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Membrane retrieval in the guinea-pig neurohypophysis

Isolation and characterization of secretory vesicles and coated microvesicles after radiolabel incorporation *in vivo*

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We have developed small-scale methods for the isolation and biochemical characterization of subcellular fractions from single guinea-pig posterior-pituitary glands. Secretory vesicles and coated microvesicles produced in this way were of similar purity to those isolated from large amounts of tissue by conventional ultracentrifugation. [^{35}S]Cysteine injected into the hypothalamus was found in the soluble contents of secretory vesicles isolated from the neural lobes 24h later. High-pressure liquid-chromatographic analysis revealed that the radiolabel was incorporated into the expected neurosecretory products (oxytocin, vasopressin and neurophysin) and also into a biosynthetic intermediate in the vasopressin system. The membranes of secretory vesicles were labelled with [^{3}H]choline 24h after its hypothalamic injection. Little or no [^{3}H]choline could be demonstrated in coated microvesicles at this time, although these structures were labelled 5 days after injection. Stimulating hormone secretion by chronic dehydration produced a significant fall in [^{3}H]choline content of the secretory-vesicle membranes without any transfer of label into coated microvesicles, suggesting that coated microvesicles are not involved in membrane retrieval in the neurohypophysis.

The neurohypophysial peptides oxytocin and vasopressin are synthesized together with their carrier proteins, the neurophysins (NPs), in hypothalamic magnocellular neurons, packaged into secretory vesicles (SVs) and transported to the nerve terminals in the neural (posterior) lobe of the pituitary gland, where they are stored for release into the bloodstream. Depolarization of the terminals causes secretion by a process of exocytosis, involving fusion of the SVs with the plasma membrane (Nagasawa *et al.*, 1970). A necessary consequence of this process is the retrieval of the SV membrane, or an equivalent area of plasma membrane, to prevent the continual expansion of the nerve terminal.

It has been suggested that membrane retrieval in the neurohypophysis occurs by uptake into a pool of coated microvesicles (CMVs) (Nagasawa *et al.*, 1971), which eventually lose their coat (Douglas *et al.*, 1971). Extracellular-fluid markers have been shown to enter CMVs, but, since the number of CMVs does not increase after stimulation, it has been argued that retrieval must proceed via another structure (Castel, 1974; Nordmann *et al.*, 1979; Morris & Nordmann, 1980, 1982). However, the lack of effect of stimulation on the uptake of external-fluid markers into CMVs could be explained if formation of CMVs was not the rate-limiting step in membrane retrieval.

In an elegant approach, Swann & Pickering (1976) showed that injection of radiolabelled compounds *in vivo* (Sachs *et al.*, 1969; Pickering *et al.*, 1975) could be used to study their incorporation into neurohypophysial subcellular structures. They reported that [^{3}H]choline and [^{35}S]cysteine injections into the cisterna magna resulted in labelling of the membranes and contents respectively of an SV-enriched fraction isolated from rat neural lobes. We have now used this approach to investigate whether any components of the SVs are retrieved directly into CMVs. A new method has

Abbreviations used: NP(s), neurophysin(s); SV(s), secretory vesicle(s); CMV(s), coated microvesicles; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulphonic acid; Mes, 4-morpholine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

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been developed for the rapid isolation of SVs from single posterior pituitary glands of guinea pigs that uses a bench-top microcentrifuge (MSE Microcentaur). Small-volume adaptors and sensitive assays also allowed the isolation and characterization of other subcellular fractions, including CMVs, by conventional ultracentrifugation.

Experimental

Materials

All reagents were of analytical grade. Percoll (Pharmacia) was dialysed for 24h against homogenization buffer containing 190mM-sucrose, 20mM-Tes, 25mM-EGTA, pH7.0 (320mosM), and the volume restored with the same buffer before use. Reagents for enzyme assays were from Sigma, except for Malachite Green (Fluka) and *o*-phthalaldehyde (BDH). [^{3}H]Choline ([*methyl*-^{3}H]choline chloride, 80Ci/mmol) and [^{35}S]cysteine (~1000Ci/mmol) were obtained from Amersham International. [^{3}H]Choline was concentrated 10-fold before injection. Polypropylene microcentrifuge tubes (1.5ml and 0.4ml) were obtained from Sarstedt, Leicester, U.K., and Alpha Laboratories, Eastleigh, Hants., U.K., respectively.

