STUDIES OF SUBCELLULAR FRACTIONS OF BOVINE POSTERIOR PITUITARY GLANDS ISOLATED BY CENTRIFUGATION

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by

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ABSTRACT

Bovine posterior pituitary glands were homogenized with teflon pestles with wide clearance designed to preserve nerve terminals. These nerve terminals are pinched off from their axons by homogenization, seal themselves at the point of rupture from their axon filaments and retain their normal complement of organelles. The homogenate was fractionated by differential centrifugation into six particulate fractions including one composed of isolated nerve endings (neurosecrosomes) and a supernatant. Each fraction was assayed for vasopressin (VP), oxytocin (OT), lactic dehydrogenase (LDH), succinic dehydrogenase (SDH), acid proteinase (AP) and nitrogen. Only 25% of the total LDH was found in the particulate fraction; the remainder was in the supernatant. The particulate LDH, an indicator for occluded cytoplasm, was highest in fractions 2 and 3 which, on electronmicroscopic observation, were found to contain neurosecrosomes and mitochondria. LDH was also found in fractions containing smaller particles and attributed to the presence of small fragments of axonal cytoplasm. The distribution of AP was similar to that of brain tissue, being parallel to the mitochondrial enzyme SDH. VP and OT were not distributed equally, supporting earlier observations that they are each contained in specific neurosecretory granules that differ in their sedimentation properties.

The rates of hydrolysis of acetylthiocholine (ASCh) and butyrylthiocholine (BuSCh) were determined in whole posterior pituitaries. The relative rates of hydrolysis of both substrates and the responses to

specific inhibitors indicated that both specific and non-specific cholinesterase (ChE) were present in the posterior lobe. The posterior pituitary hydrolyses ASCh at one-third the rate of rat brain but BuSCh at a fourfold faster rate than rat brain. The distribution of the two ChE's in the seven fractions did not parallel any of the other enzymes. Both specific and non-specific ChE are concentrated in the microsomal fraction which is made up of membrane fragments.

The neurosecrosome fraction was prepared for electronmicroscopy by two methods; the centrifugation pellet was fixed in OsO_4 and thin sectioned, and a suspension of the fraction was prepared by negative staining. Examination of the fixed pellet showed that the neurosecrosomes are similar to the nerve endings seen in fixed sections of whole tissue. However, the secretory granules (SG) in the neurosecrosomes do not readily take up stain and appear devoid of electron dense centers commonly seen in sections of whole tissue. Examination of the fraction by the negative stain technique showed that some of the SG are apparently filled and others empty. Comparison of the two staining techniques indicates that the inability of the SG to take up OsO_4 does not necessarily signify the absence of neurosecretory material.

The neurosecrosome fraction was subfractionated on a discontinuous density gradient into three particulate fractions. Evidence that each subfraction consisted primarily of neurosecrosomes was provided by electronmicroscopy and LDH determinations. The VP/OT ratios of

the three subfractions indicated that VP is associated with more dense and OT with lighter neurosecrosomes. The sedimentation properties of the hormones within the neurosecrosomes are similar to those of the hormones within SG shown previously. It was concluded that the sedimentation of neurosecrosomes is determined by the type of SG it contains. The differences in the VP/OT ratios found in the three subfractions of neurosecrosomes provide evidence that only one of the two hormones is contained in a single neurosecrosome. This provides the basis for the hypothesis that any individual neuron of the hypothalamo-hypophysial tract may contain only one of the two hormones.

The neurosecrosomes of the posterior pituitary contain microvesicles (MV) whose function is unknown. A fraction, obtained by osmolysis of the neurosecrosomes and density gradient centrifugation of the osmolysate, was seen by electronmicroscopy to contain vesicles ranging in size from 25 to 400 mμ in diameter, 41% having a diameter less than 80 mμ. It appears that the MV do not represent a discrete population of particles but are the smallest of a range of vesicle sizes continuous with SG. The MV fraction contains 2U/mg of vasopressin in contrast to about 16-30 U/mg protein for SG. The low hormone content, and electronmicroscopic evidence that SG membranes appear to form small vesicle support the hypothesis that the MV are the breakdown products of membranes of SG that have been depleted of their content of neurosecretory material.

A simple, rapid method was developed for the isolation of a fraction of relatively pure SG. Van Dyke protein is a hormone-rich (16-20 U/mg) extract of whole posterior pituitary which contains several proteins, one or more of which is presumed to be a carrier for the hormones. It is also assumed that the SG are vesicles which contain the hormones in association with carrier protein. In order to establish an identity between the Van Dyke protein and the SG, the amino acid composition of both were determined and compared. The two preparations are identical with respect to all amino acids found except cystine and glycine. The similarities indicate that the Van Dyke protein is identical to, or a major component of the SG.

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ADH	antidiuretic hormone
ACh	acetylcholine
AP	acid proteinase
ASCh	acetylthiocholine
BuSCh	butyrylthiocholine
ChE	cholinesterase
g	gravity
LDH	lactate dehydrogenase
M	moles
mag.	magnification
mg	miligrams
ml	mililiters
mμ	milimicrons
MV	microvesicles
OT	oxytocin
RSA	relative specific activity
SA	specific activity
SDH	succinate dehydrogenase
SG	secretory granules
U	units
VP	vasopressin
μ	microns
μg	micrograms
μl	microliters

TO MY WIFE

SHEILA

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GENERAL INTRODUCTION

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It is generally acknowledged that neurons exert their effect on the peripheral tissue they innervate by secreting a humoral substance. In most places, this humoral agent act upon cells that are in close proximity to the neuron terminal. However, some neurons secrete their chemical product into the circulation which carries it to distant target tissue. Although the chemical products of all neurons can be considered neuro-secretion, the term is applied to neurons which serve a glandular function and which secrete their product into the circulation. In the mammal, the only well defined neurosecretory system is the posterior lobe of the pituitary gland.

The neurosecretory neuron consists of the cell body, in which the secretory product is wholly or partially synthesized, the axon in which the secretory product is transported, and the terminal where the product is stored and released upon appropriate stimulation. In addition to its glandular function, the neuron is electrically excitable.

In the mammalian posterior pituitary gland, each of the three parts of the neuron lies in an anatomically distinct area: the cell bodies are in the anterior hypothalamus in the supra optic and paraventricular nuclei, the axons make up the hypothalamo-hypophysial tract, and the terminals make up the bulk of the posterior lobe. Thus the posterior pituitary gland is unique in that the major functional component is only a part of neuronal cells.

The nerve terminals of the posterior pituitary have been isolated in vitro by homogenization and differential centrifugation (LaBella and Sanwal, 1965). In this preparation, the terminals which are the functional elements of the gland are isolated although it is not yet certain if they retain their in vivo function. This provides an opportunity to examine several of their properties.

In the present work, the posterior pituitary was homogenized and separated into subcellular fractions by differential and equilibrium centrifugation. These were examined electronmicroscopically, and assayed for the hormones oxytocin (OT) and vasopressin (VP) and for selected enzymes.

GROSS ANATOMY

The mammalian hypophysis projects down from the bottom of the midbrain on the midline between the optic chiasma and the mammillary bodies. It lies in a cavity of the sphenoid bone, the sella turcica which is roofed by a membrane, the diaphragma sellae. The body of the hypophysis is connected to the midbrain by a stalk which penetrates the diaphragma sellae. The hypophysis (pituitary body, pituitary gland) is composed of four parts, the anterior, posterior, and intermediate lobes, and the pars tuberalis. The latter two will not be discussed in here. The anterior lobe (pars distalis, adenohypophysis) is embryologically derived from ectoderm in the roof of the primitive mouth. An invagination called Rathke's pouch extends upwards, the original connection with the mouth, the craniopharyngeal canal, eventually is closed and the cells proliferate to form glandular tissue. The posterior lobe (pars nervosa, posterior pituitary) originates from a diverticulum which grows down from the floor of the midbrain and remains, in adult form, as an extension of this part of the brain. The original diverticulum from the brain remains as the infundibulum and stalk (Harris and Donovan, 1961).

The floor of the midbrain between the optic chiasma and the mammillary bodies is a hollow conical projection with the pituitary stalk emanating from its apex and enclosing the infundibular recess of the third ventricle. This area is called the median eminence. The apical portion which is continuous with the stalk is the infundibulum. The term

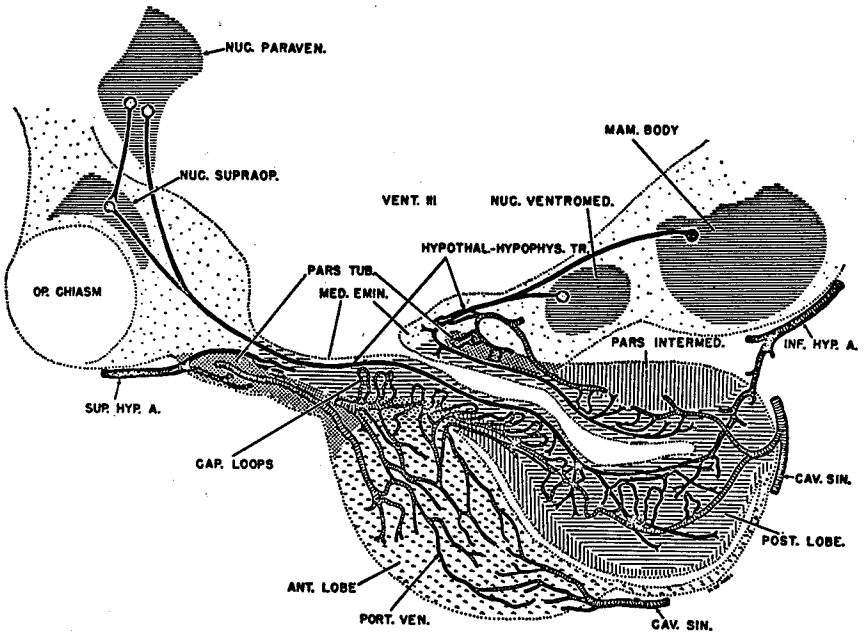


Fig. 1. Principal neural and vascular connections of hypophysis of the cat in schematic longitudinal section. (Drawn by H.W. Ades, 1960)

'neurohypophysis' refers to the complex composed of the median eminence, infundibulum, stalk, and posterior pituitary gland (Harris and Donovan, 1961) (Fig. 1).

The anterior and posterior lobes have their own blood supply from the anterior and posterior cerebral arteries respectively. In addition, venous blood from the median eminence is recirculated through the anterior lobe (Green and Harris, 1949).

HISTORY

The name pituitary is derived from the Latin "pituita" which means phlegm or slime. The pituitary body was considered by the Greeks and Romans to be an organ of excretion for the brain; excrement from the brain supposedly being collected by this appendage and secreted into the pharynx in the form of phlegm; a belief which persisted until comparatively recent times. Vesalius described a funnel (infundibulum) which drained the third ventricle into the pituitary from which four ducts (not demonstrated) carried the phlegm away. Willis in 1664 expressed the belief that the pituitary body excreted a distillate from the brain into the pharynx via the roof of the pallate or the olfactory bulbs. In 1672, Richard Lower stated that the product of the pituitary was not released into the pharynx but into the blood. In 1751 deBordeu proposed that the gland did not secrete brain excrement but produced a secretion of its own. These latter theories became more plausible with the advent of microscopy when it was found that the pituitary body possessed an extensive intricate vascular bed (Abel, 1924; Zuckerman, 1954).

In 1895, Oliver and Schafer observed the physiological effects of

extracts of the pituitary body and found a potent vasopressor action. This pressor effect was found to be restricted to the posterior lobe by Howell in 1898. It was subsequently shown to cause an increase in urine output. The observation by Frank in 1912 that patients suffering from diabetes insipidus had pathological posterior lobes led a year later to the treatment of this disease with posterior pituitary extracts which caused a remarkable decrease in urine output. It is now an accepted fact that one of the secretions of the posterior lobe of the pituitary decreases urinary output. The erroneous conclusion that the extract caused an increase in urinary output observed earlier was a reflection of the vasoconstrictor effect on animals whose renal function was severely compromised by the experimental procedure (Historical review by Dale, 1957).

In addition to the pressor and antidiuretic effects, extracts of the posterior lobe were found to produce strong contractions of the uterus (Dale, 1906) and promoted milk ejection (Ott and Scott, 1910). The action on the uterus was not altered by prior treatment of the uterus with ergot alkaloids (Dale, 1909). Abel and Kubota (1919) believed that the uterine stimulating effect was a result of histamine which is concentrated in the gland, but it was shown that histamine could not be responsible for this action (Dudley, 1919) and the theory was withdrawn (Abel and Nagayama, 1920). It was believed that all the actions of posterior pituitary extract were attributable to a single active substance (Abel et al., 1923). However, Dudley (1919) showed earlier that the pressor and oxytocic activities could be separated by butyl alcohol extraction. Similarly, Kamm et al. (1928) separated two principles by a series of extractions.

Prior to the isolation and identification of the hormones OT and VP, the development of our knowledge of the neurohypophysis was nearly exclusively based on histological studies. Cajal, in 1894, noted the abundance of nerve fibers in the stalk and the neurohypophysis. A stain for brain glial cells was used on this tissue, and a number of glial-like cells were observed. In the infundibulum, these cells were organized parallel to the fibers of the hypothalamo-hypophysial tract. In the pars nervosa, they assumed a wide variety of shapes, with extensive processes along capillaries. Bucy (1932) called these cells "pituicytes".

For many years thereafter it was thought that the pituicytes were the site of origin of the hormones, as these were the only complete cells seen that could apparently produce hormone. The pituicytes were distinguished from brain glial cells by the presence of lipid inclusions in the former. Cytological changes in the pituicytes were observed when animals were dehydrated by water deprivation. These changes were interpreted as a response to dehydration which resulted in increased production of antidiuretic hormone (Gersh, 1939; Griffiths, 1940). It was believed that a large number of nerve fibers descending from the hypothalamus to the neurohypophysis provided the innervation of pituicytes, stimulating them to secrete hormone (Gersh, 1940; Brooks and Gersh, 1941). Cutting this nerve tract by severing the stalk caused diabetes insipidus (Ranson, Fischer, and Ingram, 1938). Several workers maintained that the hormones were derived from the nerves and not the pituicytes although the evidence at that time was scanty (Palay, 1945; Bargmann, 1957).

There is now clear evidence that there are two active principles; both are octapeptides. They have been isolated as pure substances, their chemical structure determined, and have been produced synthetically (reviewed by DuVigneaud, 1954). VP was so named because of its ability to raise blood pressure. This is unfortunate terminology, as it is generally acknowledged that the pressor effect is of little if any significance under normal physiological conditions. The major action is as an antidiuretic hormone. This action is effected by concentrations in the blood that are far below those necessary to produce a rise in blood pressure (van den Velden, 1913). There is a possibility that the vasopressor effect is manifested in extreme hemorrhage (Ginsburg and Brown, 1957). The pressor activity is via a direct action on vascular smooth muscle. The activity as an antidiuretic is not clearly understood. VP promotes the passage of water across amphibian skin and bladder. A similar action on the distal portion of the nephron system is believed to promote the reabsorption of water from the tubule lumen (Thorn, 1958; Sawyer, 1961; Kleeman and Cutler, 1963). The hormone also promotes the transport of sodium across frog skin (Fuhrman and Ussing, 1951). A similar action may take place in the kidney.

OT is involved in parturition and milk ejection. In these cases the specific target tissues are the uterus and mammary alveolar myopithelium respectively. The uterus is relatively insensitive to the action of OT during gestation and at parturition it becomes 50 times more sensitive (van Dyke, 1961). During parturition, the amount of OT found in the blood

increases by a factor of at least 100 (Fitzpatrick, 1961). Stimulation of the nipple of a lactating animal by suckling causes release of OT from the neurohypophysis. This is brought about by reflex stimulation of the anterior hypothalamic nuclei (Cowie and Folley, 1956).

In general, factors causing release of one hormone cause the release of the other but the relative proportions may vary. This will be discussed in detail in section III.

MICROANATOMY

The neurohypophysis is made up of neurons of the hypothalamo-hypophysial tract. In mammals, the tract originates in the supraoptic and paraventricular nuclei which are in the anterior of the hypothalamus. The tract descends through the infundibulum and reaches the posterior lobe via the pituitary stalk. Some of the neurons terminate in the median eminence and infundibulum but most pass directly into the posterior lobe. There, the axons branch and terminate in close proximity to capillaries. If the tract is cut, retrograde degeneration takes place that can be traced to the nuclei in the anterior of the hypothalamus (Ransom, Fischer, and Ingram, 1938). With the use of the electron microscope, it was seen that the fibers of the tract are unmyelinated and contain tubules 20-30 mμ in diameter. In the posterior lobe the axon branches terminate in swellings .5 to 1.5 in diameter. These terminals, or nerve endings contain fusiform mitochondria, secretory granules (SG), and vesicles 20-40 mμ in diameter (Palay, 1957). These small vesicles shall henceforth be called micro-

vesicles (MV).

Secretory cells in general are seen in light microscopy to contain droplets of material. Neurons containing apparently secretory droplets are called neurosecretory neurons and are seen in animals from metazoa up through the entire phylogenetic scale. In mammals, this type of neuron is seen in the anterior hypothalamus, specifically in the supraoptic and paraventricular nuclei (Scharrer, 1928 reviewed by Hild, 1956; Scharrer and Scharrer, 1945). The material is seen within the perikarya in close association with Nissl substance (Scharrer et al., 1945). With hematoxylin-eosin stain, the material appears as basophilic droplets and there is no apparent distinction between this material and inclusions in epithelioid glandular cells found in other parts of the body. In 1949, Bargmann showed that Gomori's chrome-alum hematoxylin stain would specifically stain neurosecretory material (Bargmann and Scharrer, 1951). It was subsequently shown that there is a parallelism between the intensity of Gomori staining and the hormone content of the gland. Dehydration of an animal depletes the posterior lobe of VP and Gomori positive material (Ortman, 1951). When dehydrated animals were rehydrated, the content of both hormone and Gomori positive material increase (Scharrer, 1952). Painful stimuli, shown to cause release of VP (Verney, 1947), also caused depletion of Gomori positive material (Rothballer, 1953). The evidence is now clear that Gomori positivity and the neurosecretory material are directly associated. This neurosecretory material is probably the hormone in association with a carrier protein (Barnett, 1954). When the carrier protein is isolated and

separated from the hormones, the Gomori staining reaction with the protein is still positive (Acher and Fromageot, 1955). There is not necessarily a direct relationship between the hormone content of the gland and its stainability; e.g. in neonates, there is scanty Gomori positive material yet the hormone content of the gland can be relatively high. This is attributed to the fact that in neonates the hormone is not associated with carrier protein (Dicker, 1966). Sectioning the stalk caused the Gomori positive material to disappear from the posterior lobe and to accumulate in the stalk proximal to the cut (Hild and Zetler, 1953). This provided evidence that the material flows distally from the hypothalamic nuclei and that the segments of neurons distal to the cut are unable to replenish the stored neurosecretory material after it has been released. Peripheral streaming of axoplasmic material has been observed in the living pituitary stalk of the goosefish (Carlisle, 1957) and is in keeping with the fact that material flows from perikarya to terminals in all neurons (Weis, 1961). These observations were supported by experiments in which hypothalamo-hypophysial tissue was cultured (Hild and Zetler, 1953; Hild, 1954; Green and van Breeman, 1955). Cultures of tissue from the supraoptic and paraventricular nuclei survived poorly, and insufficient material was obtained for successful extraction and assay of the hormones, but material could be seen, by phase contrast microcinematography, to flow peripherally from the perikarya to the axons. Cultures of posterior lobe tissue resulted in the growth of glial cells and macrophages, but no neurons could be seen.

These cultures did not contain Gomori positive material, nor could any hormone activity be demonstrated after a few days.

