ISOLATED NERVE ENDINGS (NEUROSECRETOSOMES) FROM THE POSTERIOR PITUITARY

Partial Separation of Vasopressin and Oxytocin and the Isolation of Microvesicles

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ABSTRACT

Subcellular fractions of the bovine posterior pituitary, including one composed almost exclusively of pinched-off nerve endings (neurosecretosomes), were characterized electron microscopically, hormonally, and enzymically. 15% of the nerve terminals in the gland were isolated as neurosecretosomes, as estimated from determinations of lactic dehydrogenase, a soluble, cytoplasmic enzyme. Neurosecretosomes were subdivided into three fractions by density-gradient centrifugation. The three subfractions, each shown to be nearly homogeneous populations of neurosecretosomes by means of electron microscopic and enzymic criteria, differed from each other in their vasopressin/oxytocin (VP/OT) ratios. The VP/OT ratio increased from the lightest to the densest fraction, indicating that VP is localized to denser and OT to lighter neurosecretosomes; similar results have been obtained previously for subfractions of neurosecretory granules (NSG). No morphological differences were apparent in neurosecretosomes among the three subfractions. Although complete separation of VP and OT was not achieved, the findings suggest that VP and OT are each stored in a different species of nerve ending and support the hypothesis that a given neurosecretory cell synthesizes, stores, and secretes only one of the peptide hormones. Microvesicles, 40–80 mμ diameter and contained in typical neurosecretory cell terminals, are believed to be degradation products of membrane ghosts of depleted NSG; electron micrographs indicative of this transformation are presented. A fraction rich in microvesicles, but containing some NSG membranes, was prepared by density-gradient centrifugation of an osmolysate of neurosecretosomes. Smaller, apparently nonneurosecretory nerve endings, lacking NSG but filled with small vesicles, are occasionally seen in sections from whole gland. The vesicles in these atypical posterior pituitary nerve endings may be true neurohumor-containing, "synaptic" vesicles.

INTRODUCTION

A procedure for the isolation of pinched-off nerve endings and other subcellular components from bovine posterior pituitary glands was described by LaBella and Sanwal (40). In this early publication on isolated neurohypophysial nerve endings, estimation of fraction purity and of organelle structural integrity was made on a morphological basis by means of electron microscopy. Isolated nerve

terminals, examined both in sections of OsO_4-fixed centrifugation pellets and by negative staining of suspensions, were remarkably similar to those seen in sections of whole tissue. These pinched-off organelles apparently self-seal at the point of rupture and retain their normal complement of neurosecretory granules (NSG), mitochondria, and microvesicles (MV). Subsequent studies showed that, at least for the catabolism of glucose, cellular metabolic pathways are operative in the isolated neurohypophysial nerve endings, as in tissue slices of the posterior pituitary gland (36, 37, 41). Furthermore, a preliminary study demonstrated that lactic dehydrogenase (LDH), a soluble cytoplasmic enzyme proposed by Johnson and Whittaker (27) as a marker for "trapped" cytoplasm, was most concentrated in the particulate fraction shown to be rich in posterior pituitary nerve ending particles (41).

Nerve ending particles, which contain mitochondria and neurohumor-containing "synaptic" vesicles as exclusive particulate components, have been isolated from brain and named "synaptosomes" by Whittaker (57). In accordance with this terminology, we have coined the term *neurosecretosomes* as a more convenient, specifically descriptive alternative to "nerve ending particles isolated from posterior pituitary glands."

The neurons of the mammalian hypothalamo-hypophysial system originate in the supraoptic and paraventricular nuclei of the anterior hypothalamus and terminate as prominent swellings in the pars nervosa of the pituitary. These neurons synthesize, store, and release the peptide hormones vasopressin (VP) and oxytocin (OT). There is considerable evidence to indicate that the hypothalamo-hypophysial system has the ability to differentially control the release of the two hormones (reviewed in 3, 24, 28). The hormones are stored in NSG contained within the nerve terminals in the pars nervosa; the NSG have been shown to vary in sedimentation and density properties so that VP-containing NSG can be separated, to some extent, from those containing OT (3, 38, 39, 50). This separation of activities has led to speculation that there is a specific NSG type for each of the two hormones. The possibility that hormones are stored in separate NSG 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 given neuron, only one of the peptide hormones is present (3, 24, 38, 44, 45). If there are, indeed, separate neurons for each hormone, neurosecretosomes, like the NSG, would be expected to vary in sedimentation and density properties, thereby permitting isolation of the vasopressinergic or oxytocinergic type, according to the species of NSG contained.

Another type of inclusion in the posterior lobe nerve endings are small electron-transparent vesicles, the MV, 40–80 mμ in diameter. They have been referred to as "synaptic vesicles" (20, 49), in that they resemble acetylcholine (ACh)-containing vesicles seen in nerve terminals of motor neurons. Knowledge of the MV is, at present, limited to electron microscopic observations which have demonstrated changes in their number within the neuronal terminations as influenced by changes in the functional activity of the gland (20). The term "synaptic" vesicles is unjustified, as it implies a physiological role for which there is no experimental support; the term "microvesicles" is noncommittal and, therefore, more appropriate for the neurohypophysial organelles. There are two general theories concerning the 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 (17). The second theory postulates that they are derived from NSG and represent either hormone secretory packets, analagous to the ACh-containing synaptic vesicles (40) or breakdown products of membranes of depleted NSG (4, 25). There has been no direct experimental support for either point of view. A direct approach for obtaining further information concerning the MV is that they be isolated and examined. "Synaptic" vesicles from brain have been isolated by De Robertis et al. (16) and Whittaker et al. (58) who disrupted synaptosomes by osmolysis and obtained a fraction rich in the small vesicles by density-gradient centrifugation. A similar approach would seem feasible for the isolation of MV from neurosecretosomes.

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. Early fractionation work on this gland reported from this laboratory (38, 39) has been expanded to include a study of the properties of the neurosecretosomes, which were not present in homogenates as pre-

pared in the original procedure. VP and OT, succinic dehydrogenase (SDH), acid proteinase (AP), and LDH were estimated as markers for NSG, mitochondria, lysosomes, and trapped cytoplasm (neurosecretosomes), respectively, in the fractions obtained by centrifugation. Furthermore, the neurosecretosomes have been subfractionated by density-gradient centrifugation in an attempt to separate the vasopressinergic from the oxytocinergic type. In addition, neurosecretosomes were disrupted by osmolysis and a fraction rich in MV was isolated by centrifugation.

METHODS AND MATERIALS

BOVINE PITUITARY GLANDS: These were obtained from the slaughterhouse[1] 30–40 min 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% (0.29 M) sucrose, 10 ml per gram tissue. The total time elapsing between the death of the animals and tissue homogenization was 1–1.5 hr.

HOMOGENIZATION: As the nerve endings are fragile 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 neurosecretosomes as possiole. This was accomplished by homogenizing the posterior lobes in two stages with two teflon pestle-glass vessel homogenizers (A. H. Thomas Co., Philadelphia, Pa.), 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 passes through the grooves and disruption is performed by the central area of critical dimension (Fig. 1). During homogenization, care was taken to avoid crushing the tissue in the bottom of the tube. The first homogenization was done by using the pestle with the larger clearance. After centrifuging at 700 *g* for 10 min, the supernatant was decanted and the sediment resuspended in 15 ml of 10% sucrose and ground with the second homogenizer. The homogenate was centrifuged at 700 *g* for 10 min and the sediment recovered as fraction 1. The volume of the combined supernatants was measured, and a 1% solution of heparin in sucrose added to make a final concentration of 0.02% heparin. Heparin was added after the removal of fraction 1, because nuclear material forms a gelatinous mass in its presence (39).

DIFFERENTIAL CENTRIFUGATION: The first centrifugation of 700 *g* was done in a Servall refrigerated centrifuge. All subsequent steps were performed in a Spinco Model L ultracentrifuge with a No. 40 rotor. Each fraction was washed by decanting the supernatant, resuspending the sediment, and recentrifuging. The sediment was resuspended in 10% sucrose. The supernatant, including washings, was then centrifuged in order to prepare the next fraction. The centrifugation scheme was as follows:

Fraction	*g*-min at R_{max}	Major components
1	7,000	Nuclei and debris
2	23,300	Neurosecretosomes
3	41,100	Neurosecretosomes, mitochondria, NSG
4	164,000	Mitochondria, NSG
5	658,000	NSG, large membrane fragments
6	3,550,000	Small membrane fragments, MV
Supernatant		Soluble material

The components are assigned to the fractions on the basis of electron microscopy by LaBella and Sanwal (40) and by subsequent electron microscopy, biochemical, and hormonal studies (6, 41). Each fraction was assayed for VP, OT, nitrogen, SDH, LDH, and AP.

