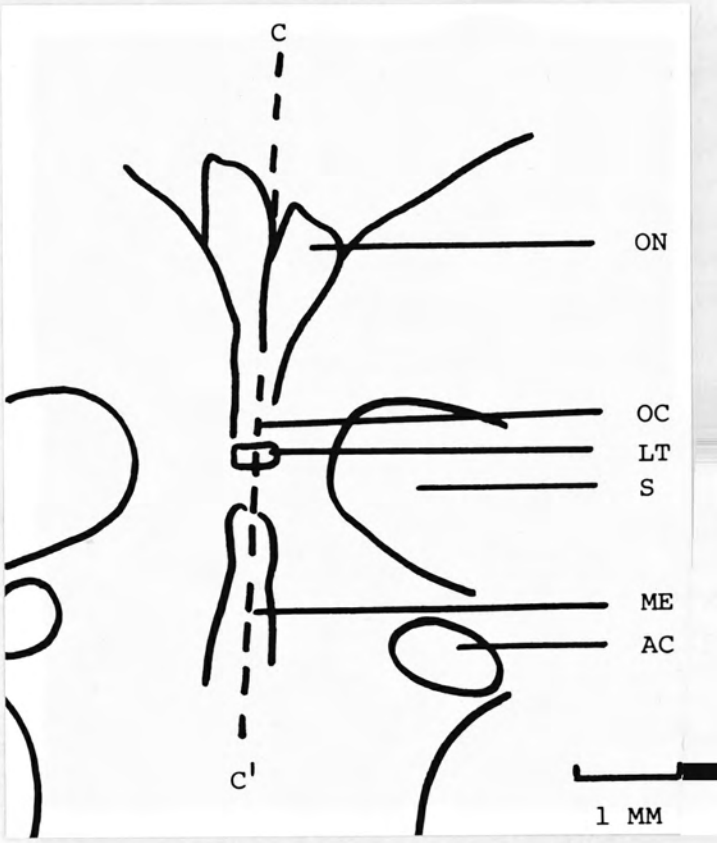


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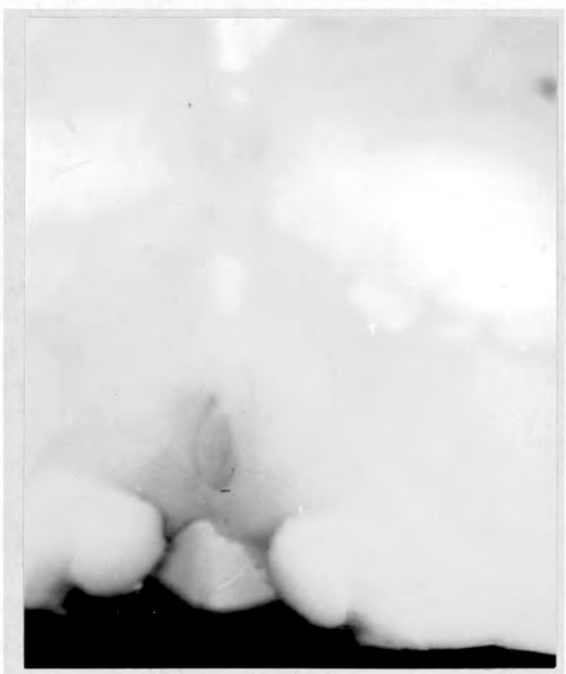
Figure 2 Bisection of hypothalamus. Entry into IIIrd ventricle from dorsal surface. The bisection was continued along line C-C'-D(see Fig. 1a).

a. Optic chiasm in focus

b. Infundibular recess in focus



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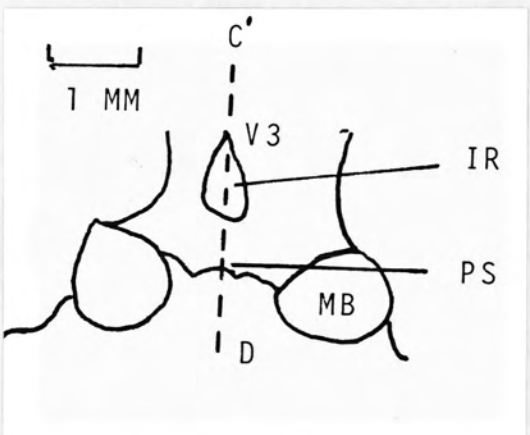
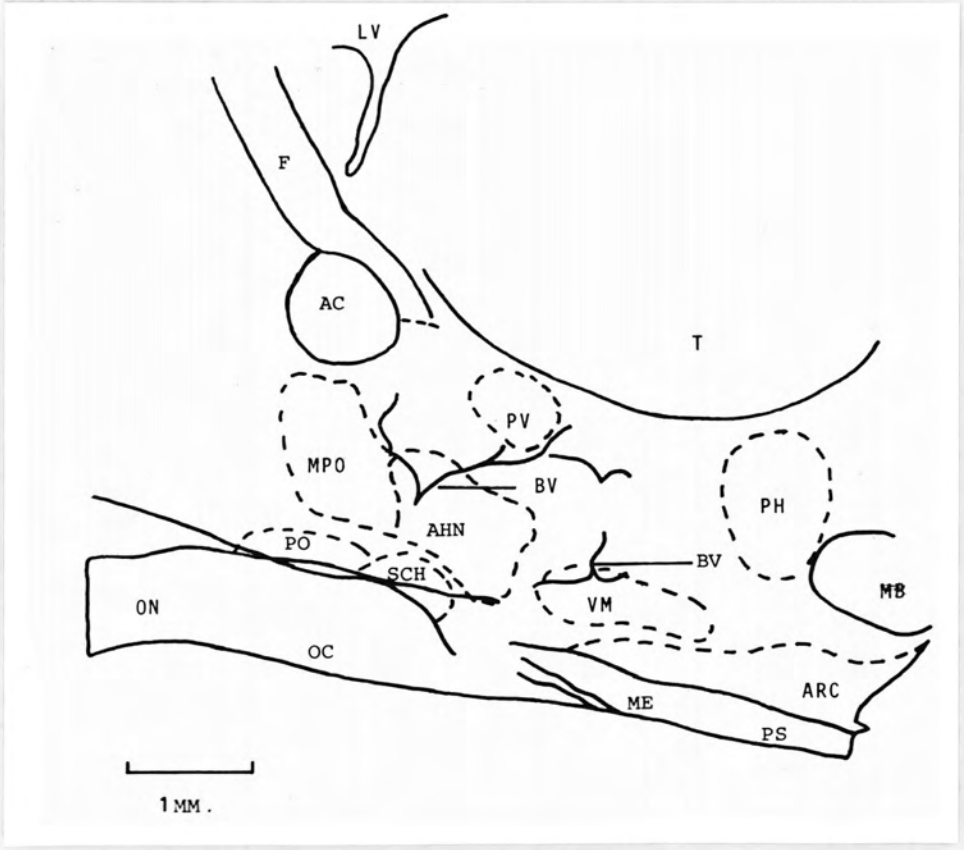


Figure 3 Medial, ventricular surface of bisected hypothalamus to show landmarks. The positions of the hypothalamic nuclei are indicated by dashed lines (after Konig & Klippel, 1963).



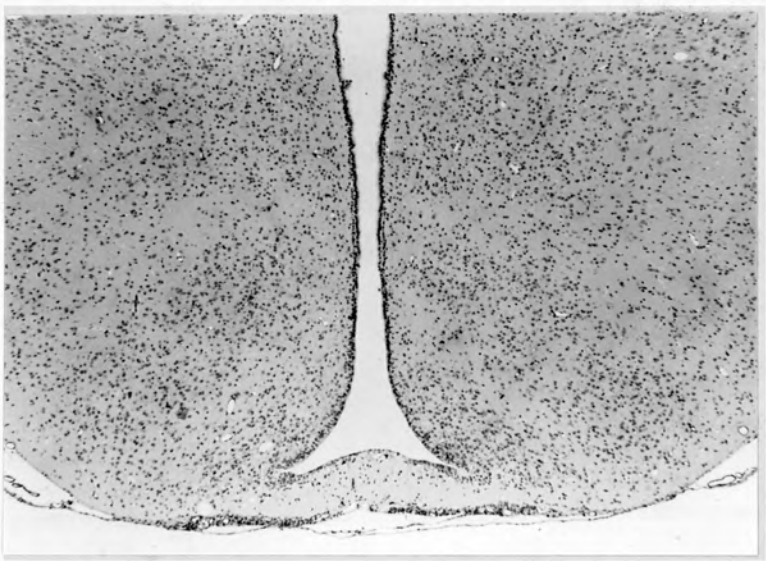
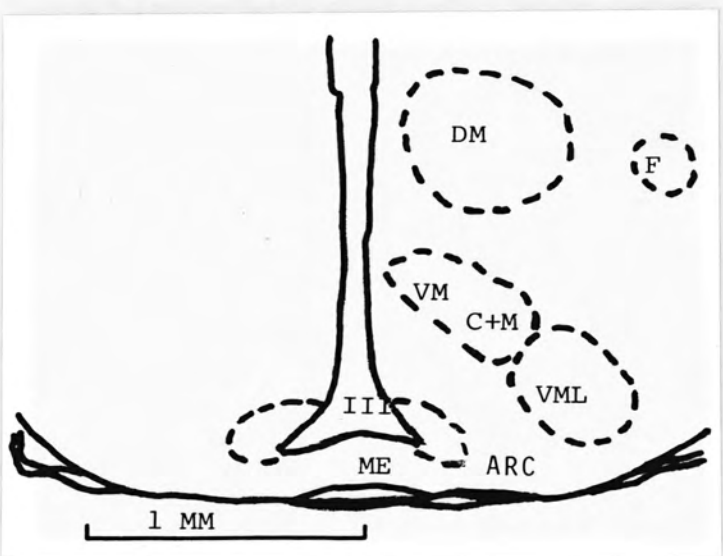


Figure 4 Transverse section of intact hypothalamus to show the ventromedial and arcuate (anterior part) nuclei. Stained with cresyl violet.



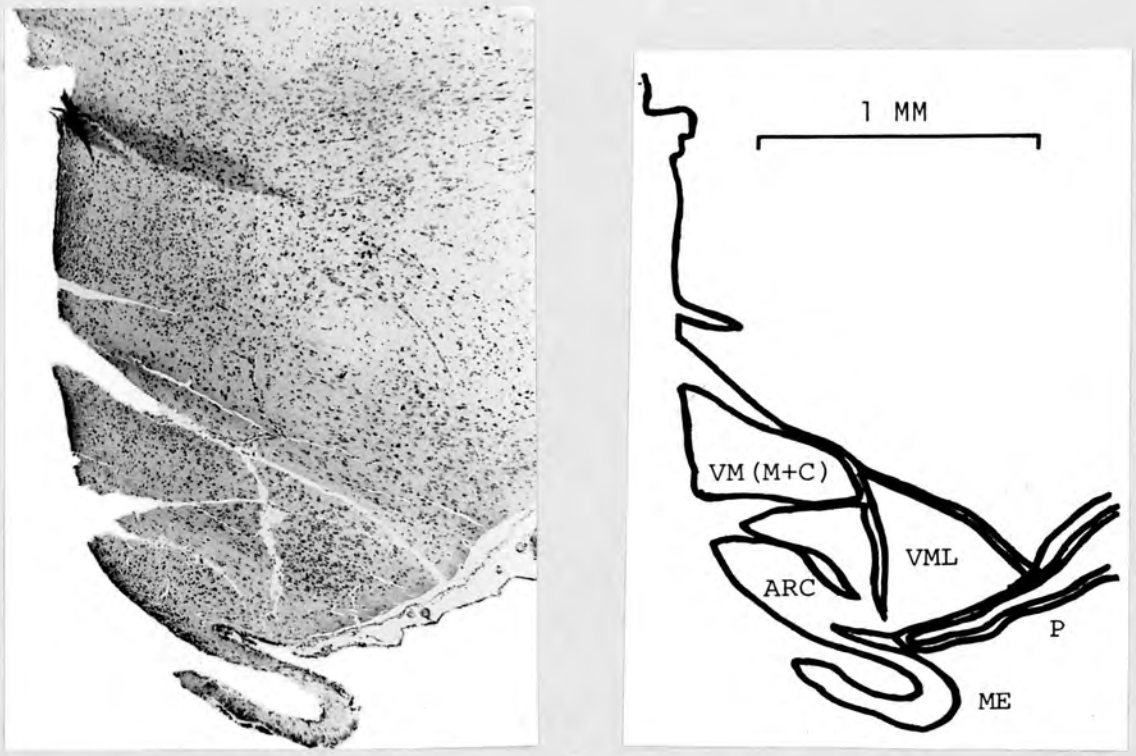


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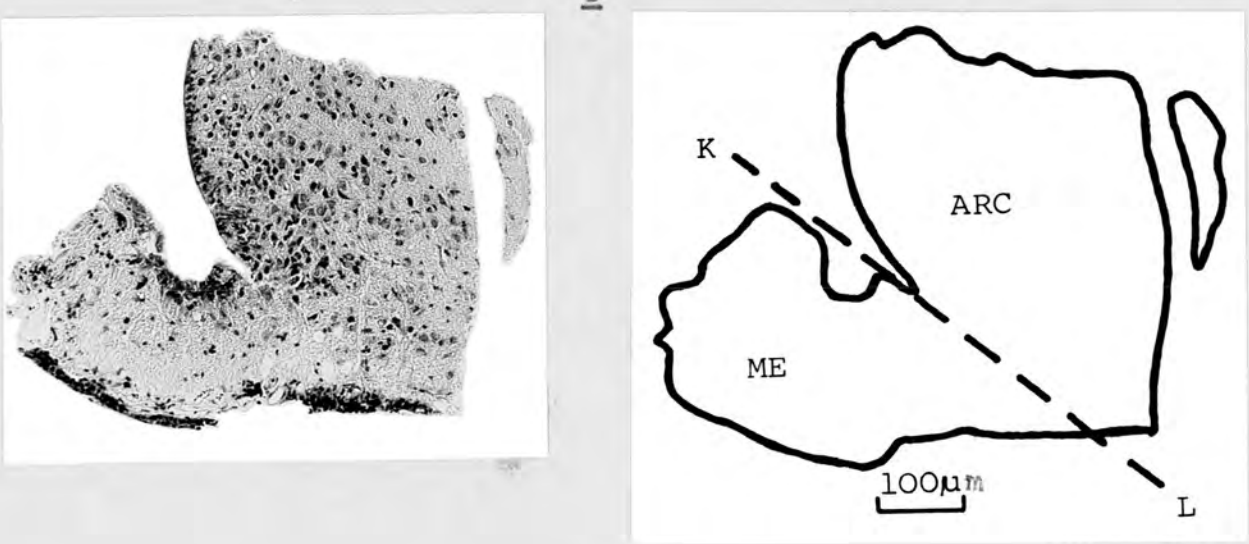
Figure 5 Dissection of ventromedial (pars centralis and pars medialis) and arcuate (anterior part) nuclei. Sections stained with cresyl violet.

a. Transverse section of ventromedial (pars centralis and pars medialis) and arcuate (anterior part) nuclei dissected and left in situ.

b. Transverse section of isolated arcuate nucleus. The median eminence was removed by a cut along line K-L.

b



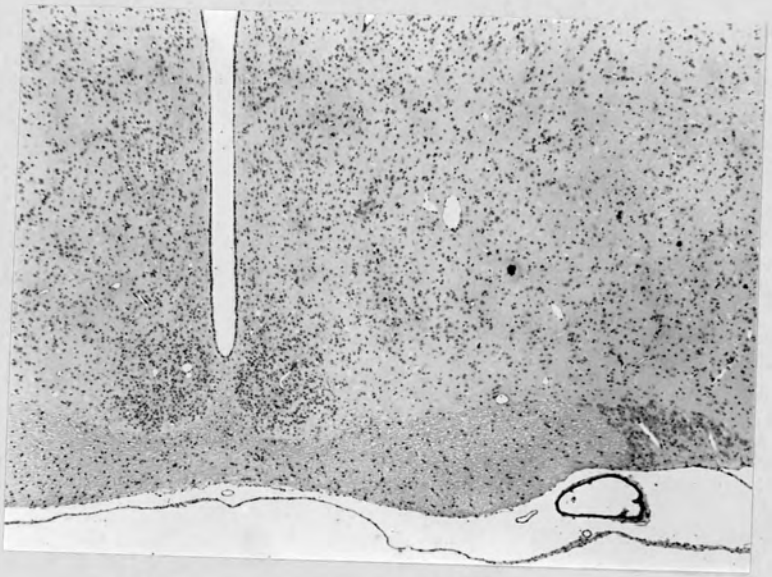
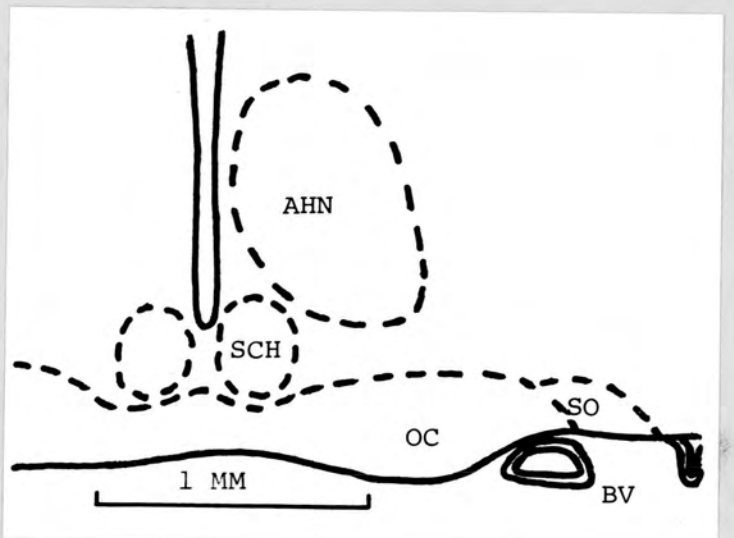
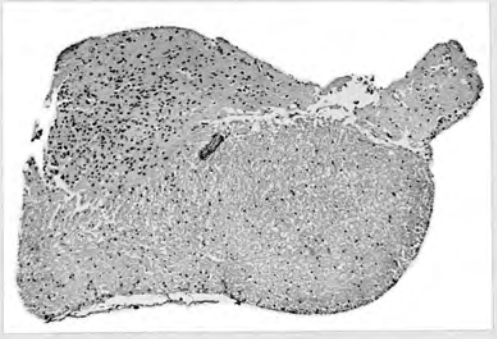


Figure 6 Transverse section of intact hypothalamus to show the suprachiasmatic, anterior hypothalamic and supraoptic nuclei. Stained with cresyl violet.

Sections through the medial preoptic and preoptic (pars suprachiasmatica) nuclei in the intact hypothalamus are not shown, but these nuclei have the same relationship to the midsagittal plane as the anterior hypothalamic and suprachiasmatic nuclei.





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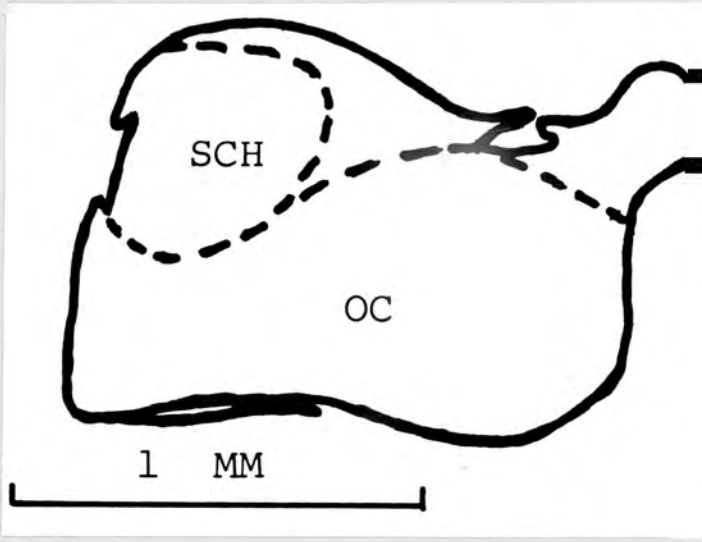
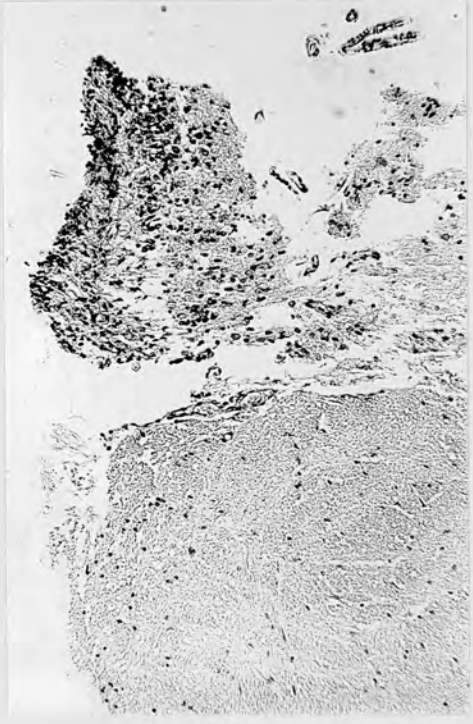


Figure 7 Dissection of suprachiasmatic nucleus. Sections stained with cresyl violet.

a. Transverse section of suprachiasmatic nucleus still attached to optic chiasm.

b. Transverse section of suprachiasmatic nucleus detached from optic chiasm.



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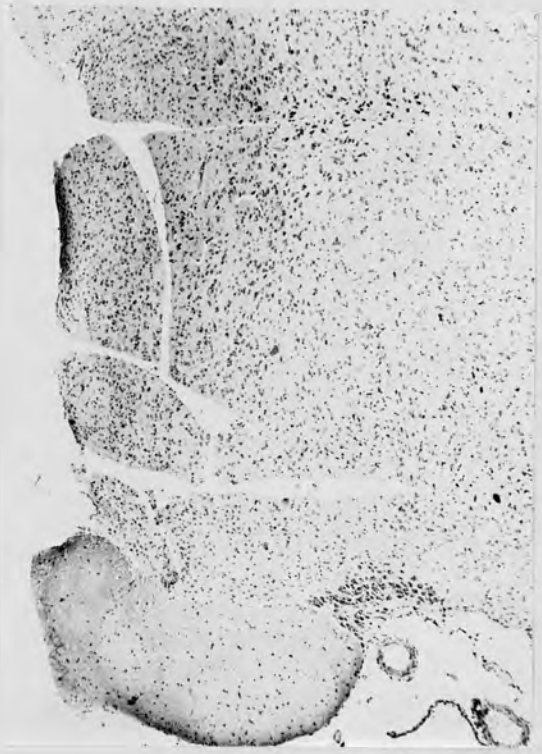


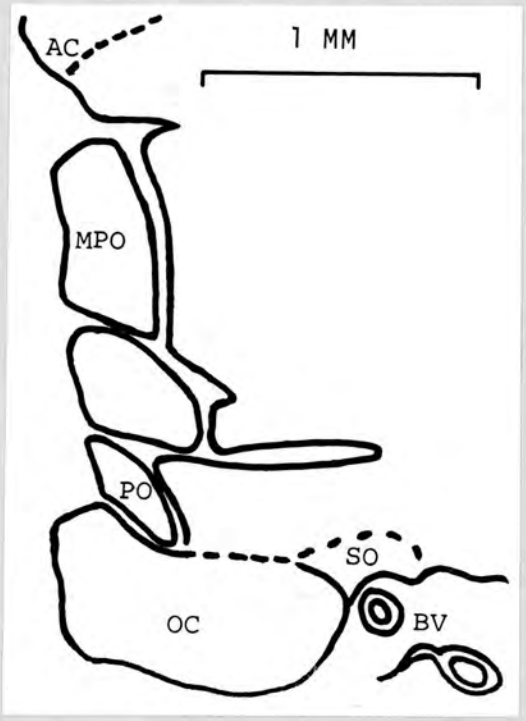
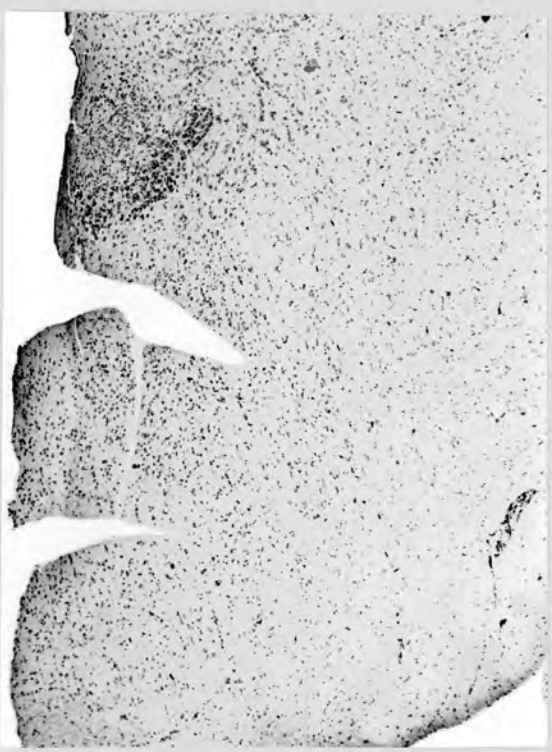
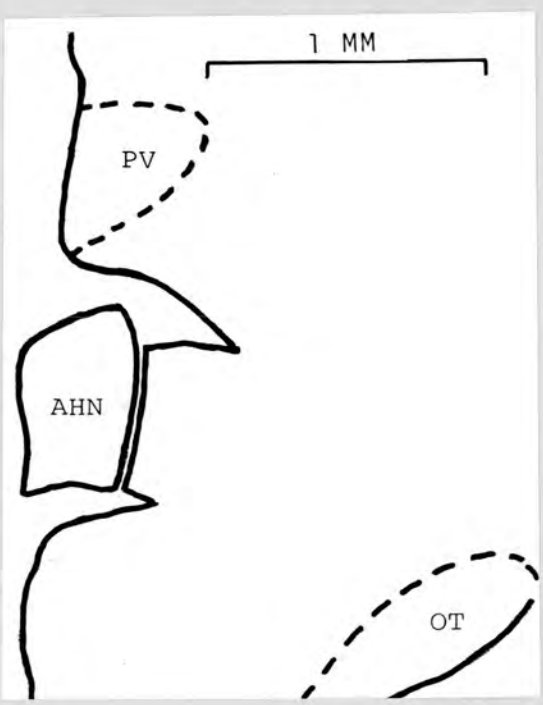


Figure 8 Transverse section of medial preoptic (including adjacent part of periventricular nucleus) and preoptic (pars suprachiasmatica) nuclei dissected and left in situ. Section stained with cresyl violet.

Figure 9 Transverse section of anterior hypothalamic nucleus (including adjacent part of periventricular nucleus) dissected and left in situ. Section stained with cresyl violet (see also Fig. 10).





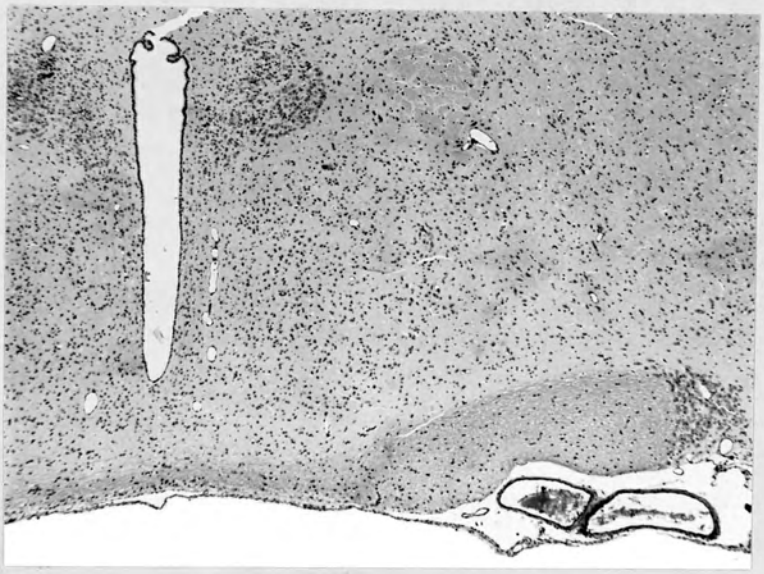


Figure 10 Transverse section of intact hypothalamus to show the anterior hypothalamic, supraoptic and paraventricular nuclei. Section stained with cresyl violet.

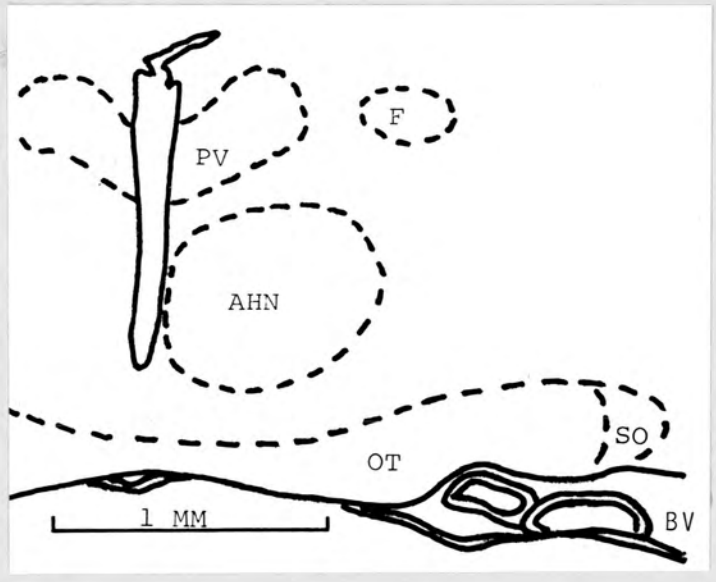
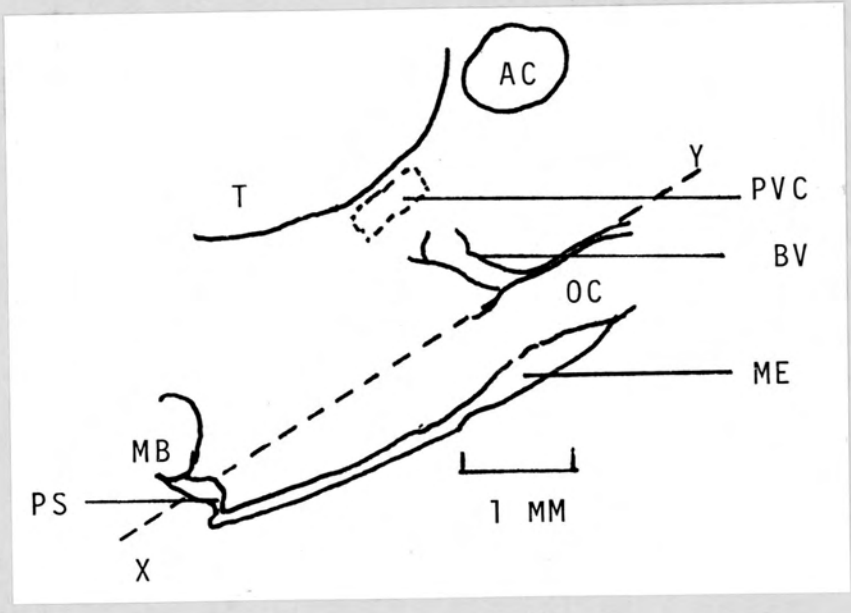
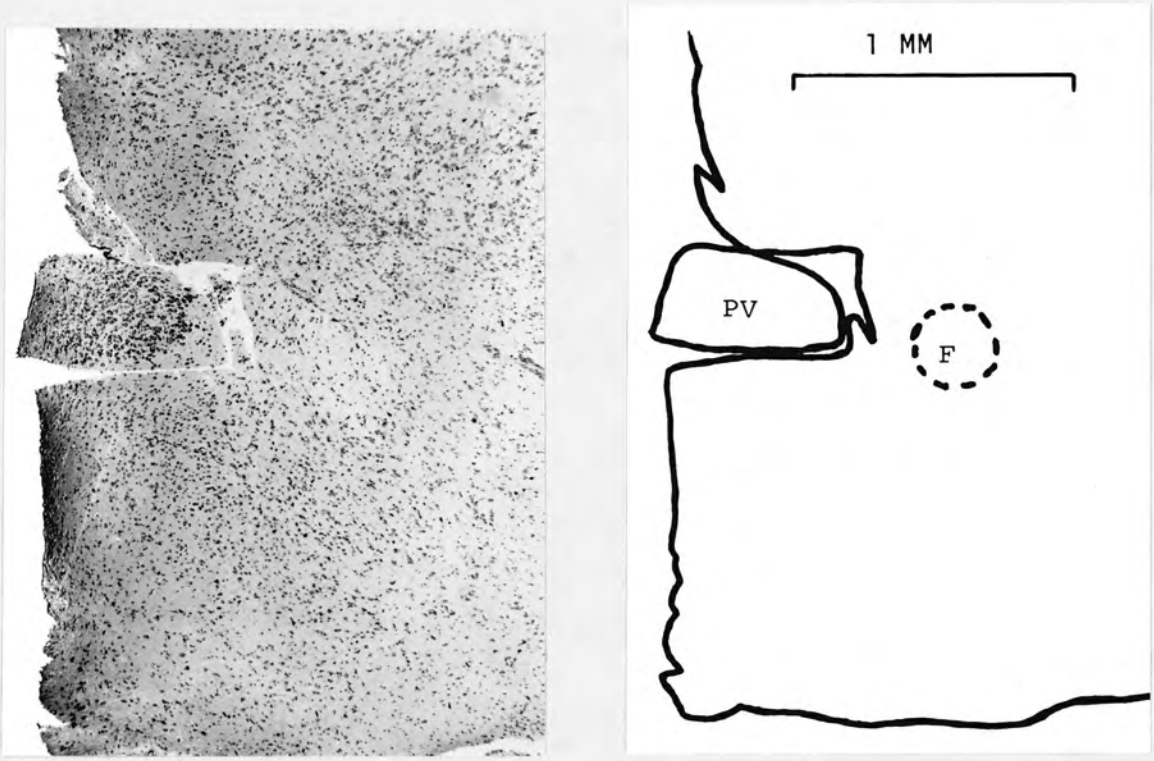


Figure 11 Medial, ventricular surface of bisected hypothalamus to show steps in the dissection of the paraventricular and supraoptic nuclei. The incisions made around the paraventricular nucleus are shown (PVC). After removal of the other hypothalamic nuclei, the base of the hypothalamus was removed along line XY. The supraoptic nucleus was then separated from the optic chiasm (Fig.15).





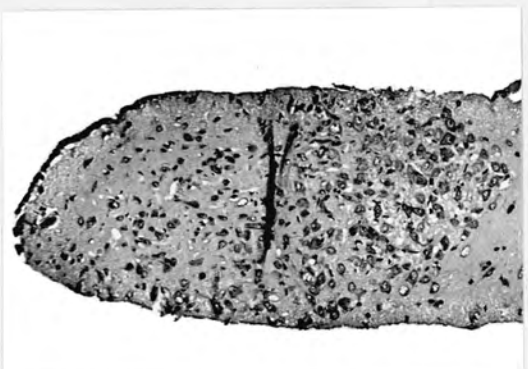
a

Figure 12 Dissection of paraventricular nucleus. Sections stained with cresyl violet.

a. Transverse section of paraventricular nucleus dissected and left in situ.

b. Transverse section of isolated paraventricular nucleus. There are large numbers of magnocellular neurones. Magnification x110.

b



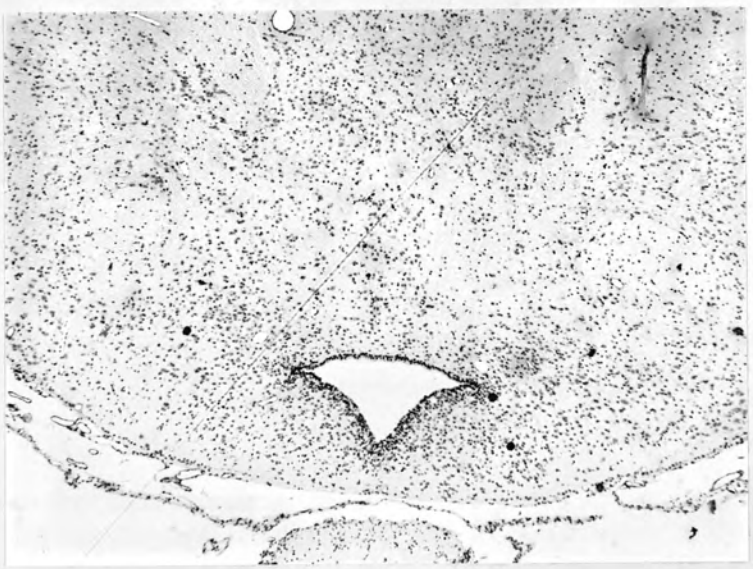
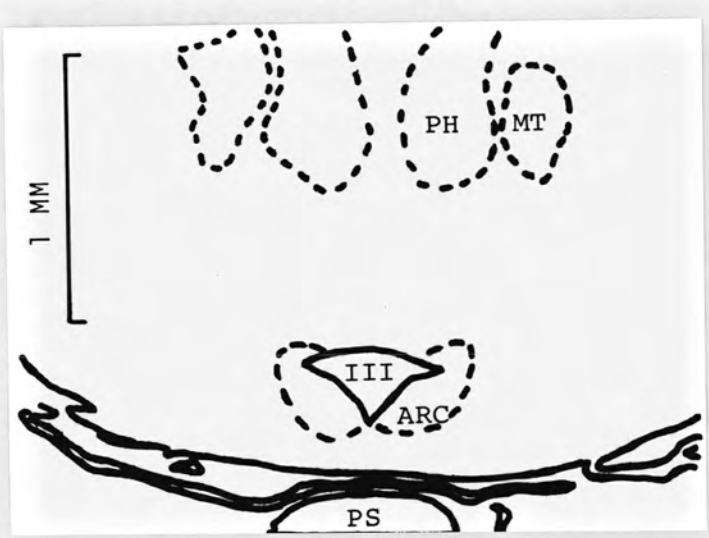
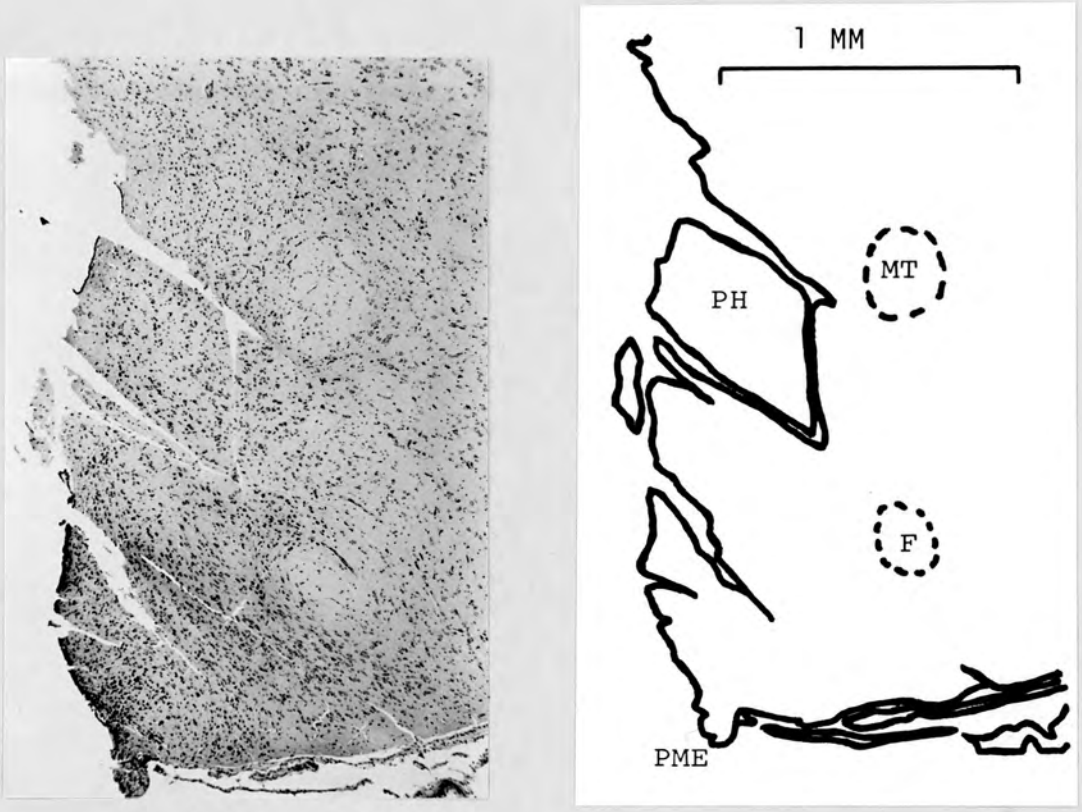


Figure 13 Transverse section of intact hypothalamus to show the posterior hypothalamic and arcuate (posterior part) nuclei. Stained with cresyl violet.





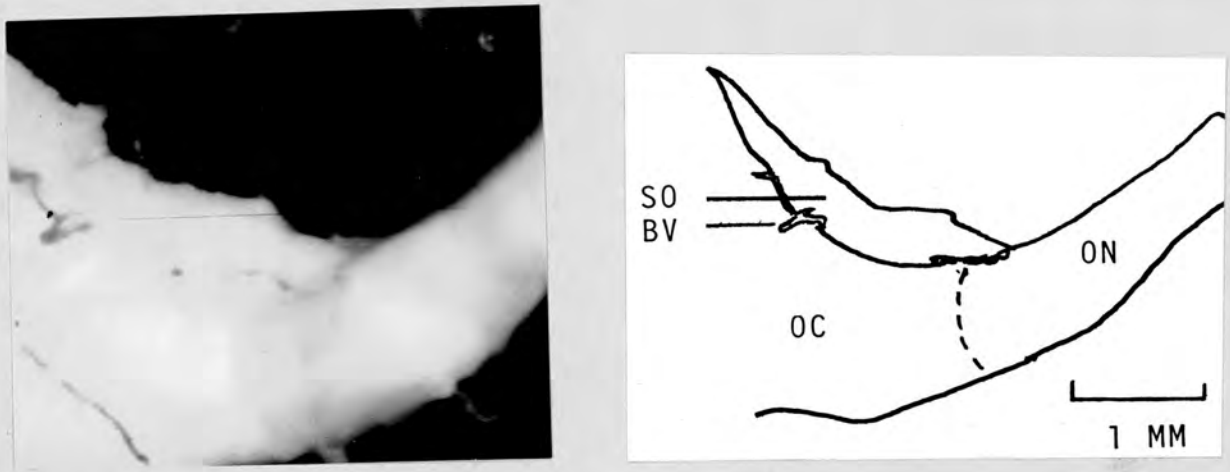
a

Figure 14 Dissection of posterior hypothalamic nucleus. Sections stained with cresyl violet.

a. Transverse section of posterior hypothalamic nucleus dissected and left in situ.

b. Transverse section of isolated posterior hypothalamic nucleus. Magnification x110.

b



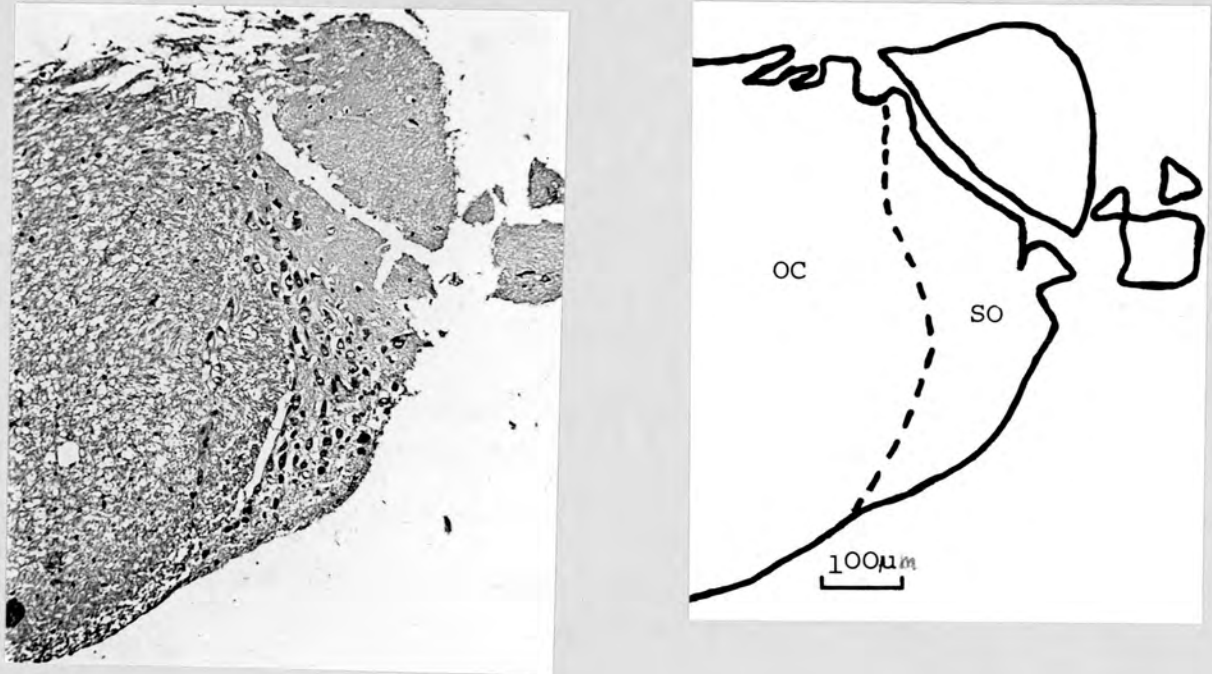
a

Figure 15 Dissection of supraoptic nucleus.

a. Optic chiasm and attached supraoptic nucleus, prepared by trimming the basal part of the hypothalamus (Fig.11).

b. Transverse section of supraoptic nucleus still attached to the optic chiasm, from which it was readily removed. Section stained with cresyl violet.

b



1. Management of animals

All experiments were performed on three month old albino rats; control and experimental animals in any experiment were of similar body weight at the start of experiments and were usually litter mates.

Animals were housed in groups of six per cage, and were fed rat pellets and water ad libitum, unless otherwise stated. The room in which the animals were housed was artificially lit for 12 hours, and dark for 12 hours each day; the temperature was maintained at 21 $\pm$ 1°C, unless otherwise stated. Cages were kept close together on metal racks.

All animals were killed between 1100 and 1300 hours. In most experiments pairs of control and experimental animals were killed on the same day. Animals were bled to death under light ether anaesthesia.

2. Dissection of hypothalamus and preparation of isolated nuclei

The preparation of isolated nuclei from discrete brain areas, and the measurement of the dry mass or nucleic acid content of the contained nucleoli are described here. The hypothalamic areas studied, and the parameters measured, are detailed in each section.

2.1 Dissection of discrete hypothalamic areas

A midline scalp incision was made and the cranial vault was removed carefully with bone nibblers. The brain was

removed, placed on a cold glass slide, and trimmed with a razor blade to leave a block containing the hypothalamus. This block was placed in ice cold homogenisation medium (vide infra) in a watch glass on the stage of a dissection microscope, used with x12 magnification. With a fragment of razor blade held in a pin vice a midline sagittal incision was made in the dorsal surface of this block of tissue. The incision passed through the interthalamic connexus, and was continued ventrally through the anterior commissure, the septum and into the IIIrd ventricle; the floor of the IIIrd ventricle was bisected from the optic chiasm to the mamillary bodies. This procedure is illustrated in Figs. 1 and 2.

Landmarks on the medial, ventricular surface of each half of the hypothalamus were identified (Fig. 3). The paraventricular nucleus was visible because it is more vascular than the surrounding tissue (Akmayev, 1971). The landmarks were used to identify the position of each of the hypothalamic nuclei (Konig & Klippel, 1963). With the mounted fragment of razor blade incisions were made around each of the hypothalamic nuclei. The nuclear areas were removed in order as follows:

1. Arcuate, in two parts: anterior or posterior to the posterior border of the junction between the pituitary stalk and the median eminence.
2. Ventromedial: pars centralis and pars medialis.
3. Suprachiasmatic.
4. Preoptic: pars suprachiasmatica.

5. Medial preoptic: including adjacent part of the periventricular nucleus.
6. Anterior hypothalamic: including adjacent part of the periventricular nucleus.
7. Paraventricular.
8. Posterior hypothalamic.
9. Supraoptic.

The accuracy of the dissection was confirmed by repeated histological examination. Examples of sections through such specimens are shown to illustrate the dissection (Figs. 5, 7, 8, 9, 12, 14). Transverse sections of the intact hypothalamus are shown for comparison (Figs. 4, 6, 10, 13). The dissection of the supraoptic nucleus is illustrated in Figs. 11 and 15.

Nuclear areas were removed from both halves of the hypothalamus; right and left nuclei were pooled in some experiments and prepared separately in others.

2.2 Preparation of isolated neuronal nuclei

The discrete hypothalamus areas were disrupted by mechanical disaggregation in a small glass homogeniser, which contained 2.5 µl of homogenisation medium. This was a buffered solution of sucrose which contained 0.34M sucrose, 0.002M $MgCl_2$, 0.001M KCl and 0.005M Tris, adjusted to pH 6.5 with 1N HCl (Hadjiolov et al, 1965).

The number of strokes of the homogeniser required to rupture the cells and release nuclei was found by preliminary experiment for each hypothalamic area.

Preparations of isolated nuclei were transferred with a Pasteur pipette to quartz slides and covered with a quartz coverslip. Nuclei were not fixed. A reference specimen of homogenisation medium only was prepared similarly.

3. Measurement of nucleolar dry mass

3.1 Interference microscopy

The method used was derived from the descriptions of Davies (1958) and Barer (1966).

3.1.1 Theory and measurement of constants

The measurement of dry mass with the interference microscope is based on the empirical finding that most important biological chemical substances have similar specific refractive increments which are practically independent of concentration, pH and temperature (Davies & Wilkins, 1952; Barer & Tkaczyk, 1954).

The interference microscope is used to measure optical path difference which is related to dry mass as follows (Appendix I - 1).

$M = \frac{DwA}{X}$, where M = dry mass, g
Dw = optical path difference in water, cm
A = area of object, cm^2
X = 100x specific refractive increment

Alternatively,

$M = \frac{Dm}{X} \cdot \frac{(nc-nw)}{(nc-nm)} \cdot A$ (Appendix I - 1, equation 6)

where Dm = optical path difference in medium of refractive index, nm
nc = refractive index of cell or nucleolus
nw = refractive index of water
A = area of object, cm^2

The dry mass of a nucleolus can be calculated if Dm, A, nc are measured and X, nm are known. X and nc are assumed to be constant for each nucleolus (Davies & Deeley, 1956).

TABLE 1

NUCLEOLAR REFRACTIVE INDEX: MEASUREMENTS ON RANDOM SAMPLES OF 50 NUCLEOLI

Hypothalamic nucleus	$\frac{Dm}{t}$*	Standard error of mean	Nucleolar refractive index nc.
Supraoptic	0.0748	0.0015	1.424
Paraventricular	0.0768	0.0013	1.426
Anterior	0.0810	0.0019	1.430
Medial preoptic	0.0821	0.0020	1.431
Preoptic, pars suprachiasmatica	0.0808	0.0022	1.430
Suprachiasmatic	0.0768	0.0021	1.426
Arcuate	0.0791	0.0019	1.428
Ventromedial	0.0800	0.0019	1.429
Posterior	0.0789	0.0021	1.428

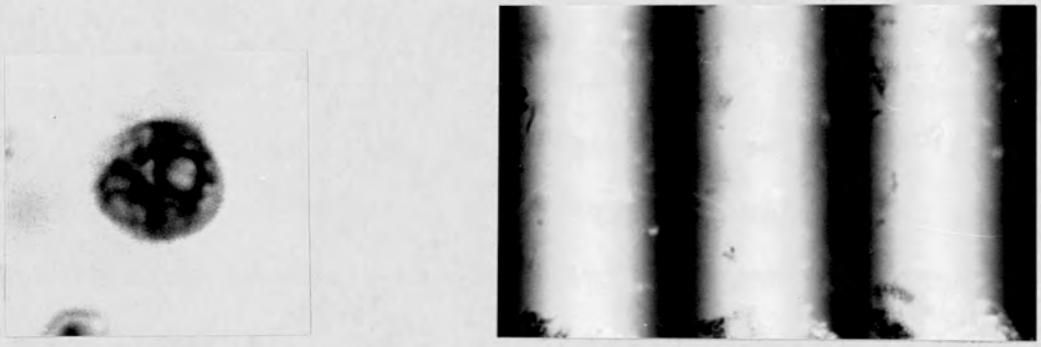
*See Appendix I-1. Equation 5.

X. For proteins, X is unaffected by concentration below 0.55 g per cc (Barer & Tkaczyk, 1954). The concentration of nucleolar dry mass in random samples, each of 50 neuronal nucleoli, from each of the 10 hypothalamic areas included in this study, was 0.52 to 0.55 g per cc (Appendix I - 1, equation 1). The dry mass concentration of liver nucleoli is about 0.4 g per cc (Stenram, 1957). A value for X of between 0.181 and 0.188 may be taken for unconjugated protein (Davies, 1958; Barer, 1966). Davies (1958) has corrected published values of X for DNA and RNA for light of wavelength 546 nm. He obtained average values of 0.187 and 0.188 respectively. For carbohydrates and lipids, X is about 0.14 (Davies, 1958), but for lipoprotein X is between 0.170 and 0.178 (Barer, 1966). For a mixture of substances X is given by the sum of the products of mass fraction and X for each constituent; protein contributes about 85% to total nucleolar dry mass (Stenram, 1958; Watson, 1969), and a value of 0.18 is consequently assumed for nucleolar dry matter.

Nucleolar refractive index, nc. This was calculated according to equation 5, Appendix I - 1 for random samples of 50 nucleoli from each hypothalamic area (Table 1).

Refractive index of medium, nm. This was measured with a sugar refractrometer. A value of 1.349 was obtained for the homogenisation medium.

Optical path difference, Dm. A light ray passing in the optical axis through the thickest part of the nucleolus has a path difference, compared with light passing through



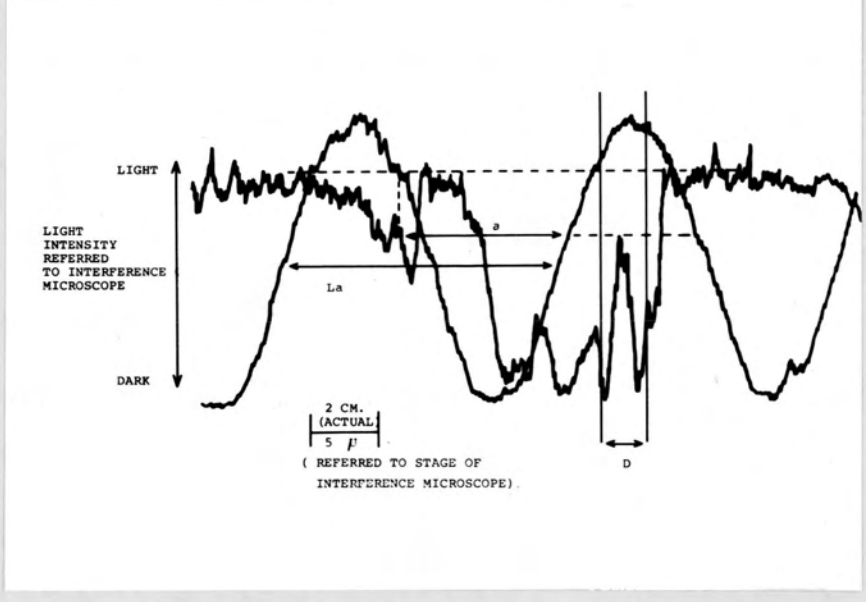
a b

Figure 16 Interference microscopy: a. Isolated paraventricular neuronal nucleus with contained nucleolus. Photomicrograph by interference contrast in an even field. b. Interference bands.

Figure 17 Microddensitometric traces of Figs. 16a, b superimposed. The densitometric scan passed through the centre of the nucleolus.

D = nucleolar diameter (d in text).

$\frac{a}{La}$ = peak nucleolar optical path difference as a fraction of 1 wavelength (546 nm).



the medium only, defined by (nc-nm)t.cm, where t is the greatest nucleolar thickness, nc is nucleolar refractive index, nm is the refractive index of the medium.

The object beam interferes with the reference beam in the image plane and optical path differences are visible as intensity changes (Fig. 16). The optical path difference between corresponding points of intensity on consecutive interference bands is one wavelength. The optical path difference for light which has passed through the nucleolus can be measured: a point of corresponding intensity on the appropriate interference band is found, the distance (Lacm) between corresponding points on adjacent interference bands is measured, and the distance, a cm, between the image of the nucleolus and its corresponding point is also measured. The optical path difference is then $\frac{a}{La}$.Lcm, where L is the wavelength of light used (Fig. 17).

Nucleolar area, Acm^2. This was measured from densitometric traces of photomicrographs of nucleoli (vide infra).

3.1.2 Technique

A Leitz double beam interference microscope was used, with objectives x50/0.85 and condensers N.A. 0.5, to measure the peak nucleolar optical path difference.

The whole number of wavelengths was determined by viewing the object with white light, from a tungsten lamp, and the number of wavelengths between the nucleolus and a band of colour corresponding to that of the nucleolus was counted. Conditions were arranged so that $\frac{a}{La}$ was always less than unity.

The optical path difference is measured with greater accuracy when the interference bands are infinitely separated and the background is of uniform intensity (Davies, 1958, Fig. 16a). The relationship between phase and intensity was found by superimposing the optical density trace of a banded field (Fig. 16b) on the optical density trace across the nucleolus in a uniform background (Fig. 17).

Each nucleolus was observed in a banded field in white light to identify the zero order band then, with monochromatic illumination from a mercury lamp, an adjacent clear field was photographed. The zero order band was infinitely separated from one of its neighbours and a photomicrograph was exposed, in monochromatic light, of a nucleolus within its nucleus. The background intensity and the maximal nucleolar intensity were adjusted, by inserting a narrow glass wedge into the path of the reference beam, so that both these intensities were on the straight part of the phase-intensity curve (Fig. 17). The film was exposed so that the phase-intensity relationship in the image plane was preserved in the relationship between light intensity and optical density of the film. In practice the film gamma is not critical for this relationship (Davies, 1958).

The peak nucleolar optical path difference was measured from microdensitometric traces of the photomicrographs (Fig. 17).

From the peak nucleolar optical path difference, the mean nucleolar optical path difference can be found as $D_{peak} \cdot \frac{2}{3} = D_{mean}$ (Appendix I - 2, equation 7), where D is optical path difference.

All photomicrographs were recorded on Kodak Plus-X Panchromatic 35 mm film and developed in Kodak 19 developer.

From each specimen of isolated nuclei, 24 nucleolar dry mass measurements were made.

3.2 Microdensitometry, technique

A Joyce-Loebl microdensitometer was used, with objective x22, and with the optical density wedge linear over the range 0-2 optical density units. The recording slit height and width were adjusted to be equivalent to 300 and 150 nm respectively in the object plane of the interference microscope. The arm ratio between the recording and specimen tables was 20 to 1.

A trace of optical density was made across each banded field, and on this was superimposed a trace across the nucleolus at its widest part in the direction of scan, which was along the long axis of the film for all specimens. As each nucleolar edge was observed to meet the recording slit the table was stopped and the nucleolar edges marked on the density trace. Nucleolar diameter, dcm, was measured as the distance between these marks (Fig. 17).

The magnification from the object plane of the interference microscope to the densitometric trace was 4.2×10^3.

3.3 Calculation of nucleolar dry mass

Substituting in equation 6 (Appendix I - 1)

$$M = \frac{Dm}{X} \cdot \frac{(nc-nw)}{(nc-nm)} \cdot A \cdot g$$

$$\text{Nucleolar dry mass } M = \frac{2}{3} \cdot L \cdot \frac{a}{La} \cdot \frac{(nc-nw)}{(nc-nm)} \cdot \frac{\pi \left(\frac{d}{2}\right)^2}{X \ Mag^2} \cdot g \quad \text{(Appendix I - 2)}$$

or $M = K \cdot \frac{a}{La} \cdot d^2 \cdot g$, where a, La and d are in

cm and measured for each nucleolus from the microdensitometry traces (Fig. 17).

$K = \frac{\frac{1}{4}L.\pi}{4X\,mag^2} \cdot \frac{(nc-nw)}{(nc-nm)}$ where L = wavelength of light at which measurements were made, 546 nm.

nc = nucleolar refractive index (Table 1)
nw = refractive index of water, 1.333
nm = refractive index of mounting medium, 1.349
X = 0.18
mag = 4.2×10^3

3.4 Sources of error

3.4.1 Object

Overlying nucleoplasm. For a nucleolus within a nucleus, measurement of nucleolar optical path difference includes a contribution from nucleoplasm in the measurement axis. Under these circumstances the true nucleolar dry mass is given by

$$\frac{A}{X} \cdot \left[\overline{Dm} - DN \left(\frac{1-d}{t}\right)\right] \cdot \frac{(nc-nw)}{(nc-nm)} \cdot g \quad \text{(Appendix I - 3)}$$

where A is nucleolar area, cm
Dm is measured nucleolar optical path difference, cm
DN is nuclear optical path difference, measured immediately adjacent to the nucleolus, cm
d is nucleolar thickness
t is nuclear thickness, measured through the nucleolus.

t is difficult to measure directly. However the contribution of overlying nucleoplasm to nucleolar optical path difference measurements has been estimated by comparing nucleolar optical path difference before and after extrusion of nucleoli from isolated nuclei: nucleoplasm contributes

less than 1% to the nucleolar optical path difference (Watt, 1972).

Nucleolar sphericity. Mean nucleolar optical path difference measurements made on the assumption that the nucleolus is spherical (Appendix I.2- equation 7) are in excellent agreement with mean nucleolar optical path difference measurements made by the more tedious technique of rotational integration which does not assume sphericity (Watt, 1972).

Nucleolar diameter. The measurement of nucleolar diameter is a possible source of large error. This measurement was reproducible for individual nucleoli within 4%, and all measurements were made by the author.

Selection of nucleoli for measurement. Measurements were not made on nucleoli which were overlain with cytoplasm, or on nuclei which contained two or more nucleoli; less than 5% of all nuclei. Glial nucleoli were not measured. Otherwise no selection was permitted; all nucleoli in the field of view were photographed; each slide was systematically scanned.

Measurements of the mean dry mass of two samples of nucleoli from each of several preparations of isolated nuclei agreed within 3%.

Losses of nucleolar constituents during the isolation procedure were probably not significant (Watson, 1968a).

3.4.2 Optical errors (Davies, 1958; Davies & Deeley,1956)

Distributional error. Nucleolar dry mass was calculated from a single measurement of peak optical path difference for each nucleolus. This measurement was made in the optical axis, and errors arising from convergent light were absent. Out of focus errors were similarly negligible.

The nucleolus was considered to be homogeneous, in the context of its refractile properties, because nucleolar intensity changes were uniform in an even field when the phase of the reference beam was changed. Nucleolar vacuoles, or nucleololi(Dutta et al, 1963) were rarely seen. Any contribution to error from heterogeneity was anyway minimised by measuring nucleolar peak optical path difference along the linear part of the optical density trace, and the recording aperture of the microdensitometer was small (Davies, 1958).

Diffraction. Light loss by scattering was reduced by making measurements on unfixed nucleoli.

The size of the object measured should be greater than 3 L.cm, where L is the wavelength of light at which measurements are made; below this limit the simple relationship between object and image breaks down (Caspersson, 1950; Davies, 1958). The smallest nucleolar diameter measured was the diameter of a suprachiasmatic nucleolus, 1.8×10^{-4} cm , which is greater than $3 \times 5.46 \times 10^{-5}$ cm. The great majority of nucleoli had diameters larger than 2.5×10^{-4} cm. The single measurement of peak nucleolar optical path difference eliminates the effects of change in refraction at the edge of the nucleolus.

Glare. This was not measured, but the effects of glare should be similar for each specimen as the background intensity was similar for each, and the condenser aperture was kept constant at 0.5.

Optically matched quartz slides and coverslips were used to reduce regional variation in optical path difference in the measuring field.

3.5 Dry mass determination by other methods

No other method was used in this study to measure nucleolar dry mass. Estimates of dry mass of isolated calf thymus nuclei by interference microscopy and by bulk estimation of protein and nucleic acid are in good agreement (Hale & Kay, 1952). Similar estimates have been made of the composition of bone, with respect to organic and inorganic constituents, by interferometry and X-ray absorption (Davies & Engstrom, 1954). These two techniques are in good agreement with respect to dry mass measurements of a variety of cell types (Davies et al, 1953).

4. Measurement of nucleolar nucleic acid content

4.1 Ultraviolet absorption microspectrography

The method used was derived from the descriptions of Walker (1956) and Freed (1969).

4.1.1 Theory

This measurement of nucleic acid or protein content depends on the specific absorption properties above 220 nm by pyrimidines in nucleic acids, and by tyrosine, tryptophan and phenylalanine in proteins. The wavelength at which

maximal absorption occurs is 260 nm for nucleic acids or 280 nm for proteins (Caspersson, 1950).

The absorbance, A, of radiation wavelength L is defined as follows:

$A_L = \log_{10} \frac{Io}{I}$, where Io is the intensity of radiation incident on the specimen,

I is the transmitted intensity.

A_L depends upon the concentration, c g per litre, and thickness, t cm, of chromophore:

$$A_L = k.c.t.$$

where k is absorptivity, and has a value of 22 for nucleic acids (Caspersson, 1936).

As $c = \frac{m}{t.a}$, where a is area of object, cm

m is mass of chromophore, g

$$c.t = \frac{m}{a}$$

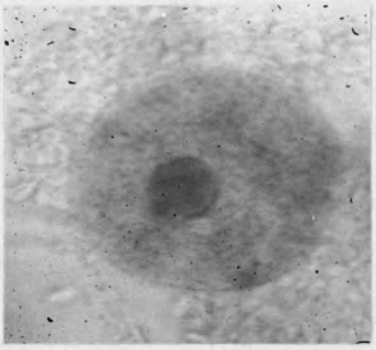
or $A_L = k.\frac{m}{a}$, for a homogenous object.

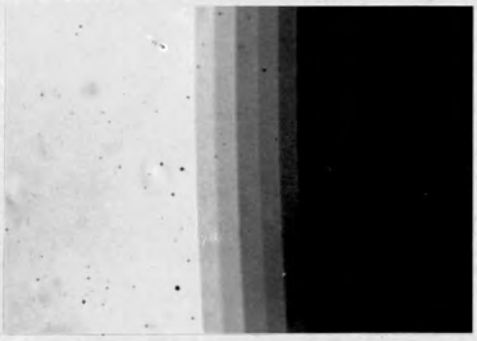
$$m = \frac{a.A_L}{k} \cdot g \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

The assumption made in measuring A_L is that the distribution of energy in the image plane of the ultraviolet absorption microspectrograph is identical with that in the object plane (Freed, 1969). The image can be recorded photographically, and provided that the relationship between optical density of the photographic plate and absorbance by the object is linear, the integrated absorbance can be measured by microdensitometry.

4.1.2 Technique

A Leitz ultraviolet absorption microspectrograph was used to obtain photomicrographs of nucleoli irradiated at





a b

Figure 18 Ultraviolet absorption microspectrography.

a. Photomicrograph of isolated supraoptic neuronal nucleus irradiated at 253.7 nm. The contained nucleolus contains more chromophore than the surrounding nucleoplasm.

b. Photomicrograph of rotating sector, calibrated in 0.1 optical density unit steps, in a clear field irradiated at 253.7 nm.

Figure 19 Nucleolar absorption spectrum.

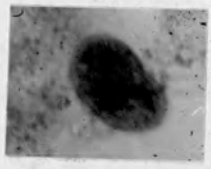
a. Photomicrograph of isolated nucleus, details as in Fig. 18a.

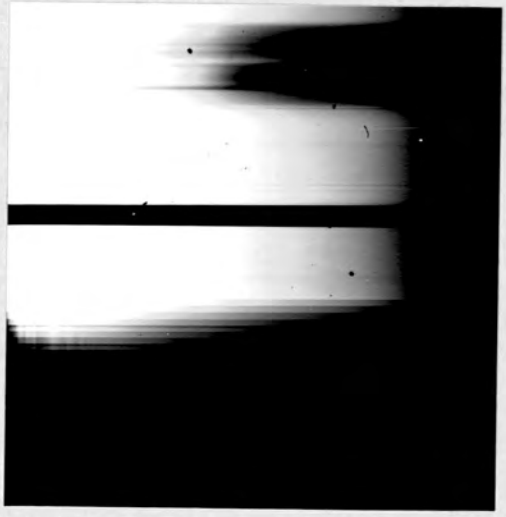
b. Microphotograph of absorption spectrum of nucleolus, irradiated over the range 235 to 320 nm. The nucleolus in a. is correctly aligned with the spectrum.

c. Microphotograph of absorption spectrum of a clear field with the rotating calibration sector inserted.

d. marks the position of the 253.7 nm line.

a





b

c

d

253.7 nm and of absorption spectra of nucleoli irradiated over the range 235 to 320 nm. A low pressure mercury lamp acted as a source for a monochromator which selected light at 253.7 nm. A water cooled hydrogen lamp provided light over the range 235 to 320 nm.

The microspectrograph was used with a reflecting objective, x300, N.A. 0.85, achromatic over the range 220 to 700 nm, and a reflecting condenser, N.A. 0.6. Both condenser and objective were used with glycerine-water immersion.

Nucleic acid and interferometric dry mass measurements were made on the same specimens. The nuclear homogenate was gently squashed between coverslip and slide before examination in the microspectrograph.