The secretory material stained by Gomori's chrome-alum hematoxylin appears as blue staining aggregates or droplets. In the perikarya, the material can be seen to be intraneuronal, but in the axons of the nerve tract and terminals in the posterior lobe this histochemical technique with light microscopy does not provide sufficient resolution to determine whether the material is intra or extraneuronal. With the use of standard electron-microscopic techniques applied to the hypothalamo-hypophysial system, these neurons are seen to contain membrane bound granules 100 to 300 mμ in diameter, the so-called neurosecretory granules (Palay, 1957). In the posterior lobe, these granules, in osmium fixed tissue, are frequently opaque to the electron beam and appear filled with dark material; varying degrees of opacity are seen in most tissue sections, ranging from dark staining to clear, with various gradations between these two extremes. Some granules have a small dense core with a clear halo between it and the membrane, but this type of granule may be an artifact produced by dehydration of the specimen. The staining properties of the granules may reflect variations in the amount, or chemical state of the granule content at the moment the tissue was fixed (Palay, 1957; Hartmann, 1958; Fujita and Hartmann, 1961). It has been observed that some nerve endings are filled with SG, and that all have the same staining property. It was postulated that there are more than one type of neuron in the posterior lobe, each type of neuron being characterized by the staining property of the SG it contained (Green and Maxwell, 1959;

Barry and Cotte, 1961; Holmes, 1964). This is doubtful, as variously stained SG can be seen within a single nerve ending. Dehydration of an animal causes depletion of the dark staining intragranular material (Palay, 1957; Gerschenfeld et al., 1960). Other factors known to cause depletion of hormone from the posterior lobe also cause this depletion, e.g., histamine which causes release of VP (Mirskey et al., 1954) depletes SG (Hartmann, 1958). The granules are seen in the perikarya, axons and nerve endings but are most concentrated in the nerve endings. In the toad, there appears to be a graded increase in size between the perikarya and the nerve endings and this is believed to reflect the fact that the material is synthesized in the cell body and upper part of the axon (Gerschenfeld et al., 1960). This observation is supported by the finding that in vitro the proximal portion of the hypothalamo-hypophysial system is capable of incorporating S-^{35} cystine into vasopressin, whereas the distal portion, the posterior lobe, cannot (Takabatake and Sachs, 1964).

The subcellular site of formation of the SG is still unclear. The nucleus, mitochondria, Nissl substance, and Golgi apparatus, have all been implicated (reviewed by Scharrer and Brown, 1961; Lederis, 1962). Electronmicroscopic observations indicate a close association between the Golgi apparatus and SG in neurons of the earthworm (Scharrer and Brown, 1961) and in the preoptic nucleus of the cod (Lederis, 1962).

It is now generally accepted that the Gomori positive material as seen in light microscopy is identical with the SG as seen by electron-microscopy, as both are depleted by the same stimuli. The SG have been

isolated by homogenization of posterior lobes and differential centrifugation. A fraction having a high hormone activity was found to contain Gomori positive material and on electronmicroscopic examination was found to be composed primarily of SG (Schiebler, 1952).

The Microvesicles (MV) are seen in many of the nerve endings in the posterior lobe and have been reported in all electronmicroscopic studies. When first observed (Palay, 1957), they were called 'synaptic vesicles' as their size and appearance are similar to the well-established vesicles seen at synapses of the myoneural junction. In the absence of evidence other than their appearance, this term is not justified and most electronmicroscopists who subsequently observed these vesicles have deferred acceptance of the term (Lederis, 1962).

The function of the MV has been a subject for considerable speculation but remains unknown. This matter will be dealt with in detail in section III.

Pituicytes

The nerve endings in the gland are surrounded by pituicytes, which resemble brain glial cells but have several distinguishing characteristics. They have long protoplasmic processes that surround nerve fibers and nerve endings and are also interposed between nerve endings and the basement membrane of the capillaries. The glial cytoplasm is rich in endoplasmic reticulum associated with ribosomes (rough endoplasmic reticulum) and also contains large osmiophilic lipid droplets. The rough endoplasmic reticulum and droplets are present in the processes as well;

therefore, the processes are easily distinguishable from nerve endings (Palay, 1957). The function of these cells remains unknown, and the application of specific stains for neurosecretory material failed to provide evidence that they are involved in hormone production (Leveque and Scharrer, 1953), nor could such activity be demonstrated by tissue culture of pituicytes (Hild, 1954; Green and van Breeman, 1955). The pituicytes are involved in some process associated with hormone release as they have been observed to be altered by conditions stimulating hormone release (Gersh, 1939; Brooks and Gersch, 1941). They also increase in mitotic activity when the posterior pituitary is stimulated to release ADH (anti-diuretic hormone) by water deprivation or the substitution of saline for drinking water (Leveque and Small, 1959). It has been postulated that the pitucytes may act on the nerve endings to promote the release of hormone, or, as they are interposed between the neurosecretory nerve endings and the capillaries, they may process the neurosecretory material passing through them, possibly separating the hormone from the carrier protein (Rennels and Drager, 1955; Leveque and Small, 1959). However, nerve endings that abut directly on capillaries can also be seen.

PROBLEMS AND APPROACHES

(1) The hormonal and some biochemical properties of fractions of posterior pituitary homogenates have been described by LaBella et al., (1963), who separated the homogenate into four particulate fractions and a supernatant. It was subsequently shown that with milder homogenization methods, an additional subcellular component, could be isolated. These are

nerve endings which have been separated from their axons and remain intact (LaBella and Sanwal, 1965). The name 'neurosecrosomes' is hereby presented as a useful term to define isolated nerve endings from the posterior pituitary. These are analogous to the presynaptic nerve terminal that have been isolated from brain tissue and called "synaptosomes" (Whittaker et al., 1964). In the posterior pituitary, neither the neurosecrosomes or the MV have been investigated with regard to their sedimentation, biochemical or hormonal properties. In the present work, the neurosocrosomes, SG, and MV were isolated and some of their properties examined. However, before individual species of subcellular components could be isolated, it was necessary to determine their distribution in centrifugal fractions of the homogenate. This was accomplished by dividing the homogenate into six particulate fractions and a supernatant. The enzymes succinic dehydrogenase (SDH), lactic dehydrogenase (LDH) and acid proteinase (AP), and the hormones OT and VP were determined in each fraction as reference markers for mitochondria, neurosecrosomes, lysosomes and SG respectively. MV were presumed to sediment in the microsomal fraction and, as there is at present no biochemical property referable to these particles, they were identified by electronmicroscopy.

(2) The presence of a cholinergic mechanism for the release of the hormones VP and OT has been postulated (Gerschenfeld et al., 1960; Koelle, 1961). This was based on the observation that the MV resemble synaptic vesicles found in brain nerve ending which are known to contain acetylcholine (ACh), and that specific cholinesterase (ChE) was histo-

chemically demonstrated to be present in the posterior lobe of the cat (Koelle and Geesey, 1961). The presence of the enzyme that metabolizes ACh may be offered as presumptive evidence that this mediator is present in the tissue; however, there is pharmacological evidence that ACh is not a mediator for the release of hormones (Douglas and Poisner, 1964). Histochemical technique has been used to characterize ChE in the posterior lobe of a variety of species. The results of these studies indicate that the presence and activity of specific ChE is variable from species to species. No attempt has been made to characterize ChE in the bovine posterior lobe histochemically; however, there are two reports concerning the ChE in this species, both investigated by the manometric technique. These two reports are in direct conflict. One was unable to demonstrate specific ChE, all activity being attributable to non-specific ChE (Parmar et al., 1963). The other found specific ChE only, with very little non-specific ChE activity (Pasetto, 1954). It therefore remains unclear as to whether specific ChE is present in this species. In the present work, ChE in the bovine posterior lobe was reinvestigated in order to determine if specific ChE is present. The type of ChE activity was characterized by determining the responses of the enzymes to specific inhibitors. In addition, an attempt was made to determine the localization of the ChE enzymes in the fractions.

(3) There is considerable evidence that the hypothalamo-hypophysial system can exert a differential control over the release of VP and OT.

Knowledge concerning the storage and release of the peptide hormones provides no basis for speculation on how differential control can be exerted. One hypothetical mechanism is that the hormones are each stored in neurons that contain only one of the hormones. If such a division between the hormones exists, it is possible to separate the neurosecrosomes into those containing VP and those containing OT. This was attempted by fractionating neurosecrosomes by density gradient centrifugation.

(4) The origin and function of the MV remain unknown. All speculation extant on these particles is based on electronmicroscopic observation of their presence in neurosecretory nerve endings. In the present work, an attempt is made to isolate these particles. Their sedimentation properties and electronmicroscopic observations are reported here and a proposal of their origin is presented.

(5) The SG are known to be the storage site for both hormones. A protein has been extracted by other workers from posterior pituitary tissue which is biologically inactive but binds the hormones. This protein is presumed to be the carrier for the hormones. It is reasonable to assume that the carrier protein is localized in the hormone storage sites, the SG, but it has not been established that this protein is indeed native to the SG. Evidence to establish this fact was provided by isolating SG in a relatively pure fraction and then comparing the amino acid composition of this fraction with that of preparations of the chemically extracted, presumed carrier, protein.

DISCUSSION OF METHODS

Two tools basic to this investigation, tissue fractionation and electron microscopy, are fraught with difficulties in interpretation. Only with a full understanding of the limitations of this type of work, and the proper reservation in the interpretation of results, can the contributions made be properly evaluated. Nevertheless, much of our understanding of the basic physiology of the cell is validly based on such experimentation. Their usefulness cannot be denied.

Homogenization and Fractionation

> "The performance of a fractionation experiment involves three successive steps. The first one is destructive and converts a tissue or cell suspension into the so-called 'homogenate'. The second one reintroduces a new kind of order into the system, by grouping together in separate fractions those components of the homogenate of which certain physical properties, such as density or sedimentation coefficient, fall between certain limits set by the investigator. The third step consists in the analysis of the isolated fractions." (DeDuve, 1963).

The homogenate is a 'soup' containing all components of the tissue. Some components are broken to such a degree that they bear little resemblance to their form in situ, e.g., endoplasmic reticulum on homogenization is isolated as small vesicles, the 'microsomes'. The localization of the so-called lysosomal enzymes on microsomal particles has been shown to be an artifact of this soup; enzymes from heavier particles go into solution and are preferentially adsorbed onto lighter particles.

The homogenization procedure can produce an infinite range of particle sizes depending on how rigorously one grinds the tissue. It is

essential to reproduce the method of homogenization as precisely as possible. Furthermore, it is obvious that the homogenization medium cannot simulate the physical and chemical conditions in which an organelle resides in the cell. Changes in function and morphology brought about by removing the organelle from its natural environment may alter its properties.

Centrifugation

There are two basic means of separating particles in suspension, by differential centrifugation or density gradient centrifugation.

A. Differential Centrifugation:

This method is the most widely used and the cruder of the two. It is based on the differences in the rates of sedementation of particles when in a gravitational field.

$$v = \frac{\phi(\rho_p - \rho_m)}{f}\omega^2 x$$

v = velocity of the sedementing particle in cm/sec
ϕ = volume of particle in cm^3
ρ_p = density of the particle in gms/cm^3
ρ_m = density of the medium in ms/cm^3
ω = angular velocity of the centrifuge in rad/sec
x = radial distance from the axis of rotation in cm
f = the frictional coefficient in gm/sec = $6\pi\eta r_o$
η = the viscosity of the medium in poises (gms/sec/cm
r_o = the radius of the particle in cm (DeDuve et al., 1959)

In conditions where $\rho_p - \rho_m$ is positive, the particle will tend to sediment in the medium at a rate v.

If a heterogeneous population of particles is suspended in the medium,

a gravitational force will cause each to sediment to the bottom of the centrifuge tube at its own rate. Small or light particles originally near the bottom of the tube will sediment at the same time as a heavier particle higher in the tube. When the centrifuge is stopped at some arbitrary time, members of all particles types present will be in the sediment but most will be of the heavier type.

Some of the heterogeneity in the sediment can be eliminated by resuspending the sediment in medium and then recentrifuging. This will dilute the lighter particles so that they will constitute a smaller contaminant of the final pellet. The advantage of this method is its ease and rapidity. It allows the initial isolation of large quantities of material which can be further purified by additional techniques. Purity of these fractions can be approached by restricting the sedimentation to a narrower range of forces (g x minutes) at the expense of yield.

B. Density Gradient Centrifugation

It can be seen that when $\rho m = \rho p$, $v = 0$. If the density of medium in a tube is graded, a particle in a gravitational field will sediment, until it reaches a level in the tube where the density of the medium is the same as the density of the particle. This results in the formation of bands of particles in the tube, each band composed of particles of the same density.

There are two basic methods of density gradient centrifugation, continuous and discontinuous. In the former, there is a continuous gradient of medium density. For any species of particle, the range of sizes and

densities can be represented by a bell-shaped curve of normal distribution. If the ranges of this distribution are reasonably restricted, distinct layers of isodense particles are formed when the particles are brought to equilibrium by centrifugation. But, if the range of particle densities is wide or there are many different species of particles present, the overlapping makes separation impossible. The second method, discontinuous density gradient centrifugation, utilizes layers of medium of differing graded density, separated by interfaces. Each particle will stop at the interface above the density that is equal to or exceeds its own. This results in more heterogenous layers but the advantage of this method is that each layer is discrete and clearly separable from adjacent layers. In both types of gradients, the distribution of particles in the bands is extremely sensitive to subtle variations in the homogenization procedure.

Identification of Fractions by Means of Biochemical Markers

In addition to direct observation of particles in a fraction by electron-microscopy, the content of a fraction can be identified by its biochemical properties. If an enzyme or a biochemical function has been clearly ascribed to a particular organelle by a variety of independent observations, the presence of that activity in a fraction can be accepted as indicative of the presence of the organelle without necessitating direct morphological observation. An enzyme that can be used for this purpose becomes a so-called 'marker' or reference enzyme. It is required that an enzyme used as a marker must be exclusive to a particular organelle and cannot be dispersed by homogenization of the tissue. It must be assumed that

"...distinct enzymic species have single intracellular locations and granules of a given class are enzymically homogeneous" (DeDuve et al., 1955). This assumption is generally supported by experimental evidence (DeDuve, 1964).

In the following investigations, in addition to enzymes, a marker is used that is not enzymic. In the posterior pituitary, it has been established that the hormones VP and OT are present in two forms; one is sedimentable and the other is not. The sedimentable hormones appear to be localized in the SG. Therefore, the hormones can be utilized as a marker for these organelles.

Although markers can be used to identify organelles in a fraction, the concentration of another enzyme in the same fraction as the marker does not necessarily identify the enzyme as a component of the same organelle. The fraction may be composed of several organelles and only after additional fractionation techniques are applied to the fraction and the distribution of the enzyme whose localization is unknown is consistently found to parallel that of the marker, can the two be localized to the same organelle. A typical example of this problem is the distribution of certain hydrolytic enzymes. When rat liver was divided into the classical four fractions, nuclei, mitochondria, microsomes and supernatant, hydrolytic enzymes were found in the mitochondrial fraction. However, when an additional fraction between mitochondria and microsomes was added to the differential centrifugation scheme, the hydrolytic enzymes were separated from those present in mitochondria. It was clear that an additional organelle was present and was

the site of localization of the hydrolytic enzymes; these are the lysosomes (DeDuve et al., 1955). This example serves to illustrate that a full understanding of the limitations of centrifugal fractionation techniques are essential for the interpretation of enzyme localization and particle-type distribution.

Electronmicroscopy

The most widely used method of preparing biological specimens for electronmicroscopy is with the use of osmium tetroxide (osmic acid) as a fixative (Palade, 1952) or as modified by Caulfield (1957). This reagent not only hardens the tissue but osmium is incorporated into the protein structure forming electron dense sites. It is impossible, however, to relate the ability of specific structures to take up osmium with any physiological or biochemical entity. Several investigators have attempted to relate osmiophilia with protein structure, but none have succeeded in establishing a definite relationship. Although chemical reactions whereby osmium forms covalent bonds with amino acids have been shown, no explanation is yet available for incorporation into protein (Stoeckenius and Mahr, 1965; Hake, 1965).

Sediments obtained from centrifugal fractionation of homogenates can be examined electronmicroscopically by two methods, thin sectioning of fixed embedded sediment or negative staining of the resuspended sediment with phosphotungstic acid.

For thin sections, sediments of fractions are fixed in buffered osmic acid; the pellet is then treated as a piece of tissue for embedding and

sectioning. Stratification of the particles forming the pellet during centrifugation no doubt occurs. Depending on the degree of heterogeneity in the fraction, there will be a gradient of species of particles within the pellet. On sectioning the embedded pellet any one of several types of particles may be concentrated in a single section. This can lead to gross mis-identification. For this reason, it is essential that all pellets be carefully oriented when embedded and sample sections examined from various strata within each embedded specimen.

Stratification is not a problem if fractions are examined by the negative staining technique. Here a small drop of a suspension of the fraction is examined. Each drop, if the suspension is well mixed, can be assumed to contain a random sample of all types of particles present. The method also is far simpler and less time consuming than the conventional fixing and embedding procedure.

For negative staining, a small drop of suspension is mixed with a solution of a substance, most frequently phosphotungstic acid, that is electron dense when dried. This mixture is placed on a carbon coated grid, the excess liquid drained off, and the remaining fluid permitted to evaporate. When dry, the phosphotungstic acid has formed a film over the grid. Particles in the original solution are embedded in this film and form electron transparent spots where the electron dense phosphotungstate is attenuated. The image is the reverse of the conventional

method, lighter areas representing greater structural density, hence the name negative staining. This method has been very successful in examination of viruses. With larger particles, distortion frequently occurs. If the particle extends above the thickness of the phosphotungstate film, it is subject to the high pressure of the surface tension created by the dehydration of the suspension. This may very well explain the cytoplasmic processes occasionally seen streaming from nerve endings prepared in this manner (see figures in LaBella and Sanwal, 1965). Furthermore, phosphotungstic acid penetrates some structures and not others. Even where gross distortions are not seen, one can never be certain what effect the surface tension has on the particles.

In thin sections, a well supported structure is sliced, whereas in negative stain the entire structure is viewed. It is frequently difficult to compare the same particle seen by these methods quantitatively, for in negative staining, one cannot be sure if the structure has been crushed flat, expanding the observable dimensions.

II

BIOCHEMICAL AND HORMONAL CHARACTERIZATION OF NERVE ENDINGS (NEUROSECROSOMES) AND OTHER FRACTIONS ISOLATED FROM BOVINE POSTERIOR PITUITARY

INTRODUCTION

The posterior pituitary is made up primarily of terminals of neurons, the perikarya of which are situated in the supraoptic and paraventricular nuclei of the anterior hypothalamus. These terminals contain the peptide hormones OT and VP which are stored in vesicles 100 to 300 mμ in diameter, the so-called "neurosecretory granules" (Palay, 1957). An appropriate stimulus, originating in the anterior hypothalamic nuclei, is presumed to promote the release of these hormones from the nerve terminals into the general circulation. The cells which comprise the hypothalamo-hypophysial system have a dual function: as neurons, they are electrically excitable (Carlisle, 1957; Cross and Green, 1959; Suda et al., 1963; Koizumi et al., 1964; Kandel, 1964; Brooks et al., 1965) and serve to transmit information, and as secretory cells they synthesize, store, and release endocrine products (Bargmann and Scharrer, 1951).

The SG can be sedimented from homogenates of posterior pituitary by centrifugation and have been found to contain both hormones (Schiebler, 1952; Pardoe and Weatherall, 1955; Lederis and Heller, 1960; Weinstein et al., 1961; Schapiro and Stjarne, 1961; Heller and Lederis, 1961; LaBella et al., 1962; Ishii et al., 1962; Barer et al., 1963; LaBella et al., 1963). Some of these reports (Pardoe and Weatherall, 1955; Heller and Lederis, 1961; LaBella et al., 1962; 1963; Barer et al., 1963) provide evidence that there are differences in the sedimentation and

density properties of the two granule-bound hormones, and it was suggested that there may be two types of granules, one containing OT, the other VP.

LaBella et al. (1963) have estimated several enzymes and the two peptide hormones among subcellular fractions obtained by differential centrifugation of posterior pituitary homogenates. It was subsequently shown (LaBella and Sanwal, 1965) that an additional particulate component was present in posterior pituitary homogenates. Some of the nerve terminals are apparently pinched off from their axons during homogenization procedure and the rupture in the membrane is quickly sealed off. These nerve endings, herein designated as neurosecrosomes, are sedimentable at 3,000 g for 10 minutes after removal of the nuclei and debris. Electronmicroscopy showed that they closely resemble the nerve endings seen in thin sections of intact tissue, and Krass and LaBella (1965) have shown that this fraction is similar in metabolic activity to that of whole tissue slices. There are several previous reports in which similar procedures have been used to isolate nerve ending particles from cerebral cortical tissue (reviewed by De Robertis, 1964; Whittaker, 1965). The posterior pituitary differs from brain tissue in that the former contains only short segments of axons and their terminal swellings. Perikarya and dendrites are absent from the gland, and there is no evidence that the posterior pituitary nerve endings form synaptic associations with adjacent cells.

The sedimentation properties of posterior pituitary neurosecrosomes differ from brain nerve endings in that the former are more dense and the latter less dense than mitochondria. This difference is probably attributable to the presence of the relatively dense SG in the posterior pituitary nerve endings.