ELECTRON MICROSCOPY OF FRACTIONS OBTAINED BY CENTRIFUGATION: The fractions were prepared for microscopy by either osmium tetroxide fixation of pellets or negative staining of suspensions as described by LaBella and Sanwal (40).

DENSITY-GRADIENT CENTRIFUGATION OF THE NEUROSECRETOSOME 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 2 hr prior to use, and 5 ml of the neurosecretosome fraction (fraction 2) suspended in 10% sucrose was layered on top. Three such tubes were centrifuged at 60,000 *g* for 2 hr in a Spinco Model L Ultracentrifuge with an SW25 rotor. Samples from each of the resulting three bands (*A*, *B*, and *C*), in order of increasing density (Fig. 2), were pipetted off and diluted to make a final concentration of 20% sucrose. The suspensions were centrifuged at 100,000 *g* for 30 min in order to concentrate the particles.

[1] Pituitary glands were generously provided by Mr. R. Mathewson of Canada Packers, Ltd., St. Boniface, Manitoba.

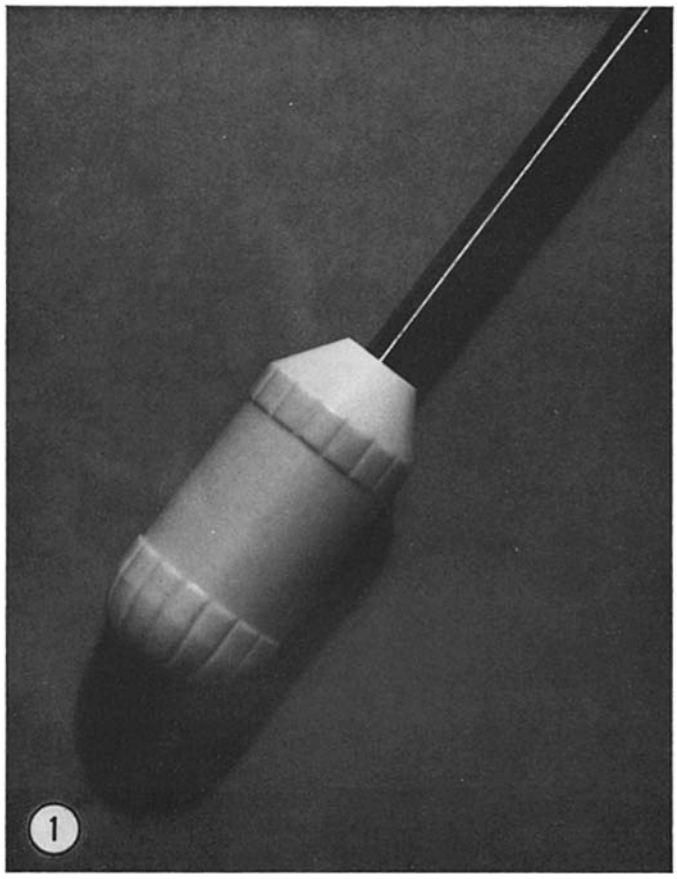

FIGURE 1 Specially constructed teflon homogenizing pestle. The middle two-thirds of the pestle was machined to provide an area of large clearance for use in the preparation of neurosecretosomes. The upper and lower segments center the pestle so that the central clearance dimension between teflon pestle and glass vessel wall is constant during homogenization. The grooves permit the pieces of tissue to pass into the central area of wide clearance where disruption occurs.

Each of the three bands obtained by density-gradient centrifugation fractions was assayed for VP, OT, nitrogen, and LDH and also examined electron microscopically. In order to account for the possibility that differences in the hormone content of the three fractions were artifacts resulting from differential release by different sucrose concentrations, aliquots of the original neurosecretosome fraction were incubated for 2 hr in 40, 50, and 60% sucrose at 4°C and the hormone content of the recentrifuged particulate material determined.

ISOLATION OF MICROVESICLES: A 0.5-ml aliquot of a suspension of neurosecretosomes was suspended in 10 ml of cold distilled water and slowly stirred in the cold for 1 hr. A continuous sucrose gradient was prepared by layering 5 ml each of 40, 30, 20, and 10% sucrose in a 30-ml cellulose nitrate tube and storing at 4° for 18 hr prior to use. 3 ml of the suspension of osmolyzed neurosecretosomes was layered on top of this gradient and centrifuged for 2 hr in a Spinco SW25 rotor at 60,000 *g*. The uppermost layer (Fig. 3) was shown by electron microscopy to contain the MV. It was pipetted off, diluted with 10% sucrose, placed in a $\frac{5}{16} \times 1\frac{15}{16}$-in. cellulose nitrate tube, and centrifuged at 100,000 *g* for 30 min in a Spinco No. 40 rotor fitted with microtube adapters.

HORMONE EXTRACTION: The hormones were extracted from the fractions by diluting 200 μl of suspension with 1.8 ml of 0.25% acetic acid in 0.9% NaCl. This was placed in a boiling water bath for 5 min, centrifuged, and the sediment discarded. The acidic supernatant was stored in the frozen state until assayed. The extracts were neutralized

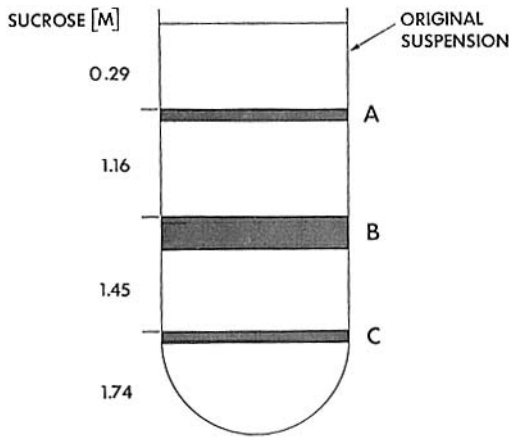

FIGURE 2 Density-gradient centrifugation of neurosecretosomes (fraction 2). 5 ml of a suspension of neurosecretosomes in 0.29 M sucrose was layered over a discontinuous gradient comprised of three 7-ml layers of 60, 50, and 40% (1.74, 1.45, 1.16 M) sucrose. Centrifugation was carried out in a Spinco SW25 rotor at 60,000 *g* for 2 hr, and this resulted in the formation of three distinct particulate bands, *A*, *B*, and *C*.

and diluted with 0.9% saline so that the hormone activity approximated that of the hormone standard used in the bioassay.

Vasopressin assay was performed by a modification of the method of Landgrebe et al. (43). Virgin female albino rats weighing 200–250 gm (Canada Breeders, St. Constant, Quebec) were anesthetized with urethane, 140 mg/100 gm, injected subcutaneously. The method was modified in that the cervical sympathetic trunks and the vagi were not cut and, instead, a ganglionic blocking agent, chlorisondamine (Ecolid, Ciba), 10 mg/100 g, 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 (14) has been tried and rejected, as this type of drug frequently causes an instability of the base line blood pressure, and, following administration of test doses of vasopressin, 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 base line and in permitting a rapid return to resting level following a pressor response. The assay was carried out against purified lysine vasopressin (260 units/mg) (obtained from NIH), using a 2 + 2 method described by Holton (26).

Oxytocin was determined on the isolated rat uterus by the method of Holton (26) against synthetic oxytocin (Syntocinon, Sandoz) as standard.

Nitrogen was determined by Nesslerization of sulfuric acid digests.

Lactic dehydrogenase was assayed by the method of Kornberg (35). The course of the reaction was recorded on a Beckman DK Recording Spectrophotometer with a fixed wave length of 340 mμ. The activity was measured by the slope of the line plotting absorbance of reduced NAD against time.

Succinic dehydrogenase assay medium, containing cytochrome *c*, $AlCl_3$ and $CaCl_2$ was prepared according to the method of Schneider and Potter (52) and the reaction followed photometrically by the method of Green et al. (21). The reduction of cytochrome *c* with time was recorded on a Beckman DK recording spectrophotometer at 550 mμ.

Acid proteinase was assayed according to the method of Adams and Smith (2), in which the hydrolysis of denatured hemoglobin at pH 3.5 is estimated by the amount of acid soluble tyrosine (absorbance at 280 mμ) liberated.

