QUANTITATIVE MICROCHEMICAL CHANGES REFLECTING FUNCTIONAL ACTIVITY IN SUPRAOPTIC NERVE CELLS OF THE RAT

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Thesis submitted for the degree of Ph.D.

UNIVERSITY OF EDINBURGH

1972

The undersigned was responsible for the composition of this thesis and for the design and execution of the research work it describes. The project was carried out under the supervision of Professor W. E. Watson, in the Department of Physiology, University of Edinburgh.

SUMMARY

1. Neurones and nuclei, isolated from the supraoptic regions of the rat's hypothalamus, have been examined by phase contrast and interference microscopy and by ultraviolet absorption microspectrography, in a quantitative study of their metabolic response to stimulation.

2. When isolated from rats dehydrated for up to five days, fresh supraoptic nuclei were larger and more likely to contain marginated nucleoli. The dry mass of fixed nuclei did not change.

The mean dry mass and nucleic acid content of supraoptic nucleoli and cell bodies increased significantly with progressive osmotic stimulation. Nucleolar changes preceded those in the cell body by 24 to 48 hours.

3. The significance of these changes is discussed in relation to published data concerning neuronal responses to injury and prolonged stimulation.

4. The distribution of dry mass among supraoptic nucleoli may be bimodal after moderate osmotic stress, but the presence of two populations of neurones within the supraoptic nucleus could not be confirmed.

5. The increases in dry mass and nucleic acid content of supraoptic nucleoli and cell bodies were both greater and faster during five days' dehydration at a raised environmental temperature ($24^{o}C$). Thus the metabolic response can be graded according to the functional load placed on the neurone.

6. Neurohypophyses which have been partially depleted of their "stores" of neurohormone contained some factor which, when injected into the lateral cerebral ventricle of normal rats, resulted in an increase in the mean dry mass and nucleic acid content of supraoptic nucleoli. These nucleolar changes seemed independent of neurohormone secretion or serum osmolality in the recipient animals.

7. Extracts of normal ("non-depleted") neurohypophyses significantly inhibited the dry mass response of supraoptic nucleoli to osmotic stress.

8. "Hormone-depleted" posterior lobe extracts did not alter the mean dry mass or nucleic acid content of nucleoli from the posterior part of the arcuate nucleus.

9. Extracts of tissue from the parietal cortex had no demonstrable effect on supraoptic nucleoli.

10. The possibility is discussed that "active" substances derived from the neurohypophysis may serve to match synthetic activity to secretory losses in supraoptic neurones.

11. The mean dry mass of pituicytes increases rapidly during the first 48 hours of dehydration. This response is as fast as any supraoptic changes detected in this investigation.

ACKNOWLEDGEMENTS

My sincere thanks are due to Professor W. E. Watson for unflagging advice and criticism which, if not always accepted gracefully, was invariably appreciated in retrospect. I am also most grateful to Mr. William Lawson and Miss Anne Robertson for dealing promptly and professionally with the photography and typing respectively.

I should like to thank the Medical Research Council and the National Fund for Research into Poliomyelitis and Other Crippling Diseases for their financial support.

Finally, I thank my wife for selfless encouragement, forbearance and assistance despite recent biological pressures.

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LIST OF ABBREVIATIONS

ADH ..... antidiuretic hormone, vasopressin

AF ..... aldehyde-fuchsin

CAH ..... chrome-alum-haematoxylin

C.S.F. ..... cerebro-spinal fluid

DNA ..... deoxyribonucleic acid

EPSP ..... excitatory post-synaptic potential

N.A. ..... numerical aperture

NSV ..... neuro-secretory vesicle

O.D. ..... optical density

O.P.D. ..... optical path difference

RNA ..... ribonucleic acid

rRNA ..... ribosomal ribonucleic acid

S.E.M. ..... standard error of the mean

Serum S.G. ..... specific gravity of blood serum

Mathematical symbols are defined where used.

SECTION I: Introduction and Historical Review

Fresh insights into the mechanism of chromatolysis have resulted from the application of quantitative techniques of cellular biology to the study of metabolic activity in single neurones (Brattgård et al 1957, Watson 1968a). Studies on the metabolic effects of stimulation, however, have been more fragmentary and contradictory (see below). Lengthy stimulation of neurones may be necessary to cause metabolic changes which these techniques can resolve. For this reason, the supraoptic neurosecretory cell was selected for this study of the metabolic consequences of nervous activity. These cells can be stimulated physiologically, for long periods, without operative interference.

Although the supraoptic cell secretes a hormone rather than a transmitter, it is undeniably a neurone in its physiological characteristics.

(A) NEUROBIOLOGY OF THE SUPRAOPTIC NEURONE

(i) Excitability

Whether or not the neurosecretory cells of the supraoptic nucleus are osmosensitive remains undecided. Finley (1938), noting the rich capillary investment of the supraoptic nuclei, suggested that they might "sense" physicochemical changes inthe blood. Verney's classical experiments (Verney 1947) traced osmoreceptors to that intracranial area supplied by the internal carotid artery. Only a small part of the hypothalamus, close to the optic chiasm, need remain attached to the hypophysis to support

normal water balance in the cat (Woods et al 1966). The isolated neurohypophysis does not respond to osmotic stimuli (Douglas and Poisner 1964a).

Von Euler (1953) recorded slow changes in potential in the supraoptic region after the injection of hypertonic saline into the common carotid artery. In the rabbit, neurones whose firing patterns were altered by such osmotic stimuli were clustered around the supraoptic and paraventricular nuclei (Cross and Green 1959). Excitatory, inhibitory and "bimodal" responses to osmotic stimuli have been described in neurones around these nuclei (Brooks et al 1962, Koizumi et al 1964, Joynt 1964). The specifity of osmoreceptors is open to question. In the supraoptic nucleus of cats, many osmosensitive cells responded also to stimulation of peripheral nerves (Brooks et al 1962, Koizumi et al 1964). More specific osmoreceptors are claimed to lie in a zone, 0.5 to 1 mm wide, around the supraoptic nucleus (Joynt 1964, Vincent and Hayward 1970).

An impaled cell which changes its firing pattern after osmotic stimuli need not be directly osmosensitive, and many supraoptic cells fail to respond (Brooks et al 1962, Koizumi et al 1964). Thus, although some neurosecretory cells can be identified by antidromic stimulation (Azuma and Matsuda 1966, Kelly and Driefuss 1970), it is not possible to demonstrate neurosecretion and osmosensitivity in the same cell.

Various observations suggest that supraoptic neurones can be excited synaptically. Acetylcholine is a potent

stimulant for supraoptic cells (Kelly and Driefuss 1970) and can produce antidiuresis when injected into the supraoptic nucleus of dogs (Pickford 1947, Pickford and Watt 1951). Adrenaline, injected into the internal carotid artery, prevented the antidiuretic response of the dog to stress (Verney 1947). Fuxe and Hökfeldt (1967, 1969) demonstrated catecholamine-rich terminals in the supraoptic nucleus by fluorescence microscopy. Excitatory and inhibitory post-synaptic potentials have been recorded from neurosecretory cells in the preoptic nucleus of the goldfish (Kandel, 1964). Axo-somatic, axo-dendritic and axo-axonic synapses have been demonstrated in electron micrographs of the supraoptic nucleus (Peterson 1965, Enestrom 1967, Rechardt 1969).

Only reflex excitation of neurosecretory cells seems adequate to account for the rapid ADH release after haemorrhage (Ginsburg and Brown 1956, Sachs et al 1967). Vagal afferents, responding to distension of the left atrium, have been suggested as the peripheral limb of such a reflex (Henry and Pearce 1956). Vagotomy, with denervation of the carotid sinuses, either reduces (Ginsburg and Brown 1956, Clarke and Rocha e Silva 1967) or abolishes (Share 1967) the antidiuretic response to haemorrhage.

(ii) Synthesis of Secretory Product

Thirty-five years after Dale's pioneering demonstration of chemical transmission in sympathetic ganglia (Dale 1937), few transmitter agents can be identified with

certainty (Hebb 1970). Chemical transmission at central synapses can be inferred from the consistent presence of presynaptic vesicles (Palay 1958, de Robertis 1958) and the similarities between central and peripheral synaptic potentials (Eccles 1964a). Electrically operated synapses have not been demonstrated in the central nervous system of mammals. Thus current information suggests that most neurones can synthesise a specific secretory product.

Evidence implicating the supraoptic nucleus in the synthesis and secretion of ADH is impressive. This nucleus sends large numbers of fibres to the neurohypophysis (Cajal 1911, Rasmussen 1938, 1939), and atrophies after lesions of the pituitary stalk (Rasmussen 1939). Pressor, anti-diuretic and oxytocic activities are present in extracts of the posterior lobe of the pituitary (Howell 1898, Dale 1909, von den Velden 1913) and are released on stimulation of the supraoptico-hypophysial tract (Harris 1946). Bilateral interruption of this tract consistently caused diabetes insipidus in cats and monkeys (Ranson, Fisher and Ingram 1936, Ingram, Fisher and Ranson 1936).

The suggestion that magnocellular hypothalamic nuclei might act as endocrine organs (Scharrer 1928) waited many years for confirmation. Bargmann (1949) and Gabe (1953) used Gomori's CAH and AF staining methods (Gomori 1941, 1950) to study neurosecretory cells. Many studies followed in which the material stained was identified with the secretory product of the posterior lobe (Ortmann 1951,

Bargmann and Scharrer 1951, Hild and Zetler 1953); its presence throughout the supraoptico-hypophysial tract lent support to Scharrer's suggestion. "Specific" stains for -SH and -S-S- groups show a similar affinity for neurosecretory cells (Adams and Sloper 1956). This may reflect the high cystine content of the neurohypophysial hormones (du Vigneaud 1954-5, Acher and Chauvet 1953, 1954) and their carrier protein (van Dyke et al 1942).

ADH and oxytocin can be extracted from the hypothalamus (Vogt 1953, Hild and Zetler 1953, Heller and Lederis 1958). Compared to the paraventricular region, supraoptic nuclei are rich in ADH (Lederis 1962). The drop in cell numbers in the supraoptic nuclei after hypophysectomy (Rasmussen 1939) coincides with a drop in local ADH activity (Melville and Hare 1945). After subarachnoid injection, ^{35}S-cysteine is incorporated into supraoptic cells up to ten hours before the label appears in the neurohypophysis (Sloper et al 1960, Flament-Durand 1967). Hypothalamic tissue can incorporate ^{35}S-cysteine and ^{3}H-tyrosine into ADH both in vivo (Sachs and Takabatake 1964) and in vitro (Takabatake and Sachs 1964).

An apparently homogeneous protein, loosely bound to oxytocic and vasopressor activities, was isolated from the posterior lobes of bovine pituitary glands (van Dyke et al 1942). Dean and his colleagues (Dean and Hope 1967, 1968, Dean et al 1967, 1968a,b, Hollenberg and Hope 1968) have isolated two hormone-binding proteins from bovine

posterior lobes. Neurophysin I was associated with oxytocin and neurophysin II with ADH. Ginsburg's claim, that these proteins form a "polymerizing equilibrium system" (Ginsburg et al 1971), may explain the original overestimates of their molecular weights. Recent estimates of the molecular weight of the monomer lie around 10,000 (Ginsburg et al 1971, Hope and Sachs 1970).

ADH and neurophysin may share a common precursor. After hypothalamic tissue has taken up ^{35}S-cysteine, there is a "lag period" of 60 to 90 minutes before labelled ADH is formed (Sachs and Takabatake 1964, Takabatake and Sachs 1964). Puromycin can prevent the appearance of labelled ADH only if administered during this "lag period" (Portanova et al 1966). Thus an early step in the synthesis of ADH may be the formation of a precursor protein from which ADH arises without ribosomal intervention. The incorporation of ^{35}S-cysteine into "rapidly transported" protein within rat supraoptic neurones shows a similar "lag period" (Norström and Sjöstrand 1971b).

(iii) Intracellular Location of Secretory Product

Circumstantial evidence makes the "packaging" of transmitter within presynaptic terminals likely. Spontaneous miniature end-plate potentials at neuromuscular junctions suggest the release of "quanta" of acetylcholine (Fatt and Katz 1952). Acetylcholine and choline acetylase can be sedimented by differential centrifugation of homogenates of brain (Hebb and Whittaker 1958, Whittaker 1959, a,b). Such fractions are rich in vesicular structures.

Accumulationsof vesicles, 20 to 65 nm in diameter, are consistently found in presynaptic nerve terminals (De Robertis 1958, Palay 1958).

Even in the light microscope, neurosecretory material appears granular (Palay and Wissig 1953). Centrifugation in sucrose solutions has been used to sediment oxytocic and ADH activities (Pardoe and Weatherall 1955, Schapiro and Stjarne 1961). Bovine neurosecretory material has been isolated in quantity and appears in electron micrographs to consist of small vesicles with electron-dense cores (Dean and Hope 1967, 1968). Palay (1955) described small round profiles 0.1 to 0.3 µm in diameter in nerve endings in the rat's neurohypophysis. Dehydration (Palay 1955) and haemorrhage (Daniel and Lederis 1966) have been reported to deplete these "neurosecretory vesicles" (NSV) of their electron-dense contents. The significance of these findings has recently been questioned. According to Douglas et al (1971), "electron-lucent" NSV are not seen when fixation with glutaraldehyde is used. The role of the "microvesicles" described by Palay (1955) and others remains obscure. The suggestion that they form a means of recapturing excess cell membrane (Douglas et al 1971, Lederis et al 1971) is attractive but needs experimental verification.

The literature now contains many reports on the electron microscopy of supraoptic tissue (Barry 1957,Duncan and Alexander 1961, Sloper and Bateson 1965, Peterson 1965,

Ishii 1966, Zambrano and De Robertis 1966, Enestrӧm 1967, Rechardt 1969). Both Enestrӧm (1967) and Rechardt (1969) described two types of neurone in the supraoptic nucleus. It is probable that the "A" and "B" cells of Enestrӧm correspond to the "dark" and "light" cells of Rechardt respectively. In tissue from normal animals, "B" neurones formed about 80% of the supraoptic population. Since this proportion increased in dehydrated animals (Enestrӧm 1967) some "A" cells may be converted to the "B" form during osmotic stimulation. NSV and microvesicles were seen in both types of cell.

(iv) Stimulus-secretion Coupling in Neurones

Katz (1962) depolarized motor nerve endings by raising the external concentration of potassium ions. This resulted in an increase in the quantal release of acetylcholine. The action potential may thus release transmitter by causing presynaptic depolarization. Depolarization of nerve terminals is effective only if other conditions are satisfied. Thus the release of acetylcholine from motor nerve endings is inhibited by a lack of calcium or an excess of magnesium in the external medium (Castillo and Katz 1954, Jenkinson 1957).

Electrical stimulation of neurosecretory axons in vivo (Harris 1946) and in vitro (Douglas and Poisner 1964a) caused the release of ADH and oxytocin. Solutions rich in potassium caused secretion of hormone from isolated neurohypophyses (Douglas and Poisner 1964a, Douglas et al 1965, Dicker 1966). The secretory effect of such solutions was

inhibited by a lack of calcium or an excess of magnesium in the external medium (Douglas and Poisner 1964a, Dicker 1966).

Calcium is taken up by the isolated neurohypophysis during the secretion of hormone (Douglas and Poisner 1964b). This prompted Ginsburg and Ireland (1966) to suggest that calcium ions might promote ADH secretion by dissociating the hormone from neurophysin. Evidence for the depletion of neurophysin from the neurohypophysis (Friesen and Astwood 1967, Rennels 1966) and for the simultaneous release of hormones and carrier (Fawcett et al 1968) makes this unlikely. The secretion of NSV contents by exocytosis has been suggested on the basis of recent electron micrographs (Douglas et al 1971, Lederis et al 1971). Arranging electron micrographs into a series of "exocytotic figures" must be subjective but the concept is compatible with the simultaneous release of neurophysin and ADH (Fawcett et al 1968).

ADH secretion in response to repeated haemorrhage in the dog suggests heterogeneity in the neurohypophysial stores of the hormone (Sachs et al 1967, Sachs and Haller 1968). Haemorrhage caused rapid secretion of ADH only until about 20% of the stored hormone had been released. Thereafter the secretion rate dropped. Repeated exposure of isolated neurohypophyses to solutions containing 56 mM potassium produced a similar biphasic pattern of secretion (Sachs et al 1967, Daniel and Lederis 1967). The relatively

high specific activity of the "readily releasable" pool of ADH after intracisternal infusion of ^{35}S-cysteine (Sachs and Haller 1968) suggests that it includes the most recently synthesised hormone. Closely similar findings have now been reported in the rat (Norström and Sjöstrand 1971b).

(v) Intra-axonal Transport

Neurosecretion requires the transport of neurosecretory material from the cell body of the neurone to its neurohypophysial terminals (Bargmann and Scharrer 1951). Experimental evidence for a centrifugal flow of axoplasm in mature axons falls into three main categories: (a) the centrifugal migration of labelled protein (Miani 1962, Ochs et al 1962, 1967, Droz and Leblond 1963, Lux et al 1970), (b) the accumulation of cholinesterase above an experimental crush of the nerve (Lubińska 1964, Lubińska and Niemierko 1971) and (c) the "damming" of axoplasm behind a chronic constriction (Weiss 1944, 1961, Weiss et al 1962). Axoplasmic streaming has been observed in vitro (Hughes 1953, Pomerat 1960).

Centripetal transport in nerve axons is less widely accepted. However the concept of "trophic" or "informational" feedback from nerve terminal to cell body is justified. The developing nerve cell depends for its maturation on a normal peripheral distribution. An enlarged peripheral field may result in hypertrophy and hyperplasia of nerve centres (Bueker 1945). Conversely, the removal of the

peripheral field, before innervation, results in cessation of growth and death of neurones around the time when connection would normally have occurred (Hamburger 1934, Harkmark 1956, Evans and Vizoso 1951). Hypoglossal nerve cells whose axons have been implanted into the sternomastoid muscle show a synthetic response when the accessory nerve is sectioned (Watson 1970). Thus denervation of the muscle is detected in some way by the hypoglossal neurones.

Acetylcholinesterase accumulates on both sides of a crushed region of nerve (Lubińska and Niemierko 1971). Time-lapse cinephotography has been used to demonstrate bidirectional movement of axonal constituents in vitro (Pomerat 1960, Hughes 1953). The centripetal movement of labelled amino acid from muscle to nerve cell has been demonstrated both in vitro (Kerkut et al 1967) and in vivo (Watson 1968c, Kristensson et al 1971).

The delay before labelled protein appears in the neurohypophysis after the intracisternal injection of ^{35}S-cysteine (Flament-Durand 1967) may represent the "transport time" from cell body to nerve ending. Neurosecretory cells accumulate neurosecretory material on both sides of a lesion of the pituitary stalk (Hild and Zetler 1953, Christ 1962) and support bidirectional transport in vitro (Hild 1954).

(vi) The Supraoptic Cell as a "Model" Neurone

In their bioelectric properties, neurosecretory cells are indistinguishable from neurones. Like neurones elsewhere

they can synthesise, "package", transport and release a secretory product. The ability to stimulate the supraoptic cell for long periods without operative interference was an overriding consideration in its selection for this study of metabolic changes in neurones.

(B) METABOLIC ACTIVITY IN NEURONES

(i) Chromatolysis

The term "chromatolysis" is an unfortunate one. Those histological changes described in injured neurones (Nissl 1892) appear to indicate augmented synthesis rather than degeneration. Swelling of the nerve cell body is an early and prominent feature after axonal injury (Brattgård et al 1957), and causes a drop in the concentrations of protein and nucleic acid in the cytoplasm (Brattgård et al 1957, Hydén 1943).

Increased amounts of RNA can be demonstrated in the nucleoli of nerve cells 1 to 5 days after axotomy (Watson 1968a). Nucleoli of injured neurones show increased turnover of ^{3}H-uridine and lose RNA faster on exposure to actinomycin D than those of control neurones (Watson 1968a). Hence the increase in RNA content of nucleoli after nerve injury is at least partly due to increased synthesis.

Ribosomal RNA (rRNA) is rich in guanine and cytosine (Edström and Gall 1963) and is quantitatively the most important class of nucleic acid within nerve cells (Edström 1957b). Synthesis of rRNA appears to be an important

function of the nucleolus. Edström and Eichner (1958b) found a linear relationship between nucleolar volume and cytoplasmic RNA content in neurones. In the growing oocyte, rRNA synthesis occurs only when nucleoli are present. At the onset of meiosis, nucleoli disappear and rRNA synthesis stops (Brown 1966). The cytoplasm of an oocyte which is not synthesising rRNA can inhibit rRNA synthesis in transplanted nuclei. Under these circumstances, the nucleolus in the transplanted nucleus disappears (Gurdon and Brown 1965). Labelled precursors which are incorporated into nucleoli appear later in cytoplasmic nucleic acid with the sedimentation constants of rRNA (Perry 1962). Anucleolate mutants of Xenopus laevis are unable to synthesise rRNA (Brown and Gurdon 1964). The dosage of the mutant gene in these animals is reflected in (a) the number of nucleoli per nucleus, (b) the number of nucleolar organisers per nucleus and (c) the number of DNA cistrons per nucleus annealing in vitro with rRNA (Birnstiel et al 1966). Such evidence strongly favours the nucleolus as the site of that DNA subserving rRNA transcription.

Thus the nucleolar findings of Watson (1968a) suggest that a major component of chromatolysis is an increase in rRNA synthesis. The RNA content in the cytoplasm of injured neurones begins to increase 24 to 48 hours later than the response seen in nucleolar RNA (Watson 1968a) and a transient increase in the rate of transfer of labelled RNA from nucleus to cytoplasm has been demonstrated (Watson 1965a).

Injured neurones incorporate ^{3}H-lysine into protein faster than intact nerve cells (Watson 1965a). A prolonged increase in the protein content and dry mass of nerve cell bodies have been reported after axotomy (Brattgård et al 1957, Watson 1969a). These findings could indicate an increased synthesis or prolonged survival of cytoplasmic protein. Evidence based on the loss of dry mass after exposure to puromycin favours the former (Watson 1969a).

Metabolic changes occurring after axotomy are not restricted to neurones. Glial cells surrounding the cell bodies of injured neurones show decreased adhesiveness (Watson 1966a), and increased oxygen uptake (Watson 1966b). Astrocytes and microglia around injured nerve cells undergo hypertrophy and hyplasia (Sjöstrand 1965, 1966, Watson 1965a) and the endothelial cells of nearby capillaries incorporate ^{3}H-thymidine into DNA (Watson 1965a).

There are few clues to the significance of the glial reaction seen around chromatolytic neurones. Neurones undergo metabolic changes similar to those of chromatolysis when their terminals are exposed to botulinum toxin. Surprisingly, these changes occur without the glial response described above (Watson 1969b).

It seems reasonable to regard chromatolysis as a growth-like response (Roberts and Mattysse 1970) involving increases in ribosome and protein synthesis. What is less clear is the nature of the stimulus which triggers this synthetic activity. Any scheme proposed should explain certain key findings:

(1) Increases in nucleolar and cell body RNA occur after a latency which depends on the distance between the axonal injury and the nerve cell body (Watson 1968a). This latency could reflect the transit time for a chemical "signal" to move centripetally from the site of injury to the nerve cell body. Alternatively the "signal" might be the absence of a normally inhibitory feedback from regions distal to the injury. Interpretation is complicated by the fact that the position of the injury also determines its severity.

(2) The duration of the nucleolar RNA response in injured hypoglossal neurones is independent of whether or not contact with muscle is re-established (Watson 1968a).

(3) When, after axotomy, nucleolar RNA levels have returned to normal in hypoglossal neurones prevented from re-innervating muscle, a second section of the nerve causes a fresh nucleolar response (Watson 1968a). Thus the "signals" for both onset and duration of the nucleolar response are unlikely to derive from muscle.

(4) Studies of central pathways using the technique of retrograde degeneration have suggested that the presence of undamaged collateral axons inhibits chromatolysis in response to axotomy (Rose and Woolsey 1949, Garey and Powell 1967).

(5) Section of the peripheral nerves of dorsal root ganglion cells produces chromatolysis whilst division of the central processes does not (Hare and Hinsey 1940, Moyer et al 1953).

(6) Hypoglossal neurones whose axons have been implanted previously into the sternomastoid muscle show a synthetic response when the accessory nerve is sectioned. This response can be inhibited by botulinum toxin (Watson 1970).

Evidence such as this prompted Watson (1969b) to suggest sprouting of the nerve fibres proximal to the site of injury as the factor common to most situations where chromatolysis occurs. The findings for dorsal root ganglion cells are probably the most difficult to interpret. Synaptic plasticity within the central nervous system might be postulated to place a continuous demand for growth on presynaptic nerve terminals. Under these circumstances, the added requirements of regeneration might be subthreshold for demonstrable chromatolysis. With this proviso, the concept of some signal generated around sprouting axons could explain the bulk of the evidence quoted. There is, however, no certainty that only a single "growth-signal" isinvolved and it is probably more realistic to anticipate an hierarchy of control systems governing ribosome and protein synthesis. Not all of these controls need be interrupted by any single experimental procedure. Nonetheless, the concept of centripetal transport of some "signal" from the site of injury to the nerve cell body seems inescapable.

(ii) Functional Activity

Nissl substance in neurones has been variously reported to decrease, increase or remain unaltered after stimulation.

Carlsson (1902) reported depletion of Nissl substance in stimulated ganglion cells in the retina of the cormorant. These findings were confirmed by Mann (1895) and extended to sympathetic ganglion cells and motor nerve cells. Bertram and Barr (1949) found depletion of Nissl substance in hypoglossal neurones of the cat after eight hours' electrical stimulation, confirming a report by Einarsson (1933). More recently, Gomirato (1954) and Gomirato and Baggio (1962) have reported depletion of ribonucleoprotein after physiological stimulation of anterior horn cells and retinal ganglion cells of the guinea-pig and rabbit respectively.

On balance, recent accounts suggest that increased cytoplasmic nucleoprotein is more characteristic of the functionally stimulated neurone. According to Brattgård (1952), the content of nucleoprotein increased in stimulated retinal ganglion cells and decreased in dark-adapted cells. The amounts of RNA in both nucleoli and cell bodies of supraoptic neurones increased after eight weeks' administration of saline (Edström and Eichner 1958, Edström et al 1961). Deiters neurones in the rabbit and rat contain increased amounts of RNA after vestibular stimulation (Hydén and Pigón 1960, Hydén and Egyházi 1962). Supraoptic neurones show increased uptake of radioactive amino acids (Murray 1967, Norström et al 1971a), and increased transfer of labelled RNA from nucleus to cytoplasm (Watson 1965b) when under osmotic stress. Spinal motor neurones of rats

incorporated more ^{3}H-leucine into protein when the label was injected during exercise (Altman 1963).

Several authors have failed to demonstrate altered basophilia or RNA content in neurones stimulated electrically (Liu et al 1950, Grampp and Edström 1963) or physiologically (Edström and Grampp 1965). Watson (1965b) found no change in the rate of transfer of RNA from nucleus to cytoplasm after five hours' electrical stimulation of cervical ganglia or the hypoglossal nerve in rats.

Liu et al (1950) believed that changes in Nissl substance occurred only after stimulation intense enough to injure the neurone. Although there seems little justification for this extreme view, it is likely that contradictions in this literature derive from the variation in duration and severity of the stimuli used by different authors. Although exceptions exist, the trend in these reports suggests that brief stimulation results in unaltered or decreased ribonucleoprotein levels in the neuronal cytoplasm. Stimulation over days or weeks permits long-term adjustment of cellular metabolism and elevated ribonucleoprotein levels are commonly found. Edström (1957) reported a decrease in cytoplasmic basophilia in motor nerve cells after brief exercise. Longer periods of activity produced changes suggesting "intense protein synthesis", notably a dramatic increase in nucleolar volume. The neurone's response to stimulation may resemble that seen after axotomy in showing an early, transient drop in cytoplasmic ribonucleoprotein concentration.

Possible links between the electrical activity of neurones and related metabolic consequences are now being clarified using the giant R2 ganglion cell of the mollusc Aplysia californica. In his in vitro preparation, Berry (1969) found a linear relation between ^{3}H-uridine uptake and the number of spikes fired per hour in response to presynaptic stimulation. With the same preparation, Peterson and Kernell (1970) and Kernell and Peterson (1970) largely confirmed these findings but failed to find an increase in the uptake of uridine when postsynaptic stimulation was used. These findings suggest that the "coupling" between electrical and metabolic activity in this preparation involves transmitter/EPSP phenomena rather than the action potential itself. It should be emphasised, however, that the in vitro situation prevents the action potential from invading the nerve terminals of the R2 cell, thus eliminating a potential source of feedback signals related to the release of transmitter.

Scope and area of investigation

The frequency of discharge, and hence the rate at which transmitter is being released, is usually dictated by factors outwith the neurone. If the supply of transmitter is to match demand, the neurone must have information about the rate at which the substance is being lost from the nerve terminals.

There is evidence for some "coupling" between synaptic activation and the synthesis of RNA in at least one neurone (Kernell and Peterson 1970). However, no system depending on activation alone can match synthesis to release consistently in all neurones. The amount of transmitter released on activation becomes unpredictable in the presence of presynaptic inhibition (Frank and Fuortes 1957, Eccles 1964b) and central facilitation (Curtis and Eccles 1960).

More accurate "coupling" of synthesis to secretion would be possible if the neurone could monitor more directly the size of its "stores" of transmitter. Of the neurosecretory cell, Daniel and Lederis (1967) wrote "... little is known at present on the relations between hormone release and synthesis .... it would be desirable to know whether releasing stimuli initiate thesynthesis immediately or whether a depletion of hormones from the storage organ provides the stimulus for synthesis."

The questions posed at the start of this investigation were:

1. What metabolic changes can be measured in single supraoptic neurones during osmotic stimulation?

2. Is the metabolic response graded according to the severity of the osmotic stimulus and, by inference, according to the amount of hormone released?

3. Is there a feedback of "information" from the nerve terminals of supraoptic neurones which can adjust synthetic activity to compensate for changes in the stored "pool" of neurohormone?