The present investigation is a study of the enzymic and hormonal properties of fractions obtained by differential centrifugation of homogenates of bovine posterior pituitary glands. The work previously reported from this laboratory (LaBella et al., 1963) has been expanded to include a study of the properties of the neurosecrosome fraction. LDH has been proposed to be a useful marker for the presence of "trapped cytoplasm", i. e., nerve endings (synaptosomes) in homogenates of cerebrocortical tissue (Johnson and Whittaker, 1963). The applicability of this enzyme as a biochemical means of identifying posterior pituitary neurosecrosomes was investigated. ChE activity was measured, because of a cholinergic mechanism of hormone release has been proposed (Koelle, 1961). The basis for postulation of such a mechanism was that: 1) small vesicles are seen in posterior pituitary nerve endings that resemble synaptic vesicles which are present in presynaptic terminals where ACh is known to be a mediator (De Robertis and Bennet, 1954) and in the brain where vesicles of similar size have been isolated and shown to contain ACh (De Robertis et al., 1963; Whittaker et al., 1964), 2) specific ChE has been identified histochemically in the posterior pituitary of the cat (Koelle and

Geesey, 1961), 3) stimulation of secretion by the posterior pituitary by depriving animals of water causes an increase in the number of MV in the nerve endings (Gerschenfeld et al., 1960). This last phenomenon is similar to the increase in the number of synaptic vesicles seen when cholinergie neurons are stimulated (De Robertis and Vas Ferreira, 1957). The function of the small vesicles remains unknown, and the significance of specific ChE is unclear. According to Parmar et al. (1963), the ChE in the posterior lobe of bovine pituitary is predominantly non-specific, whereas Pasetto (1954) reported that it was exclusively specific. In addition, AP was investigated to localize lysosomal particles and SDH to identify mitochondria. VP and OT activity were assayed in the fractions to examine further the sedimentation properties of SG and neurosecrosomes, both of which contain these hormones.

METHODS AND MATERIALS

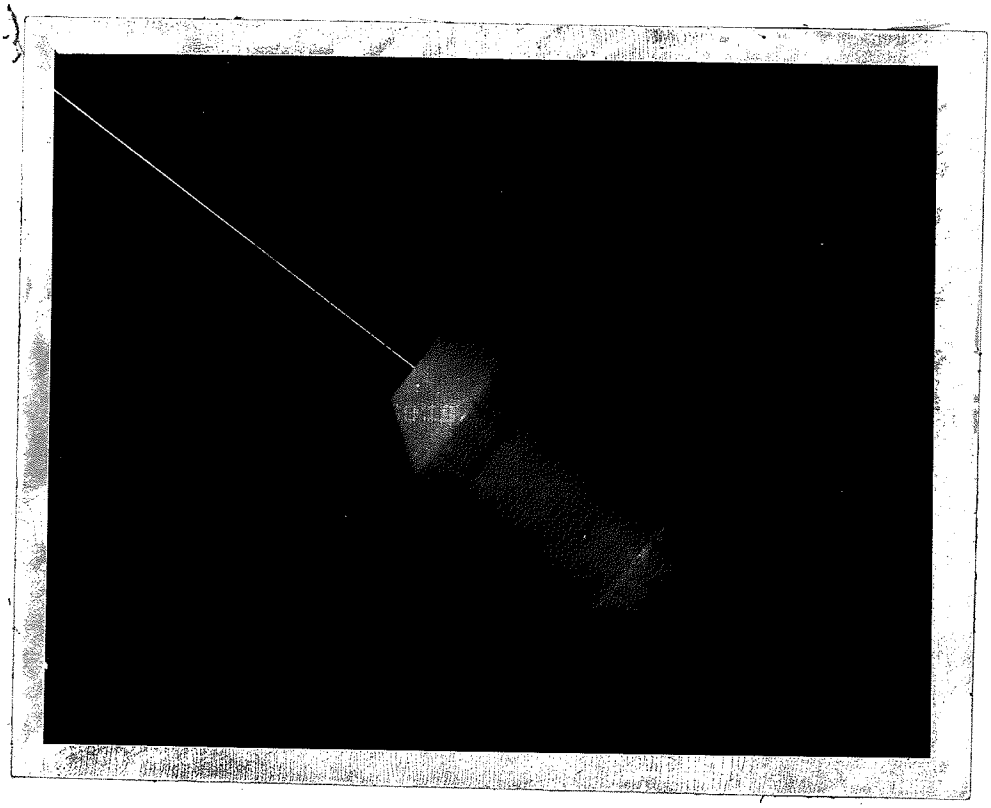
Bovine pituitary glands, obtained from the slaughterhouse 30 to 40 minutes after death, were placed in vessels surrounded by ice and transported to the laboratory. All subsequent procedures were carried out at 4°C. The posterior lobes were minced with scissors, weighed, and suspended in 10% (O. 29M) sucrose, 10 ml per gram tissue. The total time elapsing between the death of the animals and tissue homogenization was 1 to 1. 5 hours.

Homogenization. Synaptosomes from brain are believed to be separated from their axons by the shearing force created in the sucrose medium by the rotating homogenizer (Whittaker, 1965). As the nerve endings are delicate and easily destroyed by any direct grinding process, it was desirable to design a homogenizer that would produce the required shear force, yet permit the formation of as many free neurosecrosomes as possible. This was accomplished by homogenizing the posterior lobes in two stages with two teflon pestle-glass vessel homogenizers (A. H. Thomas Co.), modified in the following manner (suggested by Dr. L. H. Cohen): The middle two-thirds of the pestle was machined to a clearance of 0. 635 mm for the first and to 0. 279 mm for the second homogenizer. The remaining surface of the pestle, in closer contact with the glass walls, was grooved longitudinally to a depth greater than the clearance of the central area. This design permits the pestle to remain centered in the glass tube while tissue disruption is performed

by the central area of critical dimension (Fig. 2). During homogenization, care was taken to avoid crushing the tissue in the bottom of the tube. The first homogenization was done using the pestle with the larger clearance. After centrifuging at 700 g for 10 minutes, the supernatant was decanted and the sediments resuspended in 15 ml of 10% sucrose and homogenized with the second homogenizer. The homogenate was centrifuged at 700 g for 10 minutes and the sediment recovered as fraction 1. The volume of the combined supernatants was measured, and a 1% solution of heparin in 10% sucrose added to make a final concentration of 0.02% heparin. The addition of heparin was reported to be effective in avoiding clumping of particles during centrifugation (LaBella et al., 1963), and all sucrose media used after obtaining fraction 1 contained heparin. Heparin was added after the removal of fraction 1, as nuclear material forms a gelatinous mass in the presence of heparin.

Centrifugation. The first centrifugation of 700 g was done in a Sorvall refrigerated centrifuge. All subsequent steps were done in a Spinco model L preparative ultracentrifuge with a No. 40 rotor. Each fraction was washed by decanting the supernatant and resuspending the sediment in 5 ml of 10% sucrose containing heparin. The sediments were resuspended after washing and the final volume recorded. The combined supernatant was then centrifuged for the next step and so forth:

Figure 2. Modified Teflon pestle. The critical dimension is the clearance of the central area and the inside of the vessel wall. Grooves are cut in the remaining surface to a depth greater than that of the central area.



Fraction	g	Time (min.)	Operational Definition
1	700	10	Nuclei and debris
2	1700	10	Nerve endings
3	3000	10	Heavy mitochondria:SG
4	12000	10	Light mitochondria:SG
5	48000	10	SG;membrane fragments
6	104000	60	Small membrane fragments; MV
S			Supernatant

Gravitational forces are all calculated at R =average. The operational definitions are assigned to the fractions on the basis of electronmicroscopy by LaBella and Sanwal (1965), electron microscopic examination Section III of this thesis, and enzyme analysis of the present study. Each fraction was assayed for nitrogen, SDH, LDH, AP, ChE, VP, and OT.

Assays:

Hormone extraction: The hormones were extracted from the fractions by diluting 200 µl of the suspension with 1.8 ml of 0.9% saline containing 0.25% acetic acid. This was placed in a boiling water bath for five minutes, centrifuged and the sediment discarded. The supernatant was stored in the frozen state until assayed. No sample was thawed more than once. The extracts were diluted with 0.9% saline to a concentration that approximated that of the standard used for the assay.

VP: Assays were performed by a modification of the method of Landgrebe et al. (1946). Virgin female albino rats weighing 200 to 250 gms (Canada Breeders, St. Constant, Quebec) were anesthetized with urethane, 140 mg/100 gms, injected subcutaneously. The trachea was cannulated to provide an airway, and the blood pressure recorded from a cannulated carotid artery. The cannula was a No. 20, one inch hypodermic needle that had been filed to remove the sharp point and edges while retaining the beveled shape of the tip, and bent to a convenient angle to permit insertion into the artery. The cannula was attached to a mercury manometer by tubing filled with isotonic saline containing 0.01% heparin and the blood pressure recorded on a Palmer kymograph. A No. 27 needle was inserted into the femoral vein and connected through a four-way junction with three 1 ml syringes that were held in a rack fitted with screws to enable delivery of small quantities of fluid. On completion of surgery, 0.1 ml of 1% heparin in 0.9% saline per 100 gm was injected intravenously. This method differs from that of Landgrebe et al. (1946) in that, in addition to minor mechanical refinements, the cervical sympathetic trunks and the vagi were not cut, but a ganglionic blocking agent, chlorisondamine, (Ecolid^{R}, Ciba), 10 mg/100 gms, was given intravenously to the rat several minutes prior to the assay. The use of an adrenergic blocking agent such as dibenamine, as in the modification of Dekanski (1952), has been tried and rejected, as this type of

drug frequently causes an instability of the baseline blood pressure and following administration of test doses of VP much time is consumed in waiting for the blood pressure to return to a stable level. Chlorisondamine was found to be superior in maintaining a stable baseline and in permitting a rapid return to resting level following a pressor response. The assay was carried out against lysine VP (NIH) using a 2 + 2 method described by Holton (1948).

OT was determined by the method of Holton (1948).

Nitrogen was determined by a modified Nessler reaction according to the method of Johnson (1957). This was further modified in the following manner: The concentration of the digesting solution was doubled and only half the volume (0.25 ml) added to the digestion tubes. This decreased the final volume digested and minimized the danger of loss from liquid boiling over the tops of the tubes. To each tube including the reference standards, 0.25 ml of 40% H_2O_2 was added. This promotes the complete oxidation of the sucrose in the sample so that at the completion of digestion, a clear solution remains. The H_2O_2 must be added to the tubes prior to heating, as it will boil off too rapidly to be effective if digestion has already started. Repeated comparisons with and without H_2O_2 on nitrogen standards demonstrated that this treatment does not cause a loss of nitrogen. Protein concentration was estimated by multiplying the nitrogen values by 6.25.

LDH was assayed by the method of Kornberg (1955). The course

of the reaction was recorded on a Beckman DK Recording Spectrophotometer using a fixed wave length of 340 mμ. The activity was measured by the slope of the line plotting the optical density of reduced NAD against time.

SDH Assay medium, containing cytochrome C, $AlCl_3$, and $CaCl_2$, was prepared according to the method of Schneider and Potter (1943) and assayed colormetrically by the method of Green et al. (1955). The reduction of cytochrome C was recorded on a Beckman DK recording spectrophotometer at 550 mμ and calculated as described above for LDH.

AP was assayed according to the method of Adams and Smith (1951), in which the hydrolysis of denatured hemoglobin at pH 3.5 was observed by measuring the amount of tyrosine liberated.

Calculation of Relative Specific Activity (RSA). All data obtained was expressed in terms of percentage recovered in any fraction of the total recovered in the homogenate. RSA was calculated by dividing the percentage of substance found in a single fraction by the percentage of nitrogen found in that fraction.

ChE activity was assayed by the method of McOskar and Daniel (1959), in which the hydrolysis of thiocholine derivatives was measured colorimetrically. The method was modified slightly in that measurements were made on a recording spectrophotometer graphing optical density (OD) against time at a fixed wave length. The following

solutions were added to 4 ml tubes:

0.5 ml	0.125 M tris buffer pH 7.4
0.2 ml	3.5 M NaCl
0.2 ml	0.015 M acetylthiocholine (ASCh) or butyrlthiocholine (BuSCh).

H_2 0 to make a final volume of 1.3 ml.

This was equilibrated for 10 min. in a 37° water bath, 0.1 ml of a suspension of a fraction or tissue added, and the mixture incubated for 30 minutes or one hour. This long incubation period was essential for posterior pituitary tissue, whereas similar concentrations of homogenized rat brain suspension would hydrolize comparable amounts of substrate in 10 to 20 minutes.

Incubation was stopped by adding 0.1 ml of 50% trichloroacetic acid. Blanks were prepared by incubating the medium and substrate without the tissue which was added after the addition of trichloroacetic acid and by incubating tissue without substrate. The preparations remained in the water bath an additional 10 minutes and were centrifuged.

The following solutions were added to a 3 ml quartz cuvette in sequence:

1.0 ml saturated solution of NaCl

0.4 ml of solution containing Na_2C0_3 21.2 gms. and NaCN

0.4 ml Na Nitroprusside 2.7 gms/100 ml

The cuvette was placed in a Beckman model DK recording spectrophotometer and read at a wave length of 520 mμ using a saturated

solution of NaCl in the reference cell. The OD of the reagent solution changes spontaneously, and it is necessary to select an arbitrary point which is used as a zero reading. All subsequent determinations in any single experiment were started after waiting until the OD reached the chosen point. At the moment the OD reached the starting point, 0.2 ml of supernatant from the incubated material was added to the cuvette, stirred and the recording started. At the onset of the color reaction there was a sudden increase in OD that decayed steadily thereafter. A point on the recorded curve that corresponds to 30 seconds after the addition of the incubation medium was taken as the reading. Blanks were determined on all samples in the same manner and the reading subtracted. Although a double beam instrument was used, the blank could not be placed in the reference cell as the OD of the blank as well as the sample varied with time. The concentration of free sulfhydryl groups liberated by the hydrolysis of ASCh or BuSCh was determined by comparison with 0.1 and 0.05 μM cysteine. Samples were prepared for assay in the following manner: Individual whole glands and samples of rat cortex were homogenized in a ground glass homogenizer in 1 to 2 ml distilled water. Fractions, isolated as described above, were suspended in 10% sucrose and centrifuged at 100,000 g for 30 minutes, the supernatant pipetted off and the pellet homogenized in 1 to 2 ml of distilled water using a ground glass homogenizer. When inhibitors were used, they were added in a total

volume of 10 µl to the incubation medium containing the tissue or fraction sample and incubated for 15 minutes prior to the addition of substrate. Inhibitors used were, diisopropylfluorophosphate (DFP, Merck Sharp and Dohme) 3.4×10^{-8} M and BW 284 C 51 (1-5-bis (4-allyl dimethylammonium phenyl) pentan-3-one dibromide, Burroughs Wellcome) 2×10^{-6} M.

RESULTS

The distributions of Nitrogen, LDH, SDH, AP, OT and VP in terms of percentage recovered and RSA, are presented in Figure 3.

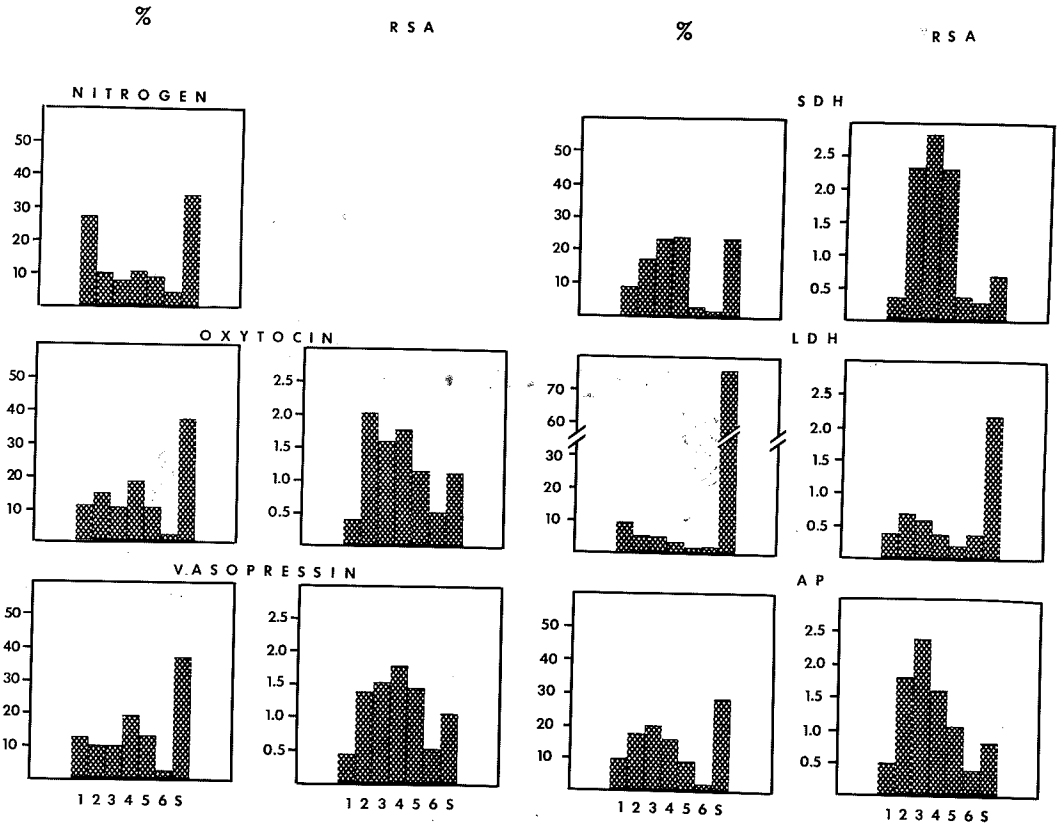
The distribution of LDH is unlike any of the other activities measured. Most of the LDH (75%) appears in the supernatant. Of the remaining 25%, nearly 10% is in fraction 1, which contains the nuclei and debris. Of the particulate fractions, the highest RSA is in fractions 2 and 3. These two fractions together make up the nerve ending fraction of LaBella and Sanwal (1965). The distribution of LDH in the remaining fractions shows a progressive decrease in activity in the lighter fractions. There is a discontinuity in this progression as fractions 4 and 6 have nearly equal activity; fraction 5 has less activity than any of the other particulate fractions.

The mitochondrial marker SDH is most active in fraction 3 and is of nearly equal activities in fraction 2 and 4. About 23% of the SDH was found in the supernatant.

The distribution of the lysosomal marker AP is similar but not parallel to that of SDH. The differences are most apparent in fractions 4 and 5. The distribution of OT and VP activities suggests that the two hormones are contained in particles that differ in their sedimentation properties. In three separate fractionations, VP exceeded OT in fraction 5, and OT exceeded VP in fraction 2.

The ChE activity of whole posterior pituitary and whole rat brain and the effect of specific inhibitors is presented in Table 1. The

Figure 3. The percentages recovered and relative specific activities of various substances in the six particulate fractions and the supernatant.

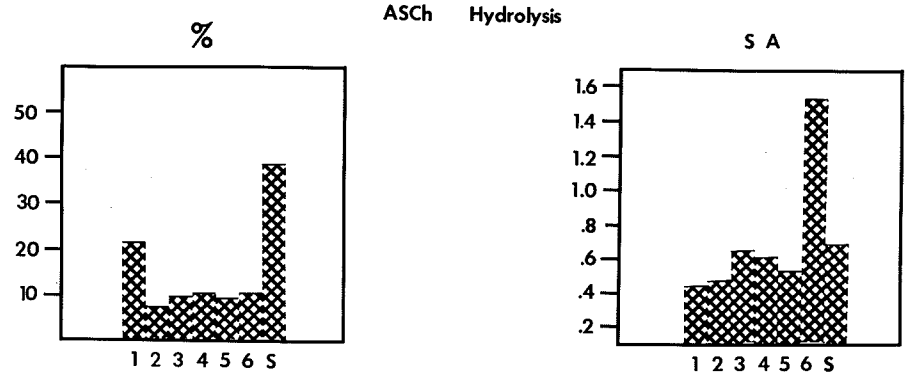

%
RSA
%
RSA
NITROGEN
SDH
OXYTOCIN
LDH
VASOPRESSIN
AP
1 2 3 4 5 6 S
1 2 3 4 5 6 S
1 2 3 4 5 6 S
1 2 3 4 5 6 S

hydrolysis of ASCh by the posterior pituitary is 0.4 and BuSCh is 3.0 times that of rat brain (Table 1). Preliminary experiments with purified enzymes indicate that, under the conditions of the assay used here, non-specific ChE will hydrolyze ASCh at 1/2 to 2/3 the rate at which it will hydrolyze BuSCh, whereas specific ChE will not hydrolyze BuSCh. These findings are in agreement with those of Koelle (1950). The concentrations of inhibitors used in these experiments were predetermined by their effects on purified enzymes. BW 284C51 at 2×10^{-6} M inhibited specific ChE 100% but had no effect on non-specific ChE, whereas DFP at 3.4×10^{-8} M inhibited non-specific ChE 80 to 90% and specific ChE by only 5 to 10%. Assuming that 1/2 to 2/3 of the hydrolysis of ASCh is attributable to the action of non-specific ChE, it can be estimated that in the posterior lobe, approximately 70% of the ASCh hydrolyzed is a result of enzyme or enzymes other than non-specific ChE. This is supported by the observation that DFP inhibits the hydrolysis of ASCh by approximately 30%. The remaining 70% of the total activity of the posterior lobe can be accounted for by that activity which was inhibited by BW 284C51.