4.1.2.1 Integrated nucleolar absorbance: nucleolar nucleic acid content

Each nucleolus was focused in phase contrast and a single exposure was made at 253.7 nm onto an Ilford N40 plate (Fig. 18a). The nucleus was then moved out of the optical axis, and a spinning calibration sector was introduced into the path of the object beam and a second exposure made (Fig. 18b). The sector was so graduated that a linear relationship between optical density of the photographic emulsion and absorbance by the object could be established. The appropriate exposure and development were previously determined to ensure a linear relationship between optical density of the emulsion and absorbance intervals of the sector over the range 0 to 0.8 optical density units.

Measurements were made on 18 nucleoli from each specimen.

4.1.2.2 Nucleolar absorption spectra

After the monochromatic absorption microphotographs were exposed, the nucleolus was irradiated by the hydrogen lamp. The radiation which passed through the nucleolus was focused onto a slit, 50 nm wide referred to the object plane. Each nucleolus was positioned so that the image of a diameter lay along the slit. The emerging beam was dispersed by the quartz spectrograph and the absorption spectrum was recorded photographically (Fig. 19b).

The position of the 253.7 nm line in the spectrum was recorded (Fig. 19d).

The absorption spectrum was calibrated by inserting the rotating sector in the beam path and photographing a clear field (Fig. 19c).

Absorption spectra were recorded for six nucleoli from each preparation. The plates were developed in Kodak D19 developer.

4.2 Microdensitometry: technique

The microdensitometer was used with objectives x22, and with the optical density wedge linear over the range 0 to 2 optical density units. The recording slit was 200 nm high and 100 nm wide referred to the object plane of the microspectrograph. The arm ratio between the recording and specimen tables was 50 to 1.

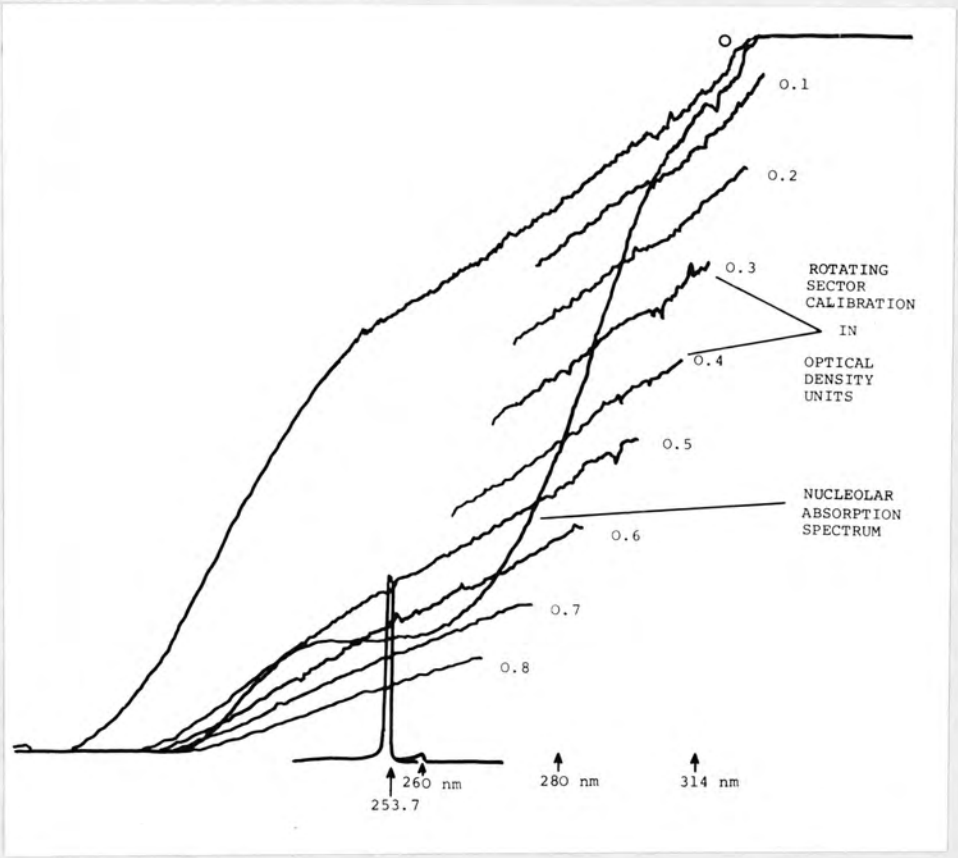
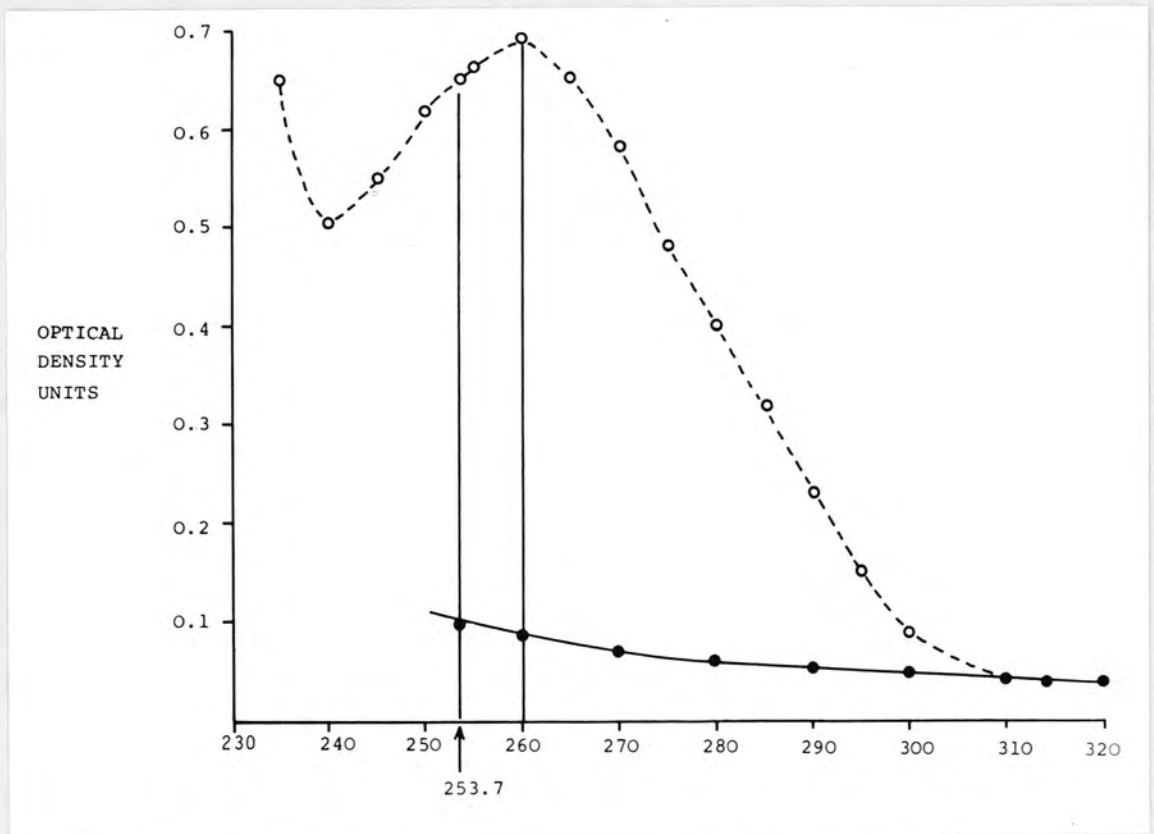


Figure 20 Nucleolar absorption spectrum: microdensitometry. Superimposed densitometric traces of nucleolar absorption spectrum and calibration spectrum(Fig.19).

Figure 21 Nucleolar absorption spectrum: optical density plotted against wavelength, nm (abscissa).

Open circles: optical density at each wavelength measured from Fig. 20.

Closed circles: estimated apparent density due to scatter.



4.2.1 Integrated nucleolar absorbance

The nucleolus cannot be assumed to be homogeneous with respect to its absorption properties. To integrate nucleolar absorbance the size of the recording slit was kept small with respect to the resolving power of the microspectrograph. The distance between densitometric scans was also small.

The microdensitometer was used to measure the integrated nucleolar absorbance, from which the nucleic acid content was calculated using equation (1) (Appendix II - 1). Details of the densitometric procedure are given in Appendix II - 1.

4.2.2 Absorption spectra

Densitometric traces were made across the absorption spectrum of the nucleolus and across the calibrated spectrum along each 0.1 optical density unit step. The 253.7 nm line was located and the optical density at selected wavelengths was read off (Fig. 20). Optical density was plotted against wavelength (Fig. 21). This absorption spectrum was used to estimate scatter (Fig. 21) and to correct measured absorption to 260 nm (Appendix II - 2).

The protein to nucleic acid ratio (Caspersson, 1950) was not measured because it is unreliable (Walker, 1956; Freed, 1969).

4.3 Sources of error

4.3.1 Object

The criteria for selecting nucleoli for measurement were the same as for interference microscopy.

An unknown thickness of nucleus lies in the optical axis of the nucleolus. This is unlikely to contribute more than

6-11% to the measured nucleolar nucleic acid content (Watson, 1968a).

No attempt was made to determine nucleolar composition chemically. The contribution to the measured absorption at 253.7 nm by constituents other than nucleic acids was unknown. From the results of others this contribution is likely to be small (Caspersson, 1950; Watson, 1968a).

4.3.2 Optical errors

The major errors arise from the redistribution of radiant energy in the image plane, with breakdown of the object plane-image plane identity.

Absorption inhomogeneity. Heterogeneous distribution of chromophore within the nucleolus may give rise to distributional error unless absorption is integrated over infinitesimal areas. To avoid this error, the microdensitometer was used with a recording aperture smaller, referred to the object plane, than the smallest resolvable object (Walker, 1956; Freed, 1969); similarly the distance between densitometric scans was small.

Out-of-focus errors. The redistribution of energy by absorbing objects outside the focal plane was minimised by compressing the homogenate (Walker, 1956).

Lateral inhomogeneity. Because of the difference in refractive index between nucleus and nucleolus diffraction occurs at the nucleolar edge. This source of light loss was reduced by using unfixed nuclei (Walker, 1956; Freed, 1969).

The correction applied for light scattering is maximal (Watson, 1968a). The relationship between wavelength and scatter is not established for the conditions within cells (Walker, 1956; Freed, 1969).

Glare. This was not measured, but the use of reflecting objectives, with glycerine-water immersion and condenser N.A. 0.6, should restrict error from this cause which should be constant between specimens.

5. Statistical analysis

The measurements of nucleolar dry mass and nucleic acid content were analysed according to the null hypothesis that the individual nucleolar measurements for an experimental group of animals were members of the same population as the individual nucleolar measurements of the compared group of animals. This hypothesis was tested by calculating Student's t, unless otherwise stated.

Because of the uncertainties in several of the constants and corrections for both dry mass and nucleic acid measurements, the measured nucleolar parameters are intended to be used to demonstrate changes, rather than to establish a set of absolute values.

SECTION III

CHANGES IN SUPRAOPTIC AND PARAVENTRICULAR NEURONES IN PREGNANCY AND LACTATION: EFFECTS OF A GRADED SUCKLING STIMULUS

1. Aims of investigation

The aims of this part of the study were:

1. To measure changes in the dry mass of nucleoli of PV and SO neurones during lactation.

2. To relate these changes either to suckling or to the preceding pregnancy.

3. To see if a graded suckling stimulus results in a similarly graded nucleolar response in PV and SO neurones.

In this section the nucleolar dry mass changes in PV and SO neurones are described. In Section IV these changes are discussed in relation to the associated peripheral stimuli and secretory responses.

2. Methods

2.1 Management of animals

2.1.1 General

The conditions under which these animals were housed have been described previously (Section II - 1).

2.1.2 Pregnancy and lactation

Twenty groups of six virgin females were used. Each group of six animals was caged with a male for 3 to 7 days, depending on the number of desired pregnancies. Pregnant animals were removed from the communal cages about five days before parturition and were caged individually with ample wood shavings for nest-building. On the day of expected parturition the animals were inspected at intervals of 3 to 4 hours. Soon after the birth of a litter, but not before the mother had eaten the placentae, the pups were removed, counted and weighed; within 3 minutes they were returned to the mother, or putative foster mother, who was also weighed.

There were four groups of animals.

Late pregnancy (Group 1). Six virgin female rats, shown by examination of vaginal smears to be in proestrus, were caged with a male rat for 30 hours. The day after proestrus was counted as the first day of pregnancy. Four animals became pregnant and were killed on the 20th day of pregnancy. The foetuses were removed post mortem and counted.

Normal lactation (Group 2). The litter size was unadjusted; each litter was returned to its mother. Lactating animals were killed from 1 to 27 days post partum.

TABLE 2

DISTRIBUTION OF ANIMALS WITH RESPECT TO NUMBER OF PUPS BORN AND NUMBERS NURSED

No. of pups nursed	Day post partum killed	0 = 1 POST PARTUM FEMALE	Totals
22-24	12	9: 0 0 0 0 15: 0	5
	7	8: 0 9: 0 0 12: 0 13: 0	5
	5	11: 0 12: 0 0	3
10	12	6: 0 9: 0 12: 0 13: 0	4
	7	8: 0 9: 0 11: 0 0	4
	5	3: 0 4: 0 12: 0	3
1	12	3: 0 4: 0 9: 0 10: 0	4(+1)*
	7	6: 0 11: 0 13: 0	3
	5	10: 0 11: 0 13: 0	3
0	12	6: 0 11: 0 12: 0	3
	7	3: 0 9: 0 10: 0 11: 0	4
	5	10: 0 11: 0 11: 0	3(+1)*
		1 2 3 4 5 6 7 8 9 10 11 12 13 14 15	44 + 2*

Number of pups born

* = Size of litter born not recorded.

Lactation and imposed variation in litter size (Group 3). The litter size was altered on the day of birth to 1, 10 or 22-24 pups. The litters of two or more mothers were pooled and redistributed. As far as possible, litters were redistributed so that each group of post partum females was similar with regard to the number of pups born to each mother (Table 2). Placentae were not redistributed. Some animals received no pups; these litters were removed as soon as the birth of the whole litter was complete. Some of the pups may have suckled for a few minutes before removal.

Animals in this group were killed on the 5th, 7th or 12th day post partum.

Controls. Groups of 7, 9 and 11 virgin female rats were used as controls for the animals in Groups 1, 2 and 3 respectively. Vaginal smears were taken from these animals each day for at least 10 days before they were killed. The smears were fixed in alcohol, stained by the Papanicolou method, and examined. The stage of the estrous cycle was recorded as estrus, metestrus, diestrus or proestrus. Only animals with 4 or 5 day estrous cycles were used.

2.2 Measurement of PV and SO neuronal nucleolar dry mass

The PV and SO nuclei were prepared, and PV and SO neuronal nucleolar dry mass measured as described previously (Section II - 2, 3 and 5). In the group nursing their own, unadjusted litters, only PV neurones were studied systematically. Right and left PV and SO nuclei were prepared and

TABLE 3

NUMBER OF NUCLEOLAR DRY MASS MEASUREMENTS:

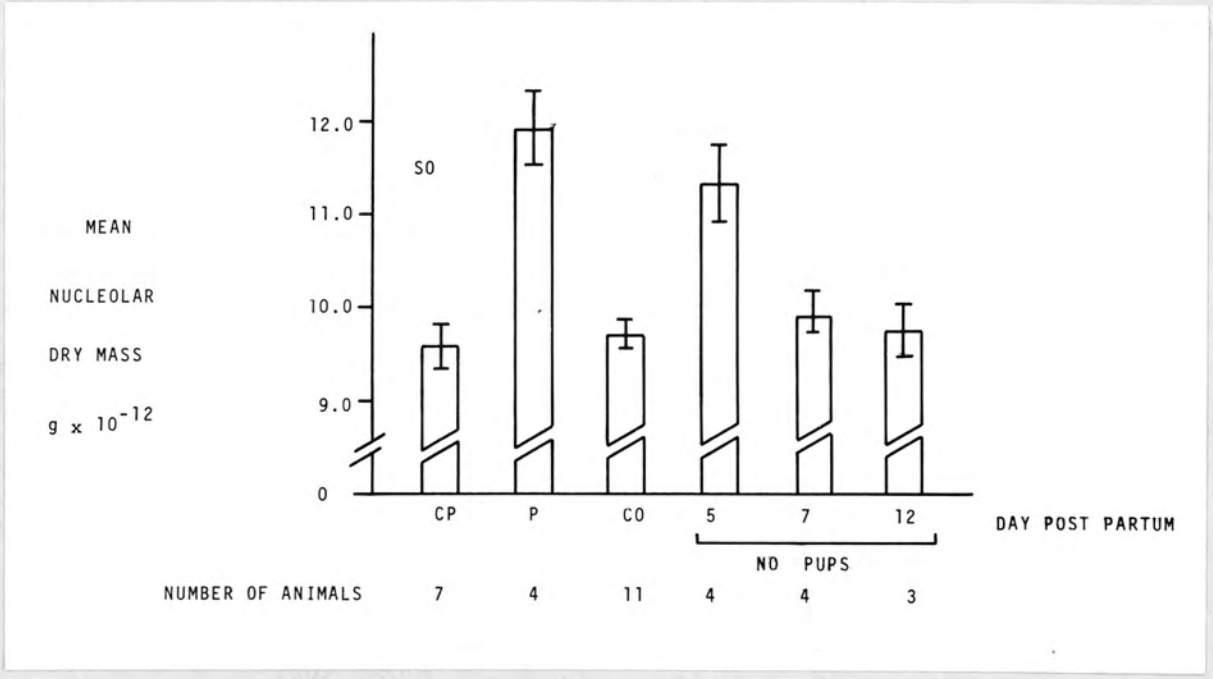
DAYS 5, 7, 12 POST PARTUM POOLED

Experimental Group	PV neuronal nucleoli	SO neuronal nucleoli
1 pup	506	430
10 pups	470	438
22-24 pups	546	574
0 pups	485	415
Virgin control	407	422
TOTALS	2414	2279

measured separately in the group in which litter size was altered and in their controls. The number of nucleolar dry mass measurements made in this group are shown in Table 3.

Other procedures are described in Section IV - 2.

3. Results



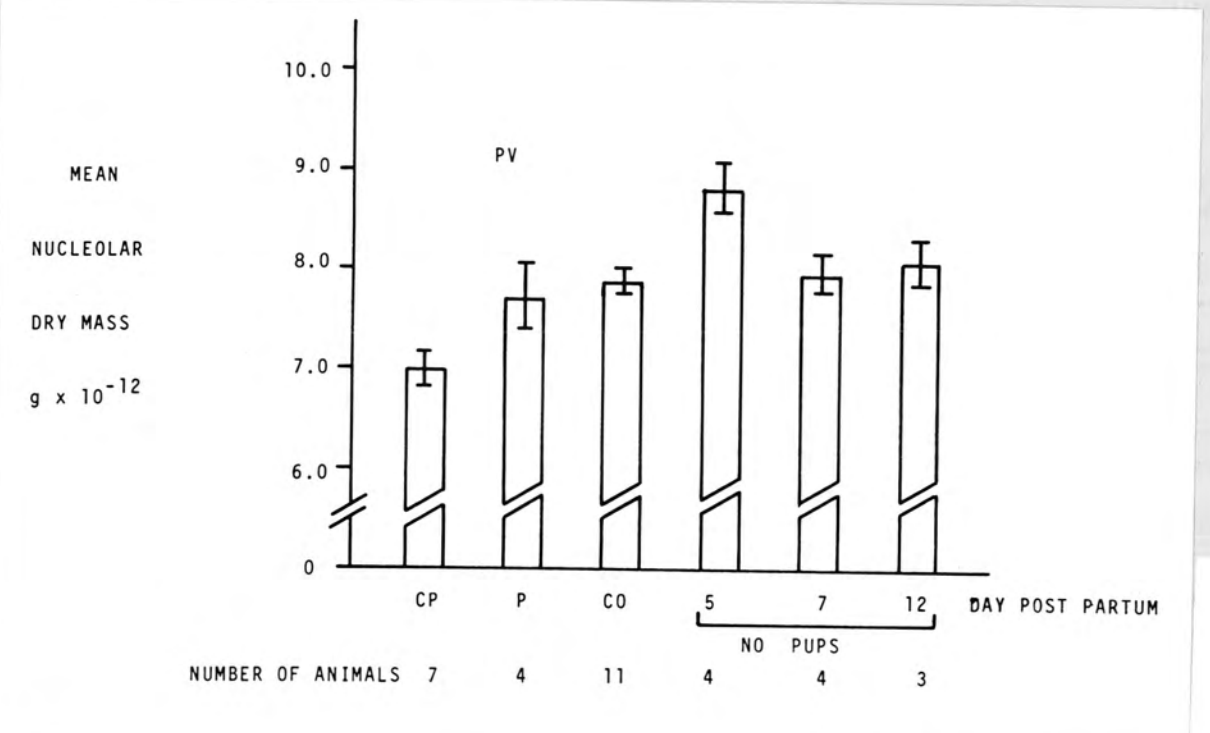
a

Figure 22 Mean neuronal nucleolar dry mass: day 20 of pregnancy (P) and virgin controls (CP). Days 5, 7 and 12 post-partum with pups removed at birth and virgin controls (CO).

Each vertical line indicates standard error of the mean.

a. SO neurones. b. PV neurones.

b



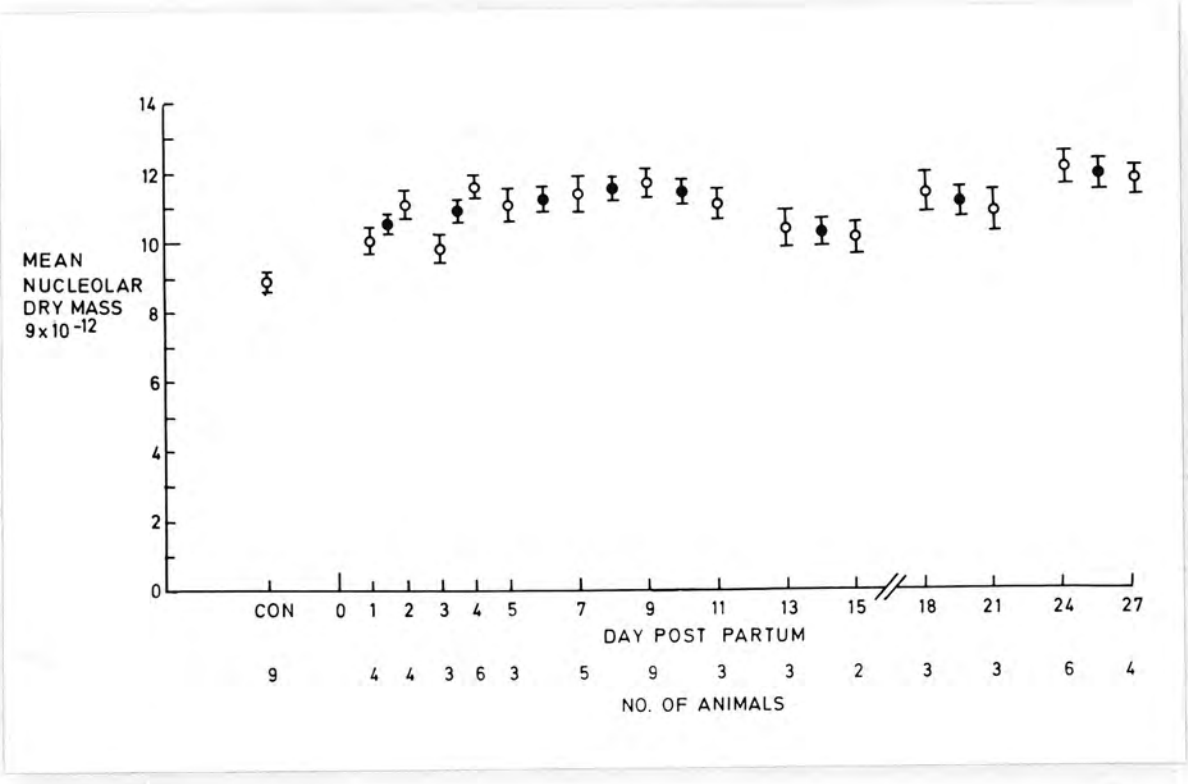


Figure 23 Normal lactation: mean dry mass of paraventricular neuronal nucleoli.

CON: Virgin controls.

Open circles: Data for individual days.

Closed circles: Data pooled for paired consecutive days.

Each vertical line indicates standard error of the mean.

TABLE 5

NEURONAL NUCLEOLAR DRY MASS: COMPARISONS BETWEEN PREGNANT GROUP AND VIRGIN CONTROLS. POST PARTUM GROUP WITHOUT LITTERS AND VIRGIN CONTROLS

Experimental group		PV neurones Virgin control	SO neurones Virgin control
Day 20 pregnancy		$0.05 > p > 0.025$*	$p < 0.001$***
Litters removed at birth	Day 5 post partum	$p < 0.001$*	$p < 0.001$***
	Day 7 post partum	$0.7 > p > 0.6$	$0.5 > p > 0.4$
	Day 12 post partum	$0.5 > p > 0.4$	$0.9 > p > 0.8$

TABLE 6

PV NEURONAL NUCLEOLAR DRY MASS: SELECTED COMPARISONS AT DIFFERENT TIMES DURING NORMAL LACTATION

Days post partum	1 + 2	13 + 15
3 + 4	$p > 0.95$	
5 + 7	$0.3 > p > 0.2$	
9 + 11	$0.05 > p > 0.025$*	$0.05 > p > 0.025$*
13 + 15	$0.6 > p > 0.5$	
18 + 21	$0.20 > p > 0.10$	$0.2 > p > 0.1$
24 + 27	$0.005 > p > 0.001$**	$0.005 > p > 0.001$**

TABLE 7

LITTER SIZES OF ANIMALS USED IN NORMAL LACTATION STUDY

Days post partum	Number of lactating animals	Mean litter size	Standard error of mean
1 + 2	6	9.2	0.87
3 + 4	5	9.8	0.37
5 + 7	7	9.3	1.41
9 + 11	8	8.9	0.78
13 + 15	5	7.6	0.68
18 + 21	6	9.3	0.67
24 + 27	8	8.9	0.58

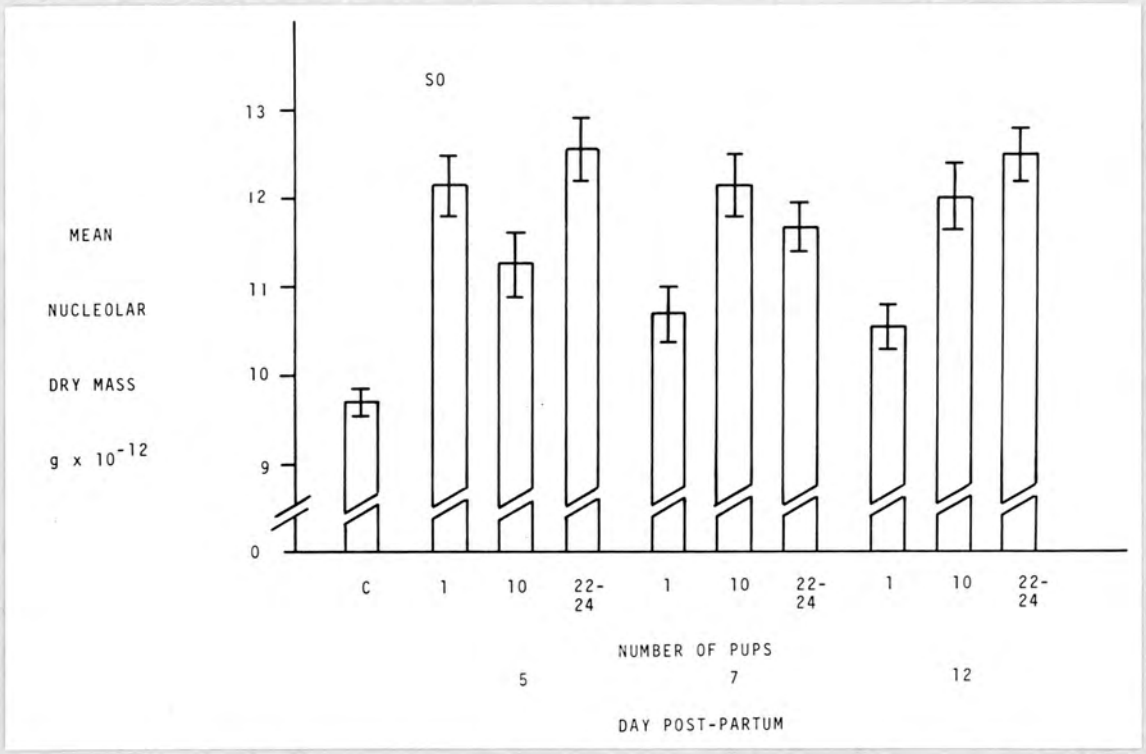


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Figure 24 Lactation with varied litter size: mean dry mass of neuronal nucleoli. Measurements expressed against day post-partum and imposed litter size.

Each vertical bar indicates the standard error of the mean.

C: virgin controls.

a. SO neurones. b. PV neurones.

b

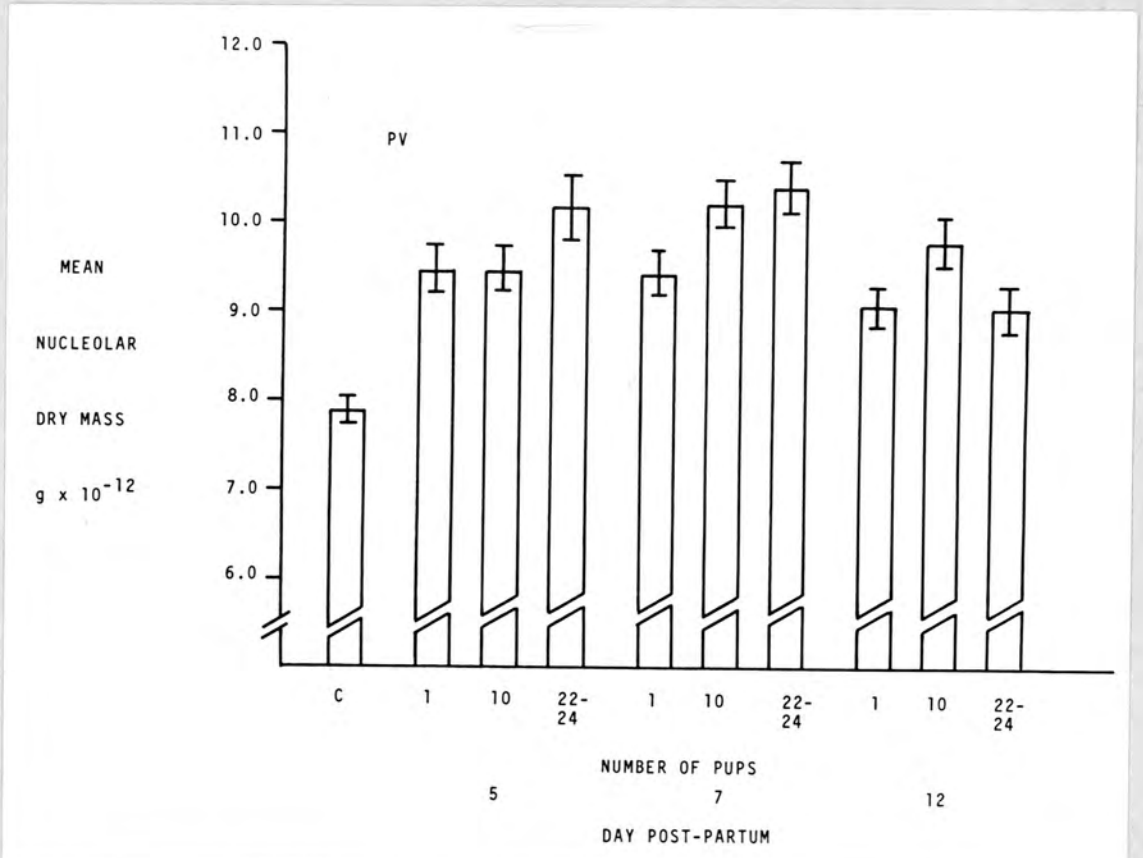


TABLE 8

NEURONAL NUCLEOLAR DRY MASS MEASUREMENTS: COMPARISONS BETWEEN GROUPS NURSING DIFFERENT SIZES OF LITTER

SO mean nucleolar dry mass

Compared groups		Litter size, pups			
		0	1	10	22-24
Day 5 post partum					
Virgin control		$p < 0.001$***	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***
Litter size, pups	22-24	$p = 0.025$*	$0.6 > p > 0.5$	$0.02 > p > 0.01$*	
	10	$p > 0.9$	$0.1 > p > 0.05$		
	1	$0.2 > p > 0.1$			
Day 7 post partum					
Virgin control		$0.5 > p > 0.4$	$p < 0.005$**	$p < 0.001$***	$p < 0.001$***
Litter size, pups	22-24	$p < 0.001$***	$0.05 > p > 0.025$*	$0.3 > p > 0.2$	
	10	$p < 0.001$***	$p < 0.005$**		
	1	$0.05 > p > 0.025$*			
Day 12 post partum					
Virgin control		$0.9 > p > 0.8$	$p < 0.005$**	$p < 0.001$***	$p < 0.001$***
Litter size, pups	22-24	$p < 0.001$***	$p < 0.001$***	$0.4 > p > 0.3$	
	10	$p < 0.001$***	$p < 0.001$***		
	1	$0.1 > p > 0.05$			

TABLE 9

NEURONAL NUCLEOLAR DRY MASS MEASUREMENTS: COMPARISONS BETWEEN GROUPS NURSING DIFFERENT SIZES OF LITTER

PV mean nucleolar dry mass

Compared groups		Litter size, pups			
		0	1	10	22-24
Day 5 post partum					
Virgin control		$p<0.001$***	$p<0.001$***	$p<0.001$***	$p<0.001$***
Litter size, pups	22-24	$0.005>p>0.001$**	$0.2>p>0.1$	$0.2>p>0.1$	
	10	$0.2>p>0.1$	$p>0.95$		
	1	$0.2>p>0.1$			
Day 7 post partum					
Virgin control		$0.7>p>0.6$	$p<0.001$***	$p<0.001$***	$p<0.001$***
Litter size, pups	22-24	$p<0.001$***	$p<0.001$***	$0.8>p>0.7$	
	10	$p<0.001$***	$0.1>p>0.05$		
	1	$p<0.001$***			
Day 12 post partum					
Virgin control		$0.5>p>0.4$	$p<0.001$***	$p<0.001$***	$p<0.001$***
Litter size, pups	22-24	$0.01>p>0.005$**	$p>0.95$	$0.1>p>0.05$	
	10	$p<0.001$***	$0.05>p>0.02$*		
	1	$0.005>p>0.001$**			

TABLE 10

NEURONAL NUCLEOLAR DRY MASS MEASUREMENTS: GROUPS NURSING LITTERS OF IDENTICAL SIZE COMPARED AT DIFFERENT TIMES

Day post partum	PV neurones		SO neurones	
	5	7	5	7
1 pup litters				
12	$p = 0.3$	$p = 0.3$	$p < 0.001$***	$0.8 > p > 0.7$
7	$p = 0.98$		$0.005 > p > 0.001$**	
10 pup litters				
12	$0.4 > p > 0.3$	$0.3 > p > 0.2$	$0.2 > p > 0.1$	$0.9 > p > 0.8$
7	$0.05 > p > 0.02$*		$0.1 > p > 0.05$	
22-24 pup litters				
12	$0.02 > p > 0.01$*	$p < 0.001$***	$p > 0.95$	$0.1 > p > 0.05$
7	$0.7 > p > 0.6$		$0.1 > p > 0.05$	

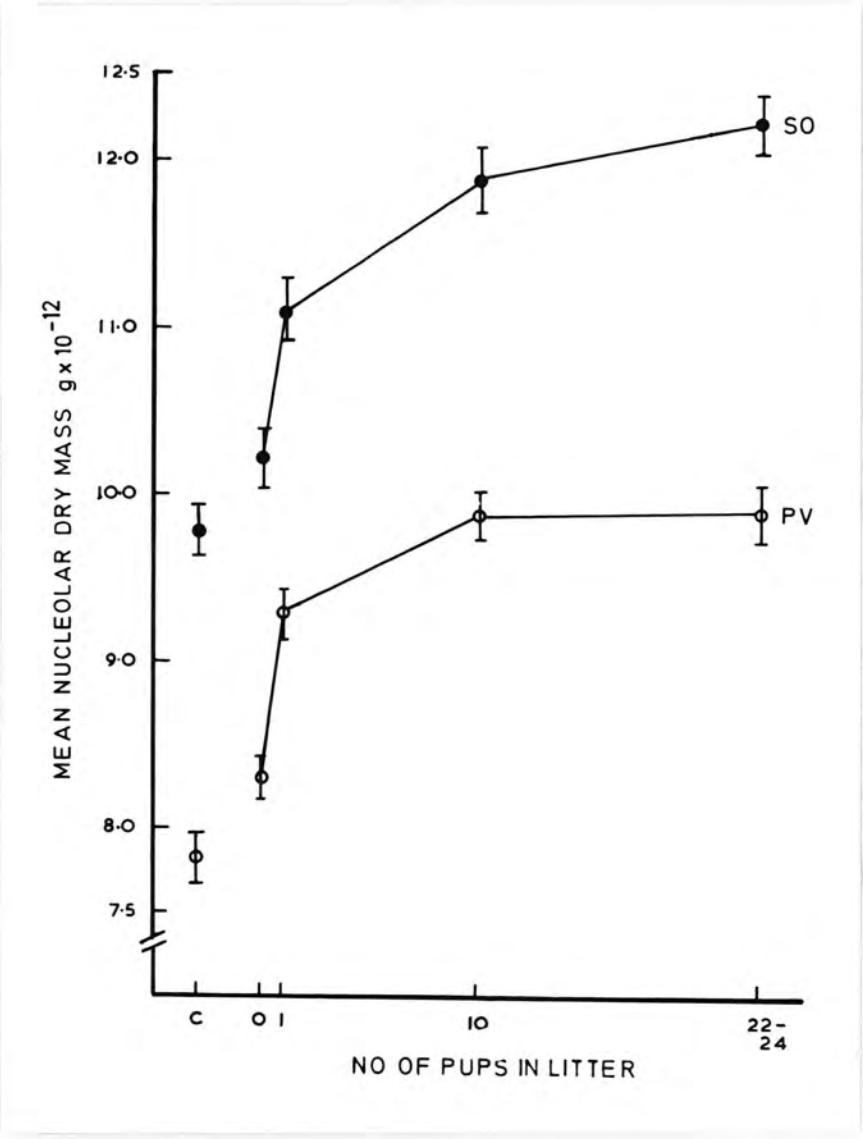


Figure 25 Lactation with varied litter size: mean dry mass of neuronal nucleoli. Data from days 5, 7 and 12 post-partum have been pooled for each litter size.

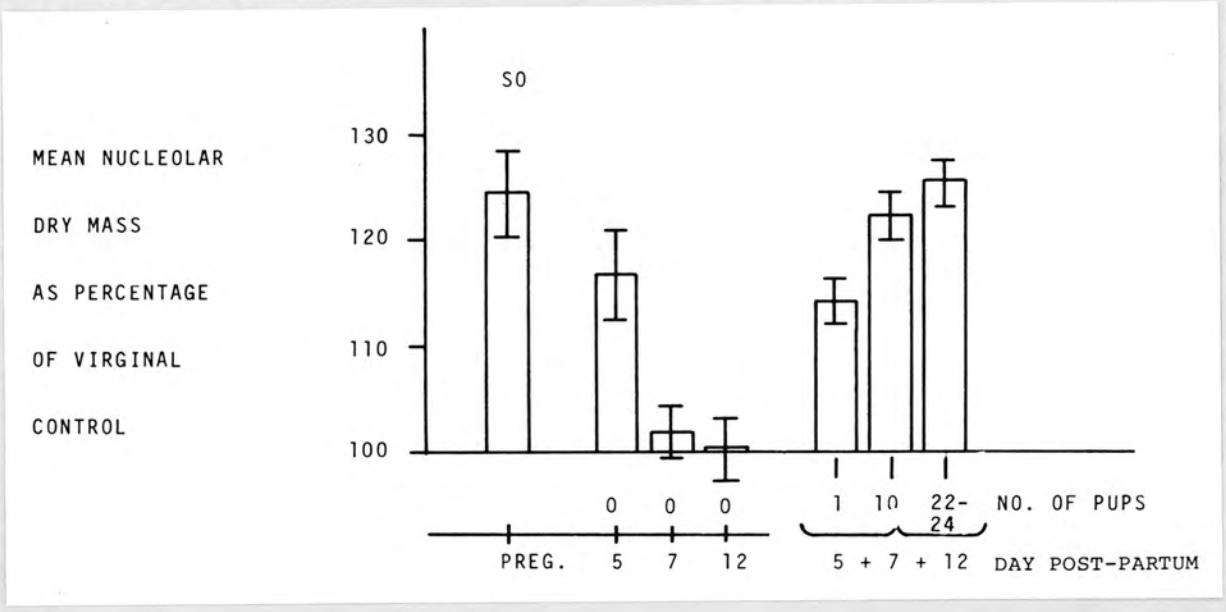
C: virgin controls.

Each vertical line indicates the standard error of the mean.

TABLE 11

NEURONAL NUCLEOLAR DRY MASS MEASUREMENTS, AVERAGED OVER 7 DAYS. COMPARISONS BETWEEN ANIMALS NURSING DIFFERENT SIZES OF LITTER

Neuronal nucleoli	PV			SO		
Litter size, pups	0	1	10	0	1	10
22-24	$p<0.001$***	$p<0.001$***	$0.95>p>0.9$	$p<0.001$***	$p<0.001$***	$0.2>p>0.1$
10	$p<0.001$***	$0.005>p>0.001$**		$p<0.001$***	$0.005>p>0.001$**	
1	$p<0.001$***			$p<0.001$***		



a

Figure 26 Mean dry mass of neuronal nucleoli in late pregnancy and post-partum expressed as percentages of values for respective virgin controls.

PREG: Day 20 of pregnancy.

5 + 7 + 12: Data from days 5, 7 and 12 post-partum pooled for each litter size.

Each vertical line indicates the standard error of the mean.

a. SO neurones. b. PV neurones. Data from paired consecutive days has been pooled for animals nursing normal litters.

b

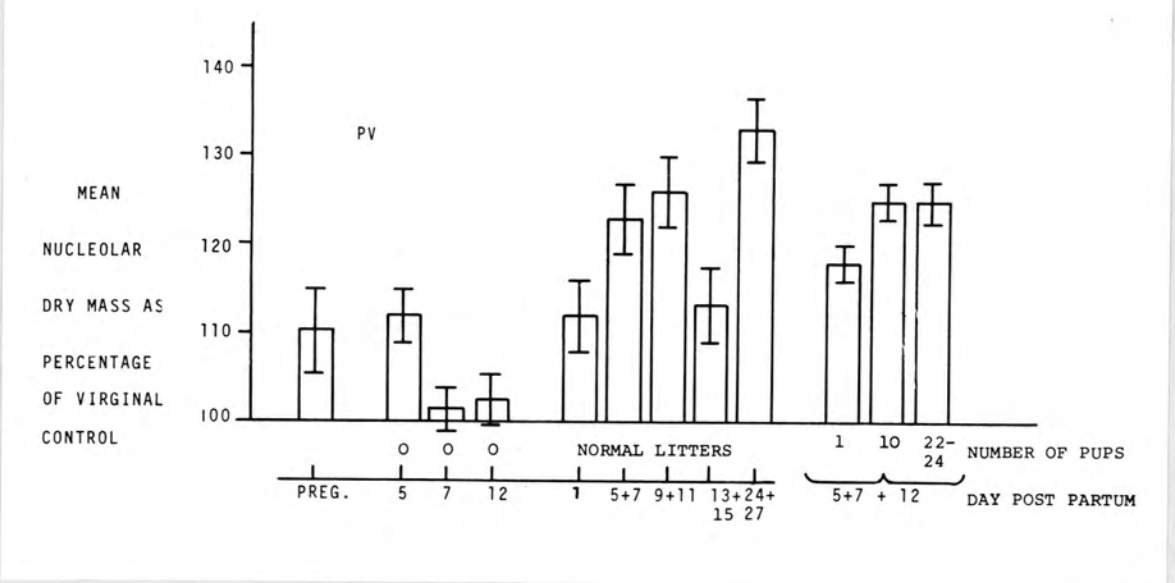


TABLE 4

COMPARISONS BY STUDENT'S t-TEST OF MEAN NUCLEOLAR DRY MASS MEASUREMENTS OF PREGNANT AND LACTATING GROUPS AS PERCENTAGE OF RESPECTIVE VIRGIN CONTROLS

Compared groups		Day 20 of pregnancy	
Experimental Group	Days post partum	PV	SO
Litter size unaltered	1	$0.8 > p > 0.7$	
	1 + 2	$0.2 > p > 0.1$	
	3 + 4	$0.02 > p > 0.01$*	
	5 + 7	$0.05 > p > 0.01$*	
	9 + 11	$0.005 > p > 0.001$**	
	13 + 15	$p > 0.4$	
	18 + 21	$0.025 > p > 0.02$*	
	25 + 28	$p < 0.001$***	
0 pups	5	$0.8 > p > 0.7$	$0.2 > p > 0.1$
	7	$0.05 > p > 0.025$*	$p < 0.001$***
	12	$0.1 > p > 0.05$	$p < 0.001$***
1 pup	5 + 7 + 12	$0.1 > p > 0.05$	$0.02 > p > 0.01$*
10 pups	5 + 7 + 12	$0.005 > p > 0.001$**	$0.7 > p > 0.6$
22-24 pups	5 + 7 + 12	$0.01 > p > 0.005$**	$0.9 > p > 0.8$

3. Results

For comparisons between all groups, the PV and SO neuronal nucleolar dry mass measurements are shown as percentages of the relevant controls in Fig. 26 a, b and Table 4 (page 84).

3.1 Late pregnancy

The mean nucleolar dry mass of PV or SO neurones was increased on the 20th day of pregnancy, compared with virgin controls (Fig. 22a, b and Table 5). Females killed on the 20th day of pregnancy each had 9, 10, 6 or 13 foetuses.

3.2 Normal lactation

The mean nucleolar dry mass of PV neurones on each day are shown in Fig. 23. For statistical comparison the data from paired consecutive days have been pooled (Fig. 23 and Table 6). At all times post partum that were studied, the nucleolar dry mass of PV neurones was greater than in virgin animals. A biphasic change occurred in PV nucleolar dry mass. It increased from the first day post partum, significantly by 7 to 9 days, and the first peak was reached between the 9th and 11th days. PV neuronal nucleolar dry mass then decreased until the 15th day, and rose steadily thereafter. The range of litter size was 3 to 12 pups; mean litter sizes are shown in Table 7.

3.3 Lactation and imposed variation in litter size

All the post partum animals accepted the cross-fostered pups. Details of nursing behaviour and lactational performance are described in Section IV - 3.

The mean nucleolar dry mass measurements of SO and PV neurones of these animals are shown in Figs. 22a,b, 24a,b and 25, and statistical comparisons in Tables 5, 8, 9, 10 and 11.

The significant features are:

1. If the pups were removed at birth, PV and SO neuronal nucleolar dry mass remained high for five days, and then decreased to virgin control values (Fig. 22a,b, Table 5).
2. At all times the mean nucleolar dry mass of PV and SO neurones was greater in all animals nursing pups than in virgin animals (Fig. 24a,b, Tables 8 and 9).
3. After the 5th day post partum the mean nucleolar dry mass of PV and SO neurones was greater in all animals nursing pups than in post partum females not nursing pups (Figs. 22a,b, 24a,b; Tables 8 and 9).
4. The mean nucleolar dry mass of PV neurones of animals nursing 22-24 pups did not alter between days 5 and 7, when it was greater than the same parameter in animals nursing one pup, but decreased in the next five days (Fig 24b, Tables 9 and 10).
5. The mean nucleolar dry mass of PV neurones of animals nursing 10 pups increased between the 5th and 7th post partum days, and did not subsequently change. On the 12th day it was greater than the same parameter of animals nursing one pup (Fig. 24b, Tables 9 and 10).
6. PV neuronal nucleoli of animals nursing one pup did not change with time (Fig. 24b, Table 10)

7. The PV neuronal nucleolar dry mass of animals nursing 10 pups was never different from that of animals nursing 22-24 pups (Fig. 24b, Table 9).
8. The dry mass of SO neuronal nucleoli of animals nursing 22-24 pups was greater only on the 5th day post partum than in animals nursing 10 pups (Fig. 24a, Table 8).
9. The only change in SO neuronal nucleolar dry mass with time was the fall in this parameter after the 5th day in animals nursing one pup. This parameter was thereafter smaller than in animals nursing 10 or 22-24 pups (Fig. 24a, Table 10).
10. The differences between each litter size group are seen more clearly if the SO and PV nucleolar measurements are averaged for each litter size over days 5 to 12 post partum (Fig. 25, Table 11).

Expressed as percentages of the respective virgin control parameters (Fig. 26b, Table 4), mean nucleolar dry mass of PV neurones was similar at the end of pregnancy, on the first day post partum, and on the fifth day post partum in animals not nursing pups. The mean nucleolar dry mass of PV neurones, averaged over days 5 to 12 post partum, increased if post partum animals nursed pups, and by a similar amount, regardless of whether the litter averaged 9 pups, or was adjusted to 10 or 22-24 pups; but a smaller change occurred with only one pup. In animals nursing their own litters, PV neuronal nucleolar dry mass decreased between days 13 and 15 to a value similar to that of late pregnancy.

Expressed as percentages of the respective virgin control measurements, the increase in SO neuronal nucleolar dry mass was as great in late pregnancy as in lactation if 10 or 22-24 pups were nursed, but was less in lactation if only one pup was nursed. The percentage change was less 5 days post partum if the pups were removed at birth, and was not significant thereafter (Fig. 26a, Table 4).

4. Discussion

4.1 Interpretation of nucleolar dry mass changes

The role of the nucleolus in the synthesis of ribosomes has been discussed (Section I - 2.2.1). Increased perikaryal nucleic acid content and dry mass and nucleolar nucleic acid content always accompany increased nucleolar dry mass (Watson, 1968a, 1969; Watt, 1970). These changes reflect increased rates of RNA and protein synthesis measured by other techniques such as autoradiography (Watson, 1965b, 1968a, 1969) and confirm previous assertions that neuronal nucleolar size or nucleic acid content increase when protein synthesis increases (Hyden, 1943; Caspersson, 1950; Edstrom & Eichner, 1958).

Nucleolar dry mass is a measure of the pool of protein, RNA and other constituents of the nucleolus. It may increase as a result of increased synthesis within, decreased release from or increased entry into the nucleolus of any of these nucleolar constituents. Which of these processes accounts for the changes seen in this study cannot be distinguished, because isotopes or inhibitors were not used (Watson, 1968a, 1969). Within these limitations, and by extrapolation from

previous studies, increased mean nucleolar dry mass of a group of neurones is interpreted as a sign of increased ribosomal, and consequently protein, synthesis.

Measurement of nucleolar dry mass has some advantages over measurement of other parameters. The nucleolus is a clearly defined intranuclear body, solitary in 95% of nuclei (Section II - 3.4.1). Nucleolar nucleic acid content is in principle of similar meaning to nucleolar dry mass, but is a more tedious measurement to make. However nucleolar dry mass and nucleolar nucleic acid content consistently change together (Section VI - 5.2.2, and unpublished). Interpretation of measurements of dry mass or nucleic acid content of isolated perikarya is limited by the unknown loss of cytoplasm in the dendrites and axon, while the distribution of cytoplasmic RNA between dendrites and perikaryon may change upon stimulation (Watson, 1973a). The errors involved in measuring perikaryal dry mass or nucleic acid content are greater than the errors incurred in measuring nucleolar dry mass (Section II - 3.4, 4.3). Nucleolar changes are an earlier indication of increased ribosome synthesis than are perikaryal changes (Watson 1965a,b, 1968a, 1969; Watt, 1970). It is possible that increased ribosome synthesis is accompanied by matched breakdown, so that perikaryal RNA content is not altered, as in mouse SO neurones during lactation (Edstrom, Eichner & Schor, 1961).

Measurements on individual cells in fixed tissue sections are unsatisfactory because of variable tissue shrinkage within and between samples, while the measured cells of a

nuclear group in a section may not be representative of the whole nucleus, different parts of which may be in different functional states (Zambrano & Mordoh, 1966). There are some disadvantages with respect to exact reproducibility of the dissection, but dissection of a nuclear group and homogenisation of the neurones, and measurements on fresh nuclei, overcome the difficulties associated with fixed tissue sections.

The general relationship between nucleolar dry mass changes and functional activity will be discussed in Section IV - 4.2.

The consistent finding that the mean nucleolar dry mass of SO neurones is greater than that of PV neurones may reflect the presence of nucleoli of parvicellular PV neurones in the latter (Section VI - 6).

4.2 Pregnancy

There is little information on changes in PV and SO neurones during pregnancy. The amount of neurosecretory material in the posterior pituitary increases between the second and third weeks of pregnancy in the rat (Stutinsky, 1953; Malandra, 1955, 1956a,b) but does not change in the cat (Racadot, 1957). Gland contents cannot be interpreted as reflecting altered synthesis. The perikarya of SO neurones enlarge during pregnancy (Stutinsky, 1953), but PV neuronal perikarya do not (Malandra, 1955), or both PV and SO neuronal volume increase during pregnancy (Ananieva, 1968). The amount of thiamine pyrophosphatase in PV and SO neurones

increases during the second week of pregnancy, and then declines until parturition (Swaab & Jongkind, 1970). These limited findings are consistent with increased synthetic activity of PV and SO neurones during pregnancy. This is confirmed by the nucleolar dry mass measurements made in this study (Fig. 22a,b). However, increased neurophysin synthesis at the end of pregnancy has not been demonstrated although neurophysin synthesis is increased 24 hours after parturition, even if the pups are not nursed (Norstrom & Sjostrand, 1972b).

It is not clear how increased synthetic activity in PV and SO neurones is related to oxytocin and ADH secretion during pregnancy. Possibly the increased body water(Dewar, 1969) and blood volume of pregnancy (Van Donk et al, 1934; Newcomer, 1947; Bond, 1948) result partly from increased ADH secretion, although the excretion of a water load is not altered by pregnancy (Hofman et al, 1954).

Oxytocin may be reflexly released by the maternal posterior pituitary during parturition (Ferguson, 1941), and high concentrations of oxytocin in maternal plasma have been measured during parturition in the guinea pig (Burton et al, 1972). However,foetal oxytocin may be more important during parturition (Bell & Robson, 1938; Chard et al, 1971), and oxytocin antibody injections do not interfere with parturition in the rat (Kumaresan et al, 1971). Lesions in the PV nucleus cause severe dystocia in the cat (Nibbelink, 1961), but posterior hypophysectomy does not affect parturition in a subsequent pregnancy in the rat (Benson & Cowie, 1956), although regeneration of functional neurosecretory endings may account for this finding (Billenstein & Leveque, 1955).

The observed nucleolar changes in PV and SO neurones on the 20th day of pregnancy obviously preceded the release of oxytocin at parturition. These nucleolar changes may result from increased ADH and oxytocin secretion during pregnancy, or from the actions of the hormones of pregnancy (Section V). A store of oxytocin could be prepared for labour in this way, although the posterior pituitary content of oxytocin at the end of pregnancy is not greater than in non pregnant animals (Heller, 1959). It is not known whether self-licking during pregnancy causes oxytocin to be secreted (Roth & Rosenblatt, 1966).

4.3 Lactation

The nucleolar changes at the end of pregnancy define a baseline against which the changes in lactation must be considered (Fig. 26a,b).

The nucleolar responses of SO and PV neurones were different insofar as SO neuronal nucleolar dry mass, as a percentage of virgin control values, did not increase further after parturition, even with a large litter, whereas PV neuronal nucleolar dry mass increased by a further 15%. This may imply activation of the PV and SO neurones by different stimuli and is discussed further in Section VI - 6.

There is substantial evidence that protein synthesis is increased in SO and PV neurones during lactation.

Some authors have reported increased nucleolar volume in SO and PV neurones 8 or 10 days post partum (Flament-Durand, 1966; Swaab et al, 1971) or noted a chromatolytic change in PV and SO neuronal perikarya (Cavallero & Malandra,

1958). Rinne (1960) found that PV and SO neuronal nucleolar volume is not significantly increased five days post partum. These discrepancies may be explained by variable tissue shrinkage during histological preparation, or by the different stages of lactation at which the measurements were made.

The nucleolar dry mass changes demonstrated in the present study indicate increased ribosome and protein synthesis in PV and SO neurones during lactation compared with virgin females (Figs. 23, 24a,b). These changes were maintained post partum only if the animals nursed a litter (Fig. 22a,b). The synthetic changes may have been related to the release of oxytocin or ADH, or both, or alternatively were caused by other hormones of lactation. It is unlikely that stimuli other than suckling were important with respect to nucleolar changes dependent on the presence of pups, because the cages containing animals nursing different litter sizes were kept close together. Olfactory, visual or auditory stimuli from the pups were shared by all the lactating animals.

The amount of neurosecretory material in the SO and PV axons apparently increases on the day after parturition; but not if the litter is prevented from suckling (Stutinsky, 1953). Enlargement of SO and PV neuronal nuclei and nucleoli after parturition, and the more rapid accumulation of ^{35}S-cysteine in the posterior pituitary similarly depend on suckling (Flament-Durand, 1966, 1967). In the absence of suckling, the nucleolar dry mass changes subsided over a period of 5 to 7 days (Fig. 22a,b). This slow decline may

reflect continued synthesis to repair depleted stores, or the turnover time of components which regulate ribosomal synthesis.

By the 10th day of a normal lactation, the activity of thiamine pyrophosphatase in SO neurones, and the amounts of this enzyme in SO and PV neurones, increase by 80% compared with the measurements in non pregnant females (Swaab & Jongkind, 1970; Swaab et al, 1971). These changes are interpreted as signs of increased secretory activity. This is confirmed by measurement of increased turnover of ^{35}S-cysteine labelled neurophysin on the sixth day post partum (Norstrom & Sjostrand, 1972b). Mean nucleolar dry mass of PV neurones, from animals nursing their own litters, was greater on the first day post partum than in virgin animals (Fig. 23). However, the mean nucleolar dry mass of PV neurones from animals killed on the 20th day of pregnancy or on the first day post partum was increased by a similar percentage compared with virgin animals. A significant suckling dependent change in PV neuronal nucleolar dry mass did not occur until the fifth or seventh day post partum (Figs. 23, 26b). This lag, after the onset of stimulation by suckling, is similar to that found for nucleolar dry mass of SO neurones during dehydration (Watt, 1970), or of hypoglossal neurones after axotomy (Watson, 1969) and for ribosomal RNA synthesis, measured autoradiographically, in SO neurones during salt loading (Watson, 1965b).

In lactating animals nursing their own litters, PV neuronal nucleolar dry mass was decreased between days 13 and 15 post partum (Fig. 23). A similar decrease occurs in

nucleolar dry mass of hypoglossal neurones 15 days after axotomy (Watson, 1969), and in the rate of RNA synthesis, measured autoradiographically, in SO neurones on the 15th day of salt loading (Watson, 1965b).

Lactation ceases after 28 days in the rat (Blaxter, 1962). No measurements were made of nucleolar dry mass after this time. It is not known if PV neuronal nucleolar dry mass returns to a virgin control value, as when the litter is removed at birth. The second increase in nucleolar dry mass of PV neurones, after the 15th day post partum, is possibly related to axonal hypertrophy (Fig. 23). The oxytocin content of the posterior pituitaries of multiparous rats is greater than that of nulliparous female or male rats (Acher & Fromageot, 1957; Rennels, 1958). These findings may indicate a permanently increased synthetic capacity of PV, or SO, neurones after a normal lactation.

The low PV neuronal nucleolar dry mass on days 13 to 15 post partum was measured in animals with small litters (Table 7, Fig. 23). The possible effect of litter size on PV and SO neuronal nucleoli was systematically investigated.

The lowest ovarian weights were also recorded in animals killed on days 13 to 15 post partum (Fig. 34a) and the possible effects of ovarian hormones on SO and PV neuronal nucleoli were also investigated (Section V).

Three aspects of nucleolar changes were examined in animals with varied litter size. These were the rate of increase, the average value in the period studied, and the time of decline of nucleolar dry mass. From the measurements

on PV neurones during normal lactation the period between the 5th and 12th days was deemed the appropriate period to study.

The peak PV neuronal nucleolar dry mass occurred two days earlier in animals nursing 22-24 pups, but declined more rapidly compared with animals nursing 10 pups; whereas the same parameter in animals nursing one pup did not change with time (Fig. 24b). The different rates of change of nucleolar dry mass may reflect rates of ribosome synthesis. The peak rate of transfer of ^{3}H-uridine labelled RNA from nucleus to cytoplasm in SO neurones occurs earlier, and the rate declines more rapidly, with a more severe osmotic stimulus (Watson, 1965b). Nucleolar nucleic acid content of hypoglossal neurones increases more rapidly the more proximal is the axotomy (Watson, 1968a), and the nucleolar changes of SO neurones in dehydration are accelerated at a high environmental temperature (Watt, 1970).

There was no evidence for different rates of onset or decline of nucleolar changes in SO neurones of animals nursing 10 or 22-24 pups, and as already noted, the nucleolar dry mass of SO neurones was no greater in lactation than at the end of pregnancy (Figs. 24a, 26a). However the nucleolar dry mass of SO neurones of animals nursing only one pup declined between the 5th and 12th post partum days. This decline was less rapid than when all the pups were removed at birth (Figs. 22a, 24a).

These nucleolar changes may indicate identical rates of ribosome synthesis in SO neurones of animals nursing 10

or 22-24 pups, but a lower, or decreasing, rate of ribosome synthesis in animals nursing only one pup.

The nucleolar dry mass measurements, averaged over five days, bore a non-linear relationship to litter size for both SO and PV neurones (Fig. 25). Whether this relationship depends on similar changes in secretory activity, or represents the limited capacity of the nucleolar apparatus, or of groups of neurones, to respond to a supranormal stimulus, is explored in Sections IV and VI.

5. Summary

Nucleolar dry mass of both SO and PV neurones was increased on the 20th day of pregnancy; during lactation changes in SO neurones were maintained by suckling, but PV neuronal nucleoli responded further. Compared with pregnancy, PV neuronal nucleolar dry mass did not change in lactation with only one pup suckling, but SO neuronal nucleolar dry mass decreased, although less rapidly than if all the pups were removed at birth.

Nucleolar dry mass of PV neurones rose more rapidly in lactation the larger the litter, but no further change occurred in SO neurones. The nucleolar dry mass measurements of PV and SO neurones were averaged over days 5, 7 and 12 for each litter size. The averaged nucleolar dry mass of PV or SO neurones in animals nursing 10 pups was the same as in animals nursing 22-24 pups; but the averaged nucleolar dry mass of PV or SO neurones in the animals nursing one pup was less than in those nursing 10 or 22-24 pups, yet greater than in post partum animals not nursing pups.

These nucleolar changes probably reflect rates of ribosome synthesis, which in lactation depend on and are related quantitatively to the number of pups suckling, but in pregnancy are related to unknown factors.

SECTION IV

RELATIONSHIP OF NUCLEOLAR CHANGES IN PARAVENTRICULAR AND SUPRAOPTIC NEURONES DURING PREGNANCY AND LACTATION TO NEURONAL FUNCTION

1. Aims of investigation

The nucleolar changes described in Section III are probably a result of increased ribosome synthesis, which may occur to supply the increased amounts of neurosecretory materials secreted during lactation (Section I - 2.4.3, 2.6.2).

To investigate the relationship between nucleolar dry mass changes and secretory activity an attempt has been made to measure the output of oxytocin under the conditions of the experiment in which litter size was varied.

Although the concept that synthetic activity is directly linked to secretory activity is attractive (Watt, 1970, 1971; Watson, 1973a), it is possible that synaptic activity on the perikarya of PV and SO neurones controls ribosome synthesis (Kernel & Peterson, 1970; Gisiger, 1971). An attempt has also been made to measure stimulus intensity.

Oxytocin is not secreted in amounts sufficient to cause milk ejection in the absence of suckling (Lincoln et al, 1973). The post partum animals deprived of pups probably did not secrete more oxytocin than virgin animals. The nucleolar changes in these animals are consistent with a synthesis-secretion interrelationship.

Some other parameters which are suckling dependent, or reflect ADH secretion, have also been used to compare responses to different intensities of suckling.

2. Methods

2.1 Management of animals

These animals were managed in the same manner as previously described (Section III - 2.1).

2.2 Stimulus parameters

2.2.1 Intensity of suckling stimulus

A method of estimating stimulus intensity was devised to see if lactating animals nursing litters of various sizes were exposed to stimulus intensities in proportion to the litter size.

The 'volume' of the suckling stimulus (Edwardson & Eayrs, 1967) depends on the number of nipples exposed to suckling and to the time for which they are exposed, on the age of the pups, and the drive to suck. Previously these variables have been studied mainly by the response to suckling, measured as milk yield or oxytocin output. There is no systematic study which relates these parameters to impulse activity in the afferent arc of the milk ejection reflex. These stimulus parameters are those which are effective for milk secretion or ejection, rather than those effective for neural responses alone.

The number of pups is obviously measurable and controllable. It does not follow that the volume of the suckling stimulus is necessarily increased by enlarging the litter; the mother may determine the suckling period.

The stimulus intensity was measured by observing litters of 1, 10 or 22-24 pups with their foster mothers. Two

litters of each size were studied and each litter was observed for six periods of 30 minutes. During each observation period the number of pups attached to nipples, and the time for which they were attached, was recorded. The integrated stimulus intensity was measured in nipple-minutes (n.t.), where n was the number of nipples with pups attached for t minutes.

n varied from 0 to 12; t varied from 0 to 30.

The stimulus intensity was expressed as a percentage of the maximum, 360 nipple-minutes, in each observation period.

It was difficult always to determine the exact number of pups attached to mothers nursing large litters, as the nursing mothers could obviously not be disturbed. However, the number of pups attached to these animals was shown to be correctly determined on other occasions when checked by lifting the mothers. Observations were made at various times during the daylight period only, between the 5th and 11th days post partum.

The mean stimulus intensities for each litter size group were compared by Student's t-test.

The average pup weight gain probably indicates the drive to suck. It seems reasonable to assume that pups not gaining weight normally will be hungrier.

2.2.2 Serum osmolality

This is not a simple measure of the osmotic stimulus for secretion of posterior pituitary hormones in prolonged experiments. Serum osmolality is a measure of the efficiency

with which it is controlled. During a period of water deprivation it is assumed, within these limitations, that the increment in serum osmolality is a measure of stimulus intensity (Moses & Miller, 1970). It can be argued that milk secretion increases serum sodium ion concentration, and reduces blood volume (this section - 4.1). It was of interest to see if a change could be measured in serum osmolality during lactation.

At the time the animals were killed, blood was collected by cardiac puncture and allowed to clot. Serum was separated by centrifugation at 4°C and stored at -4°C. Serum osmolality was measured by the freezing point depression method, using an 'Osmette' (Precision Systems Inc.). The osmometer was calibrated at 100 and 500 mOsm per Kg at the start of each batch of measurements. Each 0.2 ml sample of serum was measured once. The calibration was then checked and each sample measured again. If the duplicate measurements did not agree within 1%, the measurements were repeated. Sera were measured in batches of 20 samples in a session.

Serum sodium ion concentrations were not measured.

2.3 Response parameters

To see if oxytocin, and other suckling dependent hormones, were secreted in different amounts with different litter sizes, the following parameters were measured.

2.3.1 Milk yield

Milk yield depends upon both milk secretion and ejection. Since both these components are suckling dependent, milk yield

measures the total lactational response to suckling (Section I - 2.5).

Litter weight gain has been used as an index of milk yield (Cowie & Folley, 1947; Edwardson & Eayrs, 1967), but it is not a valid measure of milk yield of animals nursing different sizes of litter. Simple considerations show that the same milk yield could result in zero weight gain in a 20 pup litter and normal weight gain in a 10 pup litter. Only if the averaged daily weight gain per pup is similar for litters of different sizes could litter weight gain then be used as a measure of milk yield. Daily weight gains per pup were calculated as a percentage of the birth weight. Previous studies have shown that daily weight gain per pup is constant (Reddy & Donker, 1964) or decreases as litter size increases from 2 to 10 or 12 pups (Schultze, 1954; Edwardson & Eayrs, 1967). In general milk yield is adjusted to litter size (Bateman, 1957; Blaxter, 1961). The pup weight gains measured in this study with litters of 1, 10 or 22-24 pups (Fig. 29a,b) showed that weight gain was significantly different between these groups, and could not be used to estimate milk yield directly.

Some fraction of the daily milk yield is used for maintenance of the litter. The fraction of the milk yield required for maintenance of the pups was estimated. Fifteen litters of various sizes aged from 5 to 12 days were separated from their mothers and caged alone or with non-lactating, multiparous females for 24 hours. The weight lost by each litter in this period was measured and expressed as a

percentage of the initial weight. This percentage weight loss was bimodally distributed. The mean, $\pm$ the standard error, of each group was 4.7 $\pm$ 0.3%, based on nine observations, and 14.8 $\pm$ 0.6%, based on six observations. This distribution was not related to the age of the pups or to litter size, but the weight loss was greater when litters were with animals which displayed maternal behaviour. Others have recorded weight losses by pups separated from their mothers for 24 hours of 7-8% (Brody & Nisbet, 1938; Lincoln et al, 1973) and 12% (Hofer, 1970) of the initial body weight. It is assumed that the metabolic rate of litters does not change when they are deprived of milk. Even if kept warm, pups undergo metabolic changes similar to entry into hibernation if separated from their mothers (Hofer, 1970), so milk yield is likely to be underestimated by the method used here. It is also assumed that faecal and urinary losses are the same whether the litters are fed or not. It is likely that faecal and urinary losses decrease during a period of separation: to this extent milk yield will be underestimated. Within these limitations milk yield was calculated.

$$\text{Milk yield} = w_2 - (w_1 \frac{K}{100}) g \text{ per day}$$

where w_2 = litter weight at end of 24 hours,

w_1 = litter weight at start of 24 hours,

K = (100-14.8) or (100-4.7); and $\frac{K}{100}$ is either estimate of the fraction of the initial body weight remaining had the litter been starved for 24 hours (vide supra).