SECTION II: Supraoptic changes during functional activity

METHODS

Plate 1

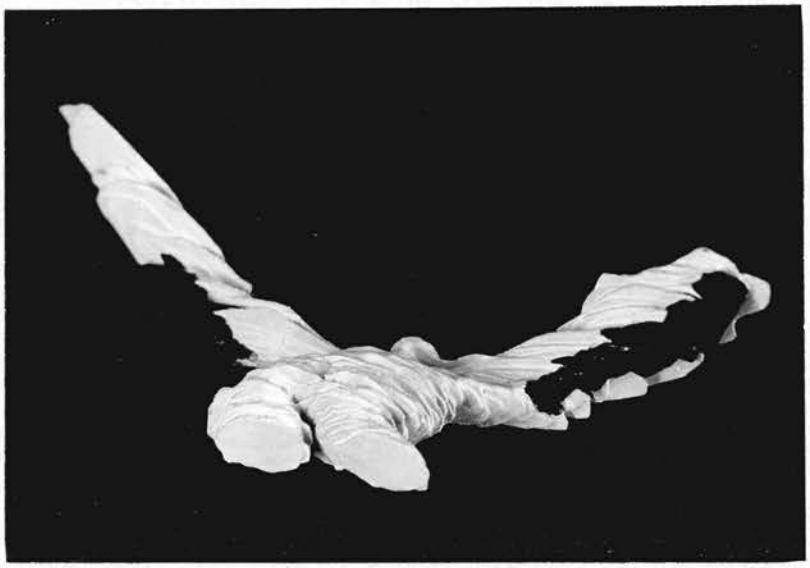
Model showing relationship of supraoptic nuclei (black) to optic chiasm and tracts.

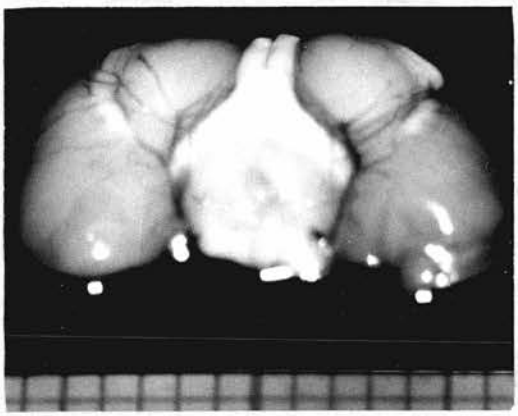
Plate 2 (a-e)

Stages in the dissection of supraoptic nuclei.

N.B. Anterior cut in Plate 2(b) is about ½ mm in front of its correct position. Where necessary, this was corrected by trimming at stage (d).

Each scale division is 1 mm.





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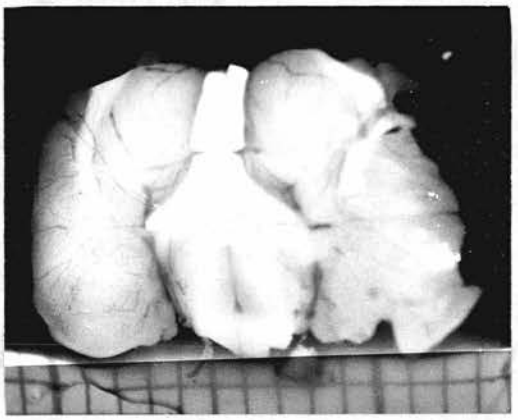

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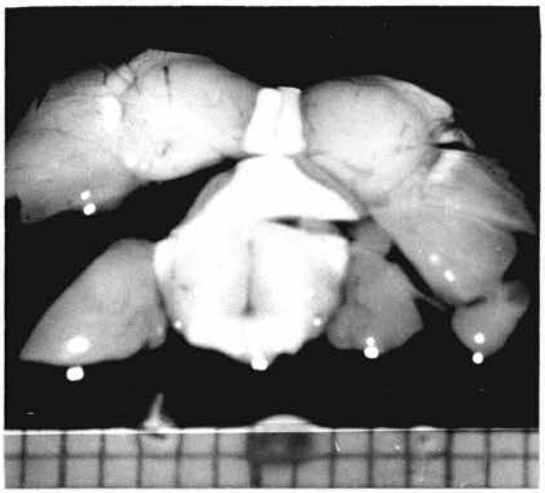

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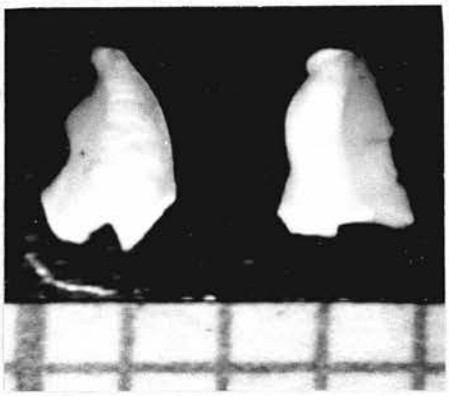

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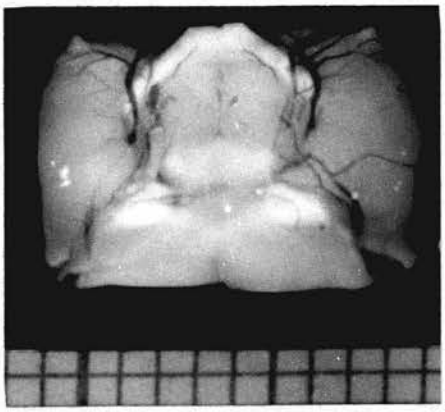

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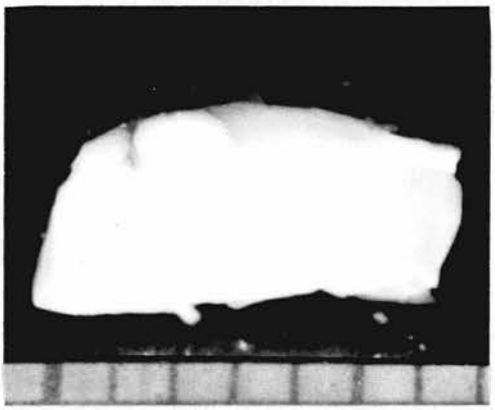

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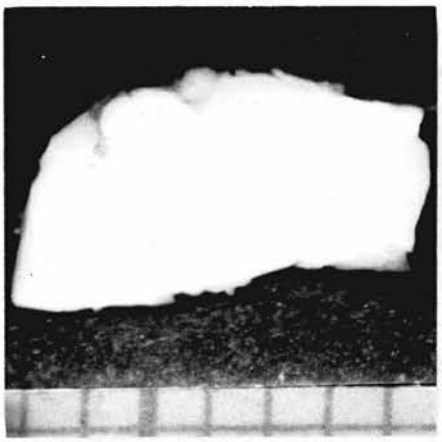

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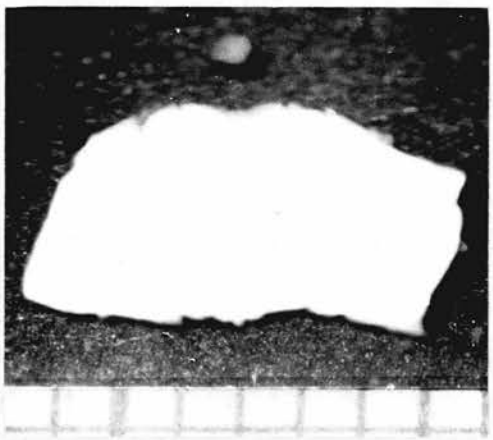
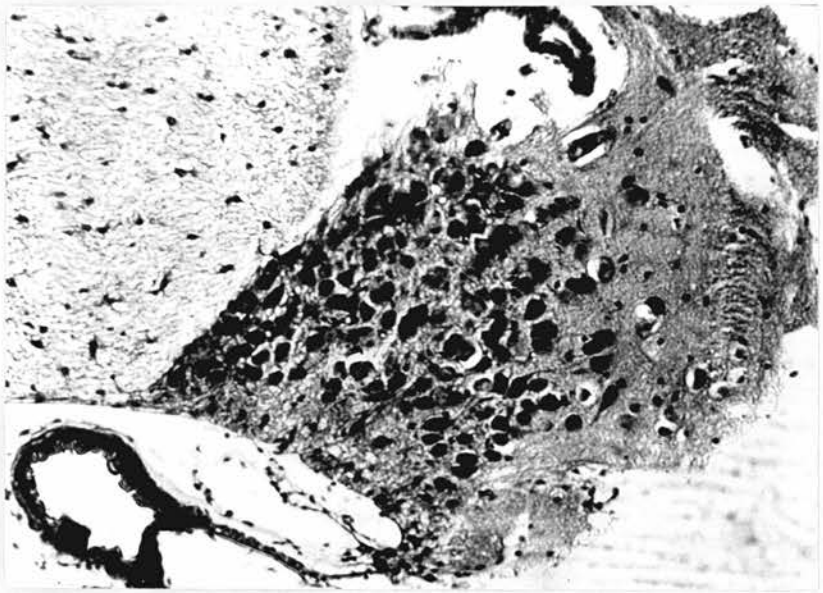


d.

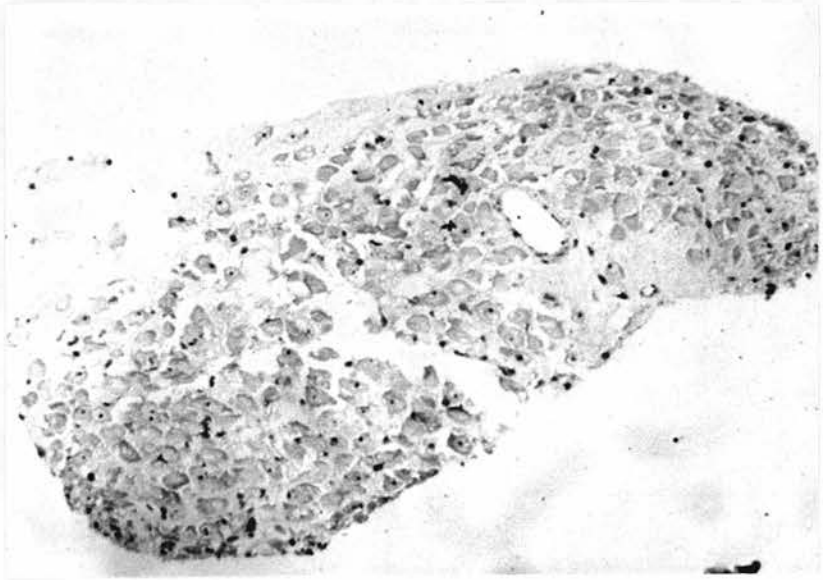
Plate 3 (a-d)

Stages in the dissection of the posterior part of the arcuate nucleus. (b), (c) and (d) show the medial surface of a midline section of the rat's hypothalamus.

Each scale division is 1 mm.



a.



b.

Plate 4

(a) Supraoptic nucleus of normal rat just before separation from the optic chiasm (Stage (e), Plate 2).

(b) Isolated supraoptic region from a rat deprived of water for five days.

Both sections were fixed in Carnoy's fluid and stained with cresyl fast violet.

Magnification x 250 (approximate).

METHODS

All neurones examined were isolated either from the supraoptic nuclei or from the posterior part of the arcuate nuclei of male albino rats aged three months. Where it was necessary to stimulate supraoptic cells physiologically, animals were deprived of drinking water, for periods up to five days, at an ambient temperature of either 20°C or 24°C.

(i) Preparation of tissues

Except where perfusion with fixative was necessary, all animals were killed by rapid exsanguination under light ether anaesthesia. Blood was collected at the time of death for the measurement of serum specific gravity or, when possible, serum osmolality.

Cell body parameters and nuclear dry mass were measured on fixed specimens; nuclear diameters and all nucleolar parameters refer to fresh tissue.

(a) Supraoptic nucleus

Identification of landmarks: The whole rat brain was fixed in Carnoy's fluid (glacial acetic acid 10%, absolute alcohol 60%, chloroform 30% by volume), embedded in paraffin wax and sectioned at 7 µm. A magnified projection of every fourth section was traced on to card from which a three-dimensional model was constructed (Plate 1).

Dissection: A thick (about 1 mm) slice was taken from the base of a freshly-removed rat brain and included the optic chiasm anteriorly and the median eminence posteriorly. The slice was placed, basal surface

up, on a clean glass slide under the dissection microscope (Plate 2a). The tissue was cut transversely at the junction of the posterior two-thirds and anterior one-third of the optic chiasm, and at the anterior border of the median eminence (Plate 2b). A third cut was placed bilaterally, parallel to and about 0.25 mm from the lateral border of the optic chiasm (Plate 2c). Two medial cuts produced two blocks of tissue, each composed of a portion of optic chiasm, with attached supraoptic region, and a variable thickness of unwanted hypothalamic tissue. After transfer to a fluid-filled watch glass, excess tissue was removed from the deep surface. Any tags of arachnoid were removed and the supraoptic region was separated from the optic chiasm (Plate 2e).

(b) Posterior part of the arcuate nucleus

The excised hypothalamus was bisected sagittally in the midline (Plate 3b). Under the dissecting microscope, a wedge of tissue was removed from the angle between the mammillary body and the floor of the third ventricle (Plates 3c and d). The knife was inclined to avoid including cells from the ventro-medial nucleus in the sample.

Early attempts at the dissection of hypothalamic nuclei were checked by histology (Plate 4).

(ii) Preparation of suspensions of isolated cells

Isolation of cells before fixation: Supraoptic regions were dissected out in 0.33 M sucrose solution and transferred to droplets of the same fluid on an agar-covered glass slide. Under the dissecting microscope, cell

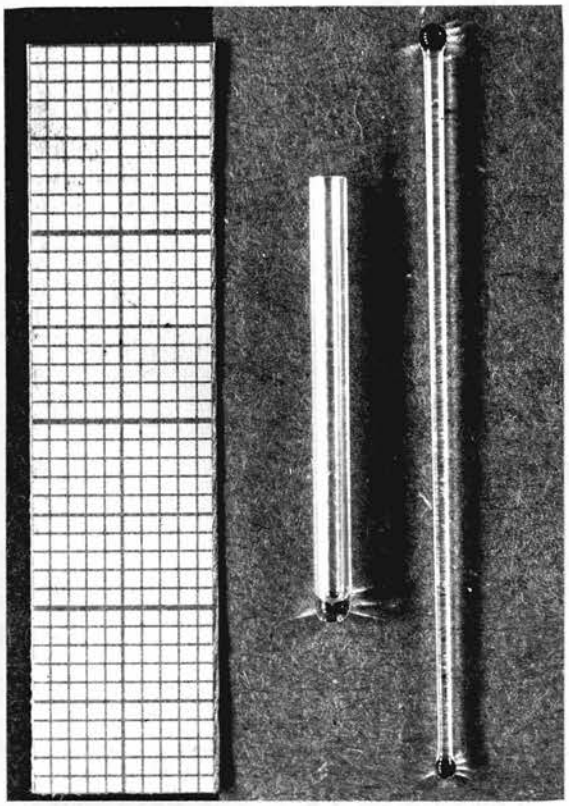
bodies of supraoptic neurones were "teased" out of the tissue by means of two tungsten needles. Fifty to sixty cell bodies were selected in the order in which seen and transferred by micropipette to a drop of 0.33 M sucrose on one half of a clean glass slide. The other half-slide was set aside as a reference for interference microscopy.

Those cell bodies which adhered to the slide after 15 minutes in a moist petri-dish were fixed by immersion for 24 hours in 10% buffered formalin. After fixation, the slide was submerged for two to four hours in two changes of distilled water, then allowed to dry at room temperature. Before interference microscopy, a coverslip with a drop of 1.55 M sucrose solution was applied to each half-slide.

Fixation of cells before isolation: Whilst the procedure described above gave good yields of cell bodies (and some nuclei), many neurones from dehydrated animals fragmented. For this reason it was necessary to isolate neurones from pre-fixed supraoptic regions.

Under ether anaesthesia, the descending aorta was clamped and 40 to 60 ml of 10% buffered formalin was infused above the block. The right atrium was opened to allow fixative to leave the circulation. Supraoptic regions were then dissected out and left in fixative for 24 hours.

After fixation, each piece of tissue was immersed for two hours in two changes of 1.55 M sucrose, then disrupted in 0.035 ml of this solution in a small glass "homogenizer"



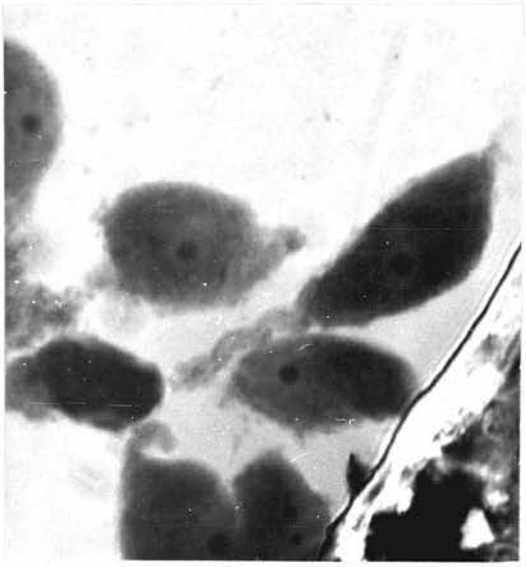


Plate 5

Glass "homogenizer" used when preparing suspensions of fixed cells or unfixed nucleoli. Each small scale division is 1 mm.

Plate 6

Neuronal cell bodies isolated from formalin-fixed supraoptic regions.

Photographed under even-field illumination in the interference microscope. Magnification x 720.

(Plate 5). The resulting "homogenate" contained large numbers of isolated cell bodies which appeared free of attached glia when examined in the interference microscope (Plate 6).

(iii) Preparation of suspensions of isolated nuclei

Supraoptic and/or posterior arcuate regions were dissected out fresh in 0.34 M buffered sucrose solution (sucrose 0.34 M, $MgCl_2$ 0.002 M, KCl 0.001 M, Tris 0.005 M, IN.HCl to pH 6.5 - Hadjiolov et al 1965). Disruption of this fresh tissue produced a suspension of nuclei, stripped of their surrounding cytoplasm. Nuclear preparations were mounted and examined in 0.34 M buffered sucrose.

(iv) Nuclear and cell body suspensions from the same animal

Both supraoptic regions were dissected out fresh. One was processed as above to prepare a suspension of nuclei, whilst the other was fixed by immersion in 10% buffered formalin for 24 hours before disruption into isolated cells. Since perfusion with fixative was not required, blood could be collected, by cardiac puncture, for the measurement of serum specific gravity.

(v) Nuclear diameter

Estimates of the diameters of nuclei within supraoptic neurones proved unreliable if fixed histological sections were used. The main difficulties were uneven shrinkage and an apparent rostro-caudal gradient of nuclear size within the supraoptic region. The measurement was therefore performed on freshly-isolated nuclei. Since the volume of an isolated nucleus can be influenced by the ionic

composition of the suspending medium (Ambrose and Forrester 1968), all measurements were made in buffered 0.34 M sucrose of constant composition.

Strips of glass cemented to the slide supported the coverslip and prevented compression of nuclei. Under these conditions, nuclei adhere either to slide or coverslip. As no estimate of the resulting deformation was made, nuclear diameter rather than volume was measured. Nuclei were photographed in positive contrast in the interference microscope and the negatives were scanned, in two directions at right angles, in the Joyce-Loebl microdensitometer Mk. IIIC. Overall magnification was x 2080.

(vi) Nucleolar margination

This was defined as contact between the nucleolus and nuclear membrane sufficient to deform the smooth outline of either (Plate 7). About 120 nuclei from each rat were examined in phase contrast and the result was expressed as "percentage of nuclei showing nucleolar margination".

(vii) Serum specific gravity

Until facilities for measuring serum osmolality were available, serum specific gravity was adopted as an approximation to the osmotic stimulus applied to the supraoptic neurones. Although indirect, this is probably a better index of dehydration than total body weight since rats deprived of water eat little after the second or third day (Dicker and Nunn, 1957). The decrease in total body water in dogs dehydrated for five days could account for only 45% of the observed loss of weight (Painter et al 1948).

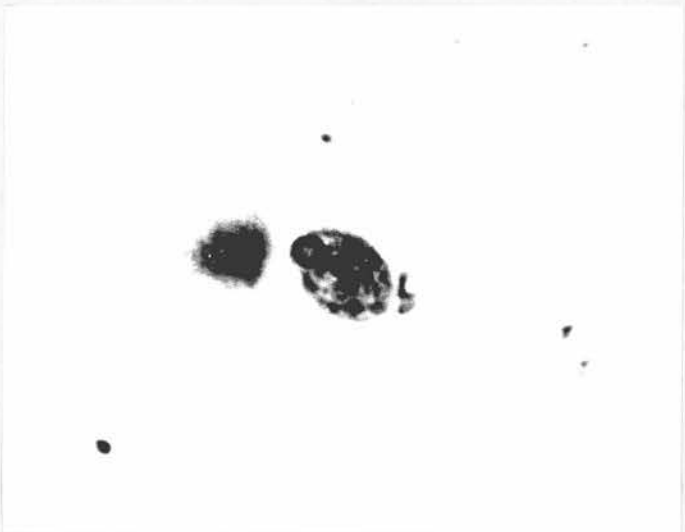


Plate 7

Nucleolar margination.

Deformation of the nuclear outline was a rare form of nucleolar margination.

Serum specific gravity was measured by means of a kerosene/bromobenzene density gradient column, buffered against swings in temperature by immersion in a 340-litre water-bath at room temperature. The column was calibrated with sucrose solutions of known specific gravity and remained stable and linear over a period of several weeks.

(viii) Dry mass measurement by interference microscopy

(a) Theory (Davies 1958)

The mass of dry substance in a transparent object immersed in water is given by the equation:

$$M = \frac{Dw.A}{X} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$ (Appendix i)

where M ..... dry mass in gm,

Dw ..... optical path difference due to the object,

A ..... area of the object in cm^2 in a plane normal to the optical axis,

X ..... 100 x (specific refractive increment).

Optical path difference (O.P.D.) can be measured more accurately when the refractive index of the object is close to that of the immersion medium. Fixed cells, with a refractive index around 1.54 (Davies 1958, Watson 1969b), must be immersed in a solution of higher refractive index than water. Equation (1) becomes:

$$M = \frac{Dm.A}{X} \cdot \frac{Np-Nw}{Np-Nm} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$ (Appendix ii)

where Dm ..... O.P.D. in medium of refractive index Nm,

Np ..... refractive index of the fixed cell,

Nw ..... refractive index of water.

(b) Evaluation of constants

An overall constant, K, may be defined as:

$$K = \frac{1}{X} \cdot \left(\frac{Np-Nw}{Np-Nm}\right) \quad \ldots\ldots\ldots\ldots \quad (3)$$

X for fresh tissue:

X = 100x, where x is the specific refractive increment, defined as the increase in refractive index of a solution when the solute concentration is raised by 1 gm per 100 ml. For soluble, unconjugated proteins, X lies in the range 0.181 to 0.188 (Davies 1958, Barer 1966), and is almost independent of concentration up to 50 gm per 100 ml (Adair and Robinson 1930, Barer and Tkaczyk 1954). When measured using light of 546 nm wavelength, the mean value of X for DNA is 0.187 (Davies 1958). The few figures available for RNA lie between 0.168 and 0.194 (Barer 1966).

The value of X for carbohydrates and lipids is usually close to 0.14 (Barer 1966, Davies 1958), although X = 0.15 has been reported for glycogen (Barer and Joseph 1957).

The nucleolus is rich in protein and is the densest cellular organelle (Sirlin 1962). So the assumption in this investigation of a value for X of 0.18 should not introduce serious error in determining the dry mass of nucleoli.

X for fixed tissue:

The true value of X for dry protein remains uncertain. Depending on the figure taken as the refractive index of

dry protein, calculated values for X either group around 0.15 or lie in the range 0.19 to 0.21 (Davies 1958). For this reason there is no authoritative figure for X in fixed tissue. The dry mass of fixed neurones and nuclei measured in this study was calculated with an assumed value for X of 0.18. This may underestimate dry mass by as much as 20%.

Refractive index:

Mounting media (Nm). All measurements of dry mass were made using sucrose solution as the mounting medium. For fixed nuclei and cell bodies, Nm was 1.4262; for unfixed nucleoli, Nm was 1.349.

Fixed cells (Np). The O.P.D. (Dm) due to a cell of "effective" thickness te (Appendix ii) and refractive index Np is given by:

$$Dm = (Np-Nm)te.$$

Thus, when $Dm = 0$, $Nm = Np$.

Figure 1 shows the change in O.P.D. at 22 points in eight different neurones placed successively in mounting media of refractive indices 1.333, 1.375 and 1.416. Inaccuracy in measuring such small phase changes has obscured the anticipated family of straight lines (Watson 1969b). A "best estimate" of Np was obtained by extrapolating the calculated regression line for all 66 points plotted in Fig. 1.

The refractive index of fixed supraoptic neurones was found to be 1.519 and 1.5207 in two separate experiments.

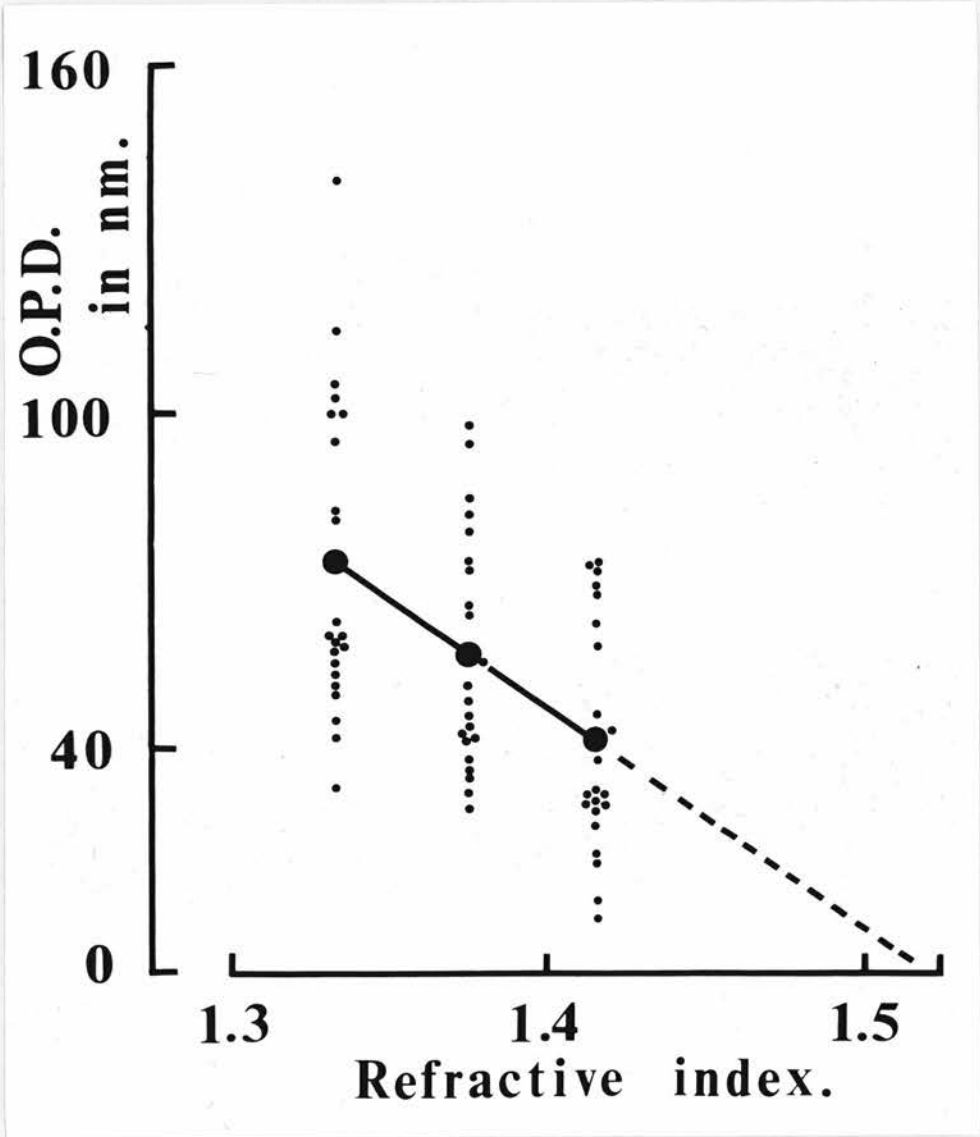

160
O.P.D.
in nm.
100
40
0
1.3
1.4
1.5
Refractive index.

Figure 1

Refractive index of fixed supraoptic neurones

O.P.D. was measured at 22 points, in eight different neurones, immersed successively in media of refractive indices 1.333, 1.375 and 1.416.

By extrapolation of the computed regression line, the refractive index of fixed supraoptic cells is 1.5207.

Unfixed nucleoli (Nc). For light rays passing through the centre of a spherical nucleolus, $Dm = (Nc-Nm)d$, where d is the nucleolar diameter.

Hence, $Nc = \frac{Dm}{d} + Nm$.

Experimental justification for assuming nucleoli to be spherical is given below. In practice, Dm may be overestimated due to nuclear substance above and below the nucleolus. The change in Nc resulting from a 10% overestimate of Dm will affect the value obtained for nucleolar dry mass by less than 2%. The nuclear contribution to measured O.P.D. has been assessed at about 1% (below) and should have negligible effect on dry mass values.

Interferometry on 100 supraoptic nucleoli gave a mean value for $Dm/d \pm$ S.E.M. of 0.077 ± 0.0013.

Hence, $Nc = (0.077 + 1.349) \pm 0.0013$
$= 1.426 \pm 0.0013$.

Substituting these values in equation (3) above,
K for fixed nuclei and cells = 11.14, and
K for unfixed nucleoli = 6.70.

(c) Nuclear contribution to nucleolar dry mass

Measurements of nucleolar O.P.D. will include a contribution due to the thickness of nuclear substance above and below the nucleolus. The true nucleolar dry mass is given by:

$$M = \frac{A}{X} \cdot \frac{D1-D2\ (1-t/d)\ (Nc-Nw)}{(Nc-Nm)} \quad \ldots\ldots\ldots (4)$$ (Appendix iii),

where D1 ..... mean O.P.D. due to nucleolus and nucleoplasm,

Table 1

Nuclear contribution to nucleolar O.P.D.

	Before extrusion	%	After extrusion	%	% change
No.of observations	45		42		
Measured diameter ± S.E.M. (μm)	3.94±0.07	100	4.20±0.12	106.8	+ 6.8
Calculated thickness	-	100*	-	88	- 12
Predicted change in O.P.D.	-		-		- 12
Measured O.P.D. ± S.E.M. (nm)	293.3±5.5	100	255.4±6.6	87	- 13
Nuclear contribution to measured O.P.D.	-	1	-	0*	- 1

* Assumed values. Nucleolar diameter was measured normal to the optical axis. Nucleolar thickness after extrusion was calculated by assuming that compression would deform the nucleolus from a sphere to an oblate spheroid. A change in diameter of + 6.8% would result from a decrease in thickness of 12%. Thus only 1% of the observed 13% decrease in O.P.D. could be attributed to the loss of overlying nucleoplasm.

D2 ..... mean O.P.D. through nucleus alone,

t ..... mean nucleolar thickness,

d ..... mean nuclear thickness.

Thus, the nuclear contribution to the measured O.P.D. (D1) is given by "D2(1-t/d)".