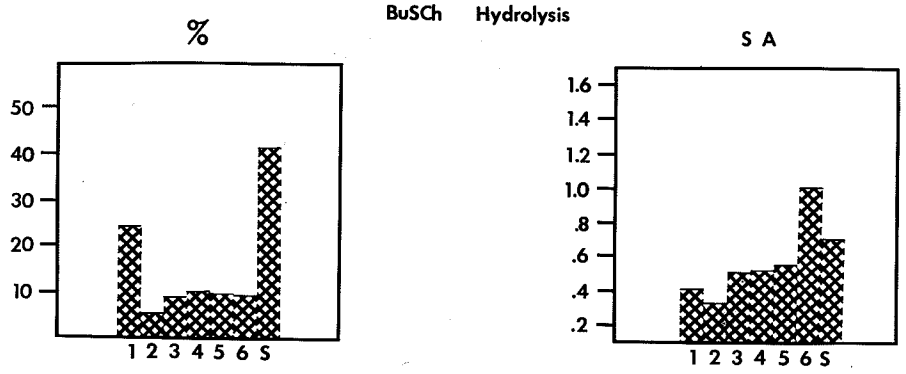
The distribution of activities in the fractions expressed in terms of percentage recovered and specific activities is presented in Figure 4 which are the averages of four experiments. Table 2 presents the specific activities of the subcellular fractions together with the inhibition data of one of three experiments. There was considerable

Figure 4. The distribution of ChE in the six particulate fractions and the supernatant expressed in terms of percentage activity in each fraction and specific activity (SA) for each of the two substrates. SA units are um/hr/mg protein (ordinate). Numbers on abscissa refer to the fractions and s = supernatant.

ASCh Hydrolysis



BuSCh Hydrolysis



variability in the specific activities as can be seen by the large standard deviations and little significance can be placed on them. However, it is apparent that the activity of enzymes hydrolyzing both substrates is concentrated in fraction 6, the microsomal fraction. The effect of the two inhibitors indicates that the enzyme activity which hydrolyzes ASCh is inhibited by BW 284C51 in all fractions except 5. The inhibition of ASCh hydrolysis by DFP is relatively low in fractions 2 and 6.

TABLE 1

RATES OF HYDROLYSIS OF ASCh AND BuSCh BY BOVINE POSTERIOR PITUITARY AND RAT BRAIN

	ASCh	BuSCh[a]	ASCh/BuSCh
	μm/100 mg/hr		
Brain	2.67 ± 0.40	0.17 ± 0.02	15.7
Post. Pit.	1.04 ± 0.05	0.56 ± 0.05	11.9

a Means and SD of eight experiments for Post. Pit. and four for rat brain

TABLE 2

EFFECTS OF ChE INHIBITORS

	BW284C51		DFP	
	ASCh	BuSCh	ASCh	BuSCh
	% Inhibition			
Brain[b]	82.4	0	7.8	100
	88.9	0	4.6	100
Post. Pit.[c]	77.4 ± 7.9	0	28.1 ± 15.0	86.6 ± 10.7

b Results of individual experiments

c Means and SD of four determinations

TABLE 3

RATES OF HYDROLYSIS OF ASCh AND BuSCh BY FRACTIONS OF POSTERIOR PITUITARY

Fraction	% Total Activity[a] ASCh	% Total Activity[a] BuSCh	µM/mg protein/hr[a] ASCh	µM/mg protein/hr[a] BuSCh	% Inhibition by BW284C51[b] ASCh	% Inhibition by DFP[b] ASCh
1	20.7	22.8	.42 ± .05	.41 ± .09	61.5	58.3
2	6.5	3.9	.49 ± .15	.37 ± .15	50.0	27.4
3	8.5	7.9	.67 ± .25	.54 ± .26	65.1	58.3
4	9.1	8.6	.61 ± .40	.55 ± .27	59.9	46.7
5	7.6	8.2	.56 ± .57	.59 ± .27	0	86.0
6	9.7	8.1	1.56 ± .71	1.09 ± .39	73.4	28.9
S	38.0	40.6	.76 ± .40	.74 ± .59	83.8	86.5

a Means of four determinations

b Results of a single experiment

DISCUSSION

Isolation of Neurosecrosomes

Electronmicroscopic studies of the posterior pituitary gland show that the great bulk of this gland is made up of nerve terminals; capillaries, pituicytes, and other supporting elements constitute a relatively minor proportion. A persistent problem in interpreting centrifugation data is the difficulty in distinguishing between certain activities which reside in organelles that have been released from neurons by homogenization, and organelles that remain within the neurosecrosomes. Although any subcellular component can be highly enriched in a specific fraction by centrifugation procedures, cross-contamination among organelle types always occurs to some degree. It can usually be determined, on the basis of distribution patterns of specific activities among various fractions, whether multiple activities are present in the same or different particles.

LDH is a soluble enzyme present in cytoplasm. Upon homogenization of cells, this enzyme would be released into the suspending medium wherever the cytoplasm is not enclosed by a plasma membrane. Neurosecrosomes are formed when the nerve terminals are sheared from their axons during homogenization. The point of rupture of the neurosecrosome from its axon filament is apparently sealed off, so that the particle is enclosed by a permeability barrier which is the plasma membrane. Sedimentable LDH, therefore, has been proposed as a

'marker' for membrane enclosed fragments such as the neurosecrosome and has been used for this purpose in studies of isolated brain nerve endings (synaptosomes) isolated from guinea pig cortex (Johnson and Whittaker, 1963).

From the data it can be observed that LDH exhibits a distribution pattern distinct from any other activity measured. Although LDH has been shown to be present in synaptosomes obtained from brain homogenates (Johnson and Whittaker, 1963), it has not been clearly established that the enzyme is associated with these particles exclusively, as its presence in several crude fractions was noted. In the present investigation, the diffuse distribution of LDH suggests that this enzyme is associated with more than one species of particle, possibly pinched-off bits of axons or fragments of neuronal membrane on which the enzyme is adsorbed. It is presumed that the activity in fraction 2 and to a somewhat lesser extent in 3 and 4, is a reflection of the presence of membrane enclosed particles. Fraction 2 is known to contain neurosecrosomes with relatively little contamination with other particles. This conclusion was based on electronmicroscopic studies (LaBella and Sanwal, 1965) and Section III of this thesis. Furthermore, it was found that the glucose metabolism of fraction 2 resembles that of posterior pituitary slices, whereas that of fractions 3 and 4 resemble isolated mitochondria (Krass and LaBella, 1965), indicating that the neurosecrosomes of fraction 2 maintain their metabolic integrity.

Hormonal Activity of Neurosecrosomes and SG. The distribution of OT and VP among the various centrifugation fractions are not parallel. The specific activity of VP is highest in fraction 4, whereas OT activity is most concentrated in fraction 2. A similar difference is the sedimentation of OT and VP on differential centrifugation of bovine posterior pituitary homogenates has been reported (LaBella et al., 1963). However, in this earlier work a ground-glass homogenizer was used so that it is doubtful that neurosecrosomes were preserved, and a single mitochondrial fraction was isolated at 6,780 g for 15 minutes. The 6,780 g fraction of LaBella et al. (1963) had more oxytocic than vasopressor activity. They also found that, on equilibrium centrifugation, OT containing particles were less dense than those containing VP. Barer et al (1963) also found differences between oxytocic and vasopressor activity on equilibrium centrifugation of homogenates of rabbit posterior pituitary glands, but in these species OT was associated with denser particles than those containing VP. In the present investigation, the RSA for OT exceeds that of VP in fraction 2 which contains primarily neurosecrosomes. This fraction, as seen by electronmicroscopy (LaBella and Sanwal, 1965) and Section III of this thesis is relatively devoid of free SG. These data suggest that OT is localized in a neurosecretory granule distinct from that which contains VP, and, furthermore, that each peptide hormone is localized in a specific type of nerve ending. If individual

nerve endings contain only one of the two hormones, it is reasonable to assume that any single neuron synthesizes, stores, and releases only one hormone. There is a body of evidence concerning the ability of the hypothalamo-hypophysial system to differentially control the release of VP and OT (reviewed by Heller, 1961; Kleeman and Cutler, 1963; Barer et al., 1963). If any individual neuron contained either hormone exclusively, differential control would be exerted by selective neural pathways stimulating or inhibiting specific neurons in the hypothalamus.

Lysosomes in Posterior Pituitary. The lysosomal enzyme AP is distributed similarly but not parallel to the mitochondrial enzyme SDH. In this respect, the posterior pituitary resembles brain tissue (Koenig et al., 1964; Whittaker, 1959). Electronmicroscopic studies of brain tissue indicate that, when stained for acid phosphatase activity, lysosomal enzymes are in dense bodies found in glial cells and in neuronal cell bodies but not in nerve terminals (Torack and Barrnett, 1962). In the present work it cannot be definitely determined whether AP in fraction 2 is intra or extra neurosecrosomal, but we have done some preliminary studies on the ultrastructural localization of another lysosomal enzyme, acid phosphatase and found free dense bodies among the neurosecrosome particles in the 1700 g fraction but not within neurosecrosomes. It appears that lysosomal particles are not a component of the neurosecrosomes but are probably derived from pituicytes and other non-neural cells.

ChE in the Posterior Pituitary. Some specific ChE activity has been identified histochemically in the neurohypophysis of the rabbit (Dumont, 1956), the cat (Koelle and Geesey, 1961), the newt, frog, carp, and rat (Uemura, 1964; 1965). Only in the newt was there a strong positive reaction, although traces were found in the other species. The enzyme was assayed manometrically in the posterior lobe of the dog (Burgen and Chipman, 1951), where a small highly variable activity of specific ChE was found in only three of five animals studied. ChE of bovine posterior lobes was investigated manometrically by Pasetto (1952), and Parmar et al. (1963). The two reports present conflicting data. Pasetto found that the posterior lobe hydrolyzed beta-methacholine and not benzoylcholine and concluded that the tissue contained specific ChE only. Parmar et al. compared the hydrolysis of butyrylcholine to acetylcholine and found a 1:1 ratio for posterior pituitary; brain tissue by comparison had an activity ratio of 10:1. The use of eserine as an inhibitor of specific ChE failed to provide a distinction between the two enzymes. Parmar et al. concluded that the posterior pituitary was "deficient in specific ChE". Holmes (1962) attempted to identify specific cholinesterase in the neurohypophyses of various mammals and could find it only in the hedgehog. In the ferret it was found to be "very inconsistent and equivocal".

The results of the present investigation on whole posterior pituitaries indicate that specific ChE is present, and the activity per unit

weight of tissue is approximately 1/3 that of rat brain. The enzymes responsible for the hydrolysis of ASCh react to inhibitors in a manner indicating that specific ChE is present in the gland. However, there is as yet insufficient information to estimate this enzyme quantitatively. In comparison to the rat brain, the rate of hydrolysis of BuSCh by posterior pituitary is considerably higher. Non-specific ChE has been investigated histochemically in the brain and spinal cord, and the low activity found was associated with vascular endothelium (Koelle, 1952; Brightman and Albers, 1959). The posterior pituitary is highly vascular which can account for a considerable amount of this enzyme. However, the enzyme may also be present in pituicytes.

The distribution of hydrolysis of ASCh and BuSCh indicates that most of the activity recovered is in the supernatant. It has been shown in the rabbit brain, that specific ChE was primarily in particulate fractions and could not be washed out (Nathan and Aprison, 1955). The specific activities of the fractions were highly variable. However, all four experiments showed considerably higher activity in the microsomal fraction.

The inhibition data in the experiment shown in Table 2 indicate that ASCh was inhibited to a greater extent by BW 284C51 than by DFP in fractions 2 (neurosecrosomes) and 6 (microsomes) indicating that these fractions have a higher activity of specific ChE. However, the percentages of inhibition in the fractions, like the specific activities, were highly variable and the evidence concerning specific ChE in

fraction 2 must be considered equivocal. In the brain, specific ChE was found to be localized in the microsomal fraction (Toschi, 1959; Aldridge and Johnson, 1959), which was found to be made up of membrane fragments (Hanzon and Toschi, 1959). Evidence has been obtained that ChE is present in neurons at two sites, intracellular and in association with the external membrane (Koelle and Steiner, 1956). It was also shown that specific ChE is found in the mitochondrial fraction as well as the microsomes (Nathan and Aprison, 1955). When brain was homogenized with the intent to preserve the nerve terminals, specific ChE was found in both the microsomal fraction and the mitochondrial fraction (De Robertis et al., 1962). The mitochondrial ChE was attributable to the presence of the nerve terminals. The posterior pituitary appears to resemble brain in that AChE is concentrated in the microsomal fraction. There is a suggestion that it is also present in the neurosecrosomes.

Cholinergic Mechanisms and Neurohypophysial Hormone Release. The mechanism whereby VP and OT are released from posterior lobe nerve endings into the circulation is unknown. It is probably initiated by an action potential originating in the perikarya within hypothalamic nuclei and propagated along the axons to depolarize the nerve terminal (Haller et al., 1965). Release of hormone may then result directly from depolarization or indirectly by the release of a humoral mediator. The presence in neurosecretory nerve terminals of small vesicles that are

similar to synaptic vesicles suggests that ACh may be a mediator for the release of posterior pituitary hormones. A substance fulfilling several of the criteria for ACh has been identified in the bovine posterior lobe (Uemura et al., 1963), but this activity may be a result of interfering substances (Kobayashi, 1964). An attempt to confirm this observation is currently in progress in this laboratory. Cholinacetylase was assayed in the posterior lobes of three dogs (Feldberg and Vogt, 1948). The results were variable and significant activity was present in only one animal.

Douglas and Poisner (1964) have presented evidence that a cholinergic mechanism is not involved in the release of hormones from posterior lobe. ACh applied to posterior lobes in vitro did not initiate the release of hormones, whereas depolarization of the tissue by the application of high extracellular potassium was effective. Cholinomimetic agents were equally ineffective in stimulating the release of hormone (Mikiten, 1966).

It is likely that the release of hormones is initiated by depolarization of the secretory neuron membrane originating in the cell bodies, and there is no evidence of synaptic transmission within the posterior lobe. The possibility also exists however that the nerve endings are sensitive to direct chemical stimulation and hormone release may be initiated by this alternate mechanism. ACh (Uemura et al., 1963), choline acetylase (Feldberg and Vogt, 1948), and ChE (Burgen and Chipman, 1951) may all

be present in the posterior lobe in smaller quantities than those found in tissue where a cholinergic mechanism is present. Small amounts of these substances have been shown to be present in all conducting tissue, even where ACh mediated transmission does not take place (Nachmansohn, 1963). Although these three substances must apparently be present at synapses where ACh acts as a mediator, caution is necessary in interpreting their presence as evidence that a cholinergic mechanism for the release of hormones exists.

It is also possible that there are neurons in the posterior lobe that do not serve a neuroendocrine function (Daniel and Lederis, 1966). Some nerve endings are seen to be devoid of SG and contain MV only (see Section III). These MV may be truly synaptic vesicles in neurons that originate somewhere other than the hypothalamo-hypophysial system. If cholinergic neurons are present among the neuroendocrine neurons of the posterior pituitary, there is no information at present to provide a basis for speculation concerning their function.

III

ISOLATED NERVE ENDINGS (NEUROSECROSOMES) FROM THE POSTERIOR PITUITARY: PARTIAL SEPARATION OF VASOPRESSIN AND OXYTOCIN AND THE ISOLATION OF MICROVESICLES

INTRODUCTION

The neurons of the mammalian hypothalamo-hypophysial system originate in the anterior hypothalamus in the supraoptic and paraventricular nuclei and terminate as prominent swellings in the pars nervosa of the pituitary. These neurons synthesize, store, and release the peptide hormones VP and OT (Bargmann and Scharrer, 1951). There is now considerable evidence to indicate that the hypothalamo-hypophysial system has the ability to differentially control the release of the two hormones (reviewed by Heller, 1961; Kleeman and Cutler, 1963; Barer et al., 1963). The hormones are stored in neurosecretory granules in the nerve terminals in the pars nervos; these granules have been shown to differ in their sedimentation properties so that VP-containing granules can be separated, to some extent, from those containing OT (Pardoe and Weatherall, 1955; Heller and Lederis, 1961; LaBella et al., 1962; LaBella et al., 1963; Barer et al., 1963). This difference in sedimentation of activities has led to speculation that there is a specific SG for each of the two hormones. The possibility that hormones are stored in separate SG and that each can be released relatively independently of the other in response to specific stimuli has led several investigators to propose that, in any one neuron, only one hormone is present (Heller, 1961; Lederis, 1961 b; Barer et al., 1963; Lederis, 1964 a; Lederis, 1964 b; Rothballer, in press, cited by Brooks et al., 1966).

It has also been shown that in posterior pituitary homogenates some of the particles isolated by centrifugation are nerve endings (neurosecrosomes) which have apparently been pinched-off from their axon filaments, retain their complement of organelles, and resemble nerve endings seen in electronmicroscopic examination of thin sections of whole tissue (LaBella and Sanwal, 1965). If there are, indeed, separate neurons for each hormone, isolated neurosecrosomes like the SG would be expected to vary in sedimentation and density properties and thus be separable into vasopressinergic or oxytocinergic according to the species of SG they contain.

Another inclusion in the posterior lobe nerve endings are small electron transparent vesicles (microvesicles, MV) 20 to 60 mμ in diameter. They have been called 'synaptic vesicles', (Palay, 1957; Gerschenfeld et al., 1960), in that they resemble acetylcholine-containing vesicles seen in nerve terminals of motorneurons. Knowledge of the MV is at present limited to electronmicroscopic observations which demonstrated changes in their concentration as influenced by changes in the functional activity of the gland, (Gerschenfeld et al., 1960). The term 'synaptic vesicles' is unjustified as it implies a physiological role for which there is no experimental support; the term microvesicles is non-commital and, therefore, more appropriate for the neurohypophysial organelles. There are two general theories concerning MV; the first postulates that they contain a humoral mediator, either ACh or some other putative transmitter, that plays a role in the release of

the hormones from the nerve terminals or the transfer of hormones from the neuron into the capillary lumen (De Robertis, 1964). The second theory postulates that they are derived from SG, and represent either hormone secretory packets analagous to the ACh-containing synaptic vesicles (LaBella and Sanwal, 1965) or breakdown products of the depleted neurosecretory granule membranes (Holmes and Knowles, 1960; Bern, 1963; Knowles, 1963; Lederis, 1964 a, 1964 b, 1965). There is, at present, no direct experimental support for either point of view. The most direct approach for obtaining further information concerning the MV is that they be isolated and examined. The "synaptic vesicles" from brain have been isolated by De Robertis et al. (1963) and Whittaker et al. (1964), who disrupted isolated nerve endings (synaptosomes) by osmolysis and obtained a fraction rich in synaptic vesicles by density gradient centrifugation. A similar approach would seem feasible for the isolation of MV from neurosecrosomes.

The neurosecrosomes fraction has been examined electronmicroscopically (LaBella and Sanwal, 1965) and biochemically (Section II of this thesis). In the present work, the neurosecrosomes were subfractionated by density gradient centrifugation in an attempt to separate vasopressinergic from oxytocinergic activity. In an effort to isolate MV, the neurosecrosomes were subjected to osmolysis and subsequent centrifugation, and a fraction rich in MV was obtained. However, there are several dissimilarities between brain and posterior lobe that make

the isolation of MV from the latter tissue difficult. In addition, electronmicroscopic observation has led us to hypothesize that the MV are derived from the membrane ghosts of depleted SG.

METHOD

Isolation of Nerve Endings. The neurosecrosomes were isolated as the 1,700 g fraction (fraction 2) as described in Section II.

Density Gradient Centrifugation of the Neurosecrosome Fraction. A density gradient was prepared by layering 7 ml each of 60%, 50% and 40% sucrose in 30 ml cellulose nitrate centrifuge tubes. The gradient was placed in the cold for two hours prior to use and 5 ml of the neurosecrosome fraction suspended in 10% sucrose layered on top. Three such tubes were centrifuged at 60, 000 g for 2 hours in a Spinco Model L Ultracentrifuge using an SW-25 rotor. Samples from each of the three bands formed (Fig. 5)) were pipetted off and diluted to make a final concentration of 20% sucrose. The suspensions were centrifuged at 100, 000 g for 30 min. and the pellets resuspended in 10% sucrose and recentrifuged. Each of the three fractions was assayed for VP, OT, nitrogen, and LDH, and samples were prepared for electronmicroscopy. To account for the possibility that differences in the hormone content of the three fractions obtained is an artifact resulting from differential release by different sucrose concentrations, aliquots of the original neurosecrosome fraction were incubated for 2 hours in 40, 50, and 60% sucrose at 4^{o} and the hormone content of the recentrifuged particulate material was assayed.