Isolation of SVs

Guinea-pig neural lobes were homogenized in buffer at 0°C (300μl/lobe) with a glass homogenizer and a Teflon pestle (0.08mm clearance) rotating at 1000rev./min. The homogenate (H) was centrifuged at 900$g_{av.}$ for 10min (MSE Chilspin). The pellet (P_1) was saved and the supernatant centrifuged for 10min at 13000$g_{av.}$ in the MSE Microcentaur in a 1.5ml polypropylene microcentrifuge tube. The supernatant (S_2) was saved, and the pellet (P_2), reconstituted in 100μl of buffer, was layered on top of a dialysed Percoll gradient composed of 50μl of undiluted Percoll and 250μl of 50% (v/v) Percoll, in a 400μl polypropylene microcentrifuge tube. After microcentrifugation (13000$g_{av.}$, 10min), 150μl was collected from the bottom of the tube (SV), the remaining 250μl (S_3) being saved for analysis of recovery. In some experiments, the material from Percoll gradients was further fractionated on sucrose gradients. The SV or S_3 fractions were diluted 1:3 in 300mM buffered/(1mM-EDTA/1mM-Mes, pH6.5) sucrose and centrifuged at 140000$g_{av.}$ for 20min to remove most of the Percoll. The loose membrane pellet and supernatant was recovered, centrifuged at 13000$g_{av.}$ for 10min (Microcentaur), the pellet resuspended in 250μl of buffered sucrose and layered on a linear 0.85–1.2M-sucrose gradient. A cushion of 0.5ml of 2M buffered sucrose prevented the material pelleting at the bottom of the tube. The gradients were centrifuged for 1.5h at 50000$g_{av.}$ in the Beckman SW 55 rotor (5.5ml tubes).

Isolation of CMVs

CMVs were isolated by two different procedures. By using the approach of Pearse (1975), as modified by Torp-Pedersen *et al.* (1980), the S_2 (see under 'Isolation of SVs') was diluted 1:4 in 100mM-Mes/1mM-$MgCl_2$/0.5mM-EGTA, pH6.5, and centrifuged for 30min at 30000$g_{av.}$ at 4°C in a Beckman SW55 rotor; 600μl Ultroclear tubes were used in adaptors. The pellet (P_{30}) was saved and the supernatant placed on a gradient composed of 1ml each of 60%, 50% and 40% (w/v) sucrose in Mes buffer, followed by 0.5ml each of 30% and 20% sucrose. The tubes were standard 5.5ml Beckman tubes. The gradients were centrifuged for 16h at 50000$g_{av.}$. In some experiments the CMVs were diluted 1:3 in Mes buffer and centrifuged at 80000$g_{av.}$ for 30min, followed by centrifugation for 45min at 50000$g_{av.}$ on a continuous 5–30%-sucrose gradient (Pearse, 1975). CMVs were recovered as a single band from the middle of this gradient. For [^{35}S]cysteine incorporation studies, CMVs were isolated rapidly by a modification of the method of Nandi *et al.* (1982). The procedure to obtain P_{30} was as described above. The supernatant was centrifuged at 80000$g_{av.}$ for 30min to produce a supernatant, S_{80}, which was saved. The pellet was placed on top of 200μl of Mes buffer in ^2H_2O and centrifuged at 80000$g_{av.}$ for 2h at 10°C using the 600μl adaptors. The pellet (CMV) was used for further experiments, the supernatant ('Rest') being saved for calculations of recovery.

Assays

Acid phosphatase (EC 3.1.3.2) activity was measured with Malachite Green (Saermark *et al.*, 1983; ATPases (EC 3.6.1.3) were assayed as previously described (Saermark & Vilhardt, 1979). Rotenone-insensitive NADH reductase (NADH dehydrogenase, EC 1.6.99.3) was assayed as described by Omura & Takiesue (1970) in a total incubation volume of 500μl. Succinate dehydrogenase (EC 1.3.99.1) was assayed in 100μl of 25mM-NaH_2PO_4, pH7.4, containing 0.5% *p*-iodonitrotetrazolium, 250mM-sodium succinate and 100mM-EDTA. The formazan formed was extracted in 500μl of ethyl acetate and its A_{490} read. Protein was determined with *o*-phthalaldehyde (0.8mg/ml), dissolved in 0.2M-sodium borate buffer, pH10.4, containing 0.1% Brij and 0.2% mercaptoethanol (Benson & Hare, 1975). Samples (100μl) containing 1% SDS were mixed with 1ml of buffered *o*-phthalaldehyde reagent and fluorescence determined at 340nm (excitation) and 435nm (emission). Bovine serum albumin was used as standard. Oxytocin, vasopressin and NP

were measured by radioimmunoassay as previously described (Robinson, 1980; Robinson *et al.*, 1981; Robinson & Jones, 1982). Radioactivity in material incorporating [^{35}S]cysteine or [^{3}H]choline was measured by liquid-scintillation counting. Reverse-phase high-pressure liquid chromatography was carried out on a Spectraphysics 8700 system with a column (70mm × 4.6mm) of Nucleosil 5μC_8 eluted at 1ml/min with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The eluate was monitored at 215nm and 1ml fractions were collected. All values are expressed as means ± S.D. unless otherwise stated. Mean differences were assessed by Student's *t* test.

Radiolabel incorporation in vivo

Guinea pigs (Hartley strain, 500–700g body wt.) were given bilateral injections of [^{35}S]cysteine or [^{3}H]choline (2μl, 40μCi) in the paraventricular nuclei under halothane anaesthesia (Robinson & Jones, 1983). Animals were killed at different times after injection and the neural lobes rapidly removed. On some occasions animals were dehydrated by giving 2% NaCl in place of drinking water for 5 days.

Electron microscopy

Electron micrographs of CMV preparations were obtained by the method of Pearse (1975) after concentration of the samples in an Airfuge (Beckman). The samples were stained with 1% uranyl acetate and examined in a Philips EM 300 electron microscope.