RESULTS

Distribution of "Markers" among Centrifugation Fractions

The distributions of nitrogen, OT, VP, SDH' LDH, and AP among the centrifugation fractions are shown in Fig. 4. LDH, a soluble cytoplasmic enzyme, shows a distribution distinct from any of the other marker constituents of cellular particulates. About 75% of LDH activity is found in the

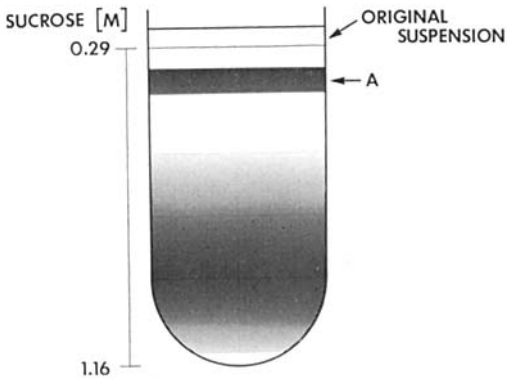

FIGURE 3 Density-gradient centrifugation of a neurosecretosome osmolysate. A suspension of neurosecretosomes (fraction 2) was mixed with cold distilled water and stirred slowly in the cold for 1 hr. 3 ml of this mixture was layered over a continuous gradient ranging from 10 to 40% (0.29 to 1.16 M) sucrose and centrifuged in a Spinco SW25 rotor at 60,000 *g* for 2 hr. A narrow distinct band (*A*) was localized to the upper part of the tube, and the bulk of the particulate material was present as a wide, diffuse lower band.

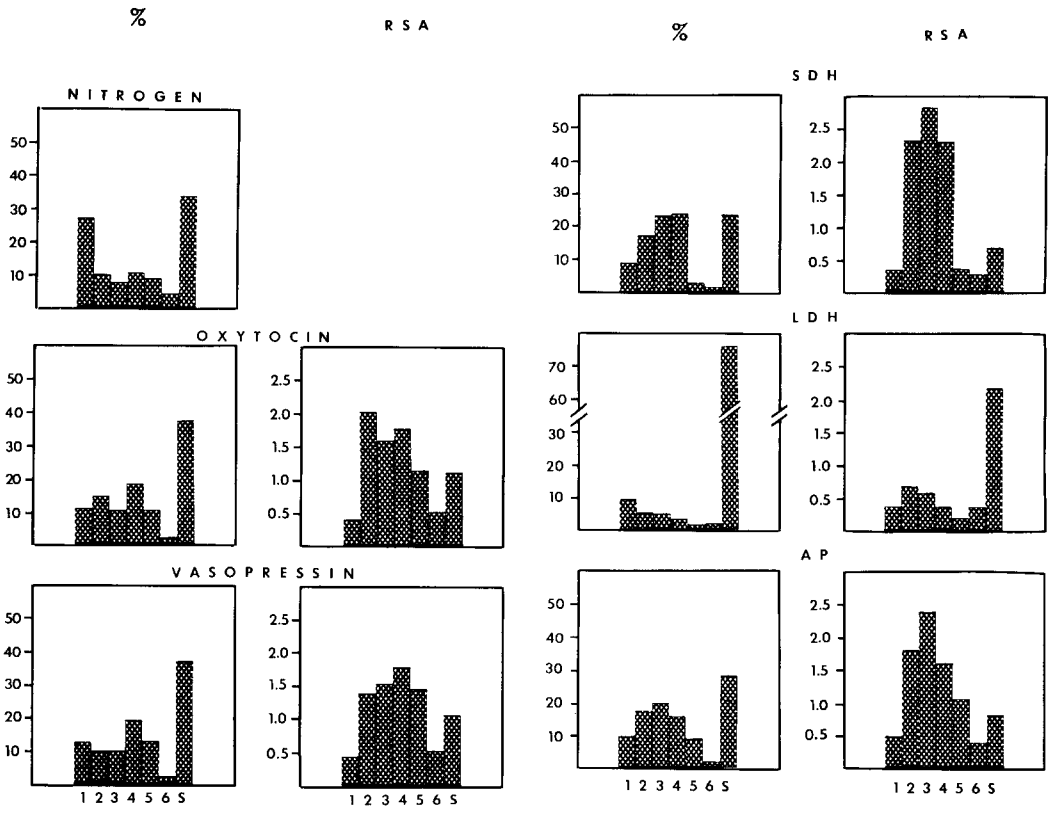

FIGURE 4 The distribution of nitrogen, oxytocin, vasopressin, succinic dehydrogenase (*SDH*), lactic dehydrogenase (*LDH*), and acid proteinase (*AP*) among fractions obtained by centrifugation. For each hormone and enzyme the activity in a given fraction is expressed (1) as the percentage of the total activity recovered in all the fractions and (2) as the relative specific activity (*RSA*) which is defined as the percentage of the total activity in a given fraction divided by the percentage of the total nitrogen content of the fraction.

supernatant, indicating that 25% of the total tissue cytoplasm is in sedimentable, "trapped," form. 10% of the latter LDH fraction is found in fraction 1, "nuclei and debris," which would be expected to contain undisrupted tissue fragments and cell aggregates. The remainder of the particulate LDH is spread over fractions 2, 3, and 4, with small amounts in fractions 5 and 6. Electron microscopy in our earlier work (40) supports this LDH data, by showing the same relative concentrations of neurosecretosomes in these fractions. The highest concentration, i.e., relative specific activity (RSA), defined as percentage of total activity in a given fraction divided by the nitrogen content of that fraction, for LDH is found in fractions 2 and 3, corresponding to morphological evidence that these fractions are the most concentrated in neurosecretosomes.

OT and VP are very widely distributed and of quite uniform concentration among the fractions, because these hormones are associated with two major species of particles, the neurosecretosomes and NSG. SDH, similarly, reflects free mitochondria and those contained within neurosecretosomes. AP is uniquely distributed, in accordance with its presumed localization in a distinct species of particles, the lysosome. On the basis of these data alone, it cannot be established whether lysosomes are present within neurosecretosomes.

OT is most concentrated in fraction 2 and VP in fraction 4; although this difference may not be a significant one, considering the degree of error

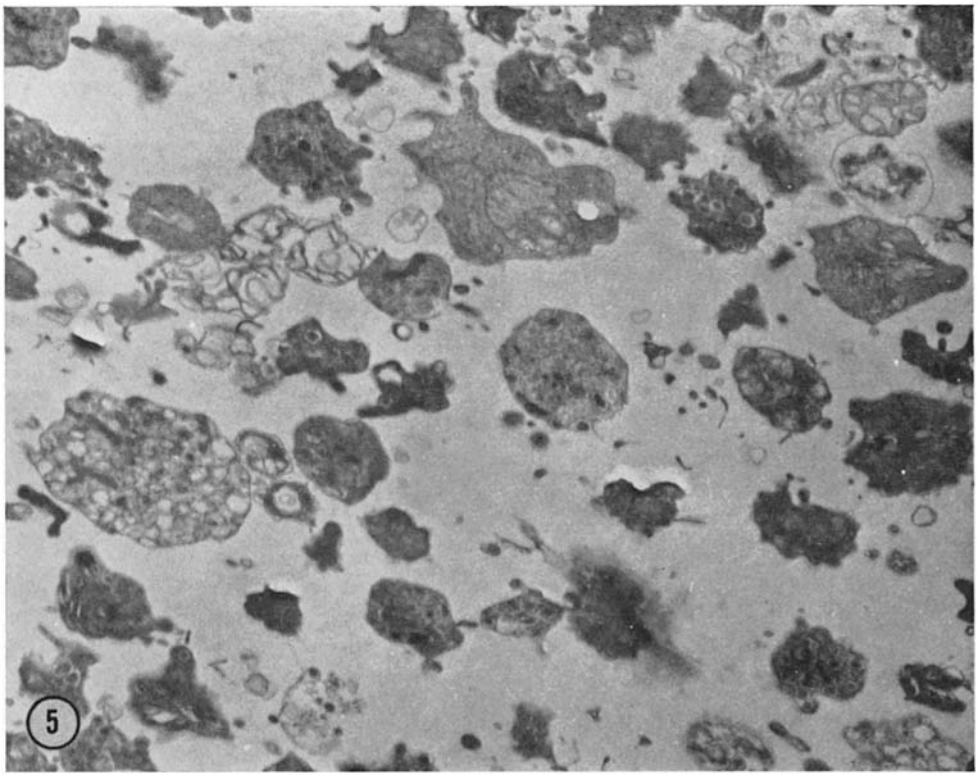

FIGURE 5 Section through an OsO_4-fixed pellet of neurosecretosomes (fraction 2). × 10,000.

in bioassays, it does suggest that the two hormones are not associated with the same particle species. This finding confirms previous findings from our laboratory (38, 39), although in the earlier work neurosecretosomes were not present in the posterior pituitary homogenates. Because fractions 2 and 3 contain few free NSG, the present findings suggest that the different localization of OT from that of VP reflects different neurosecretosome species, whereas in the former study NSG species were apparently segregated by centrifugation.

Electron Microscopy of Neurosecretosomes

The 23,300 *g*-min fraction (fraction 2) is made up nearly exclusively of isolated neurosecretosomes and occasional bits of debris; no nuclei and few free mitochondria or NSG are seen. In OsO_4-fixed sections from centrifugation pellets of this fraction (Fig. 5), the NSG within the neurosecretosomes usually have electron-transparent centers; the dense-staining NSG seen in sections of whole tissue are not seen as frequently in this type of preparation.