Isolation of Microvesicles. The neurosecrosome fraction was suspended in 10 ml water containing 0. 02% heparin, and slowly stirred for

one hour. A continuous sucrose gradient was prepared by layering 5 ml each of 40%, 30%, 20% and 10% sucrose in 30 ml cellulose nitrate tubes and stored at 4^{o} for 18 hours prior to use. Three ml of the suspension of the neurosecrosome fraction was layered on top of this gradient and centrifuged for 2 hours using a Spinco SW25 rotor at 60, 000 g. The topmost layer (Fig. 6) was pipetted off and placed in 5/16" X 1 15/16" cellulose nitrate tubes (Beckman) and centrifuged at 100, 000 g for 30 minutes in a Spinco No. 40 head fitted with tube adapters.

Chemical Determination and Assays. Nitrogen, LDH, VP, and OT were determined as described in Section II.

Electronmicroscopy of the Fractions. The fractions were prepared by either positive or negative staining technique as described by LaBella and Sanwal (1965).

Figure 5. Subfractionation of neurosecrosomes by discontinuous sucrose density gradient centrifugation. Markings at left indicate the positions of the interfaces at onset of centrifugation. A B and C are the positions of the particle bands at equilibrium.

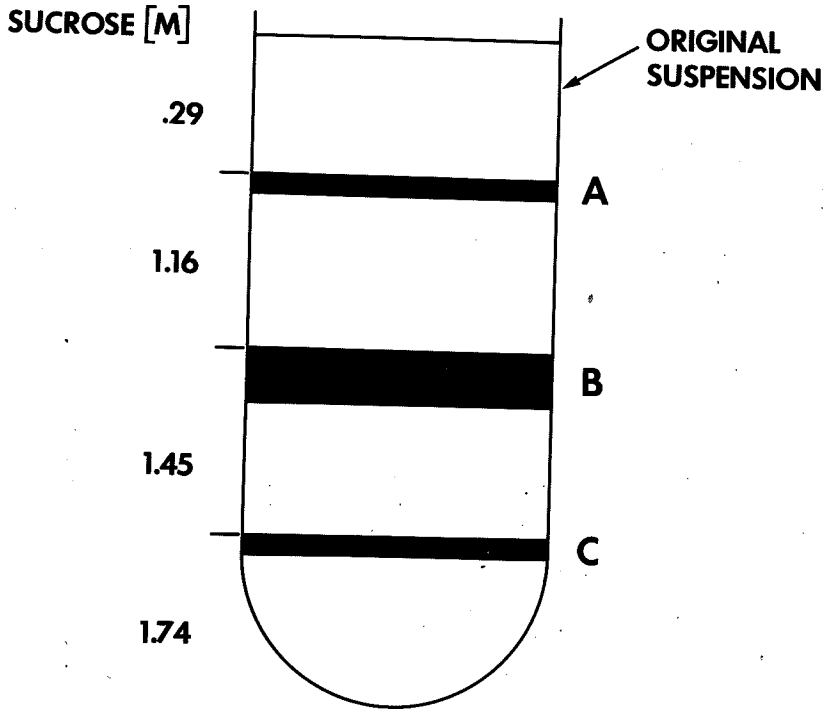
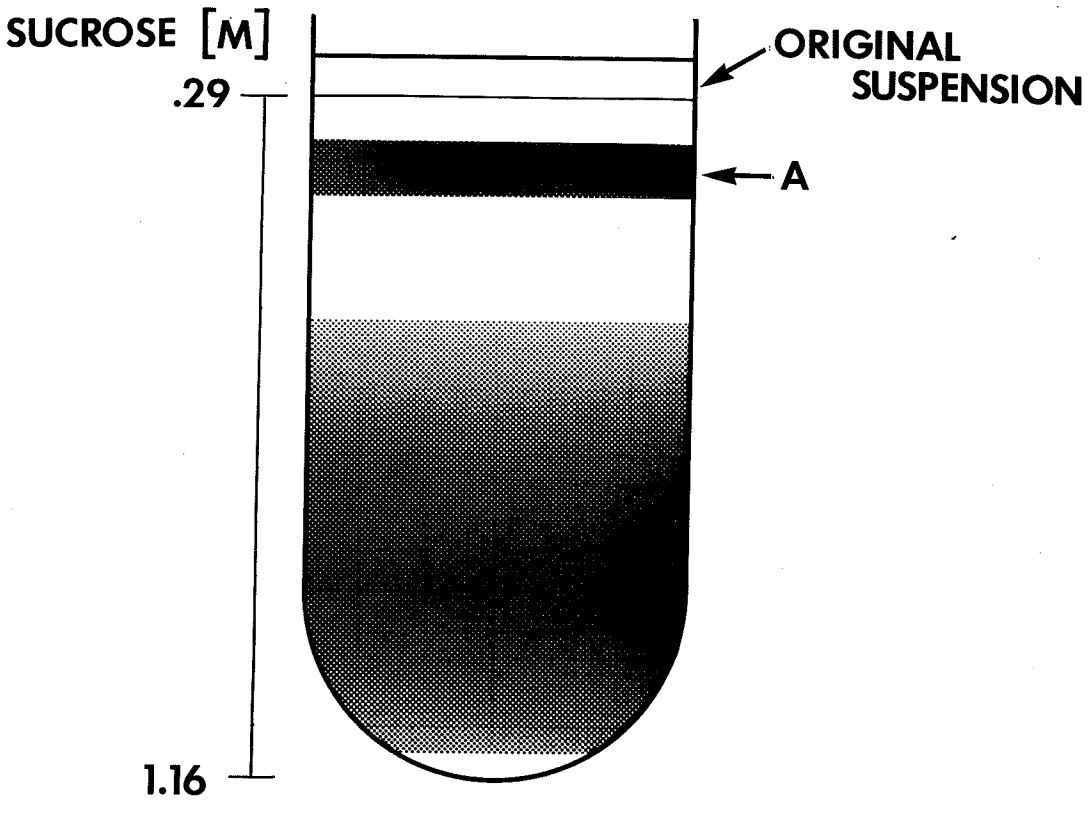

SUCROSE [M]
ORIGINAL SUSPENSION
.29
A
1.16
B
1.45
C
1.74

Figure 6. Fractionation of a suspension of lysed neurosecrosomes by centrifugation on a continuous sucrose density gradient. At equilibrium, a band of small particles rich in MV is formed near the top of the gradient. The remainder of the particulate material forms a single diffuse band whose opacity corresponds to that of the diagram.


SUCROSE [M]
.29
1.16
ORIGINAL
SUSPENSION
A

RESULTS

Electronmicroscopy of Neurosecrosomes

The neurosecrosome fraction is made up nearly exclusively of isolated neurosecrosomes with occasional bits of debris; no nuclei and few free SG are seen. In fixed sections of this fraction (Fig. 7), the SG within the neurosecrosomes usually have electron-transparent centers. The dense staining SG seen by others in sections of intact tissue, are not seen in this preparation. The negative stain technique in electronmicroscopy provides some concept of the three dimensional configuration of particles. The space occupied by particulate material embedded in the phosphotungstic acid film appears electron-transparent, whereas smaller or thinner particles are more electron dense. Although all SG within neurosecrosomes are electron-transparent in positive stain, two types of granules are apparent in negatively stained preparations; one type is the electron-transparent circular profiles, which are more transparent in their centers than at their periphery and are probably spherical granules that are filled with material. The other type of granule within the neurosecrosome have electron dense centers with an electron-transparent halo. These appear to be mono- or biconcave discs and are probably spherical bodies, presumably depleted of their content, that have collapsed. Both types of granules are seen in single neurosecrosomes (Figs. 9, 11).

The smaller vesicular inclusion collectively referred to as MV, do not appear as uniformly circular profiles. In both negatively stained (Fig. 12) and positively stained preparations (Fig. 13) some of the MV are elongated, appearing tubular or rod shaped. Occasionally, large clusters of MV are seen (Fig. 15). SG are always present in neurosecrosomes. However, in sections of whole tissue, nerve endings are seen that contain MV only (Fig. 14). These nerve endings are smaller than those containing SG.

In several positively stained sections of the neurosecrosome fraction, the membrane of the electron-transparent SG are not intact. These are always found in regions where MV are present. The finding that these granules with disrupted membranes are associated with MV suggests that they are not artifacts, although this possibility remains. In several sections broken SG membranes can be seen curling at their edges to form circular structures that are similar in size to MV (Fig. 7, 8).

Density Gradient Centrifugation of Neurosecrosomes

The vasopressor and oxytocic activities of the three fractions obtained from density gradient centrifugation of neurosecrosomes are presented in Table 4, together with the VP/OT ratio. In all six experiments, hormone activity in fraction B is intermediate between A and C. In all six experiments there is a lower activity of VP in fraction A than in the other two fractions, whereas the activity of OT shows considerable variation. The LDH activity in these fractions indicates

that the concentration is fairly constant in the three fractions.

The VP/OT ratios of neurosecrosomes incubated in four concentrations of sucrose are shown in Table 5. There are no differences among the ratios, indicating that increasing sucrose concentrations do not selectively deplete the particles of their hormone content.

Electronmicroscopy of various levels of fixed pellets, as well as negative staining of the three fractions, indicate that free SG are rare (Figs. 16, 17, 18). The neurosecrosomes appear to have deteriorated by their long immersion in sucrose (4-5 hrs. in concentrations ranging from 0.29 M to 1.74 M). In addition, it has been observed that OsO_4 tends to destroy isolated nerve endings (Whittaker, 1965). Sections of all three samples seen at low magnification show two types of neurosecrosomes which are distinguishable by the electron density of the cytoplasm. The electron-dense neurosecrosomes are smaller than the electron-transparent ones. These smaller particles appear to be fragments of neurosecrosomes whose plasma membranes are not clearly outlined. It is possible that sucrose can penetrate the particles whose membranes have been broken and thereby alter the cytoplasm to increase their affinity for osmic acid. The sizes of vesicular components presumed to be SG have been measured. Table 6 presents the mean diameters of 300 - 600 vesicular particles from each of the three fractions. There is no significant difference in the size of the SG in the three fractions, nor can any consistent difference

in the staining properties of the SG be noted.

Isolation of MV

The MV fraction prepared by positive stain is shown in Figs. 19, 20 and by negative stain of suspensions in Figs. 21, 22. The isolation of MV is made difficult by the presence of larger vesicles that sediment together with the MV. The larger vesicles are probably the membrane 'ghosts' of SG that have been depleted of their content by osmolysis. The distribution of the diameters of a sample of particles in the MV fraction seen by negative staining is presented in Fig. 23. There is a population of particles 40 to 80 mμ in diameter with a mean diameter of 54 mμ, which constitutes 41.2% of all the particles in this fraction. The remaining particles have diameters ranging from 80 to 300 mμ. This fraction has been assayed for VP and it was found in three experiments to have not more than 2 U/mg protein.

TABLE 4

SUBFRACTIONATION OF NEUROSECROSOMES BY DENSITY GRADIENT CENTRIFUGATION

Expt	Fraction	Hormone Activity U/mg Protein		V/O	LDH U/mg Protein
		VP	OT		
I	A	6.7	22.3	.30	6.50
	B	12.6	21.3	.59	9.86
	C	21.2	30.1	.70	8.25
II	A	4.7	9.4	.49	6.47
	B	8.3	2.3	3.57	7.42
	C	6.6	2.0	3.20	3.36
III	A	1.9	1.5	1.26	
	B	24.1	7.7	3.12	
	C	20.7	1.5	13.61	
IV	A	3.5	3.8	.91	6.10
	B	19.5	15.0	1.30	5.70
	C	13.7	5.4	2.53	6.66
V	A	.6	.7	.78	
	B	13.9	14.0	.94	
	C	3.9	2.6	1.49	
VI	A	3.3	4.3	.77	
	B	6.7	12.3	.54	
	C	6.3	5.3	1.19	

TABLE 5

VP/OT RATIOS OF NEUROSECROSOMES INCUBATED FOR TWO HOURS IN VARYING CONCENTRATIONS OF SUCROSE

% Sucrose	Experiment 1	2	3	4
	VP/OT			
10	.76	.62	.79	.66
40	.62	1.10	.72	.64
50	.67	1.10	.93	.62
60	.59	.91	.83	.57

TABLE 6

DIAMETERS OF SECRETORY GRANULES

IN SUBFRACTIONS OF NEUROSECROSOMES

Fraction	Diameter[a] (mμ)
A	180.5 ± 68.6
B	165.3 ± 55.2
C	205.3 ± 55.2

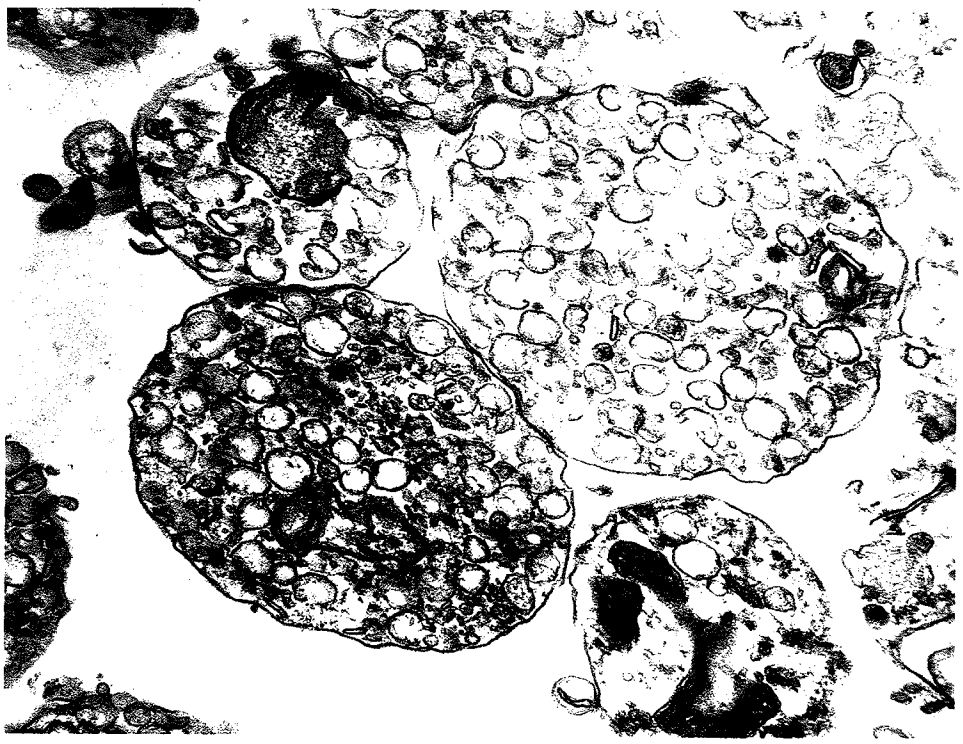
a. Mean and standard deviation of 300 to 600 granules

Figures 7 and 8. Neurosecrosome fraction. The SG have electron lucent centers. Some MV are present in each neurosecrosome. The arrows point to the ends of the broken SG membranes that appear to be curling to form small vesicles.

mag.

Fig. 7 = 23,000 x

Fig. 8 = 26,000 x



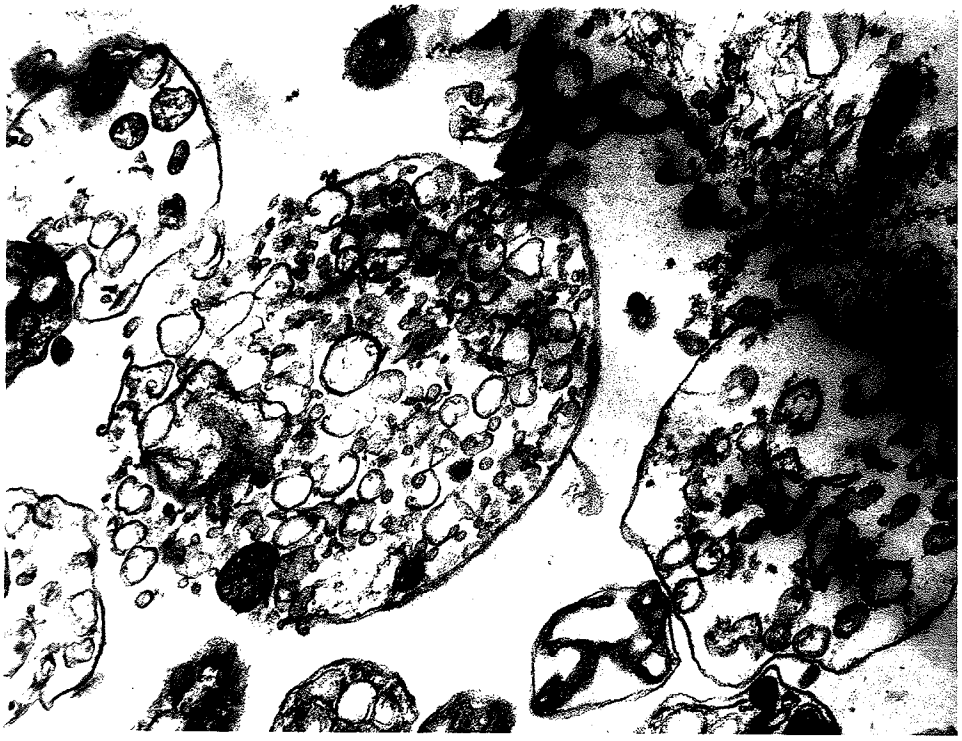


Figure 9. Negative stain of a neurosecrosome. Large white circular profiles are SG. Arrows point to SG that appear to be depleted of their content. Square at right is artifact covering damage on the film. Scale = 1μ, mag. = 27,500 x

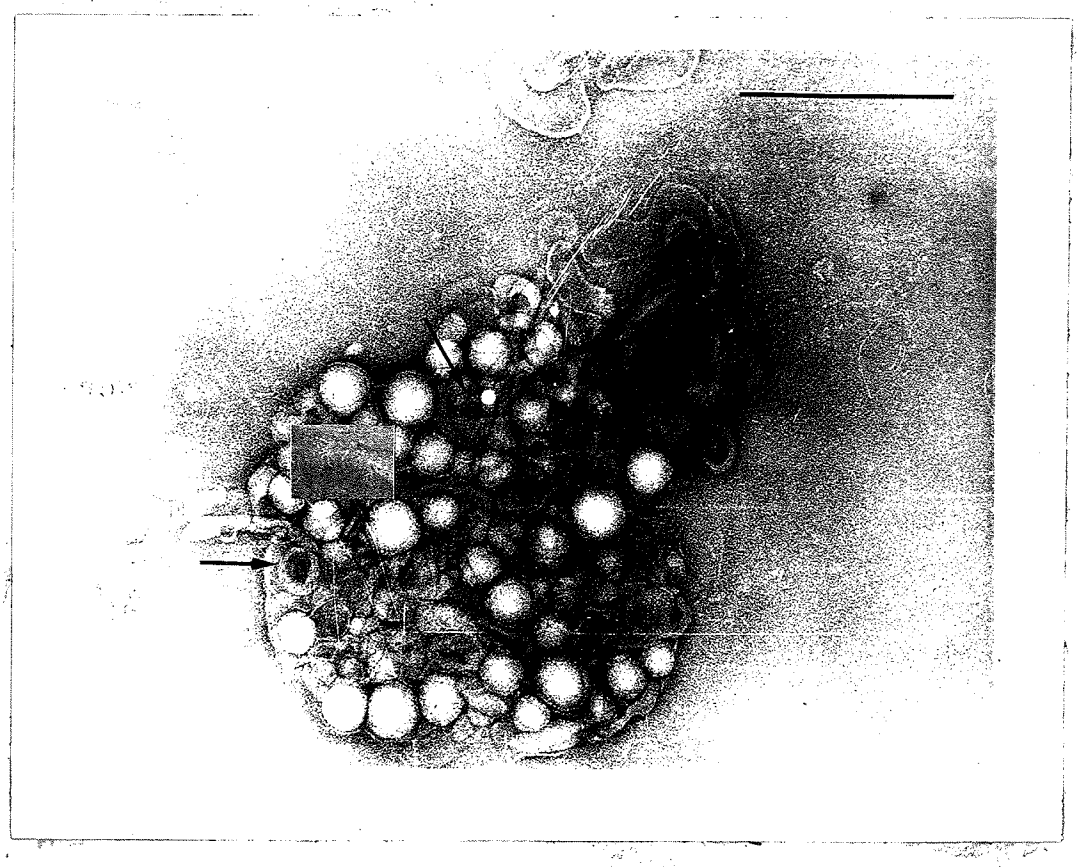
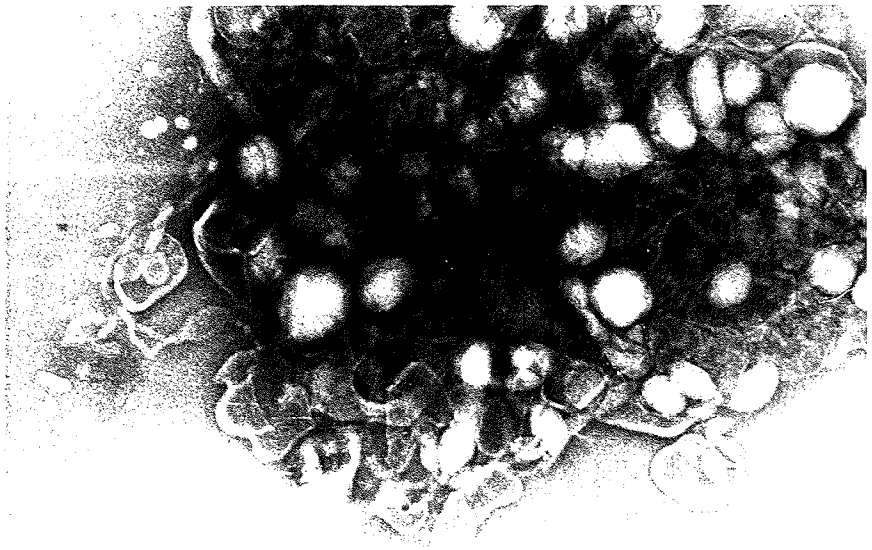


Figure 10. Negative stain of neurosecrosome.