Results

Purity of the membrane fractions

Total protein, vasopressin content, succinate dehydrogenase (mitochondrial membranes), acid phosphatase (lysosomes), rotenone-insensitive NADH reductase (endoplasmic reticulum) and Ca^{2+}/Mg^{2+}- and Na^{+}/K^{+}-dependent ATPase activities (plasma membrane) were measured in subcellular fractions of the guinea-pig neurohypophysis, and their distributions are shown in Table 1. With the exception of acid phosphatase, all the markers showed a relative purification of SVs. Varying the Percoll concentration did not change the ratio of acid phosphatase to vasopressin content. The density of guinea-pig SVs, estimated by comparison with density marker beads, was 1.13 ± 0.01g/ml ($n = 5$). The SVs were stable, releasing only 14 ± 4% ($n = 3$) of their hormone content over a period of 3h on ice.

CMVs isolated by the method of Pearse (1975) were at least 80% pure as judged by electron microscopy (Fig. 1), and almost all the coats were associated with a membrane structure. As purity was only slightly improved by a further centrifugation step (see the Experimental section), CMVs were routinely isolated by using only the 16h centrifugation procedure. The modified method of Nandi *et al.* (1982) produced a CMV fraction of approx. 50% purity, probably due to the omission of several pelleting/resuspension steps described in the original method. These produced unacceptable losses with the small amounts of neural-lobe tissue used in the present experiments.

Incorporation of [^{35}S]cysteine and [^{3}H]choline into SVs

The distributions of ^{35}S and ^{3}H radioactivity in subcellular fractions of neural lobes 24h after injection *in vivo* of [^{35}S]cysteine or [^{3}H]choline are shown in Tables 2 and 3. The distribution of ^{35}S radioactivity paralleled that of vasopressin and NP. More than 90% of the ^{35}S radioactivity was

Table 1. *Distribution of marker enzymes in fractions from the purification of secretory vesicles*
Homogenates of neural lobes were fractionated by differential and Percoll gradient centrifugation as described in the Experimental section. The activities for the various markers in each fraction are expressed as a percentage of the total homogenate activity. Also included is the relative specific activity (R.S.A.), defined as the percentage activity divided by the percentage of protein in a fraction, calculated with respect to P_2. The recovery for each marker represents the mean of the individual recoveries in separate experiments. Values given are means ± S.D.; *n* is the number of experiments, each performed on a single neural lobe.

		Activity (% of that in the homogenate)						R.S.A. relative to P_2
	n	P_1	S_2	P_2	S_3	SV	Recovery (%)	
Vasopressin	9	23 ± 9	19 ± 12	47 ± 17	29 ± 5	12 ± 2	87 ± 15	4.3
Neurophysin	4	36 ± 10	15 ± 8	44 ± 12	15 ± 7	13 ± 5	76 ± 12	5.0
Protein	5	36 ± 4	69 ± 3	25 ± 5	22 ± 5	1.5 ± 0.3	130 ± 5	–
Succinate dehydrogenase	5	43 ± 7	12 ± 1	38 ± 4	34 ± 5	0.5 ± 0.1	90 ± 7	0.2
Acid phosphatase	5	33 ± 5	50 ± 7	37 ± 10	25 ± 10	2.5 ± 0.5	111 ± 6	1.1
NADH reductase	6	35 ± 8	31 ± 3	23 ± 4	20 ± 4	0.25 ± 0.01	92 ± 18	0.2
Na^{+}/K^{+}-ATPase	3	47 ± 2	28 ± 5	17 ± 1	15 ± 4	0.2 ± 0.2	110 ± 5	0.2
Ca^{2+}/Mg^{2+}-ATPase	6	41 ± 5	44 ± 4	17 ± 5	18 ± 3	0.12 ± 0.05	110 ± 7	0.1