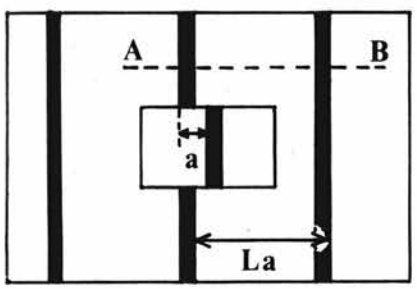
Since nuclei may be deformed when adhering to the slide or coverslip, d cannot be predicted from measurements of nuclear diameter. For this reason a practical determination of the nuclear contribution has been attempted.

The O.P.D. due to supraoptic nucleoli was measured before and after their extrusion from nuclei (Table I). The isolation of nucleoli involved compression of the sample between slide and coverslip. Compression sufficient to increase nucleolar diameter by 6.8% (Table I) should diminish nucleolar O.P.D. by about 12%. Thus only about 1% of the 13% drop in O.P.D. after extrusion could be attributed to the loss of overlying nucleoplasm.

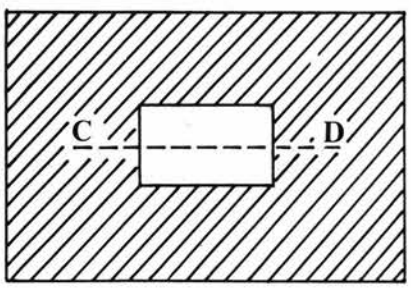
Errors are likely in distinguishing isolated nucleoli from the debris of disrupted cells and nuclei and some nucleoli may have retained adherent nucleoplasm. 1% may therefore be an underestimate of the true nuclear contribution to the nucleolar dry masses quoted in this report.

(d) The interference microscope

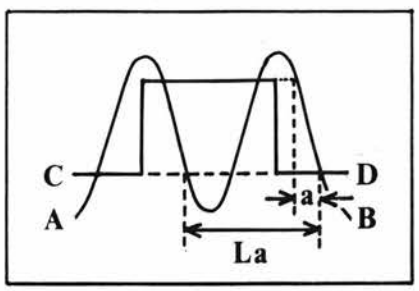
All dry masses were measured with the Leitz interference microscope (objectives x 50/0.85, condensers N.A. 0.50). Fig. 2 summarises, for a rectangular object, the



i.



ii.



iii.

Figure 2

Interference Microscopy

i. Rectangular object in banded field.

ii. Rectangular object under even-field illumination ($La = \infty$).

iii. Intensity changes along scans A-B and C-D.

With selected even-field illumination and wavelength retardation ($^{a}/La$) less than 0.2, the relationship between phase change and image intensity is approximately linear.

method used for measuring the O.P.D. of nuclei and cell bodies. In a mounting medium of refractive index 1.4262, the maximal change in phase due to the cell was about 0.2 wavelengths. Careful selection of the background intensity allowed measurement over the approximately linear part of the sine-wave relating change in phase to intensity of the image (Fig. 2 iii). A mean integrated O.P.D. could therefore be calculated by means of photoelectric scanning of a photomicrograph of the isolated cell, taken when the width of the interference fringes was infinite (Fig. 2ii).

Nucleoli were assumed to be spherical and homogeneous for the purposes of dry mass measurements. Under these circumstances, the mean "thickness" of the nucleolus is the height of a cylinder with the same radius and volume as the nucleolar sphere.

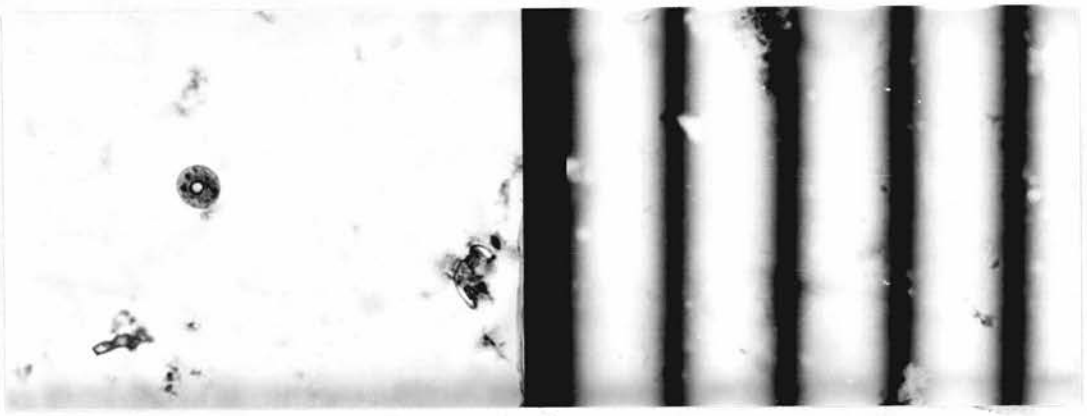
i.e. $^4/_3 \pi r^3 = \pi r^2 h$

Hence $h = {}^4/_3\ r$, or $\frac{2}{3}d$, where d is the nucleolar diameter. If the nucleolus is homogeneous, it follows that

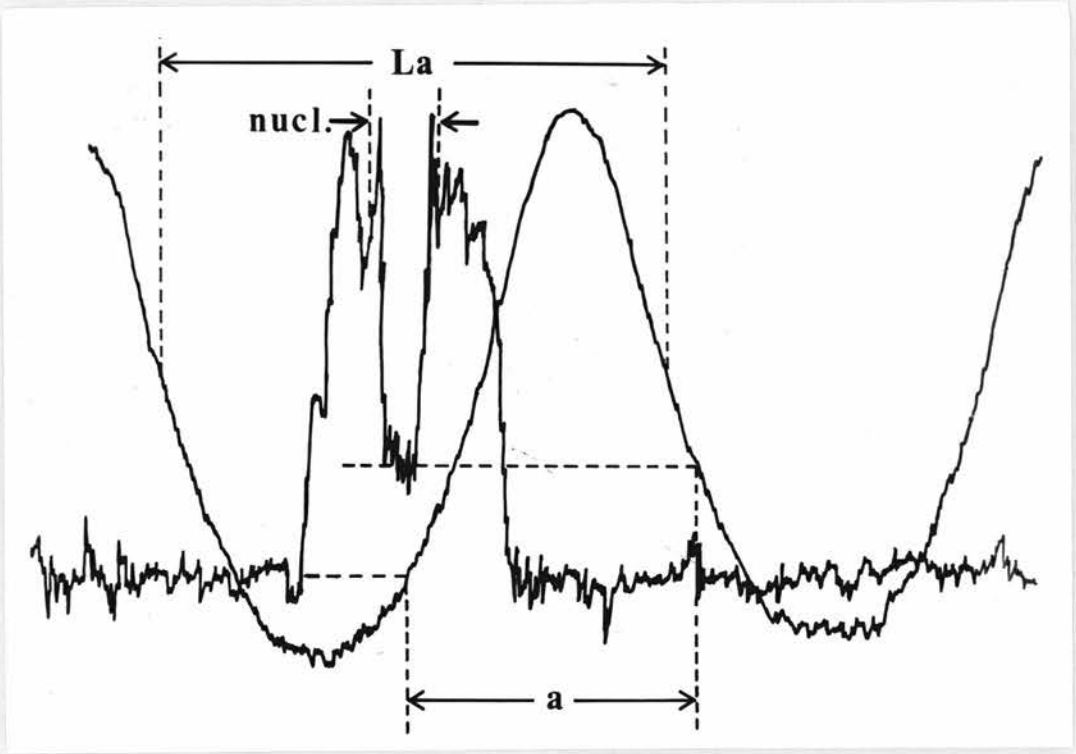
$$\underline{\text{mean nucleolar O.P.D.}} = \underline{\tfrac{2}{3} \times \text{maximal nucleolar O.P.D.}}$$

Since only maximal nucleolar O.P.D. was measured, integration was avoided and a linear relation between phase change and intensity of the image was not required. In consequence, changes in phase of up to 0.5 wavelengths could be measured accurately (Plate 8).

For maximal contrast, measurement should be restricted to the interference band which forms the centre of symmetry of the fringe pattern (Davies 1958). This "zero-



a.



b.

Plate 8

Nucleolar optical path difference

(a) Isolated nucleus under even-field illumination. Note phase reversal in the centre of the nucleolus where retardation is maximal. The adjacentframe shows interference fringes crossing the field close to the nucleus.

(b) Superimposed microdensitometer tracings of scans through the fringe pattern and the centre of the nucleolus (nucl.). Symbols as in Fig. 2.

order" band was identified using white light. Monochromatic light, of 546 nm wavelength, was used for photomicrography. Photomicrographs were recorded on Kodak Plus-X panchromatic 35 mm film, developed in Kodak D19 developer and analysed with the Joyce-Loebl scanning microdensitometer. Fifteen to twenty cells, nuclei or nucleoli were measured from each hypothalamic nucleus examined.

(e) Sources of error

1. The assumption that the nucleolus approximates to a sphere was tested by subjecting a proportion of the densitometer records to "rotational integration" (after Edström 1964). Fig. 3 shows the relationship between nucleolar dry mass calculated (a) by "rotational integration" and (b) by measurement of the maximal nucleolar O.P.D. The calculated regression line ($a = 1.006\ b + 0.118$) is a good approximation to the line of $a = b$, and the ratios $\frac{a}{b}$ all fall within the range 0.9 to 1.1.

2. Any error in measuring nucleolar diameter will be squared in calculating the dry mass. Despite individual consistency, this measurement differed sufficiently between observers to alter nucleolar dry mass by about 10%. Since all dry masses in this report were measured by the author, this uncertainty will not affect relative changes in nucleolar dry mass.

3. Since the illuminating beam is conical, light rays outside the optical axis pass through a greater thickness of specimen. The resulting overestimate of O.P.D. should not exceed 5% in the fixed flattened cells examined here

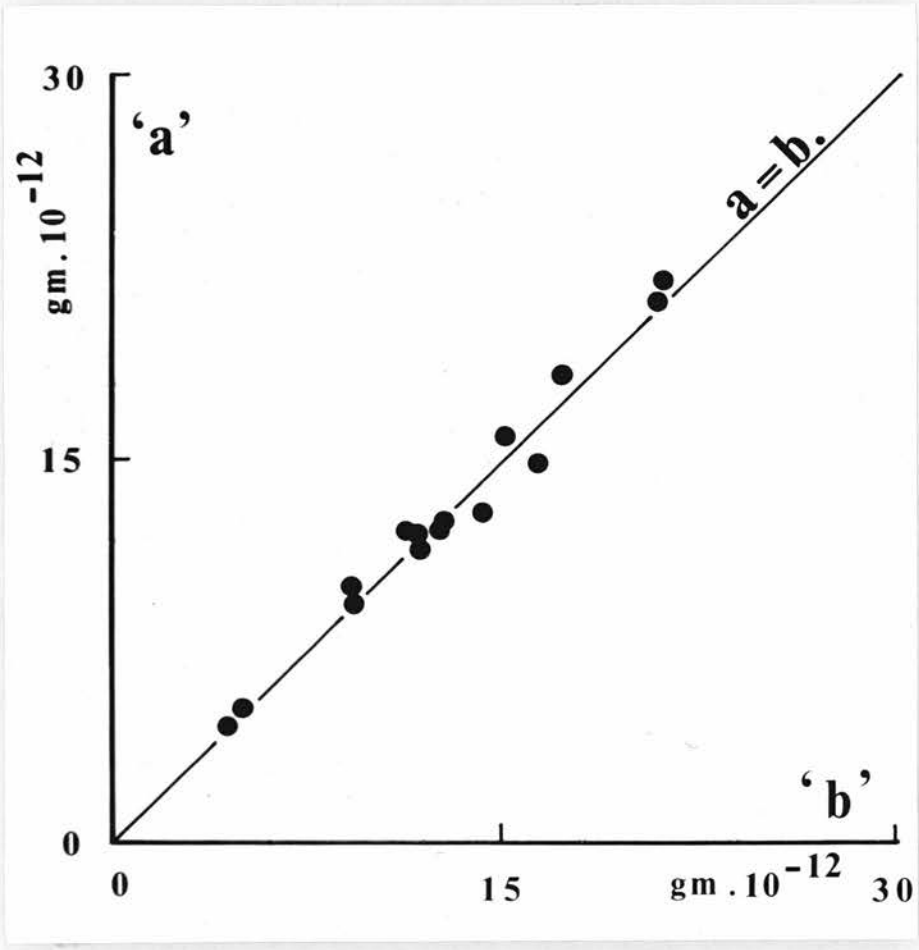

30
'a'
gm.10^{-12}
a=b.
15
'b'
0
0
15
gm.10^{-12}
30

Figure 3

Nucleolar sphericity

Nucleolar dry mass was calculated (a) by rotational integration (ordinate) and (b) from the measured maximal nucleolar O.P.D. (as Plate 7).

The computed regression line (not shown) is:

$a = 1.006\ b + 0.118$.

(Davies and Deeley 1956). Nucleolar estimations should be free from this error.

4. Errors in measuring the area of a cell on the photomicrograph can arise because of "blurring" of the image by particles lying outside the plane of focus (Davies 1958). For this reason, a rim of clear field was included in each scan across the cell during densitometry.

5. Glare in the optics of the microscope has not been estimated. Consistent even-field illumination and a small condenser aperture (N.A. 0.5) should keep this error constant between specimens.

6. The sinusoidal relationship between change in phase and the optical density of the photomicrograph is modified by the gamma of the recording film. With the change in phase restricted to 0.2 wavelength and selected even-field illumination, errors arising from the assumption of a linear relationship between O.P.D. and the optical density of the photographic image should not exceed 10% (Watson 1969, Mitchison et al 1956).

(f) Validity of interferometry of biological material

In general, interference microscopy has compared well with alternative techniques for measuring the dry mass of cells and organelles. The results of interferometry on fresh nuclei from the calf thymus correlated well with those of direct weighing after dessication (Hale and Kay 1956). The dry mass of haemolysing erythrocytes, predicted from microspectrographic measurements of haemoglobin content, showed good agreement with the results of interferometry on the same cells (Lagerlöf et al 1956).

Interferometry and X-ray absorption (Engström and Lindström 1958) give similar figures for the dry mass of rat mast cells (Ottoson et al 1958) and fresh human erythrocytes (Gamble and Glick 1960). Davies et al (1953) found good agreement between these two techniques with a wide variety of tissues. Beneke (1966a) has tabulated a selection of dry mass figures obtained by quantitative electron microscopy, X-ray absorption and interferometry. Of these techniques, the first is in its infancy and will not be considered here. Dry masses measured by the other two techniques correlate less well when concentrated or fixed tissues are examined. This may be caused by the increased inhomogeneity of fixed material (Davies 1958, Beneke 1966b) and the uncertainty of X at very high protein concentrations.

(ix) Ultraviolet microspectrography

The method relies on the absorption of ultraviolet radiation by nucleic acids (Dhéré 1906) at a wavelength (260 nm) relatively free of absorption bands due to other quantitatively important cellular constituents.

(a) Theory (Walker 1956)

The absorption law may be summarised as:

$A_L = \log Io/I = kcl,$

where A_L ..... absorbance at wavelength L,

Io ..... intensity of incident radiation,

I ..... intensity of transmitted radiation,

k ..... absorptivity,

c ..... concentration in gm/litre,

l ..... thickness of absorbing object in cm.

With biological objects, where l is difficult to measure, an alternative form is useful:

$$c = A/kl = m/al$$

where m ..... mass of absorbing material in gm,
a ..... area of object in a plane normal to the optical axis ($10^3 cm^2$).

Therefore, $\underline{m = Aa/k}$.

Mean integrated absorbance (A) was measured by ultraviolet microspectrography and area (a) by scanning microdensitometry. A value of 22 was assumed for absorptivity after Caspersson (1936).

(b) The Leitz ultraviolet microspectrograph

The instrument incorporates three alternative light sources - a low voltage tungsten lamp for focussing in visible phase contrast, a stabilized, water-cooled hydrogen lamp for "single-exposure" recording of ultraviolet absorption spectra over the range 235 to 320 nm and a monochromator, fed by a low-pressure mercury lamp, for monochromatic photomicrography at 253.7 nm wavelength.

For measurements on fixed, isolated cell-bodies in 53% sucrose solution, the x 170 objective (N.A., 0.50) was used with a condenser N.A. of 0.6. Fresh, isolated nuclei were examined in buffered 0.34 M sucrose, using the x 300 glycerine/water immersion objective (N.A. 0.85), with the same condenser. Objectives and condenser were of the reflecting type, claimed achromatic over the range 220 to 700 nm (Freed 1969).

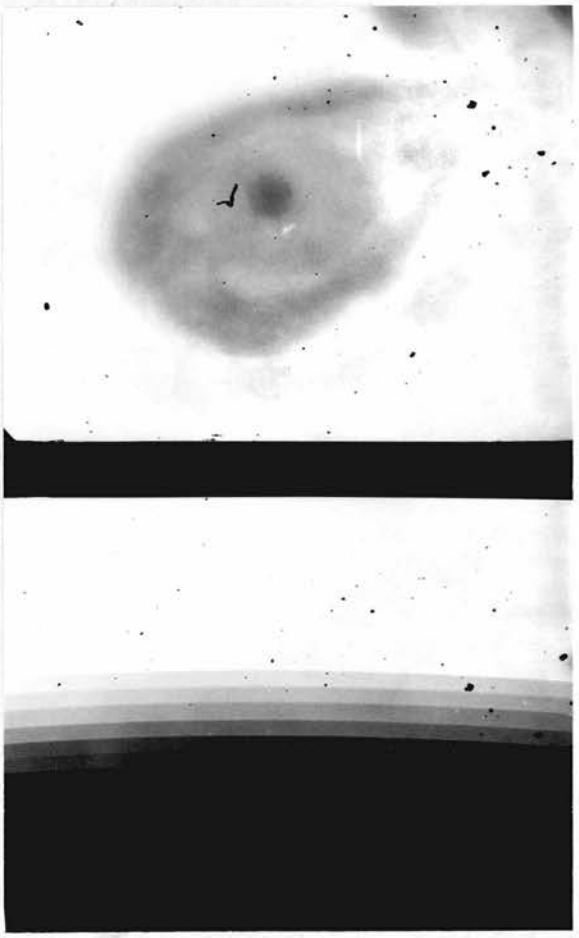


Plate 9

Photomicrograph, taken in ultraviolet light (253.7 nm), of an isolated supraoptic neurone. The adjacent frame shows the 0.1 steps in optical density derived from a rotating, stepped sector.

Magnification is x 1700 (approximate).

(c) Technique

Mean integrated absorbance. This was measured from two monochromatic photomicrographs which were recorded on Ilford N40 plates and developed in Kodak D19 developer (Plate 9).

The emulsion response was calibrated for each cell or nucleolus by means of a "clear field" photomicrograph with a rotating stepped sector in the plane of the entrance slit of the spectrograph. Each step of the sector corresponded to an apparent increase of 0.1 O.D. units in absorbance of the object. Exposure and development of the plate were adjusted to give a linear response in photographic density over the range 0 to 0.8 O.D. units.

The negatives were analysed on the Joyce-Loebl scanning microdensitometer with the digital integrator attachment.

Absorption spectra of cell structures. The cell body or nucleolus was illuminated by the hydrogen lamp and the transmitted radiation was dispersed by the quartz spectrograph. Spectrographic slit widths corresponding to 0.5 μm and 0.9 μm in the plane of the object were used for nucleoli and cells respectively.

Individual absorption spectra were calibrated using a "clear field" photomicrograph of the spectrum with the rotating sector in the beam-path. Fig. 4 shows typical spectra obtained from cell body (cytoplasm) and nucleolus.

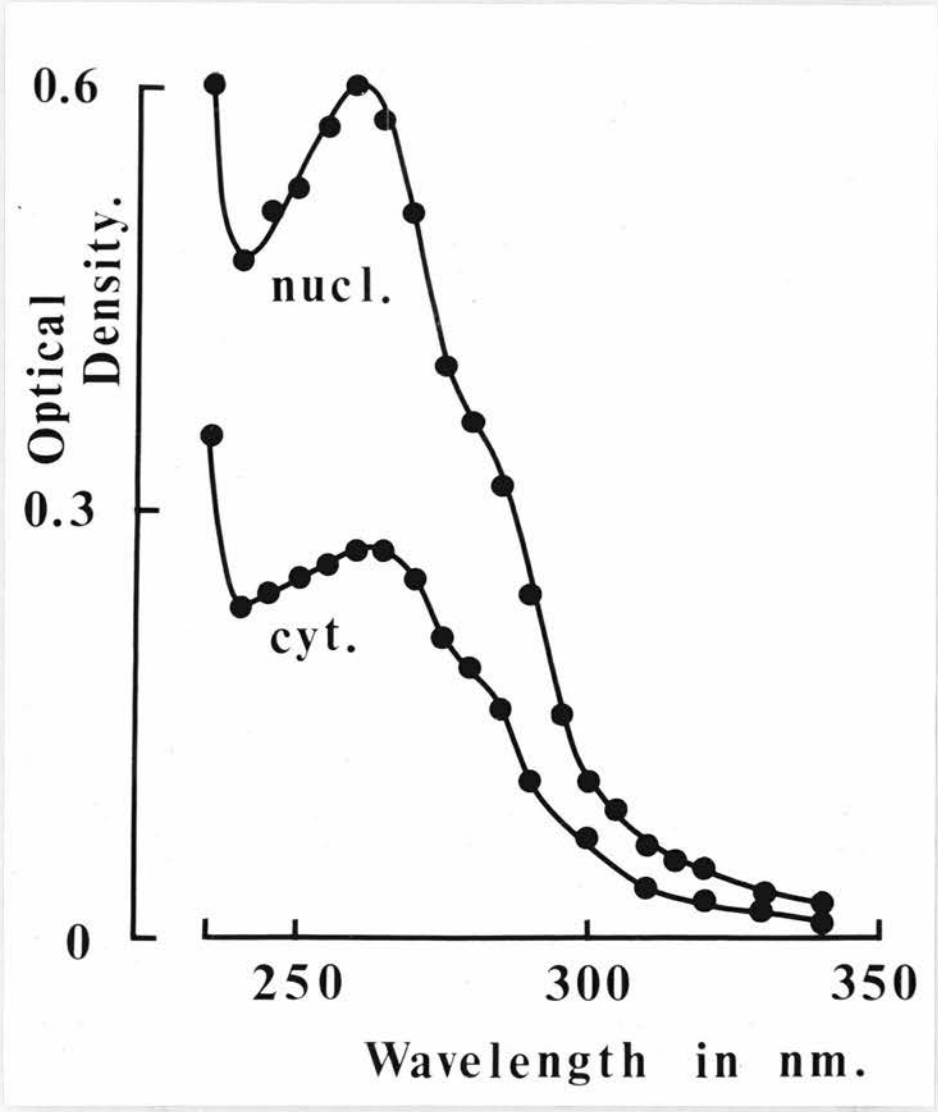

0.6
0.3
0
Optical Density.
nucl.
cyt.
250
300
350
Wavelength in nm.

Figure 4

Ultraviolet absorption spectra of nucleolar (nucl.) and cytoplasmic (cyt.) regions of supraoptic neurones.

Absorption spectra were used (a) to calculate absorbance at 260 nm from that measured at 253.7 nm, (b) to estimate the contribution of non-specific light loss to the measured integrated mean absorbance and (c) to calculate the ratio A_{260}/A_{280} as a rough indication (Walker 1956) of the relative composition of nucleoli in terms of protein and nucleic acid.

The content of nucleic acid was measured in 15 to 20 nerve cell bodies or nucleoli from each hypothalamic nucleus examined.

(d) Sources of error and corrections

1. The values quoted for nucleolar nucleic acid content will include a contribution from an unknown thickness of nucleoplasm overlying the nucleolus. The integrated absorbance decreases by 6% to 11% in nucleoli extruded from nuclei (Watson 1968a), but whether this reflects the absorbance of overlying nucleoplasm or loss of nucleolar material is uncertain. Nucleolar nucleic acid contents may thus be 6% to 11% overestimated.

2. Transmitted radiation is lost by lateral diffraction at regions of sudden change in refractive index. Assessment of the loss of light from this cause is difficult (Walker 1956, Freed 1969). In this report, it has been assumed that light loss due to scatter is inversely proportional to the fourth power of the wavelength (Rayleigh scatter law, Oster 1948). This probably represents a maximal correction (Doty and Edsall 1951, Walker 1956).

The correction factor was estimated from the absorbance at 314 nm, measured in a preliminary series of absorption spectra. At this wavelength, true absorption by quantitatively important cell constituents is believed absent (Walker 1956). For the spectra of Fig. 4, the estimated light losses due to scatter at 260 nm were 20.6% and 22.7% of the measured absorbance for nucleolus and cytoplasm respectively.

The mean factor calculated for control supraoptic nucleoli amounted to 17.8% of the measured absorbance and, for control cell bodies, to 18%. All nucleic acid results quoted here have been corrected using these figures. Dehydration of rats for five days did not alter the scatter correction for nucleoli (17.4%) but increased that for cell bodies by 9%. To this extent, 5-day figures may be overestimated. Unless stated, this additional correction does not influence the values quoted for statistical probabilities.

3. Errors arising from absorbance differences between absorbing granules and the background can be kept low by minimising the scanning aperture used when analysing the photographic plate (Walker 1956). Referred to the plane of the object, the apertures used here corresponded to 0.05 μm^2 and 0.013 μm^2 for cell bodies and nucleoli respectively.

4. Spherical aberration and glare should remain constant between specimens and no correction has been applied. The use of matched reflecting optics and measurement only over

Table II

Microdensitometer settings

	Arm ratio	Scanning aperture (width x height) µm	Scan separation µm
Cell body dry mass	10:1	0.14 x 0.28	2.6
Cell body nucleic acid	20:1	0.16 x 0.31	4.8
Nucleolar dry mass	20:1	0.14 x 0.28	-
Nucleolar nucleic acid	50:1	0.08 x 0.16	0.5

All measurements are referred to the plane of the microscopic object. "Scan separation" was the vertical distance between horizontal scans across the object.

the range 0 to 0.8 O.D. units should keep such errors to a minimum (Walker 1956).

5. Particles lying outside the plane of focus will contribute radiation over a wider than normal area in the plane of the image. No correction has been applied for this although, with structures of low absorbance, the error should be small (Walker 1956).

6. A fractional correction factor was applied to calculate absorbance at 260 nm from that measured at 253.7 nm. The factor for nucleoli was 1.026 and for cell bodies was 1.066.

7. Errors caused by damage from ultraviolet irradiation were limited by exposing a nucleolus or cell body once only.

As with dry mass values, significance has been attached only to relative changes in nucleic acid contents.

(x) Microdensitometry

Photomicrographs from the interference microscope and ultraviolet microspectrograph were scanned with the Joyce-Loebl Automatic Recording Microdensitometer, Mk. IIIc, with a digital integrator attachment. The grey-wedge range was 0 to 2 D.; objective magnification was x 22. Other instrumental settings are given in Table II.

RESULTS

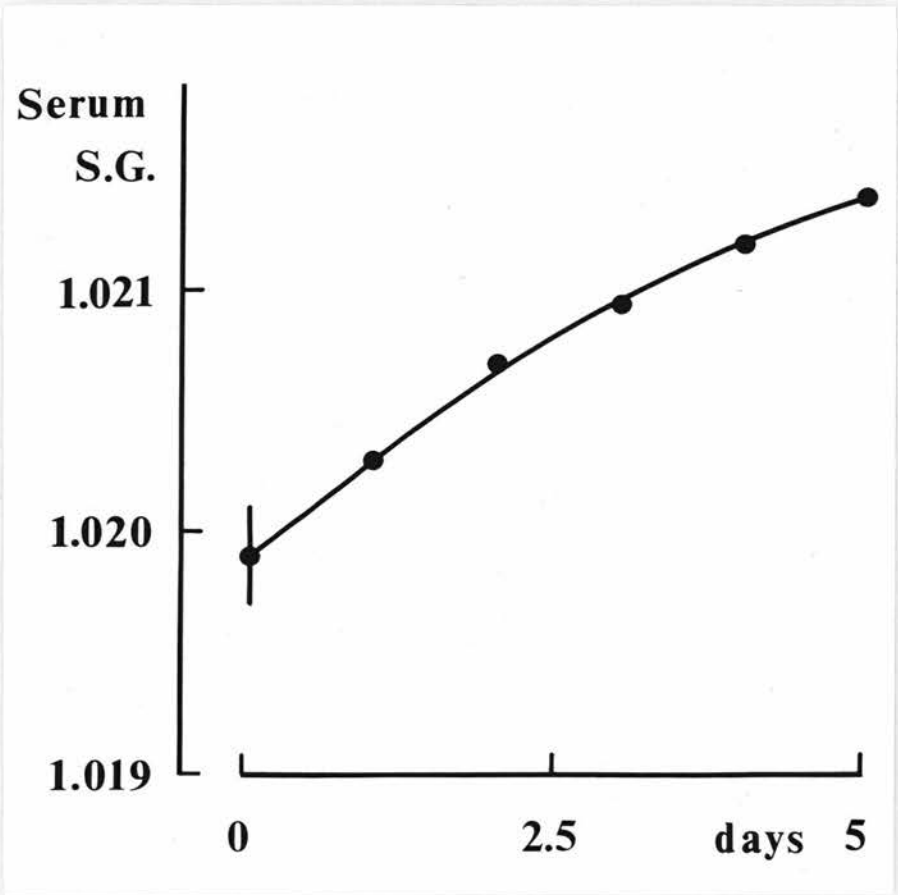
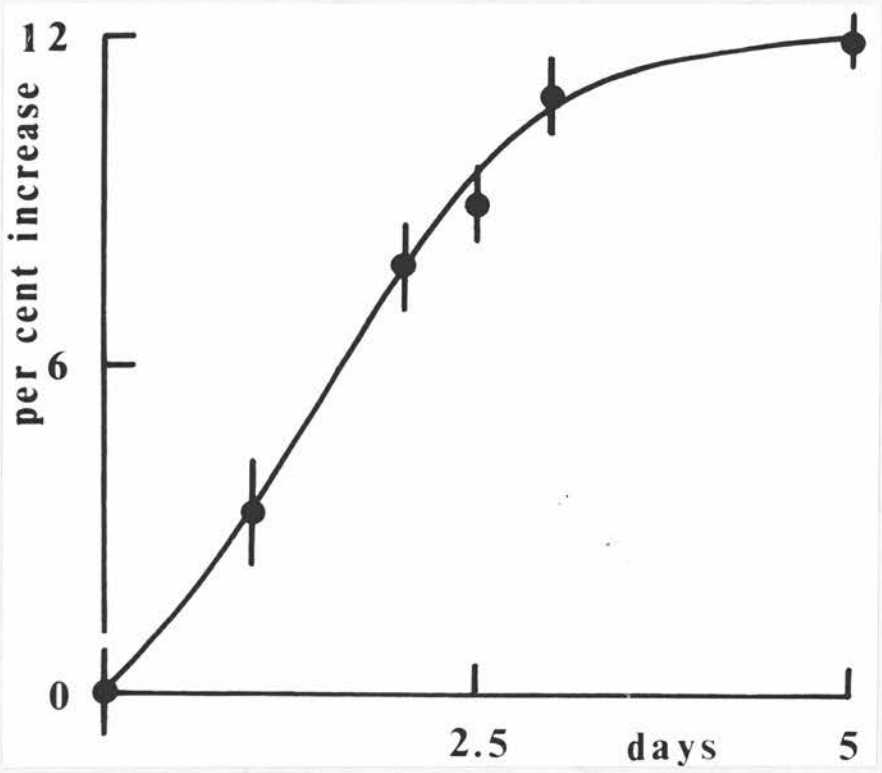

Serum
S.G.
1.021
1.020
1.019
0
2.5
days
5

Figure 5

Serum Specific Gravity

The specific gravity of blood serum is expressed against the duration of water deprivation. With the exception of day 0 (mean $\pm$ S.E.M. of eight observations), each point is the mean of 2 to 5 measurements from different rats.