Scale = 1μ, mag. = 42,500

Figure 11. Negative stain of neurosecrosome. Arrows indicate SG that have been depleted of their contents.

Scale = 1μ, mag. = 42,500 x



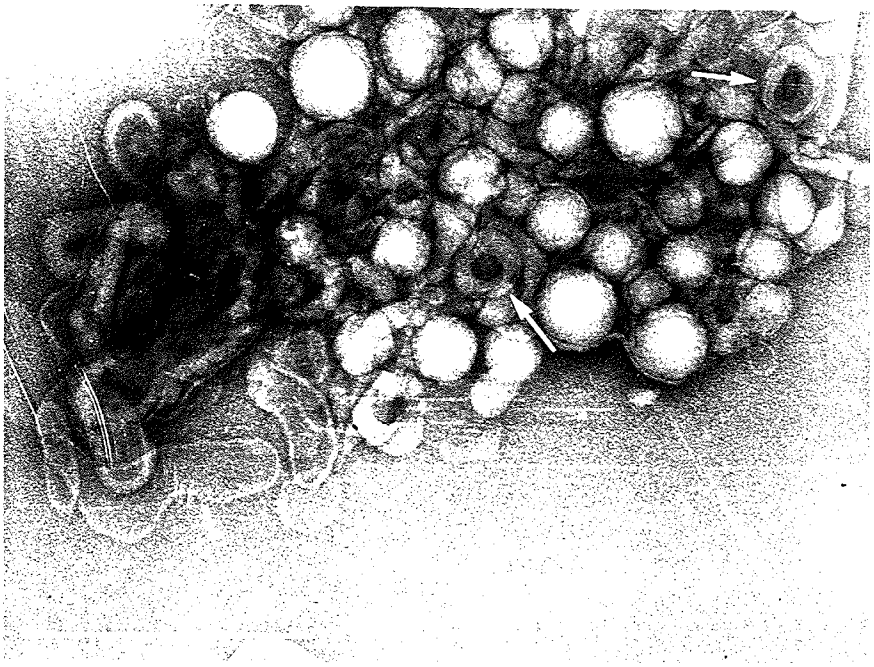
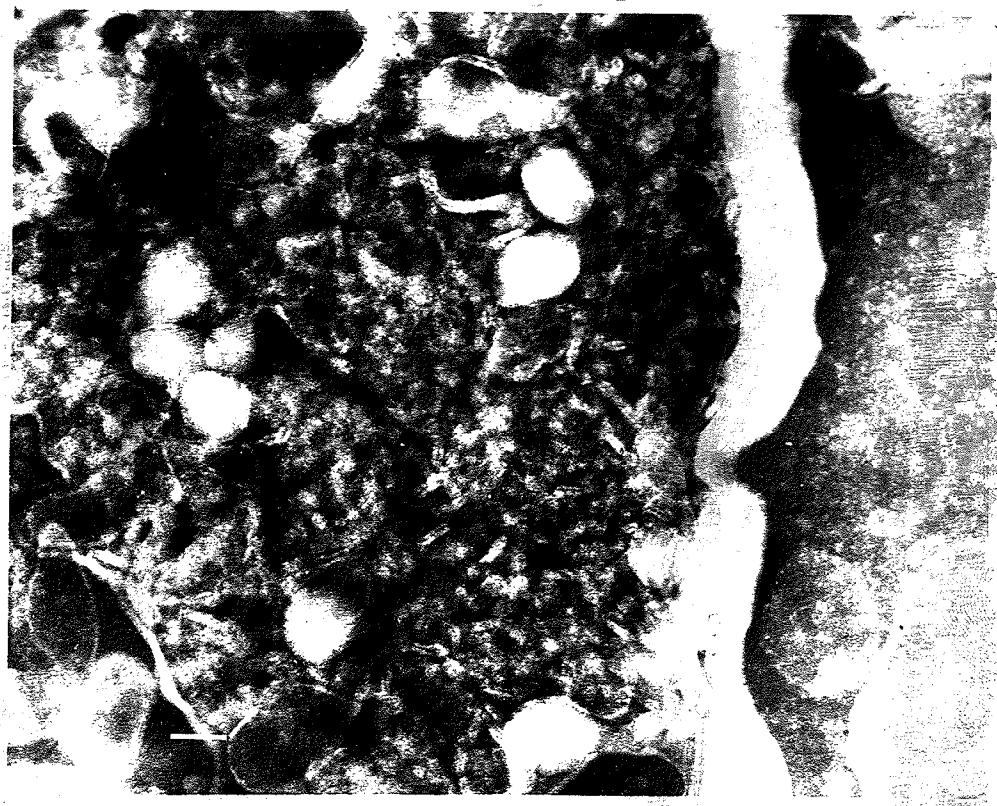


Figure 12. Negative stain of a neurosecrosome containing small SG, 100 mμ in diameter and numerous tubules and vesicles 25 to 50 mμ scale = 0.10 μ, mag. = 80,000 x

Figure 13. Positive stain of a fragment of neurosecrosome containing SG and small tubules and vesicles.
Scale 0.10 μ, mag. = 50,000 x



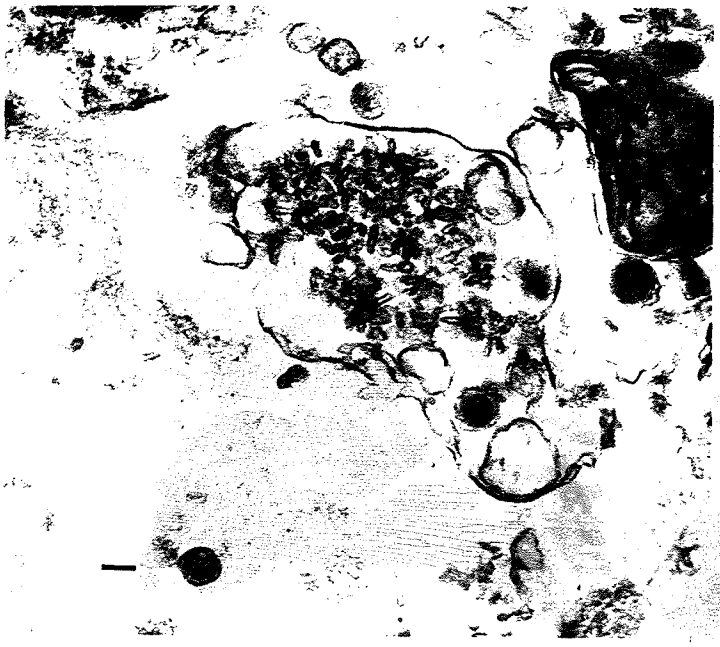
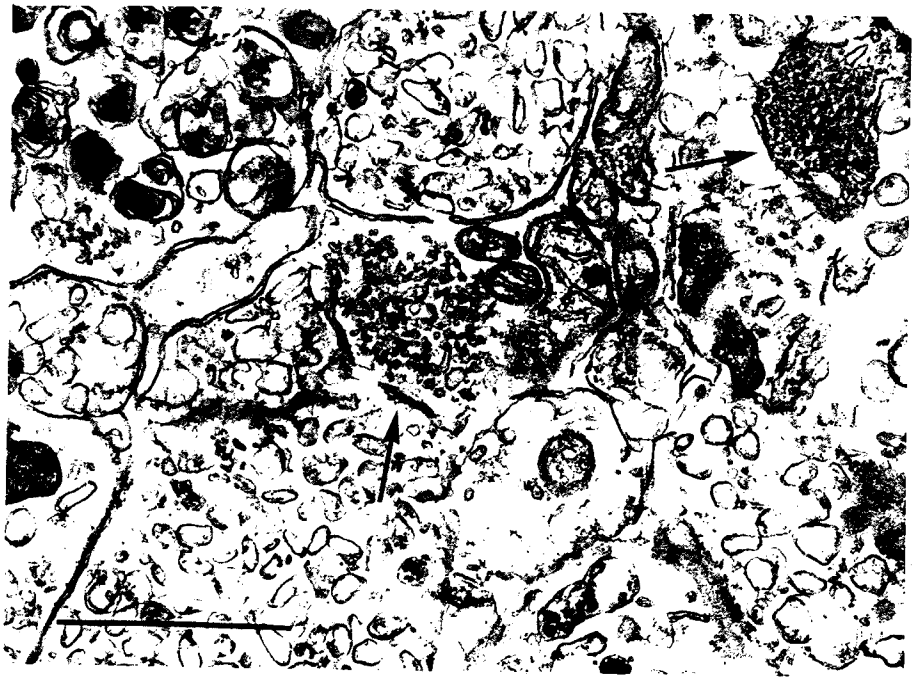


Figure 14. Section of whole posterior pituitary showing nerve endings containing SG. Two of the nerve endings (arrows) are smaller than the others and are filled with MV, no SG are present.

Scale = 1 μ, mag. = 33,000 x

Figure 15. A single neurosecrosome containing a large oval mass of MV.

Scale = 1 μ, mag. = 32,000 x



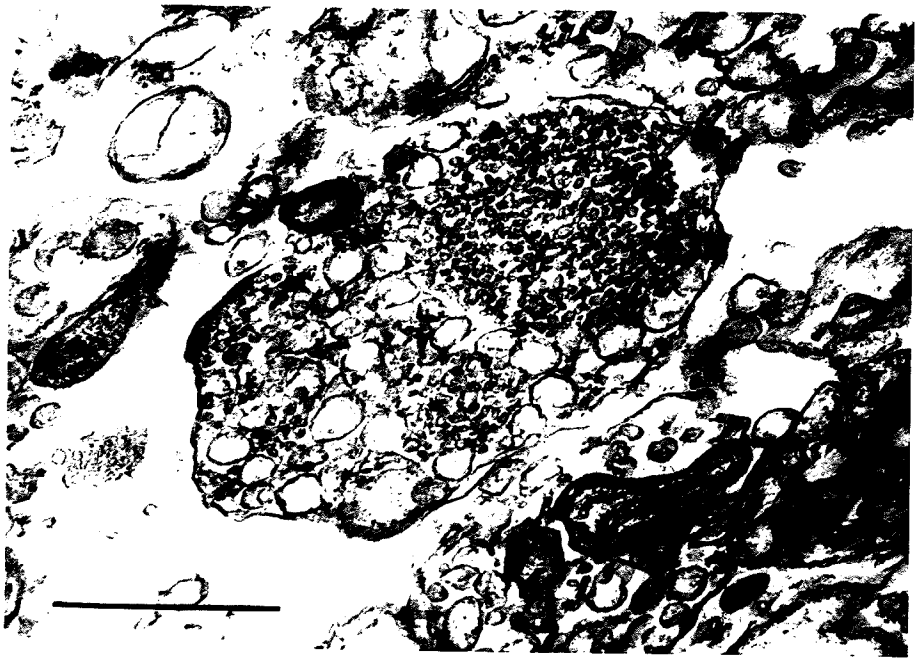


Figure 16. Subfraction A of neurosecrosomes. The cytoplasm of some neurosecrosomes are darker staining than others. Mag. = 10,000 x

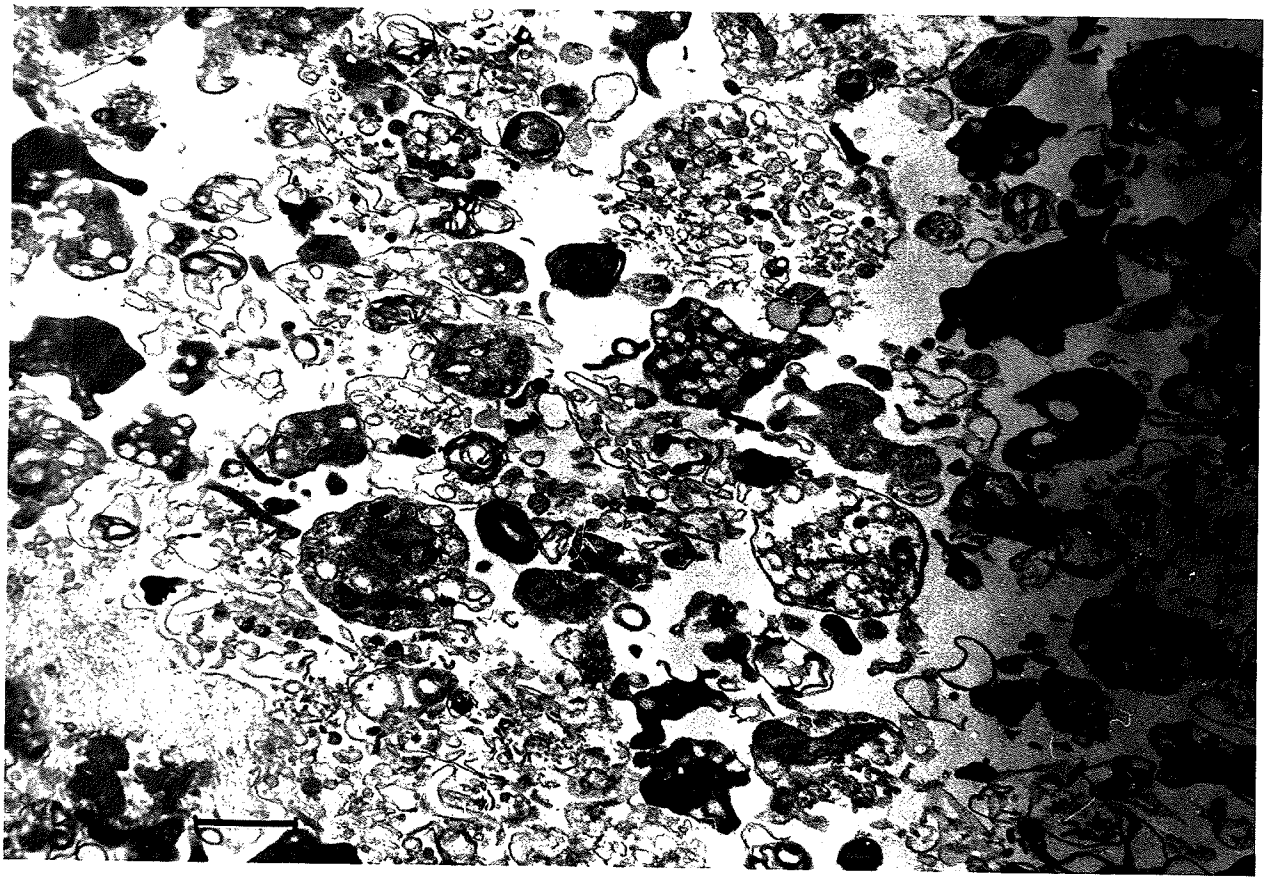


Figure 17. Subfraction B of neurosecrosomes.

mag. = 11, 000 x

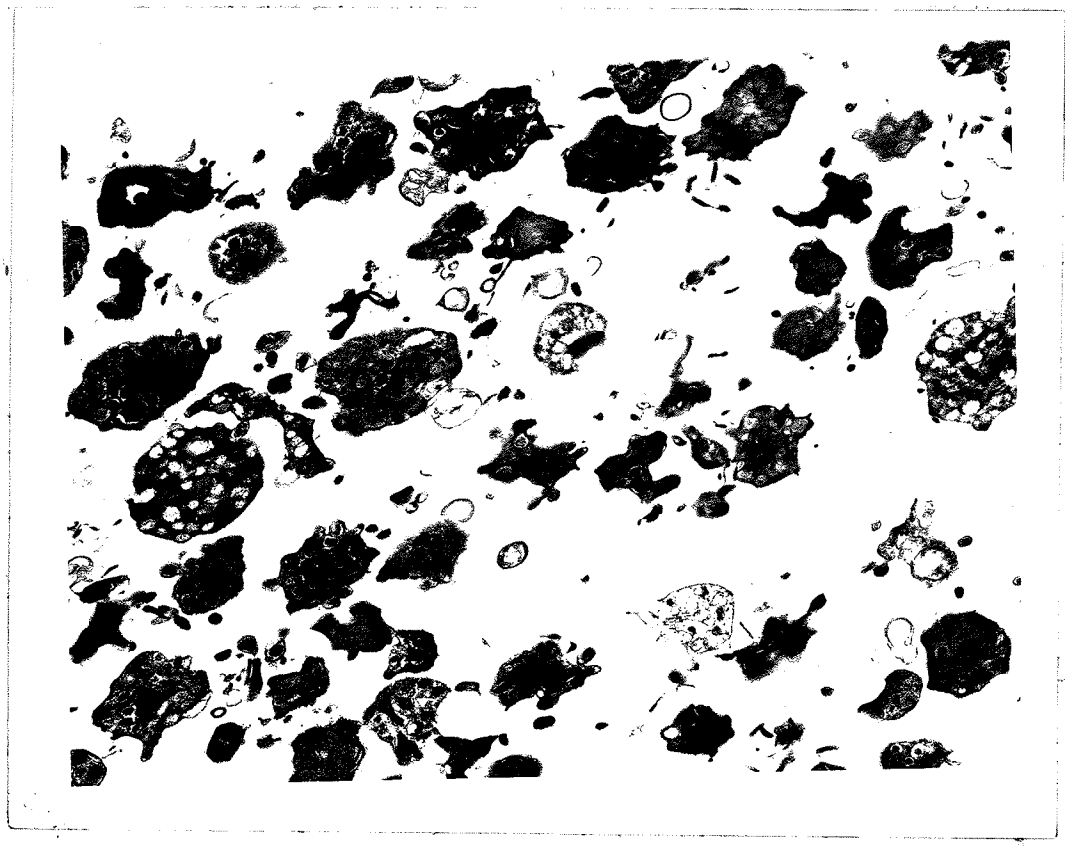


Figure 18. Subfraction C of neurosecrosomes.