Daily milk yield was calculated for each lactating female from the daily litter weights recorded at 10.00 hours;

pups were separated from their mothers or foster mothers for less than three minutes. Two estimates of total daily milk yield were made for each lactating female. The truth lies somewhere between the two.

It can be argued that because hormones secreted in response to suckling are distributed to all the mammary glands, the milk yields per gland sucked should be compared to obtain an estimate of the relative amounts of these hormones secreted by animals nursing litters of different sizes. This calculation was made: the total daily milk yield was divided by 12, 10 or 1 for animals nursing 22-24, 10 or 1 pups respectively. The divisor is the maximum number of glands from which milk can be obtained at any instant.

2.3.2 Oxytocin secretion: milk ejections

Although in general secretion of the hormones required for milk synthesis and the secretion of oxytocin alter pari passu with stimulus volume (Fuchs & Wagner, 1963; Grosvenor et al, 1968; Smith & Convey, 1970), this relationship may not apply in the conditions of the present experiments. Milk yield can not be used to distinguish between the effects of changes in anterior and posterior pituitary secretion, either of which may be limiting to milk yield.

Measurement of posterior pituitary or blood oxytocin content is of no use as a measure of oxytocin secretion over several days. The half life of circulating oxytocin is about 2 minutes (Fabian et al, 1969).

Oxytocin is excreted in urine, but the relationship between the amount secreted and that excreted is not certain (Boyd et al, 1972). The continuous collection of urine from lactating mothers in the presence of pups presents obvious difficulties. The closest approach to the continuous measurement of oxytocin secretion would be by continuous auto-bioassay, with an indwelling intramammary (Lincoln et al, 1973) or intrauterine (Fuchs & Wagner, 1963) cannula. To avoid such interference, an estimate was made of the number of milk ejections during 75 minute observation periods. The pup stretch reaction, and its relationship to oxytocin secretion, have been discussed (Section I - 2.4.3; Voorherr et al, 1967; Lincoln et al, 1973). The criteria adopted for a milk ejection response were:

1. Each pup flexed and extended the hind and fore limbs; these movements were maintained for more than 10 seconds.
2. All pups attached to nipples responded synchronously.

If the mother was crouched over the pups while nursing she was usually lifted by the combined efforts of the pups during the stretch reaction. The criteria were difficult to apply to litters of 22-24 pups as these pups competed for nipples and attached pups were frequently disturbed; however it was possible to distinguish such local disturbances from the synchronised response of the whole litter to a milk ejection.

Three groups of litters were observed; each group consisted of two litters of 1, 10 or 22 pups. Each litter was observed for 75 minutes each day from day 4 to day 12 post

partum. Because of the large variation in the number of milk ejections in a 75 minute observation period, the data from three consecutive days was pooled. The timing of each observation period was arranged so that during any three consecutive days the number of milk ejections between 1400 and 1745 hours was estimated. For each litter size there were therefore six estimates of the number of milk ejections between 1400 and 1745 hours. The mean number of milk ejections between 1400 and 1745 hours was calculated for each litter size. The means were compared by Student's t-test.

The pup stretch reaction never occurs without milk ejection, and milk ejection does not occur when pups are not attached to the nipples and always evokes a stretch reaction (Lincoln et al, 1973). Mammary gland pressure was not measured in the present study; normally between 0.4 and 0.6 mU, but up to 1.5 mU, of oxytocin may be secreted per milk ejection by lactating rats nursing 10 pups (Lincoln, 1973). The amount of oxytocin secreted per milk ejection by rats nursing 22-24 pups is not known. The number of milk ejections cannot be converted accurately to the amount of oxytocin secreted, although the stretch reaction is specifically the result of oxytocin secretion (Wakerley et al, 1973).

Neither the milk yield nor the frequency of milk ejections can be converted accurately to the quantity of oxytocin secreted in 24 hour periods; but they have been used, within the restrictions discussed, to indicate probable trends between animals nursing litters of different size.

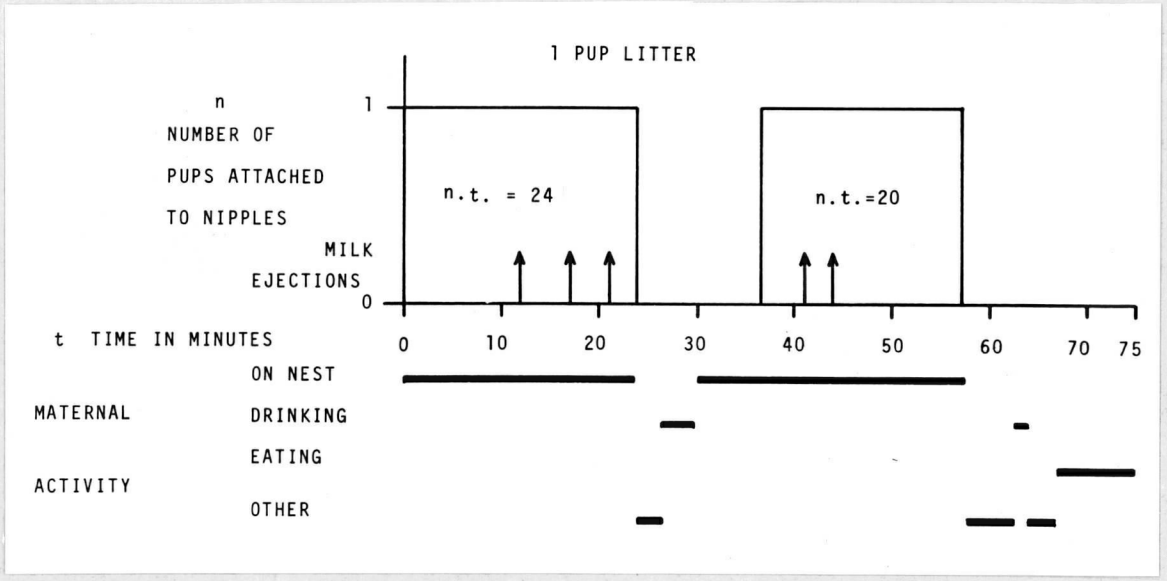
ADH secretion was not measured.

2.3.3 Ovarian weight

Gonadotrophin secretion is suppressed during lactation by suckling (Rothchild, 1960). Ovarian weight usually decreases post partum, but rapidly increases in the absence of suckling (Stotsenburg, 1923).

The ovaries of all animals were weighed to see if the animals nursing litters were exposed to a stimulus adequate to suppress ovarian growth.

3. Results



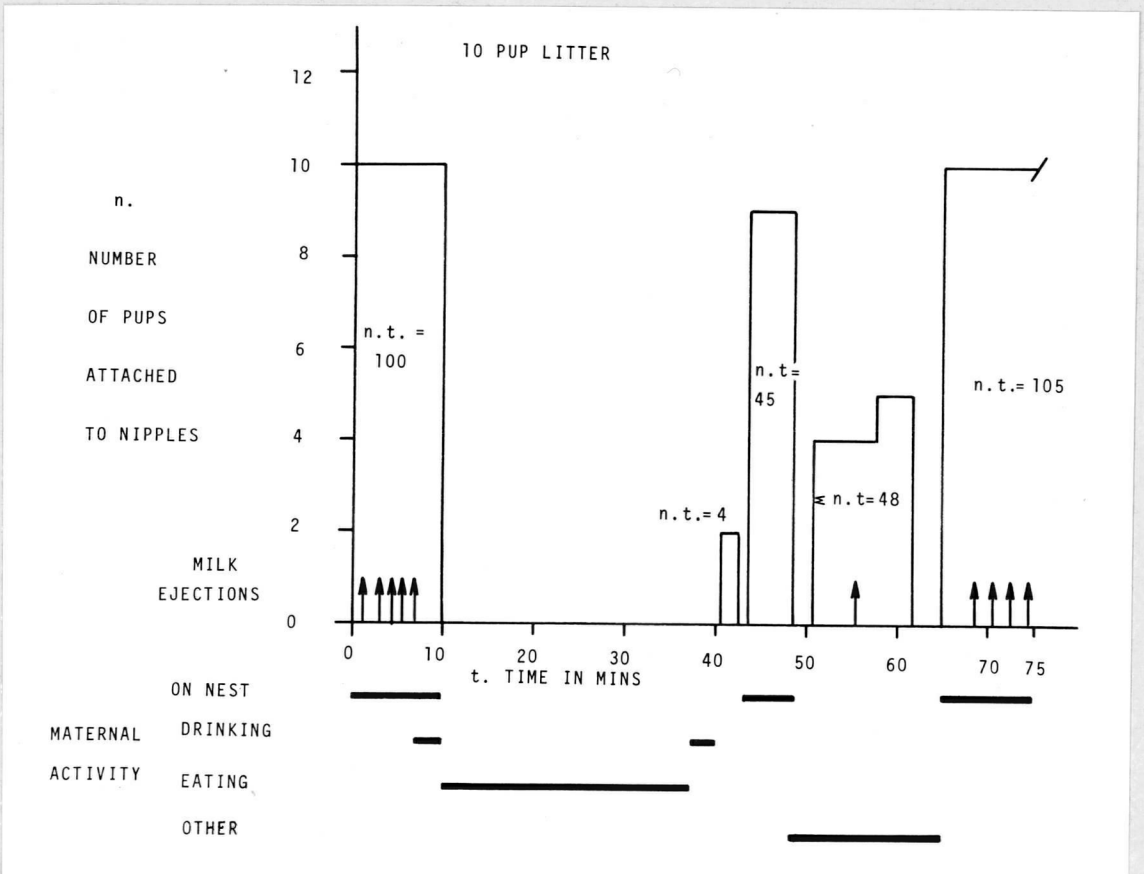
a

Figure 27 Typical records of nursing and suckling behaviour in a 75 minute observation period. Day 10 post-partum.

a. 1 pup litter. Five milk ejections in 75 minutes. Suckling intensity: 5% of maximum, 900 nipple-minutes (n.t.).

b. 10 pup litter. Ten milk ejections in 75 minutes. Suckling intensity: 45% of maximum.

b



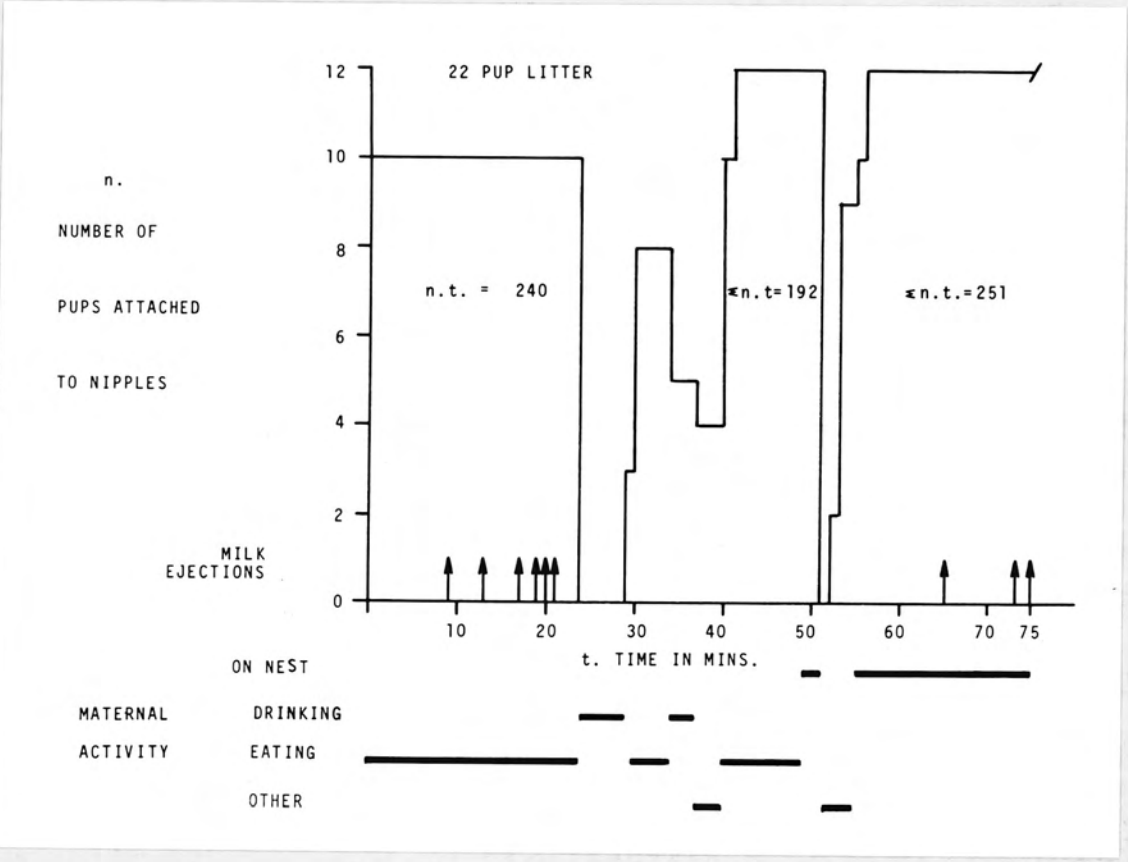
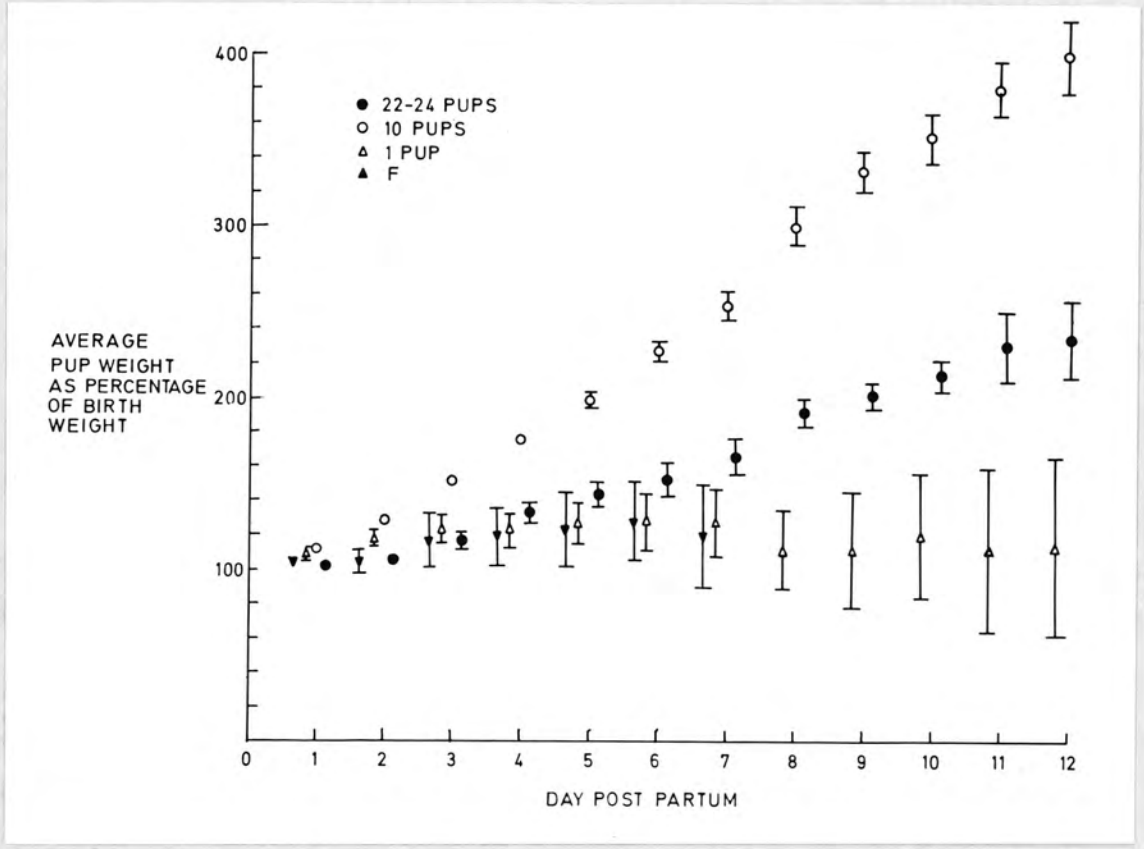


Figure 28 Typical record of nursing and suckling behaviour in a 75 minute observation period. Day 10 post-partum, 22 pup litter. Nine milk ejections in 75 minutes. Suckling intensity: 76% of maximum, 900 nipple-minutes (n.t.).



a

Figure 29 Average pup weight as percentage of birth weight expressed against time post-partum.

Each vertical line indicates standard error of the mean.

a. F = 1 pup to day 7, then 10 pups (Section V).

b. 1 pup litter weight gains as two distinct groups, A and B.

b

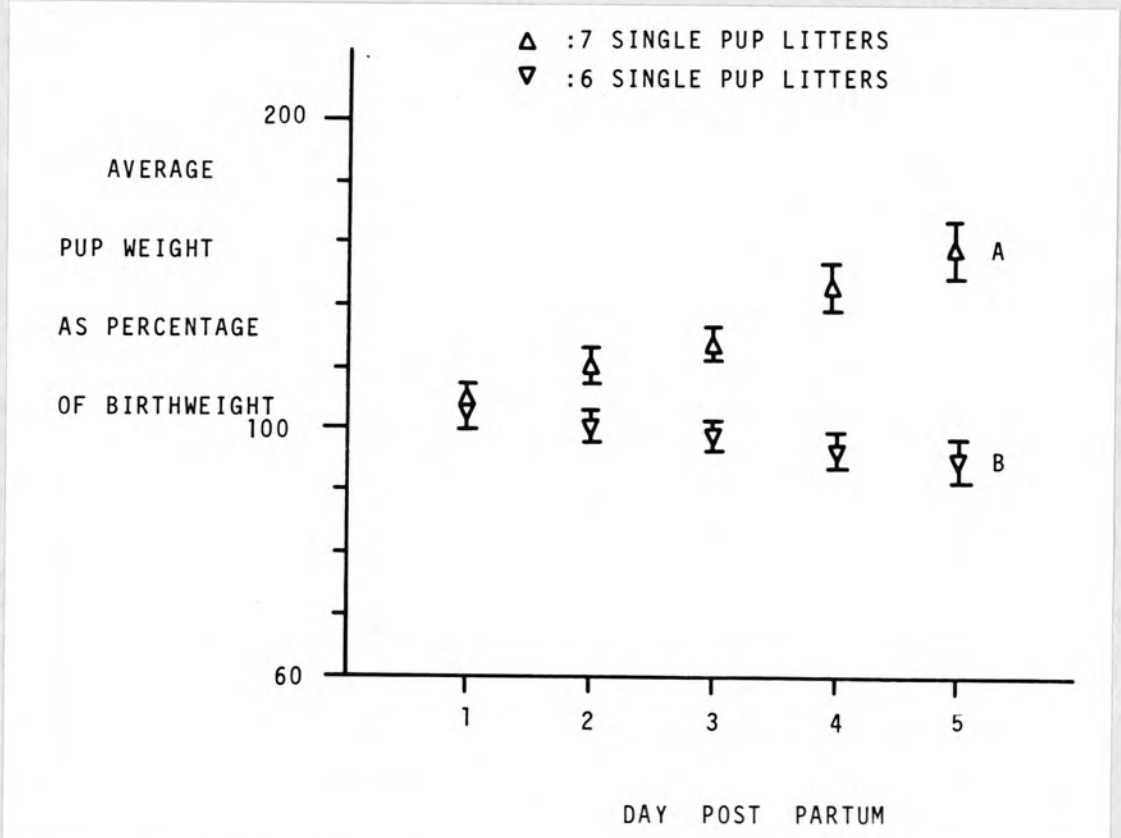


TABLE 12

AVERAGE PUP WEIGHT GAINS AS PERCENTAGE OF BIRTH WEIGHT. COMPARISONS BY STUDENT'S t-TEST BETWEEN LITTERS OF DIFFERENT SIZES. DAY 5 POST-PARTUM.

Litter size, pups	1	1, Group A	1, Group B	10
22-24	$0.3 > p > 0.2$	$0.4 > p > 0.3$	$p < 0.001$***	$p < 0.001$***
10	$p < 0.001$***	$0.005 > p > 0.001$**	$p < 0.001$***	
1, Group 'B'		$p < 0.001$***		

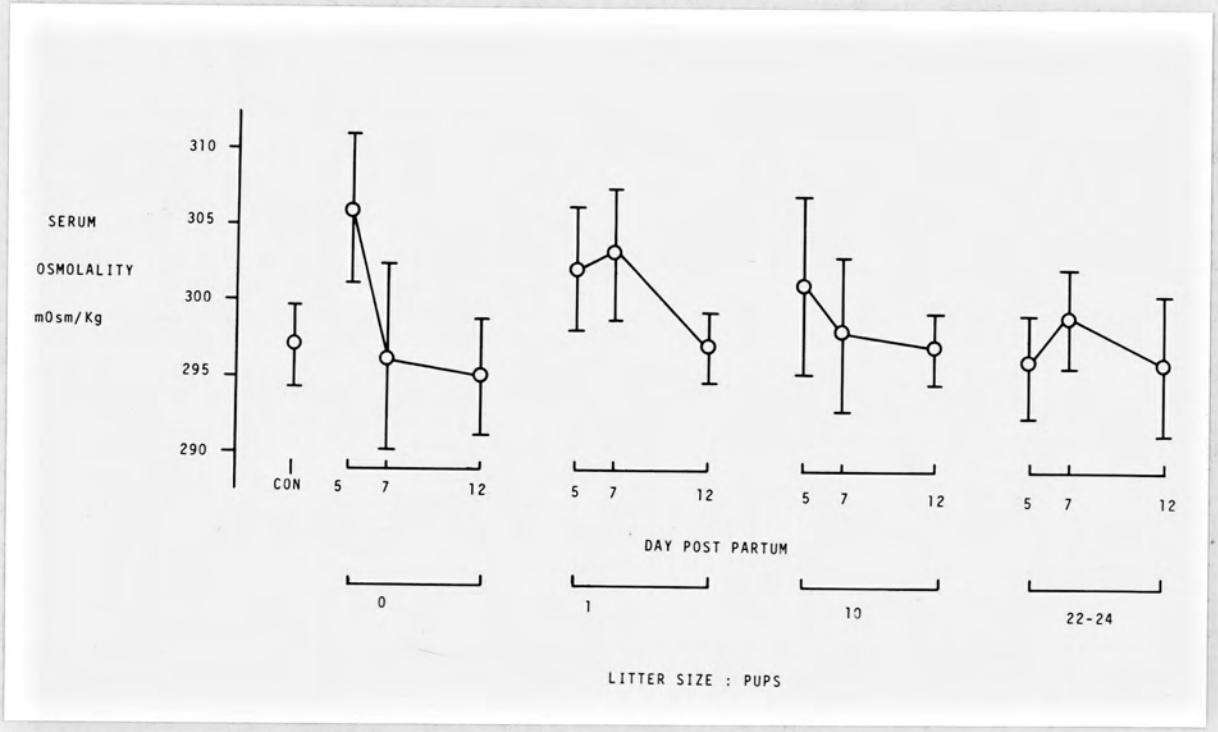


Figure 30 Serum osmolality post-partum with varied litter size.

Open circles: mean serum osmolality for each group.

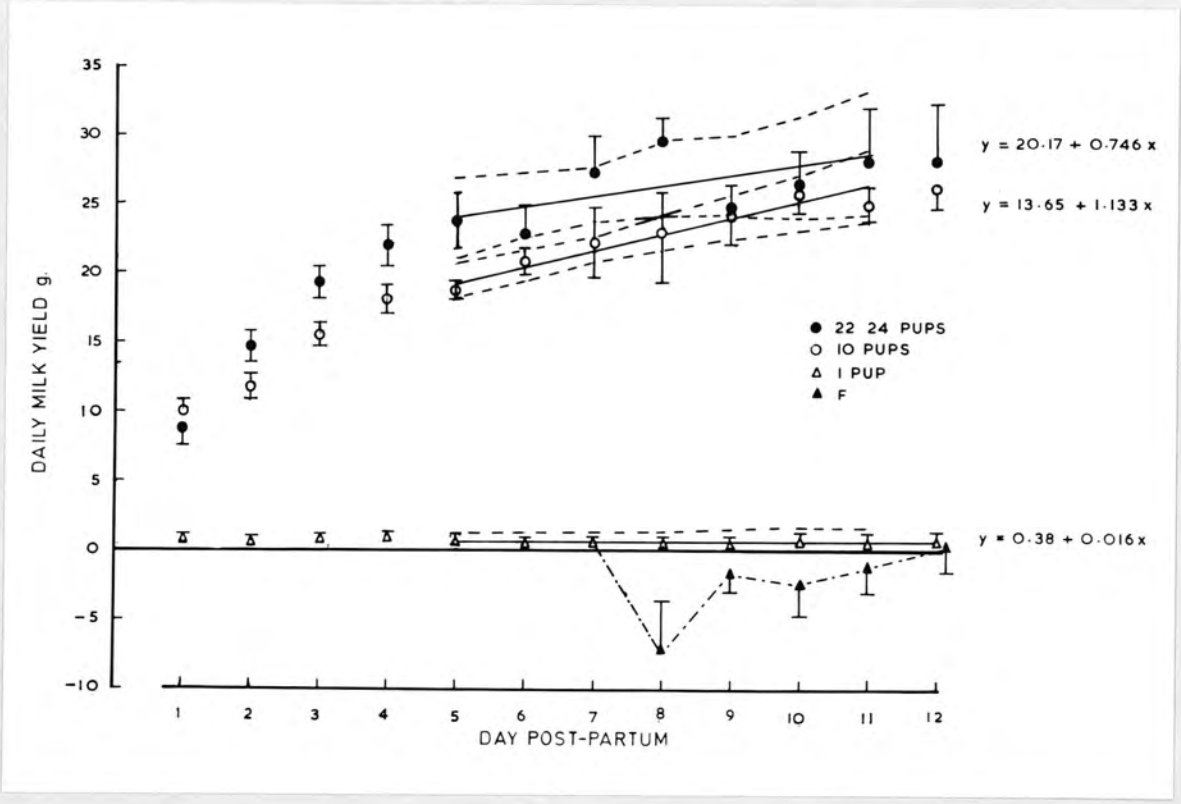
CON: virgin controls.

Each vertical line indicates the standard error of the mean.

TABLE 13

SERUM OSMOLALITY IN LACTATION WITH VARIED LITTER SIZE

Litter size	Mean Serum Osmolality mOsm/Kg Days 5,7,12 pooled ± S.E.M.	Comparisons with virgin controls: Student's t-test
Pups removed at birth	298.6 ± 3.33	p = 0.7
1 pup	299.56 ± 1.92	0.5>p>0.4
10 pups	298.08 ± 1.96	0.8>p>0.7
22-24 pups	297.14 ± 2.02	0.95>p>0.9
1 pup to Day 7, 10 pups to Day 12	289.00 ± 4.06 Day 12 only	0.2>p>0.1
Virgin control	296.8 ± 2.73	



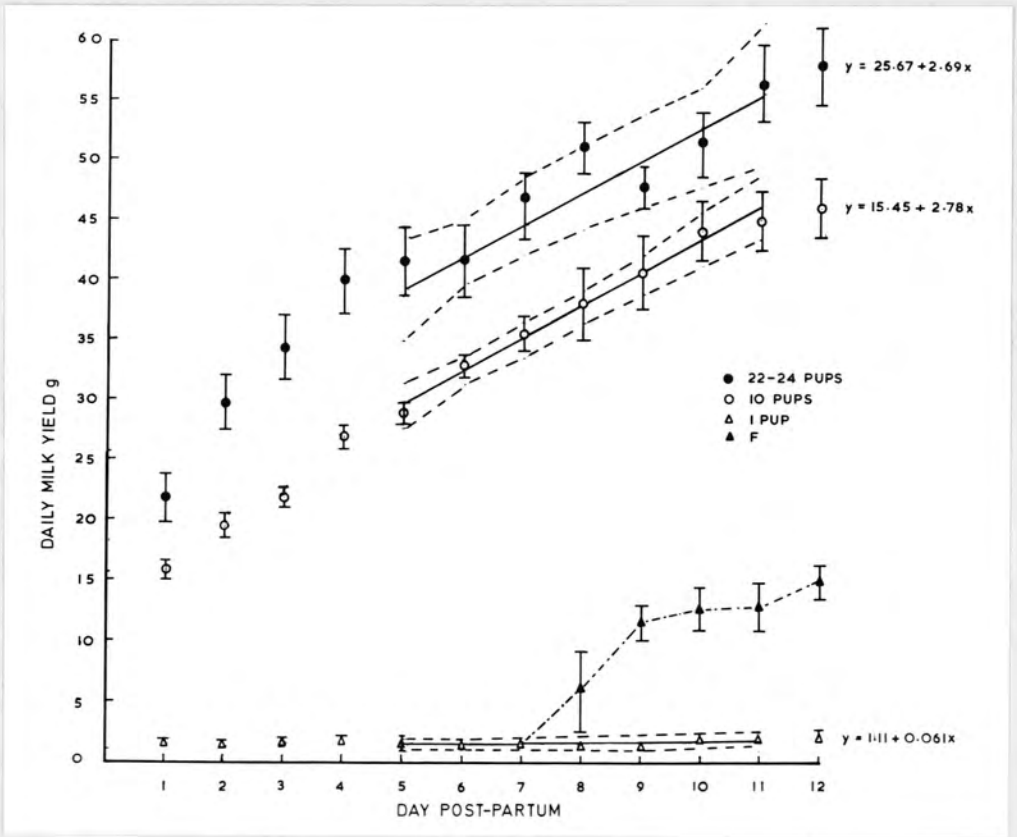
a

Figure 31 Total daily milk yield. Each symbol represents a grouped mean. Each vertical line indicates standard error of the mean. Continuous lines: calculated regression lines. Dashed lines: 95% confidence limits. F and dot-dashed lines: 1 pup to day 7, then 10 pups (Section V).

a. Litter weight gain correction factor: (100-4.7).

b. Correction factor: (100-14.8).

b



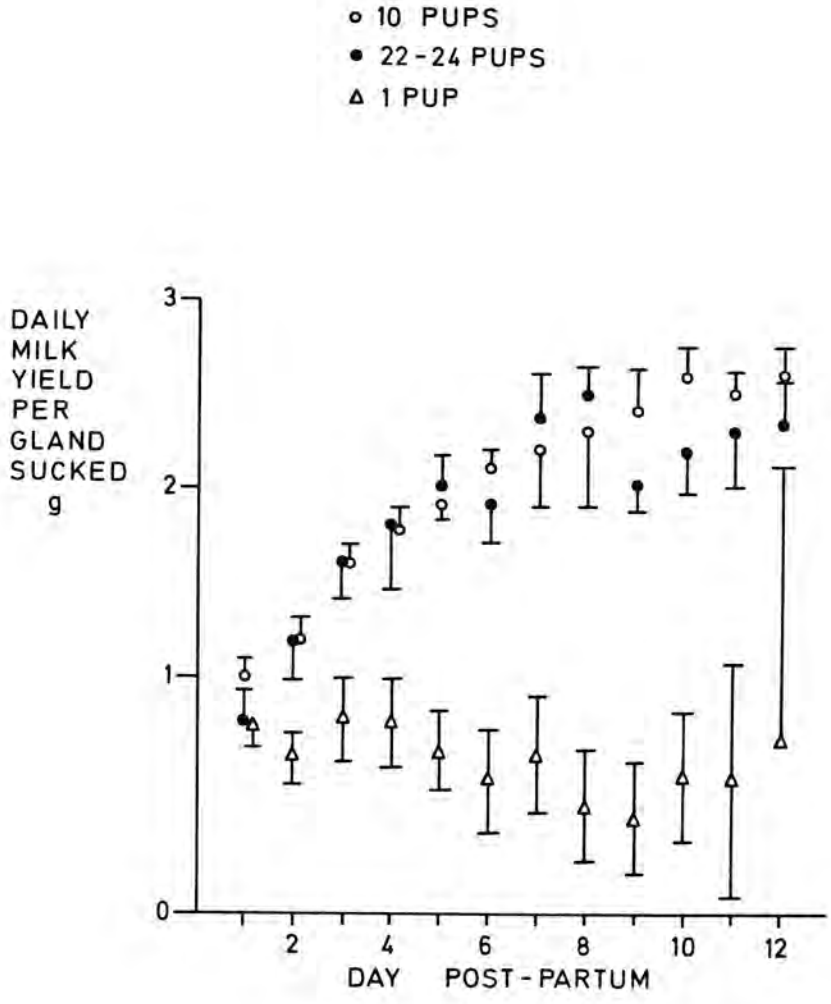


Figure 32 Daily milk yield per gland sucked. Each symbol represents a grouped mean, calculated from data of Fig. 31a.

Each vertical line indicates the standard error of the mean.

Litter weight gain correction factor: (100-4.7).

TABLE 14

TOTAL DAILY MILK YIELD WITH LITTERS OF DIFFERENT SIZES, EXPRESSED AS PERCENTAGE OF DAILY MILK YIELD OF ANIMALS WITH 10 PUP LITTERS

Correction factor used to calculate milk yield from litter weight		(100-4.7)			(100-14.8)		
Day post partum		5	7	12	5	7	12
Litter size	22-24 pups	125	123	108	141	134	117
	10 pups	100	100	100	100	100	100
	1 pup	3.1	2.6	1.7	5.2	4.6	4.3

TABLE 15

DAILY MILK YIELD PER SUCKED GLAND PER RAT: COMPARISONS BY STUDENT'S t-TEST. DAY 5 POST PARTUM

Litter size		10 pups		1 pup	
Correction factor used to calculate milk yield from litter weight		(100-4.7)	(100-14.8)	(100-4.7)	(100-14.8)
Litter size of compared group	22-24 pups	$0.9 > p > 0.8$	$0.6 > p > 0.5$	$0.05 > p > 0.02^{*}$	$0.05 > p > 0.02^{*}$
	1 pup	$p < 0.001^{***}$	$0.005 > p > 0.001^{**}$		

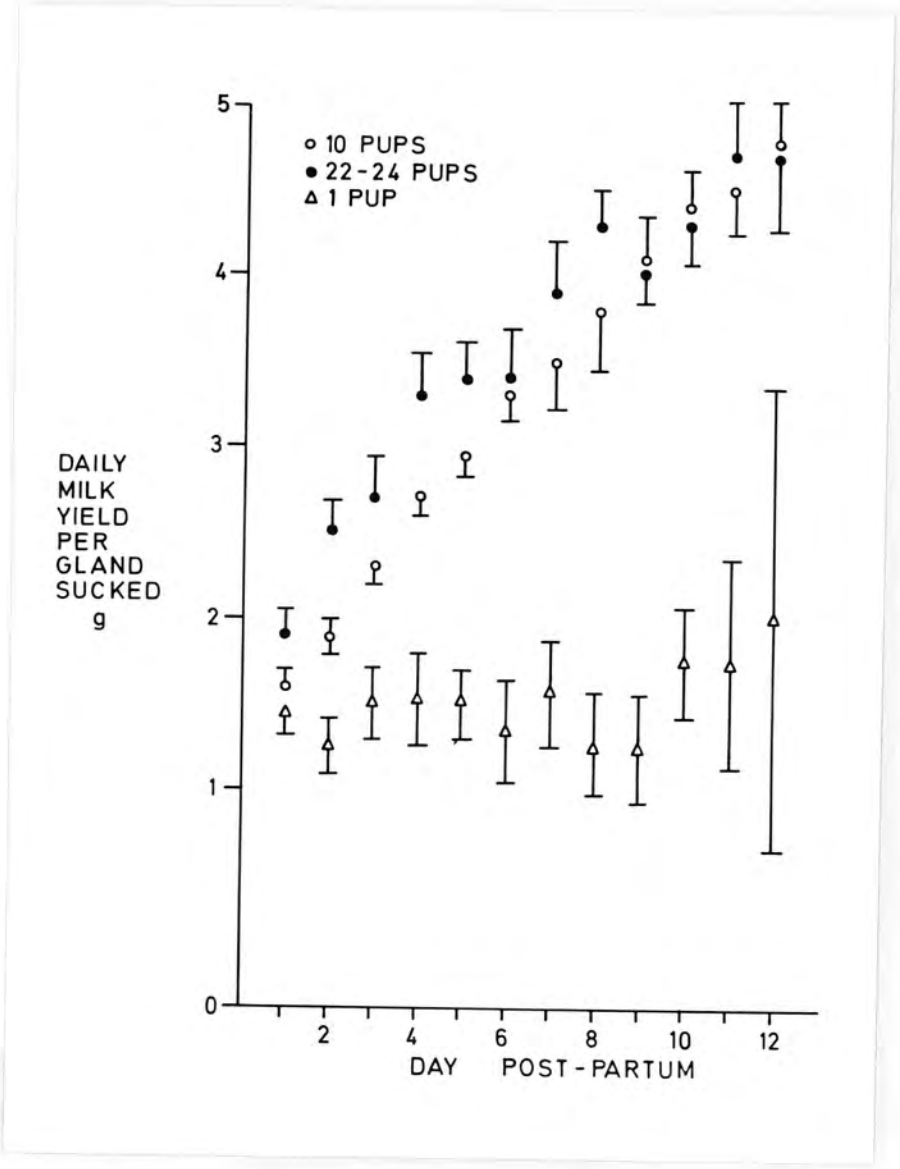


Figure 33 Daily milk yield per gland sucked. Details as in Fig. 32, but means calculated from data in Fig. 31b, correction factor (100-14.8).

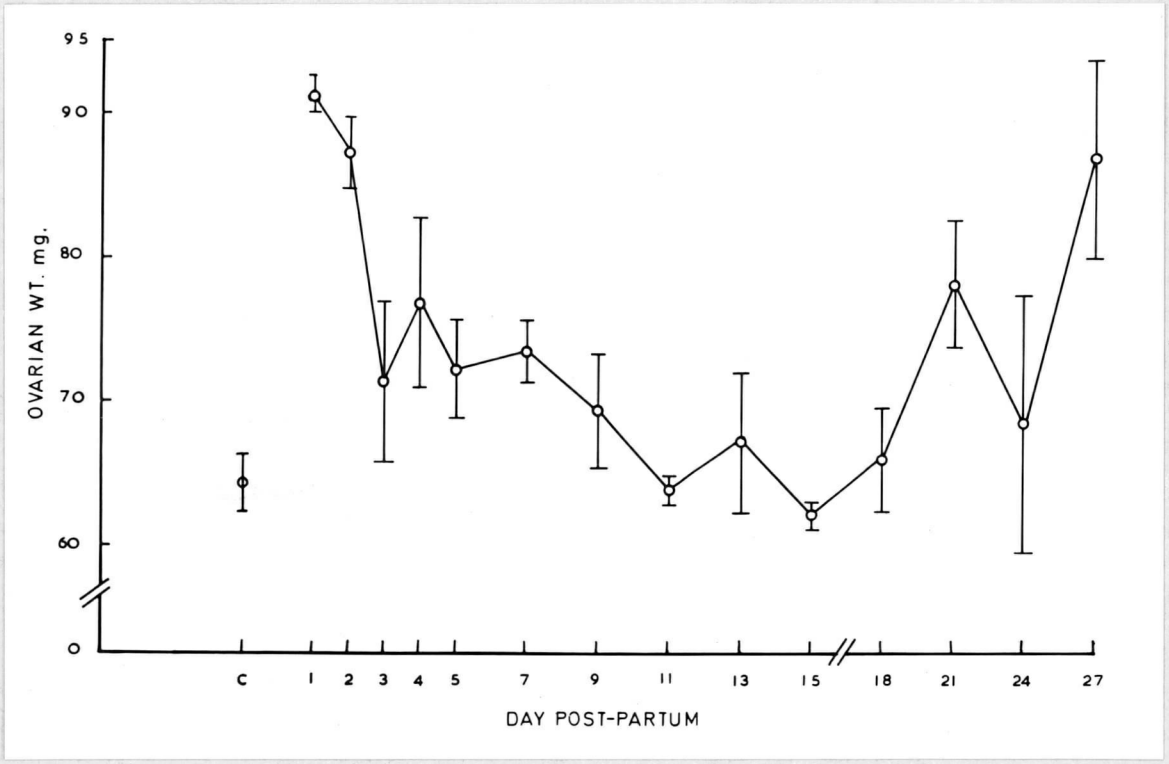
TABLE 16

FREQUENCY OF MILK EJECTIONS, MEASURED AS LITTER STRETCH REACTIONS, WITH DIFFERENT LITTER SIZES

Litter size: pups		1	1	10	10	22	22
Milk ejections between 1400 and 1745 hours	Day post partum						
	4, 5, 6	6	9	13	23	26	8
	7, 8, 9	12	10	28	5	10	34
	10, 11, 12	7	9	18	23	16	31
	Mean	8.8		18.2		20.8	
	Standard Error of Mean	0.87		4.09		4.51	

COMPARISONS OF MEAN NUMBER OF MILK EJECTIONS BY STUDENT'S t-TEST

Litter size: pups	10 pups	22 pups
1 pup	$p = 0.05$	$0.05 > p > 0.02$
22 pups	$0.7 > p > 0.6$	



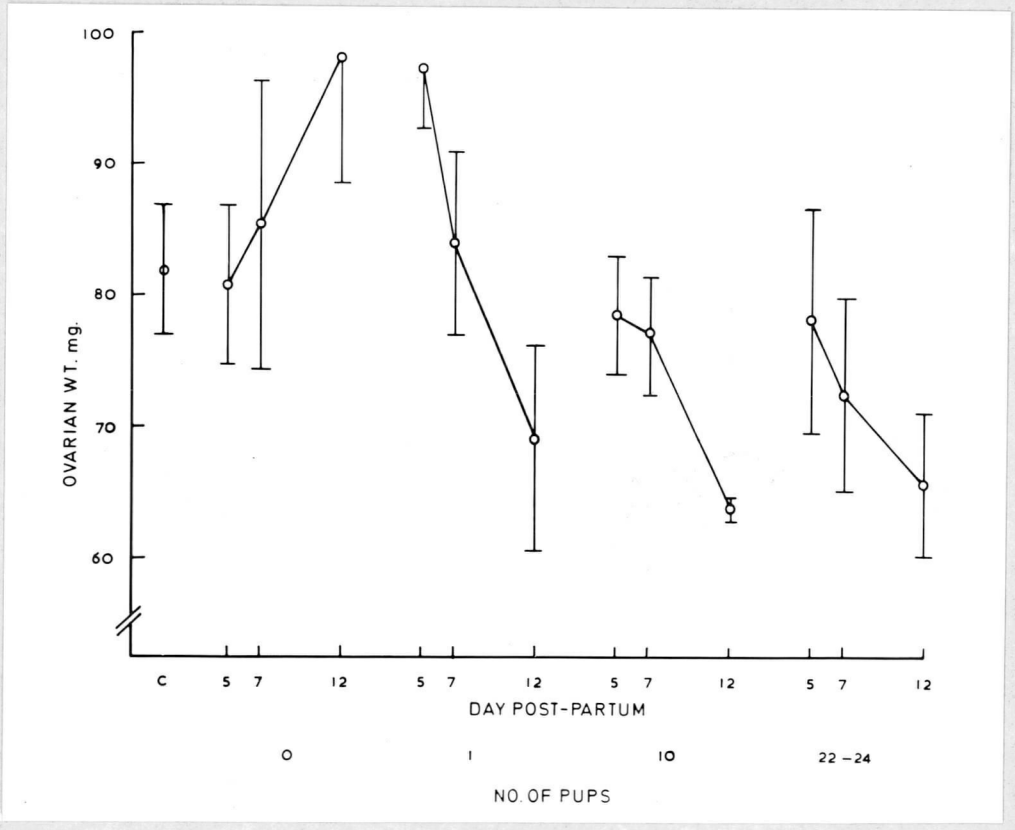
a

Figure 34 Ovarian weight post-partum. Open circles: mean ovarian weight of each group. C: virgin control. Each vertical line indicates the standard error of the mean.

a. Normal lactation.

b. Post-partum with varied litter size.

b



3. Results

3.1 Stimulus parameters

3.1.1 Intensity of suckling stimulus

The mean stimulus intensities, to which animals nursing 1, 10 or 22-24 pups were exposed, were, respectively, 4 $\pm$ 0.53%, 41 $\pm$ 6.33%, and 82 $\pm$ 8.7%, as percentages, $\pm$ the standard error of the mean, of the maximum, 360 nipple-minutes. These measurements were significantly different from each other ($p < 0.001$).

Although measurements were made in 30 minute observation periods, for comparison with the frequency of milk ejections typical records for 75 minute observation periods are shown in Figs. 27a,b, 28.

3.1.2 Average pup weight gains

Where pups had to be replaced, weight gains have been calculated and shown as if the same pups were present continuously. The daily weight gains have been expressed as percentages of the birth weights (Fig. 29a).

There was greater variation in weight gain of pups nursed singly. These pups were separated into two groups - those which clearly gained weight and those which did not. These groups were significantly different from each other five days post partum (Fig. 29b, Table 12). Six out of 13 pups nursed singly failed to gain weight. None of the 10 pup litters failed to gain weight; 1 or 2 pups in each 22-24 pup litter had to be replaced, but as a whole each litter of 22-24 pups gained weight, though at a slower rate than pups of 10 pup litters (Fig. 29a, Table 12).

3.1.3 Serum osmolality

The serum osmolality did not alter during lactation in any group (Fig. 30, Table 13).

3.2 Response parameters

3.2.1 Milk yield

Estimates of total milk yield for each litter, based on weight correction factors of (100-4.7) and (100-14.8) are shown in Figs. 31a,b.

Growth rate is linear between 5 and 11 days post partum (Cowie & Folley, 1947); linear regression lines for milk yield on time were calculated for this period (Figs. 31a,b). Total daily milk yield of animals nursing one pup was only 2-5% of the total daily milk yield of animals nursing 10 pups (Table 14).

The total daily milk yields of animals nursing 10 or 22-24 pups were different until day 8 post partum for the low estimate of milk yield, or day 11 post partum for the high estimate (Fig. 31a,b). The total daily milk yield of animals nursing 22-24 pups was not at any time more than 41% greater than that of animals nursing 10 pups. Expressed as yield per sucked gland however, the milk yields of animals nursing 10 or 22-24 pups were not different at, or before, five days post partum, when the greatest difference in total milk yield between these groups was measured (Table 15). The milk yield per sucked gland in animals nursing only one pup becomes 30 or 80%, on days 12 and 1 post partum respectively, of the yield per sucked gland of the animals nursing larger litters (Figs. 32 and 33).

3.2.2 Milk ejections

Typical patterns of milk ejection are shown in Figs. 27a,b and 28. Animals nursing 10 pups tended to nurse their young on the nest, whereas animals nursing 22-24 pups frequently nursed away from the nest as well as on the nest, and tolerated attachment of pups to the nipples much of the time they were eating or drinking. Some of these animals built two nests. Animals nursing one pup tended to spend more time on the nest than those nursing larger litters (Figs. 27a,b and 28).

The number of milk ejections between 1400 and 1745 hours was similar in animals nursing 10 or 22-24 pups, but there were fewer milk ejections in animals nursing only one pup (Figs. 27a,b, 28; Table 16).

3.2.3 Ovarian weight

Data from paired consecutive days during a normal lactation have been pooled for comparison (Fig. 34a). Ovarian weight decreased between days 1, 2 and3, 4; $0.005 > p > 0.001$. The minimum ovarian weight was found between days 13 and 15, although this could not be established statistically. The increase in ovarian weight after days 13 and 15 was not significant.

In the absence of pups, ovarian weight increased, and in the presence of 1, 10 or 22-24 pups it decreased (Fig. 34b). There was insufficient data for statistical comparison between groups at different times. With this coveat, ovarian weights tended to be lower in animals nursing 10 or 22-24 pups than in those nursing one pup.

4. Discussion

4.1 Interpretation of stimulus and response parameters

The general relationship between the number of pups suckling and the secretion of oxytocin and the other hormones of lactation has been discussed (Section I - 2.5).

4.1.1 Stimulus parameters

The number of nipples which are stimulated, the number of nerve fibres from each nipple which can carry impulses, and the duration and continuity of stimulation all contribute to the total volume of stimulus (Edwardson & Eayrs, 1967; Tindal & Knaggs, 1970; Lincoln et al, 1973). It does not follow that over the whole range of stimulation, to a maximum of 12 pups suckling continuously, the PV and SO neurones are activated in proportion to the peripheral stimulus. Inhibitory as well as excitatory synapses are found on PV and SO neurones (Section I - 2.4.2), and central inhibition of oxytocin secretion in response to suckling has been clearly demonstrated (Cross, 1955b; Taleisnik & Deis, 1964; Aulesbrook & Holland, 1969b). Recurrent inhibitory collaterals (Yamashita et al, 1970) and inhibitory interactions between PV and SO neurones (Nicoll & Barker, 1971) may also modify the secretory response to peripheral stimulation (Section I - 2.4.2). The present finding that the stimulus intensity, related to time and number of nipples sucked, is in exact proportion to the litter size must be interpreted as indicating only that the afferent activity from the nipples is in such proportion (Fig. 27a,b; 28). The suckling

stimulus may subsequently be modulated centrally by information from mammary gland volume receptors (Zaks, 1962; Lincoln et al, 1973).

Animals nursing different sizes of litter were housed in adjacent cages. It is likely that exteroceptive stimuli from the pups which may stimulate oxytocin (Deis, 1968) or prolactin (Mena & Grosvenor, 1972) secretion acted similarly on all the post partum animals.

It may be supposed that reduced weight gain is associated with hunger, and that the pups in litters of 22-24 or pups nursed singly would have sucked at least as vigorously as normal pups. A similarly decreased weight gain of pups in litters of 20-25 pups to that observed here has been reported, although unlike the present findings, single pups gained more weight than pups in litters of more than 20, but about the same amount as pups in litters of 10 (Edwardson & Eayrs, 1967; Fig. 29a,b).

4.1.2 Response parameters

All the mothers accepted their foster pups; the presence of pups is powerfully effective in eliciting maternal behaviour, even in non-lactating animals (Wiesner & Sheard, 1933; Bruce, 1961; Beach & Wilson, 1963; Richards, 1967). The growth of pups of normal sized litters is unaffected if pups are cross fostered on the first day post partum, and transfer of placentae is not necessary (Denenberg et al, 1963).

A technique similar to that used here has been used previously to estimate milk yield; total daily milk yields of up to 50 g were measured (Brody & Nisbet, 1938). The two

estimates of total daily milk yield made in this study show similar trends. Unlike the peripheral stimulus, milk yield is not linearly related to litter size (Fig. 31a,b).

Daily milk yields per sucked gland were not different for 10 or 22-24 pup litters, but daily yield per sucked gland for 1 pup litters was 30-80% of these yields (Figs. 32 and 33). The dependence of milk yield on secretion and ejection has been discussed (I - 2.5). It is not known whether oxytocin secretion is limiting to milk yield. Adrenal corticoid secretion, rather than oxytocin or prolactin, limits milk yield in prolonged lactation (Thatcher & Tucker, 1970a,b). The measured daily milk yields per sucked gland are consistent with similar daily secretion of oxytocin in animals nursing 10 or22-24 pups, but daily oxytocin secretion about half this in animals nursing one pup. Reduced milk yield is likely to be associated with reduced oxytocin secretion because oxytocin is less readily secreted in animals with emptied mammary glands (Lincoln et al, 1973). In contrast, in animals nursing 22-24 pups, the greater total daily milk yield in the first few days of lactation (Fig. 31a,b) could be attributed to a local action on the mammary gland of frequent emptying (Linzell & Peaker, 1971c); although oxytocin has a slight galactopoietic action (Morag & Brick, 1969), it does not release prolactin (Grosvenor & Turner, 1958; Kuhn et al, 1973).

The frequency of pup stretch reactions (Vorherr et al, 1967) is an exact measure of the frequency of oxytocin secretion (Lincoln et al, 1973), but unless continuously

calibrated against milk ejection pressure, an estimate of the amount of oxytocin secreted may be in error by 50% per milk ejection (Lincoln, 1973). With this reservation, the frequency of milk ejections measured in this study indicate no difference in daily oxytocin secretion between animals nursing 10 or 22-24 pups, but lower daily oxytocin secretion in animals nursing only one pup (Figs. 27a,b, 28; Table 16). Lincoln et al (1973) noted that milk ejections are rare in animals nursing less than five pups, although the amount of oxytocin secreted per milk ejection is similar with 10 or less than five pups suckling.

ADH may be released during suckling in the dog (Abrahams & Pickford, 1954; Pickford, 1960) or as a result of milk ejection in the rabbit (Cross, 1951), although simultaneous release of oxytocin and ADH in response to suckling in the rabbit has been denied (Bisset et al, 1970). Milk is isotonic with plasma, but the major osmole is lactose, and the concentrations of the major ions are similar to those of intracellular fluid (Linzell & Peaker, 1971a,b; Linzell, 1972). Milk is in osmotic equilibrium with blood flowing through the mammary gland (Rook & Wheelock, 1967). Clearly milk secretion will result in mammary venous hypernatremia which may stimulate ADH secretion. Net flux of water from blood into milk may result in a temporary hypovolemic stimulus to ADH secretion.

Serum osmolality was not altered during lactation (Fig. 30; Table 13), but as previously argued this may reflect the efficiency with which serum osmolality is controlled

(this section - 2.2.2). Serum osmolality changes in lactating animals deprived of drinking water are consistent with an osmotic stimulus arising from milk secretion during normal lactation (Section VI). The intensity of this osmotic stimulus would be expected to vary with the total daily milk yield and may have been greater early in lactation in animals nursing 22-24 pups (Fig. 31a,b).

Suckling suppresses ovarian growth during lactation (Stotsenburg, 1923; Fig. 34a) by inhibiting gonadotrophin secretion in extent and for a time related to the number of pups suckling (Rothchild, 1960; Hammons et al, 1973). Vaginal smears were not taken from lactating animals so the duration of lactational diestrus in relation to litter size is not known. The ovarian weight data suggest less effective suppression of ovarian growth by one pup than by 10 or 22-24 pups (Fig. 34b).

4.2 Summary

The intensity of the suckling stimulus was linearly related to litter size. Average pup weight gain was greater in litters of 10 pups than in either 22-24 or 1 pup litters. The responses to suckling, measured as total milk yield, milk yield per sucked gland, milk ejection or ovarian growth suppression, were not linearly related to litter size. The responses, in terms of these parameters, were similar for litters of 10 or 22-24 pups, except that total daily milk yield was greater in the latter until day 8 or 11 post partum, but the responses to a 1 pup litter were less than the responses to the larger litters. The total daily oxytocin

secretion was probably similar in animals nursing 10 or 22-24 pups, but less in animals nursing only one pup. ADH secretion probably varied with the total milk yield, which was greater at the beginning of lactation in animals nursing 22-24 pups, but there was no direct evidence of chronic osmotic stimulation.

4.3 Relationship between nucleolar changes in PV and SO neurones (Section III) and stimulus-response parameters

Associated with stimulated oxytocin and ADH secretion during lactation, protein synthesis is increased in PV and SO neurones (Section III - 4.3). Nucleolar dry mass changes have been quantitatively related to ribosomal RNA and protein synthesis in other neurones, in particular hypoglossal neurones after axotomy (Watson, 1965a, 1968a, 1969) and less directly in SO neurones during dehydration (Edstrom & Eichner, 1958; Watt, 1970). In the present study, it has been assumed on the basis of these findings that a faster rate of increase of, or a greater averaged, nucleolar dry mass indicates more rapid ribosome and protein synthesis. The quantitative relationship between rates of neurohormone and associated protein and ribosome synthesis and rates of oxytocin and ADH secretion have not been reported.

The mean nucleolar dry mass of PV neurones of animals nursing 22-24 pups reached a peak, and declined more rapidly, than that of animals nursing 10 pups, although the nucleolar responses averaged over days 5 to 12 post partum were similar (Figs. 24b, 25). These changes may reflect a faster initial

rate of ribosome synthesis in PV neurones of animals nursing 22-24 pups, but similar total ribosome synthesis in PV neurones of animals nursing 10 pups or 22-24 pups, averaged over days 5-12 post partum. The mean nucleolar dry mass measurements of PV neurones of animals nursing only one pup indicates a lower rate of ribosome synthesis compared with animals nursing larger litters.

Considered with the conclusions reached with respect to oxytocin and ADH secretion, nucleolar dry mass changes of PV neurones were quantitatively related to daily oxytocin and ADH secretion, but not to stimulus intensity (Figs. 27a,b, 28). The faster rate at which nucleolar dry mass of PV neurones initially increased in animals with 22-24 pups may have resulted from greater initial rates of neurohormone secretion in these animals. The differences in total daily milk yield were greatest early in lactation, although the milk yields per gland sucked were not different between animals nursing 10 or 22-24 pups (Figs. 31a,b; 32; 33). The different total daily milk yields are consistent with greater ADH secretion (this section - 4.1.2) early in lactation in animals nursing 22-24 pups, but the milk yields per sucked gland are consistent with similar oxytocin secretion in animals nursing 10 or 22-24 pups (this section - 2.3.1).

A similar relationship can be established between nucleolar dry mass of SO neurones and ADH and oxytocin secretion, except that the nucleoli of these neurones did not change with time in animals nursing 10 or 22-24 pups

(Fig. 24a). Nucleolar dry mass of SO neurones of animals nursing one pup decreased during lactation (Fig. 24a). This change is consistent with decreased ribosome synthesis related to reduced ADH and oxytocin secretion (Figs. 27a, 31a,b, 32, 33).

Within the limitations of the techniques of measurement of synthetic and secretory activity, ribosome synthesis and neurohormone secretion were linked in a quantitative fashion.

The process limiting the nucleolar responses was not established. It is not known whether the measured nucleolar changes represent changes in all neurones, or in a population of neurones within each nucleus. This aspect is discussed in Section VI. It is possible that the synthetic capacity of individual neurones limited secretory activity when litter size was increased to 22-24 pups. Alternatively secretory activity, restricted by central inhibition (this section - 4.1.1) with large litters may determine the nucleolar responses. Because the posterior pituitary is not depleted of hormones during lactation (Section I - 2.6.2) it is unlikely that a limited synthetic capacity prevented increased secretion of oxytocin in response to the greater suckling stimulus applied by 22-24 pups. In the discussion which follows it is argued that secretory activity determines nucleolar responses.

Increased ribosome synthesis may be required during increased neuronal activity to synthesise proteins other than those for export. This has not yet been investigated. Other proteins may serve to maintain cell structure and

energy production. The synthesis of sedentary proteins of SO neurones is increased during osmotic stimulation (Murray, 1967). The SO neuronal content of several enzymes is increased by hyperosmotic stimulation. These include thiamine pyrophosphatase (Jongkind & Swaab, 1967), acid phosphatase (Rinne, 1966; Enestrom, 1967; Bara & Skaliczki, 1968; Rechardt, 1969), acetylcholinesterase (Rinne, 1966), and enzymes of the direct oxidative pathway for glucose (Enestrom, 1967; Bara & Skaliczki, 1968).

The animals used in these experiments were nulliparous at the start of the experiments, and suckling was a novel stimulus. Use of the central pathway of the milk ejection reflex for the first time in adulthood may be associated with trophic changes at synapses in the SO andPV nuclei, or elsewhere. The establishment of new connections, or the increased use of pre-existing synapses, may involve increased, or de novo, synthesis of a class of proteins for this purpose (Roberts & Matthysse, 1970). Hypertrophy of glial cells in the PV and SO nuclei during the first lactation may reflect such synaptic changes (Watson, 1972). Self-licking during pregnancy (Roth & Rosenblatt, 1966) may act to prepare this central pathway.

There are four obvious sources of signal which may regulate neuronal ribosome synthesis. These are:

1. Chemical transmitters, or associated materials, acting upon the cell body.
2. Electrical changes in the post-synaptic cell.
3. Hormones secreted by other cells or tissues.
4. The secretory mechanism.

The possible role of hormones is explored in Section V.

There is some evidence for transsynaptic regulation of protein synthesis (Satake, 1972). RNA synthesis increases in several types of invertebrate or vertebrate neurones when stimulated with orthodox presynaptic stimuli, or by electrical stimulation of discrete brain areas (e.g. Watson, 1965b; D'Yakenova et al, 1966; Berry, 1969; Dewar & Shields, 1972). None of these studies distinguishes synaptic from post-synaptic signals.

Incorporation of tritium labelled RNA precursors is increased by presynaptic, but not by post-synaptic, electrical stimulation of neurone R_2^* of Aplysia abdominal ganglion. The change in isotope incorporation may represent increased RNA synthesis (Kernell & Peterson, 1970; Peterson & Kernell, 1970).

Post-synaptic electrical stimulation does not increase RNA synthesis in hypoglossal neurones (Watson, 1965b) or a crustacean stretch receptor (Grampp & Edstrom, 1963). However changes in RNA synthesis have usually been sought within minutes or hours of stimulation. If the mechanism whereby stimulation increases ribosome synthesis involves retrograde axonal transport (vide infra), changes would not be expected within this period.

Reserpine increases β-hydroxylase activity in adrenergically innervated tissues in the rat; this effect depends on protein synthesis and intact presynaptic innervation, although the role of post-synaptic electrical activity has not been distinguished (Molinoff, 1971). 3'-5' cyclic AMP

*one of 17 individually identifiable neurones: see Frazier, W. T. et al (1967). J. Neurophysiol. 30, 1288-1351.

may be involved in synaptic regulation of protein synthesis (Weiss & Kidman, 1969), and some transmitters stimulate adenyl cyclase (Choh, Ho & Loh, 1971). Acetylcholine stimulates RNA synthesis in sympathetic ganglion neurones and this effect does not depend on propagated action potentials or indeed on perikaryal depolarisation (Gisiger, 1971).

PV and SO neurones have receptors for ADH and oxytocin (Nicoll & Barker, 1971; Moss, Dyball & Cross, 1972), which may be released from recurrent collaterals, and regulate perikaryal ribosome synthesis. Rapid regulation of ribosome synthesis, related to secretory activity, could be achieved by such a mechanism, but it would be a positive feedback type of control system. Several transmitters, including 5-hydroxytryptamine, noradrenaline and acetylcholine, can regulate their own synthesis, but the regulation is of the end product inhibition type (Contractor & Jeacock, 1967; Spector et al, 1967; Kaita & Goldberg, 1969; Macon et al, 1971), which could subserve an intracellular control mechanism for ADH and oxytocin secretion but not a trans-synaptic mechanism. The effects of ADH or oxytocin administration on ribosome or protein synthesis have not been studied, so reported changes in the posterior pituitary content of neurosecretory material cannot be interpreted (De Groot, 1958; Fendler & Telegdy, 1962). Exogenous ADH and oxytocin, administered systemically, accumulate in the posterior pituitary (Aroskar et al, 1964; Edwards, 1971) and are rapidly broken down (Pliska et al, 1971; Edwards, 1971) as is neurophysin (Shin et al, 1970; Pickup & Hope, 1971). The nerve endings of PV

and SO neurones contain lysosomes (Whitaker et al, 1970) and may share the capacity of other nerve endings to synthesise protein (Goldberg, 1971; Cotman & Taylor, 1971; Ramirez et al, 1972). It seems likely that any regulatory signal produced by the endings will be some result of these processes.

The axotomised hypoglossal neurone has been used extensively by Watson to investigate the mechanisms which regulate ribosome and protein synthesis. This work has shown that a signal from the axon terminals controls ribosome and protein synthesis within the neurone. The lag between axotomy and onset of changes in ribosome synthesis is consistent with disto-proximal axonal transport of a signal from the site of injury (Watson, 1965a, 1968a, 1969). Botulinum toxin disrupts neuromuscular transmission without loss of axoplasm, and causes perikaryal changes similar to those of axotomy, with the exception that perikaryal dry mass increases earlier (Watson, 1969). The initial chromatolytic response to axotomy subsides if the proximal stump is transplanted into the sternomastoid muscle, but changes similar to those after axotomy recur when the accessory nerve is divided; this response can be delayed by botulinum toxin (Watson, 1970). A collision anastomosis between the proximal end of the hypoglossal nerve and the proximal end of the median nerve is followed by a chromatolytic response, which subsides after the normal duration, but subsequently ribosome synthesis is reduced. Later division of the hypoglossal or median nerves induces a chromatolytic reaction (Watson, 1970).

These results are best explained by postulating the existence of an inhibitory signal passing disto-proximally from the normal axon terminals, which is removed when the axon is sectioned, or membrane transactions at the ending are interfered with by botulinum toxin (Watson, 1969,1973a; Cragg, 1970).

In apparent conflict with this conclusion is the stimulatory action of an extract prepared from posterior pituitaries depleted of hormones in vitro, or in vivo, upon ribosome synthesis in SO and PV neurones (Watt, 1970a, 1971; Watt & Russell, unpubl.). However, synthesis of materials associated with export function, such as acetylcholinesterase or noradrenaline, is reduced after axotomy (Watson, 1966; Boyle & Gillespie, 1968; Karlstrom & Dahlstrom, 1970). Changes in ribosome synthesis after functional stimulation and after axotomy are each likely to be related to the synthesis of a different class of proteins (Watson, 1973a).

The mechanism of release of neurohormone has been discussed (Section I - 2.3.2). It was argued that the neurosecretory granule membranes are recaptured after exocytosis. Vesicles formed from recaptured neurosecretory granule membrane may have two components; one derived from the granule membrane, the other material incorporated from the pericapillary, or more generally the synaptic, space (Nagasawa et al, 1971). If these components, or their breakdown products, are transported, by a disto-proximal intra-axonal transport mechanism (Section I - 2.2.6), to the perikarya, the granule membrane component may stimulate,

and the extracellular component may inhibit, synthesis of ribosomes for export proteins and for growth proteins respectively. Nucleolar responses to increase secretory activity and to axotomy could be so explained. The amount of recaptured granule membrane returned to the perikarya would adjust nucleolar synthetic activity to the rate of secretion of neurohormone. The effects of botulinum toxin on hypoglossal neurones would result from interference with the recapture mechanism with axon terminal membrane expansion as the result (Duchen & Strich, 1968; Watson, 1969). Depressed ribosome synthesis in neurones with non-functional but intact endings (Watson, 1970) would result from return of unused transmitter vesicle membrane, which may inhibit synthesis of both export and growth proteins. The perikaryal responses which follow denervation of the sternomastoid musclewith an implanted hypoglossal nerve could result from reactivation of transmission at the terminals.

RNA synthesis is regulated by the cytoplasm in bacteria (Jacob & Monod, 1961) and in eukaryotes (Harris, 1967; Zetterburg, 1970). A cytoplasmic fraction of regenerating rat liver stimulates RNA synthesis in isolated normal liver nuclei in vitro (Thompson & McCarthy, 1968) and an intercellular acid polysaccharide from sea urchin embryos inhibits RNA synthesis in sea urchin embryo nuclei (Aoki & Koshihara, 1972).

Nuclei which have been implanted into egg cytoplasm swell before RNA synthesis increases and nuclear swelling is an essential preliminary for cytoplasm-induced RNA synthesis

(Leake et al, 1972). Nuclear swelling precedes the nucleolar changes in SO neurones during dehydration (Watt, 1970).

Disto-proximal intra-axonal transport has not been demonstrated in PV or SO neurones, but local application of colchicine to chick autonomic fibres results in changes similar to those of axotomy (Pilar & Landmesser, 1972), and after subarachnoid injection of colchicine, rat SO perikarya show ultrastructural changes consistent with increased ribosome synthesis (Norstrom et al, 1971). These changes may result from interference with disto-proximal transport of an inhibitory signal.

4.4 Summary

Increased nucleolar dry mass of SO and PV neurones during lactation is related to, and may be determined by, the secretory activity of these neurones, but is not related to the intensity of the peripheral stimulus. However, synthetic capacity may limit secretory activity, which reaches a maximum with a litter of 10 pups. A mechanism whereby secretory activity could regulate perikaryal ribosome synthesis in a graded fashion has been discussed.

SECTION V

HUMORAL INFLUENCES ON PARAVENTRICULAR AND SUPRAOPTIC NEURONES

1. Aims of investigation

Because of the widespread changes in the endocrine system in pregnancy and lactation, the experiments described in this section were performed to investigate the possible role of these changes in stimulating nucleolar responses in SO and PV neurones.

The suckling stimulus was intensified remote from the hormonal state of pregnancy, and changes in PV and SO neurones measured. To distinguish the effects of neural input from humoral influences, changes in PV and SO neurones in lactation after unilateral nipple line ablation were studied. The relationship between the number of pups born, and hence placental and corpus luteal secretory mass, to nucleolar changes in PV and SO neurones during lactation was examined. Nucleolar changes in PV and SO neurones were sought during the estrous cycle, after ovariectomy or during treatment with ovarian steroids.

2. Introduction: Possible effects of hormones on PV and SO neurones

In the previous sections attention has been focused on neural stimuli which modify SO and PV neuronal synthetic activity.

The activity of the entire endocrine system is probably altered during pregnancy and lactation. The suckling dependent hormone changes have been discussed (Section I - 2.5). Any of these hormonal changes may alter synthetic and secretory activity of PV and SO neurones, by direct or indirect actions on either process. According to the concept developed in Section I - 2.6, a change in either secretory or synthetic activity will be reflected in the nucleolar dry mass.