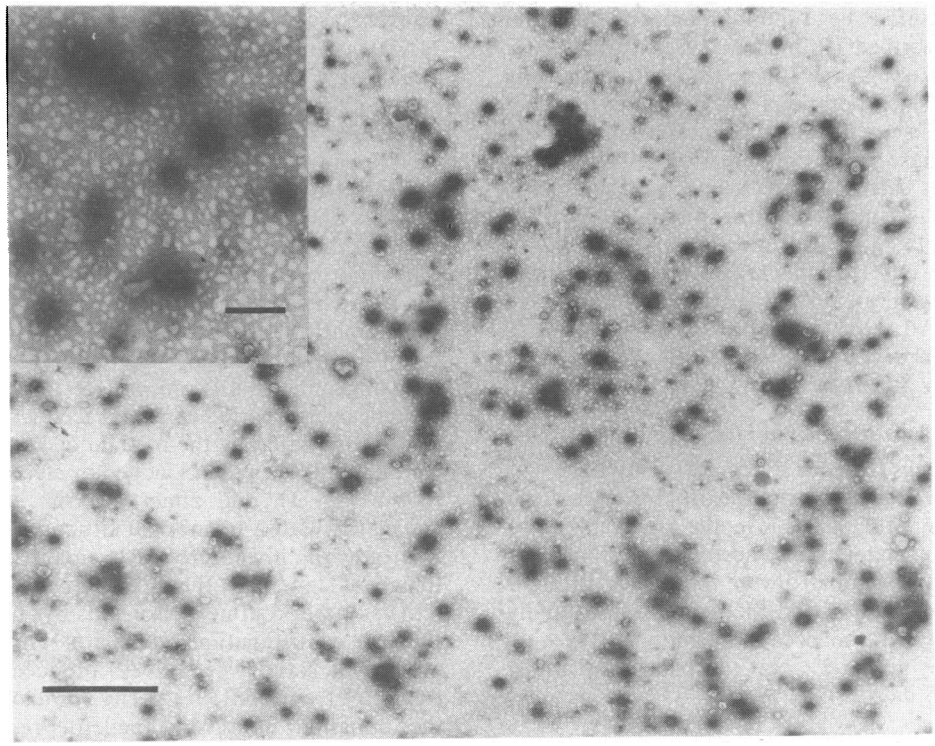

Fig. 1. *CMVs isolated from the neural lobe*
The Figure shows an electron micrograph of CMVs obtained by sucrose-density-gradient centrifugation using the modified method of Pearse (1975). The vesicles were fixed on a carbon-coated grid and stained with 1% uranyl acetate. The scale bar represents 1 μm (on the inset, 0.2 μm).

Table 2. *Distribution of [^{3}H]choline and [^{35}S]cysteine in different subcellular fractions from the neurohypophysis*
Radioactive label was injected into the hypothalamus and the neurohypophysis was removed after 24h. SVs and CMVs were isolated as described in the Experimental section, and the percentage distribution of radiolabel in each fraction determined with respect to the original homogenate (total c.p.m.: ^{35}S, 200000; ^{3}H, 100000). Results are expressed as means ± S.D. for three experiments, each performed with neural lobes pooled from three or four animals.

	Percentage distribution in:				
	SVs			CMVs	
Fraction	[^{3}H]Choline	[^{35}S]Cysteine	Fraction	[^{3}H]Choline	[^{35}S]Cysteine
H	100	100	S_2	37±10	36±6
P_1	19±5	35±10	S_{80}	6.7±1	18±7
P_2	22±7	29±11	P_{30}	4±1	10±6
S_3	19±5	19±3	P_{80}	16±3	0.7±0.1
			Rest	14±3	0.6±0.1
SV	9.8±3	7±1.4	CMV	<0.1	0.09±0.05

Recovery: [^{3}H]choline, 89%±10; [^{35}S]cysteine, 83±11

recovered in acid extracts of SVs and, when analysed by high-pressure liquid chromatography (Fig. 2), most of the radioactivity was shown to be incorporated in oxytocin, vasopressin, their respective NPs and a component which has previously been identified (Robinson & Jones, 1983) as an intermediate in the processing of the vasopressin precursor (Land *et al.*, 1982).

The SV fraction also contained ^{3}H radioactivity 24h after hypothalamic injection of [^{3}H]choline

Table 3. *Selective labelling of secretory vesicles after 24h*
The percentage distributions of [^{3}H]choline and [^{35}S]cysteine in secretory vesicles and coated microvesicles (from Table 2) were used to calculate their relative specific activities (percentage of radioactivity divided by percentage of protein, relative to the homogenate). A similar value was determined for unlabelled neurophysin (NP), measured by radioimmunoassay.

	Relative specific activity	
	SVs	CMVs
[^{3}H]Choline	6.5	<0.08
[^{35}S]Cysteine	4.7	0.15
NP	5.0	0.17

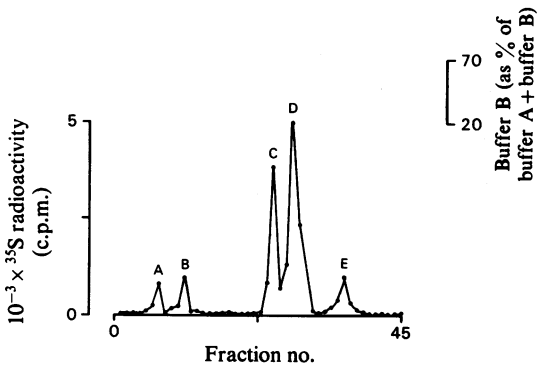

Fig. 2. *High-pressure-liquid-chromatographic analysis of ^{35}S-labelled SVs*
SVs were prepared from guinea-pig neural lobes 24h after hypothalamic injection of [^{35}S]cysteine. The SV fraction (prepared from two neural lobes) was centrifuged at 120000$g_{av.}$ for 10min (Airfuge) to remove Percoll, extracted in 100mM-HCl and subjected to reversed-phase high-pressure liquid chromatography using a gradient of acetonitrile in 0.1% trifluoroacetic acid (buffer A = 0.1% trifluoroacetic acid; buffer B = 60% (v/v) acetonitrile in buffer A). The broken line shows gradient; letters A–E indicate retention times of vasopressin, oxytocin, their related NPs and an NP-related biosynthetic intermediate respectively, as determined by radioimmunoassay.

(Table 2). The relative specific activity of [^{3}H]choline compared with the total protein content of the SV fraction was 6.5 (Table 3), but was twice as high (10–12) when expressed relative to the protein content of the SV membranes. Radiolabelled secretory vesicles were diluted with distilled water to 100mosM and centrifuged at 140000$g_{av.}$ for 30min. Osmotic lysis released 83±8% of the [^{35}S]cysteine radioactivity in the SV fraction into the supernatant, whereas 93±8% (n = 3) of the [^{3}H]choline radioactivity was retained in the membrane pellet.