mag. = 10,000 x

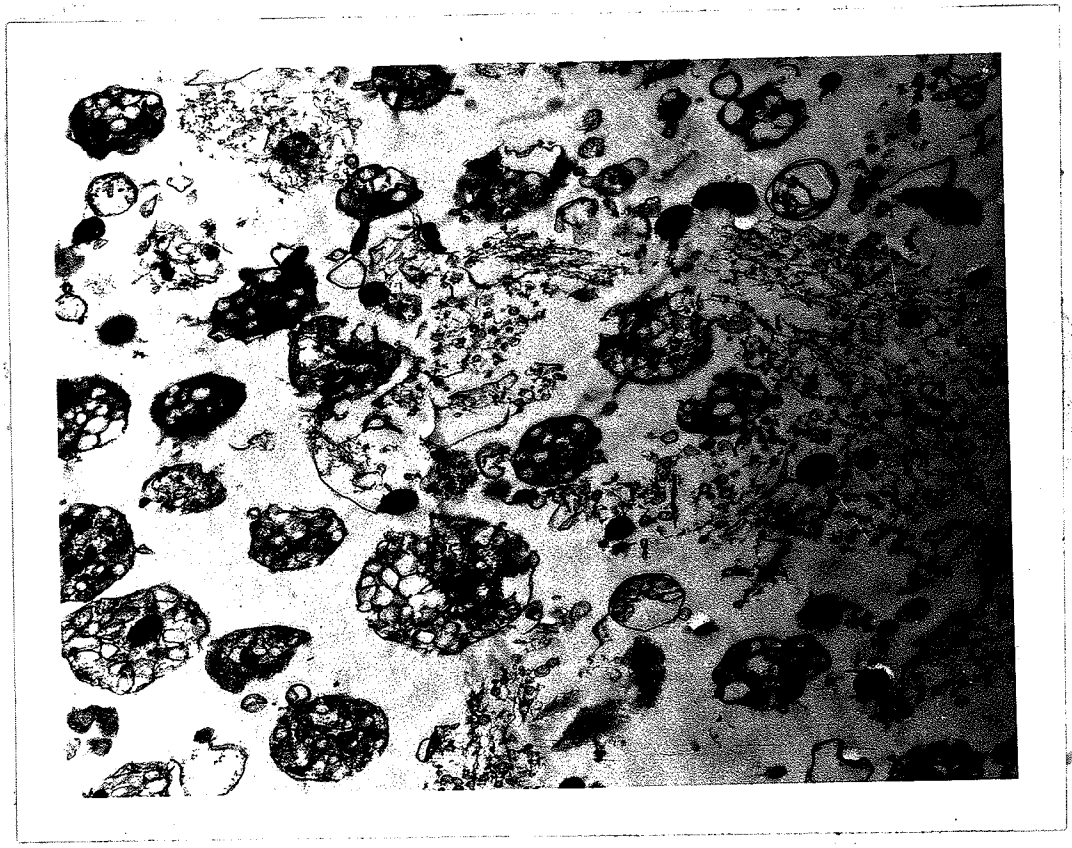
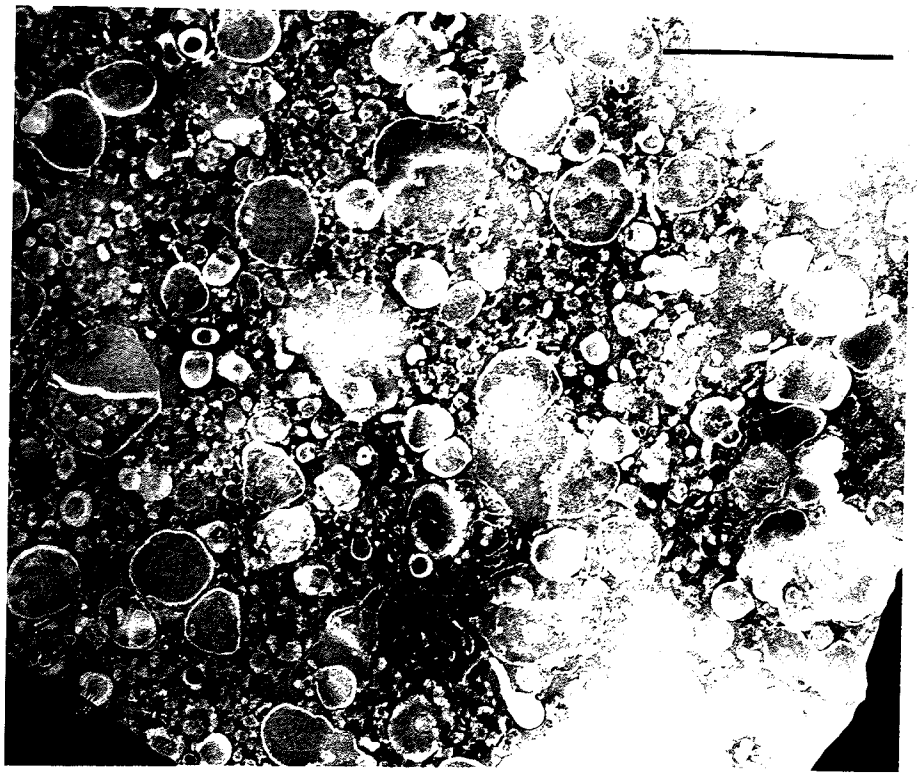


Figure 19. Positive stained section of MV fraction. A wide variety of particle sizes are present. These are predominantly MV with numerous ghosts of SG.

mag. = 21, 500 x



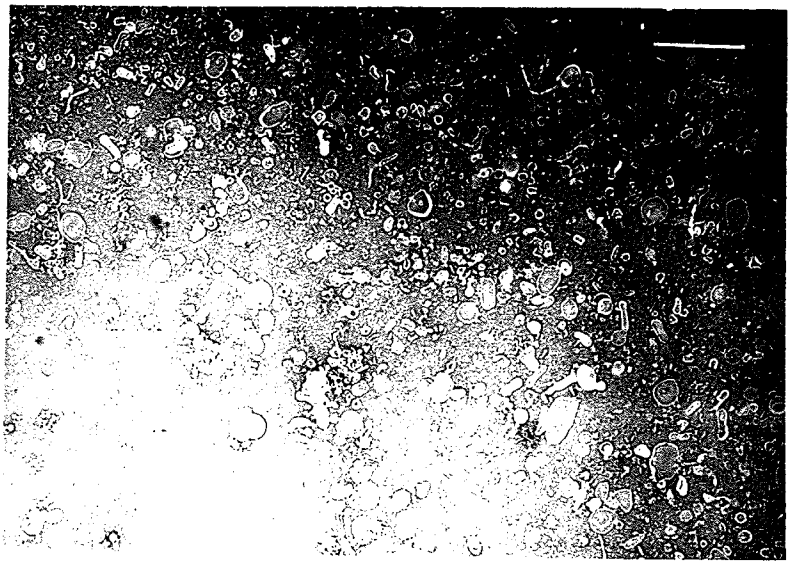
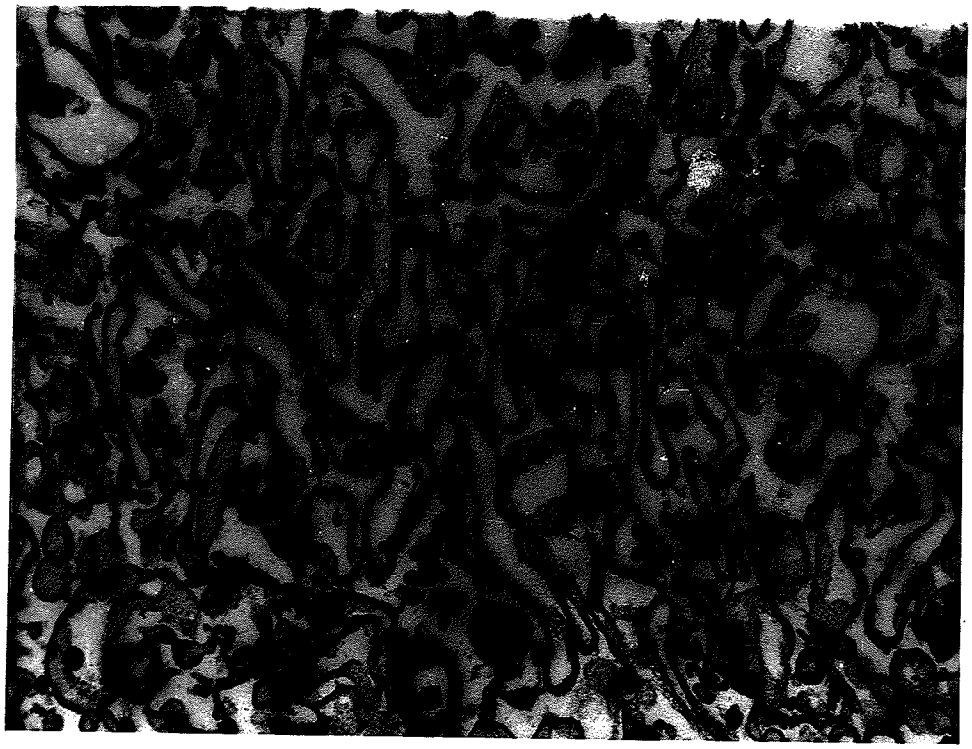


Figure 20. Positive stained section of MV fraction. This is a higher magnification of section shown in fig. 19 demonstrating the large number of MV. The MV are not of uniform size. mag. ≒ 63,000 x



Figures 21 and 22. Negative stain of MV fraction. Note the wide range of particle sizes.

Fig. 21. mag. = 14,000 x

Fig. 22. mag. = 33,000 x

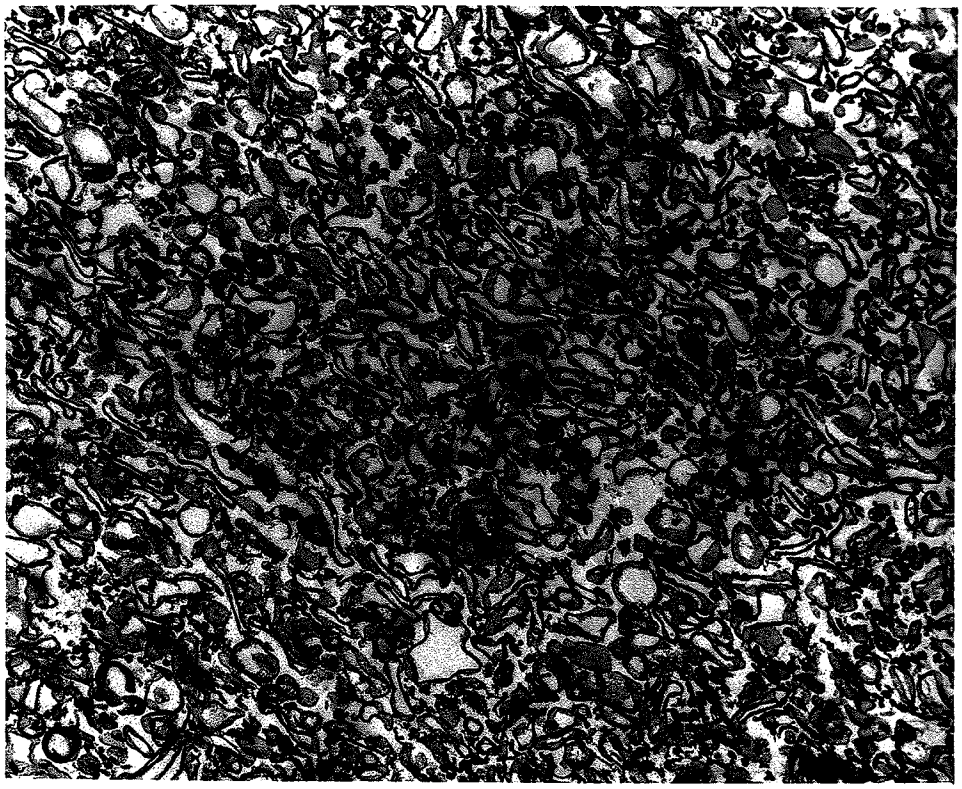
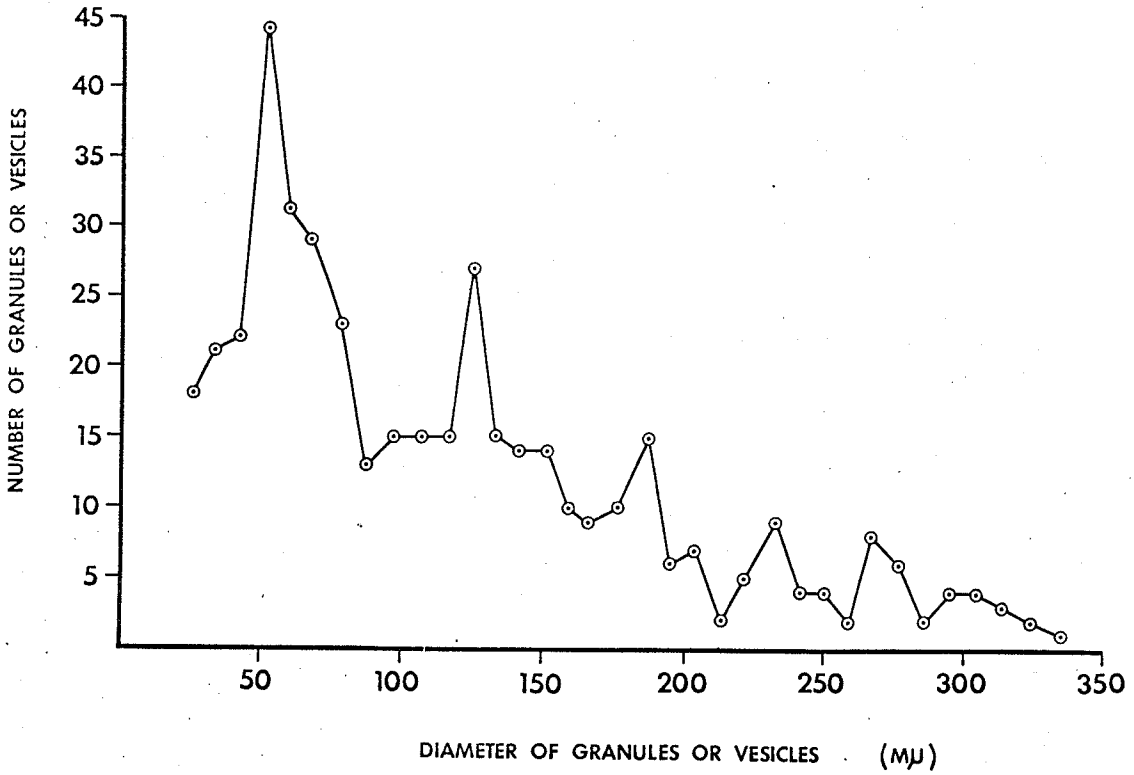


Figure 23. The number of particles seen in relation to their diameter.

450 particles were measured in a single MV fraction prepared for electronmicroscopy by the negative staining technique. 41.2% of all particles are less than 80 mμ; the peak frequency is at 54 mμ.


NUMBER OF GRANULES OR VESICLES
45
40
35
30
25
20
15
10
5
50
100
150
200
250
300
350
DIAMETER OF GRANULES OR VESICLES (mμ)

DISCUSSION

Subfractionation of Neurosecrosomes

The isolation of neurosecrosomes (LaBella and Sanwal, 1965) has provided a preparation which can be utilized to yield useful information concerning the neurosecretory process. One aspect of the subcellular morphology of the posterior pituitary which has recently been examined is the relationship between the ability of SG to take up stain for electronmicroscopy and their neurosecretory content. It is generally accepted that the SG are the storage sites for the peptide hormones VP and OT which are in association with a carrier protein (Schiebler, 1952). Electronmicroscopic studies of thin sections of whole pituitary glands from a variety of animals show that the SG vary in their staining properties, appearing as electron dense bodies, electron-transparent vesicles, or as vesicles with intermediate densities between the two extremes. Conditions such as dehydration which stimulate the gland to secrete hormone have been shown to cause a decrease in the number of dense staining granules and increase the number of electron-transparent ones (Palay, 1957; Gerschenfeld et al., 1960). It has been generally assumed that the dense staining material within the SG was the neurosecretory material. However, determination of the hormone content of the gland in conjunction with electronmicroscopic observations has recently shown that the relationship between

the two is not clear (Daniel and Lederis, 1966).

In the present findings, neurosecrosomes, prepared for electron-microscopy by positive staining, contain SG, most of which are electron-transparent. This may be an artifact produced by homogenization or centrifugation, possibly by the sucrose medium. It cannot be stated that they are not depleted of their hormone content as it is impossible to compare this fraction to a suitable control. However, if hormone is stored only in dense staining SG, it would be difficult to explain the activity of both hormones found in this fraction in the absence of any dense staining granules. In contrast, the negative stain technique reveals that there are two types of SG; the distinction between the two is based on the presence or absence of solid material dried in a phosphotungstic acid film. A similar difference between the apparent content of synaptic vesicles isolated from brain tissue has been noted (Whittaker, 1966). If the material seen to fill some of the SG is indeed neurosecretory material, these observations are in agreement with the speculation of Daniel and Lederis (1966) that some process other than the loss of neurosecretory material is responsible for the inability of the SG to take up electron dense, positive staining material. This process is probably a change in the chemical properties of the neurosecretory material that is associated with the release of hormone but which precedes the depletion of hormone from the nerve ending.

The VP/OT ratio in bovine posterior pituitary has been reported

to be 2.89 (Hild and Zettler, 1952) and 1.4 (Heller, 1961), and according to the definition of an international unit, the ratio should be 1:1, yet in the neurosecrosome fraction the VP/OT ratio was between 0.6 and 0.8. It is possible that the method of isolation of the neurosecrosome fraction does not sediment a random sample of neurosecrosomes, a greater number of particles containing OT. The higher OT content of this fraction as compared to other fractions of homogenized posterior pituitary glands has been demonstrated (Section II).

The differences in the hormone ratios of the three subfractions of neurosecrosomes indicate that more than one species of neurosecrosome are present in the posterior lobe. There is evidence that there are two types of these granules; one is the storage form of OT and the other of VP. The two types of SG can be separated to some extent by both differential and density gradient centrifugation (Pardoe and Weatherall, 1955; Heller and Lederis, 1961; LaBella et al., 1962; LaBella et al., 1963; Barer et al., 1963). The sedimentation of isolated bovine SG on a discontinuous sucrose gradient, similar to the one used here, (LaBella et al., 1963) resulted in subfractions whose VP/OT ratios were comparable to those presently found in subfractions of neurosecrosomes. This similarity suggests that the sedimentation of a neurosecrosome is largely determined by the type of SG it contains. This possibility leads to further conjecture that only one of the two types of SG and, consequently, only one of the two hormones is contained in a single neurosecrosome. If an individual

neurosecrosome contains only one hormone, it seems reasonable to conclude that a single neuron of the hypothalamo-hypophysial tract contains only one of the two hormones.

The hypothalamo-hypophysial tract has the ability to differentially control the release of the two hormones (reviewed by Heller, 1961; Kleeman and Cutler, 1963; Barer et al., 1963). Electrical stimulation of the pituitary stalk is reported to cause the release of more OT than VP (Harris, 1948). Stimulation of the paraventricular nucleus causes the release of OT as measured by milk ejection in lactating rabbits, whereas no anti-diuretic activity could be detected (Cross, 1955 a). Abrahams and Pickford (1954) used a variety of stimuli known to inhibit diuresis by causing the release of VP and found that in all cases, more OT than VP was released. During the second and third stages of labor in ewes, a 150 to 300 fold increase in OT output was observed (Fitzpatrick, 1961). The estimation of hormone release by measurements of the amount remaining in the posterior lobe is undefinitive, as synthesis may replenish the stored content despite increased rates of release. Dicker and Tyler (1953 a) and van Dyke et al. (1955) found a reduction in the OT content of the posterior lobes of lactating bitches, whereas no changes were found in the lactating rabbit (Heller, 1961) or in the lactating goat (Folley, 1952). Olivecrona (1957) found that production of lesions in the paraventricular nucleus caused a decrease in the amount of OT in the posterior lobe. Lesions in the supraoptic nucleus did not

cause a diminution of the amount of OT in the posterior lobe nor could a diminution of VP be demonstrated although polyuria did result. Olivecrona proposed that the paraventricular nucleus was concerned with the production of OT, and the supraoptic, with VP. This was supported by Bisset et al. (1963) who reported that localized stimulation of the paraventricular nucleus caused release of OT into the circulation, and stimulation of the supraoptic nucleus caused release of VP. More OT than VP was found in the paraventricular nucleus and more VP than OT was found in the supraoptic nucleus of the camel (Adamson et al., 1956) and the sheep (Lederis, 1961a). The two hormones do not appear simultaneously in the hypothalamus during embryological development (Dicker and Tyler, 1953a; 1953b) providing evidence that two separate systems are responsible for their synthesis. The electrical responses of the supraoptic nucleus to a variety of stimuli (Suda et al., 1963; Koisumi et al., 1964) differ from those of the paraventricular nucleus (Brooks et al., 1966) suggesting a separation of function of the two nuclei. Electrical activity has been recorded in the pituitary stalk (Potter and Loewenstein, 1955; Carlisle, 1957) and in presumed neurosecretory cells in the hypothalamic nuclei (Cross and Green, 1959; Brooks et al., 1962; Suda et al., 1963; Kandel, 1964; Koisumi et al., 1964). These cells resemble other neurons in their electrical activity which, in the paraventricular nucleus, has been directly related to the release of OT (Brooks et al., 1966). If release of hormone is initiated by

depolarization of neurons, any control the hypothalamus may exert over the differential release of hormones is probably in the selection of the neurons to be stimulated. This proposed mechanism requires the presence of only one hormone in a given neuron.