In the pregnant rat, estrogen is secreted by the ovaries at a low rate, comparable to that of diestrus, although the rate increases slightly at the time of implantation; but during the last three days of pregnancy estrogen secretion increases rapidly (Yoshinaga et al, 1969). Ovariectomy on day 20 of pregnancy prevents lactation, which occurs normally if estrogen is given at the time of ovariectomy (Catala & Deis, 1973), but it has not been shown conclusively whether failure of lactation is the result of inhibition of milk ejection or secretion. The rat placenta does not secrete estrogen (Townsend & Ryan, 1970), so estrogen could probably affect SO and PV neurones only at the end of pregnancy. Ovarian secretion of progesterone however is maximal at mid-pregnancy, and falls towards parturition:

the secretion of 20 ∝ hydroxyprogesterone changes reciprocally (Eto et al, 1962). Progesterone secretion increases during lactation, and is maximal on day 8 post partum, and the secretion of 20 ∝ hydroxyprogesterone alters reciprocally (Tomogane et al, 1969). The fall in progesterone secretion at the end of pregnancy triggers lactogenesis; progesterone injections at the end of pregnancy prevent, and ovariectomy precipitates, lactogenesis (Kuhn, 1969). Milk ejection after parturition is also claimed to be inhibited by progesterone treatment in late pregnancy, but the interaction of decreased milk secretion and ejection has not been satisfactorily disentangled (Herrenkohl, 1971, 1972; Herrenkohl & Lisk, 1973). Release from progesterone inhibition may facilitate oxytocin secretion during parturition (Cross, 1966).

In the pregnant rat, prolactin, FSH and LH, measured by radioimmunoassay, are at lower concentrations in blood than during diestrus, except for the first four days and the last two days of pregnancy, although blood FSH concentration rises from day 13 (Amenomori et al, 1970; Linkie & Niswender, 1972). There is lack of agreement about the role of each of these hormones in the maintenance of pregnancy (Raj & Moudgal, 1970; Takayama & Greenwald, 1973). It has long been known that the anterior pituitary is essential for the maintenance of pregnancy until the 12th day (Pencharz & Long, 1933) when placental luteotrophin takes over maintenance of the corpora lutea (Rothchild et al, 1973). There are no systematic studies on the effects of anterior pituitary

gonadotrophic hormones on ADH or oxytocin secretion. Any of the described anterior pituitary hormone changes of pregnancy or lactation may modify PV or SO neurones.

The effects of some steroids on oxytocin and ADH secretion have been studied. Oxytocin is secreted in response to vaginal stimulation in the lactating ewe or goat. This response is inhibited by a central action of progesterone, but potentiated by estrogen (Roberts & Share, 1969, 1970; Roberts, 1971). Estrogen injected into lactating rats does not inhibit the milk ejection reflex tested three hours later (Chaudhury, 1961). Cortisone may inhibit ADH secretion by a central action on cholinergic transmission (Dingman & Thorn, 1955; Dingman & Despointes, 1956).

Of other hormones, angiotensin, injected intravenously in goats, stimulates ADH secretion (Andersson & Westbye, 1970) and electrical activity of antidromically identified SO neurones is increased by angiotensin applied by microiontophoresis (Nicoll & Barker, 1971a); but angiotensin is not the cause of ADH secretion in response to haemorrhage (Claybaugh & Share, 1972), so the physiological significance of these actions is not clear.

With this limited information the possible role of hormones in causing the nucleolar changes of PV and SO neurones during pregnancy and lactation were investigated.

3. Methods

3.1 Second variation in litter size: management of animals

After a normal lactation has ceased, lactation can be restarted by suckling if successive foster litters are provided (Bruce, 1961; Ota & Yokoyama, 1965). Appropriate maternal behaviour is elicited without difficulty (Wiesner & Sheard, 1933; Bruce, 1961).

Because it was difficult to establish the time at which suckling restarted in such animals, a modified experiment was performed in which the post partum animals were maintained in lactation by a minimal suckling stimulus, applied by one pup since parturition. In five such animals, the litter size was increased to 10 pups on the 7th day post partum. The additional pups were 6 to 8 days old. The lactating females were killed on the 12th day post partum. These animals were in the same series as the animals in Section III nursing litters of various sizes. Milk yield and serum osmolality were measured as previously described (Section IV - 2.2.2, 2.3.1).

3.2 Effects of size of litter nursed and number of pups born

The data from the animals nursing litters of 1, 10 or 22-24 pups was reviewed, and an analysis of variance performed to see if the number of pups born contributed significantly to the variance. This analysis was made by the Computing and Statistics Section of the Department of Social Medicine.

Figure 35 Unilateral nipple line ablation and sham procedure.

Stages of operation:

1. In the left or right nipple line each nipple was identified and siezed with a pair of artery forceps.
2. An elliptical incision was made around each nipple.
3. Each ellipse of skin including a nipple was removed.
4. Subjacent mammary tissue was excised.
5., 6. Each incision was closed with two 3/0 silk everting sutures.
7. Sham operation consisted of making a midline ventral incision which was closed with 3/0 silk everting sutures.

The number of pups born and the number of corpora lutea of pregnancy are similar (Stotsenburg, 1923). The analysis of variance should have detected an influence on PV and SO neurones of hormones dependent on the number of corpora lutea of pregnancy and the number of foeto-placental units. The number of corpora lutea per rat was not recorded.

3.3 Unilateral nipple line ablation: operative procedure and management of animals

The afferent pathway of the milk ejection reflex is unilateral in the spinal cord (Eayrs & Baddeley, 1956). It is not known if this unilateral representation is maintained as far as the PV and SO nuclei. If it is, and if changes in PV and SO neurones depend only on afferent input from the nipples, then changes in PV and SO neurones in lactation after unilateral nipple line ablation should be asymmetric.

The right or left nipple line was ablated in eight 3-month-old virgin female rats, anaesthetised with ether. Sham operations were performed on eight similar animals. The procedures are illustrated in Fig. 35.

Three weeks after the operation the animals were mated. Management was subsequently the same as for the group of lactating animals nursing their own litters (Section III - 2.1.2). These animals were all killed on the 8th day post partum. Serum osmolality was measured as previously described (Section IV - 2.2.2).

3.4 Estrous cycle

Virgin controls used in the lactation study (Section III) were assigned to diestrus, metestrus or proestrus and estrus groups on the basis of vaginal smears (Section III - 2.1.2).

3.5 Ovarian hormones

The ovaries are a likely source of hormones which may directly or indirectly alter secretory or synthetic activity of PV or SO neurones. The effects of altering the amounts of ovarian hormones to which PV and SO neurones were exposed was investigated in virgin females.

3.5.1 Ovariectomy

Twelve virgin female rats, three months old, were ovariectomised. The animals were anaesthetised with ether, bilateral paramedian incisions were made, the ovarian vessels were ligated, and both ovaries removed. The muscle layer was closed with catgut sutures, and skin incisions were closed with 3/0 silk everting sutures. In 12 similar animals sham operations were performed, which consisted of making the skin and muscle incisions, identifying the ovaries and closing the incisions as above. Six animals in each group were killed five days after operation, the remainder 21 days after operation. The operations were checked at post mortem examination.

3.5.2 Progesterone and estradiol injections

20 mg of progesterone B.P. (4-pregnen-3,20 dione; Koch-Light), dissolved and suspended in 0.1 ml arachis oil, was

injected subcutaneously once daily into each of six virgin female rats for five days, and into each of six virgin female rats for 21 days. Similar groups were injected with 0.1 ml arachis oil only on the same regimen. In both experimental and control groups the site of injection was varied. These animals were weighed every seven days to confirm that progesterone was absorbed (Hervey & Hervey, 1967).

1 mg of 17 β estradiol benzoate B.P. (Paines & Byrne), dissolved in 0.2 ml of oil, was injected subcutaneously once daily into each of six virgin female rats for five days. Six virgin female rats were similarly injected with arachis oil only.

A group of six virgin female rats served as untreated controls for the above groups (3.5.1, 3.5.2).

After these animals were killed, the uteri were removed and slit longitudinally. Surface moisture was removed, and the uteri were then weighed to check that estrogen had been absorbed.

3.6 Measurement of PV and SO neuronal nucleolar dry mass

PV and SO neuronal nucleolar dry mass were measured as previously described (Section II - 3). In the unilateral nipple line ablation group the right and left nuclei were measured separately; in the other groups the right and left nuclei were pooled.

4. Results

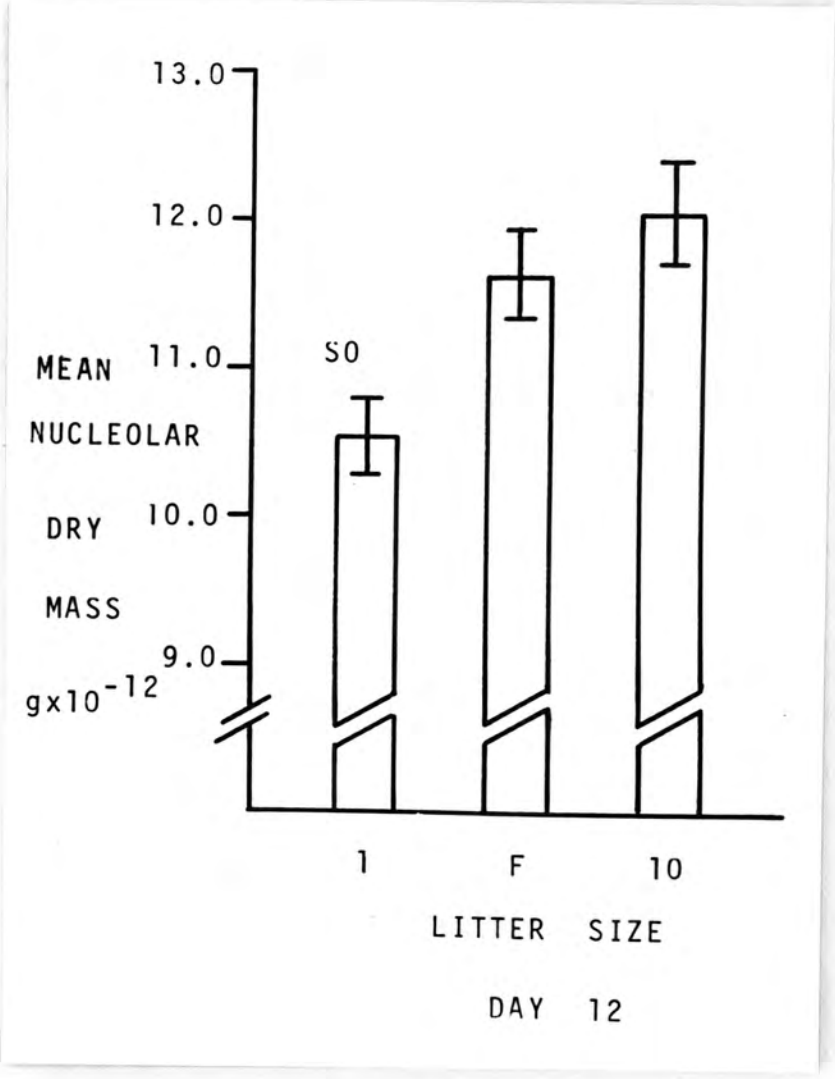


Figure 36 Second variation in litter size. Mean nucleolar dry mass of SO neurones, day 12 post-partum.

F: 1 pup to day 7, then 10 pups. 1 or 10: 1 or 10 pups continuously since day 1 post-partum.

Each vertical line indicates the standard error of the mean.

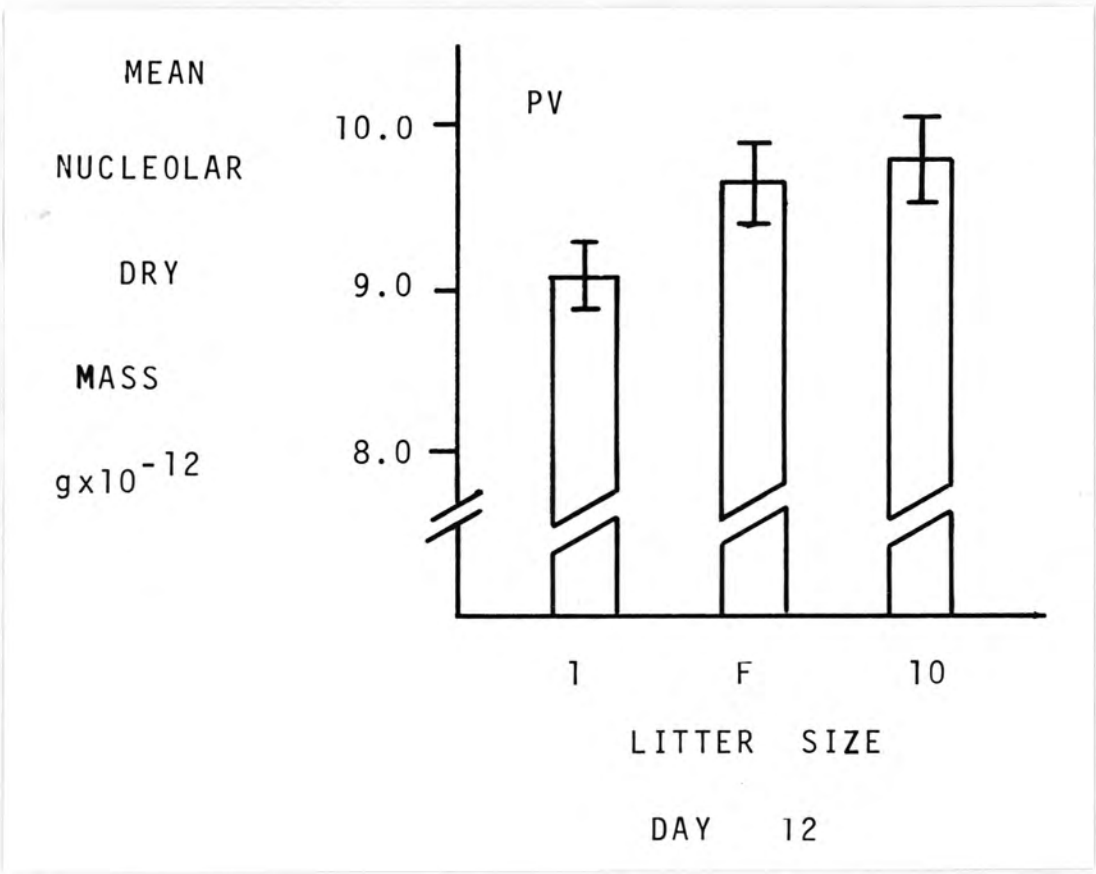


Figure 37 Second variation in litter size. Mean nucleolar dry mass of PV neurones. Details as in Fig.36.

TABLE 17

MEAN NEURONAL NUCLEOLAR DRY MASS: INCREASED LITTER SIZE DAY 7 POST PARTUM. COMPARISON WITH GROUPS NURSING 1 OR 10 PUP LITTERS

Day 12 post partum

Experimental group	PV neurones		SO neurones	
	1 pup litters	10 pup litters	1 pup litters	10 pup litters
1 pup to Day 7 post partum, then 10 pups	$0.1 > p > 0.05$	$0.8 > p > 0.7$	$0.01 > p > 0.005$**	$0.6 > p > 0.5$

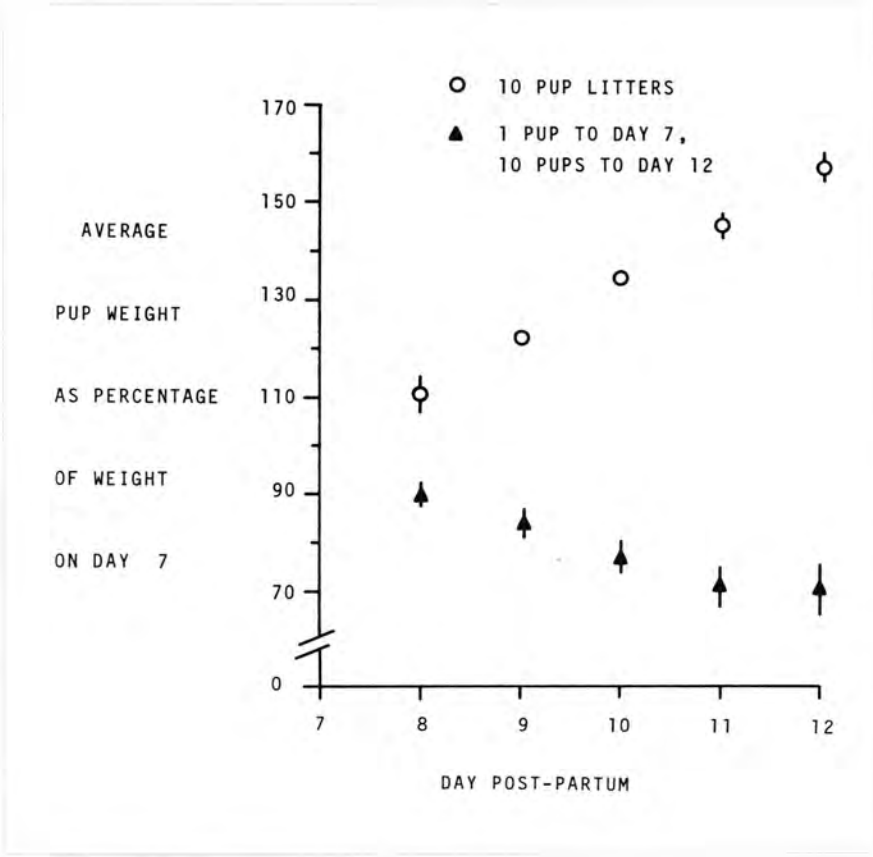


Figure 38 Second variation in litter size. Average pup weight gain as percentage of weight on day 7 post-partum.

Each vertical line indicates the standard error of the mean.

TABLE 18

TOTAL DAILY MILK YIELD WITH LATE INCREASE IN LITTER SIZE.

COMPARISONS WITH OTHER GROUPS BY STUDENT'S t-TEST

Correction factor used to calculate milk yield from litter weight	(100-4.7)			(100-14.8)		
Day post partum	5	7	12	5	7	12
Litter size, pups	10	1	1	10	1	1
Compared group: 1 pup to Day 7, 10 pups to Day 12. Milk yield Day12	$p<0.001^{***}$	$p>0.95$	$p>0.95$	$p<0.001^{***}$	$p<0.001^{***}$	$p<0.001^{***}$

TABLE 19a

NUCLEOLAR DRY MASS: ANALYSIS OF VARIANCE.

EFFECTS OF LITTER SIZE BORN AND LITTER SIZE SUCKLED

Variate	SO				PV			
	Sum of squares	d.f.	Mean square	F	Sum of squares	d.f.	Mean square	F
Litter size suckled 0,1,10,22-24	2155	3	718	4.6**	1294	3	431	3.7*
Day post partum 5,7,12	262	2	131	0.8	440	2	220	1.9
Covariate: litter size born	81	1	81	0.5	145	1	145	1.3
Interaction	657	6	110	0.7	427	6	71	0.6
Error	4647	30	154.9		3487	30	116	
Total	8482	42	202		6001	42	143	

**: significant at 0.01 level
*: significant at 0.05 level

TABLE 19b

NUCLEOLAR DRY MASS: ANALYSIS OF VARIANCE.

EFFECTS OF ESTROUS CYCLE

Variate	SO				PV			
	Sum of squares	d.f.	Mean square	F	Sum of squares	d.f.	Mean square	F
Stage of cycle	6	2	3	0.02	9	2	4.5	0.06
Error	1125	8	140.6		574	8	72	
Total	1131	10			583	10		

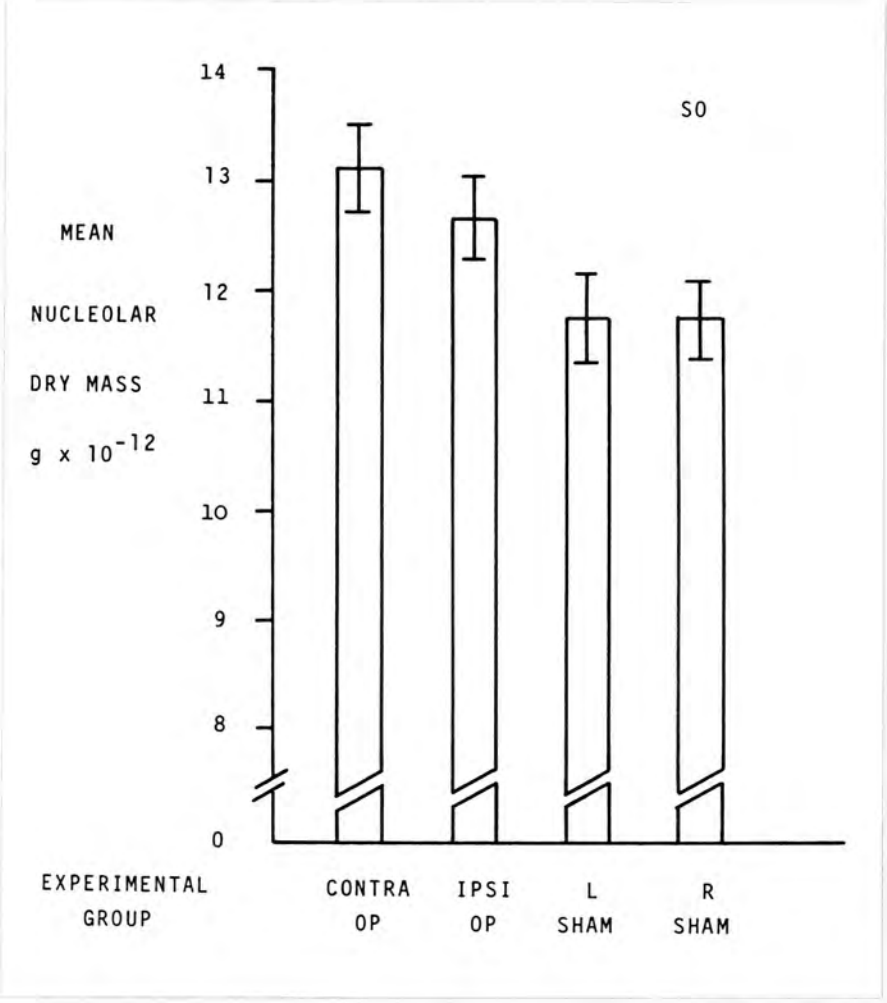


Figure 39 Unilateral nipple line ablation. Mean nucleolar dry mass of SO neurones. Day 8 post-partum.

CONTRA OP., IPSI OP.: SO neurones contra- and ipsi-lateral respectively to the nipple line ablation.

L SHAM, R SHAM: SO neurones from left and right supraoptic nuclei respectively in sham operated animals.

Each vertical line indicates the standard error of the mean.

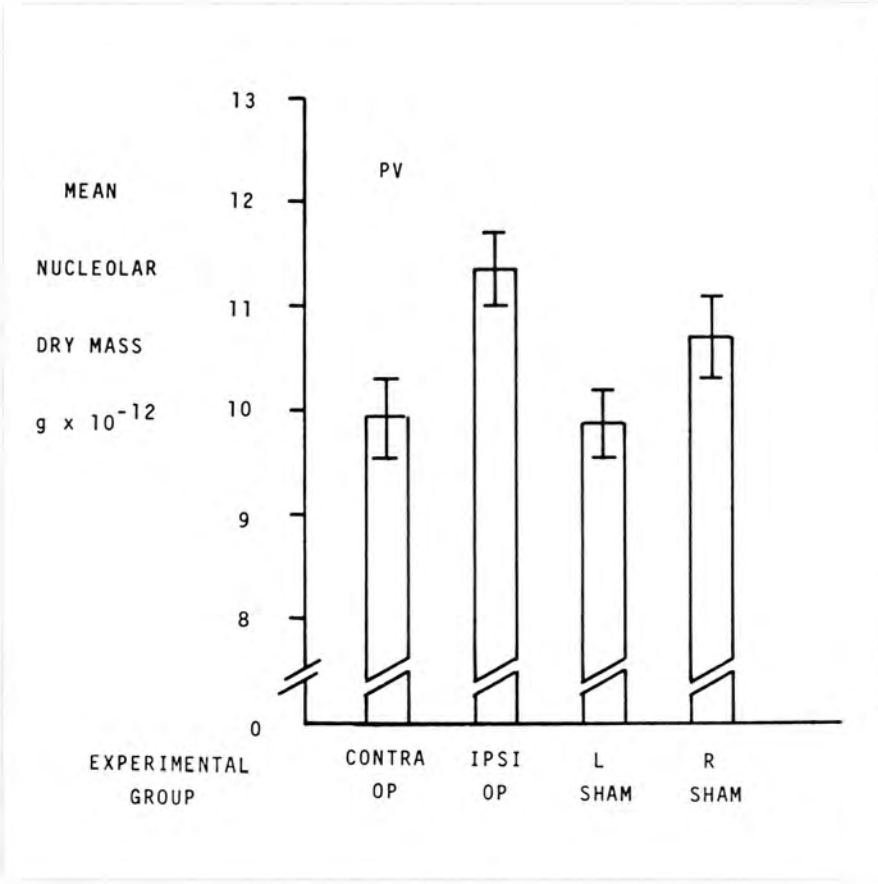


Figure 40 Unilateral nipple line ablation. Mean nucleolar dry mass of PV neurones. Details as in Fig.39.

TABLE 20

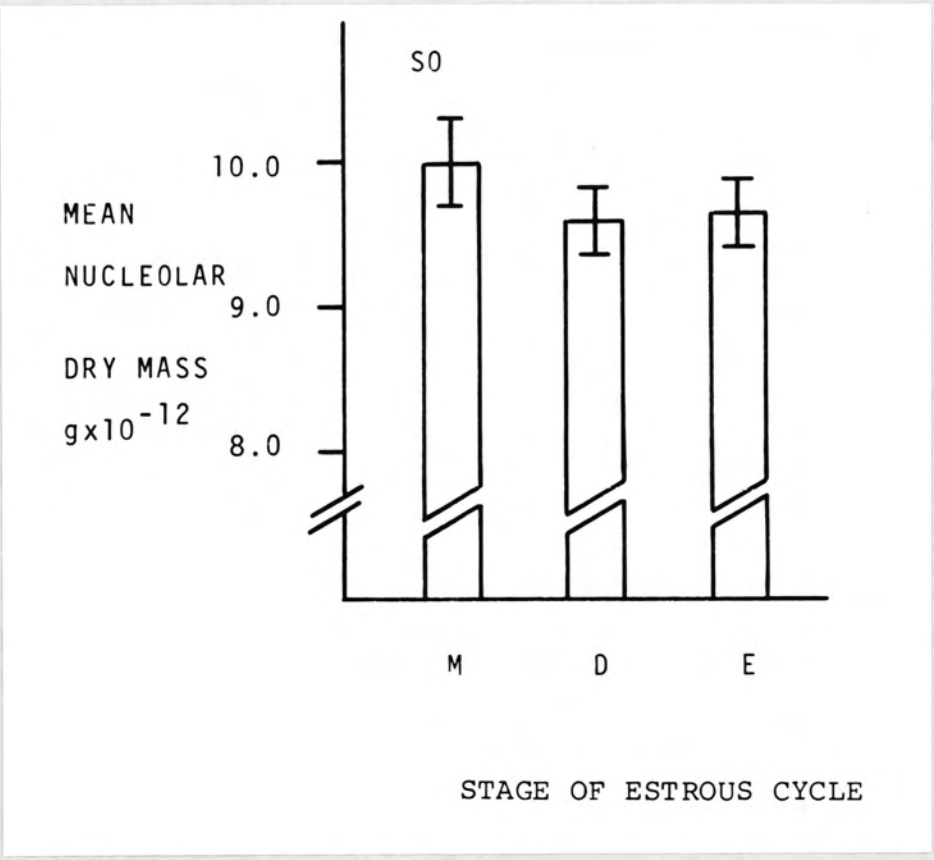
MEAN NEURONAL NUCLEOLAR DRY MASS: UNILATERAL NIPPLE LINE ABLATION. COMPARISON WITH SHAM OPERATED GROUP

Experimental Group	Unilateral nipple line ablation			Sham operation
	Side of Hypothal.	Ipsilateral	Contralateral	Left
PV neurones				
Unilateral nipple line ablation	Contra-lateral	$0.01 > p > 0.005$**		
Sham operation	Left & Right	$0.02 > p > 0.01$*	$0.5 > p > 0.4$	
	Right	$0.3 > p > 0.2$	$0.2 > p > 0.1$	$0.1 > p > 0.05$
	Left	$0.005 > p > 0.001$**	$0.95 > p > 0.9$	
SO neurones				
Unilateral nipple line ablation	Contra-lateral	$0.4 > p > 0.3$		
Sham operation	Left & Right	$0.005 > p > 0.001$**	$0.005 > > 0.001$**	
	Right	$0.005 > p > 0.001$**	$0.005 > p > 0.001$**	$0.975 > p > 0.95$
	Left	$0.005 > p > 0.001$**	$0.005 > p > 0.001$**	

TABLE 21

UNILATERAL NIPPLE LINE ABLATION. COMPARISON BY STUDENT'S t-TEST OF SEVERAL PARAMETERS WITH SHAM OPERATED GROUP

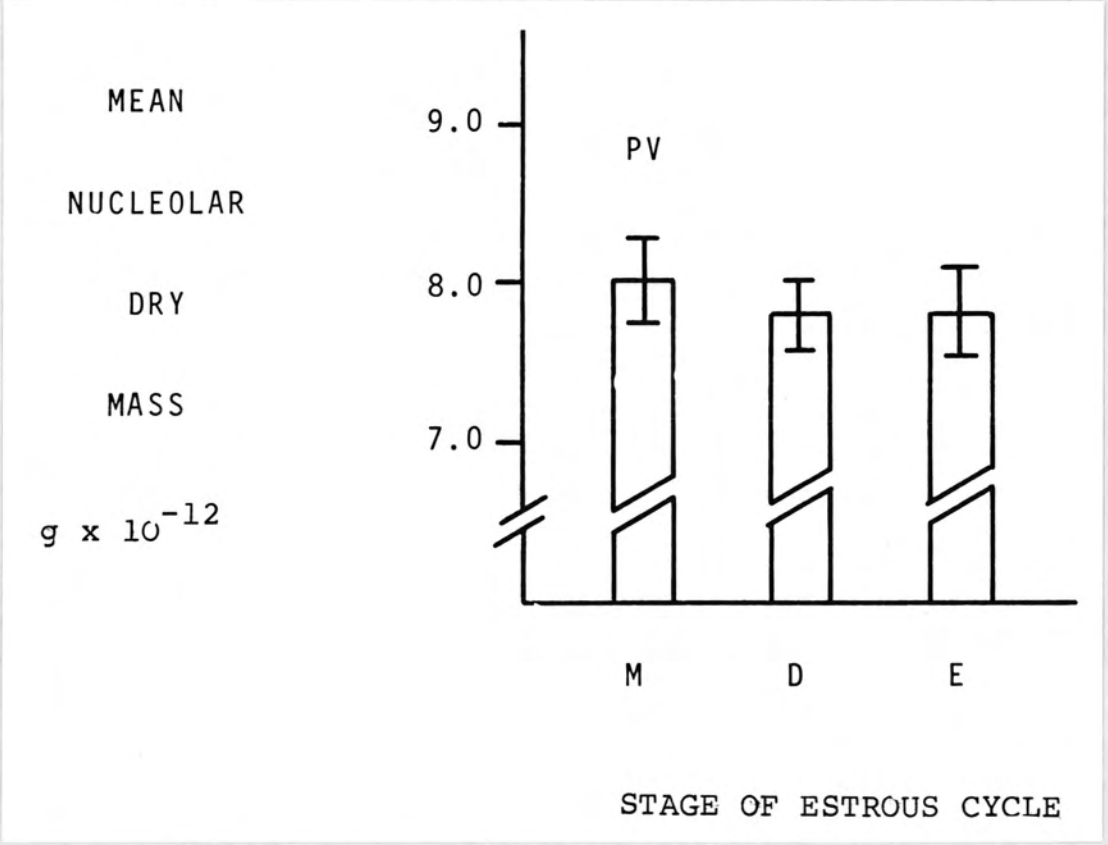
Day 8 post partum Experimental Group	Unilateral nipple line ablation	Sham Operation	p
Parameter	Mean ± Standard Error	Mean ± Standard Error	
Litter size	7.8 ± 1.11	9.4 ± 1.80	$0.6 > p > 0.5$
Pup weight g	11.3 ± 1.34	11.5 ± 0.53	$0.9 > p > 0.8$
Serum osmolality mOsm/Kg	298.6 ± 3.91	292.7 ± 2.50	$0.4 > p > 0.3$
Maternal body weight g	272.4 ± 8.20	274.0 ± 12.0	$p > 0.98$

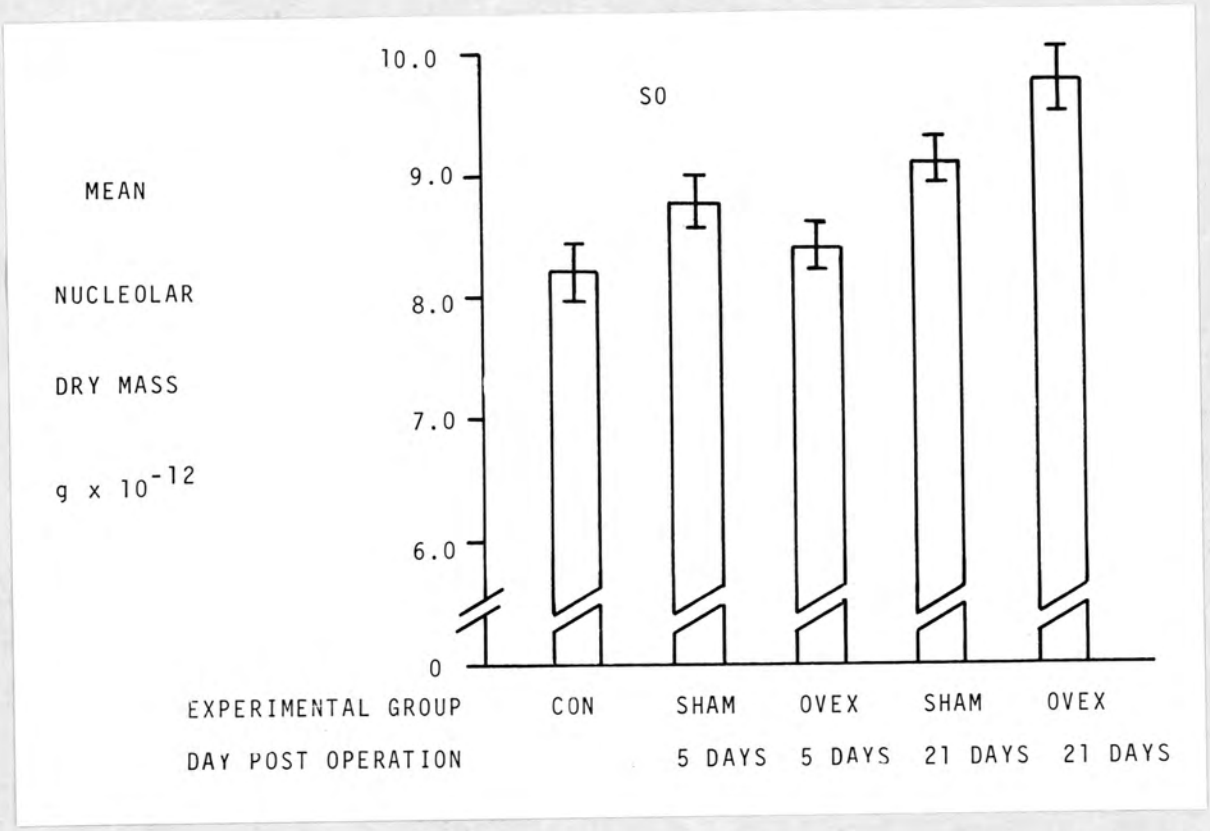


a

Figure 41 Estrous cycle and mean nucleolar dry mass.
M: metestrus. D: diestrus. E: proestrus and estrus pooled.
Each vertical line indicates the standard error of the mean.
a. SO neurones b. PV neurones

b





a

Figure 42 Ovariectomy and mean nucleolar dry mass.

CON: Virgin controls. SHAM: Sham ovariectomy.
OVEX: Ovariectomy.

Each vertical line indicates the standard error of the mean.

a. SO neurones b. PV neurones

b

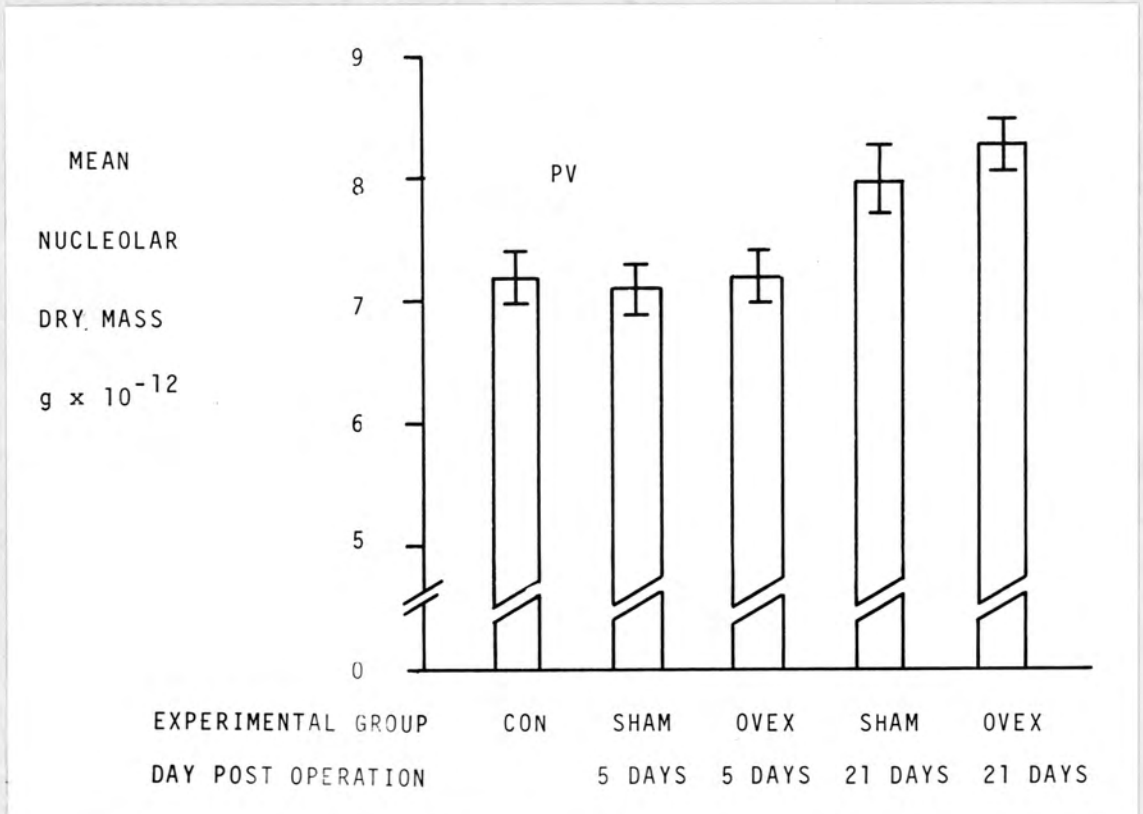


TABLE 22

MEAN NEURONAL NUCLEOLAR DRY MASS: OVARIECTOMY.

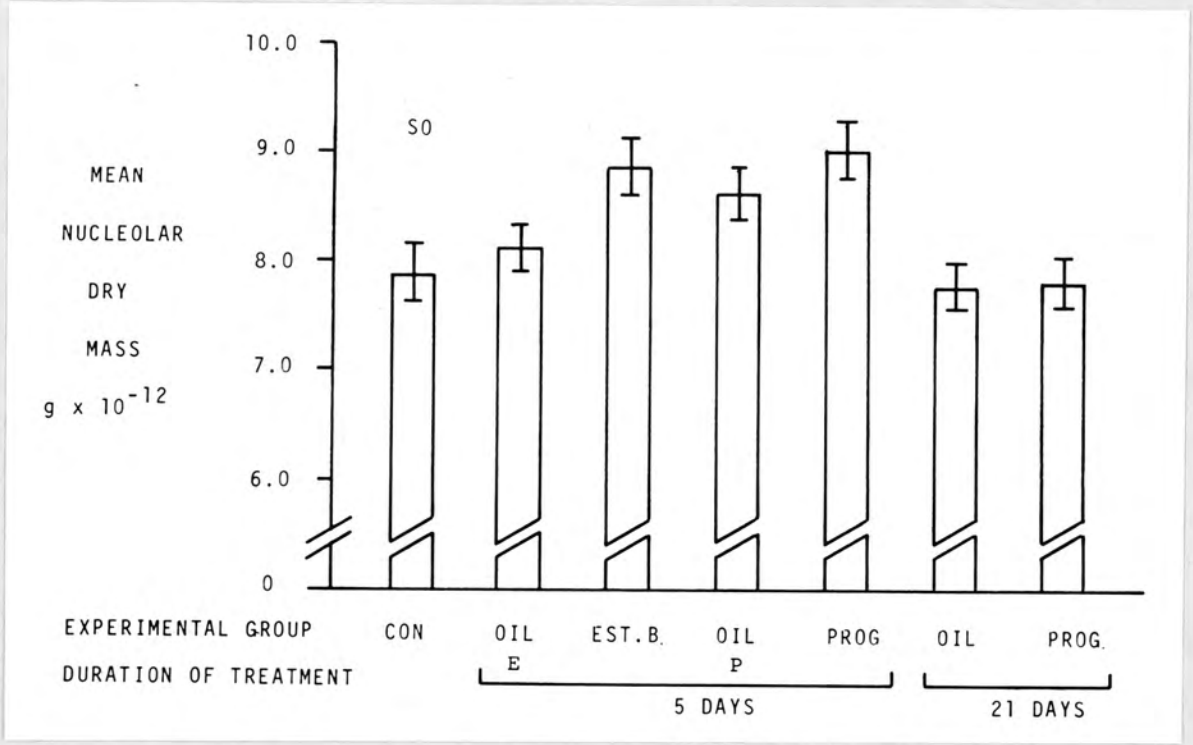
COMPARISONS WITH CONTROL GROUPS

PV neurones

Experimental Group	Ovariectomy 5 days	Ovariectomy 21 days	Sham operation 5 days	Sham operation 21 days
Virgin control	$0.99 > p > 0.98$	$p < 0.001^{***}$	$0.8 > p > 0.7$	$0.05 > p > 0.02^{*}$
Sham operation 21 days		$0.4 > p > 0.3$		
Sham operation 5 days	$0.8 > p > 0.7$			

SO neurones

Experimental Group	Ovariectomy 5 days	Ovariectomy 21 days	Sham operation 5 days	Sham operation 21 days
Virgin control	$0.6 > p > 0.5$	$p < 0.001^{***}$	$0.1 > p > 0.05$	$0.01^{**} > p > 0.005$
Sham operation 21 days		$0.1 > p > 0.05$		
Sham operation 5 days	$0.3 > p > 0.2$			



a

Figure 43 Ovarian steroid treatment and mean nucleolar dry mass. CON: Untreated virgin controls. OIL.E, OIL.P.: Oil injected control groups for estradiol (EST.B) and progesterone (PROG.) injected groups respectively.

a. SO neurones. b. PV neurones.

b

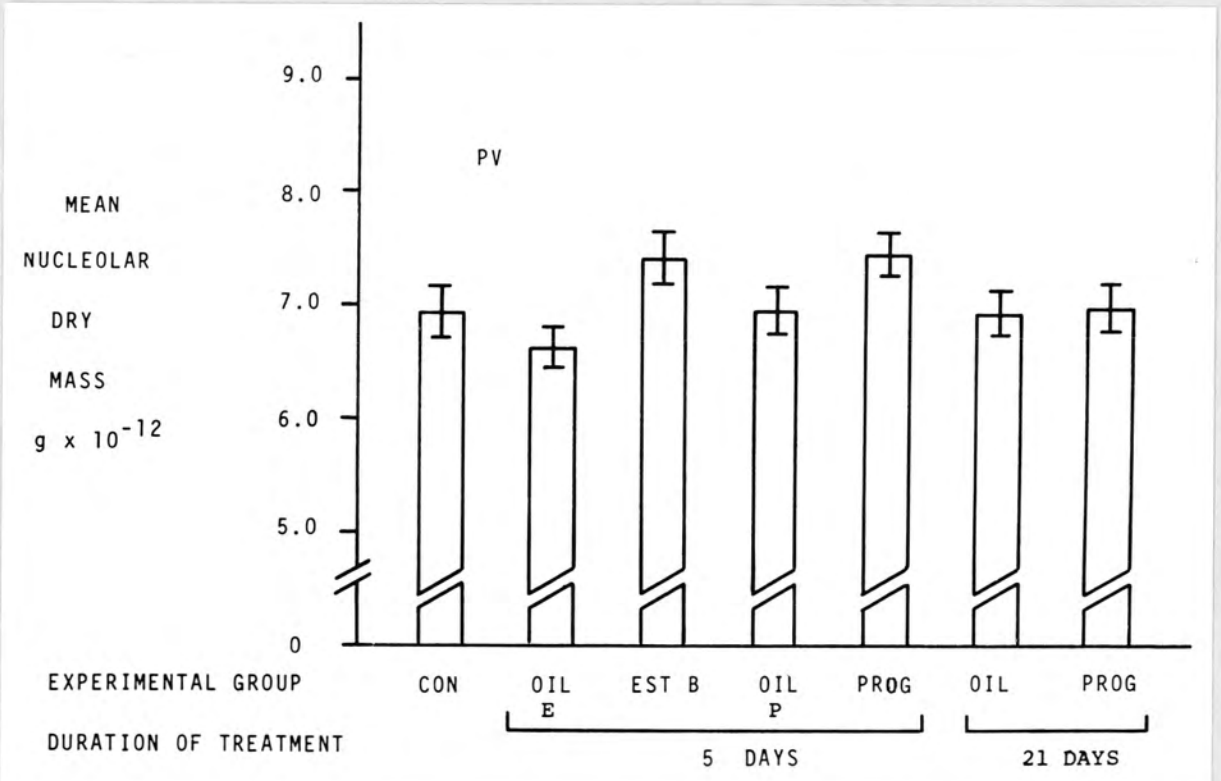


TABLE 23

MEAN NEURONAL NUCLEOLAR DRY MASS:

OVARIAN STEROID TREATMENT. COMPARISONS WITH CONTROL GROUPS

a. Estradiol benzoate

Treatment Group	PV neurones		SO neurones	
	Estradiol benzoate	Arachis oil	Estradiol benzoate	Arachis oil
Untreated control	$0.2 > p > 0.1$	$0.4 > p > 0.3$	$0.02 > p > 0.01$*	$0.4 > p > 0.5$
Arachis oil	$0.02 > p > 0.01$*		$0.05 > p > 0.025$*	

b. Progesterone

Treatment Group		PV neurones				SO neurones			
		Progesterone		Arachis oil		Progesterone		Arachis oil	
		5 Days	21 Days	5 Days	21 Days	5 Days	21 Days	5 Days	21 Days
Untreated control		$0.2 > p > 0.1$	$0.9 > p > 0.8$	$0.9 > p > 0.95$	$0.98 > p > 0.97$	$0.005 > p > 0.001$***	$0.8 > p > 0.9$	$0.05 > p > 0.02$*	$0.9 > p > 0.8$
Arachis oil	5 Days	$0.2 > p > 0.1$				$0.3 > p > 0.2$			
	21 Days		$0.9 > p > 0.8$				$0.8 > p > 0.7$		

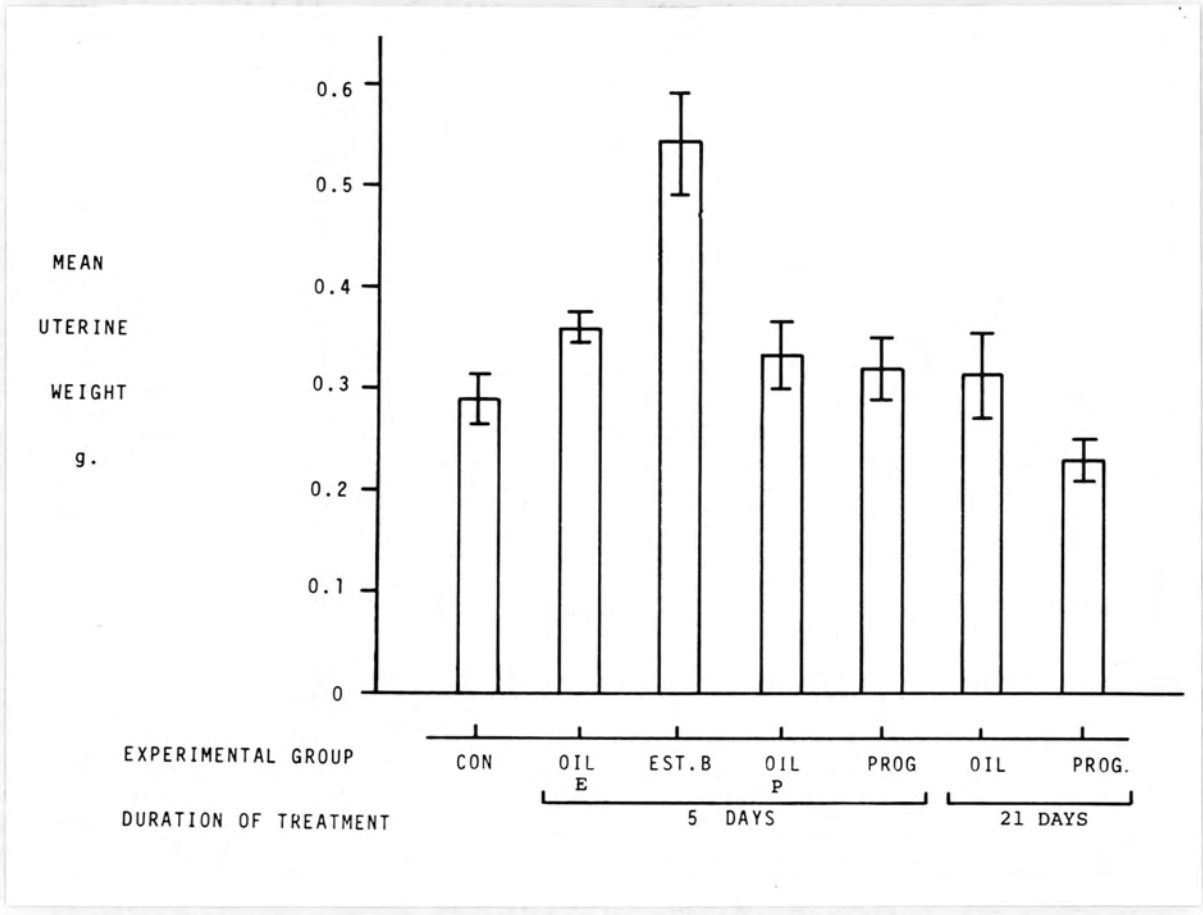


Figure 44 Ovarian steroid treatment and mean uterine weight. Details as in Fig. 43.

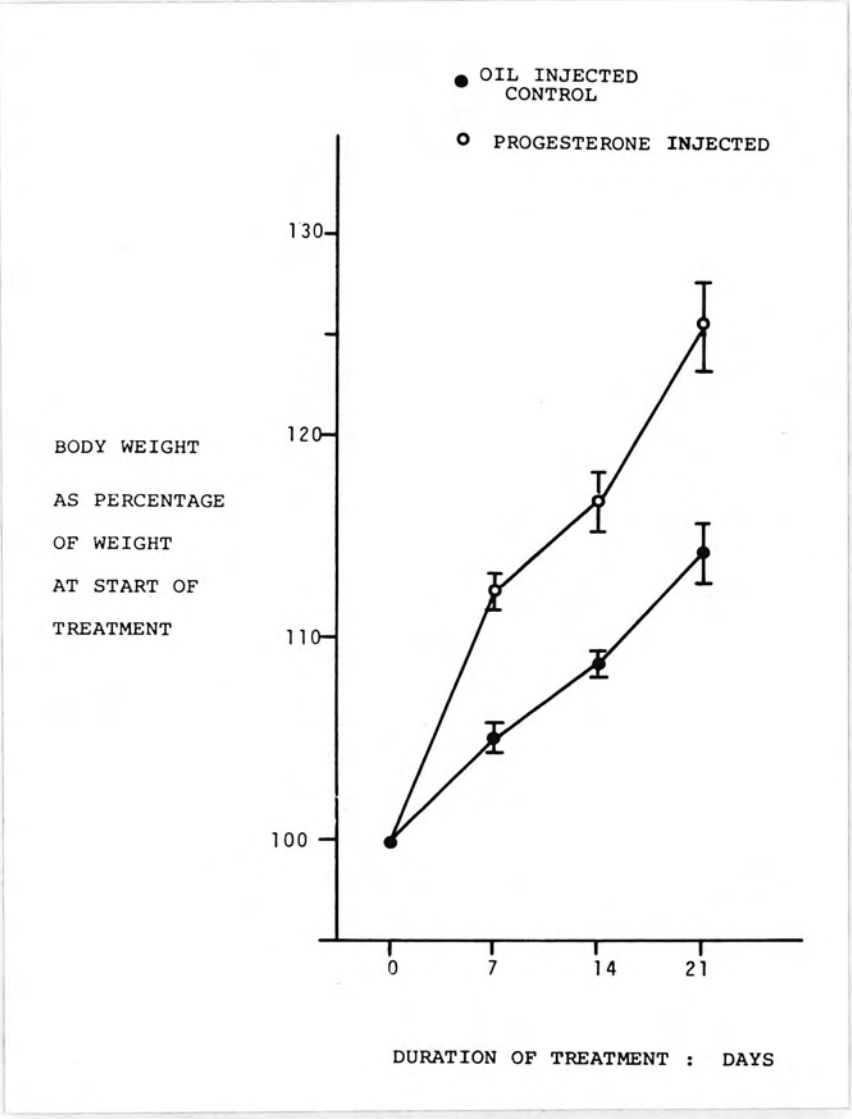


Figure 45 Progesterone treatment and body weight expressed as a percentage of body weight at start of treatment. Each vertical line indicates the standard error of the mean.

4. Results

4.1 Second variation in litter size

This experiment was performed at the same time as the experiments on litter size variation (Section III); the results may be compared.

The mean nucleolar dry mass of PV neurones of animals nursing one pup continuously or one pup until day 7 and then 10 pups were not significantly different on day 12 post partum. However the mean nucleolar dry mass of PV neurones of animals nursing 10 pups continuously or only since day 7 post partum were also not significantly different on day 12 (Fig. 37, Table 17). But the mean nucleolar dry mass of PV neurones, measured on day 12, of animals nursing 10 pups only since day 7 was more significantly different from that of animals nursing one pup continuously than it was from that of animals nursing 10 pups continuously (Table 17).

The mean nucleolar dry mass of SO neurones at day 12 post partum was greater in animals nursing one pup to day 7 and then 10 pups than in animals nursing one pup continuously, but was not different from that of animals nursing 10 pups continuously (Fig. 36, Table 17).

The pups cross fostered at day 7 to animals previously nursing one pup weighed less five days later than pups of normal 10 pup litters of the same age ($p < 0.001$, Fig. 38). Until day 7 post partum the weight gains of the single pup litters nursed by animals which continued to nurse one pup to the 12th day were identical to the weight gains of single

pups nursed by animals which nursed 10 pups from day 7 post partum (Fig. 29a). The total daily milk yield probably rose when the litter size was increased on day 7 (Fig. 31a,b; Table 18). The estimated total daily milk yield was less after five days of suckling by 10 pups if this suckling intensity was delayed until the 7th day post partum than if 10 pups had suckled for five days since birth (Fig. 31a,b; Table 18).

The serum osmolality was not altered (Table 13).

Nursing behaviour was not systematically observed, but the pups were frequently seen to suck vigorously. All the thoracic nipples and two or four cephalic abdominal teats were sucked. All the foster mothers accepted the additional pups.

4.2 Effect of number of pups born

The only significant contribution to the variance of the mean nucleolar dry mass of PV and SO neurones of animals with imposed variation in litter size was from the imposed litter size (Table 19a).

4.3 Unilateral nipple line ablation

One animal with a nipple line ablation and its control were rejected because it was subsequently found to have only five nipples in the remaining nipple line. It was, incidentally, the only such animal in this entire investigation. Five of the remaining animals in each group became pregnant and delivered litters. The results are shown in Figs. 39, 40, and Tables 20, 21.

The mean nucleolar dry mass of PV neurones was greater on the side of the nipple line ablation. Mean nucleolar dry mass of SO neurones ipsilateral and contralateral to the nipple line ablation were not different from each other.

Right and left nucleolar dry mass were not different from each other for SO or PV neurones in the sham operated animals.

Mean nucleolar dry mass of SO neurones of animals with nipple line ablations was greater than that of the sham operated animals (Table 20). The nucleolar dry mass of PV neurones ipsilateral to the nipple line ablation was greater than the mean nucleolar dry mass of combined right and left PV neurones of sham operated animals. The nucleolar dry mass of PV neurones contralateral to the nipple line ablation was not different from the latter (Table 20).

Litter size, pup weight, serum osmolality and maternal body weight were not different between the sham and experimental groups (Table 21).

4.4 Estrous cycle

Neither PV nor SO neuronal nucleolar dry mass changed during the estrus cycle in a group of 11 animals (Fig. 41, Table 19b).

4.5 Ovarian hormones

4.5.1 Ovariectomy

Mean nucleolar dry mass of PV or SO neurones was not affected by ovariectomy, compared with appropriate sham operated controls (Fig. 42a,b; Table 22). In both sham

operated and ovariectomised animals, 21 days after operation, mean nucleolar dry mass of PV and SO neurones was greater than in unoperated controls (Fig. 42a,b; Table 22).

4.5.2 Ovarian steroids

The results are shown in Figs. 43a, b, 44; Table 23a,b.

Estradiol benzoate, compared with oil alone, increased the mean nucleolar dry mass of both PV and SO neurones within five days (Fig. 43a,b; Table 23a).

Uterine weight was increased at the end of five days treatment with estradiol benzoate; $p<0.005$ and <0.02 compared with untreated and oil injected controls respectively (Fig. 44).

Progesterone treatment for five or 21 days had no effect on PV or SO neuronal nucleolar dry mass (Fig. 43a, b; Table 23b). In the group treated with progesterone or oil for five days, nucleolar dry mass of SO neurones was greater than in uninjected controls. Body weight increased more rapidly in animals injected with progesterone than in those injected with oil alone, $p<0.001$ at 7 days and thereafter (Fig. 45).

5. Discussion

With respect to PV neuronal nucleoli, a further increase in litter size onday 7 post partum had an equivocal effect. The nucleolar dry mass of PV neurones in such animals tended to be more like that of animals nursing 10 pups since birth than in animals nursing only one pup (Fig. 37). This suggests that a suckling stimulus, applied at a time remote from parturition, may cause nucleolar changes in PV neurones, but does not rule out an influence of the hormonal state at the end of pregnancy or beginning of lactation on these changes. The nucleolar dry mass of SO neurones did increase significantly when the litter size was increased on day 7 post partum (Fig. 36). The hormonal state of late pregnancy, parturition or the immediate post partum period is not essential for SO neuronal nucleolar changes in response to suckling. Repeated hyperosmotic stimulation of SO neurones, with a rest period in between stimulation periods, results in a further, but less marked, increase in ribosome synthesis (Watson, 1965b). Similar changes occur in hypoglossal neurones when axotomy is repeated (Watson, 1968a).

There is no information on the secretion of oxytocin under the circumstances of this experiment. Oxytocin secretion and milk yield may not be quantitatively related to each other in the early stages of restoration of milk yield. Milk yield was lower after five days suckling by 10 pups from day 7 than after five days suckling by 10 pups from parturition (Fig. 31a,b). Not all the nipples were suckled in the former. The integrated suckling stimulus includes

stimuli from mammary gland volume receptors, the number of pups suckling, and the duration of suckling (Section IV - 2.2.1). Because of the low milk yield the stimulus to oxytocin secretion may have been less effective in animals with litter size increased on the 7th day post partum than in animals suckling 10 pups from birth (Section IV - 4.1.2). It is possible that daily oxytocin secretion was less in the former group of animals, in accordance with the less marked PV neuronal nucleolar changes (Section IV - 4.3). Milk yield probably increased when the litter size was increased on day 7 post partum (Fig. 31a,b), although the pups did not gain weight (Fig. 38). In a similar experiment in which the litters nursed by ovariectomised animals were increased in size on day 6 post partum, litter weight gain continued normally (Hammons et al, 1973). These conflicting results may indicate that suppression of ovarian activity is a necessary prelude to adequate milk synthesis or ejection or both. In intact females, lactation can be restarted by suckling after normal weaning, or if the litters have been removed in mid-lactation (Bruce, 1961; Ota & Yokoyama, 1965). Interruption of nursing for the first 24 hours post partum permanently diminishes milk yield, judged by litter growth, in that lactation (Walsh & Tucker, 1972); this finding is in accord with the observations in the present study of the effects of reducing litter size to one pup within 24 hours of parturition (Figs. 31a,b; 29a)

Because milk yield increased, presumably oxytocin was secreted when suckling was intensified, but as suckling in

this context suppresses gonadotrophin secretion within 24 hours, and stimulates adrenal corticoid and prolactin secretion (Selye & McKeown, 1934; Bruce, 1958; Voogt et al, 1969; Hammons et al, 1973), changes in PV and SO neurones cannot be assumed to be independent of the actions of these suckling-induced hormonal changes. The limited conclusion can be drawn that suckling dependent changes in SO neuronal nucleoli, and probably in PV neuronal nucleoli (Section III), do not also depend on the hormonal state at the end of pregnancy.

This conclusion is supported by the failure of the number of pups born, and hence the number of corpora lutea and ovarian weight (Stotsenburg, 1923) to influence the PV and SO neuronal nucleolar responses after five days post partum (Table 19a). Similarly, for normal litter sizes, milk yield is regulated by the size of the suckling litter rather than by the number of pups born (Bateman, 1957).

The PV neuronal nucleolar dry mass measurements in animals with unilateral nipple line ablation indicate that these nucleolar changes are independent of a humoral factor or factors (Fig. 40); since the nucleolar dry mass of PV neurones on the contralateral side was not less than the nucleolar dry mass of PV neurones of sham operated animals, this independence may be partial. The asymmetrical changes in PV neurones of animals with unilateral nipple line ablation also indicate that more afferent fibres of the milk ejection pathway reach the contralateral than the ipsilateral PV nucleus. The afferent pathway in the spinal cord

is mainly unilateral (Eayrs & Baddeley, 1956), and inhibition by peripheral pain of oxytocin secretion in response to suckling is greater when suckling and peripheral pain are applied ipsilaterally (Taleisnik & Deis, 1964). Electrophysiological studies could confirm or refute the suggested asymmetrical distribution of the afferent fibres of the milk ejection reflex, but have not been reported (e.g. Urban et al, 1971).

The difference between right and left PV neuronal nucleolar dry mass of sham operated animals was not significant (Fig. 40). Whether one nipple line was preferentially suckled was not recorded. The mean litter size of 9.4 pups in this group could have resulted in a twofold difference in suckling intensity between the left and right sides. Rhythmic changes have been reported between right and left SO nuclei, but the PV nuclei have not been studied in this context (Zambrano & Mordoh, 1966). In the present study there were no differences between SO neurones from the right and left sides in sham operated animals or ipsi- and contra-lateral to the unilateral nipple line ablation (Fig. 39). Symmetrical changes in SO neuronal nucleolar dry mass cannot be interpreted in the context of the role of humoral factors or unilateral representation of the suckling stimulus. It should be emphasised that 'humoral factors' includes serum osmolality. In the SO nucleus about half the antidromically identified neurones which respond to ipsilateral vagus or carotid sinus stimulation also respond to stimulation of the contralateral nerves (Barker et al, 1971a,c).

In general the SO and PV neuronal nucleolar changes in the animals with unilateral nipple line ablation were greater than in the sham operated group (Table 20). This may reflect greater total oxytocin or ADH secretion, or both, in the former group if, to provide adequate milk for an average of 7.8 pups from six nipples to maintain normal pup growth (Table 21), more milk ejections were required, with consequent nucleolar changes (Section IV - 4.3). In contrast with the experiment in which litter size was varied (Section IV), the demand for more milk ejections could have been met, because the total milk yield was within normal limits. However, these two groups of animals were killed several days apart, because they did not become pregnant synchronously; an uncontrolled variation in environmental conditions, though not observed, may explain the differences in nucleolar dry mass between the sham and the operated groups.

The absence of nucleolar changes in PV and SO neurones during the estrous cycle (Fig. 41a,b) conflicts with reports of changes in other parameters in these neurones. The amounts of ADH and oxytocin in the posterior pituitary are higher during proestrus or estrus than at other stages (Heller, 1959). The percentage of SO and PV perikarya devoid of neurosecretory material, and the incorporation of ^{14}C-adenine into RNA by SO and PV neurones are greater in diestrus (Belajev et al, 1967). The amount of thiamine pyrophosphatase in PV and SO neurones is increased in estrus (Swaab & Jongkind, 1970). Rinne (1960) however did not find significant changes in PV or SO nucleolar volume during the estrous cycle, but

this parameter has been criticised (Section III - 4.1, 4.3). Rhythmic changes in secretory activity (Zambrano & Mordoh, 1966; Golubitsa & Korotchkin, 1971) may occur independently of changes in ribosome synthesis. Nucleolar dry mass changes over a period of five days (Section III - 4.3), and may adopt an average value with respect to rapid fluctuations in secretory activity within the limits of the set synthetic capacity.

Ovariectomy may slightly increase the amount of neurosecretory material in PV and SO perikarya (De Groot, 1957), or decrease the amount in the median eminence (Lisk, 1965-6). In ovariectomised rats the amount of ADH in the posterior pituitary is similar to that of diestrus, that is less than in estrus (Heller, 1959), but the amount of oxytocin is increased (Deis, 1959). After ovariectomy, the activity and content of thiamine pyrophosphatase in PV and SO neurones increase within two weeks (Swaab & Jongkind, 1970; Swaab et al, 1971); the diameter of SO and PV neuronal nucleoli increases within 20 days (Ifft, 1964), or is not altered (Flament-Durand & Desclin, 1967); but ultrastructural changes consistent with increased synthetic activity are present in SO and PV neurones within one month (Zambrano & De Robertis, 1968a). In the castrated male however, total SO perikaryal RNA decreases (Edstrom, Eichner & Schor, 1961), or is unaltered (Watt, personal communication). Decreased volumes of PV and SO neuronal nuclei after ovariectomy (Szentagothai et al, 1968) cannot be interpreted.

The nucleolar dry mass measurements made in this study conflict with some of these observations. No evidence has

been obtained which indicates that ribosome synthesis in SO and PV neurones is influenced by removal of the ovaries in virgin female rats (Fig. 42a,b).

Lactation is not affected by ovariectomy after parturition in the rat (Wiesner & Sheard, 1933; Tucker & Reece, 1963), provided the adrenals are intact (Flux,1955). Normal oxytocin secretion is implied. The effects of ovariectomy during lactation were not investigated in the present study. Ovariectomy on the first day post partum does not affect changes in nucleolar volume of PV neurones measured 15 days later, but prevents the changes in SO neurones (Flament-Durand & Desclin, 1968). Although nucleolar changes in PV and SO neurones depend on the suckling stimulus (Section III - 3.3; Flament-Durand, 1967), the effects of ovariectomy on nucleolar responses in lactation are consistent with activation of SO and PV neurones by different stimuli related to suckling (Section VI).

Progesterone treatment at doses sufficient to maintain pregnancy (Lerner et al, 1962) and probably to suppress gonadotrophin secretion (McCann, 1962) did not alter PV or SO neuronal nucleolar dry mass in intact virgin females (Fig. 43a,b). Prolactin secretion may have been increased by this treatment (Chen & Meites, 1970). Progesterone inhibits the reflex secretion of oxytocin in response to vaginal distension (Roberts & Share, 1970) and possibly suckling (Herrenkohl, 1971; Herrenkohl & Lisk, 1973), and increases (Fendler, 1961) or does not change (Deis, 1959) the amount of oxytocin stored in the posterior pituitary.

Progesterone does not alter the amount of neurosecretory material, measured subjectively, in the perikarya of SO or PV neurones or in the posterior pituitary (Gastaldi, 1952). There are no other studies of the effects of progesterone on synthetic activity in PV or SO neurones.

It would be of interest to see if PV and SO neuronal synthetic activity are affected by progesterone withdrawal, in view of the importance of this event in the initiation of lactation (Kuhn, 1969), and possibly parturition (Cross, 1966). Daily subcutaneous injections of progesterone maintain a high concentration of progesterone in blood although a circadian variation occurs (Bullock & Cook, 1967). The animals in the present study may have been exposed to repeated withdrawal of progesterone in this context. Implantation of pellets of progesterone may maintain a steadier blood concentration (Dewar, 1969). The greater change in body weight in the progesterone treated group confirmed that progesterone was absorbed (Hervey & Hervey, 1967; Fig. 45).

After estrogen treatment, the amounts of ADH and oxytocin in the posterior pituitary increase(Heller, 1959; Fendler, 1961), or the amount of oxytocin decreases(Deis, 1959). Estrogen treatment for several months increases the amount of neurosecretory material, measured subjectively, in SO and PV perikarya and axons, but does not affect the posterior pituitary(Gastaldi, 1952). Implants of 17- β estradiol in the median eminence increase the amount of neurosecretory material in the median eminence in ovariectomised, but not

in intact, rats (Lisk, 1965-6). Intracerebral implants, or subcutaneous injections, of estrogen in pregnant rats increase the amount of neurosecretory material in PV and SO perikarya and axons, measured subjectively on the 15th day post partum (Stutinsky, 1968). The amount of thiamine pyrophosphatase in PV and SO neurones of ovariectomised rats is decreased by daily treatment with estradiol benzoate (Swaab et al, 1971a). Estrogen facilitates the reflex secretion of oxytocin in response to vaginal stimulation (Roberts & Share, 1969) but does not affect the milk ejection reflex in the rat (Chaudhury, 1961).