Negative-staining electron microscopy provides some concept of the three dimensional configuration of particles (Figs. 6 and 7). The space occupied by particulate material embedded in the phosphotungstic acid film appears electron transparent, whereas smaller or thinner particulates retain the film and appear more electron opaque. Two types of NSG are apparent in negatively stained preparations: one type is the relatively electron-transparent circular profiles, which are least dense at their centers than at the periphery and are probably spherical granules filled with neurosecretory material. The other type of granule within the negatively stained neurosecretosome has an electron-opaque center with an electron-transparent halo. The latter appear to be mono- or biconcave discs and probably represent collapsed NSG, presumably depleted of their contents. Both types of NSG may be seen in individual neurosecretosomes.

The smaller vesicular inclusions, collectively referred to as MV, do not always appear as circular profiles, although this is the most common form.

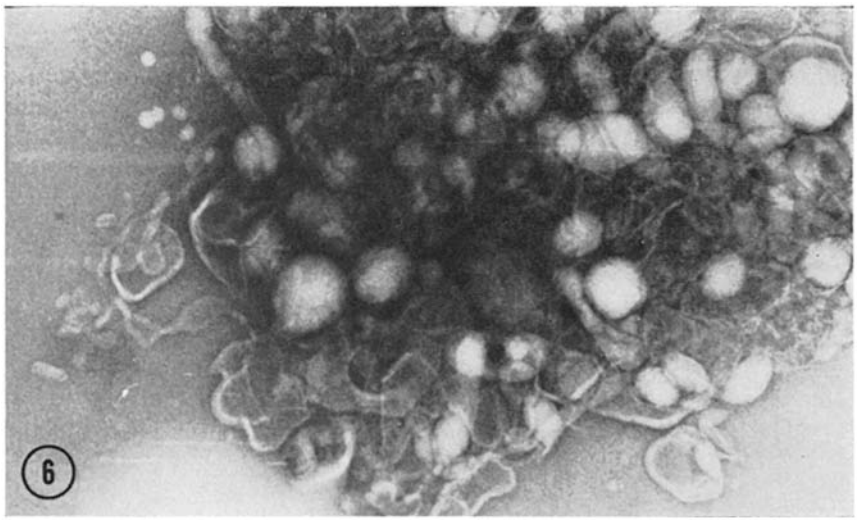

FIGURE 6 Negatively stained neurosecretosome. × 28,000.

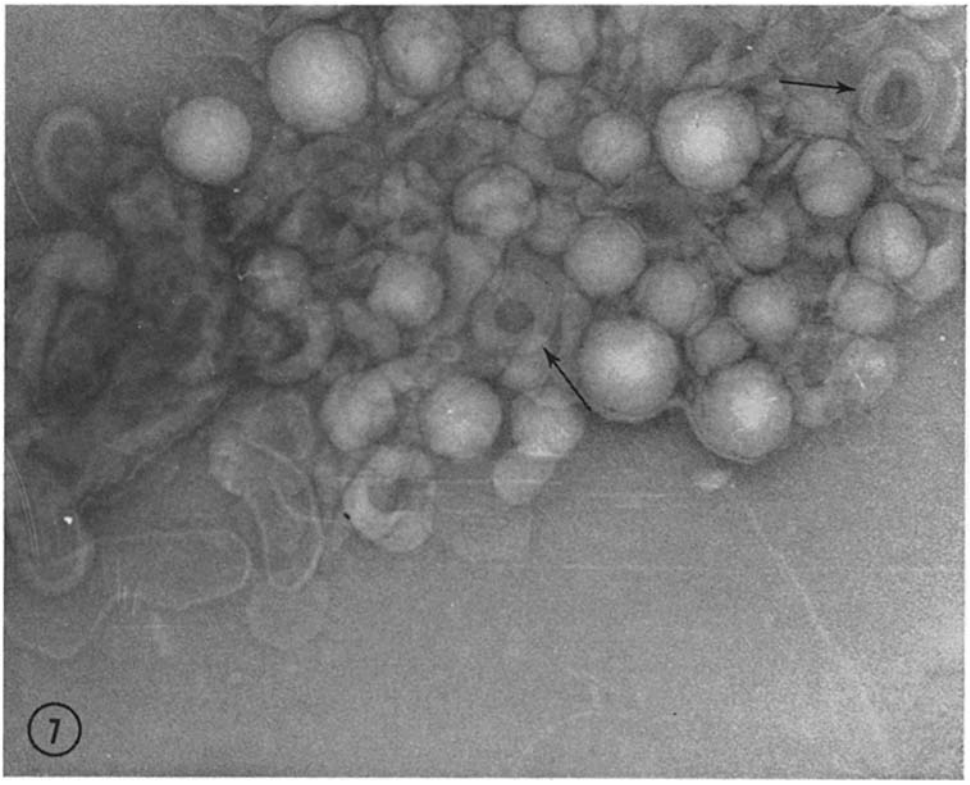

FIGURE 7 Negatively stained neurosecretosome. Arrows indicate concave central regions of apparently depleted NSG. × 42,500.

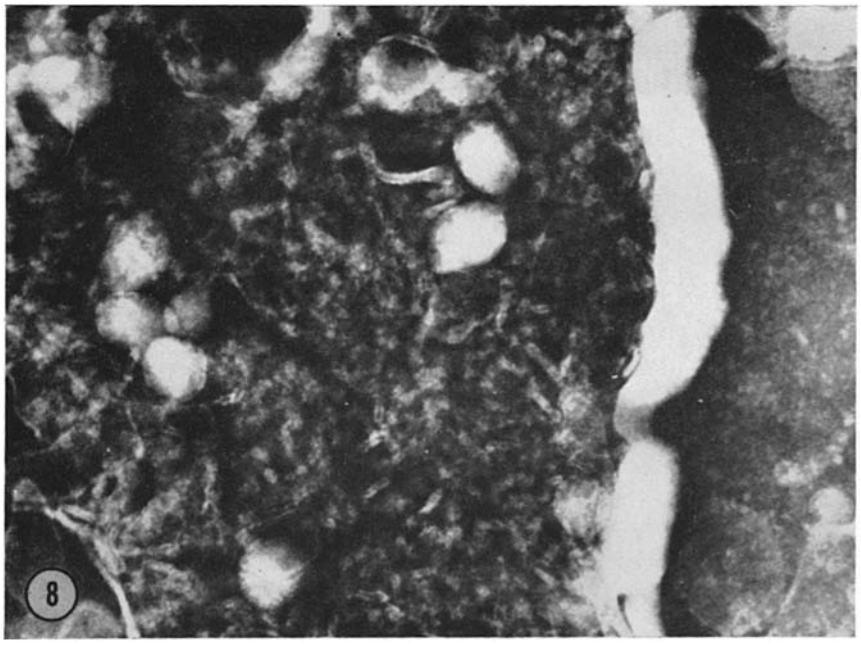

FIGURE 8 Negatively stained neurosecretosome practically filled with rod-shaped MV and a few NSG. × 80,000.

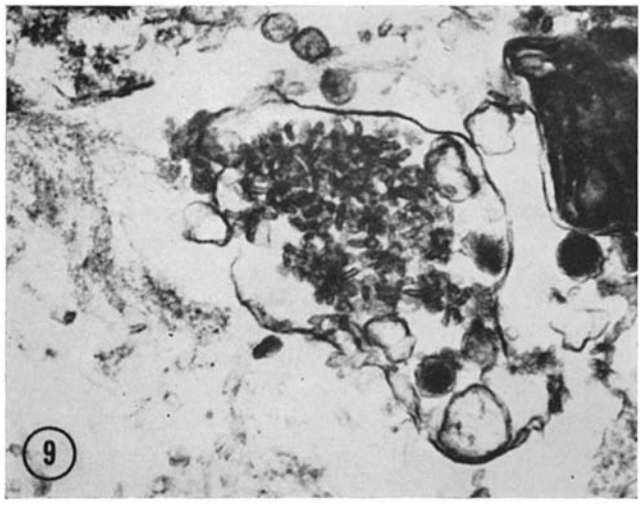

FIGURE 9 Section of an OsO_4-fixed neurosecretosome containing a large number of rod-shaped MV. X50,000.

In both negatively stained (Fig. 8) and positively stained preparations (Fig. 9), some of the MV are elongated, appearing tubular or rod shaped. The MV may be uniformly distributed within a given neurosecretosome, but large clusters of MV can also be seen (Figs. 8–10). In several positively stained sections of the neurosecretosome fraction, membranes of some of the electron-transparent, apparently depleted NSG are not intact. Frequently, broken NSG membranes can be seen curling at their edges to form small vesicular structures that are similar in size to MV (Figs. 11 and 12).