12
per cent increase
6
0
2.5
days
5

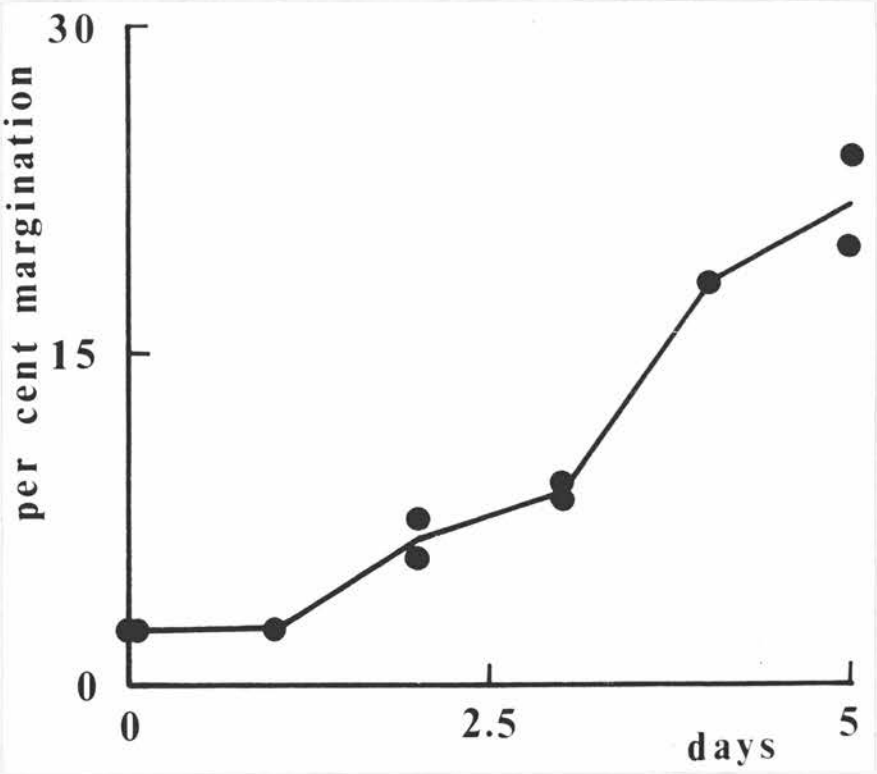

30
per cent margination
15
0
0
2.5
days
5

Figure 6 Nuclear diameter

Increase in mean diameter (%) of supraoptic nuclei isolated from rats dehydrated for up to 5 days at 20°C. Each point is the mean ± S.E.M. of 120-200 measurements (3-5 rats). The mean diameter of control nuclei (0%) was 11.31 ± 0.10 µm.

RESULTS

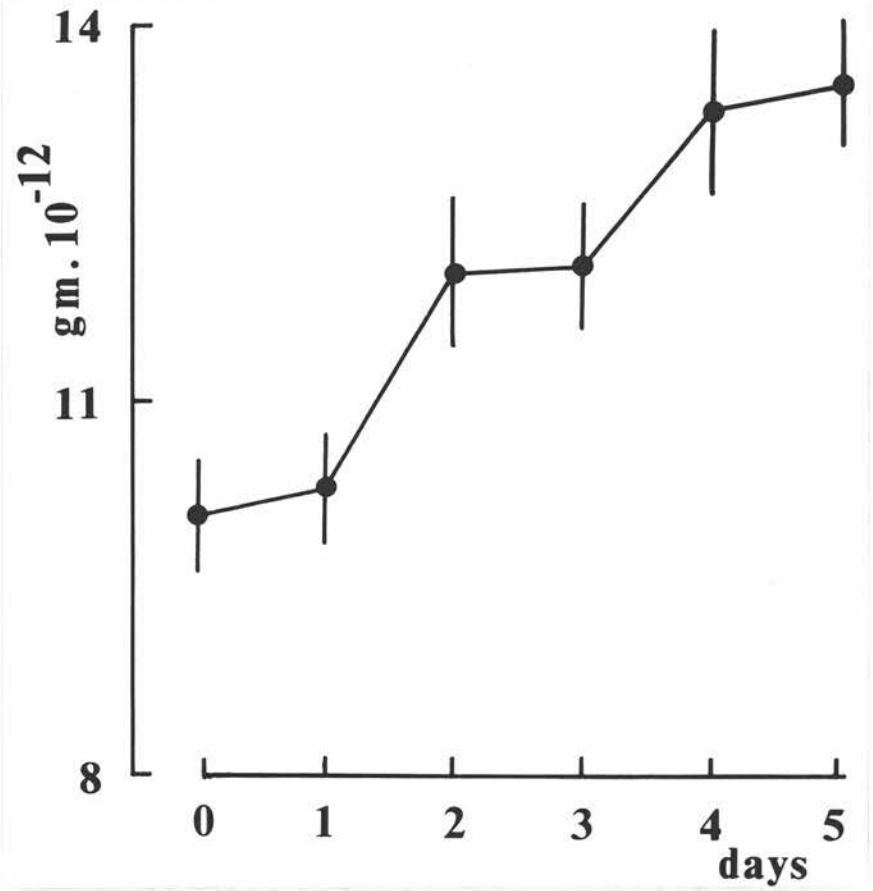
Dehydration at ambient temperature of 20°C

Five days' water deprivation caused a smooth increase in the specific gravity of blood serum (Fig. 5). Evidence from later experiments suggests that the observed increase from 1.0197 to 1.021 accompanies a drop in body weight of 25 to 30% and an increase in serum osmolality from 305 to 332 m.Osm./L.

The change in the mean diameter of isolated supraoptic nuclei occurred early and was almost complete by the third day (Fig. 6). The total increase was a mere 11.8% but was highly significant ($p \ll 0.001$). Nuclei from dehydrated animals were more likely to contain marginated nucleoli (Fig.7).

Figure 7 Nucleolar margination

Percentage of isolated supraoptic nuclei containing marginated nucleoli expressed against the duration of water deprivation at 20°C. 115-120 nuclei were examined in one animal for each plotted point.


14
11
8
gm.10-12
0
1
2
3
4
5
days

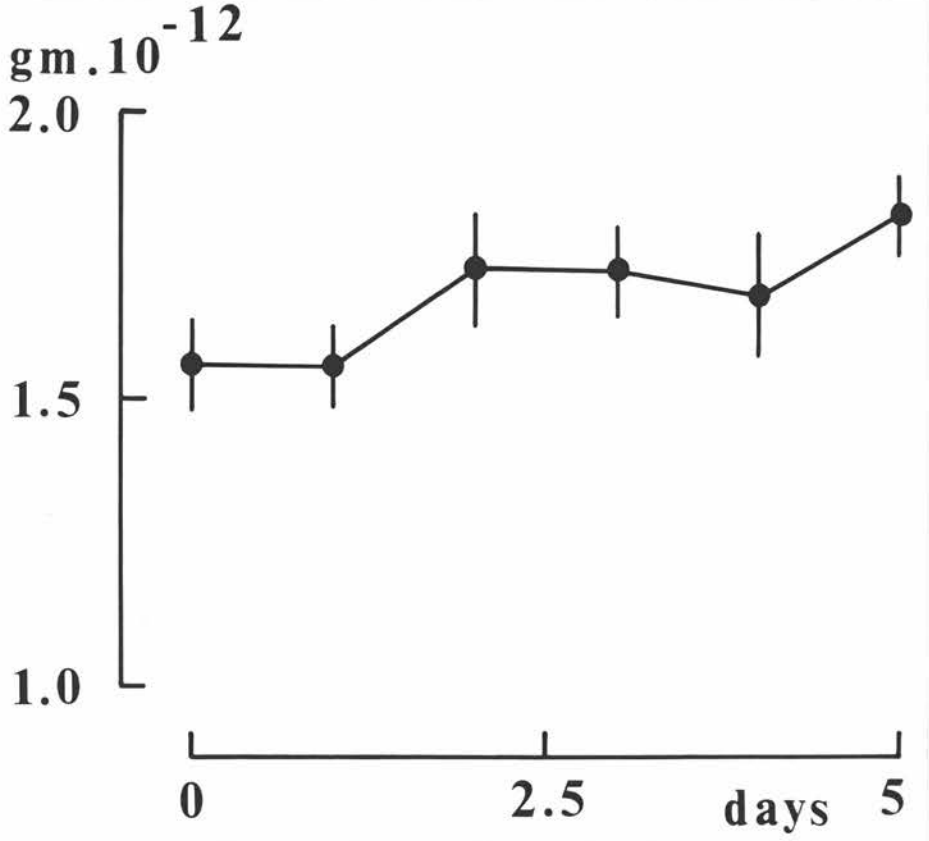

gm.10-12
2.0
1.5
1.0
0
2.5
days
5

Figure 8 Nucleolar Dry Mass

The mean dry mass of supraoptic nucleoli expressed against the duration of dehydration at 20°C. Each point is the mean ± S.E.M. for a total of 50 to 90 measurements from 3 to 5 rats.

Osmotic stress increased both the mean dry mass (34%, $p < 0.001$) and the mean content of nucleic acid (11.8%, $p < 0.025$) of supraoptic nucleoli (Figs. 8 and 9). Thus the contribution of nucleic acid to the dry mass of the average supraoptic nucleolus decreased from 15.4% on day 0 to 13.7% on day 5. This change in nucleolar composition was apparent also in the A_{260}/A_{280} ratios calculated from ultraviolet absorption spectra. For nucleolar spectra from normal and osmotically stressed rats, the respective ratios were 1.66 and 1.61.

When nerve cell bodies were isolated from supraoptic tissue fixed by immersion, their mean dry mass was about 95% greater than that of cell bodies fixed <u>after</u> isolation (Fig. 10). Intermediate values were obtained when <u>perfusion</u> with fixative was used (474 ± 11.0 $gm.10^{-12}$:

Figure 9 Nucleolar Nucleic Acid Content

The mean content of nucleic acid of supraoptic nucleoli expressed against the duration of dehydration at 20°C. Each point is the mean ± S.E.M. for a total of 50 to 85 measurements from 3 to 5 rats.

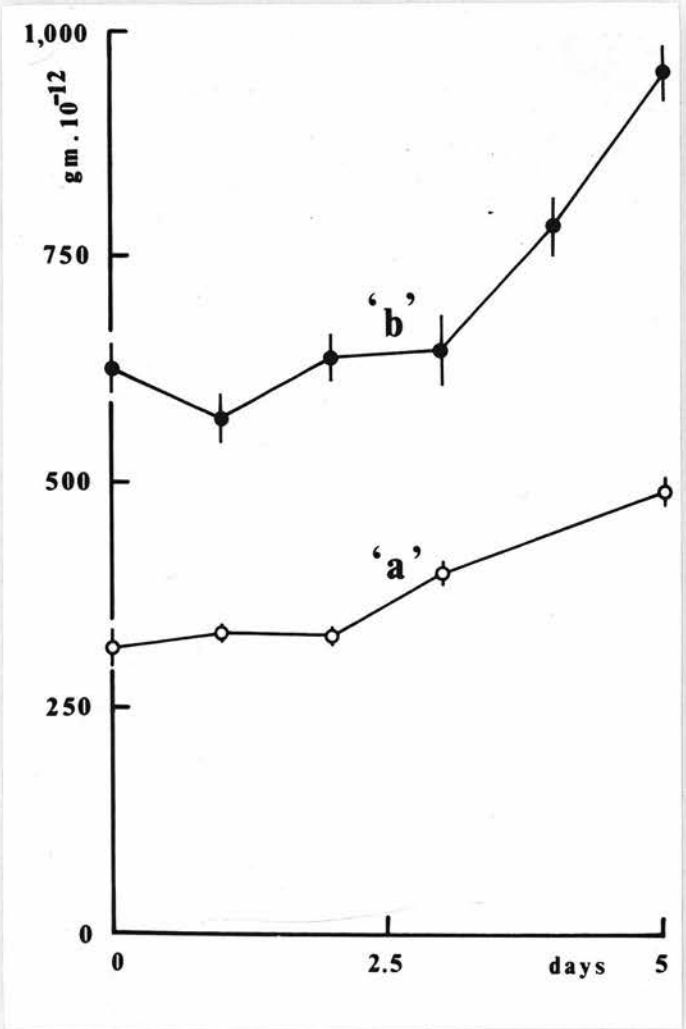
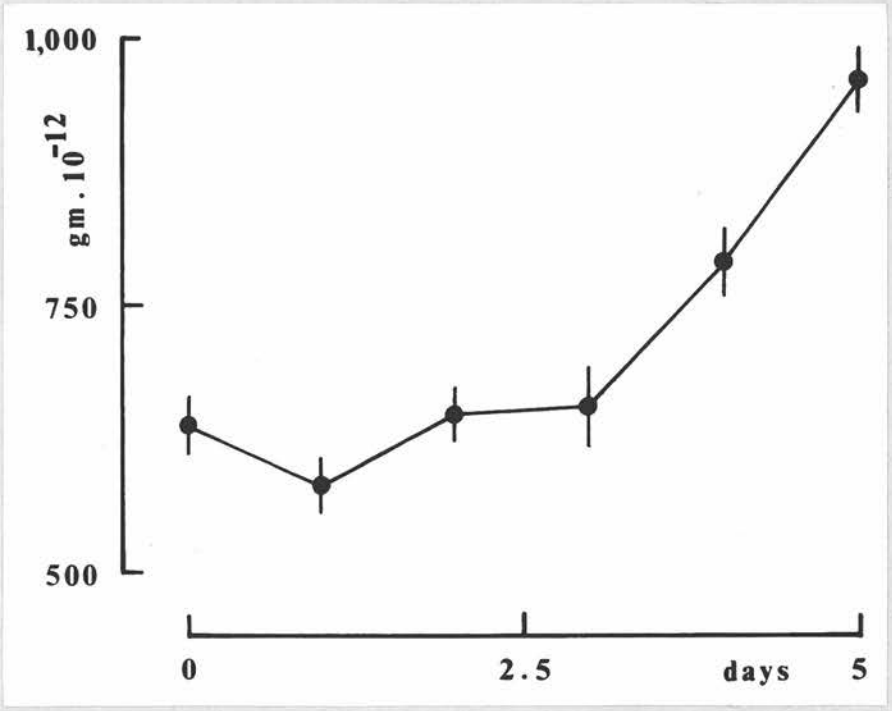

1,000
gm.10-12
750
500
250
0
'b'
'a'
0
2.5
days
5

Figure 10 Cell-body Dry Mass

The mean dry mass of supraoptic neuronal cell-bodies expressed against the duration of dehydration at 20°C. Curve 'a' was obtained when cell bodies were isolated before fixation and curve 'b' when fixed (by immersion) before isolation. Each point is the mean ± S.E.M. for a total of 35 to 136 measurements from 2 to 7 rats.


1,000
gm.10-12
750
500
0
2.5
days
5

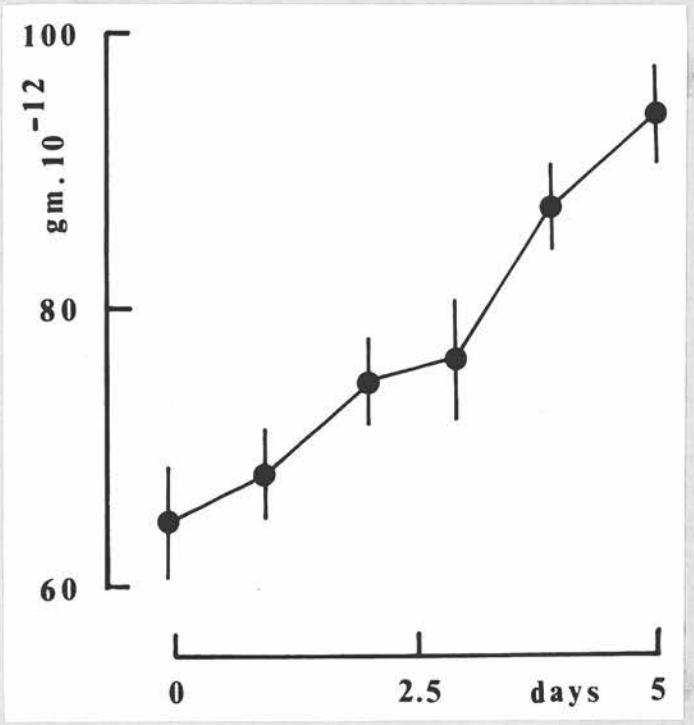

100
gm.10-12
80
60
0
2.5
days
5

Figure 11 Cell-body Dry Mass

The mean dry mass of supraoptic neuronal cell bodies expressed against the duration of dehydration at 20°C. Fixation was by immersion before the isolation of cell bodies. Each point is the mean ± S.E.M. for a total of 35 to 42 measurements from two rats.

---

mean of 158 observations in 5 normal rats). Of the procedures tried, fixation by immersion involved the minimum loss of mass and, except for the data behind Fig. 13, was used throughout this investigation.

Dehydration significantly increased both the dry mass (51.8%, $p < 0.001$) and the content of nucleic acids (46.5%, $p < 0.001$) of supraoptic cell bodies (Figs. 11 and 12). The major increase in both parameters occurred between the third and fifth days, but a definite latent period was demonstrated only in the dry mass response (Fig. 11).

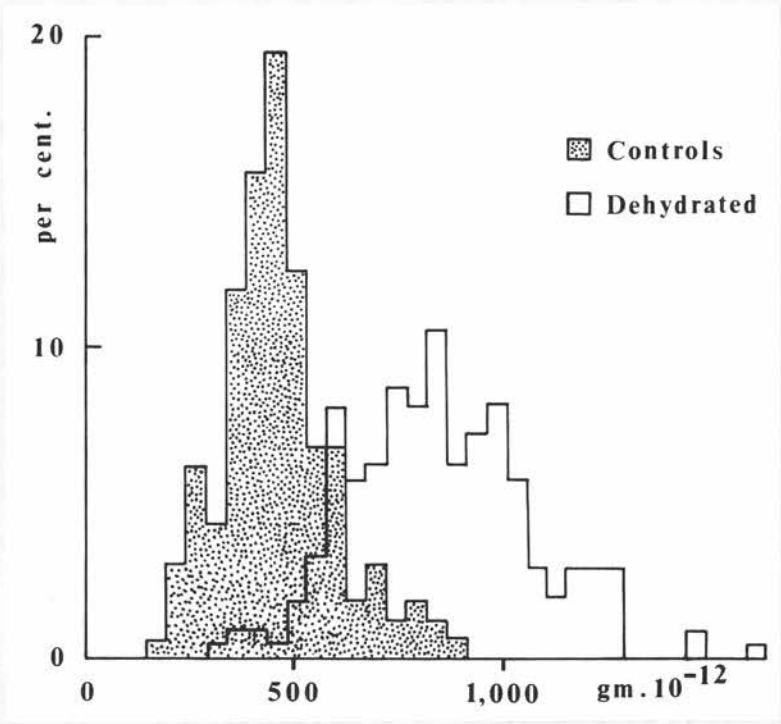
Figure 12 Cell-body Nucleic Acid

The mean content of nucleic acid of supraoptic neuronal cell bodies expressed against the duration of dehydration at 20°C. Fixation was by immersion before the isolation of cell bodies. Each point is the mean ± S.E.M. for a total of 32 to 36 measurements from two rats.

Table III

Dry mass of nuclei from supraoptic neurones

Days without water	No.of animals	No. of observations	Mean nuclear dry mass $\pm$ S.E.M.($gm10^{-12}$)	p
0	7	120	48.0 ± 2.6	
3	6	107	50.4 ± 1.2	$0.2 > p > 0.1$
5	9	166	45.6 ± 1.1	$0.2 > p > 0.1$


20
per cent.
10
0
Controls
Dehydrated
0
500
1,000
gm. 10^{-12}

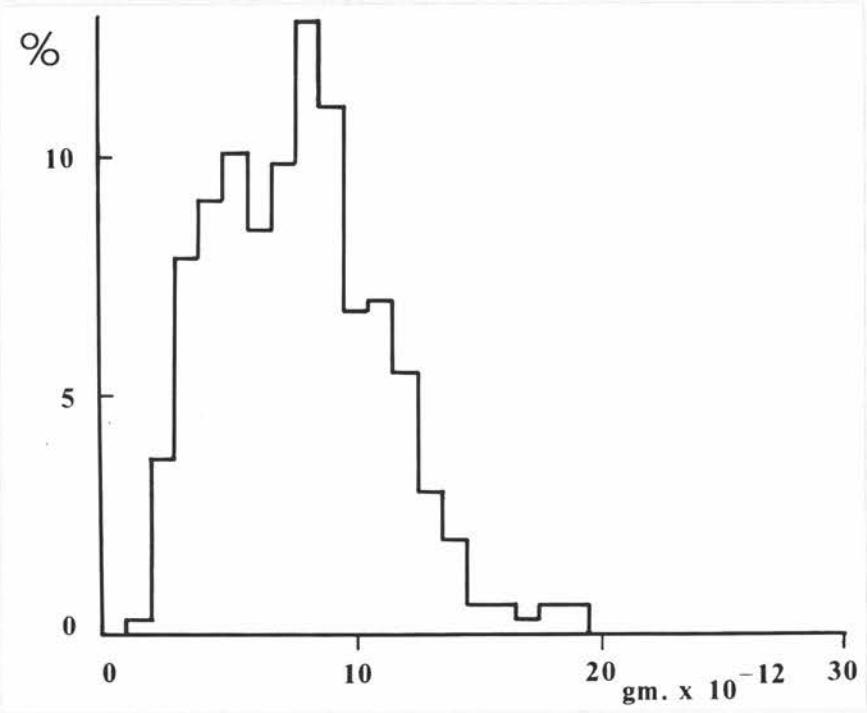
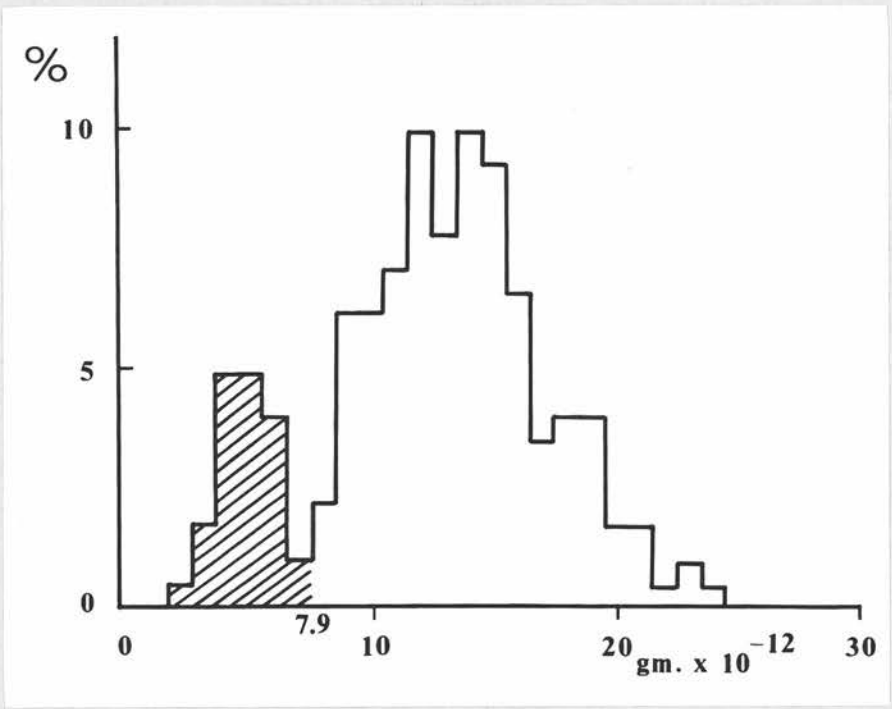

%
10
5
0
0
10
20
30
gm. x 10^{-12}

Figure 13 Cell-body Dry Mass

Distribution of dry mass among supraoptic neuronal cell bodies before (controls) and after 5 days' dehydration at 20°C. Sample size was 158 cell bodies for the control population and 201 for the dehydrated group. Mean dry masses for the control and dehydrated samples were 474 ± 11 and 873 ± 16 gm.10^{-12} respectively.

Figure 14 Nucleolar Dry Mass

Distribution of dry mass among supraoptic nucleoli from normal rats. Sample size was 317 nucleoli.


%
10
5
0
0
7.9
10
20
30
gm. x 10 −12

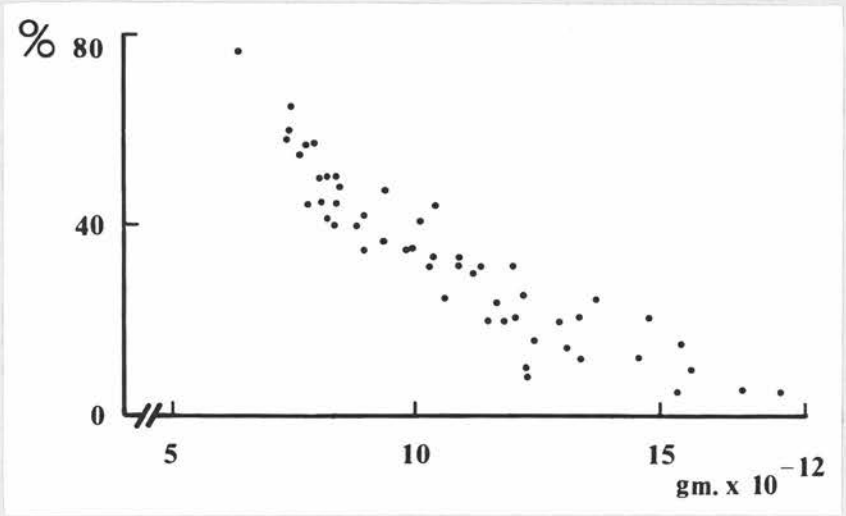

%
80
40
0
5
10
15
gm. x 10 −12

Figure 15 Nucleolar Dry Mass

Distribution of dry mass among supraoptic nucleoli from rats deprived of water for three days. Sample size was 228 nucleoli. The "hatched" area indicates a group of observations almost separate from the major population.

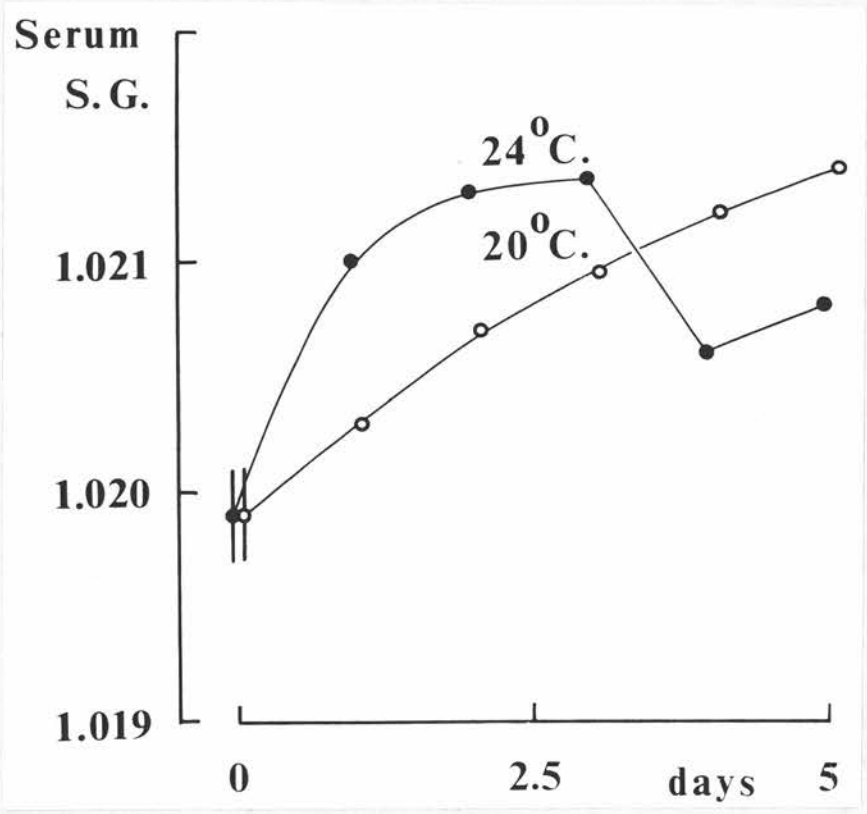
The mean dry mass of fixed nuclei from supraoptic neurones did not change significantly during five days of dehydration (Table III).

The scatter of individual dry masses about the mean was considerable for both cell bodies and nucleoli (Figs. 13 and 14), but in only one instance (Fig. 15) did the histogram suggest bimodality. About 17% of the observations contributing to this histogram appeared separate from the major population.

The percentage of observations falling within the range 1 to 7.9 gm.10^{-12} declined almost to zero as the mean dry mass of nucleoli increased (Fig. 16).

Figure 16 Nucleolar Dry Mass

Percentage of observations falling in the dry mass range 0 to 7.9.10^{-12} gm. expressed against the mean dry mass of a sample of 20 supraoptic nucleoli.