In general, these observations suggest that physiological doses of estrogen may stimulate secretory and synthetic activity in PV and SO neurones.

In rats in constant estrus, induced by continuous illumination for eight weeks, the nucleolar volumes of SO, but not PV, neurones increase (Flament-Durand & Desclin, 1960), and the rate of ^{35}S-cysteine turnover in SO neurones increases (Flament-Durand, 1965). If these changes are related to increased estrogen secretion (Flament-Durand & Desclin, 1967), then they are consistent with the results of the present study, that estradiol administration increases the mean nucleolar dry mass of SO and PV neurones (Fig. 43a, b). Swaab & Jongkind (1971a) have interpreted their results as evidence for an action of gonadotrophic hormones on SO and PV secretory activity. Daily injections of human menopausal gonadotrophin or human chorionic gonadotrophin, each together with 2 µg of estradiol benzoate, increase the amount

of thiamine pyrophosphatase in SO and PV neurones of ovariectomised rats, whereas prolactin and estradiol have no effect (Swaab & Jongkind, 1971a). If thiamine pyrophosphatase content can be regarded as an indicator of synthetic and secretory activity, these findings are in conflict with those of the present investigation and the other reports of a stimulatory action of estrogen (vide supra).

The uterine weight changes confirmed that estradiol was absorbed (Fig. 44) and it is likely that the large doses of estrogen used in this study suppressed gonadotrophin (Ramirez & McCann, 1963) and prolactin secretion (Voogt et al, 1970). The hypothesis that SO and PV neuronal synthetic activity is stimulated by anterior pituitary gonadotrophins would not explain the increased activity in these neurones during pregnancy, when FSH and LH secretion are low (Linkie & Niswender, 1972), at least at the time when thiamine pyrophosphatase changes in PV and SO neurones are evident (Swaab & Jongkind, 1970). Nor would PV and SO neuronal changes in lactation be so explained, since gonadotrophin secretion is inhibited (Rothchild, 1960; Hammons et al, 1973).

Investigation of the effects of 'physiological' doses of estrogen on PV and SO neuronal nucleoli is required, but complicating stimulatory actions on anterior pituitary hormone secretion (Chen & Meites, 1970; Meites, 1972) would have to be controlled. The present results are consistent with a facilitatory action of estrogen on oxytocin and ADH synthesis or secretion or both. Estrogen,

which is secreted in increased amounts towards the end of pregnancy (Yoshinaga et al, 1969) may partly cause the nucleolar changes in SO and PV neurones measured at the end of pregnancy (Section III - 3.1).

The SO and PV neurones do not have the properties of neurones with estrogen-specific receptors (Stumpf, 1968), but estrogen treatment may induce the synthesis of such receptors in cells which do not normally display these properties (Jensen & De Sombre, 1971). A direct action of estrogen on RNA synthesis in SO and PV neurones is possible, as in classical target tissues (Hamilton, 1968). Alternatively estrogen may modulate ADH or oxytocin secretion by a direct action on the PV and SO nerve endings, or on the synapses on the perikarya of these or other neurones. Estrogen and progesterone modify the uptake of tritiated noradrenaline by synaptosomes in vitro (Janonsky & Davis, 1970) and alter the activity of hypothalamic tyrosine hydroxylase (Beattie et al, 1972). Estradiol lowers, and progesterone raises, the threshold for electroshock induced convulsions (Woolley & Timiras, 1962) and progesterone produces electroencephalographic changes similar to those of sleep (Ramirez et al, 1967).

The various actions of ovarian steroids on synaptic and synthetic processes, as well as actions mediated via anterior pituitary hormones, are all ways in which secretory and synthetic activity in SO and PV neurones could be modulated. The only effective hormone shown in the present study is estradiol.

Local implantation or microinfusions of hormone in the region of the perikarya or nerve endings could be used to investigate further the mechanism of the action of estrogen on PV and SO neuronal ribosome synthesis.

The actions of other specific hormones were not studied. After pinealectomy, the amount of thiamine pyrophosphatase in SO and PV neurones decreases, which finding, if the pineal inhibits gonadotrophin secretion, conflicts with the thiamine pyrophosphatase changes after gonadotrophin treatment discussed above (De Vries & Kappers, 1971). Diurnal variation in the volume of the nucleoli of, and the amount of neurosecretory material in, PV and SO neurones has been described and attributed to the diurnal adrenocorticoid rhythm (Rinne & Sonninen, 1964). Cortisone decreases SO and PV neuronal nucleolar volume (Kivalo & Rinne, 1960), increases the amounts of oxytocin and ADH in the posterior pituitary (Rennels, 1958), and inhibits the antidiuretic response to nicotine (Dingman & Thorn, 1955). Although ADH secretion increases during adrenocortical insufficiency, and can be reduced by adrenocorticoid therapy, the changes in ADH secretion are caused by volemic or osmotic stimuli, and not by a direct action of corticosteroids on ADH secretion(Travis & Share, 1971). Adrenocorticoids are secreted in response to suckling(Voogt et al, 1969), and since PV and SO neuronal synthetic and secretory activity are increased by suckling (Section I - 2.6.2; Section III) adrenocorticoids probably do not have important inhibitory

effects on ADH and oxytocin secretion in lactation. It would be of interest to examine the nucleolar responses of PV and SO neurones to changes in adrenocorticoid secretion during lactation (see also Section VI - 6).

6. Summary

The hormonal state which immediately precedes normal lactation is not essential for the synthetic changes in PV and SO neurones which occur during lactation. No effect on PV or SO neuronal nucleolar changes could be attributed to the preceding pregnancy after the 5th to 7th days post partum (see also Section III - 3.3).

The afferent input from each nipple line is probably distributed mainly to the contralateral PV nucleus. There is no unequivocal evidence for any such distribution to the SO nuclei. A humoral factor, which may be a circulating hormone or serum osmolality, effective during lactation, may contribute to the PV and SO nucleolar changes. There is no direct evidence for such a factor.

In virgin females, PV and SO neuronal nucleoli did not change during the estrous cycle, after ovariectomy or after progesterone treatment. Large doses of estrogen increased nucleolar dry mass in both PV and SO neurones. Estrogen may partly cause the nucleolar dry mass changes in SO and PV neurones at the end of pregnancy, or during lactation.

SECTION VI

COMBINED EFFECTS OF LACTATION AND DEHYDRATION ON SUPRAOPTIC AND PARAVENTRICULAR NEURONES

1. Prologue

The experiments described in this section were performed to examine whether the limited nucleolar responses during lactation when the suckling stimulus was intensified were due to limited secretory activity of the SO and PV neurones or to a limited synthetic capacity of the nucleoli. The nucleolar responses of SO and PV neurones to different combinations of stimuli were compared to characterise the stimulus inputs to each nucleus.

To provide a framework for the hypotheses proposed later (this section - 3) and the experiments, the literature on the synthesis of ADH and oxytocin in separate neurones and the separate release of ADH and oxytocin is discussed in the Introduction. The distinction between these two aspects is not always expressed by others but is emphasised here in order to clarify the questions which can be asked with the methods used in this study.

2. Introduction

2.1 Synthesis and secretion of ADH and oxytocin by separate neurones

It is not clear whether there is a mixture of neurones, some which synthesise ADH and oxytocin, others which synthesise only ADH or oxytocin, or whether the PV and SO nuclei contain homogeneous populations of any of these types of neurone.

Dale (1935) proposed that a neurone could synthesise and normally release only one transmitter; the inhibitory or excitatory actions of transmitter released by a neurone depend partly on different responses of post-synaptic receptors to the same transmitter (Kandel & Gardner, 1972). To establish that magnocellular hypothalamic neurones secrete either oxytocin or ADH but not both, the criteria which must be satisfied are that in any one of these neurones only ADH or oxytocin, but not both, is (1) present, (2) synthesised, and (3) released when the neurone is stimulated.

(1) Oxytocin and ADH are not distinguished by stains for neurosecretory material. ADH, but not oxytocin, contains arginine which can be demonstrated histochemically in rat SO and PV neurones, but arginine is similarly demonstrated in pig posterior pituitaries, so the test is not specific for arginine vasopressin (Bock & Schluter, 1971). Rabbit antibody to porcine neurophysin II does not react with all PV and SO cell bodies and endings in frozen sections of pig hypothalamus and posterior pituitary. This is

consistent with the presence of neurophysin II in one pool of neurones (Livett et al, 1971), but does not exclude rhythmic changes in synthetic and secretory activity in an otherwise homogeneous population. Antibodies to other neurophysins were not raised. Antibodies to ADH and oxytocin (Boyd, 1972) have not been used, but could be.

(2) ^{3}H-arginine is incorporated into protein after intracisternal injection into rats but is not found in all the neurosecretory nerve endings in the posterior pituitary. This may indicate different rates of arginine vasopressin turnover in different neurones at the time of exposure to isotope, or that ADH is synthesised in a distinct pool of neurones (Nishioka et al, 1970). However, because arginine is not incorporated only into ADH (Bock & Schluter, 1971) the identify of the labelled material would have to be established before the results of this study can be interpreted as evidence for synthesis in separate neurones. There is no difference in the posterior pituitary between the turnover rates of ^{3}H-tyrosine labelled ADH and oxytocin (Jones & Pickering, 1972).

(3) It is impractical to stimulate a single PV or SO neurone and measure its secretory output, but if neurones which secrete oxytocin or ADH are not only separate but are segregated into the PV and SO nuclei, then stimulation of each nucleus would test thiscriterion (vide infra).

Although crucial tests of the proposed criteria have hardly been performed, there is circumstantial evidence that oxytocin and ADH may be synthesised and stored by separate neurones.

Oxytocin and ADH are separate entities (Acher et al, 1956) and have been synthesised (Du Vigneaud et al, 1953). They may be stored in separate neurosecretory granules; neurosecretory granules associated with either ADH or oxytocin can be partially separated from homogenised posterior pituitaries by density gradient centrifugation (Barer et al, 1963). Electrophoretic separation of rat neurophysin into three fractions has been reported (Burford et al, 1971; Watkins & Ellis, 1971) and disputed (Norstrom & Sjostrand, 1971a), although in this study only SO neuronal neurophysin was separated. Differential binding of separated neurophysin with ADH or oxytocin has been suggested (Burford, Jones & Pickering, 1971), but not confirmed (Watkins, 1972; Watkins & Evans, 1972; Ellis et al, 1972). Oxytocin is released from neurosecretory granules in vitro more readily than is ADH: this is consistent with storage in separate granules (Barer et al, 1963).

Rats of the Brattleboro' strain, with familial hypothalamic diabetes insipidus (Valtin & Schroeder, 1964), are unable to synthesise ADH (Valtin et al, 1965) and the putative ADH neurophysin (Burford et al, 1971). When osmotic stress is removed, by treatment with exogenous ADH, neither ADH (Valtin et al, 1965) nor putative ADH neurophysin (Burford et al, 1971) accumulates in the posterior pituitaries of these animals. Brattleboro' rats synthesise oxytocin (Valtin et al, 1965) and neurophysins other than putative ADH neurophysin (Burford et al, 1971). These studies indicate that oxytocin and its neurophysin may be synthesised

separately from ADH and its neurophysin, but not necessarily in separate neurones.

If Brattleboro' rats are treated with exogenous ADH, discrete clusters of neurosecretory material accumulate in the posterior pituitary. This has been interpreted as evidence for segregation of ADH from oxytocin secreting endings, of separate neurones, in the posterior pituitary (Orkand & Palay, 1967; Sokol & Valtin, 1967). Nerve endings, or neurosecretosomes, isolated from normal posterior pituitaries, can be partially separated into fractions containing mainly oxytocin or ADH (Bindler et al, 1967). However, segregated endings containing ADH or oxytocin need not arise from separate neurones.

2.2 Synthesis and secretion of oxytocin or ADH by SO or PV neurones

The simplest hypothesis, that ADH or oxytocin, but not both, are synthesised in either PV or SO neurones, but not both, has been tested. The criteria defined for individual neurones apply to groups of neurones in the PV and SO nuclei on this hypothesis. An additional criterion can be defined with respect to separate secretory function of PV and SO neurones; lesions of either nucleus should abolish secretion of the hormone it is supposed to produce.

2.2.1 Distribution of ADH and oxytocin in the hypothalamus

The ultrastructural features of PV and SO neurosecretory neurones are similar (Flament-Durand, 1971; Kalimo, 1971), and the size spectra of neurosecretory granules in PV and SO neuronal perikarya are similar (Kalimo, 1971), but

neurosecretory granules in PV perikarya are more electron dense than those in SO perikarya (Cannata & Morris, 1973).

In many mammals the ratio of the content of ADH to that of oxytocin, or V/O ratio, is greater in the SO nucleus than in the PV nucleus; this ratio may reflect the rates of ADH and oxytocin synthesis in each nucleus (Lederis, 1962; Van Dyke et al, 1957). Because the V/O ratio approaches unity in the posterior pituitary, but is greater than unity in the hypothalamus (Lederis, 1962), the difference in V/O ratio between the SO and PV nuclei may simply reflect different stages of maturation of the neurosecretory granules within SO compared with PV perikarya (Cannata & Morris, 1973).

In frozen sections of pig hypothalamus, antibody to porcine putative ADH neurophysin reacts with more neurosecretory neurones in the SO nucleus than in the PV nucleus (Livett et al, 1971), but neurosecretory granules, identified with Gomori's stain, are present in the perikarya of SO neurones of Brattleboro' rats, which cannot synthesise ADH or its putative neurophysin (Valtinet al, 1965; Sokol & Valtin, 1965; Burford et al, 1971).

Certainly, because both ADH and oxytocin are present in both the SO and PV nuclei, separation of secretory function between these two nuclei is not absolute.

2.2.2 Synthesis of hormones by PV or SO neurones

Incorporation of ^{35}S-cysteine into putative oxytocin neurophysin is greater when the label is injected into the PV nuclei than when injected into the SO nuclei (Burford et al, 1972).

There are no other quantitative data which compare synthesis parameters in the two nuclei under normal conditions.

2.2.3 Electrical stimulation of PV or SO neurones

Only direct stimulation of the neurones in the PV or SO nuclei is a valid test of this criterion. The separate release of ADH or oxytocin with qualitatively different stimuli, arising outwith the PV and SO nuclei, is a matter of interneuronal circuitry. If neurosecretory granules containing oxytocin or ADH are synthesised in any one neurone, the granules could be segregated among the axonal branches of the neurone; separate release of ADH and oxytocin could result from selective preterminal inhibition. There are axo-axonic contacts in the SO nucleus (Rechardt, 1969).

Only studies in which the SO and PV nuclei have both been stimulated will be discussed.

In the rabbit, antidiuresis results from SO, but not PV, nucleus stimulation (Harris, 1947). Electrical stimulation of either the PV or SO nucleus causes milk ejection and antidiuresis in lactatinggoats or rabbits (Anderssen & McCann, 1955; Cross, 1958). Alternative combinations have been reported: in the lactating rabbit, electrical stimulation of the SO nucleus releases ADH and oxytocin, but stimulation of the PV nucleus releases only oxytocin (Aulesbrook & Holland, 1969a). In the cat ADH, but not oxytocin, is secreted when the SO nucleus is stimulated; ADH and a small amount of oxytocin are secreted in response

to stimulation of Greving's tract from the PV nucleus, but no oxytocin and little ADH are secreted when the PV nucleus is directly stimulated (Bisset et al, 1967). Alternatively, stimulation of the PV nucleus, but not the SO nucleus, in the lactating cat causes milk ejection: in general secretion of ADH or oxytocin excludes release of the other hormone (Bisset et al, 1970).

These variable results may be explained because the authors used different assay procedures, depths of anaesthesia, and patterns of stimulation.

The results of stimulating the SO nucleus must be interpreted with caution, as most of the axons of the PV neurones pass through or close to the SO nucleus (Laqueur, 1954; Bisset et al, 1971; Flament-Durand & Dustin, 1972). In view of this arrangement it is perhaps surprising that stimulation of the SO nucleus does not always release both ADH and oxytocin.

Neurophysin is secreted after either the PV or SO nucleus is stimulated, but the neurophysin has not been identified as oxytocin or ADH binding (Cheng et al, 1972).

2.2.4 Lesions of PV or SO nuclei

Oliverona (1957) placed bilateral electrolytic lesions in the PV or SO nuclei of rats and then measured the amounts of ADH and oxytocin in the posterior pituitary. The amount of ADH was not altered after lesions of either the PV or the SO nuclei, but oxytocin was absent after PV but not SO nucleus lesions. None of the animals developed diabetes insipidus, but lactation was not tested. The PV nucleus

lesions were large and may have damaged axons of the supraoptico-hypophyseal tract. Although the lesions did not extend into the SO nuclei, the number of SO neurones decreased by 20% after PV nucleus lesions.

The conclusion based on these findings, that oxytocin is only synthesised by PV neurones, can no longer be accepted; in the rat complete destruction of the PV nuclei only prevents lactation if lesions are placed in the fornix (Averill & Purves, 1963). Complete deafferentation of the PV nuclei in the rat with the Halasz knife does not prevent milk ejection in response to suckling, which can be prevented by caudal hypothalamic deafferentation (Voloschin & Tramezzani, 1973). Since milk ejection is effected specifically by oxytocin (Wakerley et al, 1973), these studies imply that the SO nucleus synthesises oxytocin. The reduced amounts of oxytocin in the posterior pituitary after PV nucleus lesions (Olivecrona, 1957; Nibbelink, 1961) may reflect increased turnover of a reduced pool of oxytocin, synthesised by neurones of the SO nucleus which has a limited capacity for oxytocin synthesis.

Twenty-four hours after intracisternal injection of ^{35}S-cysteine the ratio of labelled putative ADH neurophysin to putative oxytocin neurophysin in the posterior pituitary is normally 1·5. This ratio is increased to 2·2 in animals with bilateral lesions of the PV nuclei, and decreased to 0·98 in animals with bilateral SO lesions. Synthesis of oxytocin- and ADH-neurophysin by both PV and SO nuclei is implied; but the PV nuclei synthesise more oxytocin-

neurophysin than ADH-neurophysin, and vice versa for the SO nuclei (Dyball et al, 1973). Because Brattleboro' rats cannot synthesise ADH or its putative neurophysin, posterior pituitary neurosecretory material in these animals probably represents oxytocin-neurophysin (Burford et al, 1971). One to three months after bilateral destruction of the PV nuclei in two of these animals, neurosecretory material is still present in the posterior pituitary (Sokol, 1970). If this neurosecretory material represents stored oxytocin, then oxytocin synthesis by the SO nucleus is implied.

2.2.5 Summary

Direct evidence that ADH and oxytocin are synthesised in separate neurones is incomplete. There is no evidence that they are synthesised in the same neurones. ADH and oxytocin are both synthesised by both SO and PV neurones, although the SO nucleus may synthesise more ADH, and the PV nucleus more oxytocin.

For the purpose of subsequent discussion, the traditional assumption will be made that ADH and oxytocin, like other neurohumours (Dale, 1935), are synthesised and released by separate neurones, which may have their cell bodies in the SO or PV nuclei or both.

2.3 Separate secretion of oxytocin and ADH

A question distinct from that of the synthesis of ADH and oxytocin in separate neurones is that of the selective release of ADH and oxytocin by different stimuli.

A particular difficulty in this area is the interpretation of bioassay data at the lower limits of

sensitivity (Denamur, 1965; Chard, 1971). The secretory responses to several stimuli will be discussed in the context of separate release of ADH and oxytocin.

2.3.1 Suckling

The secretion of oxytocin in response to suckling has been discussed (Section I - 2.4.3). In the water-loaded rabbit, suckling causes milk ejection and antidiuresis. The magnitude of antidiuresis is not related to the number of young suckling, to the duration of suckling, or to the milk yield, but does not occur if milk withdrawal is prevented (Cross, 1951). Antidiuresis occurs after suckling in the lactating dog (Pickford, 1960). But ADH cannot be detected in jugular venous blood of lactating rabbits suckled for 85 minutes unless the concentration of oxytocin in blood, and the milk yield, are low (Bisset et al, 1970). If ADH is released in response to suckling, it is not secreted in amounts sufficient to cause milk ejection (Wakerley et al, 1973). ADH may be released during lactation in response to osmotic and volemic stimuli resulting from milk secretion (Section IV - 4.1.2) rather than as a direct result of the suckling stimulus (Cross, 1951).

2.3.2 Hyperosmotic stimulation

Water deprivation decreases the amounts of ADH, oxytocin and neurosecretory material in the posterior pituitary (Ortmann, 1951; Dicker & Nunn, 1957; Jones & Pickering, 1969; Moses & Miller, 1970), but changes in gland content do not distinguish between altered secretion and synthesis. However, increased ADH secretion during

water deprivation is confirmed by other evidence, including a five-fold increase in the concentration of ADH in blood within 48 to 72 hours of water deprivation (Little & Radford, 1964b). Acute hyperosmotic stimulation via the internal carotid artery releases both ADH and oxytocin in the dog (Abrahams & Pickford, 1954), anaesthetised rats (Dyball, 1971) and lactating rabbits, with spinal cord transections to reduce inhibition of the peripheral actions of oxytocin (Holland et al, 1959).

When Brattleboro' rats are treated with ADH, the amounts of oxytocin and neurosecretory material in the posterior pituitary increase; this indicates that oxytocin is normally secreted by these animals in response to chronic hyperosmotic stimulation (Valtin & Schroeder, 1964; Valtin et al, 1965).

2.3.3 Hypovolemic stimulation

A fall in blood volume is a potent stimulus to ADH release (Share, 1969). In anaesthetised cats, haemorrhage increases the concentration in peripheral blood of ADH but not oxytocin (Beleslin et al, 1967). In anaesthetised rats, a haemorrhage of 40% of the blood volume results in increased concentration in peripheral blood of ADH and oxytocin (Dyball, 1968; Fabian et al, 1969) or ADH only if the blood is reduced to zero (Forsling et al, 1973).

The percentage increase in the concentration of oxytocin in blood in response to osmotic stimulation is relatively greater than the increase in ADH concentration (Dyball, 1971), but in haemorrhage the reverse is found (Dyball, 1968;

Fabian et al, 1969). Haemorrhage stimulates oxytocin secretion in Brattleboro' rats (Forsling et al, 1973).

Variability in the results of studies on the secretion of ADH and oxytocin in similar circumstances may arise from variability in the assay systems used.

The trend of the evidence is that preferential, but not absolutely selective, release of ADH or oxytocin occurs in response to qualitatively different stimuli. Dicker's statement (1961) that 'one thing is certain, any stimulus that releases one hormone releases the other simultaneously' can no longer be accepted.

2.3.4 Interaction of stimuli

It is difficult to apply only a single stimulus to the magnocellular neurosecretory system.

Osmotic and hypovolemic stimuli which may result from milk secretion have been discussed (Section IV - 4.1.2). If lactating animals are deprived of drinking water, and if milk secretion continues, serum osmolality can be expected to increase, and blood volume to decrease more than if non-lactating animals are deprived of drinking water. The effects of interaction of the suckling stimulus with hypovolemic or hyperosmotic stimuli upon ADH or oxytocin secretion have not been systematically investigated.

The rise in concentration in venous blood of ADH and neurophysin after haemorrhage in anaesthetised rats is smaller if the rats are previously dehydrated or given a water load. Prior to haemorrhage the amount of ADH in the posterior pituitary is only 10% of normal in dehydrated rats,

but is greater than normal in the water loaded rats. In the former the smaller response to haemorrhage can be attributed to lack of available ADH; in the latter, the threshold to hypovolemia is raised because plasma osmolality is low (Forsling et al, 1971). Conversely in the conscious dog, the rise in concentration of ADH in blood in response to an osmotic stimulus is reduced if blood volume is kept constant (Sczepanska-Sadowska, 1972a). In the human, the osmotic threshold for ADH release is reduced by previous dehydration; this effect can be attributed to an interaction of hyperosmotic and hypovolemic stimuli (Moses & Miller, 1971).

These studies demonstrate summation of stimuli from volume and osmoreceptors in a similar manner to the interaction between these two stimuli in mechanisms regulating thirst (Stricker, 1969).

2.4 Electrical activation of individual neurones by different stimuli

To interpret the responses to different stimuli of an anatomically defined pool of neurones, such as the PV or SO nucleus, it is of advantage to know (a) whether the same neurohormone is secreted by all neurones, (b) whether the neural input to each neurone is similar. These are measures of homogeneity within the pool.

The heterogeneous nature of the PV and SO neuronal pools with respect to oxytocin and ADH synthesis and secretion and with respect to the actions of transmitters have been discussed (this section - 2.1, 2.2; Section I - 2.4.2). The evidence that ADH and oxytocin are released

selectively in response to qualitatively different stimuli is most easily interpreted if neural input is selectively distributed, either between the PV and SO nuclei, or between populations of neurones within each nucleus.

Osmoresponsive neurones may or may not be identical with central osmoreceptors concerned with neurohormone release, which are situated in or near to the PV and SO nuclei (Section I - 2.4.1). In contrast, volume receptors are situated peripherally in the distribution of the vagi (Dyball, 1968; Saito et al, 1969; Sczepanska-Sadowska, 1972b), probably in the left atrium (Ledsome & Linden, 1968).

In the cat, 75% of osmosensitive SO neurones increase their discharge rate when a limb afferent nerve is stimulated electrically (Koizumi et al, 1964). In lactating cats, 58% of neurones in the PV nucleus are osmosensitive, most of which are not sensitive to peripheral nerve stimulation, but 83% of these osmosensitive neurones are sensitive to nipple suction or uterine distension (Brooks et al, 1966). Unfortunately these neurones were not antidromically identified as neurosecretory. In the rat, 70% of the fast firing antidromically identified SO neurones and 20% of similar neurones in the PV nucleus increase their firing rates in response to acute hyperosmotic stimulation, but the PV neuronal responses are nonspecific, and can be produced by isotonic saline. The extent of change in firing rate in response to acute osmotic stimulation is greater in SO than in PV neurones (Dyball, 1971). Although acute hyperosmotic stimulation produces specific responses only in SO neurones, after rats have drunk 2% sodium chloride solution instead of water for

three days the proportion of fast firing antidromically identified units is increased in both SO and PV nuclei (Dyball & Pountney, 1973). However, chronic salt loading may be expected to have effects other than on serum osmolality; these changes in electrical activity in PV and SO neurones cannot be specifically related to chronic hyperosmotic stimulation.

About 80% of antidromically identified SO neurones in the cat respond to either carotid sinus nerve or vagus nerve stimulation, if intermittent stimulation is used to avoid hypotensive effects. Seventy per cent of these units respond both to carotid sinus nerve stimulation and to vagus nerve stimulation; convergence is indicated. Only 3% of antidromically identified PV neurones respond to stimulation of either nerve (Barker et al, 1971a,c). Hypothalamic units in the cat respond to both acute hyperosmotic stimulation and to left atrial stretch, but these units were not antidromically identified as neurosecretory (Menninger & Frazier, 1972).

In the lactating rat nursing 10 pups, 58% of antidromically identified PV neurones increase their firing rate by 340 to 11,000% immediately before each milk ejection in response to suckling (Wakerley & Lincoln, 1973). Activity in SO neurones has not been reported.

Separate central pathways for selective ADH or oxytocin release have been described. In lactating rabbits, oxytocin is released without ADH by electrical stimulation of the periventricular grey matter and part of the limbic system,

whilst oxytocin and ADH are secreted together when other hypothalamic areas are stimulated (Aulesbrook & Holland, 1969a). Areas have been located in the lateral tegmental reticular formation which, when stimulated electrically, inhibit oxytocin secretion but excite ADH secretion (Aulesbrook & Holland, 1969b). A similar reciprocal relationship between oxytocin and ADH secretion during suckling has been described (Bisset et al, 1970). A localised pathway in the spino-thalamic system specific for oxytocin release has been described in the guinea pig (Tindal & Knaggs, 1970), but a more diffuse pathway in the lemniscal and reticular systems, specific for oxytocin release, has been found in the rabbit (Urban et al, 1971).

2.5 Summary

The afferent connections of SO and PV neurosecretory neurones have not been fully characterised. There are osmosensitive neurones in the SO and PV nuclei, but not all antidromically identified neurosecretory neurones are osmosensitive. Two or three different stimuli may converge on individual neurones, but neurones responsive to stimulation of nerves from blood volume or vascular stretch receptors are rare in the PV nucleus, but form a large proportion of SO neurones. About 60% of antidromically identified PV neurones respond to suckling in an explosive manner immediately before each milk ejection. Responses of SO neurones during suckling have not been reported.

The evidence relating to segregation of function between the SO and PV nuclei may be summarised together with the

evidence for the separate release of ADH and oxytocin. The PV nucleus is heterogeneous, contains neurones which secrete ADH and neurones which secrete oxytocin, but more of the latter; it contains most of the effector neurones of the milk ejection reflex, but also contains osmosensitive neurones, and very few neurones sensitive to blood volume changes. The SO nucleus contains neurones which secrete ADH and neurones which secrete oxytocin, but more of the former; it contains a large proportion of osmosensitive neurones, most of which also receive afferents from blood volume receptors. Osmoreceptive and volume receptive inputs summate. The SO nuclei can sustain the milk ejection reflex in the absence of the PV nuclei.

It remains possible that the heterogeneous responses of the PV and SO neurones, determined electrophysiologically during recording periods of only several minutes, may reflect temporary modulation of an underlying homogeneous population.

3. Aims of investigation: Hypotheses

The nucleolar responses of PV and SO neurones to graded intensities of the suckling stimulus have been explained in terms of a relationship to secretory activity (Section IV - 4.3). It was mentioned in that discussion that the secretory activity may have been limited by synthetic capacity. The failure of the nucleoli of PV and SO neurones to show a sustained increase in dry mass when the intensity of the suckling stimulus was doubled may have been due to:

1. dependence on secretory activity, which did not increase pari passu with the stimulus;

or

2. dependence of secretory activity on limited nucleolar synthetic capacity, due to -
   a. inability of nucleolar synthetic activity of all the neurones, all of which responded, to further increase;

   or

   b. inability of the nucleoli of a population of neurones within the SO and PV nuclei to further increase their synthetic activity, while the remaining neurones did not respond at all.

The first possibility has been discussed (Section IV - 4.3), but the responding population may be either all the neurones in each nucleus, or only some of the neurones in each nucleus.

To test these possibilities the suckling stimulus and a qualitatively different stimulus, or stimuli, resulting from water deprivation were applied alone and simultaneously.

If possibility 2a. is correct, convergence of these two stimuli should not alter the nucleolar responses, if the whole population responds to either stimulus; but if the nucleoli have not reached maximum synthetic capacity with either stimulus alone, the nucleolar responses may be greater when the stimuli are applied together.

If possibility 2b. is correct, then each population should respond when both stimuli are applied together, but each population should respond no more than when either stimulus is applied alone, regardless of the intensity of each stimulus.

As a further test of the three possibilities the dehydration stimulus was intensified by simultaneously administering a diuretic, frusemide.

It was anticipated that responses of separate populations of neurones responding to either suckling or dehydration might be distinguished in histograms of nucleolar dry mass. It can be concluded from the introduction that study of nucleolar changes in PV and SO neurones in response to different stimuli yields information about afferent input to these neurones and not information about whether these neurones secrete ADH or oxytocin.

PV and SO neuronal nucleolar dry mass are maximal between days 5 and 10 post partum in the first three weeks of lactation, and are independent of the preceding pregnancy after day 5 post partum (Section III - 3.2, 3.3). If the suckling stimulus is removed nucleolar changes in PV and SO neurones take seven days to subside. The dehydration period

was therefore started on day 5 and lasted for three days. It was reasoned that if suckling ceased to stimulate SO and PV neurones during this period, then the suckling dependent nucleolar changes would be maintained for this period, and could sum with changes dependent on dehydration.

There is no previous report of the effects of combined lactation and water deprivation on PV or SO neuronal activity.

4. Methods

These experiments were performed on two separate series of animals, several months apart. The numbers of animals in each group and in each series are shown in Table 24.

4.1 Management

4.1.1 General

These animals were housed as previously described (Section II - 1).

4.1.2 Lactation alone

These animals were managed in the same fashion as those in the study on lactating animals nursing their own litters (Section III - 2.1). These animals were killed on day 8 post partum. Total daily milk yield was measured as previously described (Section IV - 2.3.1).

4.1.3 Dehydration alone

Three month old virgin female rats were used. The water bottle was removed from each cage of six animals 72 hours before the animals were to be killed. These animals and their controls, which had water ad libitum, were weighed daily.

4.1.4 Dehydration and lactation together

Lactating females were managed as above (4.1.2) for the first five days post partum, then the water bottle was removed from each cage. The lactating females and their pups were weighed daily. The pups were inspected twice daily for the presence of milk in their stomachs. These animals were killed on the 8th day post partum. The mammary

TABLE 24 DEHYDRATION AND LACTATION: NUMBER OF ANIMALS IN EACH GROUP; LITTER SIZE

a. First Series

Experimental Group		Lactation	Dehydration	Lactation + Dehydration	Virgin control
Number of animals		14	6	11	6
Litter Size	Mean ± S.E.	11.1 ± 0.83		11.1 ± 0.81	
	Range	6-15		7-15	

b. Second Series

Experimental Group		Lactation + Dehydration	Dehydration	Dehydration + Frusemide	Dehydration + Water injection	Virgin control
Number of animals		6	6	6	6	7
Litter size	Mean ± S.E.	10.3 ± 0.80				
	Range	7-13				

glands were incised post mortem and examined for the presence of milk. Total daily milk yield was measured as previously described (Section IV - 2.3.1).

4.1.5 Dehydration and frusemide

Frusemide was used to increase the rate of water lost by animals deprived of drinking water and so to increase the intensity of associated hyperosmotic and hypovolemic stimuli. Seven virgin animals deprived of drinking water as above were each injected intramuscularly once daily with 20 mg of frusemide in 0.5 ml of water warmed to body temperature. The first injection was given at the time the water bottle was removed. Six controls, also deprived of drinking water for the same period, were injected once daily with 0.5 ml of water.

4.2 Measurement of nucleolar dry mass and nucleic acid content

Nucleolar dry mass of PV and SO neurones was measured in all these animals as previously described (Section II -3). Right and left PV or SO nuclei were pooled. In the second series nucleolar nucleic acid content of SO neurones of some groups was measured as previously described (Section II - 4).

4.3 Other parameters

Serum osmolality was measured in all animals as previously described (Section IV - 2.2.2). A rough estimate was made of blood volume at the time of death by comparing volumes of blood obtained by bleeding from cardiac puncture.

5. Results

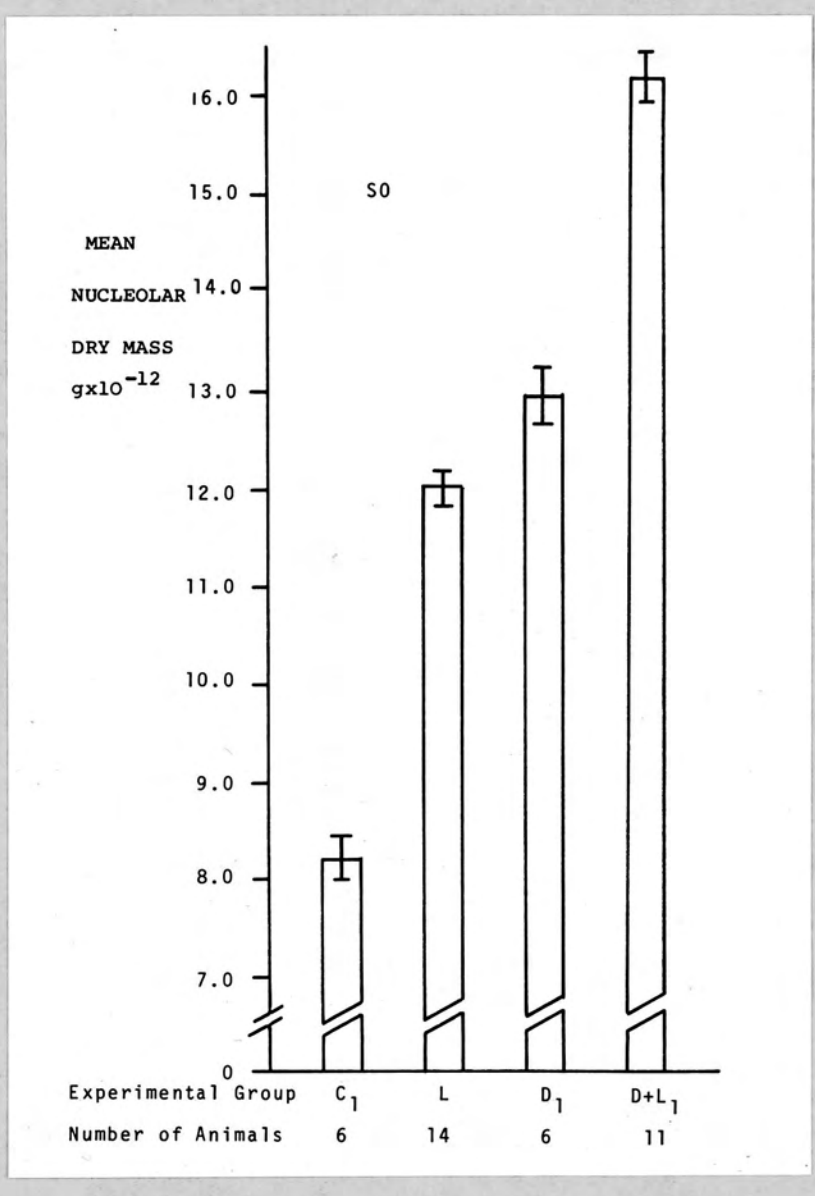


Figure 47 Dehydration and lactation: mean nucleolar dry mass of SO neurones. First series.
C_1: Virgin control group. L: Lactation alone
D_1: Dehydration alone. $D + L_1$: Dehydration during lactation.
Each vertical line indicates the standard error of the mean.

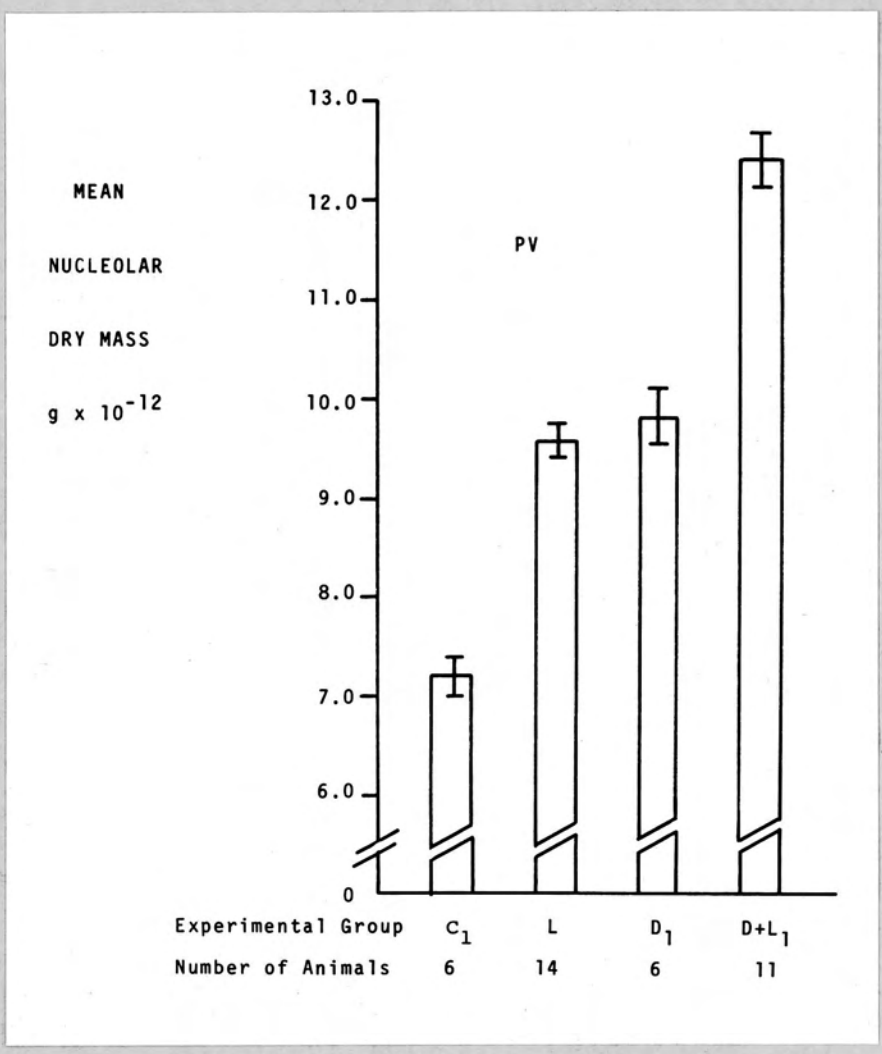


Figure 48 Dehydration and lactation: mean nucleolar dry mass of PV neurones. First series. Details as in Fig. 47.

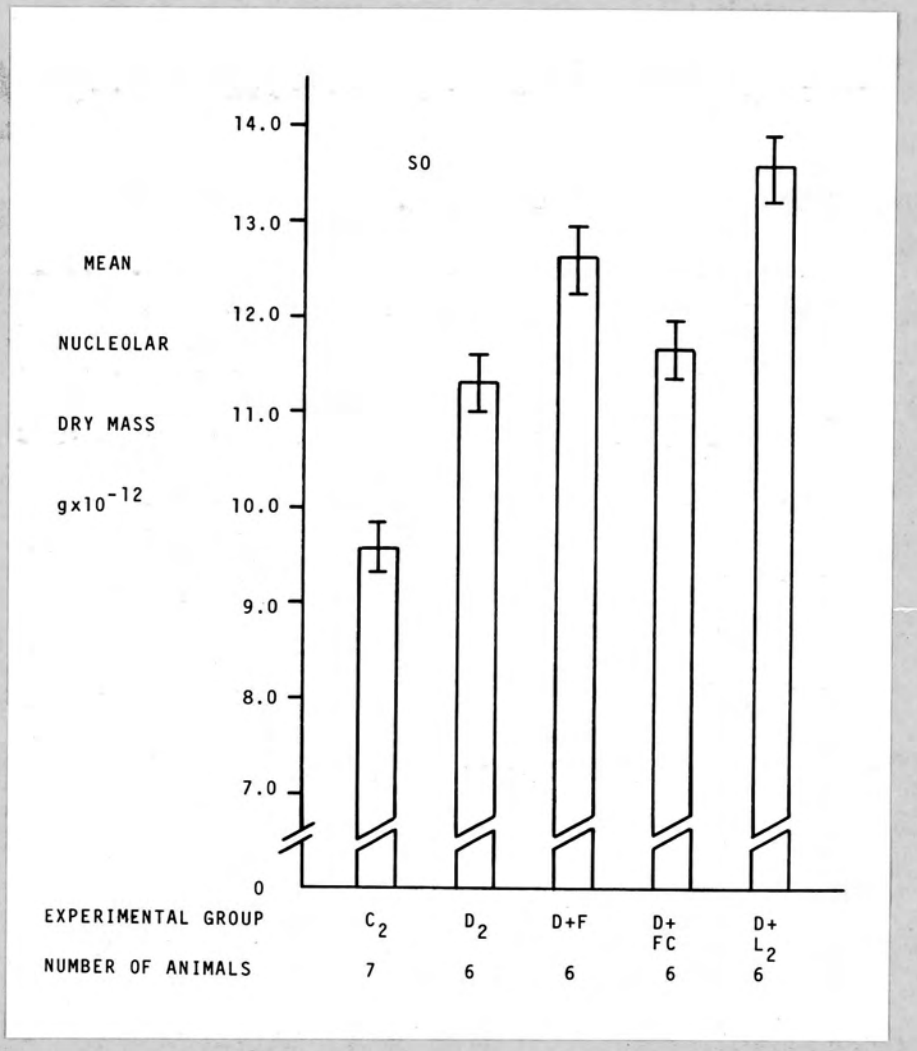


Figure 49 Dehydration, lactation and frusemide: mean nucleolar dry mass of SO neurones. Second series.
C_2: Virgin control group. D_2: Dehydration alone.
D + F: Dehydration and frusemide injections.
D + FC: Dehydration and water injections; control group for D + F.
D + L_2: Dehydration during lactation.
Each vertical line indicates the standard error of the mean.

TABLE 25

MEAN NEURONAL NUCLEOLAR DRY MASS: LACTATION, DEHYDRATION. COMPARISONS BETWEEN GROUPS

SO neurones a. First Series

Experimental Group	Lactation	Lactation + Dehydration	Dehydration
Untreated controls	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***
Dehydration	$0.01 > p > 0.005$ **	$p < 0.001$***	
Dehydration + Lactation	$p < 0.001$***		

b. Second Series

Experimental Group	Lactation + Dehydration	Dehydration	Dehydration + Frusemide	Dehydration + Water
Untreated controls	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***
Dehydration + Water	$p < 0.001$***	$0.5 > p > 0.4$	$0.05 > p > 0.025$*	
Dehydration + Frusemide	$0.1 > p > 0.05$	$0.005 > p > 0.001$**		
Dehydration	$p < 0.001$***			

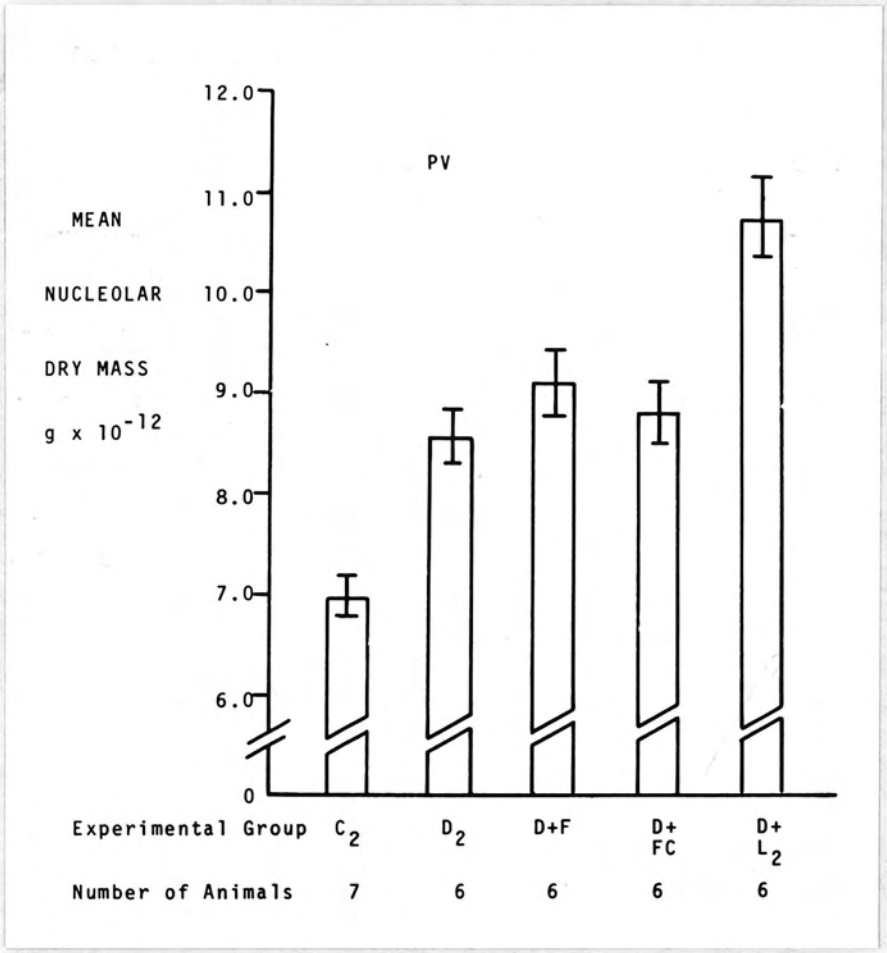


Figure 50 Lactation, dehydration and frusemide: mean nucleolar dry mass of PV neurones. Second series. Details as in Fig. 49.

TABLE 26

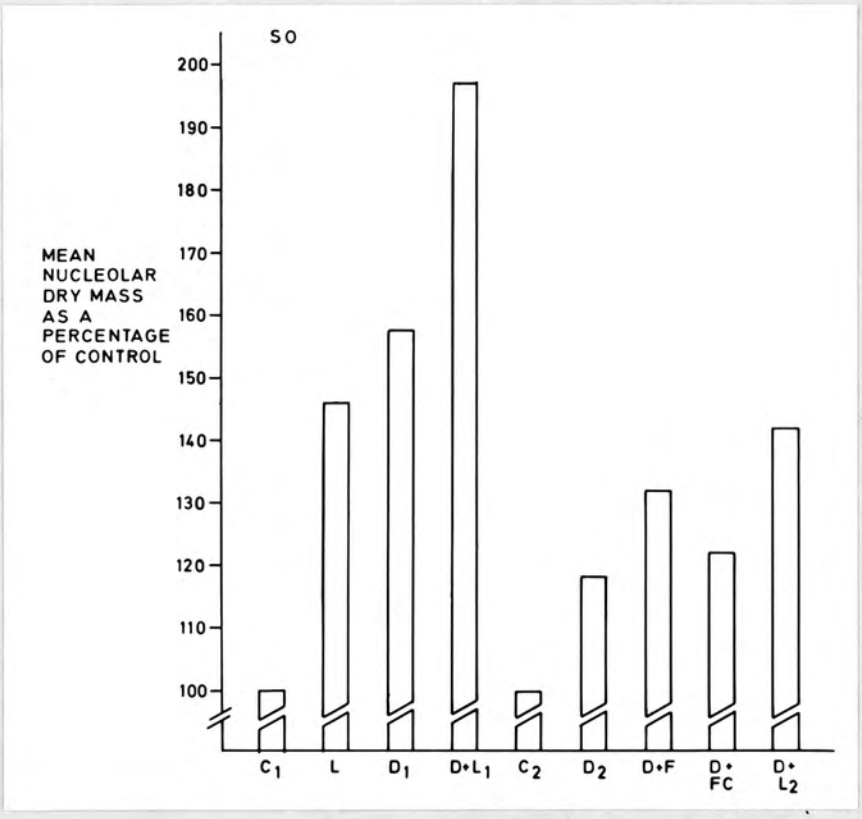
MEAN NEURONAL NUCLEOLAR DRY MASS: LACTATION, DEHYDRATION. COMPARISONS BETWEEN GROUPS

PV neurones a. First Series

Experimental Group	Lactation	Lactation + Dehydration	Dehydration
Untreated controls	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***
Dehydration	$0.6 > p > 0.5$	$p < 0.001$***	
Dehydration + Lactation	$p < 0.001$***		

b. Second Series

Experimental Group	Lactation + Dehydration	Dehydration	Dehydration + Frusemide	Dehydration + Water
Untreated controls	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***
Dehydration + water	$p < 0.001$***	$0.6 > p > 0.5$	$0.6 > p > 0.5$	
Dehydration + Frusemide	$0.005 > p > 0.001$**	$0.3 > p > 0.2$		
Dehydration	$p < 0.001$***			

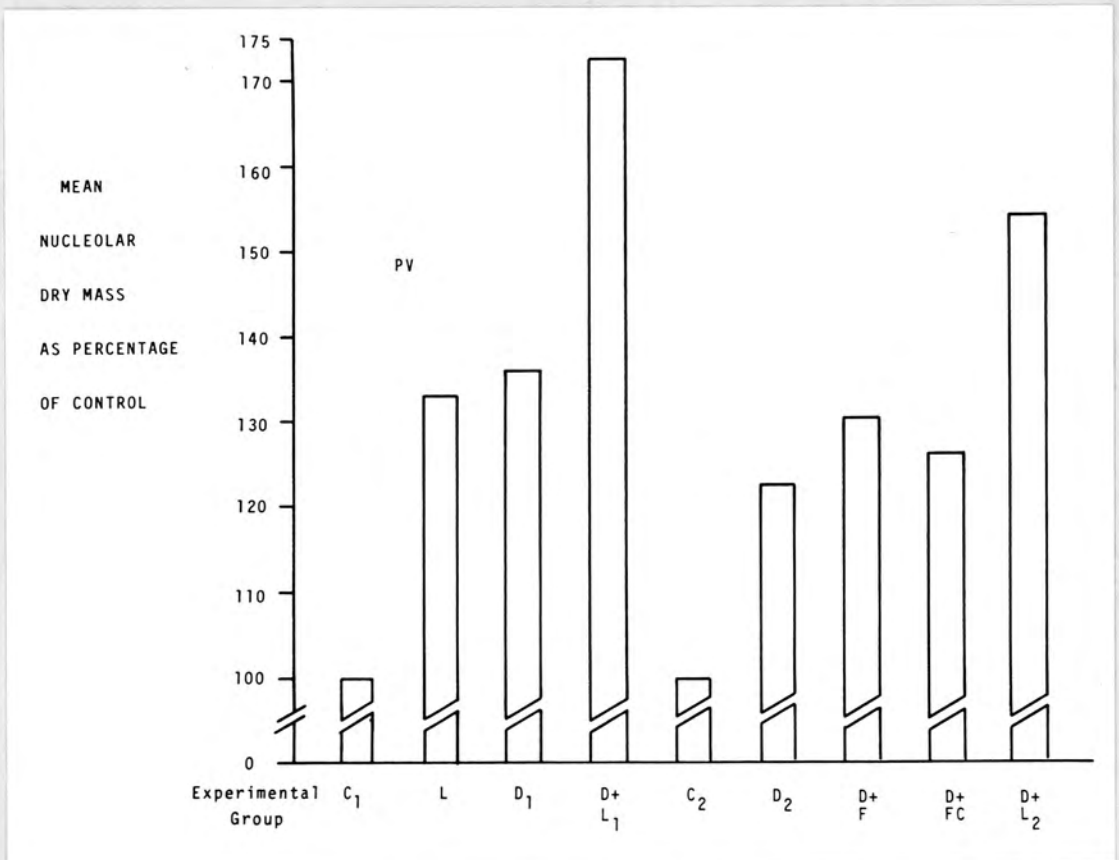


a

Figure 51 Lactation, dehydration and frusemide. Mean nucleolar dry mass expressed as percentage of respective virgin controls. C_1: Virgin controls, first series. L: Lactation alone. D_1: Dehydration alone, first series. D + L_1: Dehydration during lactation, first series. C_2: Virgin controls, second series. D_2: Dehydration alone, second series. D + F: Dehydration and frusemide. D + FC: Dehydration and water injections, controls for D + F. D + L_2: Dehydration during lactation, second series.

a. SO neurones. b. PV neurones.

b



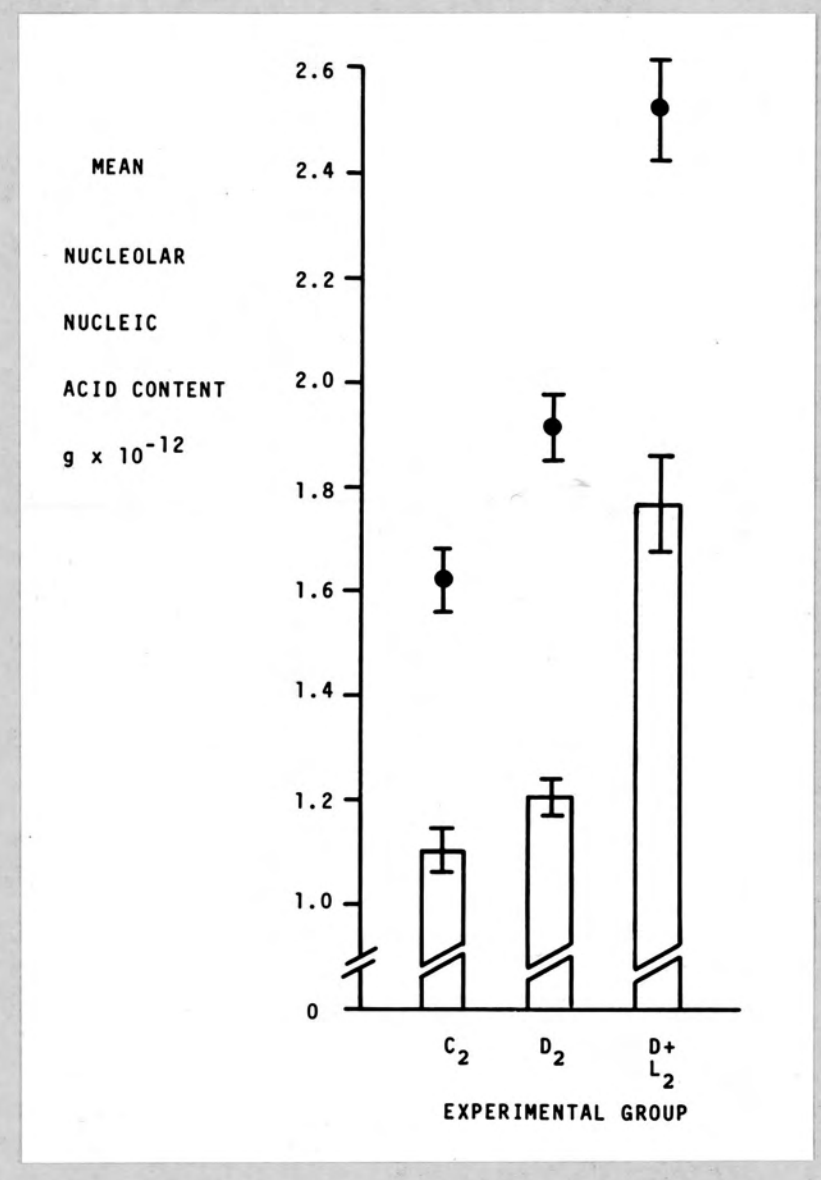
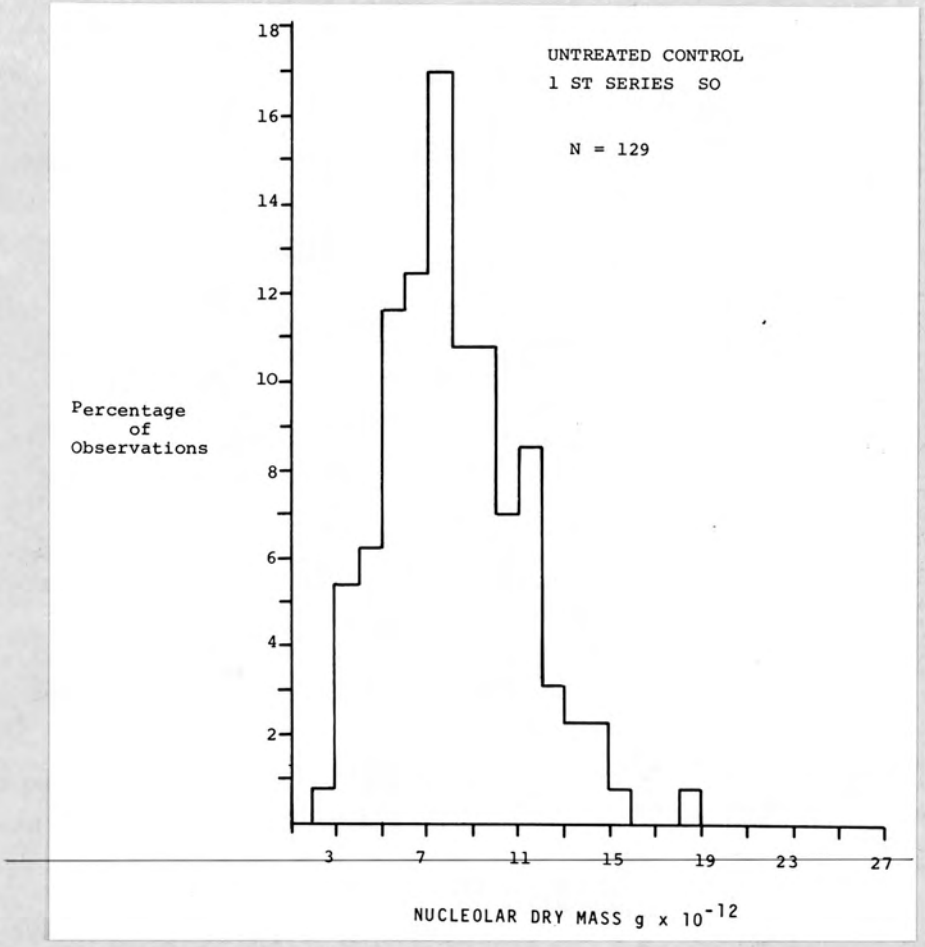


Figure 52 Dehydration and lactation. Mean nucleolar nucleic acid content of 80 neurones. Second series.
C_2: Virgin control group. D_2: Dehydration alone.
D + L_2: Dehydration during lactation.
Vertical bars: mean nucleolar nucleic acid content, corrected for scatter.
Solid circles: mean nucleolar nucleic acid content, uncorrected for scatter.
Each vertical line indicates the standard error of the mean.

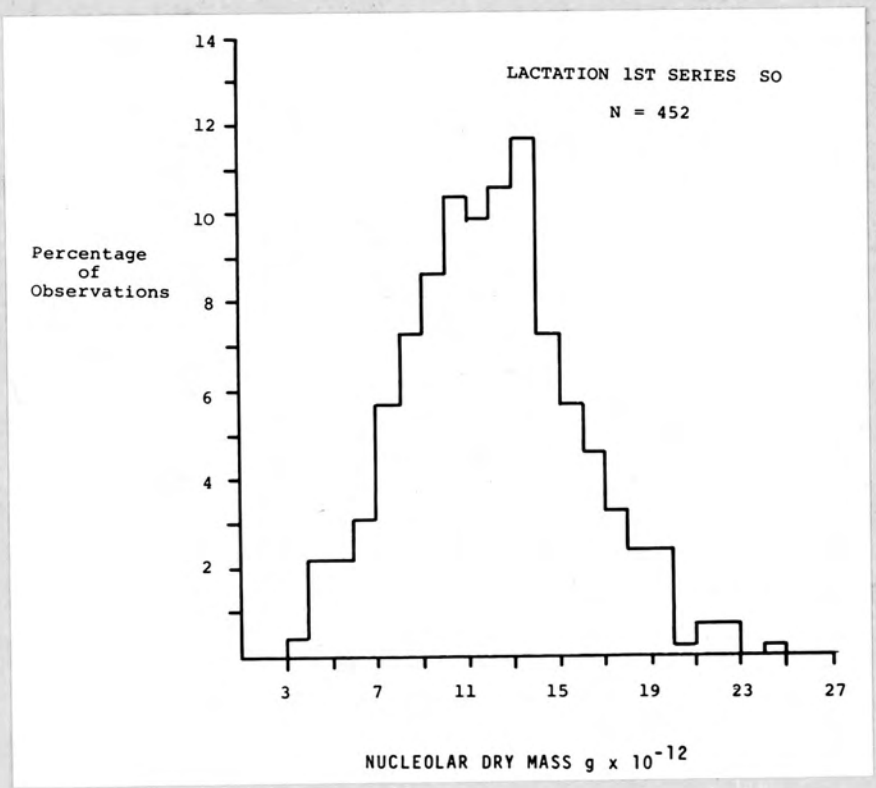


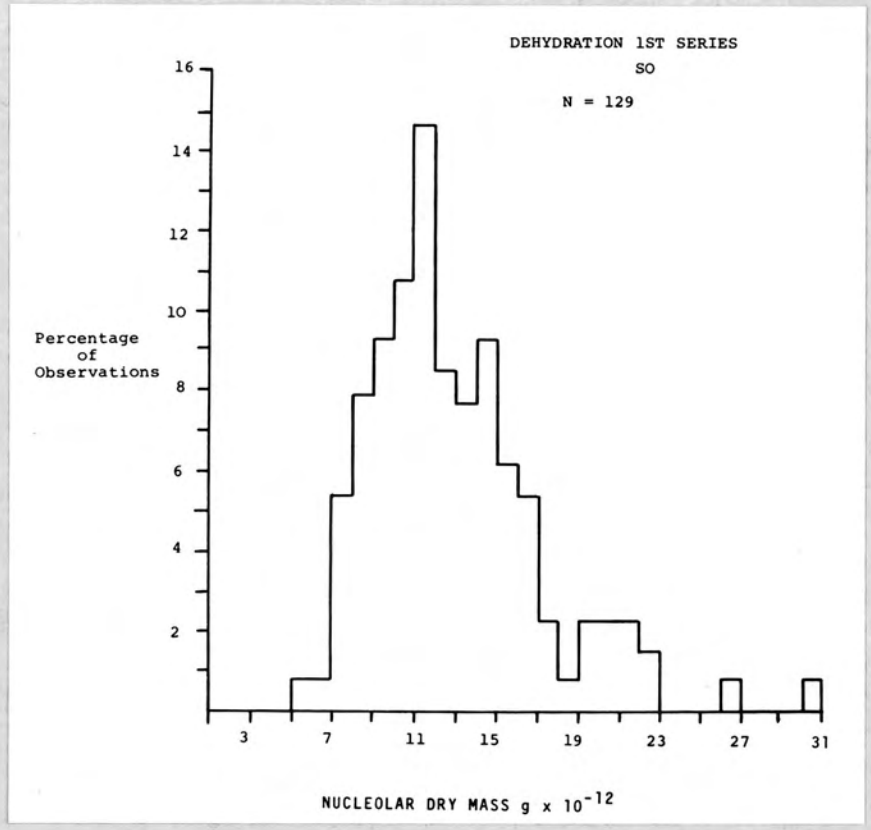
a

Figure 53 Frequency distribution of individual nucleolar dry mass measurements. Lactation, dehydration. N = number of dry mass measurements. SO neurones first series.

a. untreated virgin controls. b. lactation, day 8 post partum.

b





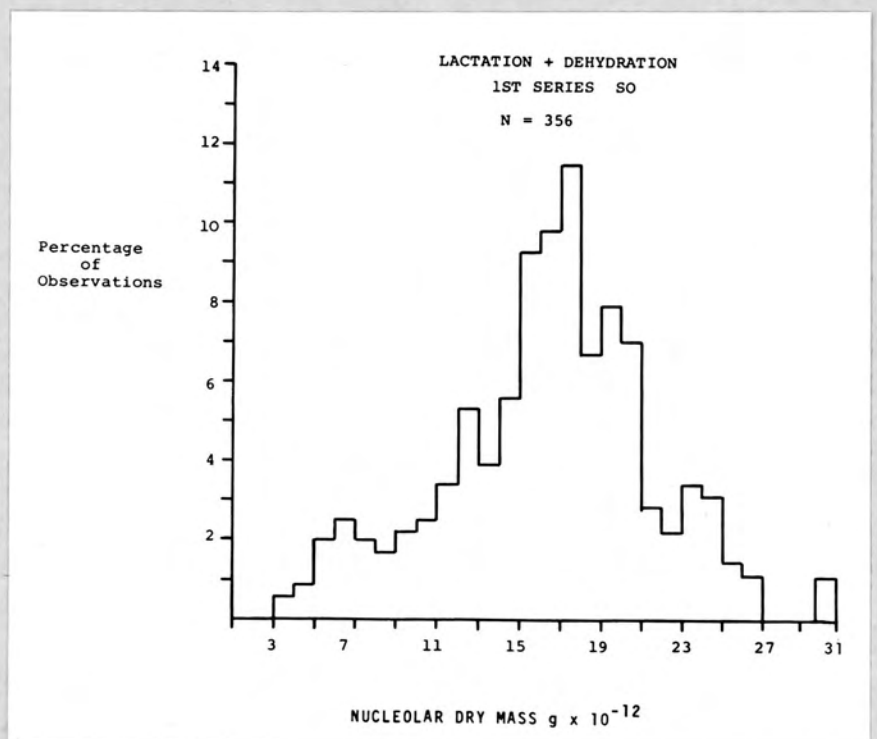
a

Figure 54 Frequency distribution of individual nucleolar dry mass measurements. Lactation, dehydration. N = number of dry mass measurements. SO neurones first series.

a. Dehydration alone for 3 days.

b. Dehydration from day 5 to 8 post partum. There is a group of small nucleoli which may represent a population separate from the remainder.

b



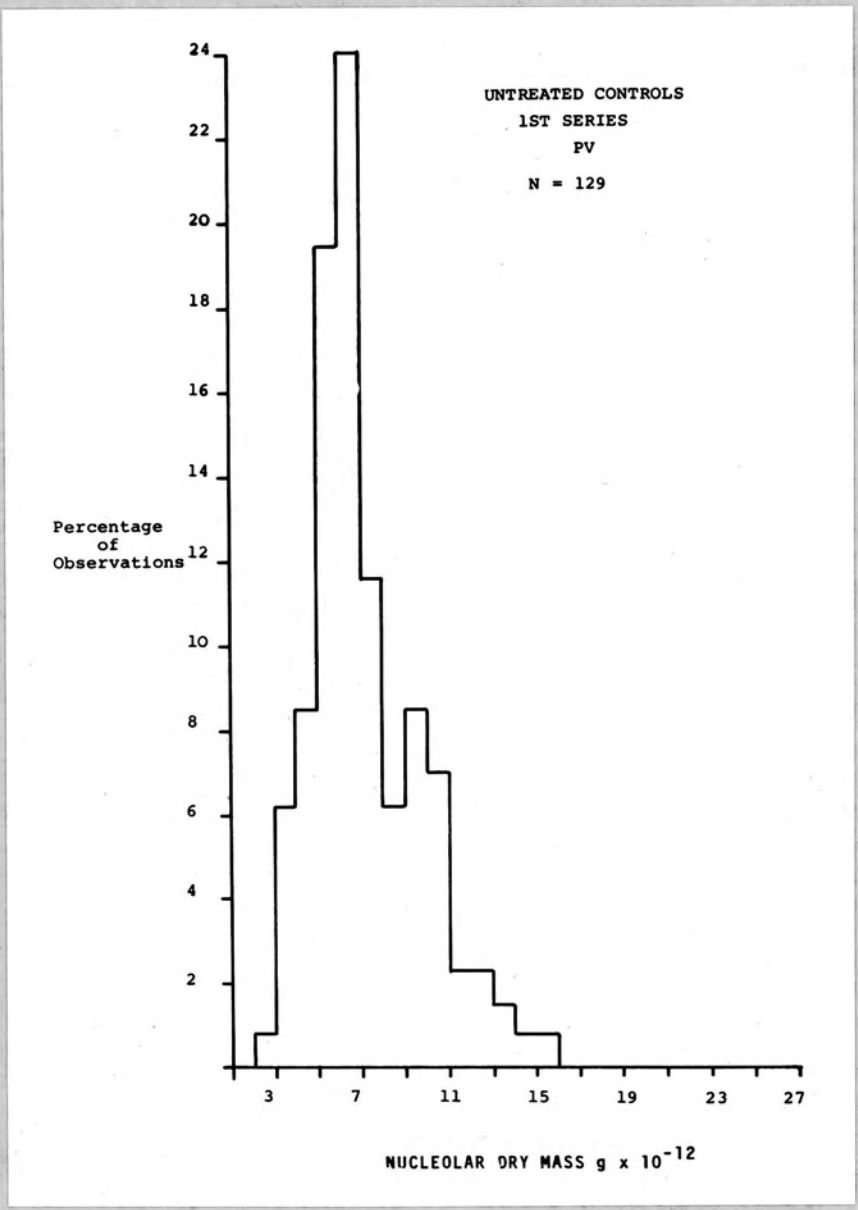
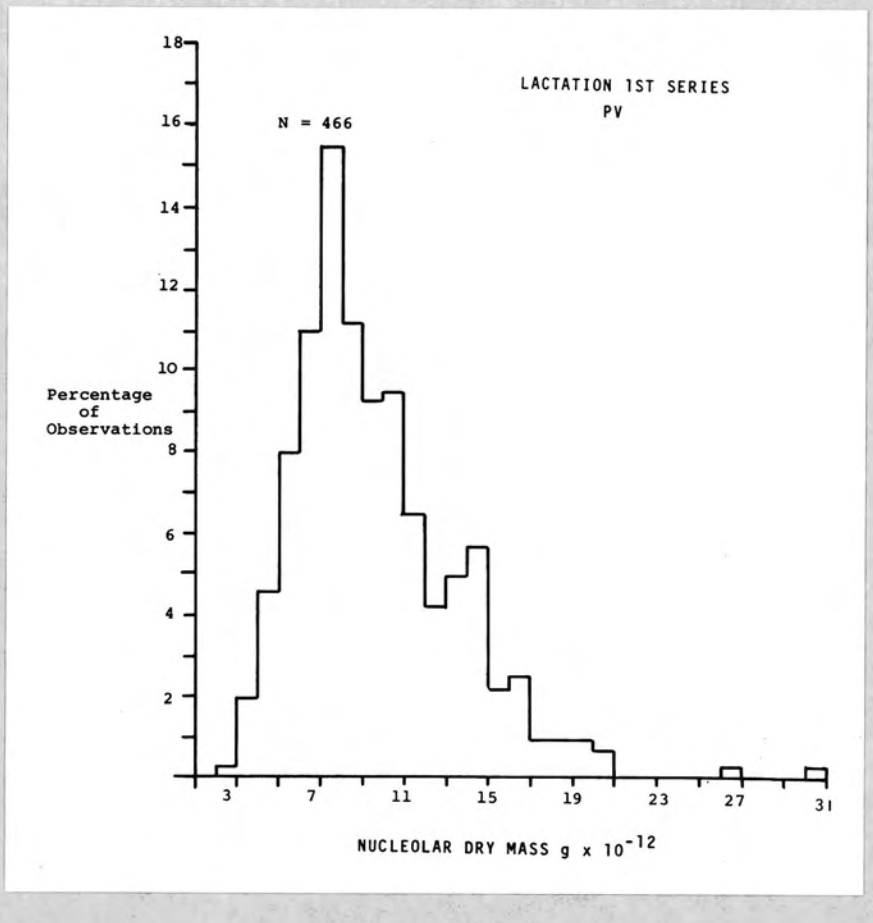


Figure 55 Frequency distribution of individual nucleolar dry mass measurements. Untreated virgin controls for lactation, dehydration groups. First series. PV neurones.
N = number of dry mass measurements.