Incorporation of [^{35}S]cysteine and [^{3}H]choline into CMVs

CMV fractions prepared from guinea-pig neural lobes 24h after hypothalamic injection of [^{35}S]-cysteine contained very little radioactivity (0.09±0.05%, Table 2). The CMV fraction had a protein content of 0.62±0.2% (n = 5) that of the homogenate, and an NP content of 87±6ng (n = 3), equivalent to 0.1% of the total homogenate content. CMV fractions were also prepared from unlabelled neural lobes by using, as homogenization buffer, the S_2 supernatant (centrifuged at 140000$g_{av.}$ for 30min) from [^{35}S]cysteine-treated animals. Under these conditions CMV fractions from the unlabelled neural lobes contained 1.5±0.1% (n = 3) of the ^{35}S present in the homogenization buffer. CMV fractions prepared from labelled neural lobes by the method of Nandi *et al.* (1982) contained even lower levels of ^{35}S (0.2±0.1%, n = 3). These results suggest that the small amounts of NP associated with the CMV fraction can be explained by adsorption during the purification procedure.

Injection of [^{3}H]choline *in vivo* produced little incorporation into CMVs 24h after injection (Table 2). Fig. 3 shows the distribution of [^{3}H]choline and protein on sucrose gradients used in the preparation of CMVs. Although most of the protein was in the fractions containing CMVs,

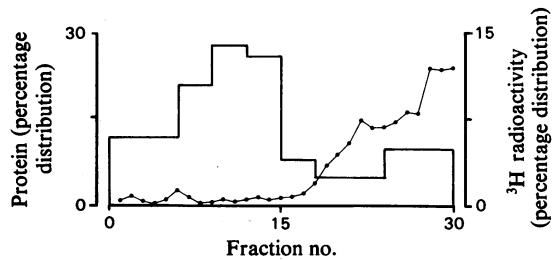

Fig. 3. *Distribution of [^{3}H]choline and protein on sucrose gradients used for preparation of CMVs*
A CMV fraction was prepared from neurohypophyses 24h after hypothalamic injection of [^{3}H]choline. The CMV-enriched pellet obtained by the initial differential-centrifugation procedure was placed on a sucrose gradient (20–60% in buffer, see the Experimental section) and centrifuged at 50000$g_{av.}$ for 16h. The gradients were fractionated from the bottom and the [^{3}H]choline content measured (●, four animals). The protein content (histogram) was determined on similar sucrose gradients using unlabelled material from six animals.

most of the ^3H radioactivity was located in a less-dense part of the gradient. At 24h after injection of the animals, the CMV fraction contained less than 0.1% of the ^3H radioactivity in the homogenate, whereas the SV fraction contained 9.8% (Table 2), giving relative specific activities of < 0.08 and 6.5, respectively (Table 3). CMV fractions prepared 5days after hypothalamic injection contained $1.5 \pm 0.4\%$ ($n = 3$) of the total ^3H radioactivity in the homogenate.

Effects of dehydration

Dehydrating guinea pigs for 5 days markedly depleted the neural-lobe stores of hormone (Fig. 4) and significantly decreased the [^3H]choline content of the SVs (control, $2.2 \pm 0.6\%$; dehydrated, $1.1 \pm 0.2\%$; $n = 3$, $P < 0.05$). Dehydration caused no change in the incorporation of [^3H]choline into CMVs (control, $1.5 \pm 0.4\%$; dehydrated, $1.5 \pm 0.2\%$; $n = 3$, $P > 0.2$). Material from the Percoll gradients was further fractionated by sucrose-density-gradient centrifugation. Fig. 5 shows the distribution of ^3H radioactivity from the upper part (S_3) of the Percoll gradient separation of material 24h after [^3H]choline labelling, compared with that of the ^{35}S radioactivity from the lower part of the Percoll gradient (i.e. the SV fraction) obtained from a single animal 24h after injection of [^{35}S]cysteine. Most of the ^{35}S radioactivity was found in a single peak corresponding to the position of SVs on this gradient. Small amounts of ^3H radioactivity were associated with this peak, but most of the [^3H]choline was found in a less-dense fraction. This was the only fraction that showed a significant increase in [^3H]choline content after chronic stimulation of hormone release (control, $4.6 \pm 0.9\%$; dehydrated, $6.1 \pm 1.0\%$; $n = 3$, $P < 0.05$).

