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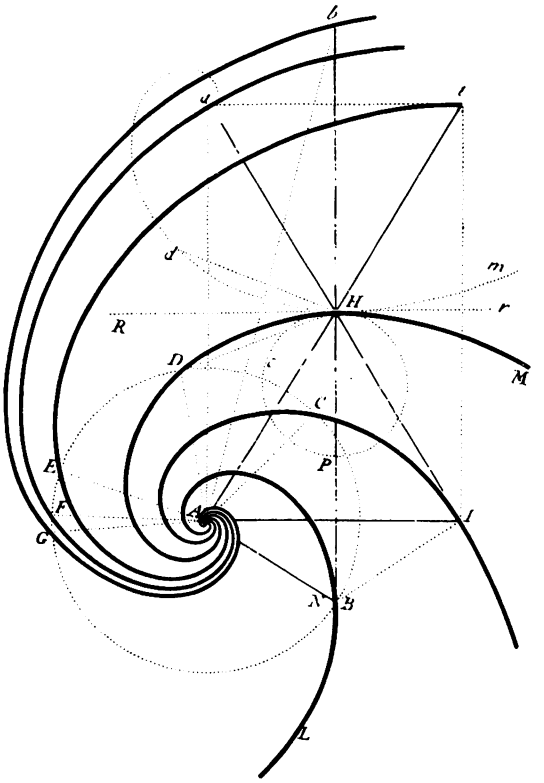

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Stimulation-dependent uptake of an extracellular marker to subcellular fractions of isolated neurohypophysial tissue[1]

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Summary. Slices of ox neurohypophyses and groups of isolated rat neurointermediate lobes were incubated in a medium containing horseradish peroxidase (HRP) and stimulated by high K^+ concentrations in the medium. After washing, the tissue was homogenized and subjected to subcellular fractionation in a Percoll/sucrose gradient. HRP was exclusively taken up by particles banding at a low density of the gradient. The HRP containing particles located to this region included vacuoles of a size comparable to secretory vesicles.

Some essential facts are known about the mechanism by which the neurohypophysial hormones vasopressin and oxytocin are released. It has been reported that on stimulation of isolated rat neurohypophyses, vesicles containing oxytocin and the binding protein (neurophysin) release their contents to the medium in a roughly stoichiometric way[4], whereas axoplasma enzyme markers are not released[5]. These facts support the hypothesis that release occurs by exocytosis. Since the area of the cell membrane in the terminal dilatations is not increased after strong stimulation[6,7] retrieval of membrane (vesicle membrane and/or cell membrane of a size similar to the total area of membrane secretory vesicles incorporated) must take place after stimulation. On the basis of morphological studies the prevailing hypothesis has been that secretory vesicles are retrieved as microvesicles[8,9], although early histological investigations have provided evidence that membrane is retrieved as vacuoles similar in size to the secretory vesicles[10,11]. In recent years, an increase in the vacuole population on stimulation without increase in the microvesicle population has been shown in several morphometric studies[6,12-14].

Stimulation-dependent uptake of isotope-labeled extracellular markers has been observed when whole rat neurohypophyses were incubated in media containing such markers[15]. No studies have been reported on larger animals and/or on attempts to isolate the vesicles retrieved after exocytosis. Such isolation would be necessary to answer the question whether the vacuole membrane is similar to that of the secretory veiscle membrane or that of the cell membrane and might be of use in studies of the fate of the vacuoles.

In the present work we report on stimulation-dependent uptake of HRP into subcellular fractions from rat and bovine neurohypophyses. As analyzed in density gradients the material containing the extracellular marker can be isolated from both species.

Materials and methods. Isolated rat neurointermediate lobes. For each of 3 experiments, 60 isolated neurointermediate lobes from female Wistar rats (300 g b.wt) killed by decapitation, were divided into 2 groups of 30. Both groups were washed twice in 'control' medium without HRP (table 1). The 2 groups were then incubated in 'control' medium and 'stimulation' medium, respectively, for 60

min. After this, both sets of neurohypophyses were washed with 15 changes of 4 ml control medium, containing no HRP, and 3 times with a solution containing 10 mM N-Tris (hydroxymethyl) methyl-2-aminoethanesulphonic acid (TES), pH 7.0, 190 mM sucrose and 25 mM EGTA after which they were homogenized. HRP was from Sigma (type II).

Slices from ox neurohypophyses. Hypophyses from cows, aged 2-5 years, killed by shooting, were obtained at the Copenhagen Public Slaughterhouse, shortly after death of the animals. The neural lobes were carefully dissected free of pars intermedia. For each of 4 experiments 2 sets, each with 2 neural lobes, were cut longitudinally with a scalpel into 5 slices (thickness approximately 0.8 mm), and placed into 2×2 ml of control medium, not containing HRP (see table 1). The material was kept at 20 °C and transported to the laboratory within 20 min. Here, 1 group of slices was incubated twice for 15 min in the control medium (table 1). The control medium in these studies contained 1 mM EGTA to reduce 'basal' release. For stimulation studies, another group of slices was incubated for 2 periods of 15 min in stimulation medium (table 1). After incubation, both groups of slices were washed once in control medium, containing no HRP and 3 times for 1 min with a solution containing 10 mM TES (pH 7.0), 190 mM sucrose and 25 mM EGTA, after which they were homogenized.