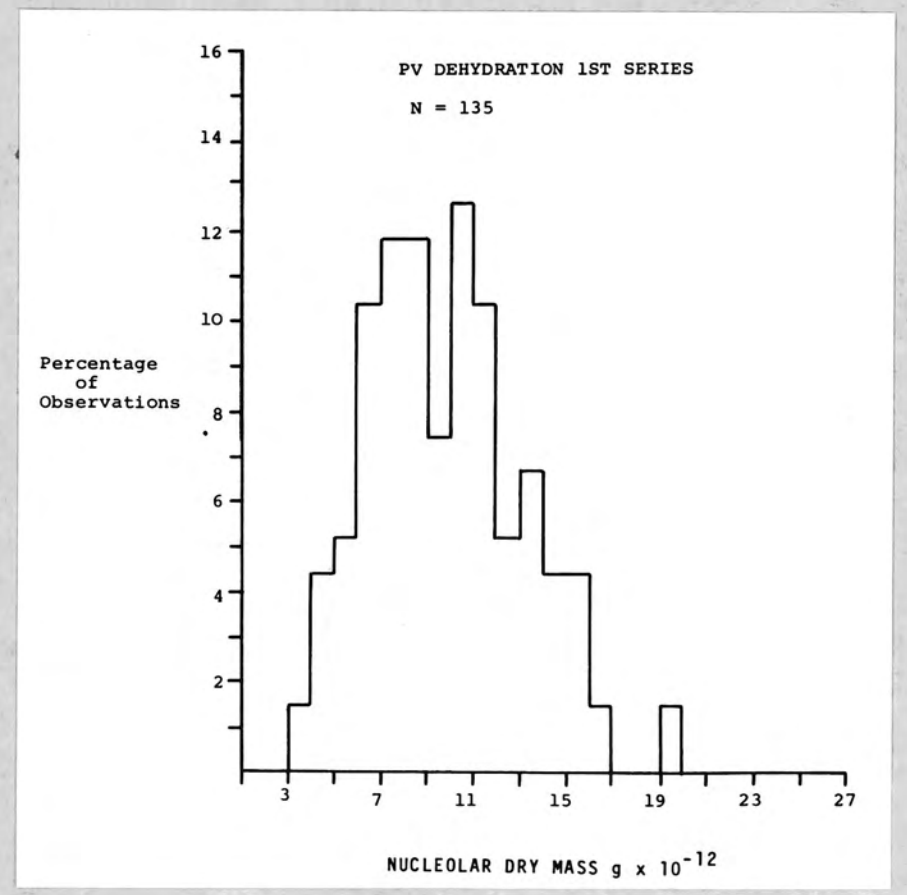


a

Figure 56 Frequency distribution of individual nucleolar dry mass measurements. Lactation, dehydration first series. PV neurones. N = number of dry mass measurements.

a. Lactation alone, day 8 post partum. b. Dehydration alone for 3 days.

b



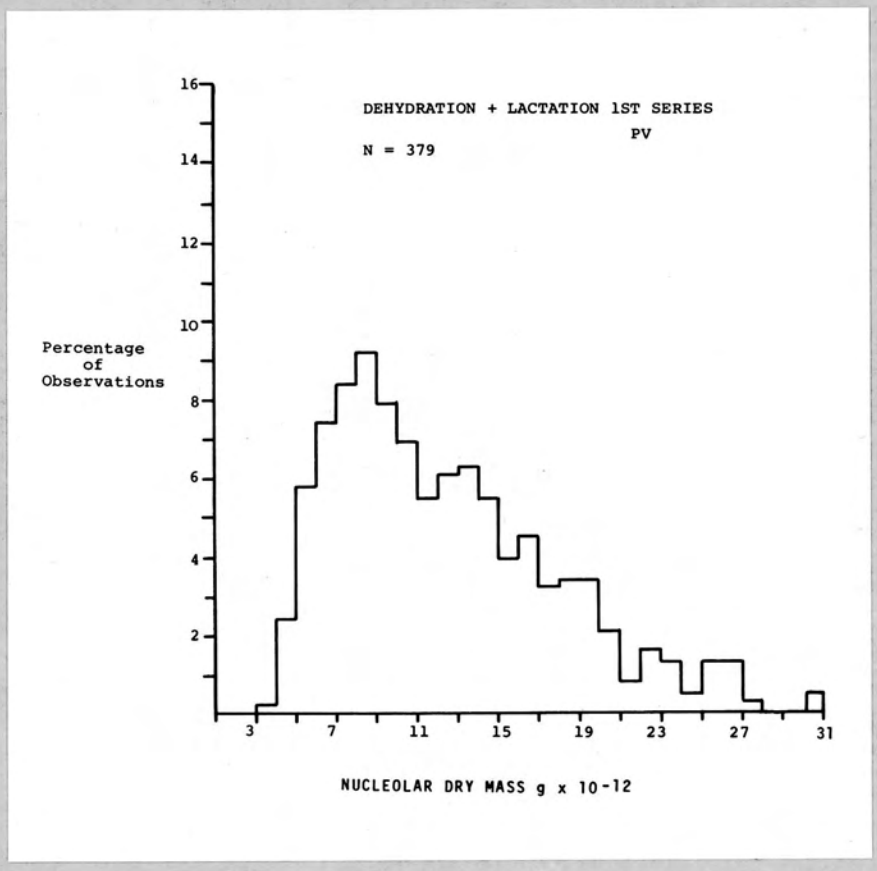
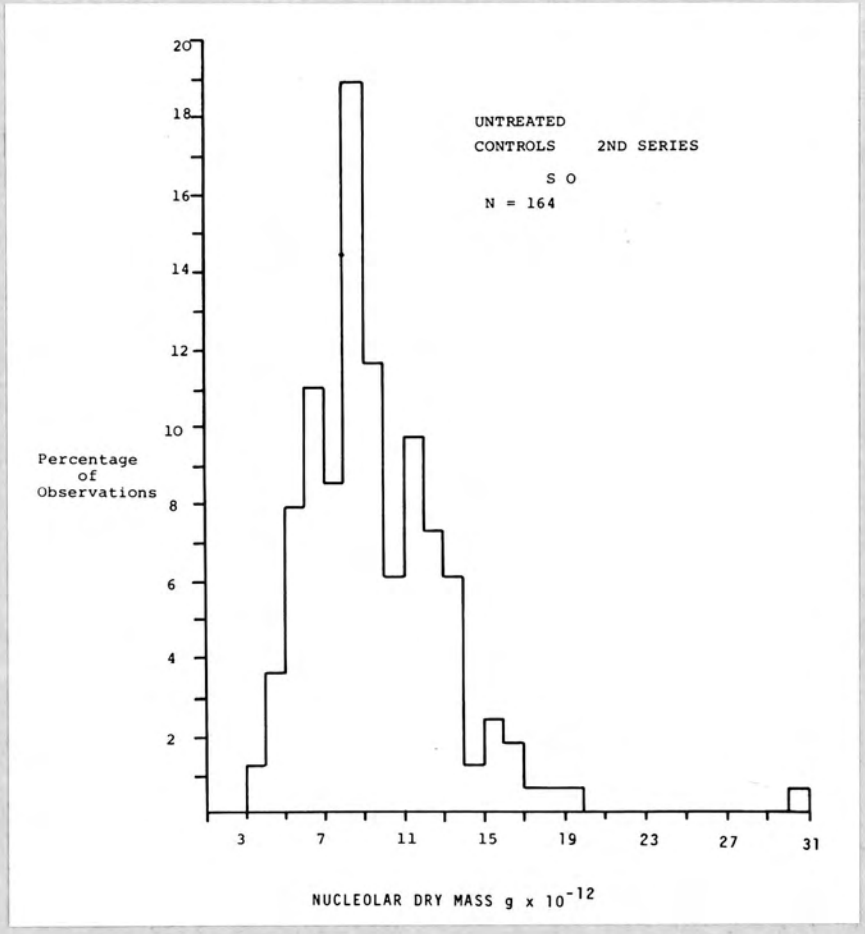


Figure 57 Frequency distribution of individual nucleolar dry mass measurements. Dehydration from day 5 to 8 post partum. PV neurones, first series. N = number of dry mass measurements.

This distribution of nucleolar dry mass is positively skewed and is consistent with two populations of nucleoli (respective modes about 9 and 17 g x 10^{-12}).

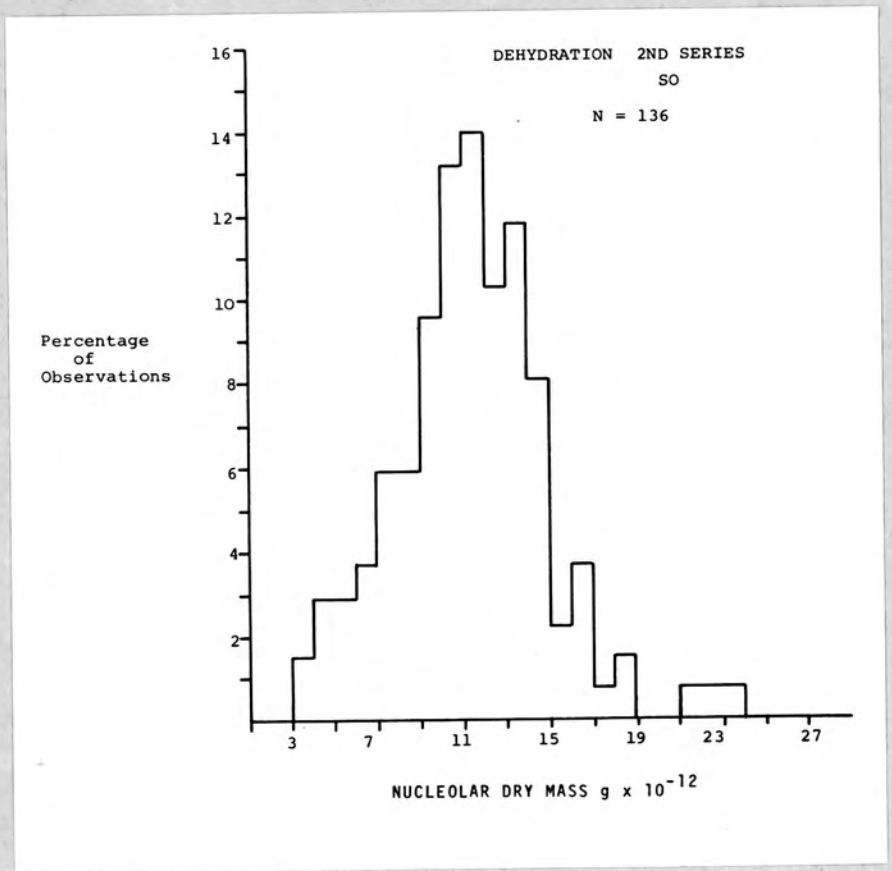


a

Figure 58 Frequency distribution of individual nucleolar dry mass measurements. Dehydration and lactation, second series. SO neurones.
N = numberof dry mass measurements.

a. Untreated virgin controls. b. Dehydration alone for 3 days.

b



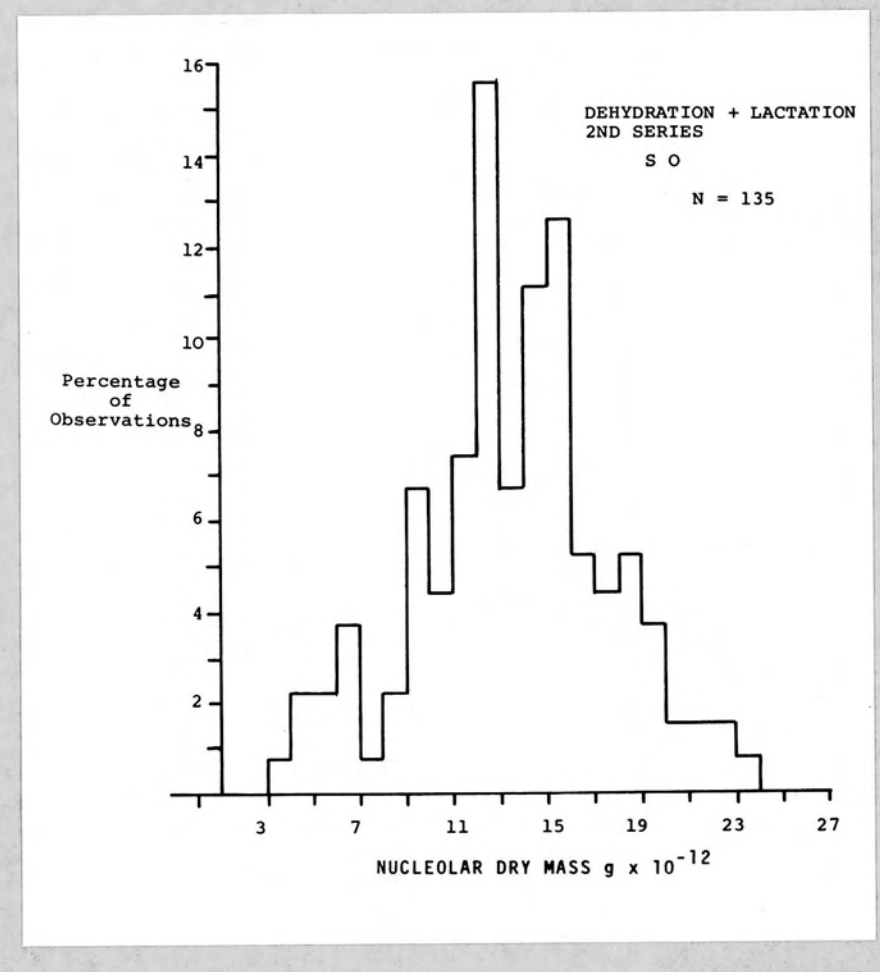
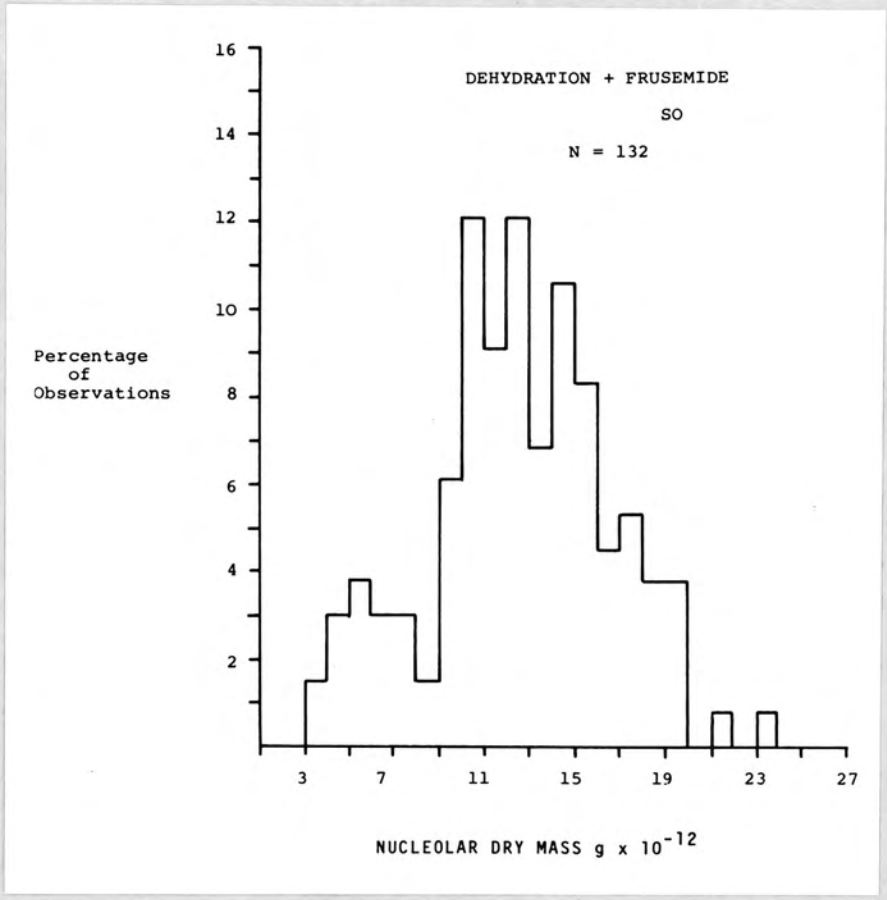


Figure 59 Frequency distribution of individual nucleolar dry mass measurements. Dehydration from day 5 to 8 post partum. Second series. SO neurones. N = number of dry mass measurements.

There is a group of small nucleoli which may represent a population separate from the remainder.



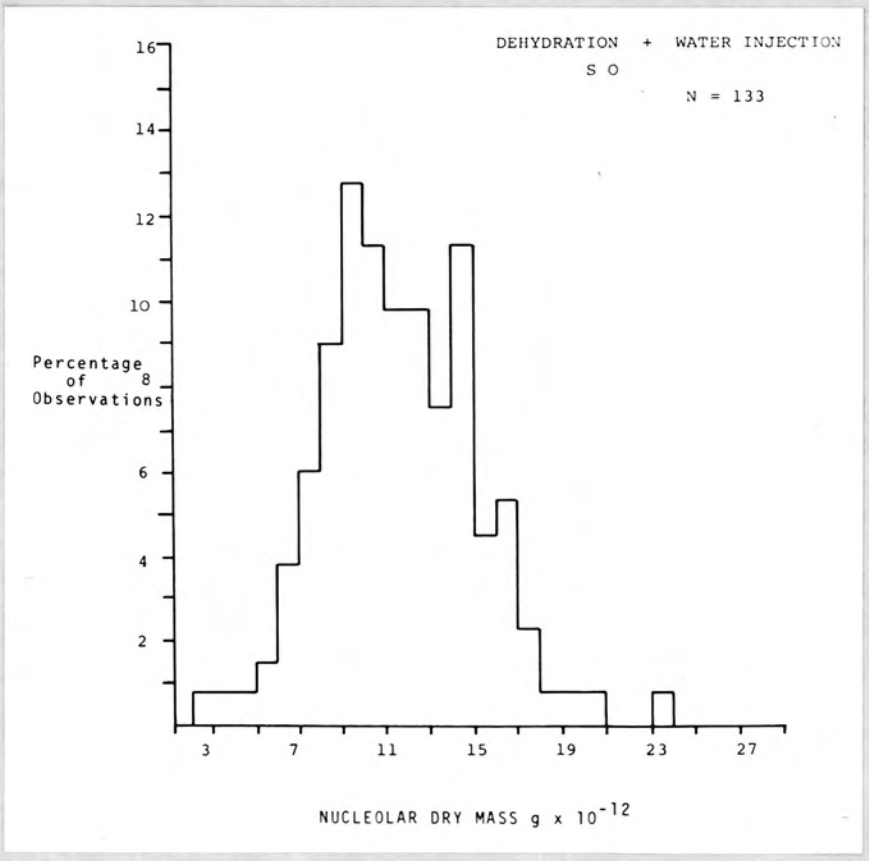
a

Figure 60 Frequency distribution of individual nucleolar dry mass measurements. Dehydration and frusemide. SO neurones. N = number of dry mass measurements.

a. Dehydration for 3 days and daily frusemide injections. There is a small group of small nucleoli similar to that in Fig. 59.

b. Dehydration for 3 days and daily water injections. Controls for a.

b



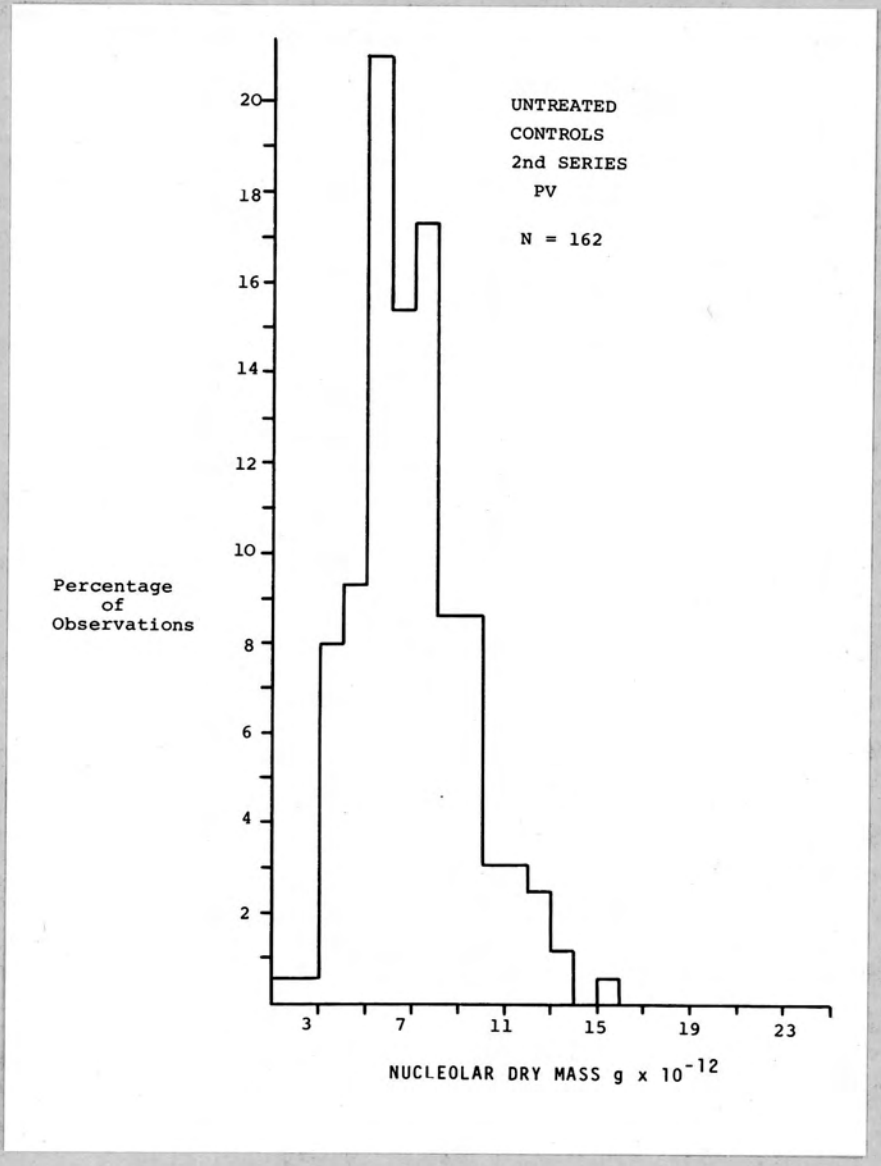
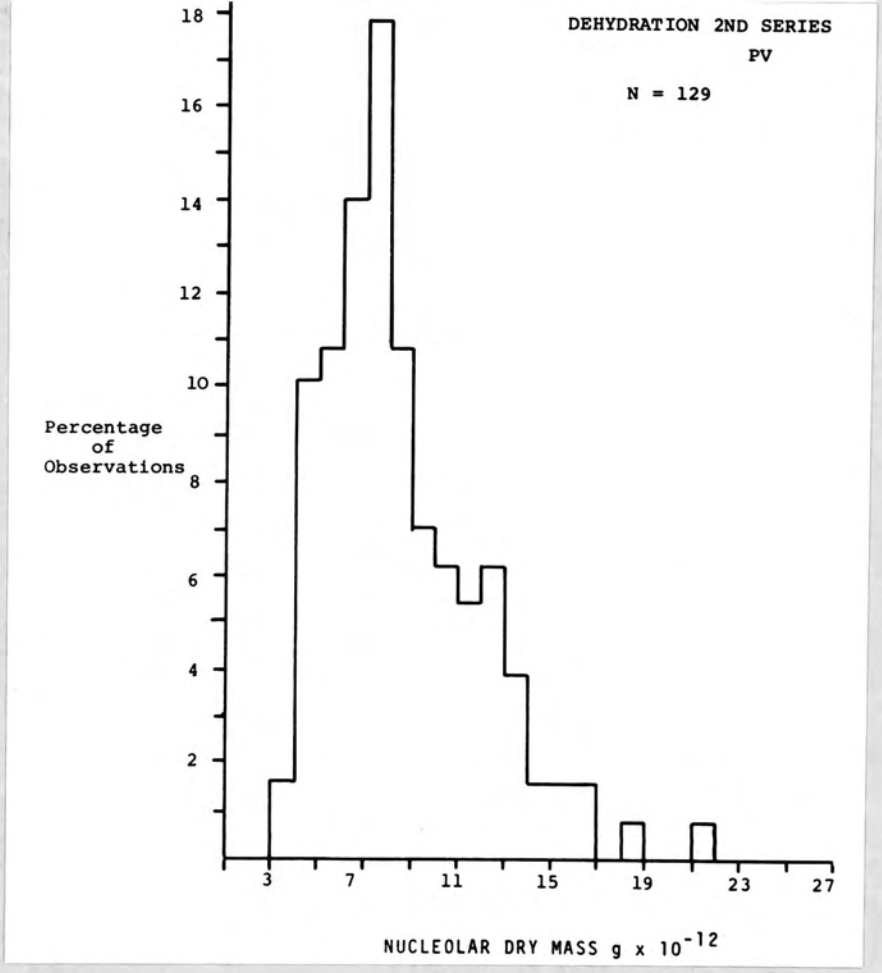


Figure 61 Frequency distribution of individual nucleolar dry mass measurements. Untreated virgin controls. PV neurones, second series. N = number of nucleolar dry mass measurements.



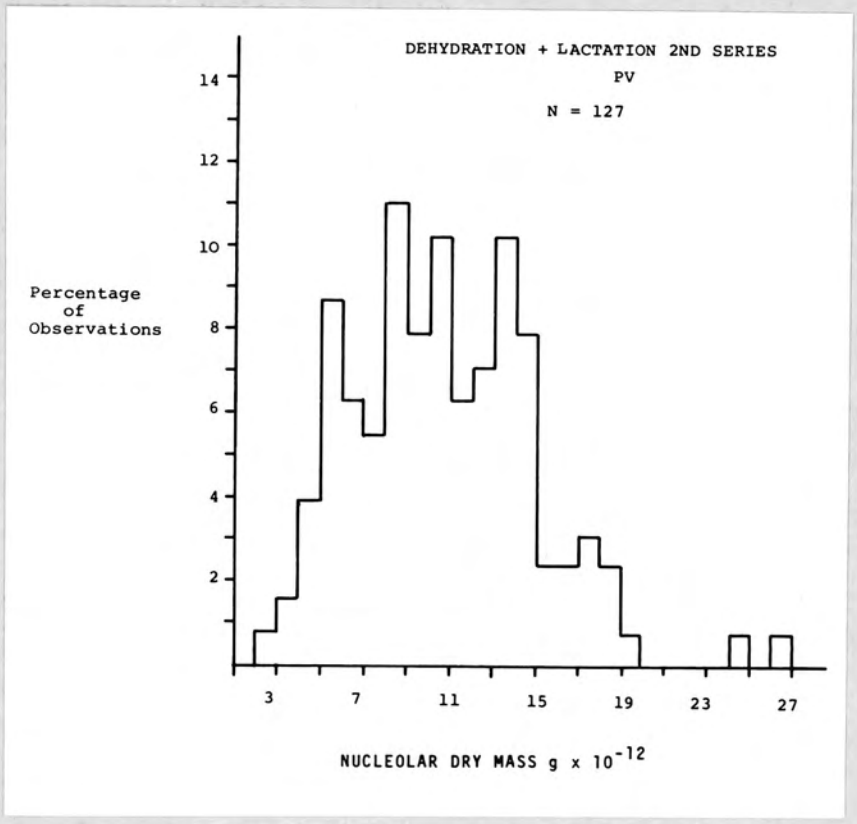
a

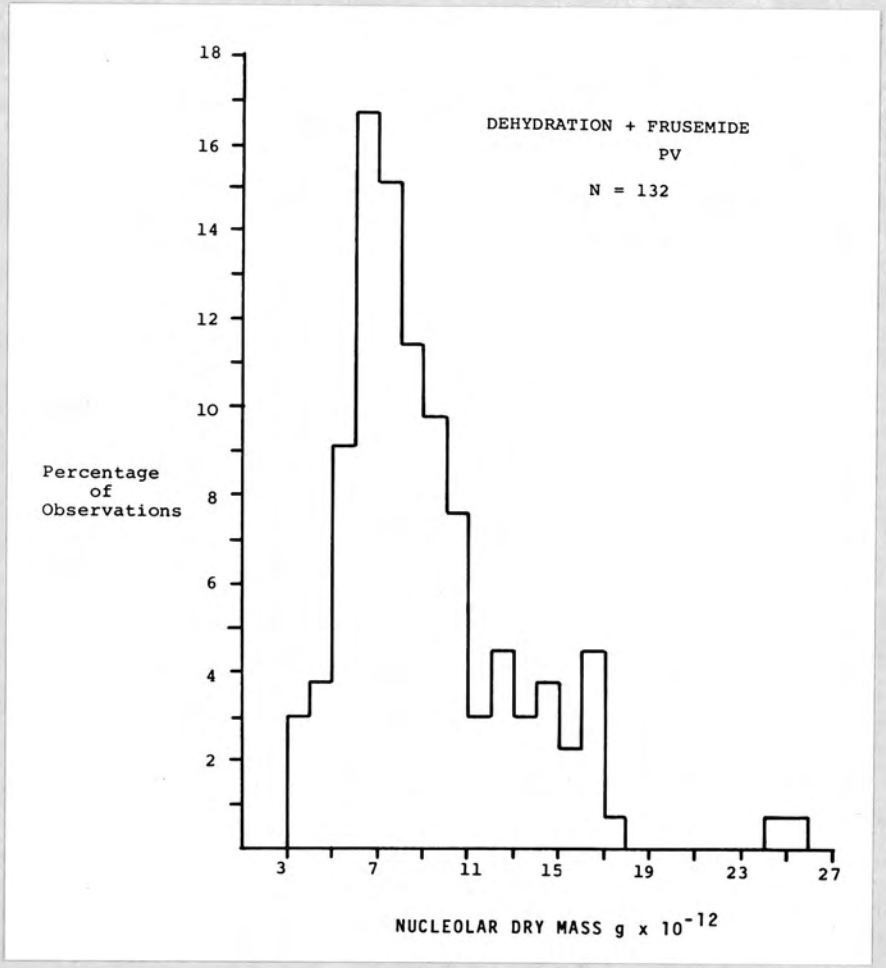
Figure 62 Frequency distribution of individual nucleolar dry mass measurements. Dehydration, lactation. PV neurones, second series. N = number of dry mass measurements.

a. Dehydration alone for 3 days.

b. Dehydration from day 5 to 8 post partum. The population of nucleoli did not respond uniformly.

b





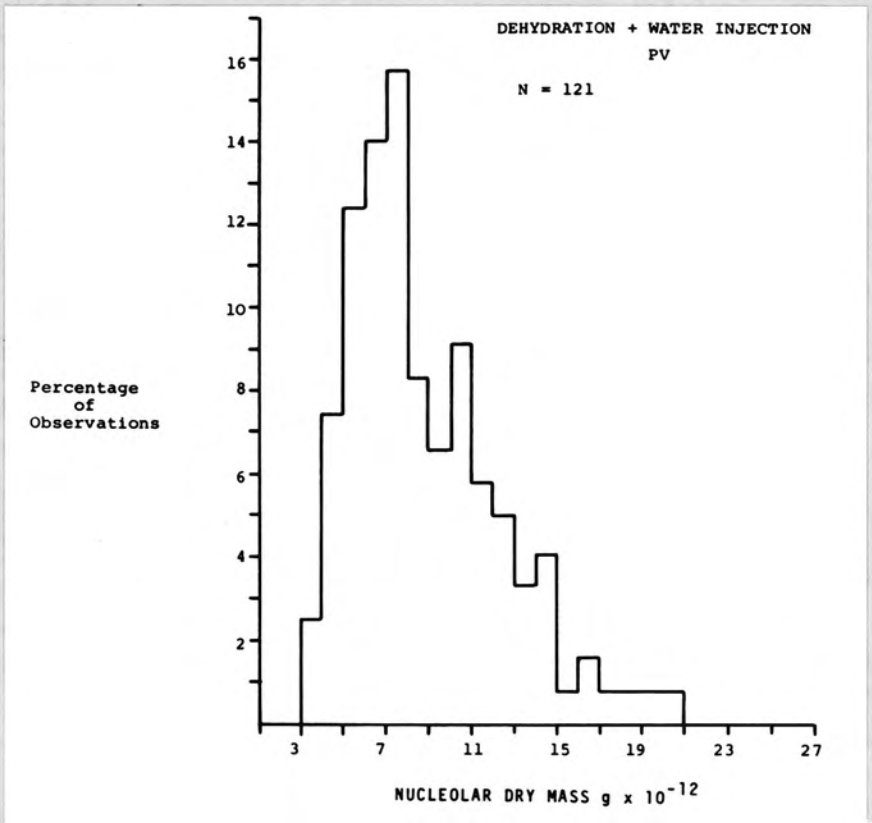
a

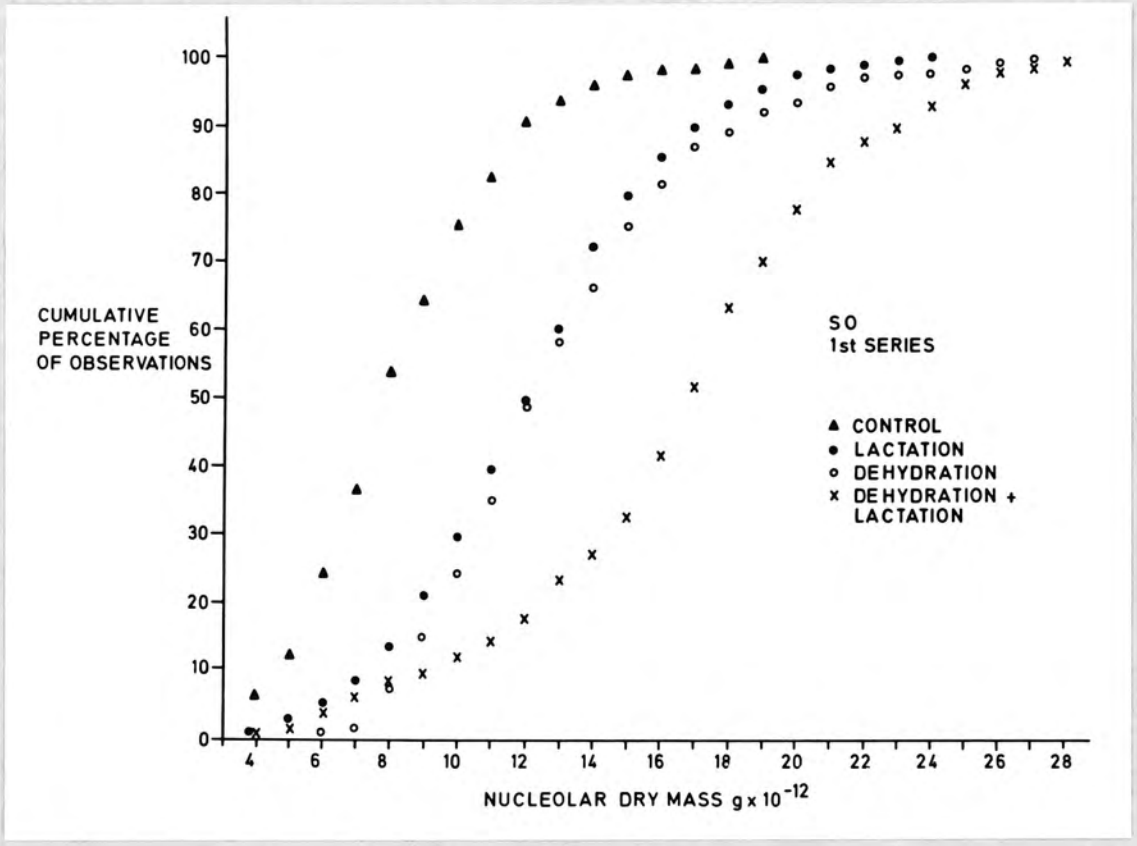
Figure 63 Frequency distribution of individual nucleolar dry mass measurements. Dehydration and frusemide. PV neurones. N = number of dry mass measurements.

a. Dehydration for 3 days and daily frusemide injections. There is a small group of large nucleoli which may represent a population distinct from the remainder.

b. Dehydration for 3 days and daily water injections. Controls for a.

b

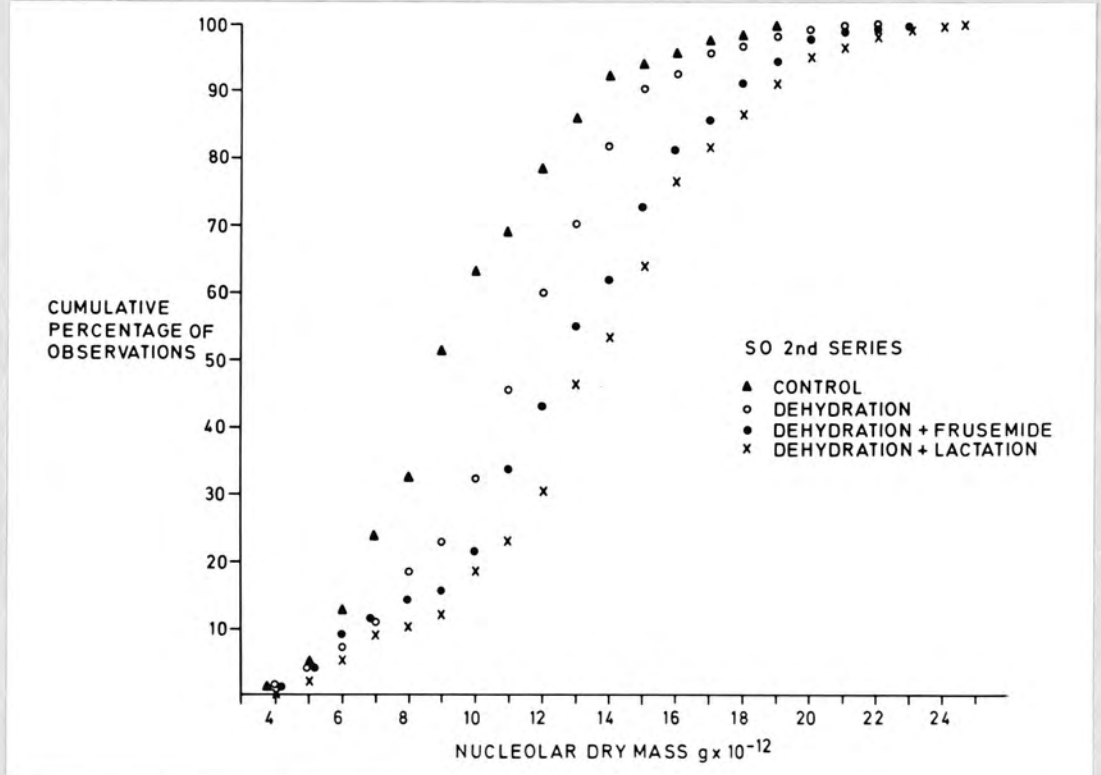


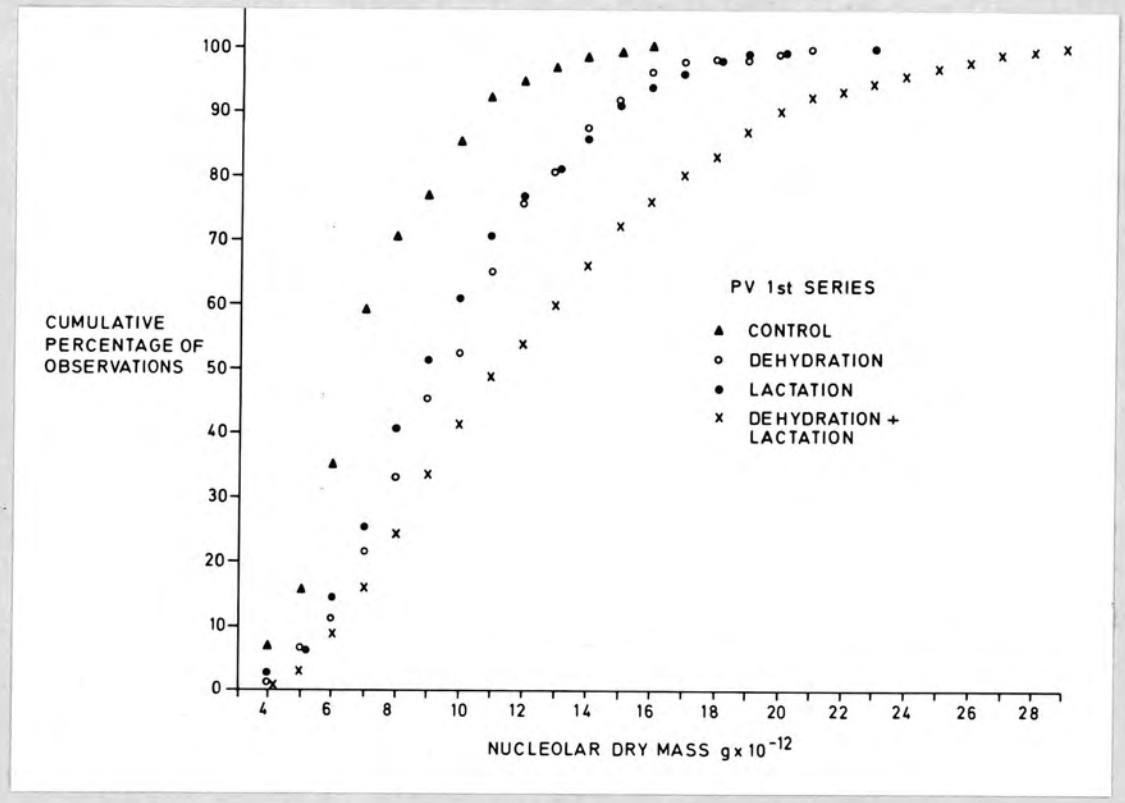


a

Figure 64 Cumulative frequency distribution of individual nucleolar dry mass measurements. SO neurones. Data replotted from Figs. 53, 54 and 58-60. a. First series. b. Second series. The curves in a. and b. are consistent with a uniform response of SO nucleoli to any stimulus combination with the exception that there is a small group of unresponding small nucleoli in all groups except dehydration in a. The curves for dehydration + frusemide and dehydration + lactation are parallel, but separated.

b





a

Figure 65 Cumulative frequency distribution of individual nucleolar dry mass measurements. PV neurones. Data replotted from Figs. 55 to 57 and 61 to 63.

a. First series. b. Second series. The curves for lactation or dehydration alone are consistent with a uniform response of a single population of PV nucleoli. The curves for dehydration + lactation are consistent with responses in more than one population of nucleoli. There are more large nucleoli in the dehydration + frusemide group than in the dehydration alone group.

b

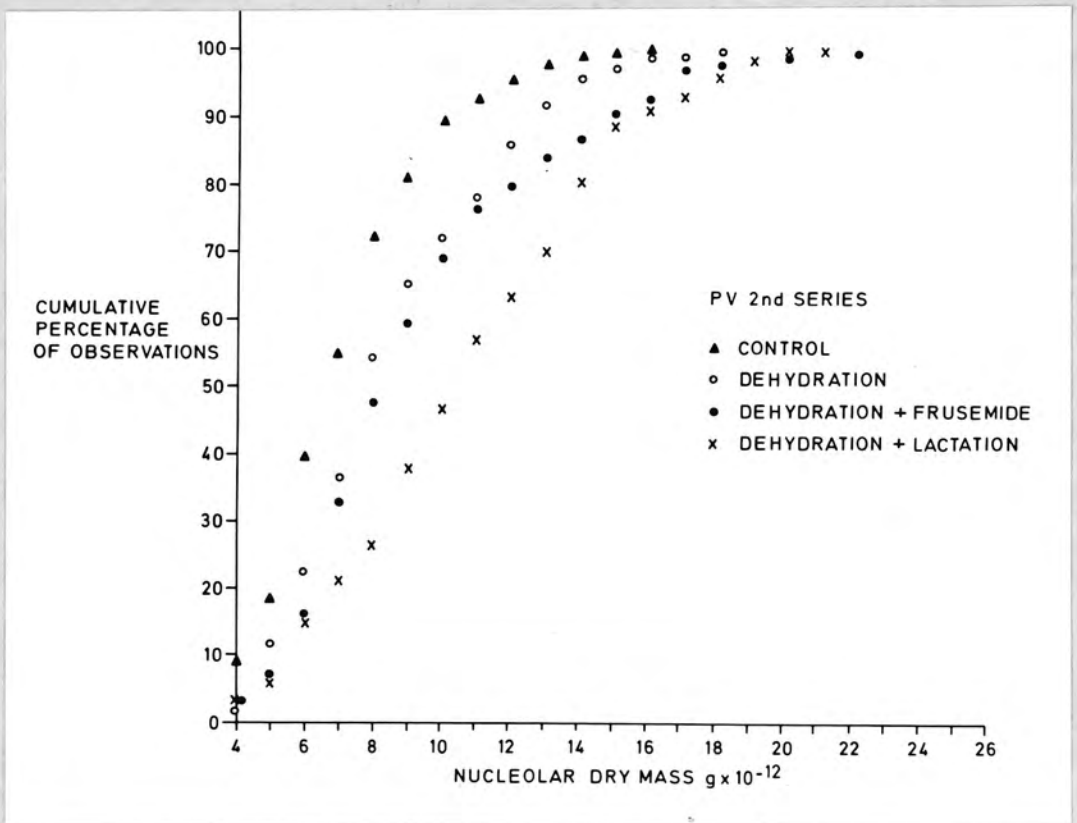


TABLE 27 NUCLEOLAR DRY MASS: MODES*, MEDIANS OF MEASUREMENTS IN LACTATION & DEHYDRATION

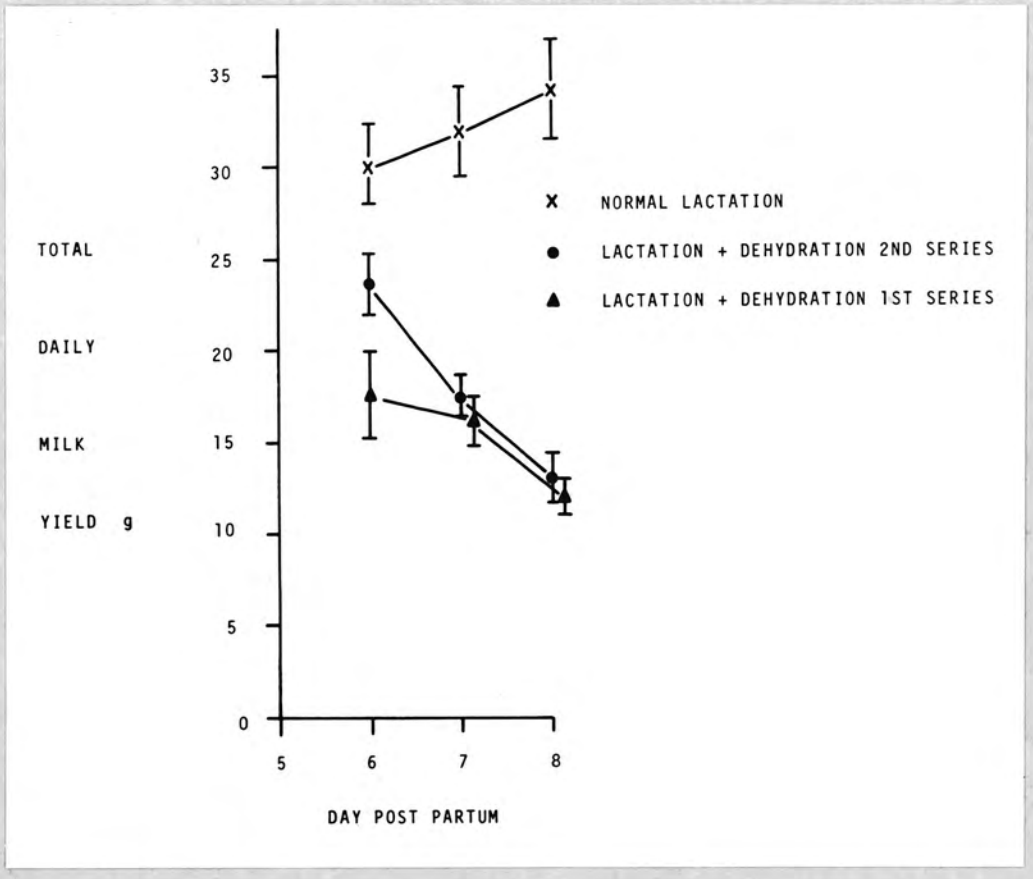
First Series

Experimental Group			Untreated controls	Lactation	Dehydration	Dehydration + Lactation
Nucleolar dry mass g x 10^{-12}	PV	Mode	5-7	7-9	7-9	7-9
		Median	6.5	8.5	9.5	11.0
	SO	Mode	7-9	12-14	10-12	16-18
		Median	7.5	12.0	12.0	16.5

Second Series

Experimental Group			Untreated controls	Lactation + Dehydration	Dehydration	Dehydration + Frusemide
Nucleolar dry mass g x 10^{-12}	PV	Mode	5-7	8-10	6-8	6-8
		Median	6.5	10.0	7.5	8.0
	SO	Mode	8-10	12-14	10-12	10-13
		Median	9.0	13.5	11.5	12.5

*Each mode is expressed as a range.



a

Figure 66 Total daily milk yield. Normal lactation and lactation + dehydration from day 5 to day 8 post partum. Each vertical line indicates the standard error of the mean.

a. Litter weight correction factor (100-14.8).

b. Correction factor (100-4.7).

b

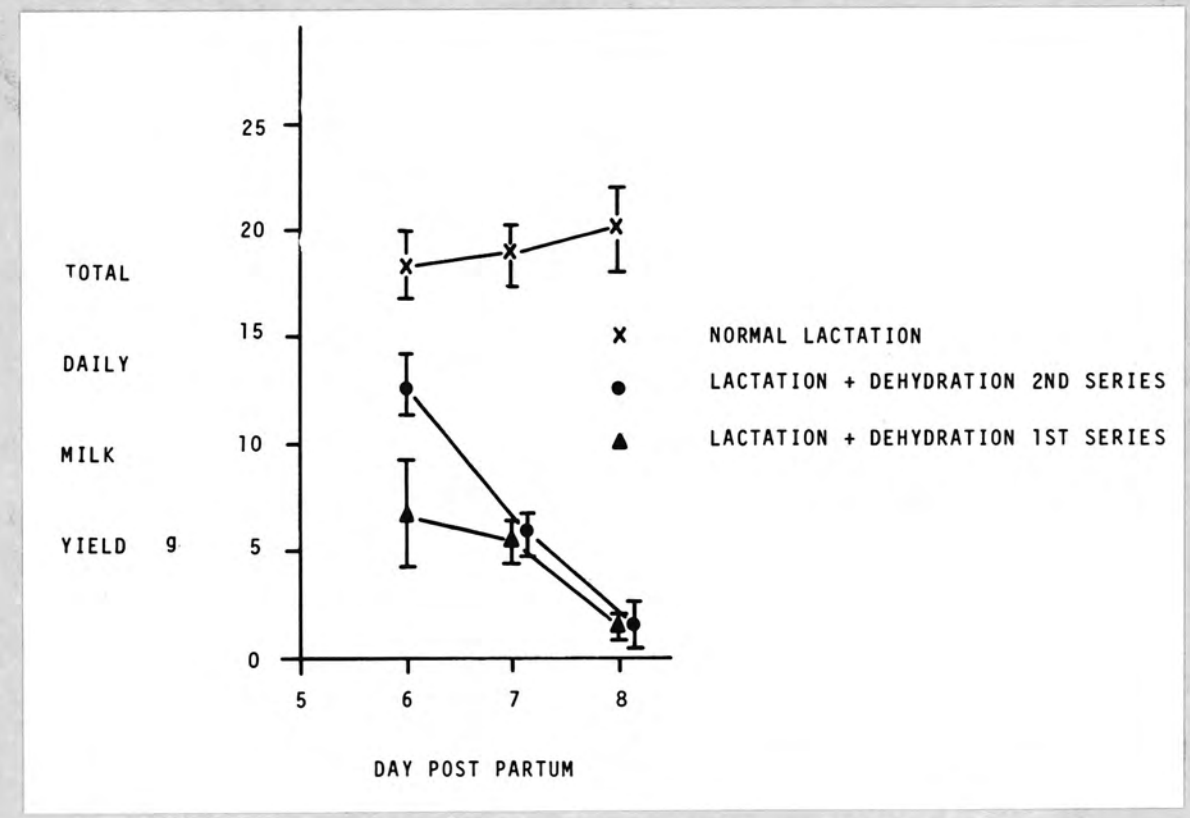


TABLE 28

TOTAL DAILY MILK YIELD: LACTATION AND LACTATION + DEHYDRATION. COMPARISONS BETWEEN GROUPS

Experimental Group			Normal lactation	
			(100-4.7)[a]	(100-14.8)[a]
LACTATION + DEHYDRATION	Day post partum	Experimental Series		
	6	1st	$0.01 > p > 0.005^{**}$	$0.01 > p > 0.005^{**}$
		2nd	$0.05 > p > 0.025^{*}$	$0.1 > p > 0.05$
	7	1st	$p < 0.001^{***}$	$p < 0.001^{***}$
		2nd	$p < 0.001^{***}$	$p < 0.001^{***}$
	8	1st	$p < 0.001^{***}$	$p < 0.001^{***}$
		2nd	$p < 0.001^{***}$	$p < 0.001^{***}$

[a] = Correction factor used to calculate milk yield from litter weight.

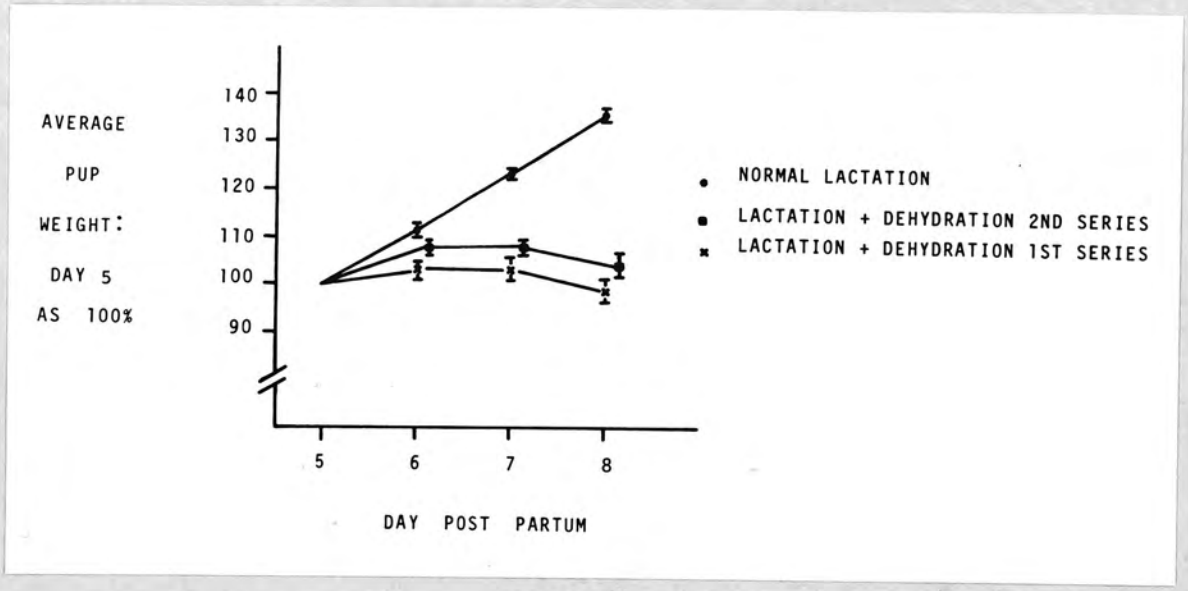


Figure 67 Average pup weight. Normal lactation and lactation + dehydration from day 5 to 8 post partum. Average pup weight expressed as a percentage of weight on day 5 post partum.

Each vertical line indicates the standard error of the mean.

Figure 68 Adult female body weight. Dehydration and lactation.

Each vertical line indicates the standard error of the mean.

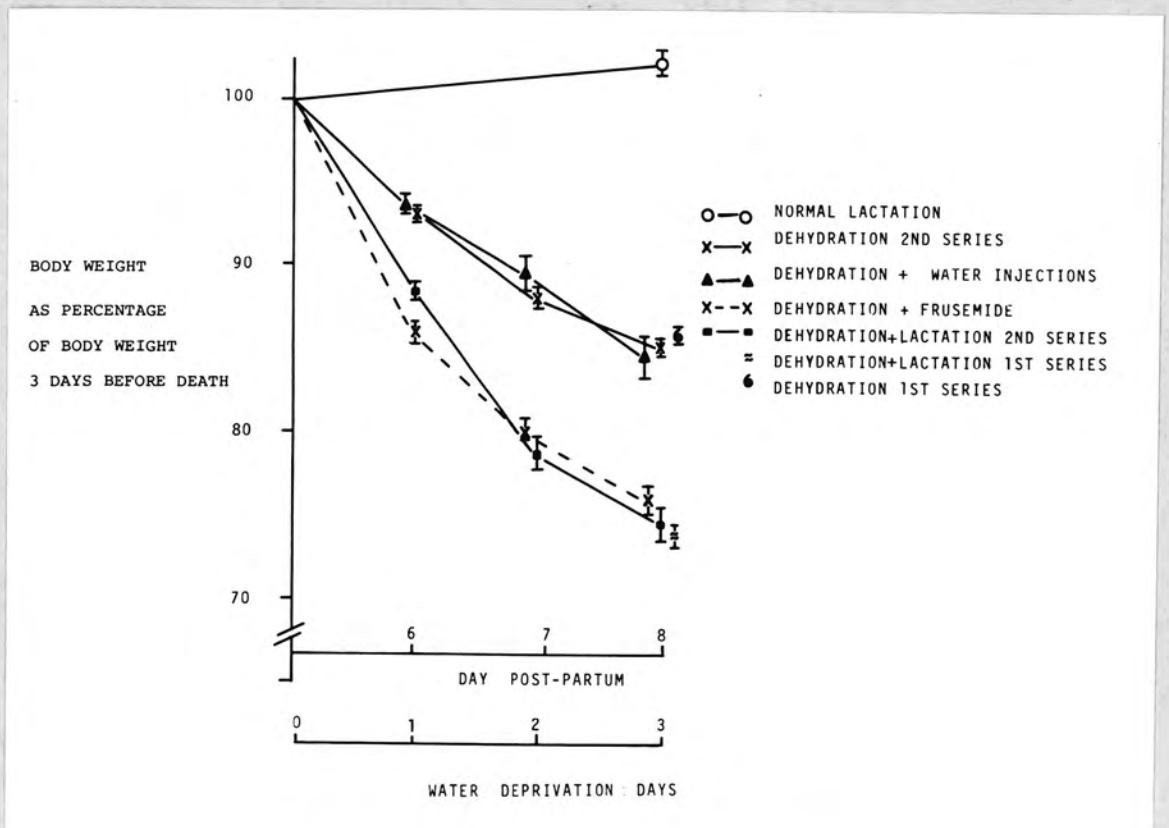


TABLE 29

AVERAGE PUP WEIGHTS: LACTATION, LACTATION AND DEHYDRATION.

COMPARISONS BETWEEN GROUPS BY STUDENT'S t-TEST

a. Average pup weight, g. Day 5 post partum

Experimental Group	Lactation + Dehydration		
	Mean $\pm$ S.E. g	First Series 10.44 ± 0.42	Second Series 10.60 ± 0.76
Normal lactation	10.85 ± 0.51	$0.4 > p > 0.3$	$0.4 > p > 0.3$

b. Average pup weight gain: Day 5 = 100%

Experimental Group	Lactation + Dehydration						
	Day post partum	6		7		8	
		Experimental Series					
		First	Second	First	Second	First	Second
Normal lactation	6	$0.01 > p > 0.005^{**}$	$0.05 > p > 0.025^{*}$				
	7			$p < 0.001^{***}$	$p < 0.001^{***}$		
	8					$p < 0.001^{***}$	$p < 0.001^{***}$

TABLE 30

ADULT FEMALE BODY WEIGHT: PERCENTAGE CHANGE IN 3 DAYS DURING LACTATION, DEHYDRATION.

COMPARISONS BETWEEN GROUPS

a. First Series

Experimental group	Lactation	Lactation + dehydration
Dehydration	$p < 0.001$***	$p < 0.001$***
Lactation + Dehydration	$p < 0.001$***	

b. Second Series

Experimental Group	Lactation + Dehydration	Dehydration	Dehydration + Frusemide
Dehydration + Water	$p < 0.001$***	$0.6 > p > 0.5$	$p < 0.001$***
Dehydration + Frusemide	$0.3 > p > 0.4$	$p < 0.001$***	
Dehydration	$p < 0.001$***		

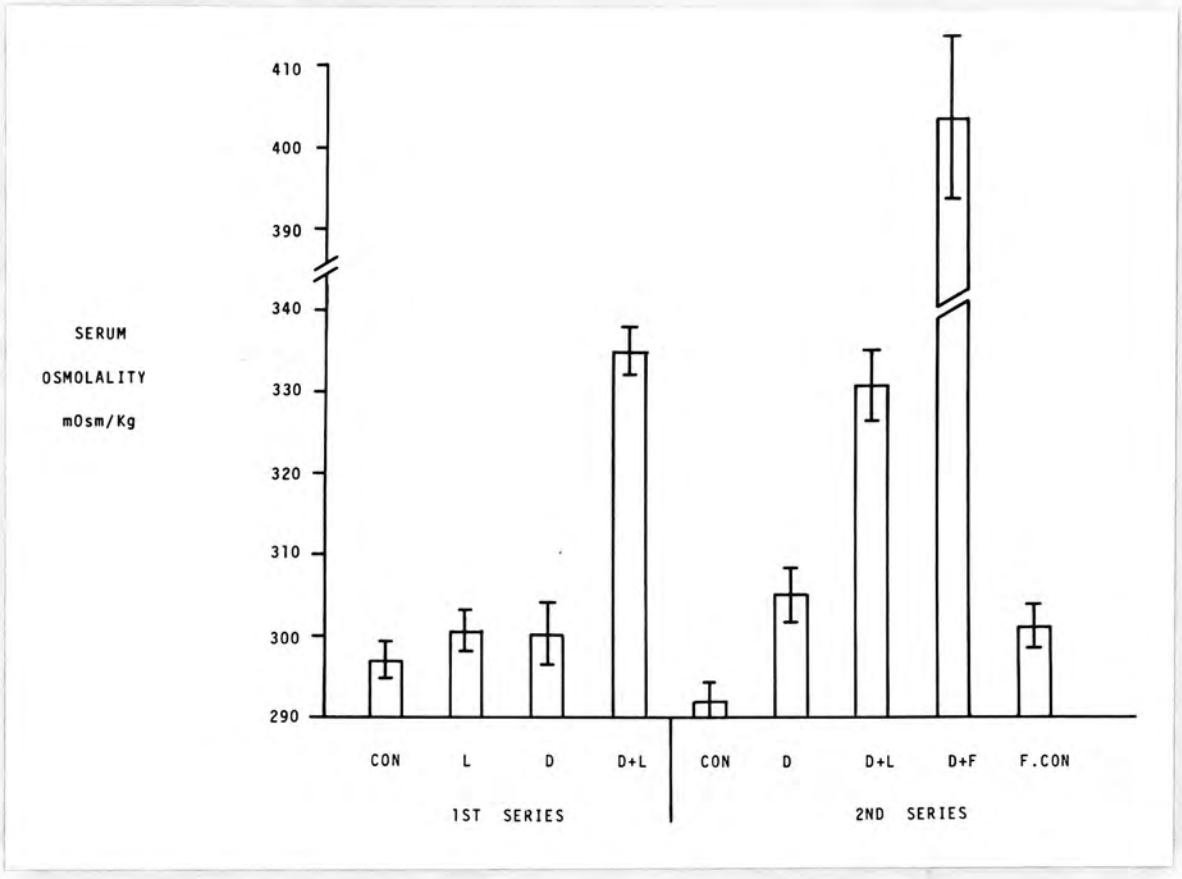


Figure 69 Mean serum osmolality. Lactation and dehydration.

CON = Untreated virgin controls.

L = Normal lactation, day 8 post partum.

D = Dehydration for 3 days, virgin females.

D + L = Lactation + dehydration from day 5 to 8 post partum.

D + F = Dehydration for 3 days + daily frusemide injections, virgin females.

F.CON = Dehydration for 3 days + daily water injections, virgin females, controls for D + F.

Each vertical line indicates the standard error of the mean.

TABLE 31 SERUM OSMOLALITY: LACTATION, DEHYDRATION. COMPARISONS BETWEEN GROUPS BY STUDENT'S t-TEST

a. First Series

Experimental Group	Lactation	Lactation + Dehydration	Dehydration
Control	$0.4 > p > 0.3$	$p < 0.001^{***}$	$0.6 > p > 0.5$
Dehydration	$0.9 > p > 0.8$	$p < 0.001^{***}$	
Lactation + Dehydration	$p < 0.001^{***}$		

b. Second Series

Experimental Group	Lactation + Dehydration	Dehydration	Dehydration + Frusemide	Dehydration + Water injection
Untreated control	$p < 0.001^{***}$	$0.05 > p > 0.025^{*}$	$p < 0.001^{***}$	$0.10 > p > 0.05$
Dehydration + water injections	$p < 0.001^{***}$	$0.5 > p > 0.4$	$p < 0.001^{***}$	
Dehydration + frusemide	$p < 0.001^{***}$	$p < 0.001^{***}$		
Dehydration	$p < 0.001^{***}$			

5. Results

5.1 First and second series: Dehydration alone; lactation alone; dehydration and lactation together

5.1.1 PV and SO neuronal nucleolar dry mass

The results are shown in Figs. 47-51, 53-65 and Tables 25, 26 and 27.