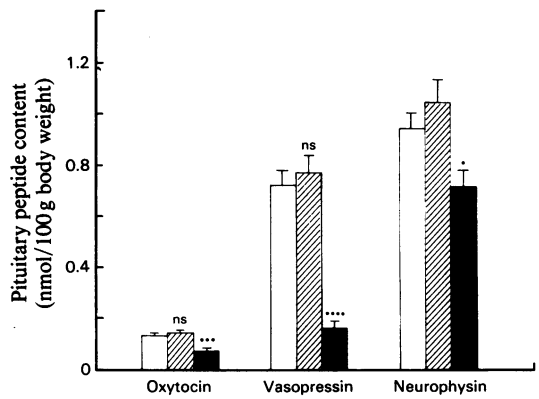

Fig. 4. *Depletion of neurohypophysial peptides by dehydration*

Guinea pigs were given 2% NaCl in place of drinking water for 5days, after which the posterior pituitary glands were removed, homogenized in 50mM-HCl, centrifuged at $8000 g_{av.}$ for 2min, and the supernatant assayed for oxytocin, vasopressin and NP. Pituitaries from dehydrated animals (■, $n = 9$) contained significantly less neuropeptides than those from normal males (□, $n = 10$) or females (▨, $n = 10$). Results are means ± S.E.M.; ...., $P < 0.001$; ..., $P < 0.01$; ., $P < 0.05$; ns, not significant.

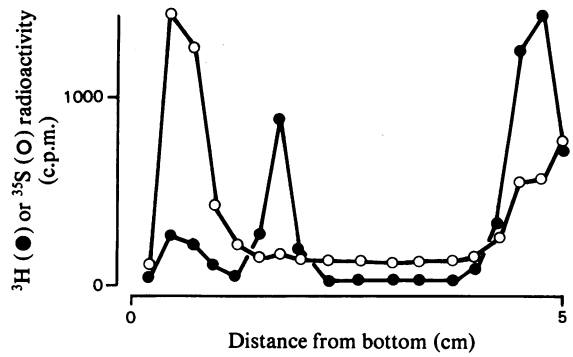

Fig. 5. *Distribution of [^3H]choline-containing membranes on a linear sucrose gradient 5 days after hypothalamic injection in dehydrated animals*

The Figure shows the distribution of ^3H radioactivity (●) from the S_3 fraction of three neural lobes removed 1 day after hypothalamic injection of [^3H]choline, fractionated on a linear gradient of 1.2–0.85M-sucrose (see the Experimental section). For comparison, ^{35}S-labelled SVs (○) from a single neural lobe were fractionated on an identical gradient. Both the gradients were fractionated from the bottom of the tube. The animals were dehydrated by giving 2% NaCl in place of drinking water for 4 days before, and 1 day after, injection of radiolabel.

Discussion

In the present paper we describe methods for the isolation and biochemical characterization of a variety of subcellular fractions from a single neural lobe of guinea pigs. By using a bench-top microcentrifuge, SVs were isolated on simple Percoll gradients under iso-osmotic conditions, with a purity similar to those obtained by others using Percoll (Gratzl *et al.*, 1980; Russell, 1981). Comparing our results with those of Gratzl *et al.* (1980) and Nordmann *et al.* (1979), it is apparent that all these methods co-purify lysosomal contaminants to a minor degree. However, SVs obtained by the Percoll method can be further purified on sucrose gradients to remove lysosomal contaminants (Vilhardt & Hope, 1974; Saermark *et al.*, 1983).

With the present methods, SVs can be prepared within 45min of the animal's death, as opposed to

several hours when bovine material is used (Gratzl *et al.*, 1980; Scherman *et al.*, 1982). The yield of SVs, assessed as the percentage of the total vasopressin recovered in the SV fraction, is slightly lower than reported for other methods using Percoll (Russell, 1981; Gratzl *et al.*, 1980), but significantly higher than reported for metrizamide (Nordmann *et al.*, 1979).

Electron microscopy of the upper part of the Percoll gradient showed the presence of numerous nerve endings (Vilhardt & Hope, 1974; Nordmann *et al.*, 1979). Increasing the first centrifugation from 900 to $3800g_{av.}$ decreased the vasopressin content in the S_3 by 60%. The density of guinea-pig SVs was similar to that reported for other species (Nordmann & Labouesse, 1981; Gratzl *et al.*, 1980), and we found no indication of separate subpopulations of SVs on these gradients (Nordmann *et al.*, 1979; Nordmann & Labouesse, 1981).

Purification of CMVs was achieved by scaling-down the methods of Nandi *et al.* (1982) and Torp-Pedersen *et al.* (1982). There are no convenient biochemical markers for CMVs, but estimates of purity using electron microscopy suggested that our modified methods gave CMVs of similar purity to the original method of Pearse (1975); scaling-down the method of Nandi *et al.* (1982) produced a CMV fraction of lower purity.

Isolation of subcellular fractions from the neurohypophysis after radiolabelling *in vivo* has been reported previously (Sachs *et al.*, 1969; Norstrom, 1975; Swann & Pickering, 1976; Nordmann & Labouesse, 1981). In the present experiments, [^{35}S]cysteine was found in the SV fraction isolated from single-guinea-pig neural lobes 24h after hypothalamic injection, and high-pressure liquid-chromatographic analysis confirmed the incorporation of radiolabelled cysteine into the expected neurohypophysial secretory products, which were quantitatively released from the SV fraction by osmotic lysis. We also confirmed that [^{3}H]choline was biosynthetically incorporated into the membrane component of guinea-pig SVs, and was not released by osmotic lysis (Swann & Pickering, 1976; Lacey & Pickering, 1982).