Serum
S. G.
24°C.
20°C.
1.021
1.020
1.019
0
2.5
days
5

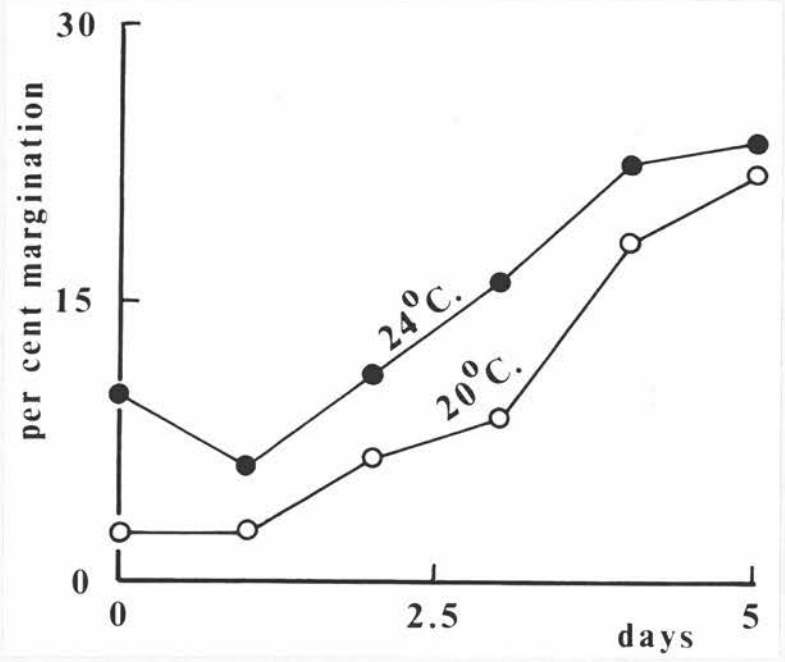

30
per cent margination
15
0
24°C.
20°C.
0
2.5
days
5

Figure 17 Serum Specific Gravity

Specific gravity of blood serum expressed against the time without drinking water at either 20°C (open circles) or 24°C (filled circles). Specific gravity at day 4 and 24°C was measured in one animal only. Otherwise legend as for Fig. 5.

Dehydration at ambient temperature of 24°C

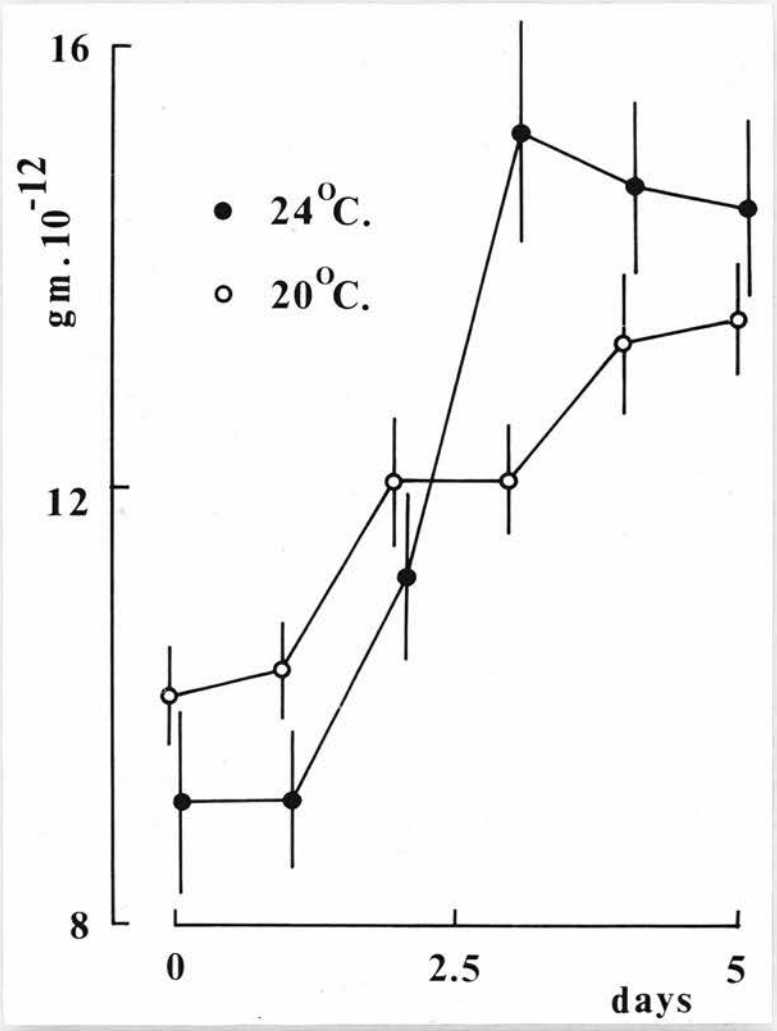
Figures 18 to 22 contrast the metabolic responses of supraoptic neurones to five days of osmotic stress at two environmental temperatures, 20°C and 24°C.

At the higher temperature, the faster increase in the specific gravity of blood serum was maintained only until the third day (Fig. 17). A decline in the specific gravity of blood plasma after three days of dehydration has been reported previously (Kurosumi et al, 1964).

There is insufficient data for statistical comparisons between the curves shown in Fig. 18. Supraoptic nuclei with marginated nucleoli were more abundant in rats kept at the higher temperature. The response to dehydration, however, seemed essentially unchanged (Fig. 18).

Figure 18 Nucleolar margination

Percentage of isolated supraoptic nuclei which contained marginated nucleoli expressed against the duration of water deprivation at 20°C or 24°C. 1 to 3 animals contributed to each point plotted. 115 to 120 nuclei were examined from each animal.


16
12
8
gm.10-12
24°C.
20°C.
0
2.5
5
days

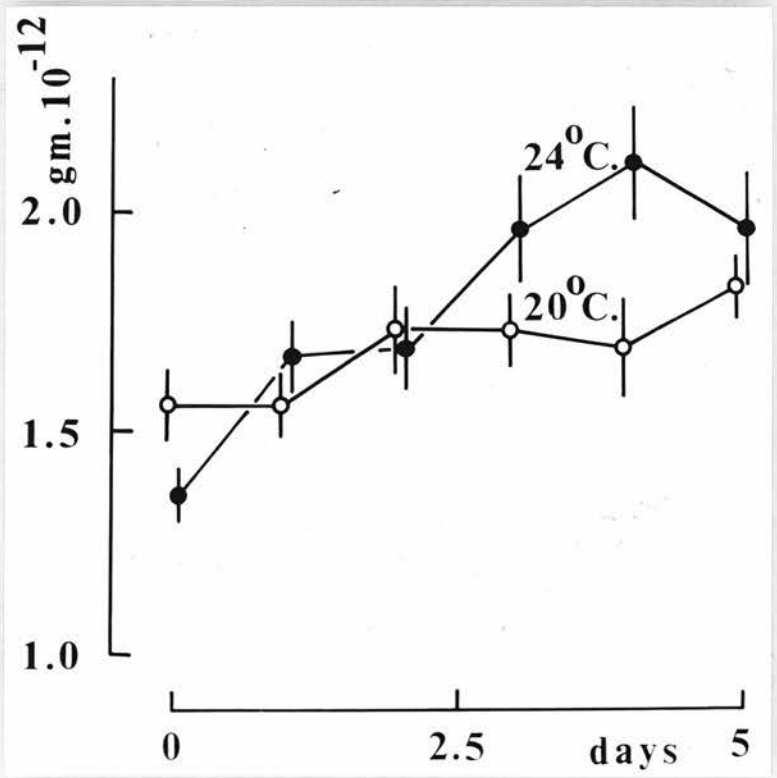

2.0
1.5
1.0
gm.10-12
24°C.
20°C.
0
2.5
days
5

Figure 19 Nucleolar Dry Mass

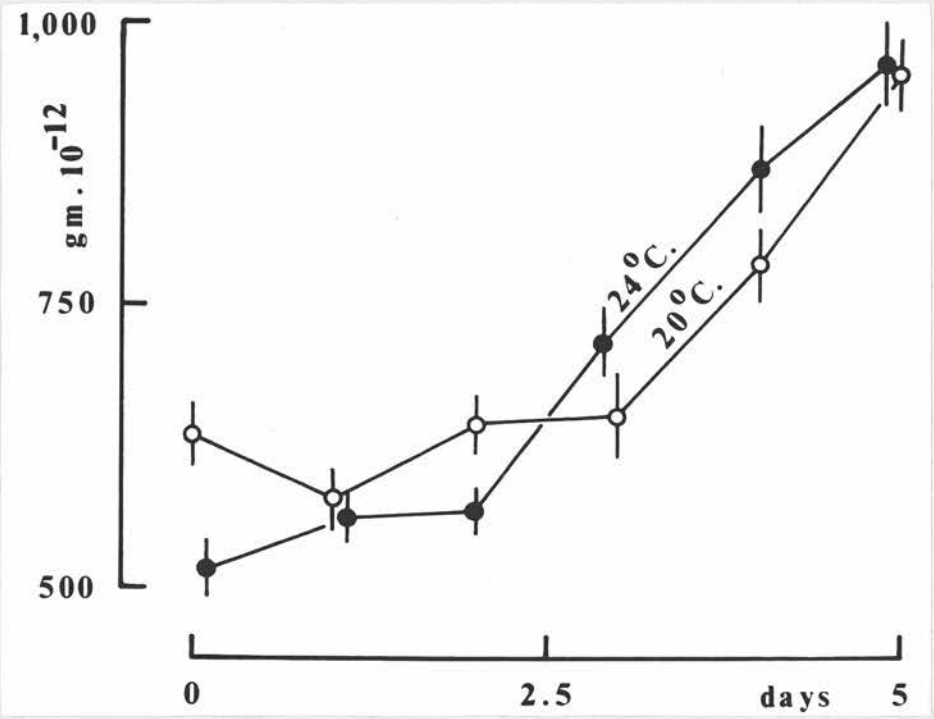
The mean dry mass of supraoptic nucleoli expressed against the duration of dehydration at 20°C (open circles) or 24°C (closed circles). Legend as for Fig. 8.

Dehydration at 24°C resulted in augmented changes in both mean dry mass and mean nucleic acid content of supraoptic nucleoli (Figs. 19 and 20). Thus, by the end of the experiment, dry mass had increased by 59% ($p < 0.001$) and nucleic acid content by 44% ($p < 0.001$). The corresponding changes at 20°C were 34% and 11.8% respectively. The slopes of the calculated regression lines over the first three days of the dehydration period were significantly greater at the higher temperature ($p < 0.01$ and < 0.05 for nucleolar dry mass and nucleic acid respectively). At 24°C neither parameter changed much after the third day of dehydration, the apparent "plateaux" coinciding with the drop in serum specific gravity (Fig. 17).

The change in nucleolar composition during dehydration seemed unaffected by the increased ambient temperature.

Figure 20 Nucleolar Nucleic Acid Content

The mean content of nucleic acid of supraoptic nucleoli expressed against the duration of dehydration at 20°C (open circles) or 24°C (closed circles). Legend as for Fig. 9.


1,000
gm . 10-12
750
500
24°C.
20°C.
0
2.5
days
5

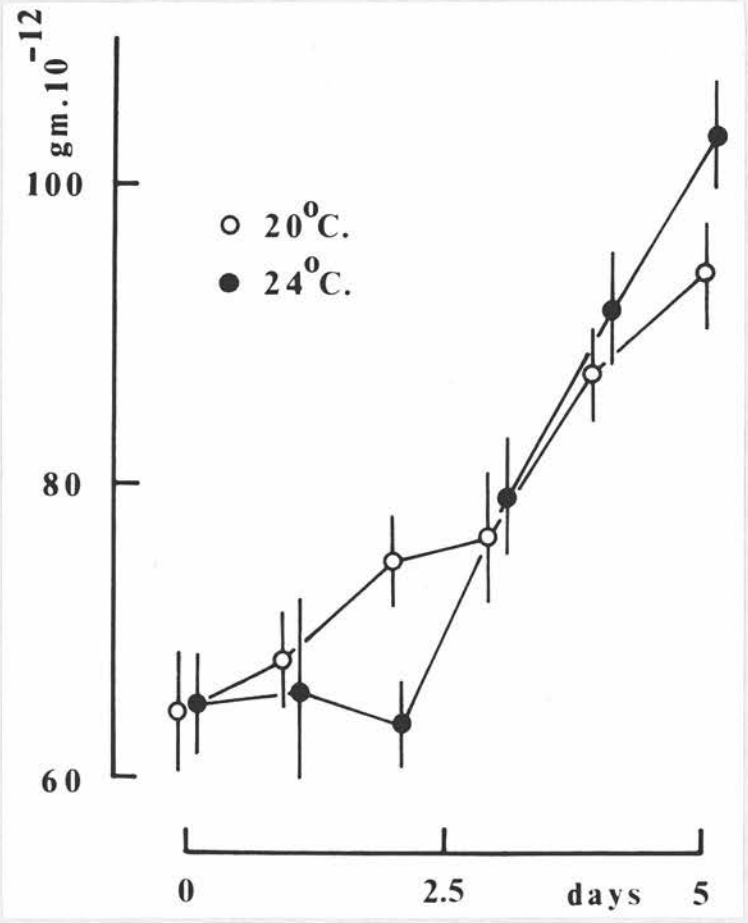

gm.10-12
100
80
60
20°C.
24°C.
0
2.5
days
5

Figure 21 Cell-body Dry mass

The mean dry mass of supraoptic neuronal cell bodies expressed against the duration of dehydration at 20°C (open circles) or 24°C (closed circles). Legend otherwise as Fig. 11.

Nucleic acid formed 14.9%, by weight, of the average control nucleolus, and 13.5% after five days' dehydration.

Osmotic stimulation at 24°C produced augmented responses in the dry mass and nucleic acid content of supraoptic cell bodies (Figs. 21 and 22). Dry mass increased earlier and was 88% greater by the fifth day ($p < 0.001$). Nucleic acid content increased by 60% ($p < 0.001$) after a latency of 48 hours.

Figure 22 Cell-body Nucleic Acid

The mean content of nucleic acid of supraoptic neuronal cell bodies expressed against the duration of dehydration at 20°C (open circles) or 24°C (closed circles). Legend as for Fig. 12.

DISCUSSION

Unconscious selection of material must always be a hazard with the techniques used here. As far as possible, the same central area was explored on each microscope slide, nuclei or cells being recorded for measurement in the order in which they appeared in the field of view. Since all nuclei seen with an intact envelope and a single nucleolus were included, some large glial nuclei may have been sampled. This may explain why the levels of nucleic acid found here in normal supraoptic nucleoli are lower than those reported by Edström and Eichner (1958), who were using fixed supraoptic neurones. Objectivity in the selection of cell bodies was less simple since they were inevitably damaged by the loss of axon and dendrites during isolation (Plate 10). All cell bodies with an otherwise unbroken outline and free from attached glia were included in the study.

Dehydration at 20°C

The osmotic stimulus of five days without water increased every supraoptic parameter examined, except for nuclear dry mass (Figs. 6 to 12).

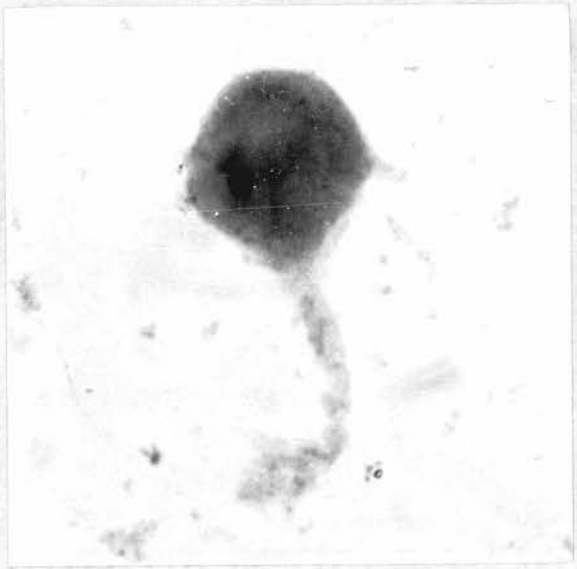
Despite the lability of the dimensions of isolated nuclei (Ambrose and Forrester, 1968), the mean diameter of those examined here was reproducible and was stable for at least two hours beyond the time required for measurement. Fresh nuclei, isolated from supraoptic

neurones, were significantly larger when obtained from dehydrated animals.

The diameter or volume of nuclei in fixed tissue has been used empirically as an index of the functional state of neurones (Szentágothai et al 1968) and increases in neurosecretory cells during osmotic stimulation (Eichner 1952, Castel and Abraham 1969). Paradoxically, the mean dry mass of fixed supraoptic nuclei did not change during dehydration. Increased volume without increased dry mass could result either from a shift of fluid into the nucleus or from accumulation of substances which are lost during fixation. Interferometry on fresh supraoptic nuclei should help to differentiate between these possibilities but has yet to be done.

The increased mean diameter of supraoptic nuclei was the earliest change detected and was significant ($p < 0.05$) after only 24 hours of dehydration. The nuclear swelling which is an early feature accompanying the induction of RNA synthesis in transplanted nuclei (Gurdon and Woodland 1968) may represent an exaggeration of the changes described here. Alone, however, the parameter is too nonspecific to support speculation about "derepression".

Apposition of nucleoli to the nuclear membrane has been dismissed as a fixation artifact, since it is rarely seen in fresh cells or nuclei under phase contrast (Busch and Smetana 1970). Yet up to 25 or 30% of the unfixed, isolated nuclei examined here contained marginated nucleoli.



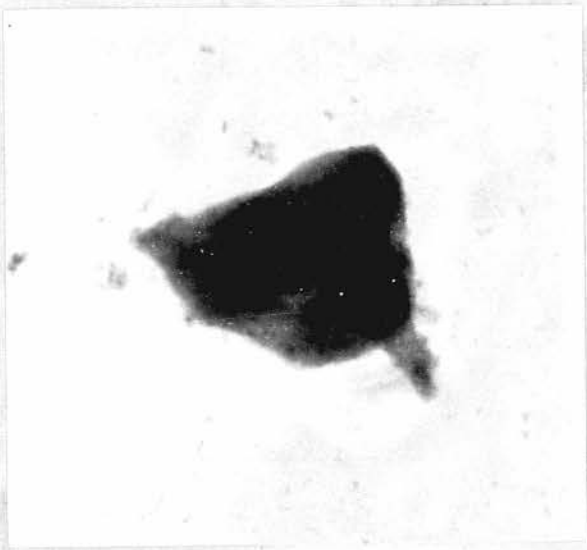


Plate 10

Supraoptic cell-bodies suspended in 1.55 M sucrose solution.

Photomicrographs were taken, under even-field illumination, in the interference microscope.

Magnification x 760.

The extrusion of nucleolar substance, in bulk, into the cytoplasm has been observed in vitro (Lettré and Siebs 1961) but is too infrequent to represent the normal means of ribonucleoprotein access to the cytoplasm. Although the evidence is tenuous, the release of ribonucleoproteins over the whole nucleolar surface seems more likely. Thus the morphological changes in liver nucleoli after small doses of actinomycin D suggest a migration of nucleolar elements from centre to periphery (Busch and Smetana 1970) and pulse-chase autoradiography has been used to show the radial movement of newly-synthesised RNA in chironomid salivary nucleoli (Sirlin 1962). The deformation of nucleoli when marginated will increase their effective surface to volume ratio only if ribosomal precursors continue to migrate from the surface apposed to the nuclear membrane. On this point evidence is lacking.

The postulate that nucleoli orientate themselves with respect to cytoplasmic structures could not be tested in these experiments. However, rotation of nuclei, with their contained nucleoli, has been demonstrated in several epithelial tissues in vitro (Pomerat 1953, Pomerat et al 1954).

Multiplication, eccentricity and margination of nucleoli have been described in supraoptic neurones during osmotic stress and have been interpreted as signs of increased nucleolar activity (Castel and Abraham 1969).

In provisional counts made by the writer, nuclei with more than one nucleolus seemed more likely to show nucleolar margination and were more abundant in preparations from dehydrated rats. This may have contributed to the increased percentage of nuclei showing nucleolar margination in the later stages of dehydration.

The functional relationship between the nucleolus and the nucleolar satellite is obscure but, in species where neuronal nucleoli are commonly eccentric (dog, skunk), it usually lies between the nucleolus and the nuclear membrane, often as a flattened, disc-shaped body (Moore and Barr 1953). The nucleolar satellite has been reported to migrate towards the nuclear membrane in up to 30% of stimulated hypoglossal neurones of the cat (Barr and Bertram 1949, 1951). Four months after axotomy, up to 45% of these neurones may show nucleolar satellites lying in contact with the nuclear membrane (Crouch and Barr 1954).

That nucleolar margination might arise from injury during isolation or exposure to the suspending medium cannot be discounted since the viability of nuclei was not checked.

Thirst or salt-loading causes an increase in the volume of nucleoli in supraoptic and paraventricular neurones (Ortmann 1951, Bachrach 1957b). Increased nucleic acid content has been demonstrated in both nucleoli and cell bodies of supraoptic neurones after eight weeks' salt loading of rats (Edström and Eichner 1958a, Edström

et al 1961), and coincides with a drop in nucleolar RNA concentration. The modest increase in nucleolar nucleic acid seen in this study after five days of water deprivation coincided with a drop in the contribution of nucleic acid to the total dry mass of the nucleolus.

During osmotic stimulation, supraoptic neurones showed a pattern of changes in the dry mass and nucleic acid content of their nucleoli and cell bodies which differed little from that described in injured neurones (Watson 1968a, 1969a). In both regenerating neurones (Watson 1968a) and liver cells (Swift 1959), nucleolar nucleic acid changes precede those seen in the cytoplasm. In this study, the time between the onset of the nucleolar and cell body responses was around 24 to 48 hours, and is best seen in the respective dry mass curves (Figs. 8 and 11). This sequence was to be anticipated, at least for changes in nucleic acid content, since the bulk of cytoplasmic rRNA is derived from the nucleolus (Section I). Evidence for the synthesis of ribosomal proteins within the nucleolus, however, remains unconvincing. Nucleoli are rich in acidic proteins (Sirlin 1962) which resemble ribosomal proteins in their amino-acid profiles (Busch and Smetana 1970). Where isolated nucleoli have been persuaded to incorporate amino acids into protein, the products resemble histones rather than acidic ribosomal protein (Maggio 1966, Zimmerman et al 1969).

Proteins can migrate between nucleus and cytoplasm in Amoeba proteus (Goldstein 1965) and the nucleolus of the rat oocyte pronucleus may accumulate cytoplasmic protein (Vincent 1955). Thus the possibility that acidic nucleolar and ribosomal proteins are synthesised outwith the nucleolus remains open. Recent evidence suggests that the fungus Neurospora crassa synthesises ribosomal proteins upon cytoplasmic ribosomes (Kuntzel 1969).

With techniques capable of measuring only the "total" dry mass or "total" nucleic acid content, the range of detectable responses which a neurone can display must be limited. The similarity between the neurone's response to stimulation and to axotomy need not extend beyond the fact that increased demand for protein is met, in part, by increased synthesis of ribosomes. The spectrum of proteins required to regenerate an axon will differ from that needed to compensate for secretory losses. Since carrier-protein is released along with ADH (Fawcett et al 1968), osmotic stress should cause an increased, but selective, loss of protein from neurosecretory cells. Stimulation augments the incorporation of both ^{35}S-cysteine and ^{3}H-tyrosine into supraoptic neurones (Flament-Durand 1967 and Murray 1967 respectively) yet appears to accelerate loss only of the former.

If protein losses are accelerated, increased protein synthesis must make a major contribution to the increased dry mass of supraoptic cell bodies from dehydrated rats.

On morphological grounds, supraoptic neurones have been divided into two categories, usually designated "A" and "B". (Enestrӧm 1967, Rechardt 1969, Ellman and Gan 1971). Osmotic stress has been reported to decrease the proportion of "A" cells from its control level of about 20% (Enestrӧm 1967). Ellman and Gan (1971) failed to find any change in the relative proportions of cell types in response to salt loading, and placed only 6% of control populations in the "A" category.

Histograms showing the distribution of dry mass among supraoptic cell bodies (Fig. 13) give no indication of more than a single population either before or after five days of dehydration. The distribution of dry mass among control populations of supraoptic nucleoli shows more "skewness" but is also unimodal (Fig. 14). Nucleolar populations in supraoptic regions from rats deprived of water for three days show an apparently bimodal distribution of dry mass (Fig. 15) with about 17% of observations contributing to a "minor population". This histogram is based on too few observations (228) to justify assertions about two populations in the sample, but raises the possibility that a proportion of the nucleoli examined is derived from non-neurosecretory cells, included during the dissection of supraoptic regions.

Fig. 16, which shows the proportion of nucleoli falling in the range 0 to $7.9.10^{-12}$gm (the "minor population") as the <u>mean</u> dry mass increases, gives no indication of a population of cells which do not respond to osmotic

stress. The ordinate in this figure is expressed as a percentage of a sample size of only 20 nucleoli. Thus one extra observation falling in the specified range will increase the apparent size of the minor population by 5%. The curve therefore becomes unreliable at low percentages and cannot exclude the possibility that some samples with a high mean dry mass are contaminated with unresponsive cells.

Dehydration at 24°C

Since insensible evaporation is the most important route of water loss in rats (Dicker and Nunn 1957), dehydration should be more rapid and severe at the higher environmental temperature. The specific gravity of blood serum rose faster and reached a maximum about 48 hours earlier than was seen at 20°C (Fig. 17). The late fall in serum specific gravity may result from changes in the concentration of serum proteins due to voluntary starvation since rats eat little after the second or third day of dehydration (Dicker and Nunn 1957). Of the weight loss observed in dogs deprived of food and water for five days only 45% was attributed to a loss of body water (Painter et al 1948). A similar but less abrupt fall in the specific gravity of plasma has been reported elsewhere (Kurosumi et al 1964). If, as has been suggested, "osmoreceptors" respond to alterations in intracellular sodium concentration (Andersson and Westbye 1970), the later stages of the curves in Fig. 17 are not a reliable

indication of the stimulus applied to the supraoptic neurones.

Faster dehydration caused augmented responses in all parameters measured except, perhaps, nucleolar margination. The increase in mean dry mass and mean nucleic acid content of supraoptic nucleoli was greater and significantly faster than at 20°C. The apparent "plateaux" between days 3 and 5 in Figs. 19 and 20 cannot be interpreted as a "maximal" nucleolar response since it coincides with a fall in serum specific gravity. Greater increases in the mean dry mass and nucleic acid content of cell bodies occurred during dehydration at the higher temperature, probably after a shorter latent period (48 cf. 72 hours).

On this evidence, it seems that the metabolic response of supraoptic neurones can be graded according to the severity of the osmotic stimulus. This suggests the existence of a quantitative link, direct or indirect, between the stimulus and the metabolic response. By analogy with the R2 ganglion cell of Aplysia (Peterson and Kernell 1970, Kernell and Peterson 1970), a "direct" link may exist between electrical activation and metabolic activity. It has been argued, however, that more accurate coupling between synthetic activity and secretory losses could result from a feedback control centred on the size of the neurone's stored "pool" of transmitter or neurohormone (Section I). For this reason, extracts of neurohypophyses have been tested for possible effects on metabolic parameters of supraoptic neurones (Section III).

Section III: Effects of extracts of posterior pituitary lobes on supraoptic neurones

METHODS

(i) Preparation of posterior lobe extracts

Neurohypophyses from which "experimental" extracts were prepared were partially depleted of octapeptide hormones by one of two procedures.

(a) Incubation in vitro. Pituitary glands were removed from normal albino rats, killed by rapid exsanguination. The posterior lobes were dissected free in chilled Locke's solution ($MgCl_2$ 1 mM; $CaCl_2$ 2.2 mM; $NaHCO_3$ 6 mM; KCl 5.6 mM; Glucose 10 mM; NaCl 154 mM - Douglas and Poisner 1964a) and as much as possible of the pars intermedia was removed piecemeal. The isolated lobes were split longitudinally for two thirds of their length and incubated, in pairs, for 90 minutes at 37°C in one of two solutions. Control extracts (AC) were prepared from posterior lobes incubated in Locke's solution and "octapeptide-depleted" extracts (AE) from those incubated in a high-potassium, low-sodium Locke's solution ($MgCl_2$ 1 mM; $CaCl_2$ 2.2 mM; $NaHCO_3$ 6 mM; KCl 56 mM; glucose 10 mM; sucrose 308 mM). Both incubating solutions were equilibrated with 5% CO_2 in O_2.

After incubation, each pair of posterior lobes was washed twice in normal Locke and homogenised in 0.035 ml of the same solution. The resulting suspension was cleared by centrifugation (1,600 g for 15 mins.) and diluted to a final volume of 0.05 ml. This represented a single dose of crude extract AC or AE for injection into a recipient

Table IV

Extracts of neurohypophysis and parietal cortex

Donor Tissue	Preparation	Status	Label
Pituitary - posterior lobe	Incubation - 90 mins. in Locke	"non-depleted"	AC
ditto.	Incubation - 90 mins. in "depolarizing"* solution	"octapeptide-depleted"	AE
ditto.	Normal rats. No incubation.	"non-depleted"	AC_o
ditto.	Dehydrated rats. No incubation	"octapeptide-depleted"	AE_5
Parietal cortex	Incubation - 90 mins. in Locke	-	BC
ditto.	Incubation - 90 mins. in "depolarizing"* solution	-	BE
ditto.	Normal rats. No incubation.	-	BC_o

* "depolarizing" solution was a sodium-deficient Locke's solution with a high (56 mM) potassium concentration (see text).

animal, and corresponded to a dilution of neurohypophysial tissue of about 1 part in 60 by volume.

(b) Hormone depletion in vivo. The donors of pituitary glands were either normal rats or rats deprived of water for five days. The posterior lobes were isolated, cleaned, divided, homogenised and centrifuged as described above. Two posterior lobes contributed to 0.05 ml of the final extract. Extract AC_0 ("non-depleted" posterior lobe extract) was prepared from normal rat pituitaries and extract AE_5 ("octapeptide-depleted") from pituitaries taken from dehydrated donor animals.

(c) Extracts of parietal cortex. Pieces of parietal cortex, similar in size to the neurohypophysis, were processed as in (a) above and yielded the parietal extracts BC and BE. A third extract of parietal cortex (BC_0) was prepared, without incubation, from normal animals.

The seven extracts used may be identified from Table IV.

(ii) Intraventricular injection

A single dose (0.05 ml) of freshly-prepared extract was injected into the right lateral cerebral ventricle of recipient rats by a modification of the technique described by Haley and McCormick (1957).

Under light ether anaesthesia, a midline incision was made in the scalp and the bony table exposed to the right of the midline. A 25 g needle was used to penetrate the skull and underlying meninges, at a point 3 mm lateral to

the sagittal suture and 3 mm posterior to the coronal suture. A long-bevelled, 25 g needle, attached by fine polythene tubing to a syringe, was inserted to a depth of 3 mm with the point inclined slightly towards the midline.

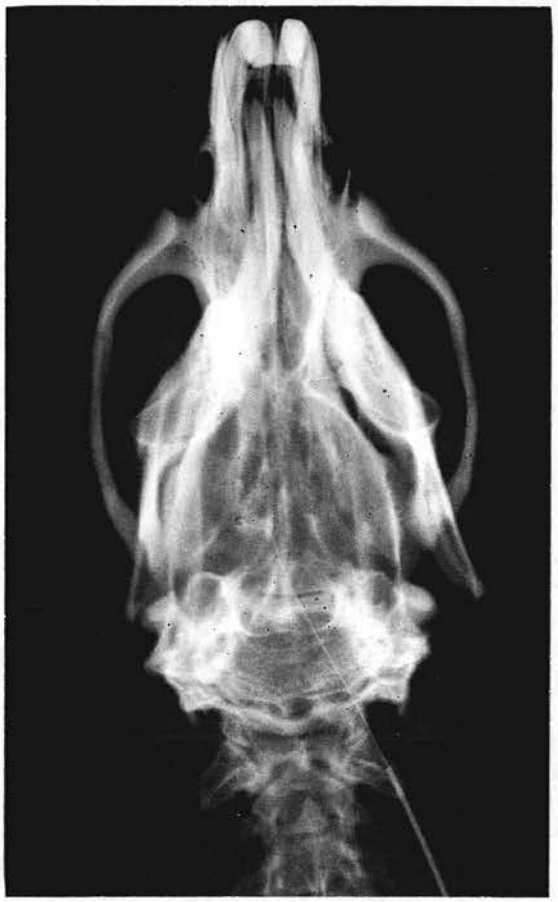
Access to the cerebral ventricles was checked in preliminary experiments by X-radiography (Plates 11 and 12), injection of indian ink and examination of the needle-track when the animal was killed.