In the second series changes in SO and PV neuronal nucleolar dry mass were similar to those in the first series with respect to the relative changes in dehydrated virgin animals and dehydrated lactating animals. Compared with untreated virgin controls, mean nucleolar dry mass did not increase as much as in the first series. The populations of nucleoli responded similarly in the two series, and the results can be described together.

Mean nucleolar dry mass of PV and SO neurones was increased on day 8 post partum in lactating animals compared with virgin controls (Figs. 47, 48; Tables 25, 26). After virgin females had been deprived of drinking water for three days, the mean nucleolar dry mass of PV and SO neurones was increased (Figs. 47, 48, 49, 50; Tables 25, 26).

When lactating animals were deprived of drinking water from day 5 post partum, the mean nucleolar dry mass of both PV and SO neurones was greater on day 8 post partum than in normal lactating animals on day 8 post partum or in virgin females deprived of drinking water for three days (Figs. 47, 48, 49, 50; Tables 25, 26).

If the nucleolar dry mass measurements in virgin females are taken as a baseline, the mean nucleolar dry mass increment, for both SO and PV neurones, after dehydration during lactation was the sum of the increments for these parameters in lactating animals and dehydrated animals (Fig. 51).

To see if the increased nucleolar response when dehydration and lactation were combined was a response of the whole population of nucleoli, histograms were constructed of the distribution of the individual measurements of nucleolar dry mass for each experimental group (Figs. 53-59, 61, 62). Comparisons between each group are more readily made from the cumulative frequency distribution curves (Figs. 64, 65). The mode and median for each population are shown in Table 27.

PV and SO neurones responded differently from each other when the nucleolar measurements were expressed as histograms. The description of these histograms must necessarily be subjective; formal analysis of the data in this form was not feasible.

PV neurones. Histograms of nucleolar dry mass measurements of PV neurones from untreated controls are shown in Figs. 55 and 61. Although there was no conclusive evidence of bimodality in any stimulated group, each histogram can be described as showing the responses of two populations of nucleoli. There was a large population of small nucleoli which did not respond during lactation, otherwise the whole population of nucleoli was enlarged on the 8th day post partum (Fig. 56a). A similar description can be applied to

the responses in virgin females to water deprivation for three days (Figs. 56b, 62a). When lactating animals were dehydrated a population of small, unresponding, nucleoli was still present and the percentage of large nucleoli was greater than in animals exposed only to suckling or dehydration. The upper limit of the nucleolar dry mass spectrum was greater in dehydrated lactating animals than in dehydrated or lactating animals (Figs. 57, 62b).

SO neurones. Histograms of nucleolar dry mass measurements of SO neurones from untreated controls are shown in Figs. 53a and 58a. A small population of small, unresponding nucleoli emerged only when lactation and water deprivation were combined (Figs. 54b, 59). Otherwise the population of nucleoli responded uniformly to all combinations of stimuli (Figs. 53b, 54a, 58b). There was no tendency for the percentage of largest nucleoli to increase out of proportion to the remaining population with any stimulus combination. The upper limit of the nucleolar dry mass spectrum was greater in dehydrated lactating animals than in dehydrated or lactating animals.

5.1.2 Other parameters

Litter size. The mean litter size of each lactating group is shown in Table 24.

Milk yield. After water deprivation, milk yield rapidly decreased in lactating animals, but lactation was maintained (Fig. 66a,b; Table 28). Until the third day of water deprivation milk was visible in the stomachs of the pups which, however, failed to gain weight(Fig.67; Table 29).

Milk was present in the mammary glands of 75% of the lactating dehydrated animals post mortem. Milk was not present in the mammary glands of virgin dehydrated animals post mortem.

No attempt was made to measure oxytocin secretion by observing pup stretch reactions, and the suckling stimulus was not quantified. The pups were observed to suckle frequently; and the mothers continued to nurse the pups to the end of the 72 hour dehydration period.

Maternal body weight. The body weight of lactating females deprived of water decreased more rapidly and to a greater extent compared with dehydrated virgin females (Fig. 68, Table 30a).

Serum osmolality. In the first series, compared with untreated controls, the serum osmolality was not increased in virgin females after three days water deprivation or in normal lactating animals. In the second series, serum osmolality of dehydrated virgin animals was significantly greater than that of untreated controls. In both series, the serum osmolality increased more after three days water deprivation in lactating animals than in similarly dehydrated virgin females (Fig. 69, Table 31a).

Rough estimates of blood volume were in the order:

$$\text{lactating} > \text{virgin} > \text{dehydrated virgin} > \begin{matrix}\text{dehydrated} \\ \text{lactating}\end{matrix}$$

Because the serum osmolality was greater, and the blood volume smaller, in lactating animals after three days water deprivation than in virgin animals after three days water deprivation, the next experiments were performed. To see

if the greater nucleolar changes in SO and PV neurones in the former were a result of greater hyperosmotic or hypovolemic stimuli, a diuretic, frusemide, was used to simulate these conditions in the absence of lactation.

5.2 Second series: Dehydration alone; lactation and dehydration together; dehydration and frusemide together

5.2.1 PV and SO nucleolar dry mass

The results are shown in Figs. 49-51, 58-65, and Tables 25, 26, 27.

The nucleolar changes in response to dehydration alone and lactation with simultaneous dehydration have been described above.

The mean nucleolar dry mass changes in PV and SO neurones of virgin animals deprived of water for three days were identical with those of animals so treated and injected with 0.5 ml of water daily (Figs. 49-51; Tables 25b, 26b).

After daily frusemide treatment during water deprivation for three days, the mean nucleolar dry mass of SO neurones increased more than after water deprivation with or without daily injections of 0.5 ml water (Fig. 49, Table 25b). The difference between the mean nucleolar dry mass of SO neurones of dehydrated lactating animals and dehydrated virgin females injected with frusemide was not statistically significant (Table 25b).

Mean nucleolar dry mass of PV neurones was not increased significantly more if dehydrated virgin animals were treated with frusemide (Fig. 50, Table 26b). The mean nucleolar dry

TABLE 32

SO NEURONAL NUCLEOLAR NUCLEIC ACID CONTENT: DEHYDRATION AND LACTATION. COMPARISONS BETWEEN GROUPS

Experimental Group	Dehydration + Lactation	Dehydration
Untreated controls	$p < 0.001$***	$0.05 > p^* > 0.025$
Dehydration	$p < 0.001$***	

mass of PV neurones of dehydrated animals treated with frusemide was significantly less than that of dehydrated lactating animals (Fig. 50, Table 26b).

5.2.2 SO neuronal nucleolar nucleic acid content

The mean nucleolar nucleic acid content of SO neurones was increased in virgin animals deprived of water for three days, and further increased in lactating animals similarly dehydrated (Fig. 52, Table 32).

Changes in nucleolar nucleic acid content were not in exact proportion to nucleolar dry mass changes. The nucleolar nucleic acid content, measurements corrected for scatter, expressed as a percentage of the mean nucleolar dry mass in untreated controls, dehydrated virgin animals and dehydrated lactating animals were, respectively, 11%, 10.6% and 13.3%. If the nucleolar nucleic acid measurements uncorrected for scatter are so expressed, the percentage nucleic acid contents were, respectively, 16.6%, 16.1% and 18.9% of the nucleolar dry mass.

5.2.3 Nucleolar dry mass histograms

Histograms and frequency distribution curves have been constructed for individual nucleolar dry mass measurements made in this series of experiments (Figs. 58-65).

The description of these figures is made with the same reservation as before: that is, the description is subjective.

Theresponses of the populations of PV and SO neuronal nucleoli to dehydration and to dehydration and lactation have been described (5.1.1).

PV neurones. Histograms of nucleolar dry mass measurements from untreated controls are shown in Fig. 61. In the dehydrated animals treated with frusemide there was a population of small nucleoli which did not respond and was distributed in a similar manner to that in the dehydrated group (Figs. 62a, 63a,b), while the distribution of the largest nucleoli was more like that of the dehydrated lactating group than the group which was dehydrated only (Figs. 57, 62a,b, 63a,b). These features are seen most clearly in Fig. 65b. However the group of large nucleoli in the frusemide treated animals was not large enough to increase significantly the mean nucleolar dry mass compared with animals which were dehydrated only (Table 26b).

SO neurones. Histograms of nucleolar dry mass measurements from untreated controls are shown in Fig. 58a. There was a small, unresponsive population of small SO neuronal nucleoli in the dehydrated animals treated with frusemide (Fig. 60a). This population was also found in dehydrated lactating animals (Figs. 54b, 59). The remaining population responded uniformly (Figs. 60a, 64b). The cumulative frequency distribution curves for the dehydrated animals treated with frusemide and the dehydrated lactating animals were uniformly separated from each other (Fig. 64b).

5.2.4 Other parameters

Lactation. Changes in maternal body weight (Fig. 68, Table 30b), milk yield (Fig. 66, Table 28) and pup weight (Fig. 67, Table 29) in the dehydrated lactating group were indistinguishable from those observed in this group in the

first series of experiments (this section - 5.1.2), with the exception that the higher estimate of daily milk yield was not significantly reduced 24 hours after water deprivation (Table 28).

Body weight. Changes in body weight of dehydrated virgin animals, with or without water injections, were similar to those in this group in the first series (Fig. 68, Table 30). Body weight, expressed as a percentage of the initial weight, decreased by an identical amount in the dehydrated group treated with frusemide and in the dehydrated lactating group, but decreased more than in the group dehydrated with or without water injections (Fig. 68, Table 30b).

Serum osmolality. This was greater, but not significantly, in the dehydrated animals injected with water than in untreated controls. The serum osmolality of animals dehydrated and injected with water was not different from that of dehydrated virgin animals not so injected (Fig. 69, Table 31b). The greatest change in serum osmolality was a large increase in the animals dehydrated and injected with frusemide (Fig. 69).

Crude estimates of blood volume were in the order: untreated controls > dehydrated or dehydrated and water injections > dehydrated lactating > dehydrated and frusemide injected.

6. Discussion

6.1 Stimulus-response parameters

Both hyperosmotic and hypovolemic stimuli cause ADH to be secreted (Verney, 1947; Share, 1969; Saito et al, 1969; Moses & Miller, 1971; Sczepanska-Sadowska, 1972a). Both of these stimuli can be expected to contribute to stimulation of ADH secretion during a period of water deprivation, interacting in a manner already discussed (this section - 2.3.4).

The crude estimate of blood volume used in the present study suggests that during a period of water deprivation the more severe the weight loss, and the greater the rise in serum osmolality, the greater will be the hypovolemic stimulus. The small rise in serum osmolality after three days water deprivation (Fig. 69), only significant in the second series, is consistent with the findings of others that normal serum osmolality may be maintained for about 48 hours after water deprivation (Jones & Pickering, 1969). Serum osmolality is clearly not an accurate indicator of the extent of dehydration, although it changes pari passu with plasma sodium ion concentration (Jones & Pickering, 1969). Hypovolemia may be a more important stimulus for ADH secretion in the early stages of dehydration, although the threshold for ADH secretion in response to serum osmolarity change is low; in man, this threshold is a 3% rise in serum osmolarity if blood volume change is prevented, or a 1.3% rise if blood volume is allowed to fall (Moses & Miller, 1970).

In virgin females, the fall in body weight was more marked than the change in serum osmolality after three days water deprivation (Figs. 68, 69). Fall in body weight results partly from fluid losses, and partly because the animals stop eating (Dicker & Nunn, 1957). The greater loss of body weight by lactating animals deprived of drinking water can be partly attributed to continued lactation. The extra weight lost by lactating animals compared with virgin animals was similar to the total milk yield, estimated on the basis of 14.8% inevitable weight loss per litter per day (Figs. 66a, 68).

The greater increase in serum osmolality of dehydrated lactating animals (Fig. 69) can be explained on the basis of the suggestion that because milk has a low sodium ion concentration, although it is isotonic with plasma (Linzell, 1972), milk secretion increases the concentration of sodium chloride in blood leaving the mammary gland (Section III - 4.1.2). Conversely, this suggestion is supported by the finding of increased serum osmolality in dehydrated lactating animals.

In goats, during a period of water deprivation milk yield is normal for the first 24 hours, but then steadily decreases, and is 50% of normal at the end of 72 hours; of particular relevance to the above remarks, the sodium concentration of such milk remains constant (Konar & Thomas, 1970). Treatment of lactating rabbits with frusemide, without water deprivation, decreases milk yield within 24 hours because, it is suggested, prolactin secretion is

inhibited (Gachev, 1969). In the present study, by either estimate milk yield fell within 24 hours of water deprivation, except that in the second series the higher estimate of milk yield was not significantly reduced at 24 hours (Fig. 66a,b). The milk yield in the final 24 hours of water deprivation was 10 to 38% of normal, calculated for the low and high estimates of milk yield respectively. Milk yield may fall during a period of water deprivation because secretion of lactogenic hormones is suppressed or because milk ejection is inhibited, or for other reasons. Adrenocorticosteroids and prolactin are both secreted in response to 'stress', and are both essential for lactation. A variety of stressful situations, including water deprivation for five days, initiates lactogenesis in estrogen primed female rats as a result of increased prolactin and ACTH secretion (Nicoll et al, 1960). However, the milk ejection reflex can be inhibited centrally and peripherally (Cross, 1955a,b). The inhibitory recurrent collateral system of the hypothalamic neurosecretory neurones, in which ADH is an inhibitory transmitter (Nicoll & Barker, 1971), may inhibit oxytocin secretion. Intravenous infusions of ADH reproduce the changes in milk yield which occur in water deprivation (Konar & Thomas, 1970), but ADH is not effective at doses which result in blood concentrations of ADH comparable with those of dehydration (Peaker & Linzell, 1973).

Although the litters nursed by dehydrated lactating animals suckled vigorously, it was not possible to assess oxytocin secretion in the manner used previously (Section IV -

2.3.1, 2.3.2). Milk yield may not have been related to oxytocin secretion, because milk ejection may have been inhibited peripherally even if oxytocin was secreted (Cross, 1955a). Central pathways which stimulate ADH secretion may inhibit oxytocin secretion (Aulesbrook & Holland, 1969b; Bisset et al, 1970). The question of the separate release of ADH and oxytocin has been discussed (this section - 2.3). However, the pups obtained milk throughout the period of water deprivation (Fig. 66), which indicates continued oxytocin secretion.

Frusemide (Peters & Roch-Ramel, 1969)stimulates sodium excretion by the kidney and abolishes the ability of the kidney to form a concentrated urine. By its actions on the proximal tubule and loop of Henlé frusemide tends to reduce urine osmolarity toward isotonicity with plasma, and induces a diuresis. The body weight changes of animals dehydrated and treated with frusemide were similar to those of dehydrated lactating animals (Fig. 68). But the serum osmolality was higher in the former, and the blood volume lower (Fig.69). The combined hyperosmotic and hypovolemic stimulus was greater in the frusemide treated dehydrated animalsthan in the dehydrated lactating animals. Between experimental groups the combined hyperosmotic and hypovolemic stimulus was in the order: dehydration alone or with water injection $<$dehydration and lactation$<$dehydration and frusemide. Serum sodium ion concentration was not measured; it is not known whether this parameter changes pari passu with osmolality under the conditions of the present experiments.

6.2 Nucleolar changes

Synthetic and secretory changes in both SO and PV neurones in response to dehydration or osmotic stimulation by salt loading are well documented. The posterior pituitary is depleted of ADH, oxytocin and neurosecretory material (Ortmann, 1951; Dicker & Nunn, 1957; Rennels, 1958; Vilhardt, 1970; Moses & Miller, 1970), the synthesis of export proteins is increased (Wells, 1961; Sloper & King, 1963; Flament-Durand, 1966; Sachs et al, 1971; Norstrom & Sjostrand, 1972a), as is ribosome synthesis (Watson, 1965b; Watt, 1970; Norstrom, 1971). Nucleolar enlargement has been frequently described (Leveque, 1953; Bachrach & Koszegi, 1957; Sloper & King, 1963) and ultrastructural changes are consistent with increased synthetic activity (Rechardt, 1969). Activity of the Golgi system increases (Jongkind, 1969). Electrical activity of PV and SO neurones increases during salt loading (Dyball & Pountney, 1973). The case for increased ribosome and protein synthesis in these circumstances has been argued already (Section I - 2.6.2).

The present study confirms that the synthetic activity of PV and SO neuronal nucleoli increases during a period of water deprivation.

Whether or not oxytocin secretion continues undiminished in response to suckling during water deprivation, nucleolar dry mass changes resulting from increased synthetic activity take seven days to regress (Section III - 3.3). The nucleolar dry mass changes measured when stimuli were combined

resulted from the effects of continually increasing hypovolemic and hyperosmotic stimulation and continued stimulation by suckling, which may have been diminishingly effective in causing oxytocin secretion.

Measurements of nucleolar nucleic acid content made on some of the preparations of SO nuclei confirmed the trend of the nucleolar dry mass measurements (Figs. 51a,b, 52). These two parameters have been previously found to change similarly (Watson, 1968a, 1969; Watt, 1970, 1970a, 1971). The percentage of the nucleolar dry mass contributed by nucleic acid increased from 11% in untreated controls to 13% in the dehydrated lactating animals. A similar increase in the nucleic acid contribution to nucleolar dry mass after a more severe hyperosmotic stimulus has been recorded (Watt, 1970). This change in nucleolar dry mass composition cannot be interpreted in the absence of kinetic studies. It may reflect utilisation of ribosomal protein at a greater rate than it is supplied at high rates of ribosome synthesis. If this is correct, measurement of nucleolar dry mass may underestimate the rate of ribosomal synthesis as nucleolar dry mass increases.

When the qualitatively different stimuli of dehydration and suckling were applied simultaneously, the changes in mean nucleolar dry mass of PV and SO neurones were double the nucleolar response to each stimulus applied separately (Fig. 51a,b). This rules out the possibility (this section - 3.2a) that the failure of the mean nucleolar dry mass of PV or SO neurones to increase further when the litter size was

increased from 10 to 22-24 pups (Section III - 3.3) was due to the inability of nucleolar synthetic activity of all SO and PV neurones, all of which responded, to further increase.

The effects of frusemide on the mean nucleolar dry mass of SO neurones confirm the previous conclusion (Section IV - 4.3) that nucleolar synthetic activity can be modified in accordance with functional demand, expressed as secretory activity, although no direct evidence was obtained that ADH secretion was further increased by frusemide treatment. Other studies indicate greater synthesis and secretion of ADH and neurophysin in response to greater hyperosmotic or hypovolemic stimulation (Saito et al, 1969; Moses & Miller, 1970; Norstrom, Enestrom & Hamberger, 1971; Norstrom & Sjostrand, 1972a; Sczepanska-Sadowska, 1972a), with an associated greater increase in ribosome synthesis (Watt, 1970; Watson, 1965b). By analogy, the restricted nucleolar response to increased suckling intensity (Section III - 3.3) is probably related to failure of the suckling responsive neurones to be electrically activated in proportion to the stimulus intensity, rather than to a restricted secretory capacity determined by limited nucleolar activity (Section IV - 4.3). The mean nucleolar dry mass changes after combined dehydration and lactation (Figs. 47-50) are consistent either with the hypothesis that nucleolar dry mass changes are dependent on and quantitatively related to secretory activity and occur in a homogeneous population of neurones, or with the hypothesis that two populations in each of the PV and SO nuclei each respond maximally to either, but not both, stimuli (this section - 3.1, 3.2b). It was

not feasible to distinguish formally between these remaining possibilities with the available data. Interpretation of the histograms and frequency distribution curves is subjective.

Responses of PV neuronal nucleoli to dehydration or lactation alone are consistent with a single responding population (Figs. 56, 62, 65a,b). There was a substantial population of nucleoli which did not respond, and since this population was still present in PV neurones from dehydrated lactating animals (Figs. 57, 62b), these nucleoli may belong to non- neurosecretory neurones, or may represent a population of neurosecretory neurones which do not receive an input for any of the stimuli associated with water deprivation or suckling. Alternatively this population of small nucleoli may be derived from neurosecretory neurones in the resting phase of a synthesis-secretion cycle (Zambrano & Mordoh, 1966; Zambrano & De Robertis, 1966b; Enestrom, 1967; Golubitsa & Korotchkin, 1971).

The upper limit of the nucleolar dry mass spectrum was greater when lactation and dehydration were combined (Figs. 57, 62b, 65a,b) compared with either lactation or dehydration alone (Figs. 56a,b; 62a; 65a,b). Intensification of the suckling stimulus, so that it is greater than that applied by 10 pups, does not alter the mean nucleolar dry mass of PV neurones (Section III - 3.3; Section IV - 3.1.1) so this change in the nucleolar dry mass spectrum was not the result of a possible intensification of suckling which may occur when lactating animals are deprived of water. The change in spectrum may have resulted from the addition of a population

of neurones responding to suckling to a population responding to dehydration, or may indicate a greater response of a population of neurones in the PV nucleus to the greater combined hyperosmotic and hypovolemic stimulus when lactating animals were dehydrated (this section - 6.1).

The mean nucleolar dry mass of PV neurones did not increase significantly more during dehydration if frusemide was administered (Fig. 50, Table 26b) which indicates that the greater response when lactation and dehydration were combined was due to addition of changes in neurones responding to suckling to changes in neurones responding to dehydration. However, the proportion of large nucleoliu was greater if dehydrated animals were treated with frusemide, and these large nucleoli were not part of a continuous spectrum (Figs. 63a, 65b). This is consistent with the above suggestion that the PV nucleus contains a population of neurones which respond in a graded manner to intensification of hyperosmotic and hypovolemic stimuli.

Clearly, the PV nucleus, with respect to neuronal nucleolar dry mass, responds to stimuli associated with both water deprivation and suckling. On the whole the responses are consistent with a homogeneous population which responds to either stimulus and a population which responds to neither stimulus. It cannot be conclusively decided whether these responses represent convergence of both stimuli on every responding neurone, or whether each stimulus is distributed to a separate population, which have similar nucleolar dry mass spectra and cannot be

separated. The only evidence in support of the latter possibility is the greater number of large nucleoli when the intensity of the hyperosmotic and hypovolemic stimuli was increased with frusemide (Figs. 63a, 65b).

The responses of the SO neuronal nucleoli during lactation or water deprivation alone, or when both conditions were combined, are consistent with a major, uniformly responding population. There is probably a small population of small nucleoli which did not respond to any combination of stimuli, to which the same considerations may be applied as were to a similar population of PV neuronal nucleoli (Figs. 53, 54, 58-60, 64). It is possible that the suckling dependent changes in SO neurones during lactation were not the result of responses to suckling per se, but resulted from hyperosmotic and hypovolemic stimuli associated with milk secretion. The changes observed when dehydration and lactation were combined, or when frusemide treatment and dehydration were combined, could be explained on this basis, if the neuronal nucleoli can respond in a graded manner to an intensified stimulus. It would be of interest in this context to investigate the effects of frusemide, or another diuretic, on SO and PV neurones in animals with free access to water. Frusemide given to guinea pigs with free access to water produces morphological changes in SO neurones consistent with increased secretory and synthetic activity (Galasinska-Pomykol & Hryszko, 1972). The difference between the mean nucleolar dry mass of SO neurones in the dehydrated animals treated

with frusemide and in the dehydrated lactating animals, although not significant, represented about 25 of the increment in the latter group from the virgin control value (Fig. 51a, Table 25b). The frequency distribution curves of the SO neuronal nucleolar dry mass of dehydrated animals treated with frusemide and dehydrated lactating animals were uniformly separated (Fig. 64b). These differences suggest that there is a small population of neurones within the SO nucleus which only respond to suckling, no matter how great the combined hyperosmotic or hypovolemic stimuli may be; the nucleolar dry mass spectrum of these neurones is similar to that of neurones which respond to dehydration. But the neuronal nucleoli which respond to hyperosmotic and hypovolemic stimuli respond in a graded fashion according to the intensity of the stimulus.

7. Summary

The mean nucleolar dry mass of PV and SO neurones was increased by dehydration and during lactation. When lactating animals were dehydrated the mean nucleolar dry mass of SO and PV neurones increased by an amount which was the sum of the increments for each stimulus alone, compared with untreated virgin controls. The nucleolar responses to combined dehydration and lactation rule out the possibility that the limited nucleolar responses to intensification of the suckling stimulus (Section III - 3.3) were due to a limited synthetic capacity of the PV or SO nucleoli. The mean nucleolar dry mass of SO neurones increased more when the hyperosmotic and hypovolemic stimuli of dehydration

were intensified by frusemide. This confirmed that SO neuronal nucleoli respond in a graded fashion to increased functional load (Section IV - 4.3), and indicates that the nucleolar changes in SO neurones during lactation, although dependent on suckling (Section III - 4.3), may be caused by hyperosmotic and hypovolemic stimuli resulting from milk secretion.

The mean nucleolar dry mass of PV neurones did not increase further when dehydrated animals were treated with frusemide. This indicates that a large proportion of PV neurones only respond to suckling.

Subjective interpretation of the responses of the populations of SO and PV neuronal nucleoli, expressed as histograms, indicates that PV neurones respond to stimuli associated with both suckling and water deprivation, and these responses may occur in separate neurones. The PV neurones which respond to stimuli associated with dehydration respond in a graded manner according to the intensity of the stimuli.

SO neurones also respond to stimuli associated with both suckling and water deprivation, but these responses may occur in a single population and may be related to hyperosmotic and hypovolemic stimuli in both circumstances. There is some evidence for a small population of SO neurones, with a similar nucleolar dry mass spectrum to the remainder, which only responded to the suckling stimulus per se. There is a population of small nucleoli in the PV and SO nuclei which did not respond to any stimulus combination.

This interpretation is consistent with descriptions of distribution of function between the SO and PV nuclei obtained with other techniques (this section - 2.5).

It is tempting to suggest that neurones responsive to hyperosmotic and hypovolemic stimuli secrete ADH, and neurones responsive only to suckling secrete oxytocin. The present findings cannot be interpreted in this context, and have no direct bearing upon the question of synthesis of ADH and oxytocin in separate neurones (this section - 3).

SECTION VII

NUCLEOLAR CHANGES IN VARIOUS HYPOTHALAMIC AREAS AFTER CASTRATION OR ADRENALECTOMY

1. Prologue

A sustained increase in the secretory activity of a group of neurones results in enhanced ribosome and protein synthesis, reflected in neuronal nucleolar dry mass changes (Section I - 2.6, Section IV - 4.3). Such changes may also result from the direct action of hormones upon synthetic mechanisms. Consequently, increased secretory activity of a group of neurones may be assumed if the mean nucleolar dry mass of these neurones increases after stimulation or after exposure to, or deprivation of, hormones.

The experiments described in this section were performed to identify pools of neurones in the hypothalamus which respond to removal of the sources of steroid hormones. The hypothalamic areas selected for study were those indicated by others to be involved in releasing factor synthesis and secretion. The criteria previously defined, which must be satisfied before a neurone or group of neurones can be accepted as the source of a neurohormone or transmitter (Section VI - 2.1, 2.2) can be applied to neurones which may secrete releasing factors. For a putative releasing factor secreting neurone or pool of neurones the criteria are as follows:

1. the releasing factor must be present;
2. the releasing factor must be synthesised;
3. on direct stimulation of the putative releasing factor neurone or pool the releasing factor must be secreted.

In addition:

4. destruction of each neurone or pool of neurones should abolish secretion of the releasing factor;
5. changes in parameters of synthetic and secretory activity in the neurone or pool should occur when releasing factor secretion alters.

The purpose of the introduction is to review, in relation to these criteria, the evidence which indicates the localisation of neurones which synthesise releasing factors. The role of these neurones in negative feedback mechanisms is discussed. The term 'releasing factor' is used for an extracted material with appropriate properties, and the term 'releasing hormone' is only used for chemically identified material with the actions of the appropriate releasing factor. In this discussion the traditional assumption is generally made that the cells which secrete releasing factors are neurones; alternative sources of releasing factors are discussed later.

2. Introduction

There are two sets of experimental findings which are relevant to the investigations described here. These are:

1. that normal anterior pituitary function depends on humoral factors secreted by hypothalamic neurones into the hypothalamo-hypophyseal portal vessels in the median eminence (Green & Harris, 1947; Harris, 1950; Harris & Jacobsohn, 1952); and
2. that steroid secretion by the gonads and adrenal cortices is regulated by negative feedback mechanisms.

The large increase in synthesis and secretion of LH and FSH which normally occurs after gonadectomy (Yamamoto et al, 1970) can be prevented or reversed by giving the female estrogen (McCann, 1962; Ramirez & McCann, 1963; Blake et al, 1972) and, partly, by giving the male testosterone (Ramirez & McCann, 1965; Gay & Dever, 1971). Similarly, ACTH synthesis and secretion increase after bilateral or unilateral adrenalectomy (Ganong, 1954; Sydnor & Sayers, 1955; Wool et al, 1961); these changes in ACTH production can be prevented by corticosteroid treatment (Tepperman et al, 1943; Sayers, 1950; Hodges & Vernikos, 1960).

The possible link between these two sets is that the hypothalamus is the site at which the steroid hormones exert their feedback effects. The result of this hypothesis is that anterior pituitary function, in the context of negative feedback control, is regulated by hypothalamic releasing or inhibitory factors. Evidence for this statement is in two parts:

1. Feedback regulation of anterior pituitary trophic hormone secretion requires intact portal vessels between the hypothalamus and the anterior pituitary; and
2. There are sites in the hypothalamus which respond to changes in concentrations of circulating steroids.

The separate hypothesis that the central nervous system modulates the negative feedback mechanisms controlling anterior pituitary secretory activity via hypothalamic releasing factors and the hypothalamo-hypophyseal portal vessels was firmly established experimentally by Harris (1948, 1950), De Groot & Harris (1950), Harris & Jacobsohn (1952) and Harris (1972).

2.1 Dependence of anterior pituitary responses to castration or adrenalectomy on contact with the median eminence

Gonadectomy does not result in the usual morphological changes in anterior pituitary gonadotrophes if the anterior pituitary is separated from the median eminence either by transplantation to a site remote from the sella turcica, or by transection of the pituitary stalk (Hohlweg & Junkmann, 1932; Westman & Jacobsohn, 1938). Similarly, compensatory adrenal hypertrophy does not occur if the anterior pituitary is transplanted to a site remote from the sella turcica (Casentini et al, 1958).

These classical studies indicate that the secretion of LH, FSH and ACTH by the anterior pituitary alters in response to reduced concentrations of circulating steroids

only if the portal vessels are intact. However, ectopically transplanted anterior pituitaries may not respond to changes in steroid levels because they are necrotic or inadequately vascularised. Parts of the anterior pituitary die when the portal blood supply is interrupted by pituitary stalk lesions (Adams et al, 1964) or after transplantation to the kidney (Nikitovitch-Winer & Everett, 1959). Other parts of such transplanted anterior pituitaries survive and are vascularised; if later replaced beneath the median eminence they support normal endocrine function (Harris & Jacobsohn, 1952), and normal anterior pituitary cytology is restored (Nikitovitch-Winer & Everett, 1959).

As well as prolactin, other trophic hormones may be secreted by ectopically transplanted anterior pituitaries; testicular activity may be maintained, but not if these animals also bear testosterone implants in the median eminence (Smith & Davidson, 1967). The peripheral plasma of hypophysectomised rats may contain LRF and CRF, but not if lesions are made in the median eminence (Nallar & McCann, 1965; Brodish & Long, 1962). There is some uncertainty about the relationship between the hypothalamus and ACTH secretion by ectopic anterior pituitaries. Dexamethasone implants in, or lesions of, the median eminence inhibit ACTH secretion in normals, but do not inhibit ACTH secretion in animals with ectopic anterior pituitaries (Kendall & Allen, 1968). Removal of the whole hypothalamus does abolish ACTH secretion in these animals (Sirett & Kendall, 1969).

Hypophysectomised rats with ectopic anterior pituitaries have been treated with median eminence extracts or LH-RH by systemic injections or perfusion close to the transplantation site. ACTH or FSH and LH are secreted by the transplanted anterior pituitaries during such treatment (Evans & Nikitovitch-Winer, 1969; Evans, 1972; Debeljuk et al, 1973). The grafts secrete ACTH immediately in response to infusion of median eminence extracts (Sirett & Kendall, 1969).

These experiments demonstrate convincingly that the anterior pituitary functions normally only when it is exposed to a trophic influence from the median eminence; this trophic effect may be exerted by releasing factors.

2.2 Hypothalamus as the site of steroid hormone feedback: possible sites of releasing factor neurones

The effects of LH-RH on grafted anterior pituitaries, and the inhibitory effects of testosterone implants in the median eminence on gonadotrophin secretion by these grafts strongly suggest that the hypothalamus mediates the negative feedback effects of steroid hormones on anterior pituitary function.

The amount of LRF in the hypothalamus increases after gonadectomy, and is decreased by testosterone or estrogen (Piacsek & Meites, 1966). Similarly, the amount of CRF in the median eminence increases after adrenalectomy and can be decreased by hydrocortisone (Vernikos-Danellis, 1965). The usual comments about the relationship between content

of hormone and secretion apply. It has been argued that because the amount of releasing factor in the median eminence increases when anterior pituitary trophic hormone secretion increases then releasing factor secretion must also be increased (Piacsek & Meites, 1966). This argument is circular in relation to the role of the hypothalamus in negative feedback regulation. Increased LRF secretion after gonadectomy is indicated by the presence of LRF in the peripheral plasma of ovariectomised, but not normal, rats (Burger et al, 1972). LRF is present in portal blood collected from the cut pituitary stalk of ovariectomised rats, and the concentration of LRF is reduced by estrogen (Fink & Harris, 1970).

Other attempts to demonstrate the site of steroid feedback are difficult to interpret because lesions of the median eminence interfere with the blood supply of the anterior pituitary, and from implants of hormone or antagonists in the hypothalamus some of the implanted material may diffuse into the portal vessels and directly affect the anterior pituitary (Bogdanove, 1963b) or reach the portal vessels by the CSF (Kendall et al, 1969).

Many investigators have used electrical or electrochemical stimulation of various hypothalamic areas to study the general relationship between the hypothalamus and anterior pituitary, and to localise areas concerned with regulation of specific endocrine glands (e.g. Harris, 1948; De Groot & Harris, 1950; Everett, 1965; Quinn, 1969). These studies demonstrate mechanisms which could be used for feedback regulation, but not that they are so used.

The Hungarian school (Szentagothai et al, 1968a,b) developed two techniques which have been used extensively to localise hypothalamic areas which regulate the anterior pituitary. These techniques are selective hypothalamic deafferentation and implantation of fragments of endocrine tissue. Others with the same aims have implanted small quantities of pure hormone or made classical lesions in various hypothalamic areas.

2.2.1 Hypophyseotropic area of the hypothalamus

The Hungarian group advanced the concept of the hypophyseotropic area of the hypothalamus, defined as the medial and ventral part of the hypothalamus which supports normal cytology and function of fragments of anterior pituitary implanted into it (Halasz et al, 1962; Flament-Durand, 1966). Maintenance of such grafts does not depend on vascularisation of the implants by the median eminence portal vessels, but is supposed to be caused by diffusion of trophic factors from surrounding neurones (Halasz et al, 1962). Such grafts respond to castration (Szentagothai et al, 1968a) and after unilateral ovariectomy ovarian compensatory hypertrophy occurs (Halasz et al, 1965). However, these implants respond to adrenalectomy only if the anterior pituitary fragments are in contact with the ventricular surface of the median eminence (Halasz et al, 1965).

The hypophyseotropic area includes the arcuate nucleus, possibly the medial part of the ventromedial nucleus, and extends into the anterior hypothalamic area. If the nervous connections of the hypophyseotropic area with the

rest of the brain are severed then testicular and adrenocortical function continue for at least a month, although ovulation stops (Halasz & Pupp, 1965). Ovulation continues if neural connections with only the anterior hypothalamus are left intact (Halasz & Gorski, 1967; Koves & Halasz, 1970); and in particular a ventral and midline pathway converging in the suprachiasmatic region is adequate for an ovulatory surge of LH to be released in response to electrical stimulation of the medial preoptic area (Tejasen & Everett, 1971).

The autonomy of hypothalami which have had neural connections with other brain areas severed has been widely studied. In male rats testicular weight is decreased after complete deafferentation of the ventral hypothalamus (Davidson & Bloch, 1969); within six weeks the concentrations of FSH, LH and testosterone in peripheral blood decrease (Blake et al, 1973).

In similarly prepared female rats the concentration of LH in peripheral blood decreases, but if the afferent input from only the anterior hypothalamus is interrupted the concentration of LH in peripheral blood increases (Weiner et al, 1972). But in the male normal gonadotrophic and gonadal hormone secretion does not depend on an input from the anterior hypothalamus to the ventral hypothalamus, but on afferent connections via the posterior hypothalamus (Voloschin et al, 1968; Blake et al, 1973); although normal testicular weight is not maintained in male rats with complete hypothalamic deafferentation, anterior or posterior deafferentation alone has no effect (Davidson &

Bloch, 1969). In the female with a ventral hypothalamic island the anterior pituitary responds to bilateral ovariectomy, but the responses to unilateral ovariectomy are impaired; although in the former the pituitary LH content rises less than when normal females are ovariectomised (Halasz & Gorski, 1967). The effects of gonadectomy in males with hypothalamic islands have not been reported.

The amounts of releasing factors have been measured in hypothalamic islands including the suprachiasmatic and arcuate regions, but excluding the PV nuclei (Mess et al, 1970). The total content of LRF in these islands is increased 8 and 15 days after preparation of the island, but FRF disappears. There is little change in the amounts of FSH or LH in the anterior pituitaries of these animals. The conclusion that FRF is synthesised outwith the island and LRF within (Mess et al, 1970; Tima, 1971) ignores the possibility that changes in synthesis or secretion of releasing factors could produce the same results. Similarly it is unnecessary to propose separate synthesising factors (Mess et al, 1970) to explain the unaltered amounts of gonadotrophins in the anterior pituitary. Indeed in the light of what is known continued secretion of FRF by the islands would be the obvious conclusion.

The literature on hypothalamic island function in relation to ACTH secretion is confused because stress responses are not always distinguished from changes in the feedback control mechanism, and dexamethasone is frequently used to test the integrity of feedback mechanisms: dexamethasone is not a normal product of the adrenal cortex,

and any results obtained with this substance may or may not have physiological significance.

Rats with forebrains removed except for the hypothalamus or median eminence secrete adrenocorticoid hormones in amounts similar to animals with intact forebrains, and respond to ether stress andto dexamethasone (Kendall et al, 1964; Dunn & Critchlow, 1969). The less severe procedure of hypothalamic deafferentation yields similar results; the diurnal rhythm of adrenocorticoid secretion is lost and basal secretion is increased, but such preparations, with the dorsal cut through or ventral to the ventromedial nucleus, respond to ether stress and to unilateral adrenalectomy (Halasz et al, 1967; Voloschin et al, 1968). Other stressful stimuli may not be effective (Feldman et al, 1970; Makara et al, 1970). The deafferented ventral hypothalamus is adequate for dexamethasone inhibition of ACTH secretion (Palka et al, 1969). It is difficult to draw conclusions from experiments with isolated pituitary islands, formed by removing the forebrain including the median eminence; although these preparations secrete ACTH at a low rate, the anterior pituitaries are partially infarcted (Kendall et al, 1964).

The general conclusion from these studies is that the ventral hypothalamus, including only the median eminence, arcuate nucleus and possibly the ventromedial nucleus, is an adequate unit with respect to the feedback regulation by steroids of gonadotrophin and ACTH secretion. It follows that the cells which synthesise releasing factors are located within this hypophyseotropic area, although there

may be cell bodies of similar neurones elsewhere in the nervous system. Alternatively the hypophyseotropic area may have a trophic action, not related to releasing factors, on the anterior pituitary; this trophic action may permit a direct action of steroids on the anterior pituitary.

Certainly the activity of the hypophyseotropic area is normally modified by the release regulating system (Szentagothai et al, 1968c); some components of this system also respond to circulating steroids.

2.2.2 Effects of hypothalamic lesions

The anterior hypothalamus is part of the release regulating system. Lesions of the anterior hypothalamus in female rats produce similar effects on ovarian function to anterior deafferentation of the ventral hypothalamus, while the normal anterior pituitary cytological responses to unilateral or bilateral ovariectomy are prevented (Flerko & Bardos, 1961; Taleisnik & McCann, 1961). Anterior pituitary histological changes occur after bilateral ovariectomy in such animals if the suprachiasmatic nucleus is spared, and can be prevented by estrogen (Bogdanove, 1963a). However, in female rats with bilateral lesions of the anterior hypothalamic, medial preoptic and suprachiasmatic nuclei the concentrations of LH and FSH, measured by radioimmunoassay, in peripheral plasma increase in the first 14 days after ovariectomy; this can be prevented by estrogen. These gonadotrophic hormone changes do not occur in animals with lesions in the median eminence region, involving the arcuate nucleus but not the portal

vessels; but lesions of only the PV nuclei do not alter FSH secretion and lesions of the dorsimedial, posterior hypothalamic or ventromedial nuclei have variable and insignificant effects on ovariectomy responses (Bishop et al, 1972a,b). In male rats, combined lesions of the suprachiasmatic, medial preoptic and anterior hypothalamic nuclei reduce the secretion of LH in response to castration: LH secretion is not increased after castration in male rats with median eminence lesions, but PV lesions have no effect (Bishop et al, 1972a). However, lesions between the optic chiasm and PV nuclei prevent the inhibitory effects of testosterone on gonadotrophin secretion after castration (Flerko & Illei, 1957). Median eminence lesions result in testicular atrophy (McCann, 1953) and lesions in the ventral hypothalamus anterior to the mamillary bodies prevent normal testicular development in young rats (Corbin, 1963). Implants of cycloheximide in the PV nuclei of male rats have been claimed to decrease the amount of FRF in the ipsilateral side of the median eminence (Motta et al, 1970b): presumably cycloheximide may diffuse into the CSF from this site and act directly on the median eminence. Lesions of the PV nuclei decrease the amount of FRF in the median eminence, but do not alter the amount of LRF. Lesions of the suprachiasmatic nuclei markedly decrease the amountof LRF, but not FRF, in the median eminence; lesions of the arcuate nuclei have similar, but less marked, effects (Motta et al, 1970b). The results of these studies are consistent with regulation

of FSH secretion by an area in the anterior hypothalamus, near to the PV nuclei, and regulation of LH secretion by the suprachiasmatic region (Flerko, 1970).

Bilateral anterior hypothalamic lesions do not prevent adrenocortical responses to operative or ether stress, but median eminence lesions do (McCann, 1953; Brodish, 1963) and partially prevent adrenal compensatory hypertrophy (Ganong & Hume, 1954; Fulford & McCann, 1955). The amount of CRF in the hypothalamus is not reduced by lesions of the PV or suprachiasmatic nuclei, but is increased by lesions of the arcuate or ventromedial nuclei, or even of the median eminence (Mess et al, 1970).

2.2.3 Hormone implants in the hypothalamus

Grafts of ovarian fragments in the female anterior hypothalamus between the optic chiasm and paraventricular nuclei result in uterine atrophy: implants in the anterior pituitary are ineffective (Flerko & Szentagothai, 1957). Testosterone secretion is inhibited by implants of testosterone placed in the arcuate nucleus, but less effectively by implants in the suprachiasmatic nucleus, and not at all by implants in the anterior hypothalamus, paraventricular nucleus or anterior pituitary (Lisk, 1962; Davidson & Bloch, 1969). Conversely implants of a testosterone antagonist, cyproterone, stimulate LH secretion in male rats, but are only effective in the sites at which testosterone inhibits gonadotrophin secretion (Davidson & Bloch, 1969). Others have found that implants of estrogen prevent anterior pituitary cytological changes after ovariectomy

if the implants are placed in the median eminence, but not in the anterior pituitary (Kanematsu & Sawyer, 1963). Bogdanove (1963b) has argued that estrogen can directly affect the anterior pituitary, but the effects are localised to the zone immediately surrounding the implant. He explained the effects of median eminence implants by suggesting that the portal system is simply a way of efficiently distributing hormone from these implants to the anterior pituitary. Others have found median eminence or anterior pituitary implants of estrogen to be equally effective in inhibiting LH and FSH secretion (Chowers & McCann, 1967). In vitro estradiol can stimulate LH secretion by the anterior pituitary, but inhibits the release of LRF from median eminence extracts (Schneider & McCann, 1969, 1970a,b,c). Conversely, testosterone depresses the stimulatory action of exogenous LH-RH on gonadotrophin secretion in intact male rats (Debeljuk et al, 1972). Gonadotrophin secretion by ectopic anterior pituitaries, as well as gonadotrophin secretion in intact animals, is inhibited by implants of testosterone in the median eminence: because such implants do not have systemic androgenising effects this is evidence that the feedback action of testosterone is on the hypothalamus (Smith & D vidson, 1967).

Implants of hydrocortisone or dexamethasone in the median eminence decrease basal corticosterone secretion, adrenal weight and compensatory adrenal hypertrophy; implants in some other areas of the forebrain have similar effects, but implants in the anterior pituitary do not (Chowers et al, 1963; Davidson & Feldman, 1963). The

effects of implants at sites other than the median eminence can be explained by diffusion into the CSF and transport to the median eminence (Kendall et al, 1969). Implants of dexamethasone in the median eminence decrease its CRF content and the amount of ACTH in the anterior pituitary, whereas intrapituitary implants of dexamethasone also decrease ACTH storage and secretion, but increase the amount of CRF in the median eminence. This effect may be due to an inhibitory action of ACTH on the median eminence (Chowers et al, 1967). Others have found that dexamethasone acts directly on the anterior pituitary and inhibits responses to CRF, andthey explain the effects of implants in the median eminence according to Bogdanove's (1963b) paradox (Russell et al, 1969; Gonzalez-Luque et al, 1970). Dexamethasone inhibits anterior pituitary responses to CRF in vitro (Arimura et al, 1969). The acute effects of systemically administered dexamethasone, however, are to inhibit CRF secretion without altering anterior pituitary responses to CRF (Smelik, 1969; Hedge & Smelik, 1969).

It would be useful if these studies were to be repeated with physiological steroids.

In summary, gonadal and adrenal steroids have inhibitory actions on the ventral hypothalamus, and act directly on the anterior pituitary. Considered with other evidence for the dependence of anterior pituitary responses to gonadectomy or adrenalectomy on contact with the median eminence, and with the changes in releasing factor content of the median eminence and the effects of releasing factors on ectopic

anterior pituitaries (this section - 2.1), it seems likely that the negative feedback control of the anterior pituitary by the gonads and adrenal cortices is effected by releasing factors.

The studies with hypothalamic islands and lesions indicate areas in which the intact hypophyseotropic system is likely to be found, and the studies with implants of steroids indicate the localisation of steroid sensitive elements of this system. The cells which synthesise releasing factors may or may not be also sensitive to steroids; other cells may have this function and be linked to the cells which synthesise releasing factors.

2.2.4 Electrophysiology: Isotopically labelled steroids

Localisation of steroid receptive neurones with isotopes or electrophysiological techniques indicates no more than that the cells so identified are sensitive to steroids. Such neurones may be part of the release regulating system, and be concerned in rhythmic or environmentally induced changes in anterior pituitary function, or they may be concerned in sex or stress behaviour. The distribution of neurones which have specific intracellular receptors for gonadal or adrenocortical steroids is far wider, extending even into the spinal cord, than the distribution of neurones essential for negative feedback control of the anterior pituitary (e.g. Stumpf, 1968; McEwen & Weiss, 1970; Zigmond & McEwen, 1970; Keefer et al, 1973; Sar & Stumpf, 1973). These studies only demonstrate neurones in which synthetic mechanisms may be directly altered by steroid

hormones, but exclude neurones on which synaptic activity may be modified by steroids (vide infra). Similarly distributed are neurones in which electrical activity is sensitive to steroids (e.g. Ramirez et al, 1967; Steiner, 1970). These studies at least demonstrate biochemical and electrophysiological means by which steroids can modify neuronal activity.

2.3 Organisation of the hypophyseotropic area

There are three ways in which the hypophyseotropic area could be organised:

1. The median eminence may contain nerve endings from neurones which secrete releasing factors but their cell bodies are widely distributed in the hypothalamus, limbic system and midbrain (Stumpf, 1970; Knigge & Scott, 1970).

or

2. All the neurones which secrete releasing factors are entirely within the hypophyseotropic area; their cell bodies are in the arcuate or ventromedial nuclei and their secretory activity is modulated by neural input from other areas (e.g. Szentagothai et al, 1968c).

or

3. The releasing factors are synthesised by ependymoglial cells in the median eminence, and secretory activity of these cells may be modified by transmitters released from nerve endings in the median eminence (Knowles, 1969).

The first concept is inherently unattractive, and the results of the experiments with hypothalamic islands and lesions do not support it. There is no evidence which distinguishes the two other concepts: the presence, release or synthesis of releasing factors has not been associated with any particular cell type.

Certainly releasing and inhibiting factors have been isolated from the hypothalamus and some have been synthesised (e.g. Matsuo et al, 1971; Harris, 1972), but although antibodies to releasing factors can be raised (Jeffcoate & Holland, 1973) no attempt at immunofluorescence localisation in the hypothalamus has been reported.

Wherever the cell bodies of the releasing factor neurones which regulate anterior pituitary activity may be, some of their endings must be in the median eminence. It has, however, been suggested that the CSF may be the route of transport to the median eminence of releasing factors secreted elsewhere (Knigge & Scott, 1970): this mechanism probably would not account for rapid responses of the releasing factor system. In Golgi preparations axons from arcuate neurones enter the median eminence as the tubero-infundibular tract and end in its external zone: axons from neurones in the anterior hypothalamus or ventromedial nucleus probably do not contribute to the tubero-infundibular tract (Szentagothai et al, 1968d). Two or three days after deafferentation of the ventral hypothalamus degenerating nerve endings are not seen in electron micrographs of the median eminence if the island contains only the suprachiasmatic

and arcuate nuclei. If either of these areas is damaged, however, degenerating endings are seen in the median eminence (Rethelyi & Halasz, 1970). Studies with microfluorescent techniques indicate that there is a ventral input, lateral to the arcuate nucleus, of noradrenergic axons to the median eminence (Bjorklund et al, 1970). Some ventromedial axons end in the arcuate nucleus (Szentagothai et al, 1968d) and if the ventromedial or arcuate nucleus is electrically stimulated then antidromically propagated action potentials can be recorded in the medial preoptic and anterior hypothalamic nuclei (Dyer & Cross, 1972).

Nerve endings in the external zone of the median eminence contain vesicles and granules smaller than those of SO or PV neurones (Rinne & Arstila, 1965; Kobayashi et al, 1970). The median eminence contains dopamine, noradrenaline and 5-hydroxytryptamine (Fuxe, 1964; Kordon & Glowinski, 1972). Monoamine fluorescence is greatest in the external zone; the endings of axons which originate in the arcuate nucleus contain dopamine (Fuxe, 1964; Hokfelt, 1967), noradrenaline is in the endings of axons of neurones with cell bodies in the midbrain (Bjorklund et al, 1973), and 5-hydroxytryptamine is probably contained in endings of raphe neurones (Kordon & Glowinski, 1972).

Although releasing factors are probably stored within granules in the median eminence (Mulder, 1970; Ishii, 1970) their origin is not known. Electrical stimulation of the preoptic area or median eminence of female rats in proestrus

increases the amount of LRF in hypothalamo-hypophyseal portal blood (Harris & Ruf, 1970). Exocytotic profiles in nerve endings in the external zone of the median eminence have been described, but the nature of the contents of the discharging vesicles is not known. It is notable that these profiles are more frequent in areas of the membranes of nerve terminals adjacent to other endings, including those of ependymal cells, than adjacent to the pericapillary basement membrane (Stoeckart et al, 1972). Release of PIF and CRF, by incubation in a medium with a high concentration of potassium, from synaptosomes isolated from the median eminence (Bradford et al, 1972), is consistent with either a neuronal or ependymal origin of releasing factors.

Against the concept of secretion of releasing factors by only arcuate neurones is the lower threshold for secretion of an ovulatory pulse of LRF to electrical stimulation of the medial preoptic region compared with the median eminence region (Everett, 1965; Terasawa & Sawyer, 1969). Electrical activity in the medial preoptic area and the median eminence but not the arcuate nucleus is increased by uterine cervical stimulation, which causes reflex ovulation (Blake & Sawyer, 1972a). However, after ovariectomy no further increase in LH secretion can be obtained by electrical stimulation of the medial preoptic area, arcuate nucleus or median eminence which indicates that all available LRF secreting neurones are maximally active after ovariectomy (Clemens et al, 1972); these effects could be due to absence of a facilitatory action

of estrogen on the medial preoptic area (Neill et al, 1971; Velasco & Rothchild, 1973) which may contain a pool of LRF neurones. LRF is not secreted in response to electrical stimulation of the medial preoptic area or median eminence of rats in estrus; but this is consistent with depletion of LRF after the proestrus LH surge (Harris & Ruf, 1970), or with removal of a facilitatory action of estrogen. Stimulation of the medial preoptic area in intact male rats causes LH and LRF to be secreted (Quinn, 1966; Burger et al, 1972; Velasco & Rothchild, 1973), but the effects in castrated males have not been reported.

LRF and FRF are found only in the ventral and medial hypothalamus, including the suprachiasmatic region, although most activity is found in the median eminence, including the arcuate nuclei (Watanabe & McCann, 1968; Schneider et al, 1969; Crighton et al, 1970). In the rabbit and dog, LRF activity is found also in the reticular formation, amygdala and anterior or posterior hypothalamus (Endroczi & Hilliard, 1965): but the usual assay system for LRF was not used in this study. In the present context, the crucial question is whether or not LRF found in these areas can be released by the median eminence (vide infra). In the hypothalamus CRF is confined to the median eminence (Vernikos-Danellis, 1964), and although material with CRF activity is present elsewhere in the brain (De Wied, 1968), this may not be released in the median eminence. Releasing factors may function as transmitters or modulators at orthodox synapses in the nervous system; by analogy, for example, with noradrenaline which is both a synaptic transmitter

and a neurohormone, or with ADH and oxytocin (Section I - 2.4.2).

Monoaminergic and cholinergic mechanisms have long been recognised tobe important in the control of gonadotrophin secretion (Everett, 1964). Fuxe has argued that dopamine is secreted by arcuate neurone endings in the median eminence and inhibits secretion of gonadotrophin releasing factors (Fuxe et al, 1968a,b); but his technique involves a battery of pharmacological agents and a subjective measurement of fluorescence to estimate dopamine content. His conclusions are in conflict with measurements of changes in the amounts of catecholamine in the arcuate nucleus after electrical stimulation of the arcuate nucleus or medial preoptic area (Keller & Lichtensteiger, 1971). FRF and LRF are secreted by the median eminence in response to dopamine in vitro and in vivo; other monoamines are ineffective (Schneider & McCann, 1969, 1970a,b; Kamberi et al, 1969, 1970a,b). In vitro studies have been used to show that dopamine acts on α receptors in the ventral hypothalamus and has no direct action on the anterior pituitary; the action of dopamine is blocked by estrogen by a mechanism which involves protein synthesis (Schneider & McCann, 1970c). It is possible that dopamine is converted to noradrenaline which then stimulates LRF release (Donoso et al, 1971). Intraventricular infusion of 5-hydroxytryptamine inhibits LH secretion in ovariectomised females, and in general it probably inhibits gonadotrophin secretion by an action on the median eminence (Schneider &

McCann, 1970a; Kordon & Glowinski, 1972). Others have suggested that the major site of action of monoamines is in the anterior hypothalamic area; that is, on part of the release regulating system for gonadotrophin secretion (Rubinstein & Sawyer, 1970). Electrical activity in the median eminence is increased after intraventricular injections of adrenaline, dopamine or noradrenaline but the active cells have not been identified (Weiner et al, 1971). Acetylcholine stimulates LRF and FRF release from the median eminence in vitro, and does not act directly on the anterior pituitary. Atropine decreases the spontaneous release of FRF and LRF from the median eminence in vitro (Motta & Martini, 1972).

Cholinergic and monoaminergic mechanisms are also implicated in the regulation of ACTH secretion (Marks et al, 1970). In general acetylcholine and monoamines may respectively stimulate and inhibit CRF secretion (Marks et al, 1970; Fuxe et al, 1970). Implants of atropine in the anterior hypothalamus block ACTH secretion in response to operative stress: the effects on negative feedback mechanisms have not been reported (Hedge & Smelik, 1968). In vitro studies of the effects of transmitters on CRF release from isolated ventral hypothalami have not been reported.

The immediate effect of systemically administered dexamethasone is to inhibit the secretion of CRF in response to stress, but CRF is secreted in response to ADH for a short period. This selective effect of dexamethasone has

been interpreted as an initial synaptic action of dexamethasone, followed by inhibition of CRF synthesis; in the initial phase the response to ADH is explained by an action of ADH at the endings of CRF neurones in the median eminence (Hedge & Smelik, 1969).

This model of the CRF neurone is similar to that of the LRF neurone (Schneider & McCann, 1970c); the perikarya, probably in the arcuate nucleus, may respond to orthodox synaptic activity, modified by the release regulating system, while synaptic activity and releasing factor synthesis may be modified by steroid hormones. Finally the releasing factor nerve endings may be bathed in monoamines in the external zone of the median eminence or acted upon by monoamines released at axo-axonic contacts, although axo-axonic contacts are not a significant feature of the ultrastructure of the median eminence (Knigge & Scott, 1970): the relative amounts of catechol- and indoleamines free in this zone may gate releasing factor secretion (Scharrer, 1965; Kordon & Glowinski, 1972). It is not known at which stages in this hypothetical system the negative feedback effects of steroid hormones are exerted. It is likely that the cells which secrete releasing factors are localised to the hypophysiotropic area. If these cells are not neurones but ependymoglial cells in the median eminence, then the monoaminergic neurones which innervate the median eminence would function as orthodox neurones, and have their endings on the secretory cells of the median eminence. The median eminence could then be compared with other glands regulated by monoaminergic innervation (Scharrer, 1965).

It has been recently claimed that unlike ADH and oxytocin the releasing factors are synthesised enzymatically, and not directly by ribosomes. The evidence is derived from in vitro studies on hypothalamic fragments in which ribosome dependent protein synthesis is inhibited, or from which ribosomes have been removed (Mitnick & Reichlin, 1972; Mitnick et al, 1973; Reichlin & Mitnick, 1973). The cells which contain the synthesising enzymes have not been defined.

2.4 Metabolic changes in putative releasing factor neurones

The experimental approach of the present investigation has been used previously. Szentagothai's group (1968e) have measured neuronal nuclear diameters in fixed sections of hypothalami after removal of various endocrine glands or hormone treatment. This parameter can only be interpreted as indicating some undefined change in the state of the neurone: endocrine gland extirpation and hormone treatment often change neuronal nuclear volume in the same direction. Ifft (1964) measured nucleolar volumes of hypothalamic neurones after similar procedures, and generally found widespread changes.

Estradiol implanted in the median eminence, but not elsewhere, reduces the nucleolar volume of arcuate neurones (Lisk & Newlon, 1963); other neurones were not studied.

Subjective interpretation of electron micrographs of arcuate neurones 1-6 months after castration indicate increased RNA and protein synthesis (Zambrano & De Robertis,

1968b), but apart from the magnocellular nuclei, other areas were not studied. Whorled bodies of membrane continuous with the endoplasmic reticulum appear in the perikarya of arcuate neurones one month after castration (Brawer, 1971); other neurones were not studied.

Although an autoradiographic technique for detecting changes in RNA synthesis in discrete brain areas has been devised (MacKinnon et al, 1969) such changes have not been sought after adrenalectomy or castration. Measured by the traditional technique of grain counting, neonatal androgen treatment of female rats decreases RNA synthesis in all brain areas except the medial preoptic and medial amygdaloid nuclei (Clayton et al, 1970). However the average grain counts reported were between 1 and 2 grains per neuronal nucleus, which seem low for reliable measurement of isotope incorporation. In vitro, estrogen increases RNA synthesis by hypothalamic fragments (Schally et al, 1969). Large doses of estrogen for four months decrease protein synthesis, measured autoradiographically, in the PV, arcuate and periventricular nuclei, but not in neurones of the medial preoptic, anterior hypothalamic, suprachiasmatic or ventromedial nuclei (Litteria & Timiras, 1970).

3. Aims of investigation

The criterion previously defined (this section - 1(5)) was used to identify neurones which may synthesise LRF, FRF or CRF. Changes in nucleolar dry mass of neurones in various hypothalamic nuclei were sought after castration or adrenalectomy. The areas selected for study were those implicated in releasing factor synthesis by the numerous studies discussed.

4. Methods

Male albino rats, three months old, were used to avoid the complicating effects associated with the estrous cycle.

4.1 Castration

4.1.1 General management of animals

These animals were not housed under conditions of controlled illumination or strictly controlled temperature, but were exposed to variations in natural and artificial lighting during normal working days in winter. Environmental temperature was about 20°C.

4.1.2 Operative procedures

Bilateral orchidectomy was performed on animals anaesthetised with ether. Suprapubic skin incisions were made over each spermatic cord; the sheath was incised and the vas deferens, testis, epididymis and fat were pulled through the incision. The testicular vessels were ligated and the caput epididymis separated from the testis which was removed. The epididymis, fat, vas deferens and blood vessels were replaced in the cord, which was closed with a silk suture. Each skin incision was closed with two silk sutures. Sham operation consisted of the above procedure except that the testicular vessels were not ligated and the caput epididymis was not detached from the testis, which was replaced in the spermatic cord with the other contents. Equal numbers of sham operated and castrated rats were prepared. These animals were killed 5, 10, 30 or 45 days after operation; at these times, respectively, 3, 6, 4 and 4 pairs of sham operated and castrated animals were killed. A separate

batch of animals was prepared for PV neurone measurements: six pairs of animals were killed 5 or 10 days after operation. Unoperated controls were not used, with the result that only changes due to castration could be detected. Operations were checked post mortem.

4.1.3 Nucleolar dry mass measurements

The hypothalami were prepared as previously described (Section II - 2). The hypothalamic nuclei from which neuronal nuclear preparations were made were (Figs. 5-14):

medial preoptic, including adjacent parts of the periventricular nucleus,

anterior hypothalamic, including adjacent parts of the periventricular nucleus,

suprachiasmatic,

preoptic, pars suprachiasmatica,

arcuate, anterior to posterior border of pituitary stalk,

ventromedial, pars medialis and pars centralis,

posterior hypothalamic,

paraventricular.

Right and left nuclei were pooled; nucleolar dry mass measurements were made as previously described (Section II - 3). About 6000 nucleolar dry mass measurements were made in this series.

4.2 Adrenalectomy

4.2.1 General management of animals

These animals were kept under controlled conditions as previously described (Section II - 1).

4.2.2 Operative procedures and post-operative care

Bilateral adrenalectomy was performed on animals anaesthetised with ether. Bilateral skin incisions were made in the lumbar region, lateral to the lumbar muscles and immediately inferior to the 13th rib; the muscle lateral to psoas was incised, then the adrenal gland was identified, separated from surrounding fat and avulsed; bleeding was slight. Muscle and skin incisions were closed with silk sutures. Sham operations consisted of the same procedure except that the adrenals were not separated from fat or avulsed.

Groups of three adrenalectomised and three sham operated animals were caged together post operatively. Each cage of animals had free access to a bottle of tap water and a bottle of saline. adrenalectomised rats controlled their own salt intake (Richter, 1936). It was hoped in this way to control possible hyperosmotic stimulation which may result from provision of saline alone.

All the animals were healthy during the post operative period. Pairs of adrenalectomised and sham operated animals were killed together. Two pairs of animals were killed each day for five days, then two pairs every two days for 10 days. Four untreated controls were included in this series. Operations were checked post mortem: one animal was discarded, on day 4, because only one adrenal had been removed.

4.2.3 Nucleolar dry mass measurements

The hypothalami were prepared as previously described (Section II - 2). The hypothalamic nuclei from which

isolated neuronal nuclei were prepared were:

paraventricular,

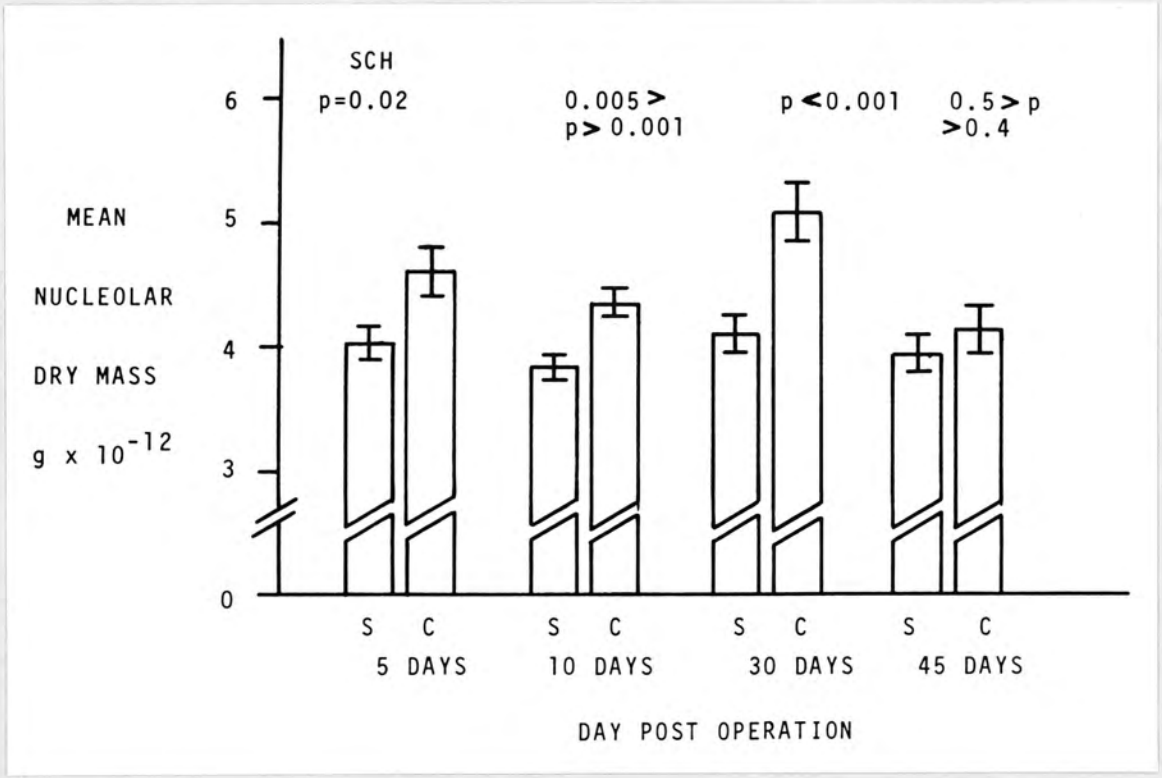
ventromedial, pars medialis and pars centralis,

arcuate, in two parts: anterior or posterior to posterior border of pituitary stalk.

Right and left nuclei were pooled; nucleolar dry mass measurements were made as previously described (Section II - 3). About 4500 nucleolar dry mass measurements were made in this series.

5. Results

5.1 Castration



a

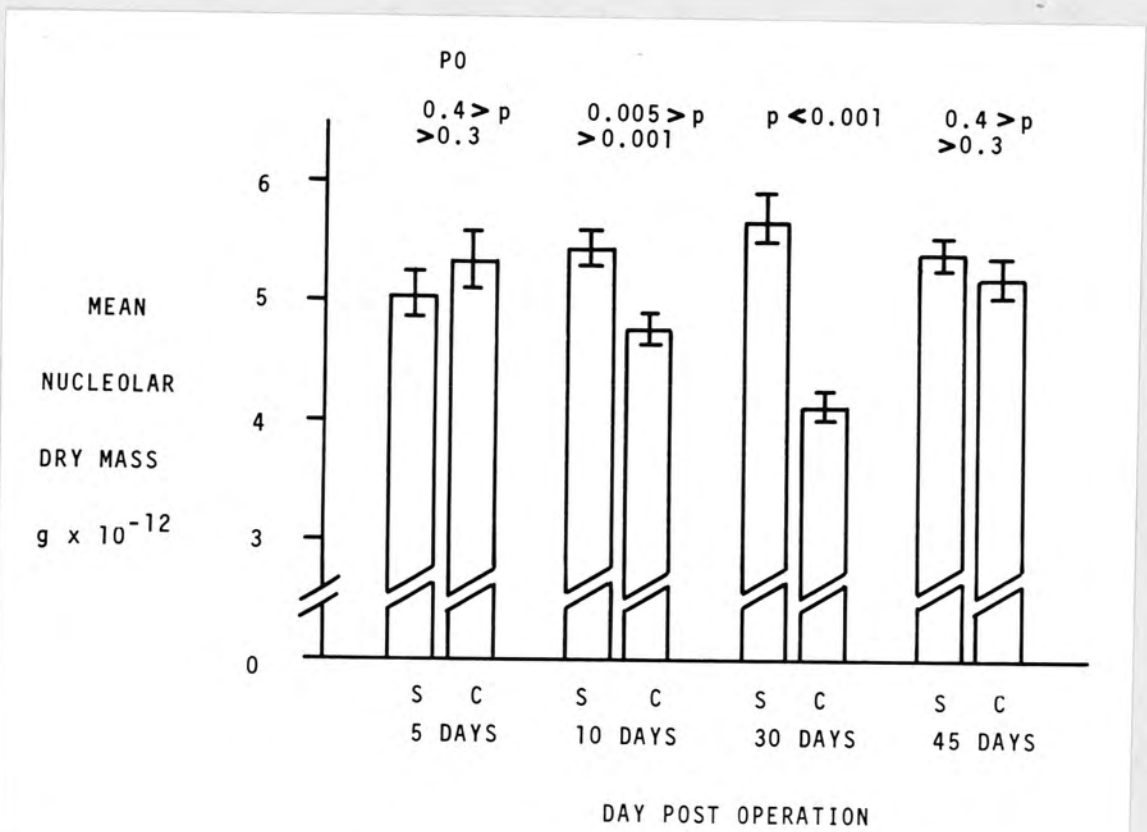
Figure 70 Mean nucleolar dry mass: Castration.
S: Sham operation. C: Castration.