At 24h after labelling *in vivo* the SV fraction was enriched in both [^{3}H]choline and [^{35}S]cysteine, as would be expected from the rapid axonal transport of SVs (Jones & Pickering, 1972; Norstrom, 1975; Gainer *et al.*, 1977). In contrast, at this time CMVs contained much less [^{3}H]choline or [^{35}S]cysteine, and the small amounts of radioactivity that were present could be explained by adsorption during homogenization. After 5 days, however, CMVs contained significant amounts of ^{3}H, indicating that these structures do incorporate [^{3}H]choline, but that the CMV pool is labelled at a much slower rate than the SVs in the neural lobe.

The idea that membrane retrieval in the neurohypophysis might occur via CMVs was first suggested by Nagasawa *et al.* (1971) and Douglas *et al.* (1971). Coated pits have been described on both SV and plasma-membrane surfaces, and clusters of CMVs have been located near sites of fusion between SVs and the plasma membrane (Douglas *et al.*, 1971). Extracellular markers are taken up into pituitary nerve terminals after stimulation (Castel, 1974; Nordmann *et al.*, 1974; Theodosis *et al.*, 1976) and have been shown to enter both CMVs and smooth vesicles (Douglas *et al.*, 1971; Nagasawa *et al.*, 1970). On the other hand, the number of CMVs was not increased by stimulation (Theodosis *et al.*, 1977; Lescure & Nordmann, 1980), nor was the uptake of extracellular markers into CMVs stimulation-dependent. However, failure to demonstrate an effect of stimulation on CMV number or uptake of extracellular markers might not be conclusive, since the interior of newly formed CMVs may not be freely accessible to all markers of the extracellular space (Willingham & Pastan, 1981). The lack of effect of stimulation could easily be explained if formation of CMVs was not the rate-limiting step in membrane recapture, and that subsequent formation of smooth vesicles represented the site of accumulation of extracellular markers, as in the adrenal medulla (Nagasawa & Douglas, 1972).

There have been few biochemical studies of membrane-retrieval structures in the neurohypophysis. Pituitary CMVs have a similar membrane protein composition to brain CMVs (Torp-Pedersen *et al.*, 1980), but differ markedly from SV and plasma-membrane fractions. Furthermore, CMVs have a low cholesterol content (Pearse, 1975) and no Na^+/K^+-ATPase activity (Saermark & Thorn, 1982), which makes it unlikely that they arise from the general plasma-membrane pool. The use of extracellular-fluid markers in pituitary subcellular-fractionation studies is not straightforward, since the markers may also be taken up into glial cells whose activity increases with chronic stimulation of the neural lobe (Sachs *et al.*, 1971; Tweedle & Hatton, 1977, 1980). This difficulty can be avoided by using an internal marker incorporated by biosynthesis into SV membranes after hypothalamic injection.

Swann & Pickering (1976) were the first to use this approach to look for the transfer of [^{3}H]choline from SVs to a microsomal fraction. We have now extended this approach in guinea pigs, by performing further fractionation studies in order to measure the relative specific activity of [^{3}H]choline in purified fractions of subcellular structures whose membrane components might interchange during membrane retrieval.

If, after exocytosis, SV membrane retrieval

occurred directly into CMVs, one would expect to see a transfer of labelled choline between the membranes of these structures. We found no evidence for this. The relative specific activity of [^{3}H]choline was at least 60-fold less in the CMV fraction than in the SV fraction, and stimulation of exocytosis did not increase the [^{3}H]choline labelling of CMVs. We conclude from these results that the formation of CMVs by direct recapture of SV membranes does not occur to any significant extent in the posterior pituitary gland.

It is unlikely that our negative results can be explained by the kinetics of CMV turnover or dilution of [^{3}H]choline during fusion with the plasma membrane. The relative specific activity of [^{3}H]choline in the CMVs should have risen markedly as the SV membranes were recaptured, especially if newly synthesized SVs were released first (Sachs *et al.*, 1969). Stimulation of exocytosis by prolonged dehydration decreased the [^{3}H]choline of the SV fraction, and it was possible to locate a subcellular fraction which showed a concomitant increase in labelling. This fraction had a density different from that of both SVs and CMVs and might include the vacuoles, which have also been suggested to be retrieval structures in this tissue (Castel, 1974; Nordmann *et al.*, 1979; Morris & Nordmann, 1982). Interestingly, chronic dehydration depleted the neural-lobe stores of oxytocin and vasopressin to a greater extent than NP, as has also been found in the rat (North *et al.*, 1983).

The role of CMVs in the posterior pituitary gland remains unclear. These structures were labelled 5 days after hypothalamic injection of [^{3}H]choline, though this labelling was not stimulation-dependent. It is therefore possible that CMVs are synthesized in the cell body and transported slowly to the neural lobe. Alternatively the slow appearance of ^{3}H in the CMVs may simply reflect redistribution of the radiolabel within the nerve terminal. CMVs contained little or no oxytocin, vasopressin or NP, which would seem to exclude the possibility (Alonso & Aschenmacher, 1979) that CMVs might secrete or retrieve these neuropeptides directly. Perhaps they are involved in the transport of other substances from the cell body to organelles in the nerve endings (Forgac *et al.*, 1983). The combination of labelling *in vivo* and subcellular-fractionation studies on single laboratory animals now allows biochemical investigation of these and other subcellular structures in the posterior pituitary gland under different physiological conditions.