Recipient animals were killed by exsanguination 48 or, rarely, 72 hours after injection. A block of hypothalamic tissue was excised, placed in 0.34 M buffered sucrose, and used to prepare suspensions of isolated nuclei from supra-optic and posterior arcuate regions as detailed previously. The parameters subsequently estimated were nucleolar margination, nucleolar dry mass and nucleolar content of nucleic acid.

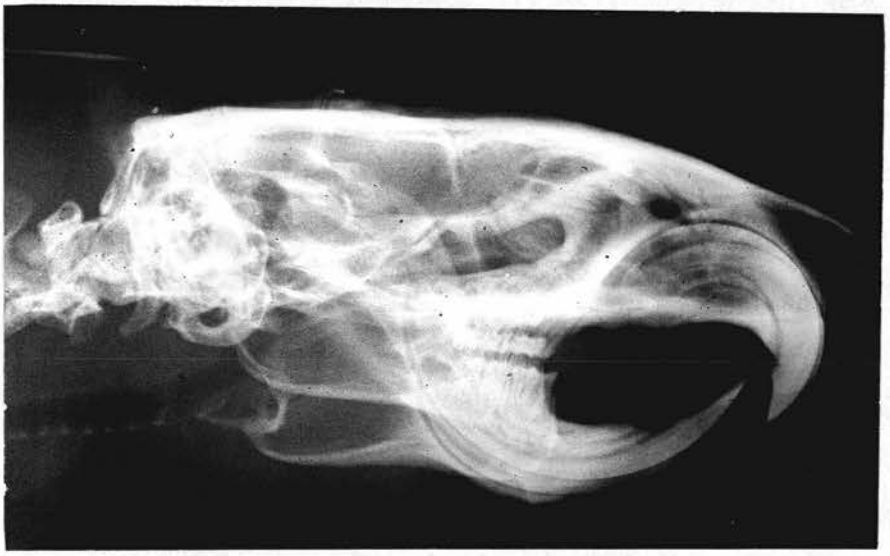
For convenience, recipient animals were identified by the extract with which they had been injected. Thus AC animals received extract AC, AE_5 animals received extract AE_5 etc. Rats' designated "controls" received no injection.

(iii) Serum osmolality

Blood was collected from control and recipient animals at the time of death for the measurement of serum specific gravity or, when possible, serum osmolality. The latter was estimated by "freezing-point-depression" using the Precision Systems "Osmette" model 2007. Sera were deep-frozen from the time of collection and all samples were



a.



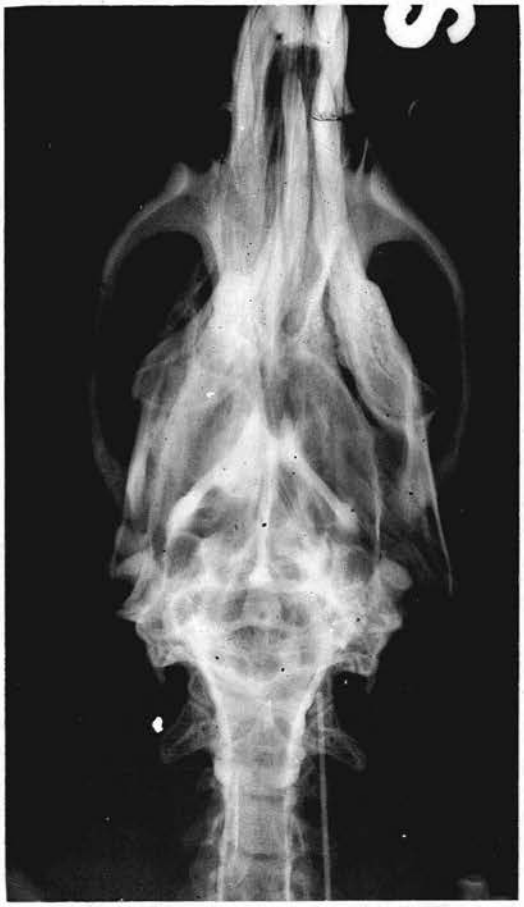
b.

Plate 11 X-radiography of rat skull

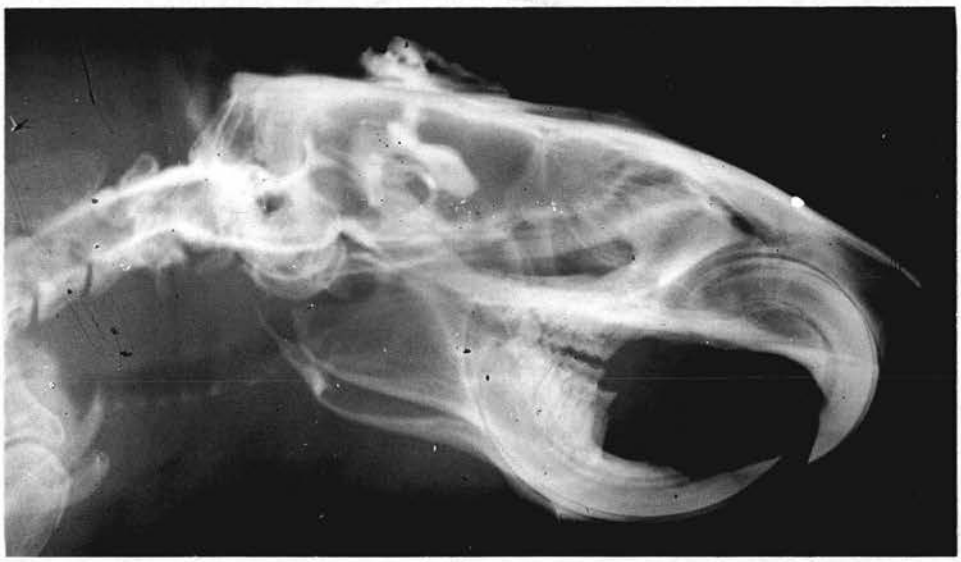
(a) Dorsal, (b) lateral.

0.02 ml "Conray" injected through cannula inserted into R. Lateral cerebral ventricle. Radio-opaque material is visible in the cannula and can be traced to the site of penetration of the skull, but has not outlined the cerebral ventricles (cf. Plate 11).

Magnification x 2 (approximate).



a.



b.

Plate 12 X-radiography of rat skull

(a) Dorsal, (b) lateral.

0.7 ml of "Conray" was injected through a cannula inserted into the right lateral cerebral ventricle. The spinal cord was sectioned in the lumbar region to allow leakage of displaced C.S.F. Note radio-opaque "Conray" filling the system of cerebral ventricles and spreading into the subarachnoid space over the brain and spinal cord (cf. Plate 10).

Magnification x 2 (approximate).

analysed on the same day. The instrument was calibrated over the range 100 to 500 m.Osm./litre, using standard solutions, and was checked at the beginning and end of each batch of estimations. Sera were analysed in 0.2 ml samples, each batch being repeated at least once to check reproducibility.

RESULTS

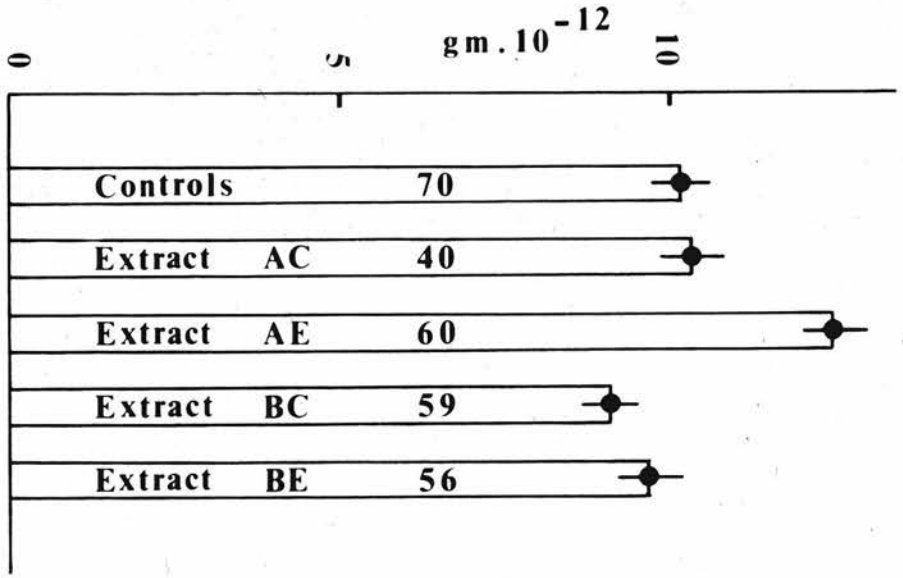

0
5
10
gm . 10 -12
Controls 70
Extract AC 40
Extract AE 60
Extract BC 59
Extract BE 56

Figure 23

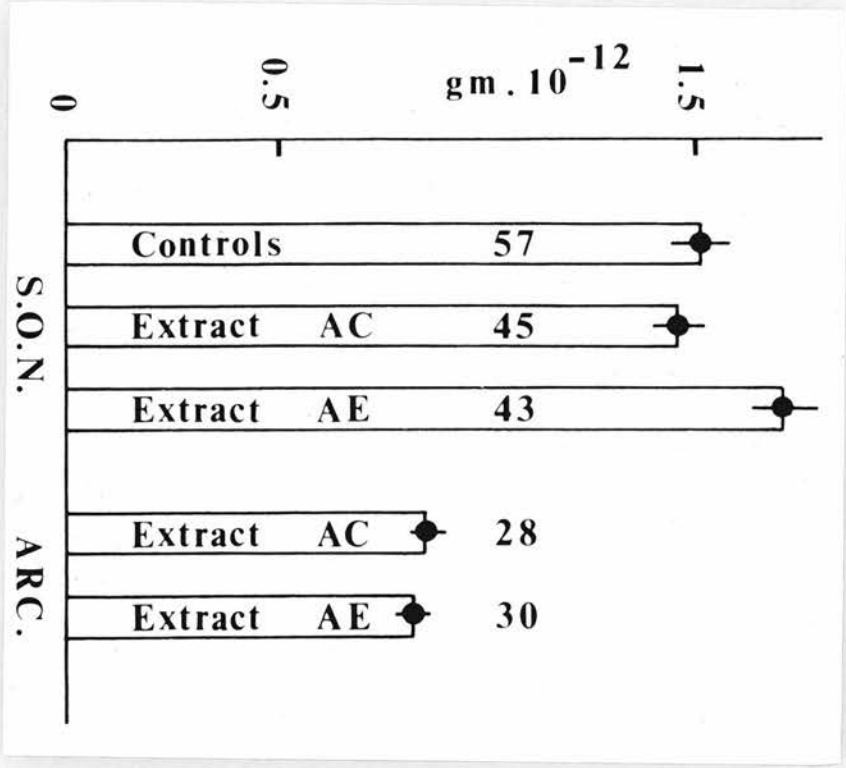
The dry mass of supraoptic nucleoli, 48 hours after the intraventricular injection of extract of posterior pituitary or parietal cortex. Each column shows the number of observations contributing to the mean which is entered ± its S.E.M. as a vertical bar. Except for the "AC" group (2 rats), 3 rats contributed to each mean.

Table V

Nucleolar margination, 48 hours after the injection of extract

Extract	No. of animals 48 h*	72 h*	Nucleolar margination (%) 48 h*	72 h*
AC	2	3	12.2	10.3
AE	3	3	20.8	15.9
BC	3	3	8.1	7.1
BE	3	3	9.2	3.3

*Time after intraventricular injection.


0
0.5
gm . 10 -12
1.5
S.O.N.
Controls 57
Extract AC 45
Extract AE 43
ARC.
Extract AC 28
Extract AE 30

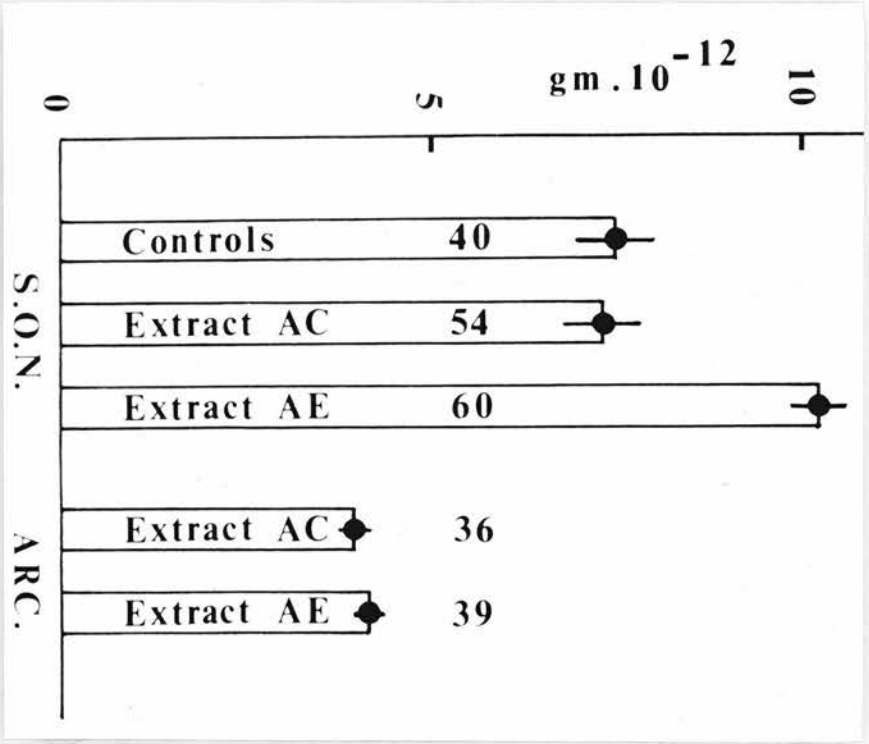

0
5
gm . 10 -12
10
S.O.N.
Controls 40
Extract AC 54
Extract AE 60
ARC.
Extract AC 36
Extract AE 39

Figure 24.

The dry mass of supraoptic and posterior arcuate nucleoli, 48 hours after the injection of posterior pituitary extracts. Three animals contributed to each supraoptic mean, two to each arcuate mean. Means are entered ± S.E.M. as a vertical bar. Each column shows the number of measurements made.

RESULTS

Extracts prepared by incubation in vitro

Of the four extracts tested (AC, AE, BC, BE), only "octapeptide-depleted" posterior lobe extract (AE) caused significant changes in the dry mass and nucleic acid content of supraoptic nucleoli, 48 hours after injection. In two separate experiments (Figs. 23 and 24), the mean dry mass increased by 24% ($p < 0.01$) and 39% ($p < 0.001$) respectively. The mean nucleolar content of nucleic acid increased by 17% ($p < 0.02$, Fig. 25). The percentage of nuclei with marginated nucleoli was also highest in those animals given extract AE (Table V).

The specific gravity of blood serum, sampled 48 hours after the injection of extract, was 1.0204 (range 1.0198 to 1.0207) and 1.0205 (range 1.0200 to 1.0211) in animals

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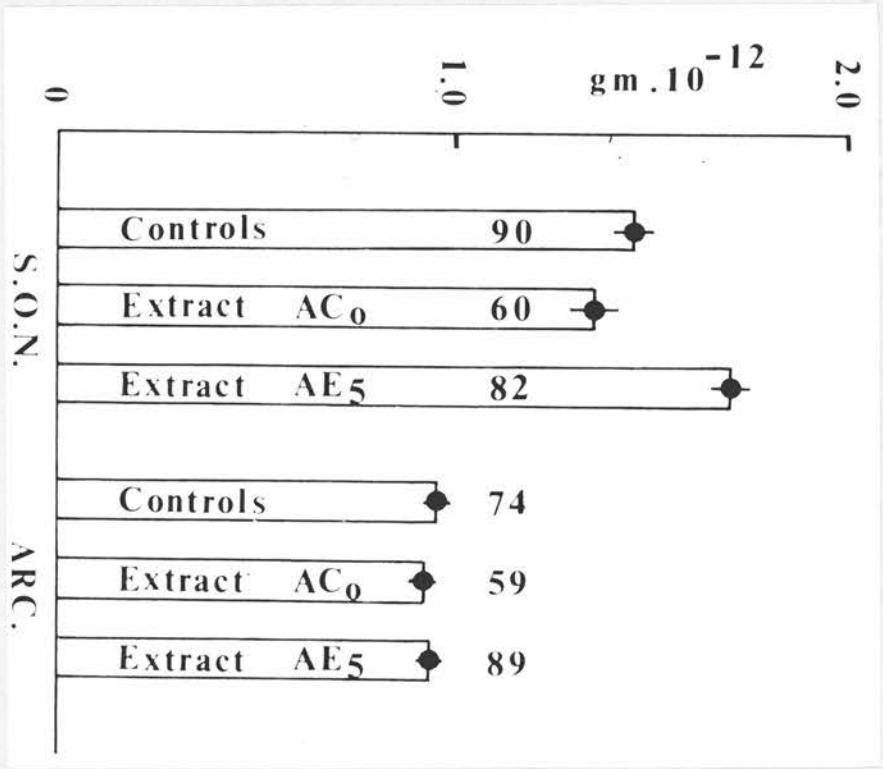
Figure 25

The content of nucleic acid in supraoptic and posterior arcuate nucleoli, 48 hours after the injection of posterior pituitary extracts. Legend as for Fig. 24.


0
1.0
2.0
gm . 10 - 12
S.O.N.
Controls 90
Extract AC0 60
Extract AE5 82
ARC.
Controls 74
Extract AC0 59
Extract AE5 89

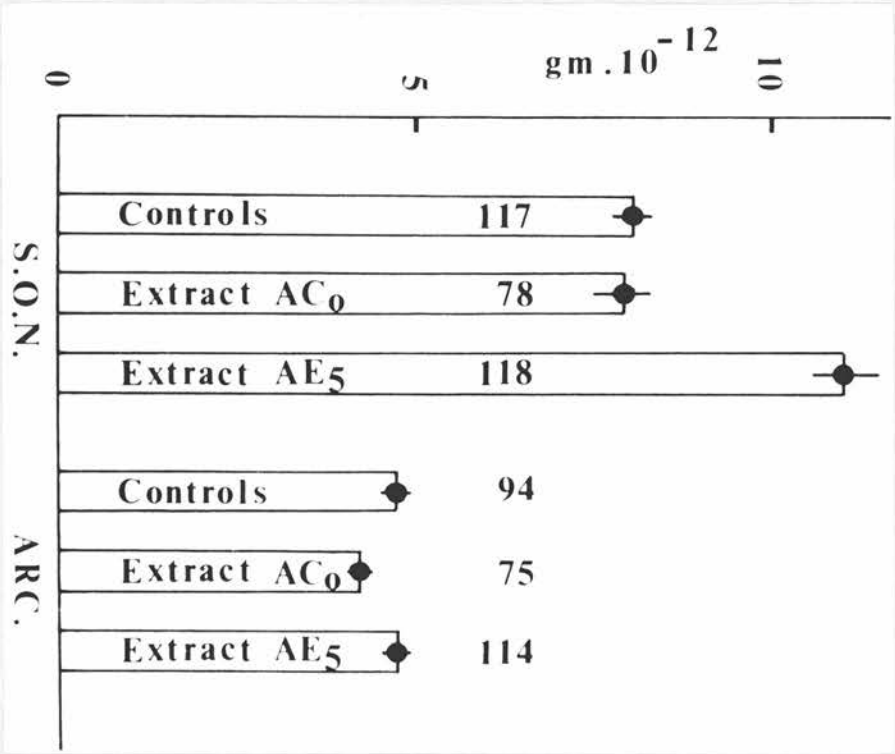

0
5
10
gm . 10 - 12
S.O.N.
Controls 117
Extract AC0 78
Extract AE5 118
ARC.
Controls 94
Extract AC0 75
Extract AE5 114

Figure 26

The dry mass ofsupraoptic and posterior arcuate nucleoli, 48 hours after the injection of posterior pituitary extracts. 4 to 6 animals contributed to each mean which is plotted ± S.E.M. as a vertical bar. The number of measurements is shown for each column.

---

given extracts AC and AE respectively.

Nucleoli from the posterior part of the arcuate nucleus showed no response to either posterior lobe extract (Figs. 24 and 25).

Extracts prepared without incubation

Forty-eight hours after the injection of 0.05 ml of a posterior lobe extract prepared from dehydrated rats (AE_5), supraoptic nucleoli showed increased mean dry mass (36%, Fig. 26) and nucleic acid content (17%, Fig. 27). Both of these changes were significant at less than one part per thousand.

Extract prepared from rats with free access to water (AC_0) altered neither of these parameters significantly.

With the possible exception of a decrease in nucleolar dry mass after the injection of extract AC_0 (p 0.05,

---

Figure 27

The content of nucleic acid in supraoptic and posterior arcuate nucleoli, 48 hours after the injection of posterior pituitary extracts.

Legend as for Fig. 26.

Fig. 26), nucleoli from the posterior part of the arcuate nucleus did not respond to either extract (Figs. 26 and 27).

Serum specific gravity was unaffected by the injection, 48 hours previously, of either extract (Table VI).

Table VI

Serum specific gravity, 48 hours after the injection of extract

	No. of animals	Serum specific gravity (range)
Controls	6	1.0200 (1.0192-1.0211)
Extract AC_0	4	1.0200 (1.0193-1.0212)
Extract AE_5	6	1.0202 (1.0190-1.0209)

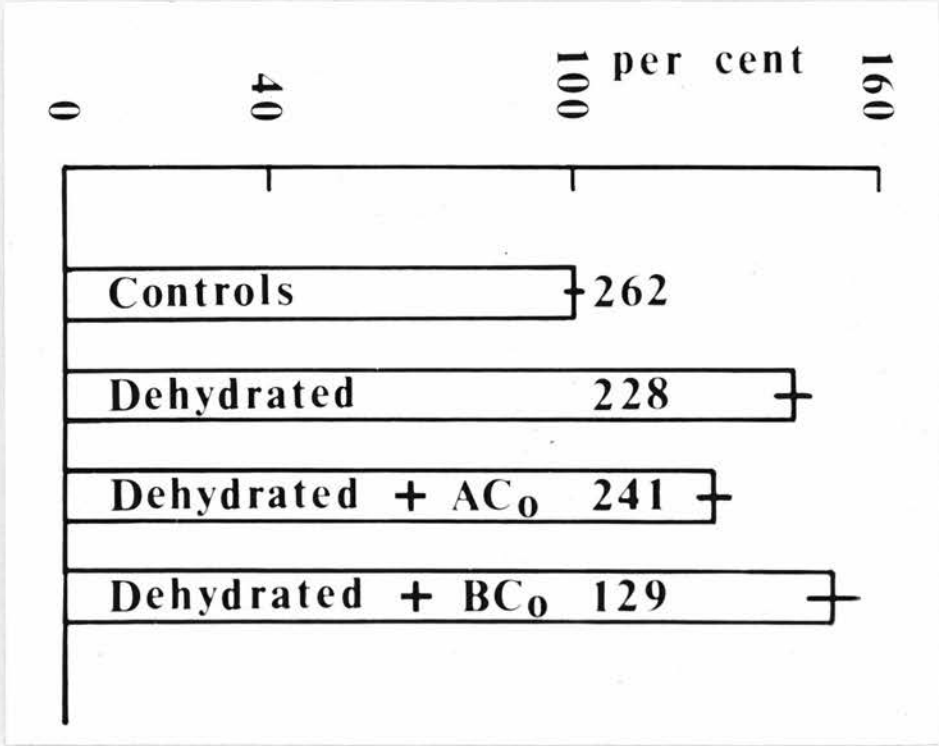

0
40
100
per cent
160
Controls
262
Dehydrated
228
Dehydrated + ACo
241
Dehydrated + BCo
129

Figure 28

Effects of extracts of posterior pituitary and parietal cortex on the dry mass of supraoptic nucleoli during dehydration.

Nucleolar dry mass is shown as a percentage of normal. Rats other than controls were dehydrated for three days. Injections of extracts were given 24 hours after the onset of dehydration. Means are entered ± S.E.M. as a vertical bar. Except for the "Dehydration + BC_o" group (6 animals), 10 to 12 rats contributed to each mean shown. Figures in each column show the number of observations made.

Table VII

Serum osmolality - dehydration + extract

	No. of animals	Serum osmolality(range) m.Osm./litre
Controls	7	298.8 (293.5-305.0)
Dehydrated	6	313.6 (295.6-324.5)
Dehydrated + AC_o	6	313.2 (292.0-325.1)
Dehydrated + BC_o	6	313.2 (292.5-328.5)

Effects of "non-depleted" extract (ACo) on dehydrated rats

The dry mass of supraoptic nucleoli after three days of dehydration was 11% less in those animals injected with extract AC_o 24 hours after the start of the experiment (Fig. 28). This inhibition of the dry mass response to dehydration was significant ($p < 0.001$).

A similar extract of parietal cortex (BC_o) had no significant effect. The increase in serum osmolality was the same in all three groups of dehydrated animals irrespective of which extract was injected (Table VII).

DISCUSSION

The metabolic response seen in supraoptic neurones during osmotic stimulation (Section II) suggested that nucleolar changes occurred earlier than those in the cell body. For this reason, nucleolar parameters were measured in this investigation into the effects of single doses of crude extract on neuronal metabolism. The forty-eight hours between the injection of extract and the examination of nucleoli was a compromise between the time necessary for nucleolar changes to become detectable and the anticipated duration of the response. In addition, it allowed time for recovery from the effects of anaesthesia (Daniel and Lederis 1966).

The release of octapeptide hormones by neurohypophyses exposed to solutions rich in potassium and deficient in sodium is now well documented (Douglas and Poisner 1964a, Sachs and Haller 1968, Douglas et al 1965, Dicker 1966). Like secretion induced by electrical stimulation, the effects of incubation in high-potassium/low-sodium media are calcium-dependent and inhibited by magnesium (Douglas and Poisner 1964a,b). By exposing neurohypophyses to 56 mM potassium, in vitro, Sachs and Haller (1968) could mimic the "biphasic" pattern of ADH secretion seen in experimentally hypotensive dogs (Sachs et al 1967). Neurophysin appears to accompany neurohormones whether secretion is stimulated in vivo (Rennels 1966, Friesen and Astwood 1967) or, by excess potassium, in vitro (Fawcett et al 1968, Cheng and Frieson 1970).

Injections of indian ink into the right lateral cerebral ventricle confirmed that the volume of each dose of extract (0.05 ml) was sufficient to flush out the entire ventricular system. The subarachnoid space overlying the supraoptic nuclei was invariably filled with the injected ink.

Reproducible increases were found in the mean dry mass and nucleic acid content of supraoptic nucleoli, 48 hours after the injection of "octapeptide-depleted" posterior lobe extracts (AE or AE_5). There was an accompanying increase in the frequency of marginated nucleoli.

Potassium, when present in high concentration, is accumulated by isolated neurohypophyses (Daniel and Lederis 1967). "Trapped" potassium in donor neurohypophyses is unlikely to explain the observed supraoptic changes since extract of parietal cortex incubated in the same solution (extract BE) was ineffective (Fig. 23). Neurohypophyses depleted of hormones without exposure to a high potassium concentration can yield an active extract (AE_5 - Figs.26 and 27).

The only consistent difference between extracts which produced nucleolar changes in supraoptic neurones and those which were inactive seems to be the prior release of neurohypophysial hormones. Thus, some aspect of the release-mechanism confers on the neurohypophysis the ability to cause metabolic changes in neurosecretory neurones. The

specificity of this "active" factor has been examined only superficially. Active extract cannot be prepared from parietal cortex (Fig. 23) and does not induce changes in the nucleoli of neurones from the posterior part of the arcuate nucleus (Figs. 24 to 27). Provisional observations suggest that active neurohypophysial extracts are capable of inducing, in the nucleoli of paraventricular neurones, changes similar to those described here (Russell 1972).

The effect of hormone-depleted extracts was never accompanied by a recorded change in serum specific gravity, although this quantity could have varied, without detection, between the injection of extract and the death of the animal, 48 hours later. Hence, active extracts may cause changes in supraoptic nucleoli by stimulating the secretion of neurohormone from the secretory terminals of the recipient rat. Using an in vivo perfusion technique, an attempt was made to distinguish between this possibility and the alternative of a more direct influence of active extracts on supraoptic synthetic mechanisms (Section IV).

"Non-depleted" extract and the response to dehydration

The injection, into rats undergoing dehydration, of a neurohypophysial extract prepared from normally hydrated animals, significantly reduced the size of the nucleolar dry mass response of supraoptic neurones. This effect appeared not to involve any change in the normal increase in serum osmolality during dehydration (Table VII) and could not be duplicated using extract of parietal cortex.

This raises the interesting possibility that neurohypophyses with well-stocked reserves of neurohormone are capable of inhibiting nucleolar synthetic activity. Such an interpretation must be guarded since it is based solely on changes in the dry mass of nucleoli of which 85% may be protein synthesised outwith the nucleolus (Section II). Cytoplasmic factors can inhibit nucleolar RNA synthesis in nuclei transplanted into amphibian oocytes (Gurdon and Brown 1965) and Yamana and Shiokawa (1966) have demonstrated a soluble inhibitor of nucleolar activity in the cytoplasm of embryonic cells of Xenopus laevis. No such inhibitor has yet been demonstrated in mature neurones.

Further consideration of the possible role of neurohypophysial "active" factors in the control of neuronal synthetic activity will be deferred until the influence of extracts on endogenous hormone release in recipient animals has been examined (Section IV).

It needs to be emphasised that the changes observed in the supraoptic cells in the course of water-deprivation could be non-specific responses to the stress of deprivation, and not necessarily indicative of increased synthesis of neuro-hypophysial hormones. Similar changes in other cells have been described following injury. In the present study no other cells were studied. Therefore similar changes observed in the supra-optic cells in response to the administration of hormone-depleted hypophysial extracts cannot be regarded as evidence bearing on ADH synthesis and secretion.

SECTION IV: Site of action of extract on the supraoptic neurone

METHODS

(i) Combined cannulation of right cerebral ventricle, left external jugular vein and urinary bladder

Anaesthesia was induced with intraperitoneal nembutal (0.18 ml) and maintained, when necessary, with ether. One of two procedures was used to insert an indwelling polythene catheter into the urinary bladder.