Much of the apparent disruption of neurosecretosomes seen in OsO_4-fixed sections is probably due to the breakdown of these structures during the

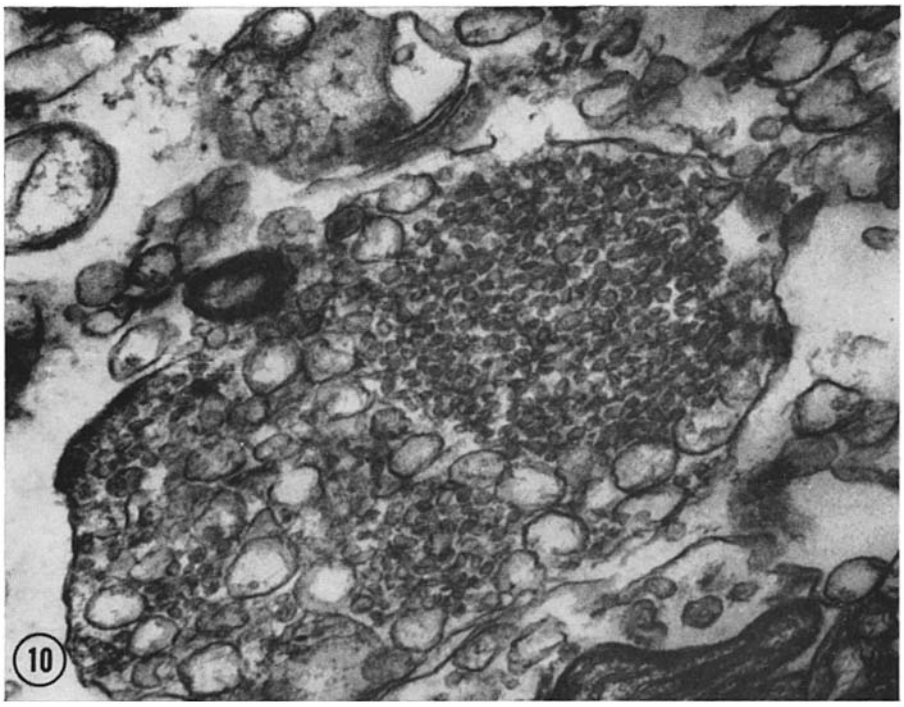

FIGURE 10 Section of an OsO_4-fixed neurosecretosome containing numerous spherical MV in addition to some apparently depleted NSG. × 32,000.

fixation process. This conclusion is based, on the one hand, by many observations on both OsO_4-fixed and negatively stained preparations. The latter procedure involves minimal manipulation and the neurosecretosomes are usually examined shortly after their isolation; neurosecretosomes visualized in this manner rarely exhibit the generalized disruption frequently seen in OsO_4-fixed preparations. The occurrence of localized aggregates of NSG interspersed with other components suggests that surrounding membrane has been destroyed by osmication. Whittaker (57) has commented that brain synaptosomes appear to be readily disrupted by fixation in osmium tetroxide.

Density Gradient Centrifugation of Neurosecretosomes

The VP, OT, and LDH content of subfractions obtained from density-gradient centrifugation are shown in Table I. The important finding is that in every case but one, the VP/OT ratio is lowest in fraction A, i.e., the least dense particulate fraction in any given experiment is most concentrated in OT. Conversely, the highest ratio is found for fraction C in every case but one, i.e., the most dense particulate fraction has the highest VP content. The 23,300 *g*-min fraction has been shown by electron microscopy to be almost completely homogeneous. This fraction contains almost pure populations of neurosecretosomes with little contamination by mitochondria, NSG, or membrane fragments. More slowly sedimenting fractions also contain neurosecretosomes, but these organelles are of a smaller variety and, therefore, more heavily contaminated with other particulates.

LDH in the three subfractions was determined in three experiments. The similar activities among the fractions, with the exception of fraction C of experiment 2 which was quite low, indicate that the neurosecretosome concentrations were similar in all fractions. On the basis of the LDH data, it appears unlikely that the different VP/OT ratios among fractions A, B, and C represent relative segregation of NSG from neurosecretosomes. Electron microscopy of fractions A, B, and C was carried out, despite the fact that microscopy of the

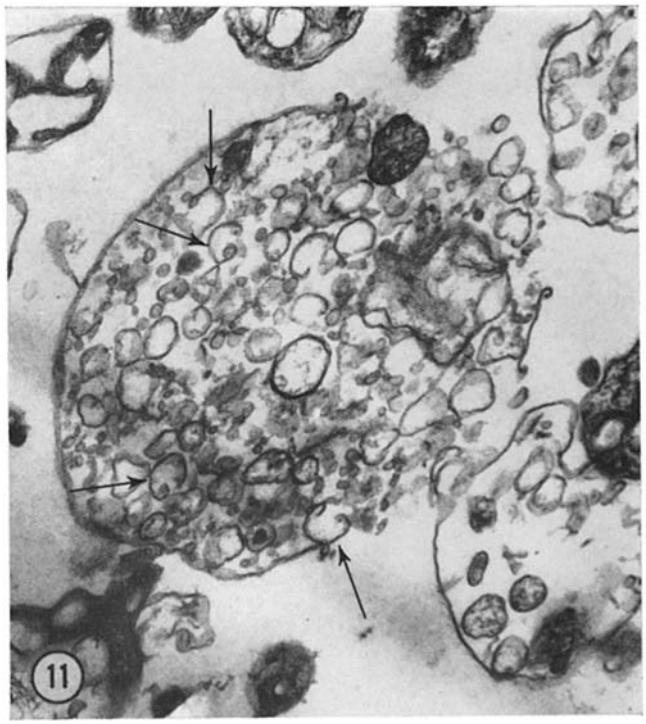

FIGURE 11 OsO_4-fixed neurosecretosomes showing "curling" of membranes of depleted NSG (arrows). × 26,000.

original 23,300 *g*-min neurosecretosome fraction has consistently been shown to be highly pure.

In one experiment, OsO_4-fixed pellets of fractions A, B, and C were randomly sectioned and examined electron microscopically. Each of these fractions contained, as did the primary neurosecretosome fraction, neurosecretosomes as the only significant particle species, and each fraction was essentially identical morphologically. A section of fraction A is shown in Fig. 13. Much of the apparent "debris" seen in the micrographs of fractions A, B, C is derived largely, we feel, from neurosecretosomes ruptured during the fixative procedure and is not due to the presence of other particulate components (see preceding section). It must be further pointed out that the neurosecretosomes shown in Fig. 13 had been initially obtained from homogenates by differential centrifugation, then subjected to concentrated sucrose for a few additional hours during density-gradient centrifugation, isolated from the density-gradient tubes and concentrated by centrifugation, and, finally, fixed in OsO_4. No doubt some of the disruption is due to these prolonged and somewhat severe procedures. The morphological, enzymic, and hormonal properties of these neurosecretosomes, however, indicate that these particles have maintained their functional capacity remarkably well.

In attempts to exclude possible artifactual causes for the differences in VP/OT ratios among the three subfractions, control experiments were carried out in which the primary neurosecretosome fraction was exposed to the same sucrose concentrations and for the same length of time as in the experimental situation (Table II). These control experiments were designed to examine the possibility that increasing sucrose concentration preferentially released OT from the neurosecretosomes, resulting in progressively higher VP/OT ratios in the particle fractions A, B, and C. There did not appear to be any change in the ratios which were consistent or of a magnitude to explain the results obtained by density-gradient centrifugation.