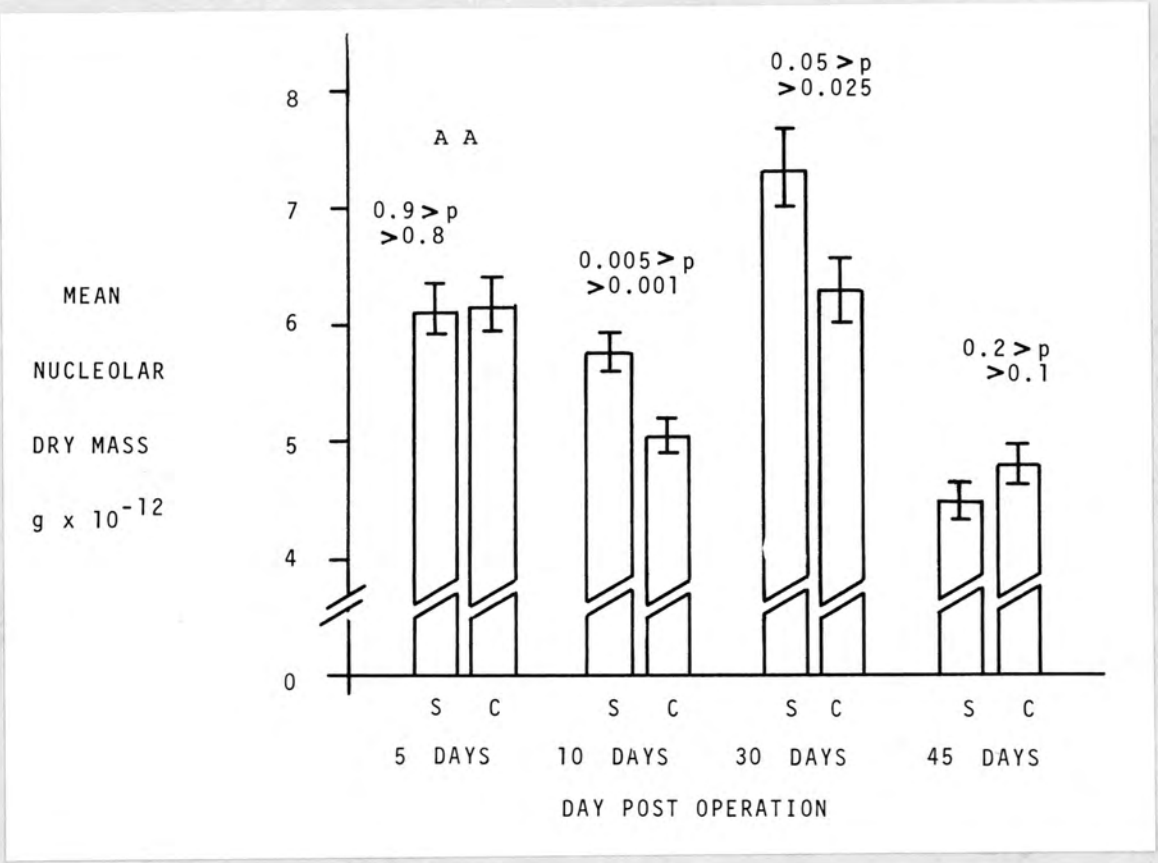
Each vertical line indicates the standard error of the mean.

Sham operated and castrated groups were compared by Student's t-test, and the appropriate p-values are shown above each pair of compared groups.

a. Suprachiasmatic neurones. b. Preoptic (pars suprachiasmatica) neurones.

b



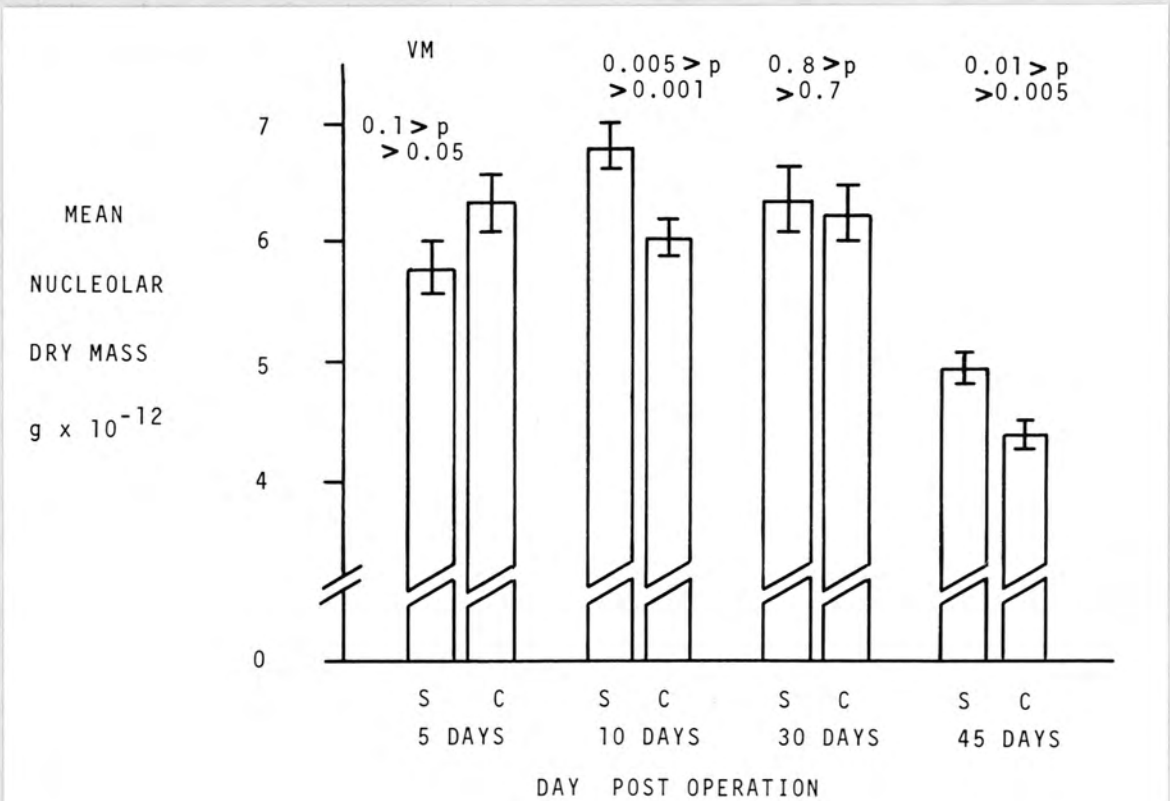


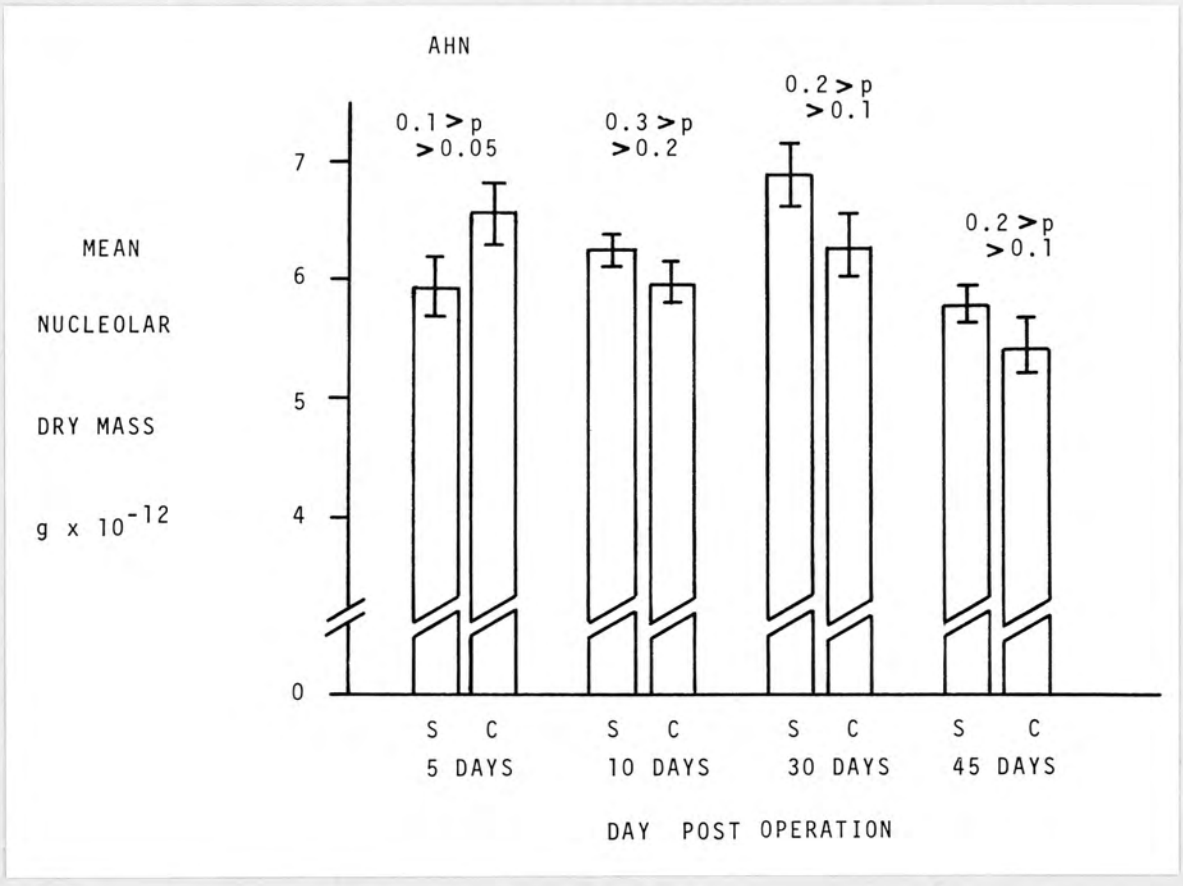
a

Figure 71 Mean nucleolar dry mass: Castration. Details as in Fig. 70.

a. Anterior arcuate neurones. b. Ventromedial (pars centralis, pars medialis) neurones.

b



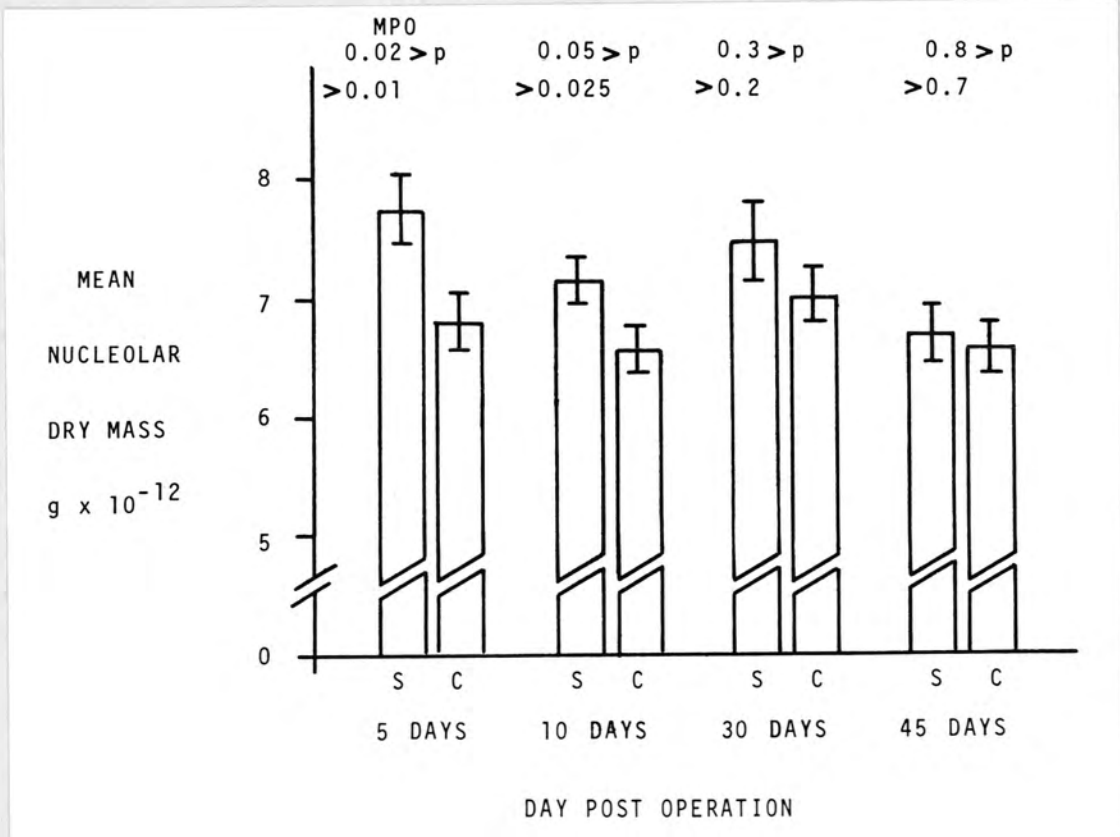


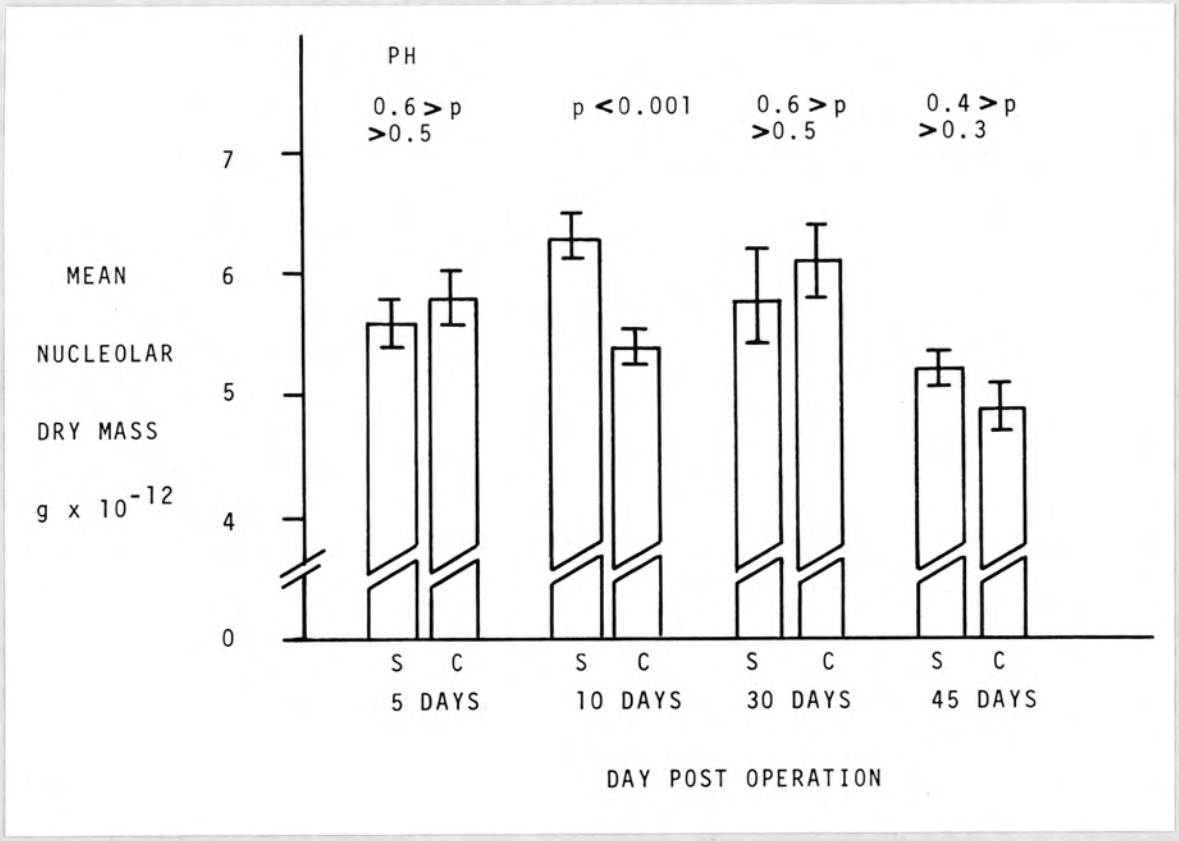
a

Figure 72 Mean nucleolar dry mass: Castration. Details as in Fig. 70.

a. Anterior hypothalamic neurones. b. Medial preoptic neurones.

b



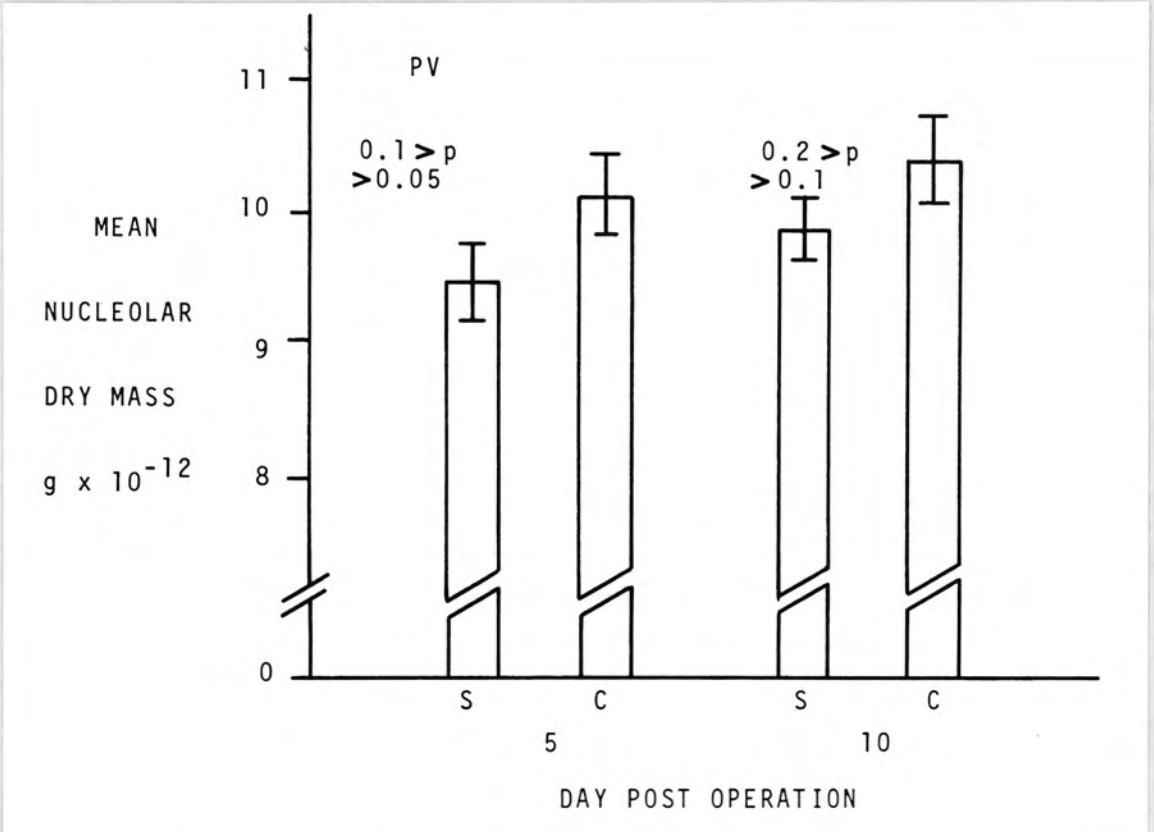


a

Figure 73 Mean nucleolar dry mass: Castration. Details as in Fig. 70.

a. Posterior hypothalamic neurones. b. Paraventricular neurones.

b



5. Results

5.1 Castration

The results are shown in Figs. 70-73. The changes in each hypothalamic area were:

a. Suprachiasmatic nucleus: Mean nucleolar dry mass of these neurones was greater in castrated than in sham operated animals five days after operation and for at least the next 25 days. By 45 days there was no difference between the sham operated and castrated groups (Fig. 70a).

   Statistical comparisons at different times have not been made (see discussion), but in the sham operated group mean nucleolar dry mass did not change with time.

b. Preoptic nucleus, pars suprachiasmatica: Mean nucleolar dry mass of these neurones was not altered by castration at five days after operation, but was decreased in the castrated group at 10 and 30 days, but not 45 days (Fig. 70b). The nucleolar dry mass of the sham operated group did not change with time.

c. Arcuate nucleus, anterior: Mean nucleolar dry mass of these neurones was decreased by castration at 10 and 30 days after operation, but not at 5 or 45 days (Fig. 71a). Mean nucleolar dry mass of the sham operated and castrated groups were lower at 45 days than at other times.

d. Ventromedial nucleus, pars centralis and pars medialis: The effects of castration were time dependent; mean nucleolar dry mass of these neurones was insignificantly increased by castration at five days after operation, but significantly decreased at 10 and 45 days, and not different from sham operated animals at 30 days (Fig. 71b). Mean nucleolar dry mass of the ventromedial neurones of the sham operated and castrated groups were lower 45 days after operation than at other times.

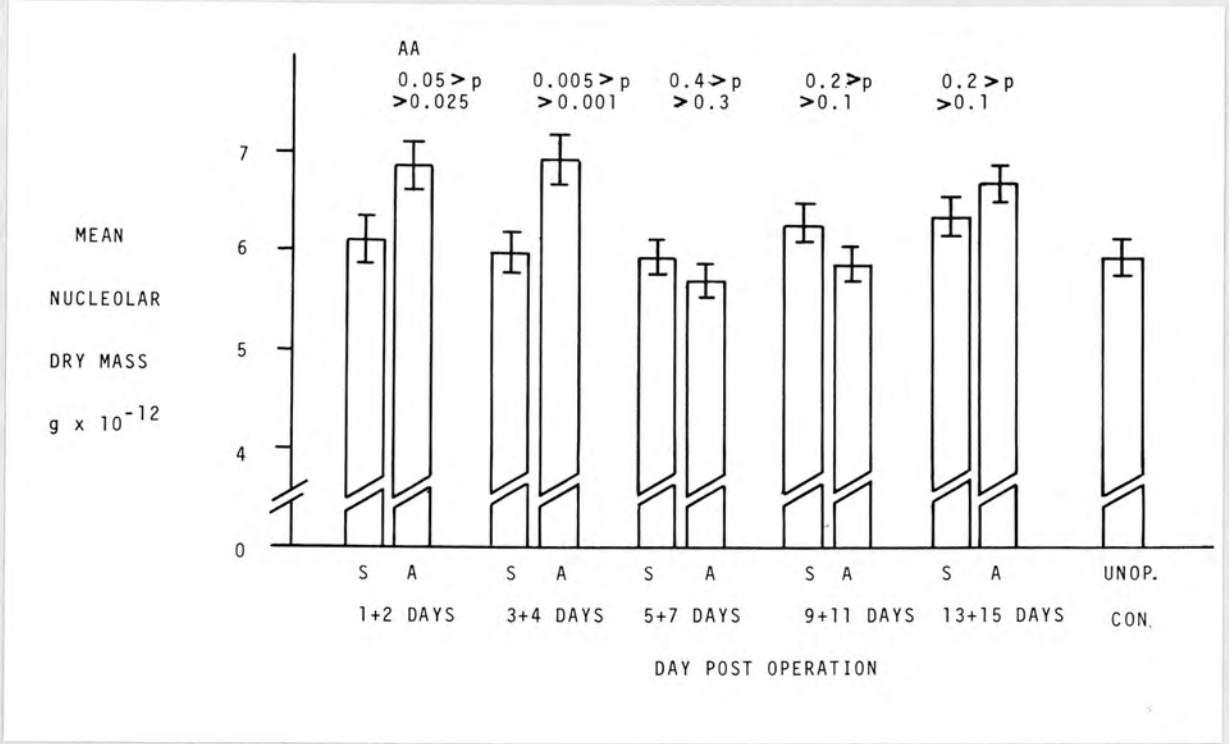
e. Anterior hypothalamic and part of periventricular nuclei: Castration had no significant effect on the nucleolar dry mass of these neurones (Fig.72a).

f. Medial preoptic and part of periventricular nuclei: Mean nucleolar dry mass of these neurones was decreased by castration 5 and 10 days after operation but not at later times (Fig. 72b).

g. Posterior hypothalamic nucleus: Castration decreased the mean nucleolar dry mass of these neurones, but only at 10 days after operation (Fig. 73a).

h. Paraventricular nucleus: Mean nucleolar dry mass of these neurones was not significantly altered by castration, although there was a tendency for this parameter to rise (Fig. 73b).

5.2 Adrenalectomy

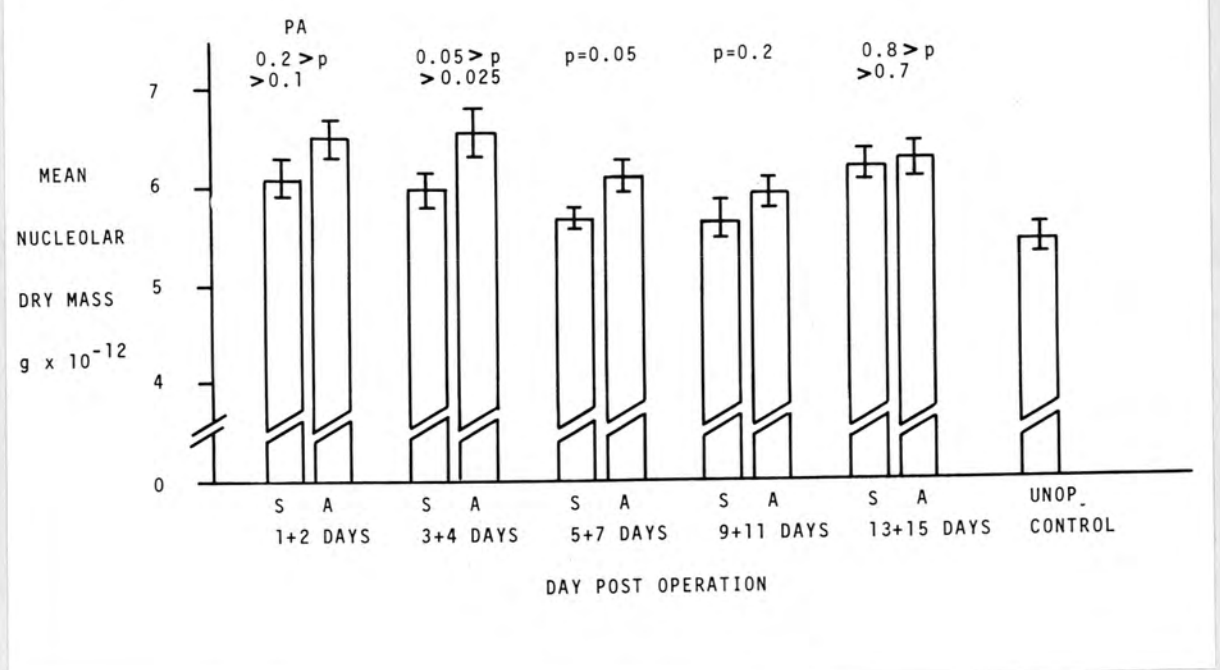


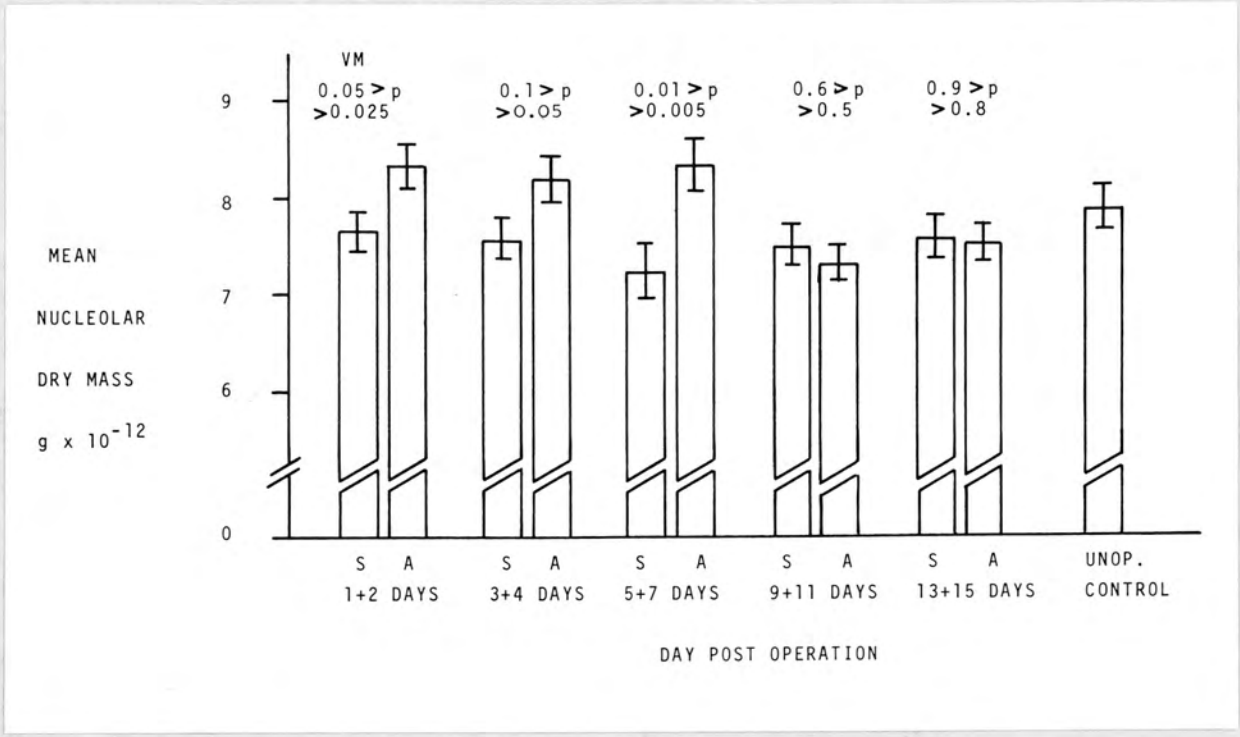
a

Figure 74 Mean nucleolar dry mass: Adrenalectomy. S: Sham operation. A: Adrenalectomy. Data from paired consecutive days have been pooled. Sham operated and adrenalectomised groups were compared by Student's t-test, and the appropriate p values are shown above each pair of compared groups. Comparisons with unoperated controls are shown in Table 33. Each vertical line indicates the standard error of the mean.

a. Anterior arcuate neurones. b. Posterior arcuate neurones.

b





a

Figure 75 Mean nucleolar dry mass: Adrenalectomy. Details as in Fig. 74.

a. Ventromedial (pars centralis and pars medialis) neurones.

b. Paraventricular neurones.

b

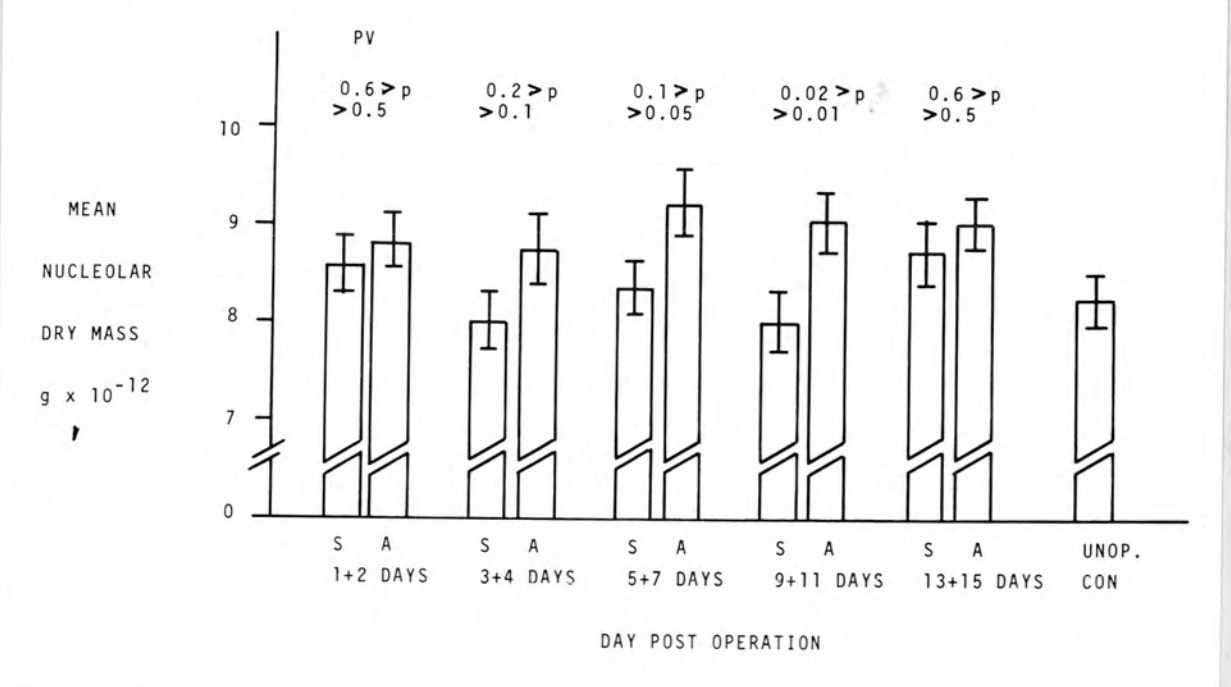


TABLE 33 MEAN NUCLEOLAR DRY MASS: ADRENALECTOMY, SHAM OPERATION. COMPARISONS BY STUDENT'S t-TEST WITH UNOPERATED CONTROLS

Neurones		PV		AA		PA		VM	
Experimental Group		Sham	Adrect.	Sham	Adrect.	Sham	Adrect.	Sham	Adrect.
Days post operation	1+2	$0.5 > p > 0.4$	$0.2 > p > 0.1$	$0.6 > p > 0.5$	$0.005 > p > 0.001$ **	$0.01 > p > 0.005$ **	$p < 0.001$ ***	$0.6 > p > 0.5$	$0.2 > p > 0.1$
	3+4	$0.6 > p > 0.5$	$0.3 > p > 0.2$	$0.9 > p > 0.8$	$0.005 > p > 0.001$ **	$0.02 > p > 0.01$ *	$p < 0.001$ ***	$p = 0.4$	$0.4 > p > 0.3$
	5+7	$0.8 > p > 0.7$	$0.05 > p > 0.025$ *	$0.95 > p > 0.9$	$0.5 > p > 0.4$	$0.3 > p > 0.2$	$0.005 > p > 0.001$ ***	$0.2 > p > 0.1$	$0.2 > p > 0.1$
	9+11	$0.7 > p > 0.6$	$0.05 > p > 0.025$ *	$0.3 > p > 0.2$	$0.9 > p > 0.8$	$0.5 > p > 0.4$	$0.05 > p > 0.025$ *	$0.3 > p > 0.2$	$0.1 > p > 0.05$
	13+15	$0.4 > p > 0.3$	$0.1 > p > 0.05$	$0.2 > p > 0.1$	$0.005 > p > 0.001$ **	$p < 0.001$ ***	$p < 0.001$ ***	$0.5 > p > 0.4$	$0.3 > p > 0.2$

Adrect.: Bilateral adrenalectomy.

5.2 Adrenalectomy

The results are shown in Figs. 74 and 75 and comparisons by Student's t-test with unoperated controls in Table 33. Data from paired consecutive days have been pooled for statistical comparison.

The changes in each hypothalamic area were as follows:

a. Arcuate nucleus, anterior: Mean nucleolar dry mass of these neurones was increased for four days after adrenalectomy, compared with sham operated or unoperated controls. On days 13 and 15 mean nucleolar dry mass was greater in adrenalectomised animals than in unoperated, but not sham operated, controls. Mean nucleolar dry mass in the sham operated group was never different from that in the unoperated group.

b. Arcuate nucleus, posterior: Mean nucleolar dry mass of these neurones was increased after adrenalectomy from the third to seventh post operative days compared with sham operated animals, but was greater at all times than in unoperated animals. In the sham operated controls, mean nucleolar dry mass was greater than in unoperated controls on days 1 to 4 and 13 to 15 after operation.

c. Ventromedial nucleus, pars centralis and pars medialis: The mean nucleolar dry mass of these neurones was increased for 1-7 days after adrenalectomy, compared with sham operated animals; but the nucleolar dry mass of neither the adrenalectomised

nor sham operated animals was different from that of the unoperated animals at any time.

d. Paraventricular nucleus: Mean nucleolar dry mass of these neurones was increased 5-11 days after adrenalectomy, compared with either sham or unoperated animals. Nucleolar dry mass in the sham operated group did not change compared with the unoperated controls.

6. Discussion

Changes in nucleolar dry mass are interpreted as reflecting changes in ribosome and protein synthesis (Section III - 4.1). In previous sections, increased nucleolar dry mass has been the usual finding. In other studies, when mean nucleolar dry mass has been found to decrease to less than normal similar changes in nucleolar nucleic acid content and perikaryal RNA and protein content have occurred: these changes indicate ribosome and protein synthesis at lower rates than normal (Watson, 1970).

The nucleolar changes observed in the present study can be strictly interpreted only in the context of dependence of ribosome synthesis in any of the affected neurones upon intact testes or adrenal glands. However, the findings will be discussed in the context of possible functions of these neurones.

6.1 Castration

According to the concept that increased secretory activity of any neurone will result in increased ribosome synthesis (Section I - 2.6), and because at least some of the neurones which secrete gonadotrophin releasing factors do so at increased rates after castration (this section - 2.2), only the suprachiasmatic neurones, of the areas studied, are a possible source of these releasing factors. Synthetic activity of other areas was decreased or not altered after castration. It remains possible that other changes occurred in these neurones at times not studied.

The anterior pituitary responds rapidly to castration; gonadotrophin secretion and synthesis increase within eight hours and are increased indefinitely (Ramirez & McCann, 1965; Yamamoto et al, 1970; Gay & Dever, 1971). If the hypothesis that steroid hormones act on the hypothalamus to regulate anterior pituitary activity is correct, then secretion of LRF and FRF will increase before LH secretion increases, and continue to be increased indefinitely. The amount of LRF in the median eminence and in hypothalamo-hypophyseal portal blood increases after castration (Piacsek & Meites, 1966; Fink & Harris, 1970), although the temporal relationship of these changes to anterior pituitary responses have not been defined.

a. Suprachiasmatic neurones

The changes in nucleolar dry mass of suprachiasmatic neurones indicate increased ribosome synthesis in these neurones between days 5 and 30 after castration (Fig.70a). LRF and FRF are present in the suprachiasmatic region but concentrated in the median eminence (Crighton et al, 1970; Watanabe & McCann, 1968): such a distribution would be expected if the perikarya of LRF and FRF neurones are in the suprachiasmatic nucleus, by analogy with the distribution of hormones in PV and SO neurones (Section I - 2.2.3). Although LH-RH releases FSH as well as LH (Schally et al, 1971; Arimura et al, 1972), it is probable that there is also a separate FRF (Johansson et al, 1973; Currie et al, 1973; Bowers et al, 1973). This uncertainty is acknowledged in the following discussion. In the male, gonadotrophin

secretion is inhibited by implants of testosterone in the anterior hypothalamus (Flerko & Illei, 1957) and excited by electrical stimulation of the medial preoptic area (Quinn, 1966; Velasco & Rothchild, 1973). However, six weeks after anterior hypothalamic deafferentation, caudal to the suprachiasmatic nuclei, in the otherwise intact male the circulating levels of LH, FSH or testosterone are normal (Blake et al, 1973). But lesions of the suprachiasmatic region diminish the gonadotrophic hormone responses to gonadectomy (Bishop et al, 1972a,b), although two months after such lesions LRF is still present in the median eminence (Schneider et al, 1969). Although axons of raphe neurones terminate in the suprachiasmatic nucleus (Fuxe, 1965), it is not known whether axons from the medial preoptic nucleus or elsewhere in the anterior hypothalamus or limbic system end in the suprachiasmatic nucleus. The suprachiasmatic nucleus is a possible source of LRF or FRF or both; although in the studies cited above the suprachiasmatic region usually includes much of the medial preoptic and anterior hypothalamic nuclei. Lesions do not distinguish between the effects of interrupting fibres passing through a damaged area, or ablating perikarya in the damaged area. Only 'a few' suprachiasmatic neurones have specific ^{3}H-testosterone receptors (Sar & Stumpf, 1973), but, as previously argued, releasing factor neurones need not also be steroid receptors (this section - 2.2.4) while testosterone may not be the only testicular hormone which inhibits anterior pituitary gonadotrophin secretion (Ramirez &

McCann, 1965; Gay & Dever, 1971). Steroid hormones may directly alter RNA and protein synthesis in neurones as in other cells (Hamilton, 1968) or act indirectly via synapses (Woolley & Timiras, 1962), perhaps by modifying catecholamine turnover (Donoso & Cukier, 1968; Beattie et al, 1972). Testosterone implants in the suprachiasmatic region decrease gonadotrophin secretion in male rats (Lisk, 1962), and of particular relevance to the present investigation, 0.5 mg of testosterone injected systemically on alternate days for 12 days into intact male rats decreases the mean nucleolar dry mass of suprachiasmatic neurones (Lloyd, O. L., personal communication). After systemic reserpine or morphine treatment, the mean nucleolar dry mass of suprachiasmatic neurones increases (Lloyd, 1973); these changes are consistent with reduced inhibition of suprachiasmatic neurones by 5-hydroxytryptamine, secreted by the terminals of raphe neurones in the suprachiasmatic nucleus (Fuxe, 1965; Kordon & Glowinski, 1972). 5-hydroxytryptamine inhibits gonadotrophin secretion in ovariectomised females (Schneider & McCann, 1970a): its effects in males have not been reported.

The anterior hypothalamus is sexually differentiated (Barraclough & Gorski, 1961; Raisman & Field, 1971) and in adult rats the mean nucleolar dry mass of suprachiasmatic neurones of male or neonatally androgenised females is greater than that of normal females; there are no sex differences in other hypothalamic areas (Russell, unpubl.). If the suprachiasmatic neurones secrete, or regulate synthesis of, gonadotrophin releasing factors, it follows

that integrated gonadotrophin secretion in males or neonatally androgenised females should be greater than in females: the concentrations of LH and FSH in the plasma of neonatally androgenised adult females are greater than in normal adult females (Barraclough, 1968).

Less than 10 hours after coitus in the rabbit, ultrastructural changes consistent with increased ribosome and protein synthesis occur in suprachiasmatic neurones (Clattenburg et al, 1972): these changes indicate involvement of suprachiasmatic neurones in the ovulatory secretion of LH. Protein synthesis in suprachiasmatic neurones of ovariectomised females is not altered by treatment with large doses of estrogen for four months (Litteria & Timiras, 1970): although this is in apparent conflict with the present results in male rats, the experimental conditions are not comparable.

If enzymatic synthesis of releasing factors (Reichlin & Mitnick, 1973; Mitnick & Reichlin, 1972; Mitnick et al, 1973) can be confirmed, increased nucleolar dry mass in putative releasing factor neurones would be related to increased synthesis of releasing factor synthetases and other products such as carriers (Section IV - 4.3).

Suprachiasmatic neurones may secrete materials other than releasing factors; but according to the present results the function of these neurones may be to regulate gonadotrophin releasing factor secretion, and the secreted materials would stimulate LRF or FRF secretion. The distribution of all the suprachiasmatic neurone axons is

not known, although degenerating nerve endings are found in the median eminence if the suprachiasmatic region is surgically separated from the median eminence (Rathelyi & Halasz, 1970) and antidromically propagated action potentials can be recorded from some suprachiasmatic neurones if the ventral surface of the median eminence is electrically stimulated (Makara et al, 1972).

b. Preoptic and medial preoptic neurones

It is remarkable that the mean nucleolar dry mass of neurones of the preoptic nucleus, pars suprachiasmatica, which lies just rostral to the suprachiasmatic nucleus, decreases after castration (Fig. 70b). It is possible that these neurones normally have an inhibitory influence on the secretion of gonadotrophin releasing factors. A similar possibility exists with respect to neurones of the medial preoptic nucleus; the mean nucleolar dry mass of neurones from this nucleus was also decreased for at least 10 days after castration (Fig. 72b). Unlike the suprachiasmatic nucleus, the medial preoptic and preoptic nuclei contain noradrenergic endings (Fuxe, 1965). Stimulation of the anterior hypothalamus increases gonadotrophin secretion in the male (Quinn, 1966; Velasco & Rothchild, 1973) as well as in the female (e.g. Everett, 1965). In contrast with the stimulation studies, complete anterior hypothalamic deafferentation in the male does not affect gonadotrophin secretion (Blake et al, 1973), and has no effect on testicular function in the normal male (Davidson & Bloch, 1969). Secretion of gonadotrophins in response to electrical

stimulation of the anterior hypothalamus may depend on testosterone, by analogy with the control of ovulation in the female (e.g. Schwartz, 1969), and the decreased nucleolar dry mass of medial preoptic and preoptic, pars suprachiasmatica, neurones after castration may reflect a dependence on testosterone of the stimulatory actions of these neurones. These neurones may be considered to be part of the release regulating system, which is only active in the presence of gonadal steroids, whereas suprachiasmatic neurones may be part of a negative feedback regulatory system, inhibited by gonadal steroids. Some medial preoptic neurones send axons into the arcuate nucleus (Dyer & Cross, 1972). The pathway from the anterior hypothalamus to the median eminence region passes ventrally in the midline in female rats, but the proportion of medial preoptic neurones contributing axons to this pathway is not known (Koves & Halasz, 1970; Tejasen & Everett, 1971). Some periventricular neuronal nuclei were included in the preparation of nuclei of medial preoptic neurones (Fig. 8). The periventricular neurones are dopaminergic and send axons to the median eminence (Fuxe & Hokfelt, 1967; Bjorklund et al, 1970). It is unlikely that nucleolar changes in these neurones contributed significantly to the measured changes because no change occurred in anterior hypothalamic neurones (vide infra) which also included some periventricular neurones (Fig. 9).

There are few studies of synthetic changes in neurones of the anterior hypothalamus. Castration decreases oxygen

consumption by the anterior or posterior hypothalamus, and these changes are prevented by testosterone (Moguilevsky et al, 1966): these experiments cannot be interpreted in the present context. In the newborn female rat, testosterone reduces RNA synthesis in all areas of the brain except the medial preoptic and medial amygdaloid nuclei (Clayton et al, 1970). Protein synthesis in medial preoptic neurones of ovariectomised rats is not altered by treatment with large doses of estrogen for three months (Litteria & Timiras, 1970). Medial preoptic neurones have other functions than the regulation of gonadotrophin secretion: testosterone implants in this area in castrated male rats restore male sexual behaviour (Lisk, 1967); so do implants in the posterior hypothalamus (Davidson & Bloch, 1969). Reduced nucleolar dry mass of medial preoptic neurones after castration is consistent with a testosterone dependent role for these neurones in male behaviour patterns.

c. Anterior hypothalamic neurones

It is difficult to localise lesions or implants to either the medial preoptic or anterior hypothalamic nucleus. Inhibition of FSH secretion by ovarian implants in the anterior hypothalamic nucleus of female rats has been described (Flerko & Szentagothai, 1957), but implants of testosterone in this area do not alter gonadotrophin secretion in the male (Lisk, 1962). The anterior hypothalamic nucleus has been implicated in female sexual behaviour (Kennedy, 1964). The absence of nucleolar dry mass changes in neurones of this area after castration (Fig. 72a) indicate that it has no function dependent on or related to the testes.

d. Paraventricular neurones

The PV nucleus has been suggested as the source of FRF (Motta et al, 1970b). Nucleolar dry mass of these neurones in males, as in females (Section V - 4.5.1), did not change after gonadectomy (Fig. 73b), and consequently did not satisfy one of the criteria for the identification of releasing factor neurones. SO neuronal nucleoli do not change after castration (Watt, R. M., personal communication).

e. Arcuate and ventromedial neurones

The circumstantial evidence that the neurones which synthesise and secrete LRF or FRF are wholly located within the arcuate or ventromedial nuclei (e.g. Halasz et al, 1962; Szentagothai et al, 1968) was not supported by the present study; if the assumptions made about the interpretation of nucleolar dry mass changes are correct.

The mean nucleolar dry mass of arcuate or ventromedial neurones was not increased by castration, but was decreased at 10 days and some time after (Fig. 71a,b). These changes are consistent with decreased but not increased synthesis and secretion of export products by these neurones. Again, these changes could be interpreted as reflecting an action of these neurones on gonadotrophin secretion, which is inhibitory or stimulatory and dependent upon testosterone. The only known secretory product of arcuate neurones is dopamine, which is probably released from the endings in the outer zone of the median eminence (Fuxe, 1964). There is conflicting evidence that dopamine inhibits (Fuxe et al, 1969a,b) and excites (Schneider & McCann, 1969; Kamberi et al, 1969; Keller & Lichtensteiger, 1971) gonadotrophin

releasing factor secretion from the median eminence: the weight of evidence favours a stimulatory action of dopamine. Because nucleolar dry mass of arcuate neurones decreases, it follows that dopamine synthesis may be decreased after castration; if so, then a dopaminergic mechanism may not be essential for the gonadotrophic changes after castration. Although changes in the amount of dopamine in arcuate neurones during electrical stimulation of the medial preoptic area are consistent with activation of arcuate neurones during increased LH secretion (Lichtensteiger, 1971; Keller & Lichtensteiger, 1971), vaginal stimulation causes electrical activation of the medial preoptic region but not the arcuate nucleus, yet LH secretion is increased (Blake & Sawyer, 1972a): these findings suggest that activation of the arcuate neurones is not an essential step in the secretion of gonadotrophin releasing factors. A similar conclusion applies to ventromedial neurones, although decreased nucleolar dry mass after castration is consistent with the involvement of the ventromedial nucleus in sexual behaviour (Kennedy, 1964).

Nucleolar volume of arcuate neurones decreases within 21 days of ovariectomy, but the nucleoli of ventromedial neurones are normal (Ifft, 1964). Protein synthesis in arcuate neurones of ovariectomised female rats is decreased by treatment with large doses of estrogen for four months; ventromedial neurones are not affected (Litteria & Timiras, 1970). Nucleolar dry mass of arcuate and ventromedial neurones would be expected to increase in castrated males

after testosterone treatment. Electron microscopic studies of arcuate neurones after castration have not included quantitative studies of parameters related to synthetic activity (Zambrano & De Robertis, 1968b; Brawer, 1971); apart from a measured increase in the number of neurones with whorled bodies (Brawer, 1971) which may or may not indicate increased protein synthesis.

Nucleolar dry mass of arcuate and ventromedial neurones in both sham operated and castrated animals were probably decreased 45 days after operation compared with other times (Fig. 71a,b). The animals in the present experiment were not kept under rigorously controlled environmental conditions and these nucleolar changes may reflect altered neuronal activity in response to some environmental change; for this reason statistical comparisons between groups at different times have not been made.

f. Posterior hypothalamic neurones

Nucleolar dry mass of posterior hypothalamic neurones was decreased only at 10 days after castration (Fig. 73a). The oxygen consumption of this area is decreased more than two months after castration (Moguilevsky et al, 1966), but neuronal nucleolar volume is not changed 20 days after ovariectomy (Ifft, 1964). This area is not usually attributed with a gonadotrophin regulating function, but implants of testosterone in this area in castrated males can restore male sexual behaviour (Davidson & Bloch, 1969). The nucleolar dry mass changes are consistent with a testosterone dependent function of these neurones.

Concluding remarks

Only neurones of the suprachiasmatic nucleus satisfy the condition that increased releasing factor secretion results in increased synthetic activity in the cells of origin. The condition has been made by analogy with other neurones, but may not be valid. If this condition is correct, the changes in suprachiasmatic neurones after castration do not necessarily mean that they synthesise LRF or FRF or both: there is no evidence that they produce any other neurohormone, and some direct evidence that they secrete gonadotrophin releasing factors. It is possible that the other pools of neurones are heterogeneous, and contain only small numbers of releasing factor neurones: the techniques used in this study would not have detected small sub-populations of responding neurones in each nuclear area (Section VI - 6.2).

The nucleolar dry mass changes may not have been the result of removing the major source of testosterone, or other testicular steroids: damage to autonomic nerve fibres, or short-loop feedbacks between the anterior pituitary or median eminence and the hypothalamus (Motta et al, 1969; Moguilevsky & Christot, 1973) may have caused the measured changes. LH decreases oxygen consumption by the anterior hypothalamus in vitro, and FSH increases oxygen consumption by the posterior hypothalamus (Moguilevsky et al, 1970) while LH-RH decreases protein synthesis in only the anterior hypothalamus in vitro (Moguilevsky & Christot, 1973).

6.2 Summary

Without further study of the responses of nucleoli of the hypothalamic neurones to various hormones and antagonists, locally or systemically administered, the firm conclusions are that in the absence of the testes the mean nucleolar dry mass of suprachiasmatic neurones increased, while the same parameter of other neurones did not change or decreased. The nucleolar changes in suprachiasmatic neurones are consistent with synthesis of gonadotrophin releasing factors by these neurones; and changes in other neurones are consistent with a stimulatory action of testosterone on synthetic and secretory activity of the medial preoptic, preoptic, arcuate, ventromedial and posterior hypothalamic neurones. Anterior hypothalamic or paraventricular neurones did not respond to castration.

6.3 Adrenalectomy

Nucleolar dry mass increased after adrenalectomy in the four hypothalamic areas studied. Ifft (1964) found the nucleolar volume of neurones in 16 hypothalamic areas to increase after adrenalectomy, but these changes also occurred if only the adrenal medullae were removed. Similarly, nuclear volume changes in only one ventromedial nucleus after unilateral adrenalectomy indicate that hypothalamic neuronal changes after adrenalectomy may result from interruption of a neural rather than a hormonal link (Szentagothai et al, 1968f). From one to seven days after bilateral adrenalectomy, neuronal nuclear volume increases in only the centromedial part of the

ventromedial nucleus, the rostral, and to a lesser extent the caudal parts of the arcuate nucleus; the effects of adrenocorticosteroids have not been reported (Palkovits & Stark, 1972). Nuclear volume changes in fixed sections cannot be interpreted in the context of ribosome synthesis. Nucleolar dry mass changes occurred in these areas after adrenalectomy in the present study. The nucleolar changes in ventromedial and anterior arcuate neurones occurred two or three days earlier than in the posterior arcuate nucleus (Figs. 74, 75). These nucleolar changes indicate an immediate increase in ribosome synthesis of ventromedial and anterior arcuate neurones after adrenalectomy, and a later increase in posterior arcuate neurones. These changes were closely related temporally to increasing CRF and ACTH secretion after adrenalectomy (Vernikos-Danellis, 1965).

There was no indication that the effects of adrenalectomy were specific for any group of neurones. The neuronal nucleoli may have responded because ribosome synthesis in all neurones is normally inhibited by adrenocorticosteroids, or because some neurones are involved in CRF synthesis and others in catecholamine synthesis which is altered by adrenalectomy (Fuxe et al, 1970), or because a neural link between the hypothalamus and the adrenal medulla or cortex, or both, was interrupted. Again, the neurones may have responded to ACTH or CRF rather than to removal of adrenocorticosteroids (Motta et al, 1970a).

In the sham operated animals only the nucleoli of neurones in the posterior arcuate nucleus increased after operation (Table 33). This indicates that these neurones may be activated by operative or ether stress; lesions in this region do not prevent responses to ether stress combined with operative stress (McCann, 1953), but do prevent adrenocortical responses to immobilisation stress (De Groot & Harris, 1950).

Nucleolar dry mass increased later in PV neurones than in other neurones (Fig. 75); the changes in these neurones may be related to increased ADH or oxytocin secretion in response to hypovolemic stimuli as a result of adrenalectomy (Share & Travis, 1970; Travis & Share, 1971). Destruction of the PV nuclei does not impair the adrenocortical responses to stress, which indicates that PV neurones are not the source of CRF (McCann, 1953).

6.4 Summary

After adrenalectomy mean nucleolar dry mass of ventromedial, arcuate and paraventricular neurones increased. The changes in ventromedial and anterior arcuate neurones occurred early. The nucleolar dry mass of posterior arcuate neurones increased after sham operation as well as after adrenalectomy. The nucleolar changes are consistent with the synthesis of CRF in any of the neurones studied, but these changes could also be due to altered catecholamine secretion by these neurones, or caused by interruption of neural or humoral links with the adrenal glands, or by direct actions of ACTH or CRF on these neurones.

The nucleolar changes may reflect inhibition of ribosome synthesis in all neurones by adrenocorticosteroids.

6.5 Concluding remarks

Many of the questions raised in discussion of the nucleolar changes after castration or adrenalectomy could be answered by further experiments: the effects could be studied of unilateral gland removal, replacement therapy with hormones, administration of hormone antagonists, anterior hypophysectomy, treatment with releasing factors and anterior pituitary trophic hormones and so on. Simple calculations reveal why these experiments have not yet been done. It is clear that nucleolar changes must be studied over a long period at frequent intervals after a procedure to capture fleeting responses; a period of 30 days, with measurements made at daily intervals for 10 days, and on alternate days for 20 days, should suffice. Five pairs of experimental and control animals should be used at each time studied. Ten hypothalamic areas are of interest, and from each preparation 24 nucleolar dry mass measurements are made. For one experiment the number of nucleolar dry mass measurements is therefore 4.8×10^4. Six obvious procedures have been described: if each is used in relation to the testes or adrenals, the number of procedures is 12, and the total number of nucleolar dry mass measurements is 5.8×10^5. To measure the dry masses of 100 nucleoli by the method used in this study is a full day's work. The time required to complete the experiments outlined above would be not less than 20 years.

APPENDIX I

INTERFERENCE MICROSCOPY

1. Derivation of formulae

The refractive index of an aqueous solution is dependent upon the concentration of dissolved substance:

$$nc = X.C + nw$$

where C is concentration g per cc,

X is 100x specific refractive increment, defined as the increase in refractive index for every 1 g per 100 cc increase in concentration of solution.

If X is known, C can be found:

$$C = \frac{nc - nw}{X} \quad \ldots\ldots\ldots \quad (1)$$

For a uniform plate-like object, which consists of such a solution, immersed in water with its long axis normal to the optical axis, the mass of dissolved substance can be found:

$$M = C.A.t,$$

where M = mass of dissolved substance, g

A = area of plate, cm

t = thickness of plate, cm

$$\text{or } C = \frac{M}{At} \quad \ldots\ldots\ldots\ldots \quad (2)$$

Substituting in equation (1):

$$\frac{M}{At} = \frac{nc - nw}{X}$$

$$\text{or } M = \frac{(nc - nw)}{X}.A.t$$

the optical path difference, Dw cm, due to the object for light parallel to the optical axis is given by:

$$(nc - nw)\ t = Dw \quad \ldots\ldots \quad (3)$$

$$\text{So, } M = Dw\ \frac{A}{X} \quad \ldots\ldots\ldots \quad (4)$$

If the object is placed in an aqueous medium of refractive index nm, then the optical path difference Dm

is given by:

$$Dm = (nc - nm)\, t \quad \ldots\ldots\ldots\ldots (5)$$

$$\text{or } t = \frac{Dm}{(nc - nm)} = \frac{Dw}{(nc - nw)} \text{ (equation (3))}$$

$$\text{or } Dw = Dm \frac{(nc - nw)}{(nc - nm)}$$

Substituting in equation (4):

$$M = \frac{Dm}{\lambda} \cdot \frac{(nc - nw)}{(nc - nm)} . A . g \quad \ldots\ldots (6)$$

2. Mean nucleolar optical path difference

If the nucleolus is assumed to be spherical, and has radius r cm, its volume is $\frac{4}{3} \pi r^3$. The height of a cylinder radius r of equal volume to a sphere of radius r is given by:

$$\frac{4}{3} \pi r^3 = \pi r^2 h, \text{ where h is height of cylinder}$$

$$\text{or } h = \frac{4}{3} r \text{ or } \frac{2}{3} d \text{ where d is diameter in cm.}$$

Peak optical path difference for a nucleolus of diameter, d, and hence thickness, d cm, is: Dpeak = (nc - nm)d, from equation (3), where nc is nucleolar refractive index. The optical path difference along the axis of a cylinder, height $\frac{2}{3}$d, is identical along all axes. Dmean = $(nc - nm)\frac{2}{3}d$ where Dmean is the optical path difference along any axis.

It follows that $Dmean = \frac{2}{3} Dpeak \quad \ldots\ldots (7)$

Mean nucleolar optical path difference can be easily calculated from a single measurement of peak nucleolar optical path difference.

3. Overlying nucleoplasm

The optical path difference, DN, for nucleoplasm immediately adjacent to the nucleolus is (nN - nm)t where nN is nuclear refractive index and t is nuclear thickness.

The thickness of nucleoplasm overlying the nucleolus is (t - d) cm. The true nucleolar optical path difference is (nc - nm) d.

The measured optical path difference Dm = (nN - nm) (t - d) + (nc - nm) d,

or $Dm = (nN - nm)\ t\ (1 - \frac{d}{t}) + (nc - nm)\ d.$

True nucleolar optical path difference, (nc - nm) d = Dm - DN $(1 - \frac{d}{t})$,

or nucleolar dry mass $= \frac{A}{\lambda} \cdot \left[\overline{Dm} - DN\left(\frac{1-d}{t}\right)\right] \cdot \frac{(nc-nw)}{(nc-nm)} \cdot g.$

APPENDIX II

ULTRAVIOLET ABSORPTION MICROSPECTROGRAPHY

1. Microdensitometry and calculation of nucleolar nucleic acid content

Nucleolar integrated optical density was measured by scanning the nucleolar photographic image in a series of scans 500 nm apart, referred to the original object plane.

The integrated optical density was recorded by a digital integrator. The total scan length was recorded.

The relationship between absorbance and optical density was determined by recording the integrated optical density of a scan along 10 cm, referred to the recording table, of each of the sector steps.

The integrated absorbance was calculated.

If T = total number of counts recorded by integrator for the nucleolar scan,

l = length of nucleolar scan,

B = counts per cm of background,

difference between background and nucleolus = Bl - T counts.

Mean nucleolar optical density = $\frac{Bl - T}{l} \times \frac{1}{k_1 l}$ o.d. units

where k_1 is the calibration factor, counts per cm per optical density unit. k_1 is obtainedfrom 10 cm scans along the steps of the sector calibration.

Mean integrated optical density = $\frac{Bl - T}{l} \cdot \frac{1}{k_1 l} \cdot \frac{a.l}{mag^2}$

where a = scan interval, cm,

mag = total magnification.

Since the absorptivity is linearly related to optical density, then mass = $\frac{Bl - T}{l} \cdot \frac{a\ a}{mag^2 k_1} \times \frac{1}{22}$ g ..... (1)

since absorptivity for nucleic acid = 22.

2. Scatter correction and extrapolation to absorption at 260 nm

The maximal contribution to absorption by scatter was estimated on the assumption that scatter is proportional, inversely, to the fourth power of the wavelength (Watson, 1968a). At 312 nm no biologically important substances absorb (Walker, 1956), so the apparent absorption at 312 nm is due to scatter.

If apparent absorption at 312 nm = S,

then scatter at 253.7 nm = $\frac{312^4}{253.7^4}$ .S

and scatter at 260 nm = $\frac{312^4}{260^4}$.S

True absorption at 260 nm if y is measured = $y-s\frac{312^4}{260^4}$

and true absorption at 253.7 nm if x is measured = $x-s\frac{312^4}{253.7^4}$

$$= x\left(1-s\frac{312^4}{253.7^4 x}\right)$$

$$= x.k_3.$$

An average value of k_3 was calculated for each preparation of nuclei from measurements on six nucleoli, and each measured nucleolar absorbance was multiplied by k_3 to correct for scatter. S, x and y were measured from absorption spectra (Fig. 21).

To correct absorption measured at 253.7 nm for each preparation to true absorption at 260 nm the average ratio

$\frac{y - s.\frac{312^4}{260^4}}{x - s.\frac{312^4}{253.7^4}}$, or k_2, was calculated for six nucleoli in each preparation, and applied to the remainder.

Multiplying measured absorption by $k_2.k_3$ corrected for scatter and to true absorption at 260 nm, $k_2.k_3$ varied from 0.67 to 0.75 between specimens.

APPENDIX III

Published Paper

'Observations on metabolic changes in hypothalamic neurosecretory neurones in lactation in the rat: effect of variation in litter size'. J. Physiol. 225, 11-13P, 1972.

[*From the Proceedings of the Physiological Society*, 3 *June* 1972]
Journal of Physiology, **225**, 11–13*P*

Observations on metabolic changes in hypothalamic neurosecretory neurones in lactation in the rat: effect of variation in litter size

By J. A. Russell. *Department of Physiology, University of Edinburgh*

The dry mass of nucleoli of paraventricular and supraoptic neurones has been measured in lactating albino rats.

Litter size was varied to study the metabolic responses of these neurones at different intensities of stimulus. Six groups of rats were used:

1. Virginal controls.

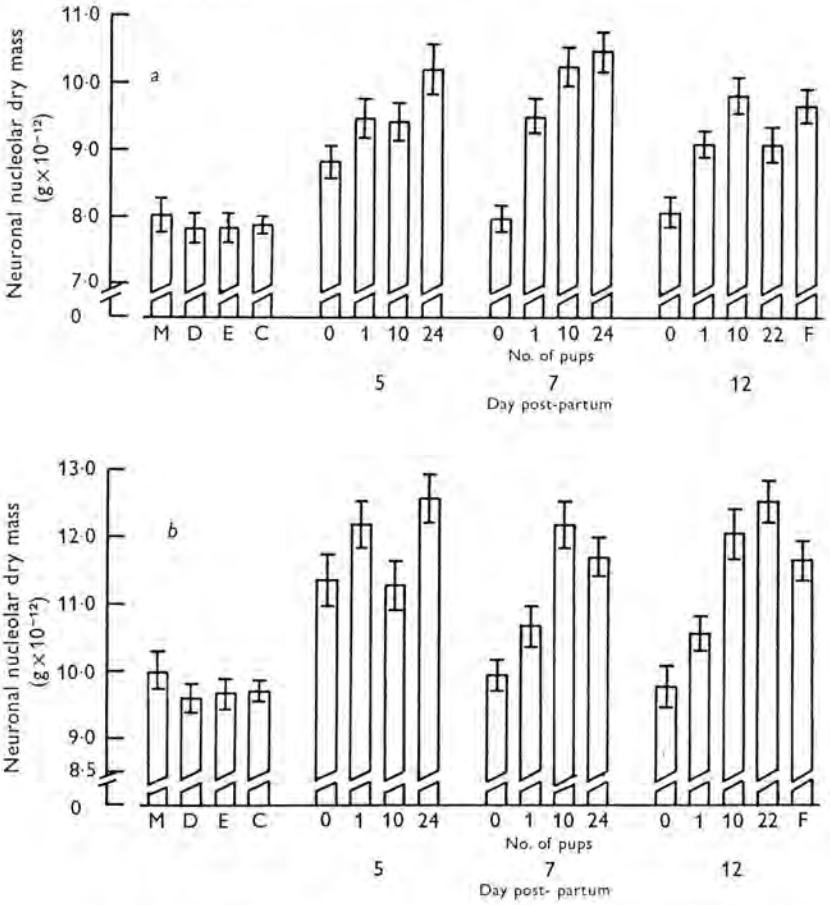


Fig. 1. Height of each vertical bar indicates the grouped mean of about 150 nucleolar dry mass measurements, from three to four animals. (*a*) Paraventricular neurones. (*b*) Supraoptic neurones. Vertical line on each bar indicates standard error of the mean. C = combined virginal females. M, D, E refer to virginal females in metoestrus, dioestrus or oestrus. F = litter of one pup to day 7, then ten pups to day 12.

[P.T.O.

2. Post-partum animals, litter adjusted to one of the following sizes on the first day:

(*a*) Nil pups,

(*b*) one pup,

(*c*) ten pups,

(*d*) twenty-two to twenty-four pups,

(*e*) one pup to day 7 post-partum, then ten pups to day 12.

On day 5, 7 or 12 post-partum the mothers were killed, neuronal nuclei were prepared from isolated paraventricular and supraoptic regions: nucleolar dry mass was measured in these preparations by interference microscopy (Watson, 1969).

Results are shown in Fig. 1*a*, *b*, and may be summarized:

1. No change occurred in nucleolar dry mass, in supraoptic or paraventricular neurones during the oestrous cycle.

2. In all animals nursing pups, nucleolar dry mass of supraoptic and paraventricular neurones was significantly greater than in controls at all times studied.

3. On the whole, the larger the litter the greater were the nucleolar changes in the supraoptic and paraventricular neurones.

4. In the absence of suckling, nucleolar dry mass in paraventricular and supraoptic neurones returned to normal by day 7 post-partum.

5. An increase in litter size at day 7 of lactation resulted in a further increase in nucleolar dry mass of supraoptic and paraventricular neurones by day 12.

These results show that the synthetic activity of supraoptic and paraventricular neurones is increased in lactation. This increase is due in part to the preceding pregnancy, but is sustained by suckling in a manner relating to its intensity.

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