(a) A midline incision was made in the lower abdomen extending from one centimetre above the pubic symphysis to the root of the penis. The symphysis was exposed and excised to about 1.5 mm on either side of the midline. The membranous urethra was incised transversely and a polythene catheter (Portex PP205) was passed into the urinary bladder. A ligature around the membranous urethra retained the catheter in position and prevented leakage of urine. A large loop of the catheter was tethered subcutaneously and the free end was "tunnelled" under the skin of the abdomen, side of thorax and dorsum of neck, to emerge through a midline scalp incision.

(b) A midline, lower abdominal incision exposed the upper margin of the pubic symphysis. The bladder outlet was identified and a ligature was tied as caudad as possible around the membranous urethra.

The peritoneum was opened in the upper part of the incision to expose the urinary bladder. A polythene catheter (Portex PP205) was inserted for about 1 cm through an incision in the crown of the bladder and

retained in place by a purse-string suture. A second ligature, below the purse-string, prevented leakage of urine. The peritoneum and abdominal wall were closed around the catheter, which was then tethered subcutaneously and brought out,as above, through a midline scalp incision.

The left external jugular vein was exposed through a midline incision in the front of the neck. A polythene cannula (Portex PP30) was inserted into the incised vein for a distance of 3 to 4 cm and retained by an encircling ligature. A loop of cannula was tethered subcutaneously and the free end "tunnelled" around the side of the neck to emerge through the scalp incision.

A 25 g needle was used to penetrate the skull as described previously. A fine, stiff polythene cannula (Portex PP10) with bevelled end, was inserted for 3 mm along a path inclined towards the midline. The cannula was secured to pericranium at the site of penetration and was looped and tethered, as above, before emerging through the scalp incision.

The bladder catheter, intravenous cannula and intraventricular cannula were individually tethered to pericranium and threaded through a short length (about 2 inches) of wide-bore polythene tubing (Portex PP325) which was subsequently sutured to the edges of the scalp incision.

The rats spent a total of about two hours under anaesthesia and were allowed to survive for a further 48 hours.

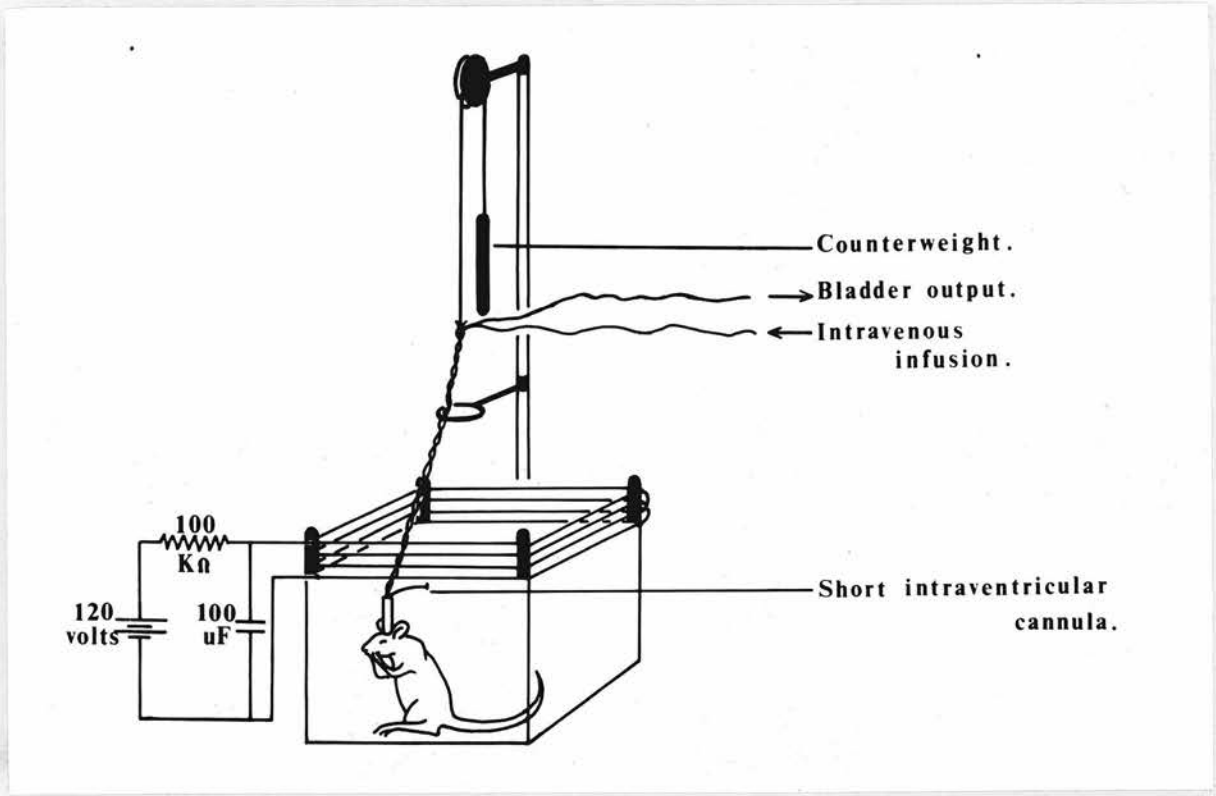

Counterweight.
Bladder output.
Intravenous infusion.
100 Kn
120 volts
100 uF
Short intraventricular cannula.

Figure 29

The apparatus which allowed continuous intravenous infusion, continuous collection of urine and intermittent injections into the cerebral ventricles of a conscious, unrestrained rat.

(ii) Continuous infusion and urine collection

A modification of the method of Edmonds and Thompson (1970) was used (Fig. 29). Rats were caged individually in wire-mesh cages, open at the top and electrified to deter escape. The bladder catheter and intravenous cannula were supported by a small counterweight mounted on a pulley to allow almost unrestricted movement of the animal. The intraventricular cannula was kept short (internal volume 0.01 to 0.02 ml) to minimise "dead space" for injections into the cerebral ventricles.

The intravenous cannula was fed by a slow-infusion pump, adjusted to deliver about 1 ml per hour. A mixture of equal volumes of 0.9% saline and 5% glucose solution was infused overnight and replaced by 5% glucose alone between 9 a.m. and 9 p.m. This regime usually established a regular background pattern of urine output on the day of the experiment. A minimum of twelve hours elapsed after operation before any experiment was started.

Test intravenous infusions of 5% glucose producing a brief, reproducible diuresis, were determined individually for each animal, then remained unchanged for all studies on that animal. Typical test infusions were as follows:

15 min. at 0.12 ml/min. = 1.8 ml total,

10 min. at 0.24 ml/min. = 2.4 ml total,

6 min. at 0.36 ml/min. = 2.16 ml total

Intravenous or intraventricular injections were administered when the infusion was two-thirds complete. Intraventricular

injections of an "artificial C.S.F." (composition: NaCl 121.5 mM; $NaHCO_3$ 25 mM; KCl 3.5 mM; $CaCl_2$ 1.3 mM; $MgCl_2$ 1.14 mM; NaH_2PO_4 0.51 mM; Urea 3.33 mM; Glucose 3.33 mM) were given to control for possible effects due to raised intracranial pressure.

Urine was syphoned continuously from the urinary bladder under a slightly sub-atmospheric pressure (Atmos.-3 cm water), and fed into a drop-recorder. The drop-recorder output, on smoke-blackened paper, provided a continuous record ofurine flow.

At the end of each experiment, the rat was killed by intravenous nembutal. The positions of the bladder catheter and intravenous cannula were checked visually, and the site of the intraventricular cannula by the injection of indian ink.

RESULTS

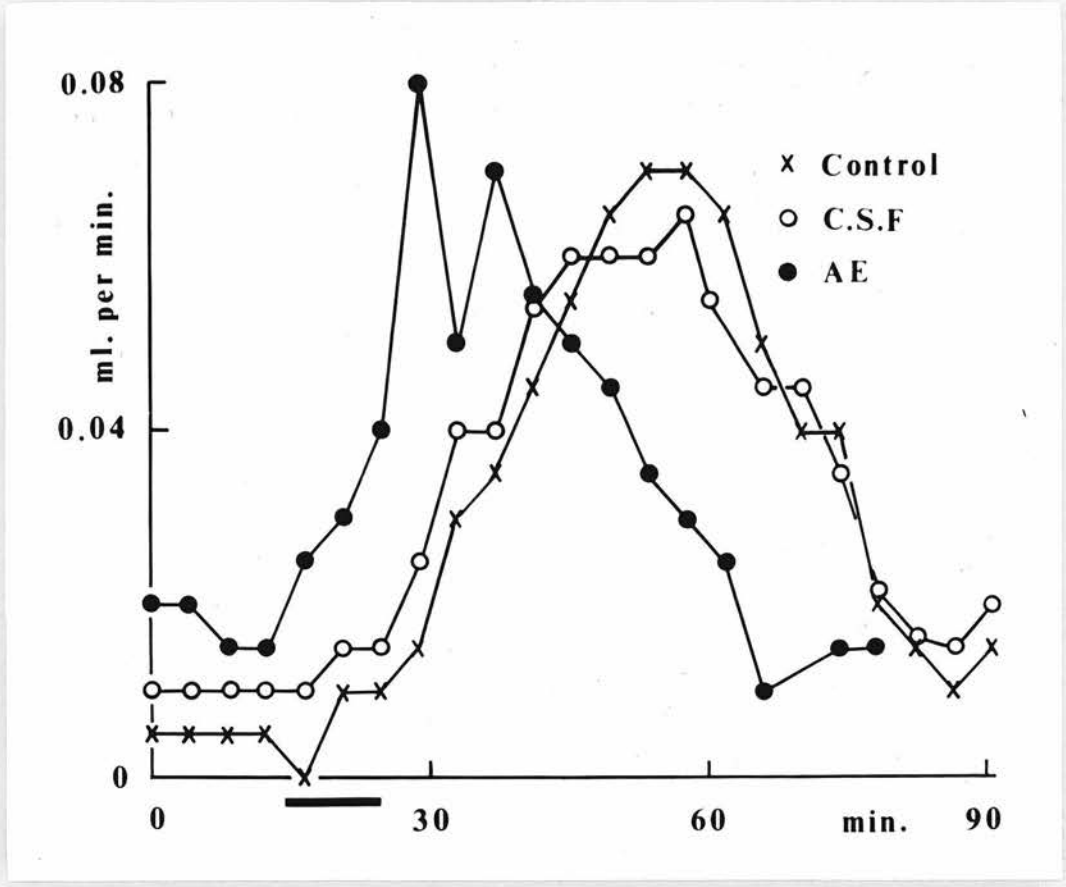

0.08
0.04
0
ml. per min.
Control
C.S.F
AE
0
30
60
min.
90

Figure 30 Urine flow during and after the injection of posterior lobe extract into the conscious rat.

Ordinate is urine flow in ml per min., abscissa is time in minutes. The horizontal bar shows the duration of the test intravenous infusion (5% glucose, 2.4 ml in 10 mins.). The responses of urine flow to three consecutive test infusions have been superimposed. 0.05 ml of artificial C.S.F. was injected into the right lateral cerebral ventricle during the second test infusion, and 0.05 ml of extract AE during the third.

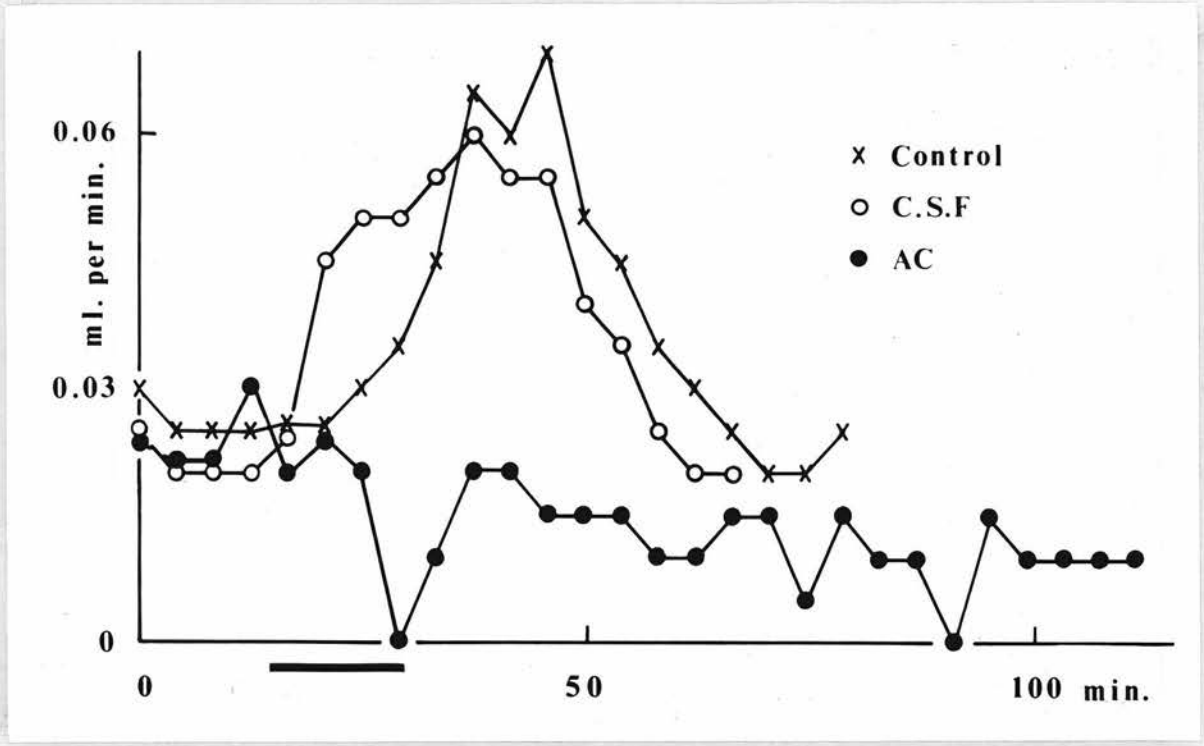

0.06
0.03
0
ml. per min.
Control
C.S.F
AC
0
50
100 min.

Figure 31 Urine flow during and after the injection of posterior lobe extract into the conscious rat.

The experimental design was as for Fig. 30, except that extract AC was injected into the lateral cerebral ventricle during the third test infusion. The test infusion consisted of 1.8 ml of 5% glucose infused over 15 minutes, and is indicated by the horizontal bar.

RESULTS

Extracts AC and AE have been tested for their effects on the urinary response to brief intravenous infusions of 5% glucose solution. Urine output was measured over successive four-minute periods and the flow-rate plotted as "ml per min."

"Octapeptide-depleted" posterior lobe extract (AE)

The responses to three consecutive test infusions, of 2.4 ml of 5% glucose, are summarised in Fig. 30. During the second infusion, 0.05 ml of artificial C.S.F. was injected into the right lateral cerebral ventricle without significant effect on the flow of urine. 0.05 ml of extract AE, injected during the third test infusion, did not inhibit the diuresis.

"Basal" urine flow increased gradually during the experiment suggesting that not all of the test infusion was cleared during the diuresis. This may explain the progressive decrease in the latency of the responses shown (Fig. 30).

Extract AE, injected into the cerebral ventricles, failed to inhibit the diuretic response in five trials in five animals.

"Non-depleted" posterior lobe extract (AC)

The injection of 0.05 ml of extract AC into the right lateral cerebral ventricle inhibited urine flow for up to two hours after the test infusion (Fig. 31). This antidiuresis was consistent in seven trials in five

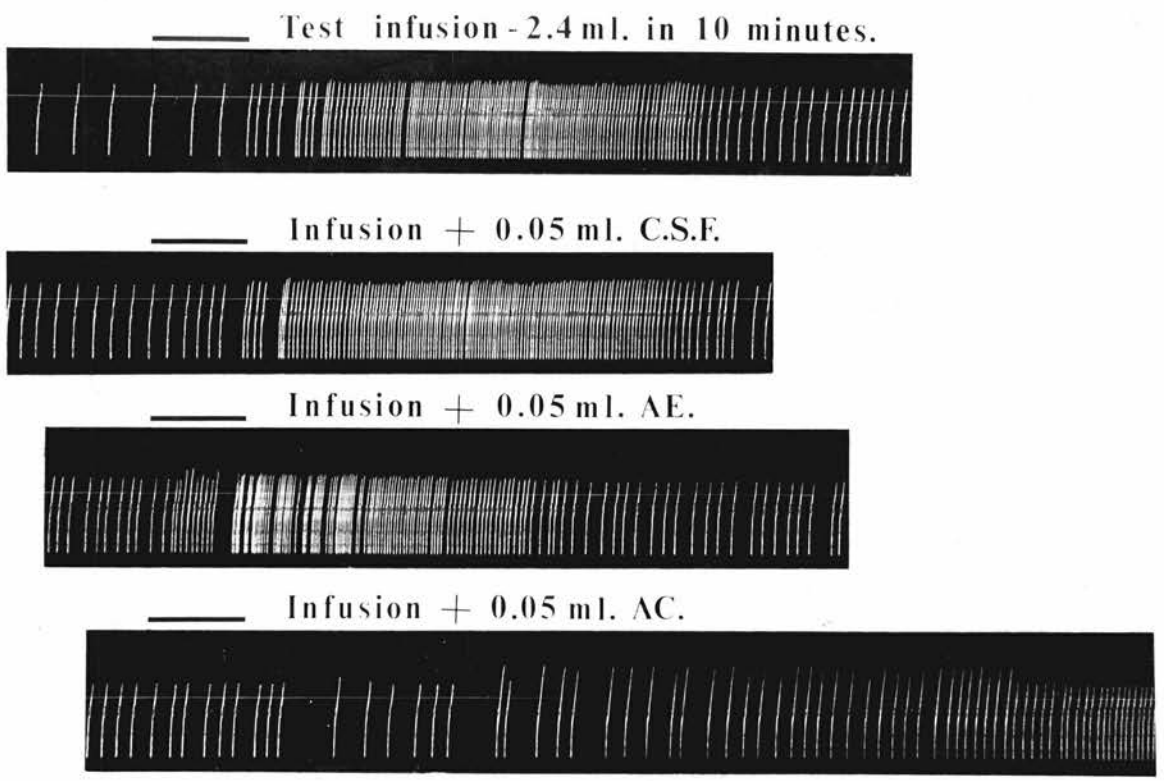

Test infusion - 2.4 ml. in 10 minutes.
Infusion + 0.05 ml. C.S.F.
Infusion + 0.05 ml. AE.
Infusion + 0.05 ml. AC.

Figure 32 Urine flow in the conscious, perfused rat.

Records show the pattern of urine output (drop recorder) in a conscious rat in response to four consecutive test infusions of 5% glucose. Artificial C.S.F., extract AE and extract AC were injected into the lateral cerebral ventricle during the second, third and fourth test infusions respectively.

Horizontal bars indicate the duration of the test infusions (2.4 ml in 10 mins.)

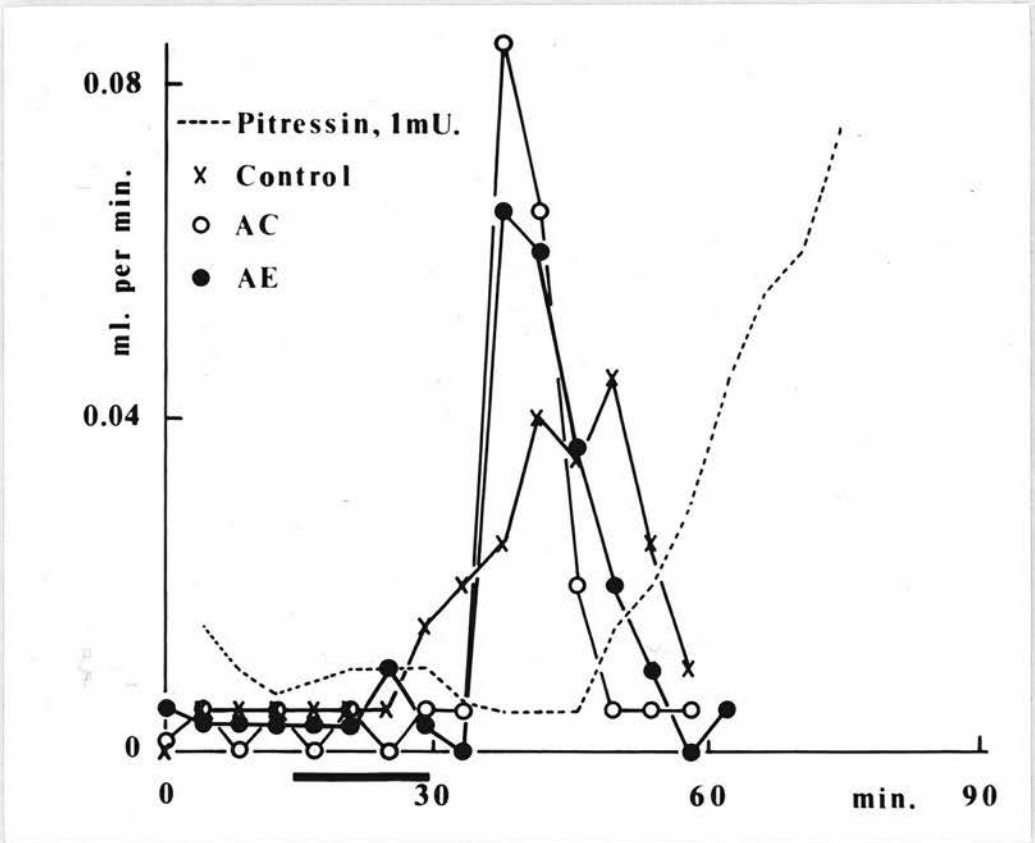

0.08
0.04
0
ml. per min.
----- Pitressin, 1mU.
x Control
o AC
● AE
0
30
60
min.
90

Figure 33 Effects on urine flow of intravenous injections of posterior lobe extracts.

Urine flow, in ml per min., is expressed against time in minutes. The horizontal bar shows the duration of the test infusion (1.8 ml of 5% glucose in 15 mins.). 0.1 ml of extract AE, extract AC and Pitressin were injected intravenously during the second, third and fourth test infusions respectively.

animals even, on one occasion, when the injection coincided with a spontaneous diuresis.

Sample records of urine output, as recorded by the drop recorder, are shown in Fig. 32. (Same experiment as Fig. 30.)

No significant antidiuretic effect was detected after the intravenous injection of up to 0.1 ml of either extract (Fig. 33).

DISCUSSION

Metabolic changes consistent with increased ribosome and protein synthesis accompany the elevated release of neurohormones during osmotic stress (Section II). A similar synthetic response might result from any procedure causing neurohormone secretion. That this is the mechanism by which "active" posterior lobe extracts exert their effects is unlikely on two counts. First, extract AE, when injected into the lateral cerebral ventricle, does not produce antidiuresis, yet does cause significant changes in supraoptic nucleoli (Figs. 22 to 25). Second, extract AC, administered intraventricularly, is a potent antidiuretic, yet does not cause demonstrable changes in the nucleoli of supraoptic neurones (Figs. 22 to 25).

Thus, the effects of "octapeptide-depleted" posterior lobe extracts on supraoptic nucleolar activity (Section III) cannot be caused by secretion of neurohormones in the recipient animals at the time of injection.

The apparent paradox, that extract AC is antidiuretic when injected into the cerebrospinal fluid, yet inactive when given intravenously, remains unexplained.

Attempts to locate the site of action of extract AE on the supraoptic neurone have been uninformative. Two approaches were tried.

(i) Autoradiography. Extracts AC and AE were prepared, using donor rats which had received an intraventricular injection of 100 μCi of ^{3}H-lysine 18 hours previously.

^{3}H-lysine, rather than ^{35}S-cysteine, was chosen to minimise the risk of non-specific localisation of label in the recipient animals. Supraoptic neurones have a high affinity for ^{35}S-cysteine (Sloper et al 1960) and metabolites of ADH may be taken up by the neurohypophysis (Pliška et al 1971). Donor neurohypophyses showed heavy incorporation of label but no radioactivity could be demonstrated in hypothalamic sections from recipient rats.

(ii) The proteins in freshly-prepared extracts were labelled by conjugation with the fluorescent dye, Lissamine Rhodamine B, as described by Nairn (1969). In hypothalamic sections from recipient rats, fluorescent dye could be demonstrated in the ependymal lining of the third ventricle and subarachnoid spaces, and infiltrated the whole hypothalamus by four to eight minutes after injection. Fluorescence was not concentrated in any specific hypothalamic areas and was noticeably absent from the neurohypophysis up to 16 minutes after injection. By this time, hypothalamic fluorescence was undetectable.

The distribution of fluorescence gave additional confirmation that, by the intraventricular route, extract constituents do reach the hypothalamic neurosecretory cells.

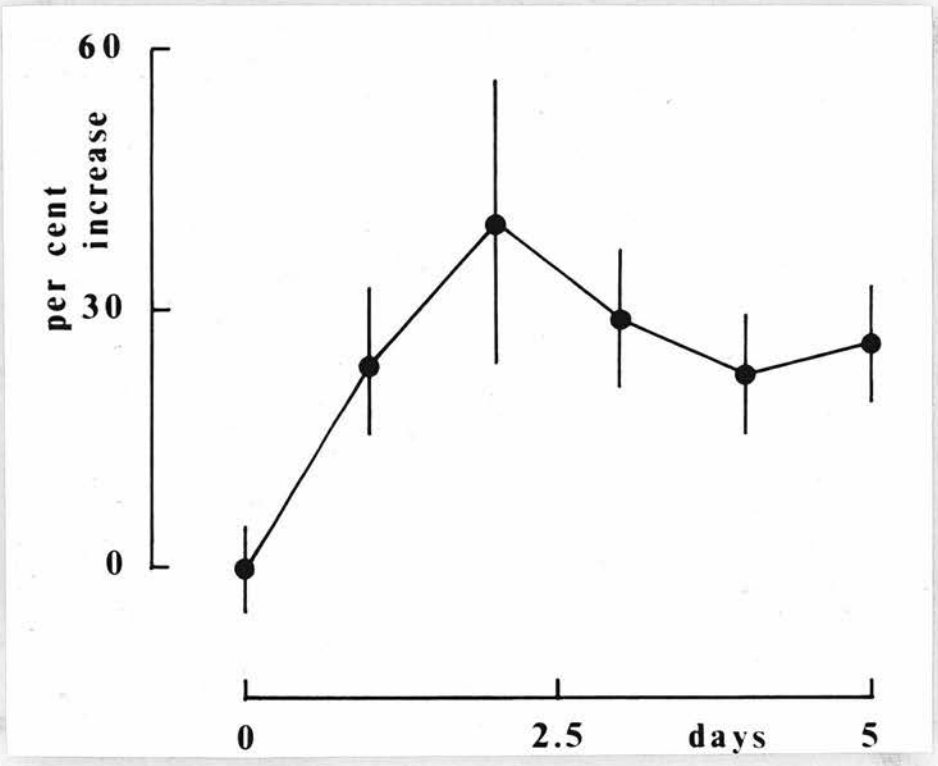

60
per cent
increase
30
0
0
2.5
days
5

Figure 34 Pituicyte dry mass during dehydration

The increase in the mean dry mass of pituicytes is expressed (as %) against the duration of dehydration. Each point is the mean ± S.E.M. of 55 to 138 measurements (3 to 7 rats). The mean dry mass of fixed, control pituicytes, calculated with an assumed value of 1.54 for the refractive index of fixed cells (Davies 1958), was 116.5 ± 5.4 gm.10^{-12}.

CONCLUDING DISCUSSION

By analogy with the changes described in neurones during chromatolysis (Section I) and stimulation (Section II), the increased dry mass and nucleic acid content of supraoptic nucleoli after the injection of posterior lobe extracts probably indicate increased ribosome synthesis. Since only posterior lobes which have been partially depleted of their stores of neurohormone give "active" extracts, some factor associated with secretion may be responsible for the observed changes.

No attempt has been made to identify, within the neurohypophysis, the source of the active agents present in extracts AE and AE_5. The two likely candidates are the nerve terminal and the enigmatic pituicyte. Pituicytes divide and hypertrophy during osmotic stress (Selye and Hall 1943, Ortmann 1951, Bachrach 1957a, Leveque and Small 1959) and incorporate ^{3}H-uridine faster (Sachs et al 1971). Despite Bodian's (1951) suggestion that this represents a glial response to injury, the pituicyte seems intimately involved in the response of the hypothalamic neurosecretory system to osmotic stimulation.

Fig. 34 shows the changes measured in the dry mass of fixed pituicytes, isolated from rats dehydrated for up to five days. The steps in fixation and isolation were as described for supraoptic cell bodies (Section II, Methods). Mean dry mass increased early and was significant after only 24 hours of dehydration ($p < 0.02$). This response is at

least as fast as that of any parameter monitored in supraoptic neurones during dehydration (Section II) and precedes measurable change in the dry mass of supraoptic cell bodies by 48 to 72 hours.

Pinocytosis has been observed at the tips of embryonic neurites in vitro (Hughes 1953) and has been postulated as a means of "membrane conservation" in the nerve endings of mature neurosecretory cells (Douglas et al 1971, Lederis et al 1971). Bovine serum albumin, labelled with Evan's blue, can penetrate the nerve terminals of hypoglossal neurones in the rat and rabbit (Kristensson et al 1971). There is, therefore, an obvious route by which chemical "signals" generated outwith the nerve terminal can penetrate its membrane.

The injection of large doses of posterior pituitary extract (Green and van Breeman 1955) or vasopressin (Dicker and Nunn 1957) does not prevent the depletion of neurosecretory material and neurohormone from the neurohypophysis during osmotic stimulation. Hollander and Blythe (1960) measured the urine volume (12 hour sample) and osmolality, serum osmolality and total body weight in rats deprived of water for up to three days. Chronic administration of exogenous ADH, up to the onset of dehydration, did not alter the normal responses in these parameters.

On balance, a direct negative feedback of circulating ADH on its own secretion or synthesis has not been excluded but seems unlikely.

Rats with hereditary diabetes insipidus have a chronically raised blood osmolality and their supraoptic (and paraventricular) neurones show the histological features of intense synthetic activity (Sokol and Valtin 1965, Valtin et al 1965). These changes in supraoptic neurones regress only partially when a normal blood osmolality is restored with exogenous ADH. Heterozygotes of this strain of rats show lesser, but significant, histological signs of activity, yet have a normal serum osmolality. Thus, in both of these situations, some stimulus in addition to blood osmolality appears to drive the supraoptic synthetic activity.

Extracts of normal neurohypophyses have been shown here to inhibit partially the dry mass response of supraoptic nucleoli to osmotic stress (Fig. 28). Since rats with diabetes insipidus lack normal stores of ADH, this potential brake to synthetic activity may be lost.

The interruption of a negative feedback from the nerve terminals might also contribute to the "signal" which triggers the synthetic response seen in neurones after axotomy (Section I).