The sizes of granules within neurosecretosomes of subfractions A, B, and C were measured, in order to ascertain whether morphological dif-

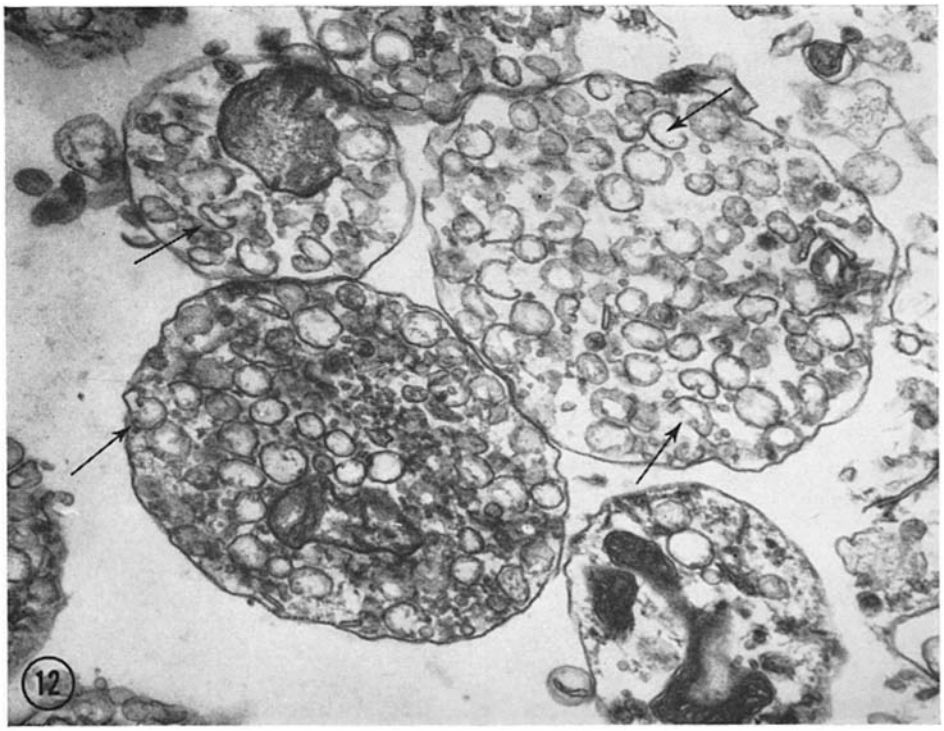

FIGURE 12 OsO_4-fixed neurosecretosomes showing "curling" of membranes of depleted NSG (arrows). × 28,000.

ferences could be detected among neurosecretosome fractions varying markedly in their hormonal activities. In Table III, the mean diameters of 300–600 NSG within neurosecretosomes in each fraction are shown. No significant differences in granule size were observed among the fractions, nor could any differences be observed in the staining properties of neurosecretosomes as determined electron microscopically.

It was concluded, on the basis of the above morphological, enzymic, and hormonal criteria, that the different VP/OT ratios among subfractions obtained by density-gradient centrifugation were the result of an actual separation of neurosecretosomes of different densities. The indication, therefore, is that VP is contained within more dense and OT within less dense neurosecretosomes.

Isolation of MV

The two density-gradient fractions obtained from osmolysates of neurosecretosomes (Fig. 3) were examined electron microscopically. The upper band, (*A*) was found to consist of MV and NSG membranes; OsO_4-fixed and negatively stained preparations are shown in Figs. 14 and 15, respectively. The wide lower band was comprised of undisrupted neurosecretosomes, mitochondria, NSG, and large membrane fragments. The distribution of diameters of several hundred particles in several fields of the MV fraction (band A) is shown in Fig. 16. There is a population of particles 40–80 mμ in diameter, with a mean diameter of 54 mμ, which constitutes 41% of the total particle population. The remaining particle diameters range from 80 to about 300 mμ and probably are NSG membranes. These larger membranes, which are somewhat distorted and compressed in the OsO_4-fixed preparation, are probably membrane "ghosts" of the depleted NSG. A few of the circular membrane structures are as large as 0.5 μ in diameter and may represent entire or large fragments of the neurosecretosome membrane. Examination of the high speed sediment of an osmolysate of the NSG fraction demonstrated the presence of similar membrane "ghosts." Attempts to separate MV from the larger membranous components are under way.

The MV, isolated as described above, are prob-

TABLE I

Subfractionation of Neurosecretosomes by Density Gradient Centrifugation

Exp.	Fraction	Hormone activity U/mg protein		VP/OT	LDH Arbitrary units/mg protein
		VP	*OT*		
1	A	6.7	22.3	0.30	65
	B	12.6	21.3	0.59	99
	C	21.2	30.1	0.70	83
2	A	4.7	9.4	0.49	65
	B	8.3	2.3	3.57	74
	C	8.6	2.0	3.20	34
3	A	1.9	1.5	1.26	
	B	24.1	7.7	3.12	
	C	20.7	1.5	13.61	
4	A	3.5	3.8	0.91	61
	B	19.5	15.0	1.30	57
	C	13.7	5.4	2.53	67
5	A	0.6	0.7	0.78	
	B	13.9	14.0	0.94	
	C	3.9	2.6	1.49	
6	A	3.3	4.3	0.77	
	B	6.7	12.3	0.54	
	C	6.3	5.3	1.19	

ably derived primarily from the terminals of neurosecretory cells which comprise the bulk of the posterior pituitary gland. However, another relatively rare type of nerve ending, quite unlike the typical NSG-containing variety which is full of MV and lacks NSG, is observed in tissue sections. These atypical nerve endings are usually smaller than the obviously neurosecretory type (Fig. 17). Presumably, the MV fraction would also contain small vesicles derived from this species of nerve ending.

DISCUSSION

Isolation and Characterization of Neurosecretosomes

LDH is a soluble enzyme within the cytoplasm. Upon homogenization of cells, this enzyme is released into the suspending medium when the plasma membrane is disrupted. Neurosecretosomes are formed when nerve terminals are sheared from their axons during the homogenization. The point of rupture of the neurosecretosome from its axon filament apparently becomes sealed off, so that the particle is completely enclosed by a permeability barrier, the plasma membrane. Sedimentable LDH, therefore, has been proposed as a marker for "trapped" cytoplasm and has been used for this purpose in studies on nerve endings (synaptosomes) isolated from guinea pig cortex (27).

From the present data, it can be observed that LDH exhibits a distribution pattern distinct from any other activity measured—notably, the high proportion of this enzyme in the supernatant. 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 even fragments of neuronal membrane on which the enzyme is adsorbed. Fraction 2 is known to contain neurosecretosomes with relatively little contamination by other particles. This conclusion is based, on one hand, on electron microscopic studies. If it is assumed that the 10% of the total LDH activity which occurs in fraction 1 represents pieces of tissue, then only about 15% of the nerve endings has survived homogenization and been isolated as occluded cytoplasm. It was found that glucose catabolism of fraction 2 is very similar to that of posterior pituitary slices (36, 37), indicating that the neurosecretosomes retain the metabolic integrity of intact nerve endings.

Subfractionation of Neurosecretosomes

The isolation of neurosecretosomes by LaBella and Sanwal (40) has provided a preparation which can be utilized to yield useful information concerning the neurosecretory process. The first application which occurred to us was the possibility of separating vasopressinergic from oxytocinergic (this terminology was proposed by Lederis, reference 45) neurosecretosomes by centrifugation procedures.

By definition (USP), the VP/OT ratio in pooled, acetone-powdered bovine posterior pituitary glands is 1.0. We have observed that the ratio in individual or a few pooled glands can vary widely, and presumably, the value 1.0 is found only for a very large number of pooled posterior pituitaries. In the neurosecretosome fraction, the VP/OT ratio usually ranged between 0.6 and 0.8. It is possible that the method of isolation of the neurosecretosome fraction does not sediment a random sample of neurosecretosomes but rather, a

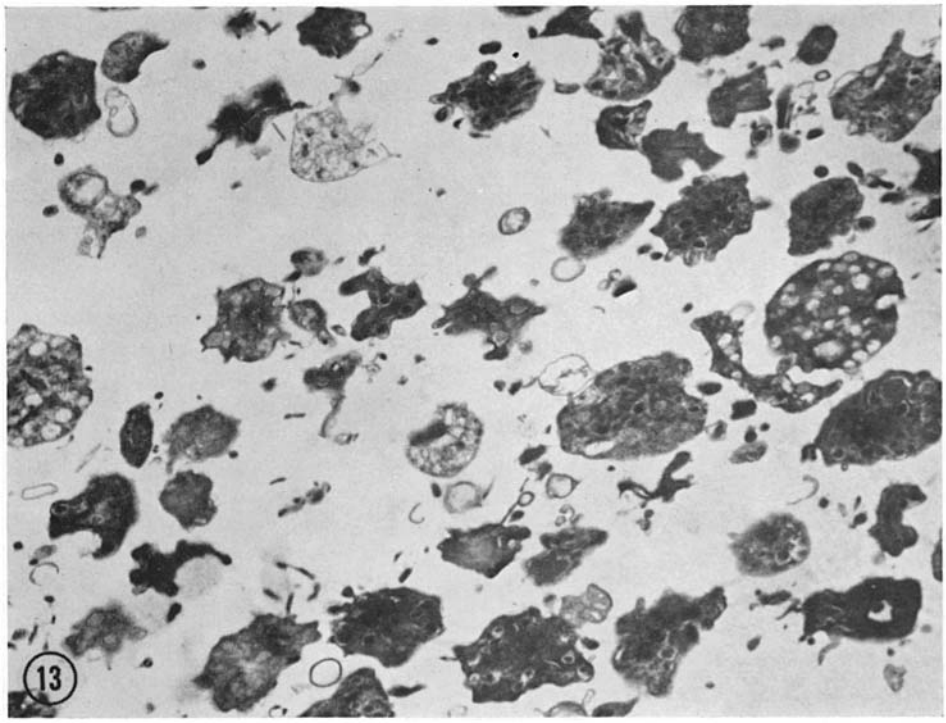

FIGURE 13 Section of OsO_4-fixed pellet of neurosecretosomes from density-gradient band *A* (see Fig. 2). × 11,000.