Homogenization, subfractionation, and other procedures used were carried out as described previously for ox neurohypophyses[16]. Briefly, the homogenate was centrifuged at $750 \times g_{av}$ 15 min at 4 °C. The supernatant (S_1) was centrifuged at $28,000 \times g_{av}$ for 15 min using a TI50 rotor in a preparative Beckman ultracentrifuge. The pellet (P_2) was resuspended, mixed with a 50% solution of Percoll® in homogenization medium and centrifuged at 60,000 (cow neurohypophyses) or $80,000 \times g_{av}$ (rat tissue) for 30 min at 4 °C using a Beckman 40.2 rotor with the brake on. After centrifugation the tubes were fractionated through a needle in the bottom. The S_2 supernatant was further centrifuged at $170,000 \times g_{av}$ for 60 min, giving a pellet (P_3) and a supernatant (S_3)[16].

The HRP activity was determined in the gradient and other fractions using 3,3'-diaminobenzidine as a hydrogen donor[17] with 0.2% Triton X-100 present in the assay.

Results and discussion. Figure A shows the distribution of HRP in the gradients containing the material from stimulated and non-stimulated rat tissue. In the fractions obtained from stimulated neurohypophyses, a symmetric peak in the gradient contained more than twice as much HRP activity as the control. In 2 more experiments, the HRP activity was increased by a factor 2.2 and 3.3, respectively. Increased uptake of HRP into subcellular particles obviously had occurred. This stimulation-dependent uptake was abolished by lack of Ca^{2+} in the medium or by D600 (a methoxy derivative of verapamil) which blocks stimulation-induced release of vasopressin[18] (data not shown). It can be concluded that the extracellular marker used in these experiments is useful to mark newly formed particles not only in the tissue[10,11,15] but it is also kept within these structures during homogenization, differential centrifugation and density gradient centrifugation.

Although fresh rat neurohypophyses can easily be obtained, a major problem in work with the gland from these animals is the minute amount of tissue (< 1 mg/neural lobe) and difficulties in separating pars intermedia from the neural lobe. Therefore, biochemical analyses and subfractionation studies have been carried out mainly with bovine neurohypophyses (150 mg/neural lobe), with the drawback that the hemorrhage during slaughtering stimulates neurohypophyses to release hormone[19]. Despite this fact, we have found an increased uptake of HRP into previously characterized subcellular fractions from bovine neurohypophyses[16] which have the advantage that they can be freed completely of tissue from the intermediate lobe.

The distribution of HRP in fractions obtained from stimulated and non-stimulated bovine neurohypophyses is shown in table 2. The high amount of enzyme activity observed in all supernatant fractions most likely results from incomplete washout of extracellular marker prior to subfractionation. We observed a small increase of HRP activity into the microsomal fraction (P_3) and a larger increase in fraction P_2. The latter subcellular fraction has been found to contain about 40-50% of the marker for secretory vesicles, mitochondria, microsomes, lysosomes and cell membranes[16]. The hormone containing material recovered in P_2 can be separated from other structures by density gradient centrifugation using Percoll®[16]. Using an identical procedure we found maximal HRP activity around fraction 23 in the gradients (see fig. B), well separated from secretory vesicles (which form a band around fraction 7), and mitochondria (centered around fraction 13)[16]. In 3 experiments, besides the one shown in figure B, the uptake of HRP was increased by a factor of 1.6, 1.6, and 1.3, respectively. However, the fractions containing HRP activity are still contaminated by lysosomes,

Table 1. Composition (mM) of media used for HRP uptake studies in rat neurointermediate lobes and in ox neurohypophysial slices. All media were kept at 37 °C and gassed with O_2

Neurointermediate lobes						pH 7.0		
Control	NaCl (140)	KCl (5)	$MgSO_4$ (1.5)		$CaCl_2$ (2.5)	Hepes (10)	Dextrose (10)	HRP (5 mg/ml)
Stimulation	- (45)	KCl (100)	- (1.5)		- (2.5)	-	- (10)	- (5 mg/ml)
Neurohypophysial slices						pH 7.3		
Control	Choline Cl (150)	- (0)	$MgCl_2$ (2)	EGTA (1)	- (1)	Hepes (10)	Dextrose (10)	- (5 mg/ml)
Stimulation	- (90)	- (56)	- (2)	- (1)	- (4)	-	- (10)	- (5 mg/ml)

Table 2. Distribution of horseradish peroxidase in bovine neurohypophysial fractions separated by differential centrifugation

	Values in percent of homogenate					
	P_1	S_1	P_2	S_2	P_3	S_3
Control	13.6 ± 3.0	94.9 ± 3.1	1.8 ± 0.2	79.9 ± 4.6	0.9 ± 0.2	71.4 ± 2.3
Stimulation	12.0 ± 1.6	82.5 ± 6.6	2.8 ± 0.3	68.5 ± 3.9	1.3 ± 0.2	63.2 ± 3.4

100% equals the total peroxidase activity in the homogenate of two neurohypophyses stimulated by 56 mM K^+ (104.7 ± 19.8 μmoles/min) or in nonstimulated neurohypophyses (137.3 ± 23.8 μmoles/min). Values are means of 5 experiments ± SD.

microsomes, and cell membrane fragments[16]. In thin section electronmicrographs of the HRP containing fractions recovered from the gradients the mentioned impurities were also observed, in addition to vacuoles which contained reaction product if the material was preincubated with 3,3'-diaminobenzidine and H_2O_2 (not shown).