The results presented here suggest that the metabolic activity of supraoptic neurones may be influenced by at least two unidentified substances produced in the neighbourhood of their nerve terminals. The secretion of neurohormones gives rise to a factor capable of stimulating

metabolic activity, and "replete" hormone stores may generate a metabolic inhibitor. Such agents could co-operate to match synthetic activity in supraoptic neurones to secretory losses.

The route by which such agents might reach the cell body has not been investigated. Axoplasmic transport mechanisms may be both complex and specific. In the "brain-nerve trunk-muscle" preparation from the snail (Kerkut et al 1967), ^{14}C-glutamate migrated centrifugally whilst ^{14}C-Xylose did not. Recently, Norström and Sjöstrand (1971a) have published suggestive evidence for three different rates of migration of labelled protein down the supraoptico-hypophysial tract. Whether individual axons, or transport channels within the same axon, subserve different rates of migration is unclear. Haemorrhage increased the amount of protein transported, but did not diminish the "transit time" between supraoptic nucleus and neurohypophysis (Norström and Sjöstrand 1971b).

Centripetal transport within nerve axons is less well established, but seems likely (Kerkut et al 1967, Watson 1968c, Lubińska and Niemierko 1971, Kristensson et al 1971) and has been described in neurosecretory cells in vitro (Hild 1954).

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APPENDIX

(i) Dry mass by interferometry (Davies 1958)

Consider a rectangular "slab"of solution or gel of refractive index Nc, area A cm^2 and thickness t cm, immersed in water of refractive index Nw.

Let M gm be the mass of substance other than water in the slab, and c its concentration in $gm.ml^{-1}$

Then $c = \frac{M}{At}$ $gm.ml^{-1}$

or $M = \frac{c}{At}$ gm ..... (1)

By definition, the specific refractive increment (x) is given by:

$$x = \frac{Nc-Nw}{100c}$$

or $c = \frac{Nc-Nw}{100x}$ ..... (2)

Combining (1) and (2),

$$M = (Nc-Nw)t.\frac{A}{100x}$$

or $M = \frac{Dw.A}{100x}$, where Dw = (Nc-Nw)t, and is the optical path difference due to the plate for light rays passing through the thickness t.

Since $X = 100x$,

$$M = \frac{Dw.A}{X} \quad \text{..... (3)}$$

(ii) Dry mass of fixed cells

Fixation converts cell substance into a three-dimensional matrix of "clumped" material, the spaces of which may be penetrated by the surrounding medium (Davies 1958). The true thickness of retarding material is therefore the

geometrical thickness of the cell, less the total thickness of the medium-filled matrix spaces. This quantity is the "effective thickness", te.

Key:

Np ..... refractive index of fixed cell,
Nm ..... refractive index of mounting medium,
Nw ..... refractive index of water,
Dm ..... O.P.D. in mounting medium,
Dw ..... O.P.D. in water,
te ..... "effective" cell thickness,
A ..... area of cell (normal to optical axis),
X ..... 100x.

In water, $Dw = (Np-Nw)te$, and in mounting medium,

$$Dm = (Np-Nm)te.$$

Hence, $$\frac{Dw}{Dm} = \frac{(Np-Nw)}{(Np-Nm)}$$

or, $$Dw = Dm.\frac{(Np-Nw)}{(Np-Nm)}$$

Since $$M = \frac{A.Dw}{X}, \text{ (Appendix i)}$$

$$M = \frac{A.Dm}{X} \cdot \frac{(Np-Nw)}{(Np-Nm)} \quad \ldots\ldots\ldots (4)$$

(iii) Dry mass of nucleoli within nuclei

Measurements of nucleolar O.P.D. will include a contribution from nuclear substance above and below the nucleolus.

Key:

Nc ..... refractive index of nucleolus,
Nm ..... refractive index of mounting medium,
Nw ..... refractive index of water,
D1 ..... mean O.P.D. due to nucleolus and overlying nucleoplasm,
D2 ..... mean nuclear O.P.D.,
Dc ..... mean nucleolar O.P.D.,
t ..... mean nucleolar thickness,
d ..... mean nuclear thickness.

Thus, the mean thickness of overlying nucleoplasm is (d-t) cm, and

$$D1 = Dc + \left(\frac{d-t}{d}\right).D2$$

$$= Dc + D2\ (1-{}^{t}/_{d})$$

or

$$Dc = D1 - D2\ (1-{}^{t}/_{d})$$

Substituting in equation (4), Appendix ii,

$$M = \frac{A}{X}.D1 - D2\ (1-{}^{t}/_{d}).\frac{(Nc-Nw)}{(Nc-Nm)}$$

(iv) Published papers (enclosed)

1. Watt, R. M. Metabolic activity in single supraoptic neurones and its relation to osmotic stimulation. Brain Research, 21 (1970) 443-447.

2. Watt, R. M. Rat supraoptic neurones: nucleolar changes following injection of posterior pituitary extracts. Brain Research, 22 (1970) 413-416.

3. Watt, R. M. Rat supraoptic neurones: nucleolar changes in response to posterior pituitary extracts. Brain Research, 29 (1971) 163-165.

Reprinted from
Brain Research
Elsevier Publishing Company, Amsterdam
Printed in the Netherlands

Metabolic activity in single supraoptic neurones and its relation to osmotic stimulation

Quantitative microchemical estimations on the protein and nucleic acid metabolism of single neurones have been successfully employed in attempting to categorise the nerve cell's response to stimulation[2–4,6,7] or injury[10–12]. Little information is available, however, on the mechanism(s) whereby this metabolic response is related to the functional demands placed upon the cell.

As a prelude to a detailed look at the coupling of protein and nucleic acid synthesis to neurohormone secretion in isolated supraoptic cells, it was necessary to demonstrate (a) the quantitative alteration in various cellular and subcellular parameters during physiological stimulation and (b) some correlation between the degree of stimulation and the concomitant metabolic response.

The results below refer to single supraoptic cells derived from the male albino rat aged 3 months (approx. 250–300 g). Physiological stimulation of these cells was achieved by depriving the animals of water for periods up to 5 days at two different ambient temperatures (20°C and 24°C). Parameters measured included nuclear volume, nucleolar margination, nucleolar dry mass and nucleic acid and cell body dry mass and nucleic acid content.

Animals were killed by exsanguination under light ether anaesthesia and a thick (1 mm) basal slice of brain excised and placed in either (a) chilled, buffered 0.34 *M* sucrose solution (composition as Hadjiolov *et al.*[5]) for subsequent isolation of cell nuclei or (b) 10% buffered formalin for isolation of supraoptic cell bodies. Supraoptic regions were then dissected free in the appropriate medium.

Mechanical disaggregation of supraoptic regions, fixed for 24 h in 10% buffered formalin, produced a good yield of isolated fixed cells, free from adherent glia when viewed in the interference microscope. The fixed tissue (approx. 0.75 mm × 0.25 mm × 0.25 mm) was placed for 30 min in 53% sucrose solution followed by mechanical disaggregation in $5 \cdot 10^{-4}$ ml of the same solution. The resultant cell suspension was then mounted on quartz slides for dry mass and nucleic acid measurements. Nuclear suspensions for nucleolar estimations were examined fresh following disaggregation of isolated supraoptic regions in $5 \cdot 10^{-4}$ ml of buffered 0.34 *M* sucrose.

In order to obviate uneven shrinkage during fixation of histological sections, the measurement of nuclear diameters was performed on freshly isolated nuclear suspensions. Because of the known sensitivity of isolated nuclear volume to the ionic composition of the suspending medium[1], all measurements were made in buffered 0.34 *M* sucrose of constant composition. Nuclei were photographed in positive phase contrast in the interference microscope and the negatives scanned in two directions at right angles in a scanning microdensitometer. Overall magnification was × 2080.

'Nucleolar margination' was arbitrarily defined as contact between nucleolus and the nuclear membrane sufficient to deform nucleolar and/or nuclear outline. Approximately 120 nuclei from each animal were examined and the result expressed as 'percentage of nuclei showing nucleolar margination'.

Serum specific gravity was adopted as an easily measured approximation to the

osmotic stimulus applied to the supraoptic cells. Although indirect this is probably a better index of dehydration than total body weight, as rats deprived of water eat little after the second or third day[8]. Measurements were made on a kerosene/bromobenzene density gradient column immersed in a 340 litre water-bath at room temperature. This column remained stable and linear over a period of weeks. Standardisation was by sucrose solutions of known specific gravity.

Dry mass measurements were made using the Leitz transmitted light interference microscope and nucleic acid contents were estimated by ultraviolet ab-

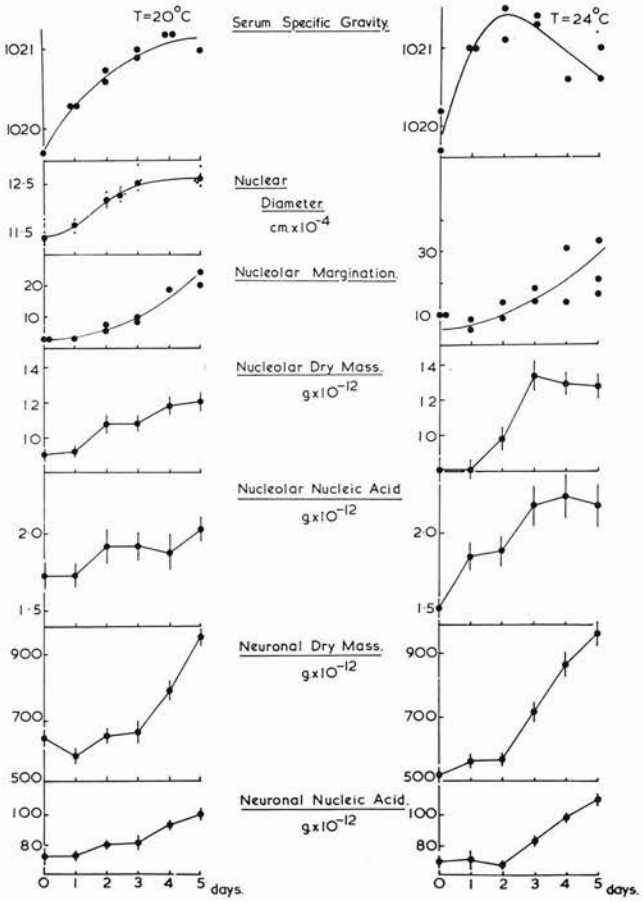

Fig. 1. Metabolic response of supraoptic cells to dehydration. T = 20°C represents the response to 5 days' water deprivation at 20°C. T = 24°C illustrates the response to 5 days' water deprivation at a temperature of 24°C.
Serum specific gravity: each point represents the mean of 2 or 3 estimations on serum derived from one animal. Nuclear diameter: · represents mean of diameter measurement on 40 isolated nuclei from one animal; ● represents overall mean value. Nucleolar margination: each point represents percentage occurrence of marginated nucleoli in 115–120 isolated nuclei from one animal. Nucleolar dry mass, nucleic acid, neuronal (cell body) dry mass and nucleic acid: each point, plotted with its standard error as a vertical bar, represents mean value for a total of 30–90 estimations derived from 2 to 5 animals.

TABLE I

SUMMARY OF MEASURED CHANGES IN SUPRAOPTIC CELLS ISOLATED FROM MALE RATS SUBJECTED TO 5 DAYS' DEHYDRATION

T = 20°C denotes dehydration at 20°C; T = 24°C signifies dehydration at 24°C. Mass and nucleic acid measurements are entered with their standard errors.

	T = 20°C			*T = 24°C*		
	Day 0	*Day 5*	*P*	*Day 0*	*Day 5*	*P*
Nuclear diameter ($cm \cdot 10^{-4}$)	11.36	12.61				
Nucleolar margination (%)	2.5	22.1		10.0	23.9	
Nucleolar dry mass ($g \cdot 10^{-12}$)	9.00 ± 0.40	12.03 ± 0.56	< 0.0005	8.12 ± 0.75	12.92 ± 0.72	< 0.0005
Nucleolar nucleic acid ($g \cdot 10^{-12}$)	1.73 ± 0.09	2.03 ± 0.08	< 0.0125	1.51 ± 0.06	2.18 ± 0.14	< 0.0005
Cell dry mass ($g \cdot 10^{-12}$)	628 ± 27.4	950 ± 29.2	< 0.0005	512 ± 22.9	960 ± 36.8	< 0.0005
Cell nucleic acid ($g \cdot 10^{-12}$)	72.4 ± 5.3	100.9 ± 3.9	< 0.0005	69.4 ± 3.5	110.7 ± 3.9	< 0.0005

sorption microspectrography at 253.7 nm. Subsequent derivation of absorption spectra permitted correction of nucleic acid figures for peak absorption at 260 nm and for non-specific light absorption. Evaluation of photographic records from the interference microscope and ultraviolet microspectrograph was carried out by means of the Joyce–Loebl scanning microdensitometer with digital integrator attachment.

Both series of curves illustrated in Fig. 1 (T = 20°C and T = 24°C) indicate a positive change in all measured parameters with progressive dehydration. Increases in nucleolar dry mass and nucleic acid precede the corresponding neuronal (cell body) changes by 24–48 h, and the earliest change detected is in nuclear diameter which appears to increase within the first 24 h. This increase is small (11.1%) but corresponds to a volume change of 23.6%. With the exception of nucleolar nucleic acid changes at 20°C the overall increases in those parameters amenable to statistical treatment proved highly significant at 5 days (Table I).

Any comparison of these two series of curves depends on the differences in osmotic stimulation inferred from the respective serum specific gravity curves. Five days' water deprivation at 20°C produces a progressive increase in serum specific gravity, reaching maximal values at 4–5 days, whilst a similar dehydration period at 24°C produces maximal values at 2–3 days with a subsequent fall. This drop in specific gravity may reflect serum protein changes following voluntary starvation in animals deprived of water. In dogs deprived of both food and water, only 45% of the weight loss at 5 days was due to loss of body water[9]. A similar fall in serum specific gravity during dehydration has been reported elsewhere[8].

On the basis of these specific gravity curves, dehydration is more rapid and severe at 24°C than at 20°C and with the exception of margination, nucleolar and cell body parameters undergo correspondingly greater increases at the higher ambient temperature. Thus, at 24°C, nucleolar dry mass and nucleic acid increase by approximately 59% and 44% respectively (*cf.* 34% and 12% respectively at 20°C). Both of these parameters show a tendency to plateau formation in animals dehydrated at 24°C (days 3–5) but whether this represents maximal nucleolar activity and ribosome synthesis is debatable as it coincides with a fall in recorded serum specific gravity. Cell body dry mass and nucleic acid undergo increases of about 88% and 60% respectively at 24°C compared with 51% and 39% at 20°C.

The unexpected finding that supraoptic cells from animals maintained at an environmental temperature of 24°C consistently demonstrated lower control (day 0) values for dry mass and nucleic acid parameters renders accurate statistical comparison of 20°C and 24°C responses difficult. Some tentative conclusions can, however, be drawn. Thus, a comparison of the calculated regression lines for nucleolar dry mass and nucleolar nucleic acid changes during days 0–3 of the dehydration period (*i.e.* excluding the 'plateau' phase of the 24°C curves) indicates significantly greater slopes under the higher temperature conditions ($P < 0.01$ and < 0.05 for dry mass and nucleic acid curves respectively). In this context, attention should be drawn to the apparent shorter latency of response in cell body dry mass and nucleic acid under the higher temperature conditions (approx. 48 h *cf.* 72 h).

The results presented here are consistent with an increase in the nucleic acid

and protein synthesising systems of the cell in response to increased functional demand and the augmented responses seen in association with dehydration of increased severity argue in favour of some quantitative correlation between the degree of stimulation and the cell's metabolic response.

My thanks are due to Professor W. E. Watson for help and equipment.

The work was supported by grants from the Medical Research Council and the National Fund for Research into Poliomyelitis and other Crippling Diseases.

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(Accepted May 20th, 1970)

Reprinted from
Brain Research
Elsevier Publishing Company, Amsterdam
Printed in the Netherlands

Rat supraoptic neurones: nucleolar changes following injection of posterior pituitary extracts

Current concepts of the mechanism underlying the neurone's metabolic response to axotomy[1,4] and stimulation[5] would be simplified by the definitive demonstration of a feedback of information from nerve terminal to the cell body. The work to be described investigates the concept of a feedback mechanism linking synthetic activity in the nerve cell body to secretory events occurring at or near the nerve terminals.

Taking advantage of techniques developed by Douglas and Poisner[2,3], a high potassium, low sodium, containing medium has been employed as a convenient means of producing octapeptide neurohormone release in isolated posterior lobes of rat pituitary glands.

Using male albino rats aged 3 months, crude extracts of posterior pituitary were prepared as follows: Normal animals were killed by exsanguination and their pituitary glands removed. Posterior lobes were dissected free in chilled Locke's solution (composition: $MgCl_2$ 1 m*M*; $CaCl_2$ 2.2 m*M*; $NaHCO_3$ 6 m*M*; KCl 5.6 m*M*; glucose 10 m*M*; NaCl 154 m*M*), and as much as possible of the pars intermedia was removed piecemeal. Posterior lobes were then split longitudinally for 2/3 of their length and incubated in pairs for 90 min at 37°C in either (a) Locke's solution or (b) high potassium, low sodium Locke's solution (composition: $MgCl_2$ 1 m*M*; $CaCl_2$ 2.2 m*M*; $NaHCO_3$ 6 m*M*; KCl 56 m*M*; glucose 10 m*M*; sucrose 308 m*M*). Both incubating solutions were equilibrated with 5% CO_2 in O_2.

Extract AC was prepared from two posterior lobes incubated in normal Locke's solution and extract AE from posterior lobes incubated in the high potassium, low sodium solution. After incubation, each pair of posterior lobes was washed twice in normal Locke's solution and homogenized in 0.035 ml of the same solution. The resultant suspension was centrifuged for 15 min at 3,000 rev./min, the supernatant collected and diluted to a final volume of 0.05 ml. This then formed a single dose of crude extract for injection into a recipient animal, and represented an overall dilution of donor posterior pituitary tissue of approximately 1 part in 60 by volume.

Small pieces of parietal cortex were used as donor material for control extracts BC and BE. These extracts were prepared from pieces of tissue of similar dimensions to the pituitary posterior lobe and incubation, homogenization and dilution steps exactly paralleled those for the preparation of extracts AC and AE. Extract BC was derived from cortical tissue incubated in normal Locke's solution and extract BE from tissue incubated in high potassium, low sodium medium.

Under light ether anaesthesia, recipient animals were injected with 0.05 ml of a freshly-prepared extract into the right lateral cerebral ventricle. Some control animals received no intraventricular injection.

Forty-eight hours after injection, recipient animals were exsanguinated and the hypothalamus excised. Supraoptic and posterior arcuate regions were dissected free and suspensions of isolated nuclei prepared for nucleolar dry mass and nucleic acid determination as described previously[5].

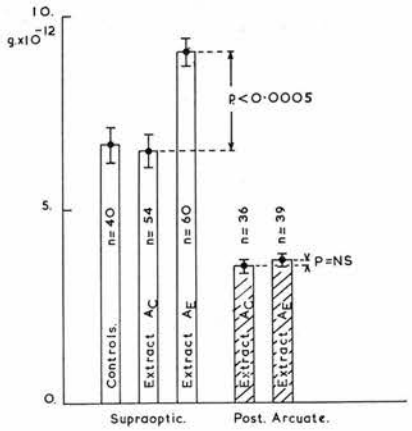

Fig. 1. Supraoptic and posterior arcuate nucleolar dry mass 48 h after injection of posterior pituitary extract. n is the number of estimations contributing to the mean value which is entered with its standard error as a vertical bar. Three recipient animals contributed to each supraoptic mean value, 2 animals to each posterior arcuate mean. Extracts are as defined in text. N.S. = not statistically significant.

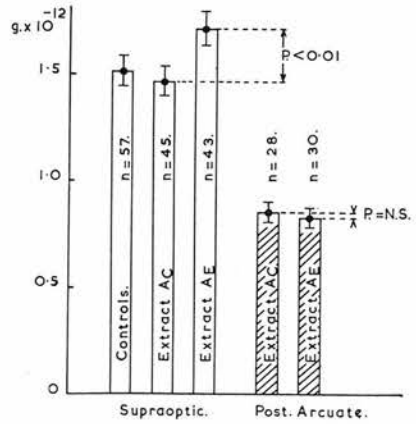

Fig. 2. Supraoptic and posterior arcuate nucleolar nucleic acid content 48 h after injection of posterior pituitary extract. Abbreviations as in Fig. 1.

The serum specific gravities of recipient animals were estimated using a kerosene/bromobenzene density-gradient column immersed in a 340 litre water-bath at 37 $\pm$ 0.001°C.

Supraoptic nucleolar dry mass remained unchanged from control values 48 h after injection of extract AC but showed a highly significant increase after extract AE (Figs. 1 and 3). Extracts BC and BE (Fig. 3) did not differ significantly in their effects on supraoptic nucleolar mass although both groups of animals exhibited lower mean values than was found in control animals. This apparent loss of mass was not statistically significant ($0.1 > P > 0.05$). Supraoptic nucleolar nucleic acid changes in response to injection of extract AE were less dramatic but significant ($P < 0.01$), the AE group showing a 17% higher value than those animals given extract AC (Fig. 2).

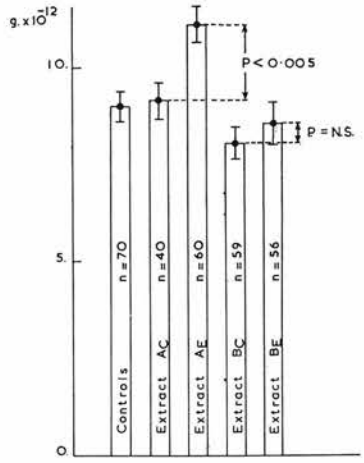

Fig. 3. Supraoptic nucleolar dry mass 48 h after injection of extract of posterior pituitary or parietal cortex. Abbreviations as in Fig. 1.

Thus, of the 4 extracts examined, only 'octapeptide-depleted' posterior lobe extract (AE) consistently produced significant elevation of supraoptic nucleolar dry mass and nucleic acid content. Serum specific gravities in animals given extracts AC and AE were 1.0204 ± 0.0001 and 1.0205 ± 0.0001, respectively, suggesting that the observed changes in supraoptic nucleoli were not reflecting changes in serum osmolarity.

As the only preparative difference between 'octapeptide-depleted' (AE) and 'non-depleted' (AC) posterior lobe extracts was in the incubation medium, the ability to reproduce some of the supraoptic nucleolar changes seen in dehydration[5] must be conferred on the posterior pituitary lobe during the high potassium, low sodium incubation. The experimental findings could, in theory, arise from a failure to remove potassium 'trapped' in the incubated tissue, producing an excessive potassium content in the resultant extract. This is effectively ruled out by the failure of extract BE (parietal cortex, high potassium, low sodium incubation) to elevate supraoptic nucleolar dry mass (Fig. 3).

It is not at present possible to state whether the active substance present in 'octapeptide-depleted' posterior lobe extract is specific either in its source or target-cell, although its failure to produce nucleolar changes in posterior arcuate cells (Figs. 1 and 2) makes a 'blanket' effect on all hypothalamic neurones unlikely. Similarly, the absence of any increase in supraoptic nucleolar mass after giving extract BE (Fig. 3) excludes the possibility that active extract can be prepared from any neural tissue.

Feedback control of cell body synthetic activity by a substance produced at or near secretory terminals requires that the 'active' substance reaches the cell body. No evidence is presented here bearing on how or even if such a centripetal transport of 'active' substance might be achieved *in vivo*. The route of administration of extract, admittedly unphysiological, was chosen to achieve close proximity between posterior lobe extract and neurosecretory cells.

Aside from considerations of centripetal transport, a feedback mechanism of the type envisaged here requires the demonstration of the ability of 'active' extract to stimulate protein and nucleic acid synthesis in the nerve cell body in the absence of secretory activity at the recipient animal's neurosecretory terminals. This aspect is currently under investigation.

My thanks are due to Professor W. E. Watson for access to equipment.

The work was financed by grants from the Medical Research Council and the National Fund for Research into Poliomyelitis and other Crippling Diseases.

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(Accepted June 22nd, 1970)

Reprinted from
Brain Research
Elsevier Publishing Company, Amsterdam
Printed in the Netherlands

Rat supraoptic neurones: nucleolar changes in response to posterior pituitary extracts

The incubation of posterior pituitary lobes in a high potassium, low sodium medium results in the release of the octapeptide hormones, vasopressin and oxytocin[1,2]. Extracts from such posterior lobes are capable of producing increases in nucleolar dry mass and nucleic acid in the supraoptic neurones of normal rats[7]. The work described here examines the question of whether similar properties are conferred on pituitary posterior lobes by the release of neurohormone *in vivo*, *i.e.*, without exposure to a high potassium, low sodium solution.

'Octapeptide-depleted' posterior lobe extract (AE_5) was prepared from 3-month-old male albino rats deprived of water for 5 days. The animals were killed by exsanguination and their pituitary glands removed. Posterior lobes were dissected free in chilled Locke's solution and the greater part of the pars intermedia removed piecemeal. The isolated pituitary posterior lobes were then homogenised in pairs in 0.035 ml of Locke's solution and centrifuged for 15 min at $1{,}600 \times g$. The supernatant, after dilution with Locke's solution to a final volume of 0.05 ml, formed a single dose of crude extract.

'Non-depleted' posterior lobe extract (AC) was prepared in an exactly parallel manner from normal male rats with free access to water.

Under light ether anaesthesia, a single dose of freshly prepared extract AC or AE_5 was injected into the right lateral cerebral ventricle of each recipient animal, according to the method of Haley and McCormick[4]. The consistency of site and depth of the needle track was checked histologically in preliminary experiments and confirmed visually at the time of death of the experimental animals used here. A third group of rats (controls) received no intraventricular injection.

Recipient animals were killed by exsanguination 48 h after injection and supraoptic and posterior arcuate regions dissected free from the excised hypothalamus. Mechanical disaggregation of such unfixed supraoptic and posterior arcuate regions in a buffered 0.34 *M* sucrose solution[3] produced good yields of fresh isolated nuclei, suspensions of which were mounted on quartz slides for the measurement of nucleolar dry mass and nucleolar nucleic acid content. The techniques currently in use in this laboratory for the measurement of dry mass and nucleic acid content by interference microscopy and ultraviolet microspectrography respectively have been described elsewhere[5,6] along with a consideration of their limitations.

Blood from recipient and control animals was used for serum specific gravity estimations as described previously[7].

Increases in supraoptic nucleolar dry mass (36%, $P < 0.001$) and nucleolar nucleic acid (17%, $P < 0.001$) were observed 48 h after injection of 'octapeptide-depleted' (AE_5) posterior lobe extract (Figs. 1 and 2). 'Non-depleted' extract (AC) did not produce significant change in either of these parameters ($P > 0.7$ for nucleolar dry mass, $P > 0.1$ for nucleolar nucleic acid).

With the possible exception of a borderline significant decrease in nucleolar dry mass after injection of 'non-depleted' extract ($P < 0.05$), nuclei derived from the

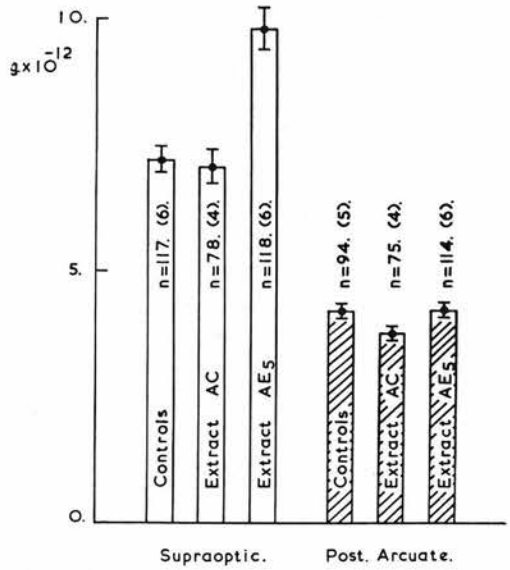

Fig. 1. Supraoptic and posterior arcuate nucleolar dry mass 48 h after injection of posterior pituitary extract. Controls: normal uninjected male rats. Extract AC: Rats injected with extract of normal posterior pituitary lobes. Extract AE_5: Rats injected with extract of posterior pituitary lobes taken from 5-day dehydrated animals (see text). n is the number of observations contributing to the mean value which is entered with its standard error as a vertical bar. Bracketed figures show the number of animals contributing to each mean.

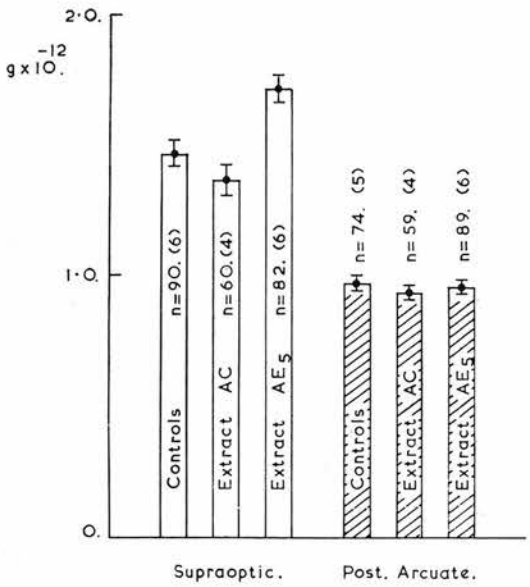

Fig. 2. Supraoptic and posterior arcuate nucleolar nucleic acid content 48 h after injection of posterior pituitary extract. Abbreviations as in Fig. 1.

TABLE I

SERUM SPECIFIC GRAVITY, IN RECIPIENT ANIMALS, 48 h AFTER INJECTION OF THE EXTRACTS INDICATED

	Number of animals	*Serum specific gravity (range)*
Controls	6	1.0200 (1.0192–1.0211)
Extract AC	4	1.0200 (1.0193–1.0212)
Extract AE_5	6	1.0202 (1.0190–1.0209)

posterior part of the arcuate nucleus did not show observable nucleolar responses to either extract (Figs. 1 and 2).

Serum specific gravity was unaffected by the injection, 48 h previously, of either extract (Table I).

No fundamental difference appears to exist between the responses obtained here and those reported previously concerning pituitary posterior lobes exposed to a high potassium, low sodium solution[7]. This suggests that the occurrence of a substance in the pituitary posterior lobe, capable of initiating the observed changes in supraoptic nerve cells, is a consequence of the release of octapeptide hormones *per se* and not an artefact associated with *in vitro* exposure to depolarizing solutions.

My thanks are due to Professor W. E. Watson for help and access to equipment.

The work was supported by grants from the Medical Research Council and the National Fund for Research into Poliomyelitis and Other Crippling Diseases.

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(Accepted March 17th, 1971)