TABLE II

VP/OT Ratio of Neurosecretosomes Previously Suspended in Varying Concentrations of Sucrose

Sucrose conc.	Experiment			
	1	2	3	4
%	*VP/OT*			
10	0.76	0.62	0.79	0.66
40	0.62	1.10	0.72	0.64
50	0.67	1.10	0.93	0.62
60	0.59	0.91	0.83	0.57

TABLE III

Diameters of Neurosecretory Granules within Neurosecretosomes

Fraction	Diameter*
	mμ
A	180 ± 68
B	165 ± 55
C	205 ± 55

*Mean and standard deviation of 300 to 600 granules.

greater number which contain OT. The higher OT content of this fraction as compared to other fractions of homogenized posterior pituitary glands has been pointed out above. The differences in hormone ratios of the three subfractions of neurosecretosomes indicate that more than one species of nerve ending are present in the posterior lobe. NSG fractions, relatively enriched in either VP or OT activity, can be separated to some extent by both differential and density gradient centrifugation (3, 38, 39, 50). The sedimentation of isolated bovine NSG on a discontinuous sucrose gradient (39), similar to the one used here, resulted in subfractions whose VP/OT ratios were comparable to those found in subfractions of neurosecretosomes. This similarity suggests that the sedimentation of a neurosecretosome is largely determined by the type of NSG it contains. This possibility leads to further conjecture that only one of the two types of NSG and, consequently, only

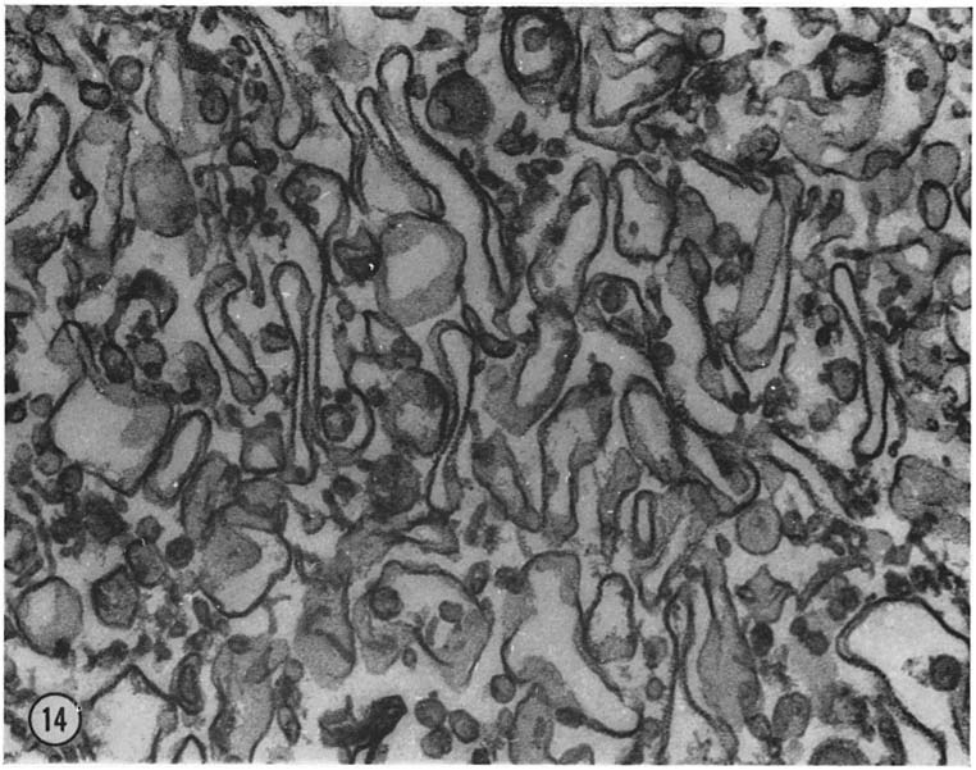

FIGURE 14 OsO_4-fixed pellet of density-gradient band *A* (see Fig. 3) derived from osmolyzed neurosecretosomes. This fraction appears to be comprised exclusively of MV and NSG membranes. × 63,000.

one of the two hormones is contained in a single neurosecretosome. The present findings support the hypothesis that any single neuron of the hypothalamo-hypophysial tract synthesizes, stores, and secretes either VP or OT.

The hypothalamo-hypophysial tract has, to some extent, the ability to control differentially the release of the two hormones. Electrical stimulation of the pituitary stalk was reported to cause the release of more OT than VP (23). Stimulation of the paraventricular nucleus caused the release of OT as measured by milk ejection in lactating rabbits, whereas no antidiuretic activity could be detected (11). Abrahams and Pickford (1) used a variety of stimuli known to inhibit urine flow by causing the release of VP and found that, in all cases, more OT than VP was released. Olivecrona (48) found that production of lesions of the paraventricular nucleus caused a decrease in the amount of OT in the posterior lobe and proposed that this nucleus was concerned with the synthesis of OT and the supraoptic nucleus with VP. This was supported by Bisset et al. (7) who reported that localized stimulation of each of the two nuclei caused release of the corresponding hormone into the blood. The two hormones do not appear simultaneously in the hypothalamus during embryological development (18), providing evidence that two separate systems are responsible for their synthesis. The electrical responses of the supraoptic nucleus to a variety of stimuli (53) differ from those of the paraventricular nucleus (10), suggesting a separation of function of the two nuclei. Electrical activity has been recorded in the pituitary stalk (57) and in presumed neurosecretory cells in the hypothalamic nuclei (12). These cells resemble other neurons in their electrical activity which, in the paraventricular nucleus, has been directly related to the release of OT (10). If release of hormone is initiated by depolarization of neurons, any control which the hypothalamus may exert over the differential release of hormones is

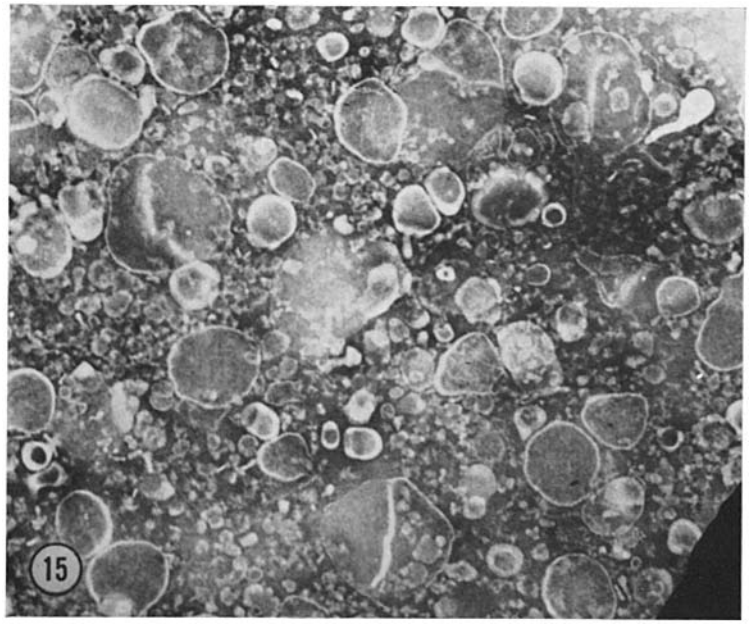

FIGURE 15 Negative-stain of density-gradient band *A* (see Fig. 3) derived from osmolyzed neurosecretosomes. This is the same preparation which was examined after OsO_4 fixation, shown in Fig. 14. The NSG membranes are spread out in this preparation in contrast to the distorted appearance in the OsO_4-fixed pellet. × 33,000.

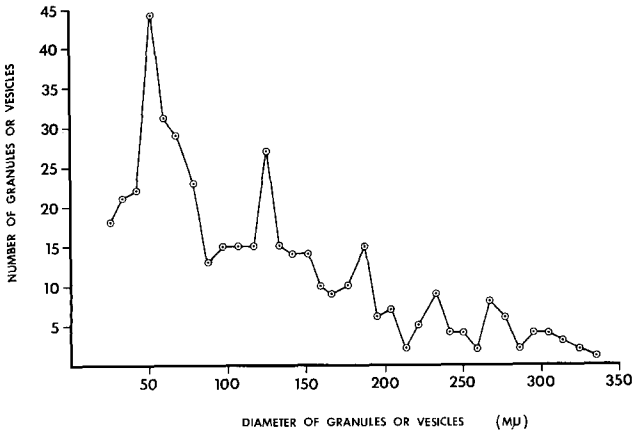

FIGURE 16 Frequency distribution of particles observed in density-gradient band *A* (see Fig. 3). A field of several hundred particles was counted. This fraction was isolated from an osmolysate of neurosecretosomes and appears to consist of NSG membranes and MV.