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References

Alonso, G. & Aschenmacher, I. (1979) *Cell Tissue Res.* **199**, 415–429

Benson, J. R. & Hare, P. E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 619–622

Castel, M. (1974) *Gen. Comp. Endocrinol.* **22**, 336–337

Douglas, W. W., Nagasawa, J. & Schultz, R. A. (1971) *Nature (London)* **232**, 340–341

Forgac, M., Cantley, L., Wiedenmann, B., Altsteil, L. & Branton, D. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1300–1303

Gainer, H., Sarne, Y. & Brownstein, M. J. (1977) *Science* **195**, 1354–1356

Gratzl, M., Torp-Pedersen, C., Dartt, D., Treiman, M. & Thorn, N. A. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1615–1628

Jones, C. W. & Pickering, B. T. (1972) *J. Physiol. (London)* **227**, 553–564

Lacey, J. H. & Pickering, B. T. (1982) *J. Endocrinol.* **88**, 115–123

Land, H., Schütz, G., Schmale, H. & Richter, D. (1982) *Nature (London)* **295**, 299–303

Lescure, H. & Nordmann, J. J. (1980) *Neuroscience* **5**, 651–659

Morris, J. F. & Nordmann, J. J. (1980) *Neuroscience* **5**, 639–649

Morris, J. F., & Nordmann, J. J. (1982) *Neuroscience* **7**, 1631–1639

Nagasawa, J. & Douglas, W. W. (1972) *Brain Res.* **37**, 141–145

Nagasawa, J., Douglas, W. W. & Schultz, R. A. (1970) *Nature (London)* **227**, 407–408

Nagasawa, J., Douglas, W. W. & Schultz, R. A. (1971) *Nature (London)* **232**, 341–342

Nandi, P. K., Trace, G., Van Jaarsveld, P. P., Lippoldt, R. E. & Edelhoch, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5881–5885

Nordmann, J. J. & Labouesse, J. (1981) *Science* **211**, 595–597

Nordmann, J. J., Dreifuss, J. J., Baker, P. F., Ravazzola, M., Malaisse-Lagae, F. & Orci, L. (1974) *Nature (London)* **250**, 155–157

Nordmann, J. J., Louis, F. & Morris, J. F. (1979) *Neuroscience* **4**, 1367–1379

Norstrom, A. (1975) *Ann. N.Y. Acad. Sci.* **248**, 46–63

North, W. G., LaRochelle, F. T. & Hardy, G. R. (1983) *J. Endocrinol.* **96**, 373–386

Omura, E. & Takiesue, A. (1970) *J. Biochem. (Tokyo)* **67**, 249–257

Pearse, B. M. F. (1975) *J. Mol. Biol.* **97**, 93–98

Pickering, B. T., Jones, C. W., Burford, G. D., McPherson, M., Swann, R. W., Heap, P. F. & Morris, J. F. (1975) *Ann. N.Y. Acad. Sci.* **248**, 15–35

Robinson, I. C. A. F. (1980) *J. Immunoassay* **1**, 323–347

Robinson, I. C. A. F. & Jones, P. M. (1982) *Neuroendocrinology* **34**, 59–63

Robinson, I. C. A. F. & Jones, P. M. (1983) *Neurosci. Lett.* **39**, 273–278

Robinson, I. C. A. F., Woolf, C. N. & Parsons, J. A. (1981) *J. Endocrinol.* **90**, 227–236

Russell, J. T. (1981) *Anal. Biochem.* **113**, 229–238

Sachs, H., Fawcett, P., Takabatake, Y. & Portanova, R. (1969) *Recent Prog. Horm. Res.* **25**, 447–491

Sachs, H., Saito, S. & Sunde, D. (1971) *Mem. Soc. Endocrinol.* **19**, 325–336

Saermark, T. & Thorn, N. A. (1982) *Cell Calcium* **3**, 561–582

Saermark, T. & Vilhardt, H. (1979) *Biochem. J.* **181**, 321–330

Saermark, T., Thorn, N. A. & Gratzl, M. (1983) *Cell Calcium* **4**, 151–171

Scherman, D., Nordmann, J. J. & Henry, J. P. (1982) *Biochemistry* **21**, 687–694

Swann, R. W. & Pickering, B. T. (1976) *J. Endocrinol.* **68**, 95–108

Theodosis, D. T., Dreifuss, J. J., Harris, M. C. & Orci, L. (1976) *J. Cell Biol.* **70**, 294–303

Theodosis, D. T., Dreifuss, J. J. & Orci, L. (1977) *Brain Res.* **123**, 159–163

Torp-Pedersen, C., Saermark, T., Bundgaard, M. & Thorn, N. A. (1980) *J. Neurochem.* **35**, 552–557

Tweedle, C. D. & Hatton, G. I. (1977) *Cell Tissue Res.* **181**, 59–72

Tweedle, C. D. & Hatton, G. I. (1980) *Neuroscience* **5**, 661–667

Vilhardt, H. & Hope, D. B. (1974) *Biochem. J.* **143**, 181–189

Willingham, M. C. & Pastan, I. (1981) *J. Histochem. Cytochem.* **29**, 1003–1013