The evidence cited above, as well as the observation that the hormones are stored in SG that appear to be distinct from one another, and the present finding that neurosecrosomes can, to some extent, be separated into vasopressinergic and oxytocinergic particles, support earlier speculation (Heller, 1961; Lederis, 1964a; 1964b; Rothballer, in press, cited by Brooks et al., 1966) that there are separate neurons for each hormone. The present finding that both hormones were found in all three subfractions of neurosecrosomes, although in different ratios, indicates that the density of each population of neurosecrosomes is distributed over ranges that differ only slightly from one another. It is possible that differences in the electronmicroscopic staining properties of SG may reflect their content of either VP or OT (Green and Maxwell, 1959). It has also been noted that, in fixed sections of whole tissue, some nerve endings contain larger SG than others (LaBella and Sanwal, 1965). In the present work, electronmicroscopy failed to show any morphological distinctions between the SG in the neurosecrosomes of the three subfractions. The possibility still remains that both hormones are present in each neurosecrosome but in varying ratios.

Another interpretation of these results is that there is a fixed VP/OT ratio in all the neurosecrosomes and the differences found here are the result of free SG which were isolated together with the original neurosecrosome fraction. This possibility is not likely, as there are few free SG seen in the electronmicroscopic photographs of these fractions and the differences in size between SG and neurosecrosomes is such that they are not likely to sediment in the same fraction. The LDH activity is similar in all three fractions indicating the absence of significant contamination of these fractions by non-neurosecrosomal elements. Furthermore, as the neurosecrosomes are composed primarily of SG, it would require a considerable amount of free SG contamination to account for the differences in the VP/OT ratios found.

Isolated Microvesicles

At present, knowledge concerning the neurohypophysial MV is limited to the electronmicroscopic observations that they are of a size similar to that of synaptic vesicles seen in cholinergic neurons and that they increase in number when the posterior pituitary neurons are stimulated to release hormones (Gerschenfeld et al., 1960). Similarly, an increase in synaptic vesicles has been reported on stimulating the cholinergic nerves to adrenal medullary cells (De Robertis and Vas Ferreira, 1957). These similarities have led to the speculation that the MV contain a neurohumoral mediator such as ACh for the release of VP and OT (Gerschenfeld et al., 1960). Koelle and

Geesey (1961) have identified ChE histochemically in the posterior lobe of the cat. Most of the activity was that of non-specific ChE, but, with the use of a specific inhibitor, a small component of the total activity was attributed to specific ChE. On the assumption that ACh is present wherever specific ChE is found, they proposed that ACh is present in the gland and acts back on the nerve endings, from which it is released to cause the release of hormones. This action is in keeping with a generalized theory for a presynaptic action of ACh (Koelle, 1961). A substance with "acetylcholine-like" activity was reported to be present in extracts of bovine neurohypophysis (Uemura et al., 1963), but one of the authors subsequently attributed this activity to possible interfering substances (Kobayashi, 1964).

Others have postulated that the MV contain a humoral mediator, although not necessarily ACh, which may act on pituicytes to initiate an action that is yet unknown (Bern, 1962); or it may act on capillary endothelium to promote the passage of hormone into the vascular system (Bodian, 1963). Noradrenaline is present in small vesicles in the anterior hypothalamus (De Robertis et al., 1965), and sympathetic fibers have been identified in the posterior lobe (Dandy, 1913; Fuxe, 1964). However, in preliminary experiments, we were unable to detect catecholamine in whole posterior lobes assayed by a fluorometric method (Bertler et al., 1958) which can detect as little as 0.026 µg/gm tissue.

Small vesicles, resembling MV have been observed inside SG in

the neurohypophysis of the rat and ferret (Holmes and Knowles, 1960). It has been proposed that the MV are an additional storage site for the hormones, possibly more readily releasable than the SG (LaBella and Sanwal, 1965). If this were true, the MV would be expected to have a high specific activity of hormones, at least 16 to 20 U/mg protein, which was reported for SG (LaBella et al., 1963). We have assayed the MV fraction for its VP content using the rat blood pressure method and found a specific activity of 2 U/mg. It cannot be concluded, however, that the hormone is not concentrated in the MV, because firstly, the fraction is not pure MV and contamination by larger particles can greatly inflate the estimate of total protein and secondly, osmolysis may have removed hormone from the MV. Kobayashi et al. (1961) graphed the diameter-frequency relationship of vesicular structures in the pars nervosa of the parakeet and showed the presence of a continuous range of particle sizes. A similar observation was made in the neurohypophysis of the trout (Lederis, 1964 a). Our MV fraction also contains a range of sizes, rendering the distinction between a large MV and a small SG arbitrary. Our inability to obtain a pure MV fraction may indicate that there is no discrete population of MV that is distinct from the larger vesicles.

Several investigators have proposed the MV may be a product of the breakdown of the SG (Bern, 1963; Knowles, 1965; Lederis, 1964 a; 1964 b; 1965; Bodian, 1966). The present results are interpreted as

supporting this theory. MV are seen in the vicinity of SG that appear to be in the process of breaking apart, and conversely, broken SG are not seen in areas where MV are not also present. It is proposed that there is a sequence of morphological changes that corresponds to the release of hormone followed by dissolution of the granule. This sequence includes the formation of MV by the free edges of broken membranes. It is likely that physical forces acting on free membrane edges will cause them to curl forming tubules or spheres. Erythrocyte membranes have been observed to form cellules in this manner (Miller and Hannay, 1965).

Samples of the entire population of neurosecrosomes in the homogenate are not necessarily isolated by the present method of differential centrifugation. This is indicated by LDH activity in fractions 3 and 4 in section II (Fig.3). The smaller nerve endings that are filled with MV and do not contain SG, seen in sections of whole tissue, have not been seen in the neurosecrosome fraction. The absence of SG in these nerve endings may reduce their density so that they sediment at a higher centrifugal force than the one presently used to isolate neurosecrosomes. Nerve endings that contain MV only, may be neurosecretory nerve endings that have been completely depleted of their hormone content and the SG have disintegrated, or these nerve endings are the terminals of a type of neuron other than those containing OT and VP.

These presumably non-hormonal neurons may contain MV that are indeed synaptic vesicles and release a humoral transmitter that serves a yet-undefined function.

IV

AMINO ACID COMPOSITION OF THE PROTEIN OF VAN DYKE AND OF NEUROHYPOPHYSIAL SECRETORY GRANULES

INTRODUCTION

The posterior pituitary is now known to secrete two hormones, the octapeptides VP and OT. However, some early attempts at extracting the active principle from the gland resulted in the isolation of what appeared to be a single substance possessing both oxcytocic and vasopressor activity (Abel, 1930). Rosenfeld (1940) subjected posterior lobe press juice to ultracentrifugation and found both hormone activities associated with material that sedimented as a single protein. Van Dyke et al. (1942) isolated a substance by chemical extraction that was soluble in dilute acid and contained high activity of both hormones in a 1:1 ratio. The solubility, ultracentrifugation behavior, and electrophoretic properties of this material indicated that it was a single protein with a molecular weight of approximately 30, 000. The Van Dyke protein was later found (Acher and Fromageot, 1957), to be a complex consisting of the two hormones in association with a protein which has been called 'neurophysin' (Chauvet et al., 1960). It was postulated that neurophysin is a carrier protein to which the hormones are bound when stored in the neurosecretory neurons of the gland.

The subcellular site of storage of the hormones in the posterior lobe is the SG which are membrane bound vesicles 100 to 300 mμ in diameter (Palay, 1957). The SG have been isolated by centrifugation and found to contain most of the sedimentable hormones (LaBella et al., 1963; Barer et al., 1963). The distribution of neurophysin in centrifugal fractions was found to accompany the distribution of both VP and OT (Ginsburg, 1964).

It appears that the SG may be packets of neurophysin in which the hormones are bound.

The present investigation further elucidates the relationship between carrier protein and the SG by providing evidence that the crude Van Dyke protein and the predominant protein of the SG are identical. This was done by comparing the amino acid composition of the SG with that of the Van Dyke protein. It was found that the two are similar in their amino acid composition supporting the assumption that the SG and neurophysin are composed of essentially the same protein.

METHODS

Preparation of Tissue.

Bovine posterior pituitaries were prepared and homogenized as described in Section II. The homogenate was centrifuged at 1, 700 g for 10 minutes and washed. The supernatant was centrifuged at 48, 000 g for 10 minutes and the pellet suspended in 15 ml of 10% sucrose containing 0.02% heparin.

Purification of SG.

A density gradient was prepared by layering 10 ml of 22.5% sucrose onto 10 ml of 45% sucrose in 30 ml cellulose nitrate tubes and permitting them to stand at 4^{o} for 2 hours. The suspension of the 48, 000 g fraction was layered over the gradient, 5 ml per tube, and centrifuged at 64, 000 g for two hours in a Spinco Model L Ultracentrifuge with a SW 25 rotor. Samples of the resulting three bands (Fig. 24) were pipetted off and centrifuged, the pellet recovered by decanting the supernatant. Only the pellet fraction (D) was recovered in its entirety. Each sample was washed by suspending it in 10% sucrose and centrifuging at 100, 000 g for 30 minutes.

Biochemical and Hormonal Assays.

Fractions were assayed for VP, OT, LDH, SDH, and nitrogen. All determinations were performed as described in Section II.

Determination of the Amino Acid Composition.

Fraction D, the pellet at the bottom of the density gradient was

suspended in water and aliquots hydrolyzed in 6N HCl at 110° for 48 hours sealed in glass vials under N_2, and analyzed for total amino acid on a Technicon instrument using a 5 1/2 hour accelerated system with type B resin. Cystine was determined separately by oxidizing cystine to cysteic acid according to the method of Moore (1963).

Van Dyke protein was prepared from posterior pituitary glands by the method of Van Dyke et al. (1942) and assayed for VP and total amino acid.

One, SG preparation was dialyzed against 20 volumes of water for 24 hours at 4° and one Van Dyke protein preparation was dialyzed against 20 volumes of 0.25% acetic acid. The amino acid compositions were determined before and after dialysis.

RESULTS

Isolation of SG.

Four fractions were obtained from the density gradient centrifugation of the 48,000 g fraction (Fig. 24). The results of analyses of these fractions are presented in Table 7. LDH is used as a marker for particles containing cytoplasm such as the neurosecrosomes and other cell fragments, and SDH for mitochondria. It can be seen that fraction D contains the hormone concentrated two-fold as compared to other fractions, whereas LDH and SDH, although present in this same fraction, are relatively less concentrated. This system of density gradient was designed to concentrate the SG in the pellet, where it can be recovered in its entirety by simply decanting the overlying solutions. The data indicate that fraction D is rich in hormone, while having less of the non-SG particulate material.

The Amino Acid composition of SG and Van Dyke protein.

Table 8 presents the amino acid composition of fraction D and the Van Dyke protein in terms of moles percent of 15 of the amino acids found in these preparations and does not include cystine and methionine. The cystine content of each preparation was determined separately and is presented at the bottom of the table as percent of total nitrogen recovered. Methionine is partially destroyed by hydrolysis and was not determined separately in this work, but the partial values found in the preparations indicate that it is present in small amounts. In preparation 1, the SG

were dialyzed in distilled water prior to assay in order to remove small peptides or free amino acids that may be released by osmolysis. This preparation does not differ from those that were not dialyzed. Determination 3 is of an aliquot of the SG sample analyzed in determination 4, but has been washed in ether and ethanol prior to analysis to remove possible lipid contamination. This procedure did not alter the amino acid composition of the fraction. Determinations Nos. 8 and 9 are of the same preparation of Van Dyke protein but 8 was dialyzed against 0.25% acetic acid and 9 against water. Acetic acid dialysis removed all detectable hormone from sample No. 8, while dialysis against water did not alter the hormone content appreciably. It can be seen that there is no appreciable difference in the amino acid composition of these preparations.

Comparison of the amino acid composition of the SG with that of the Van Dyke protein indicates a general similarity between the two; the only notable difference is in the glycine content. In the Van Dyke protein, the cystine content was highly variable. In two determinations of sample 6 the cystine content was 17.6 and 16.1%, whereas in samples 8 and 9 it was 6.4 and 5.2% of the total nitrogen respectively. The SG on the other hand were fairly uniform in their cystine content, from 2.6 to 4.2%. It appears that Van Dyke protein and SG differ markedly only in their glycine and cystine content.

Figure 24. Isolation of SG by discontinuous sucrose density gradient centrifugation. Figures at left denote sucrose concentrations. Letters ar right refer to the individual band locations at completion of centrifugation. The pellet (D) is composed primarily of SG

TABLE 7

ISOLATION OF SG BY DENSITY GRADIENT CENTRIFUGATION

Fraction	Expt. 1	2	3	4
		SDH[a]		
A	.93	0	0	.82
B	7.83	6.23	8.63	6.35
C	8.34	8.35	7.02	5.75
D	3.55	4.54	3.90	4.45
		LDH[a]		
A	3.92	0	1.40	2.80
B	6.76	3.39	4.30	5.58
C	7.05	2.25	3.14	2.13
D	0.94	1.13	1.39	1.01
		Vasopressin[b]		
A	6.65	1.55	4.63	2.35
B	5.56	7.73	8.34	7.41
C	6.01	11.75	8.19	10.53
D	18.89	20.66	19.97	18.12

a. Arbitrary units per mg protein

b. International units per mg protein

TABLE 8

AMINO ACID COMPOSITION OF SG AND VAN DYKE PROTEIN

	SG						van Dyke Protein						
Expt.	1	2	3[a]	4	$\bar{x}$	SD	5	6	7	8[b]	9	$\bar{x}$	SD
	Residues per 100 total residues[c]												
Asp	10.8	10.0	8.9	9.8	9.8	0.8	9.9	7.1	7.8	8.5	10.3	8.7	1.4
Thr	4.4	3.9	4.5	4.1	4.2	0.3	3.1	2.7	5.7	4.1	5.0	4.1	1.2
Ser	6.8	6.6	5.6	5.6	6.2	0.6	3.8	4.7	6.0	4.8	5.8	5.0	0.9
Glu	13.3	14.5	12.5	11.7	13.0	1.2	11.7	15.1	16.4	14.3	12.8	14.1	1.9
Pro	6.8	7.0	5.3	9.1	7.1	1.6	8.7	11.8	9.9	7.0	6.1	8.7	2.3
Gly	11.5	12.2	9.2	10.7	10.9	1.3	15.2	20.8	18.4	16.5	13.5	16.9	2.8
Ala	9.6	9.4	10.4	9.6	9.8	0.5	8.8	7.2	6.4	8.8	8.8	8.0	1.1
Val	6.4	6.4	8.8	5.9	6.9	1.3	5.8	4.6	6.3	5.3	5.9	5.6	.7
ILeu	3.9	3.7	4.6	3.8	4.0	0.4	3.8	3.5	3.3	3.8	4.4	3.8	.4
Leu	8.6	9.6	9.8	9.0	9.3	0.5	8.3	7.2	7.2	8.1	8.8	7.9	.7
Tyr	1.4	0.9	2.7	2.9	2.0	1.0	1.8	1.6	1.4	3.0	2.6	2.1	.7
Phe	3.6	3.9	4.6	4.1	4.1	0.4	3.7	3.4	3.6	4.1	4.0	3.8	.6
Lys	5.4	5.1	5.7	5.0	5.3	0.3	4.9	3.5	2.7	4.3	5.0	4.1	1.0
His	2.0	1.8	2.1	1.6	1.9	0.2	1.6	0.9	0.5	1.2	1.5	1.1	.5
Arg	5.4	5.4	5.0	7.2	5.8	1.0	5.9	6.5	6.6	6.1	5.5	6.1	.5
Cys 1/2[d]	4.2		2.6	3.7				16.5		6.4	5.2		
VP U/mg	19.4	22.7		16.3			16.6	13.4	14.9		17.4		

a. 3 is an aliquot of 4 that was washed in ether and ethanol.
b. 8 is an aliquot of 9 that was dialised against dilute acetic acid.
c. excluding cystine and methione.
d. percent of total nitrogen recovered.

DISCUSSION

The amino acid composition of the two preparations is highly suggestive that the major protein in each is identical. The accuracy of the amino acid determinations allows for an error of up to 2% and only two of the amino acids, glycine and cystine, differ by more than this amount. These two preparations are not likely to be unrelated as it has been shown that the distribution of neurophysin and SG is similar in centrifugation fractions (Ginsburg, 1964).

Neither preparation can be considered to be a pure substance. The SG contain some contaminating material such as neurosecrosomes as indicated by the presence of LDH, and mitochondria as indicated by the presence of SDH. Furthermore, there is undoubtedly some additional protein material in the SG such as the granule membrane. No effort has been made to ascertain the purity of the Van Dyke protein other than the determination of the VP activity. This protein was reported to have an activity of 16U/mg protein (Van Dyke et al., 1942) and 18 to 20U/mg (Acher et al., 1956; Chauvet et al., 1960). Van Dyke et al. (1942) found a high sulfur content in their protein which was all attributable to cystine. Block and Van Dyke (1952) analyzed the amino acid composition of the Van Dyke protein and found 16.9% cystine. In the present analysis, the cystine content was comparable with the reports cited above in one preparation but not in another. Although the hormones contain cystine, the high cystine

content of the protein is not attributable to the hormone bound to it as in these preparations the hormone contents are similar despite the differences in their cystine content. In contrast to neurophysin, SG consistently gave a cystine content of 3 to 4 %.

Neurophysin has been isolated from the posterior pituitary by gel filtration and found to be a mixture of two or more proteins (Ginsburg and Ireland, 1964; Hope and Hollenberg, 1966); only one component binds the peptide hormones and can be considered to be the carrier protein. A possible explanation for the differences between the amino acid compositions of the two preparations is that a component rich in these amino acids, especially cystine, is not present in the SG but is isolated together with the neurophysin.

V. SUMMARY AND CONCLUSIONS

1. Bovine posterior pituitary glands were homogenized with a teflon pestle designed to preserve nerve ending particles (neurosecrosomes). The homogenate was fractionated into six particulate fractions and a supernatant by differential centrifugation and the distributions of vasopressin (VP), oxytocin (OT), and certain enzymes among the fractions determined. The neurosecrosomes, secretory granules (SG) and microvesicles (MV) were further purified for more detailed investigation.

2. Lactic dehydrogenase (LDH) is a soluble intracellular enzyme indicative of trapped cytoplasm and was found in the neurosecrosome and mitochondrial fractions. However, it is also found in lighter fractions indicating a variety of particles containing trapped cytoplasm.

3. Acid proteinase is distributed similarly but not identically to the mitochondrial marker succinic dehydrogenase, indicating that this lysosomal enzyme cannot be localized to a particular fraction in which these particles can be isolated.

4. OT and VP are not distributed equally in the fractions, supporting the earlier observation that VP and OT are in different types of secretory granules. Sub fractionation of the neurosecrosomes on a density gradient resulted in a partial separation of VP containing neurosecrosomes from those containing OT. It is concluded that the sedimentation properties of

the neurosecrosomes are largely determined by the type of SG they contain. Some neurosecrosomes contain only VP containing SG and others only OT. As neurosecrosomes are the terminal portions of the hypothalamo-hypophysial tract, the localization of a single hormone to a neurosecrosome indicates that the entire neuron contains only one hormone. A division of hormones into separate neurons can explain the mechanism whereby the hypothalamo-hypophysial tract differentially controls hormone release.

5. Whole posterior pituitary glands hydrolyzed acetylthiocholine (ASCh) at twice the rate they hydrolyzed butyrylthiocholine (BuSCh). Compared to rat brain, the posterior pituitary was 1/3 as active in hydrolyzing ASCh and 3.3 times as active in hydrolyzing BuSCh. The use of inhibitors, BW 284C51 for specific cholinesterose (ChE) and DFP for non-specific ChE, as well as the differences in the rates of hydrolysis of both substrates indicate that specific ChE is present in the posterior lobe. Non-specific ChE is present in a higher concentration than in brain. The activity of the seven fractions was highly variable in replecate experiments but the specific activities for both substrates was highest in the microsomal fraction indicating that the enzymes are concentrated in membrane fragments. A difference in the distribution of activities of the two enzymes could not be demonstrated.

6. The neurosecrosomes were examined electronmicroscopically by both positive and negative staining. In negatively stained preparations, some

SG within a single neurosecrosome are seen to be filled with material whereas some are apparently depleted of their content. In positively stained preparations all SG are electron-transparent.

7. A fraction rich in MV was isolated by osmolysis of neurosecrosomes and subsequent density gradient centrifugation of the osmolysate. This fraction contained SG ghosts and vesicles ranging in size from 25 to 350 mμ. This range of particle sizes suggests that the MV are not a discrete subcellular entity but are the lower limit of a continuous range of sizes. It is concluded that the MV are the breakdown product of SG membranes.

8. A simple rapid method of isolating SG was developed wherein homogenate of posterior pituitary is centrifuged on a modified density gradient. A fraction was obtained that consisted of relatively pure SG with a high specific activity of VP.

9. The amino acid composition of isolated SG was compared to that of Van Dyke protein. The latter was extracted from whole pituitary glands and contains VP and OT in association with an inactive protein moiety which is presumed to be a carrier for the hormones in vivo. Similarities between the amino acid compositions indicate that their major protein components are identical.

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