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, including the observation that each of the hormones is apparently stored in a distinct type of NSG (38, 39) and the present finding that the neurosecretosomes can, to some extent, be separated into vasopressinergic and oxytocinergic varieties, support repeated speculation that there are neurons specific for each hormone. The present finding that both hormones were found in all three subfractions of neurosecretosomes, although in different ratios, indicates that the density of a specific population of neurosecretosomes is distributed over a range which overlaps that of other populations. It has been

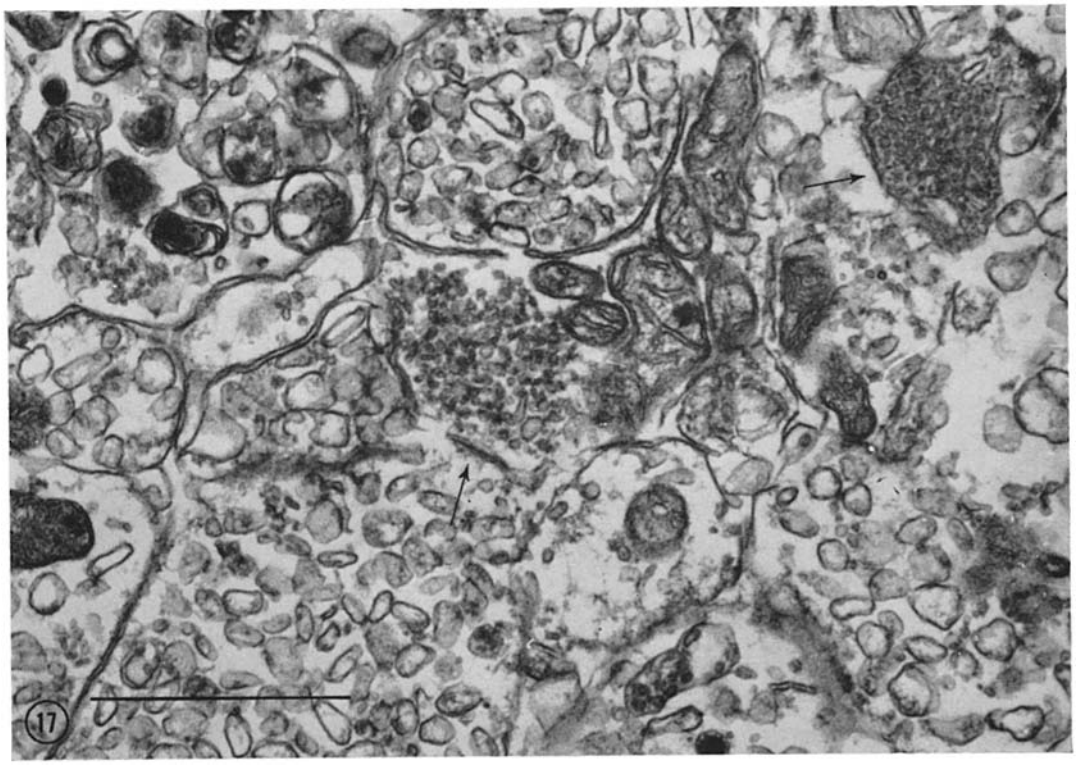

FIGURE 17 Section of whole, OsO_4-fixed posterior pituitary gland. Two nerve endings (arrows) are present which contain mitochondria and small vesicles and lack NSG. The small vesicles within these terminals seem to be more densely staining than the MV in surrounding typical neurosecretory nerve endings. × 59,000.

proposed that differences in the staining properties of NSG may reflect their content of either VP or OT (22). It has also been noted that, in fixed sections of whole tissue, some nerve endings contain larger NSG than others (40). However, in the present study, electron microscopy of the three fractions failed to show any morphological distinctions between the NSG in the neurosecretosomes of the three subfractions. The possibility remains that both hormones are present in all neurosecretosomes, but in varying ratios.

Another interpretation of the present results is that there is a fixed VP/OT ratio in all of the neurosecretosomes and the differences found among the density-gradient subfractions are due to the presence of free NSG which were present in the original neurosecretosome fraction. This possibility seemed unlikely, as there are few free NSG seen in the electron microscopic photographs of these fractions and, besides, the differences in size between NSG and neurosecretosomes is such that they would not be expected to sediment in the same fraction. Furthermore, as the neurosecretosomes are comprised primarily of NSG, it would require a considerable amount of the free NSG to account for the differences in the VP/OT ratios found. The LDH activity is similar in all three fractions, indicating the absence of significant contamination of these fractions by nonneurosecretosomal elements.

Lysosomal Enzymes in Posterior Pituitary

The lysosomal enzyme, AP, was distributed similarly to the mitochondrial enzyme, SDH. (Lysosomes tend to sediment with mitochondria from homogenates of tissues in general.) In this respect, the posterior pituitary resembles brain tissue (34, 56). Electron microscopic–histochemistry of brain indicates that acid phosphatase activity, presumably of lysosomal origin, is localized

to dense bodies found in glial cells and in neuronal cell bodies but not in nerve terminals (54). In the present work, it cannot be definitely determined whether AP present in fraction 2 is intra- or extra-neurosecretosomal, but we have done some preliminary studies on the ultrastructural localization of acid phosphatase and found a positive reaction in supporting cells, but not in the nerve terminals of the posterior lobe. It appears that lysosomal enzymes in homogenates of the posterior pituitary are not a component of the neurosecretosomes, but are probably in lysosomes derived from pituicytes and other nonneural cells.

Isolated Microvesicles

At present, knowledge concerning the neurohypophysial MV is limited to the electron microscopic 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 (20). Similarly, an increase in synaptic vesicles has been reported on stimulating the cholinergic nerves to adrenal medullary cells (15). These similarities have led to the speculation that the MV contain a neurohumoral mediator such as ACh for the release of VP and OT (20). Koelle and Geesey (33) have identified cholinesterase (ChE) histochemically in the posterior lobe of the cat. Most of the activity was that of nonspecific 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 (32). A substance with "acetylcholine-like" activity was reported to be present in extracts of bovine neurohypophysis (55), but one of the authors subsequently attributed this activity to interfering substances (31).

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 (5) or on capillary endothelium to promote the passage of hormone into the vascular system (8). The identification of sympathetic fibers (13) and a positive fluorescent reaction for sympathomimetic amines (19) have been claimed for the posterior lobe. However, in preliminary experiments, we were unable to detect catecholamine in whole posterior lobes assayed by a sensitive fluorometric method.

Small vesicles, resembling MV, have been observed within apparent NSG in the neurohypophysis of the rat and ferret (25). It has been proposed that the MV are an additional storage site for the hormones, possibly more readily released than the NSG (40). If this were true, the MV would be expected to have a high specific activity of hormones, at least 16–20 U/mg protein which we have found for NSG (39). We have assayed the MV fraction for its content of VP and have found a specific activity of about 2 U/mg protein. It cannot be absolutely concluded, however, that the hormone is absent from the MV, because the fraction is not pure MV and contamination by larger particles can greatly inflate the estimate of total protein, and, alternatively, osmolysis may have removed hormone from the MV. Kobayashi et al. (30) 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 (46). Our MV fraction also contains a wide range of particle diameters, and the distinction between a large MV and a small NSG becomes arbitrary. Our inability to obtain a pure MV fraction may indicate that there is no discrete population of MV distinct from the larger membranous components.

Several investigators have proposed that the MV may be a product of the breakdown of the NSG (4, 9, 25, 29, 40, 46). The present results are interpreted as supporting this theory: however, not breakdown of the NSG per se, but of NSG membranes. MV are often seen in the vicinity of NSG that appear to be in the process of breaking apart, and, conversely, disrupted NSG are almost always associated with the presence of MV. It is proposed that there is a sequence of events in which depletion of neurosecretion from the NSG is followed by dissolution of the NSG membrane. This sequence includes the formation of MV from the free edges of broken membranes. It is likely that physical forces acting on free membrane edges will promote the formation of bodies whose surface volume ratio is minimal, i.e., spheres. Erythrocyte membranes have been observed to form cellules in this manner (47).

The method of isolating neurosecretosomes may

not permit the isolation of atypical nerve endings, seen in whole tissue sections. The latter have not been definitely identified in centrifugation fractions, but this may reflect their relatively rare occurrence in the posterior lobe as a whole. These structures are filled with MV, lack NSG, and are further distinguished from those containing NSG by their smaller size. It is possible that these nerve endings are the terminals of a nonneurosecretory type of neurons. These presumably nonhormonal neurons may, in fact, contain acetylcholine or other humoral transmitter that plays a yet undefined role in the posterior pituitary.

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