The distribution within the gradients for rat and ox neural lobes were very similar. The peak in the rat experiments (using $80,000 \times g_{av}$ for 30 min) was at fraction 18, in the ox experiments (using $60,000 \times g_{av}$ for 30 min) it was at fraction 23.

The exclusive uptake of HRP into the material banding at low density of the gradient argues against a non-specific adsorption of HRP to membranes. If non-specific adsorption had occurred, enzyme activity should also have been observed in the secretory vesicle fraction which is centered around fraction 7 of the gradient used.

There was some uptake of HRP to non-stimulated rat neurohypophyses and a considerable uptake by non-stimulated ox neural lobe slices, even if they had been incubated in a control medium with a very low concentration of free Ca^{2+}. This is probably due to the fact that even in the control circumstances there is some stimulation of the tissue.

The increased uptake of marker into fractions from bovine neurohypophyses stimulated with potassium was smaller than observed in experiments with rat neural lobes. This is probably due to the massive release of hormone induced by bleeding of the animals during slaughtering. However, an increased uptake of HRP could still be observed, which encourages further work on the isolation of these particles.

The demonstration in the present experiments that HRP containing particles could be isolated after stimulation adds independent support to the hypothesis that release occurs by exocytosis.

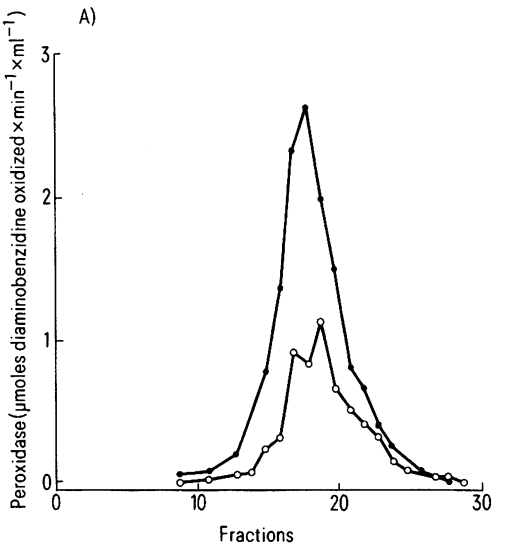

Figure A. Distribution on a Percoll/sucrose gradient of HRP taken up by subcellular fractions of stimulated (●) and non-stimulated (○) rat neurointermediate lobes. Fractions were collected from the bottom.

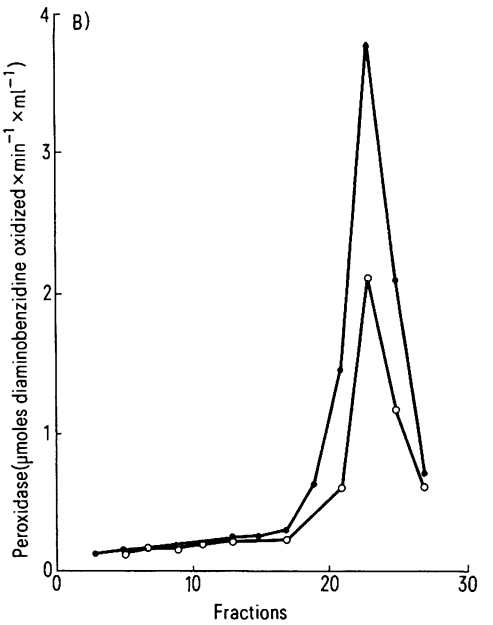

Figure B. Distribution on a Percoll/sucrose gradient of HRP taken up by subcellular fractions of stimulated (●) and non-stimulated (○) bovine neurohypophysial slices.

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4 Matthews, E.K., Legros, J.J., Grau, J.D., Nordmann, J.J., and Dreifuss, J.J., Nature New Biol. *241* (1973) 86-88.
5 Edwards, B.A., Edwards, M.E., and Thorn, N.A., Acta endocr. *72* (1973) 417-424.
6 Morris, J.F., and Nordmann, J.J., Neuroscience *5* (1980) 639-649.
7 Pickering, B.T., in: Essays in Biochemistry, vol. 14, pp. 45-81. Eds P.N. Campbell and W.N. Aldridge. Academic Press, London 1978.
8 Nagasawa, J., Douglas, W.W., and Schulz, R.A., Nature *227* (1970) 407-409.
9 Nagasawa, J., Douglas, W.W., and Schulz, R.A., Nature *232* (1971) 341-342.
10 Gerschenfeld, H.M., Tramezzani, J.H., and De Robertis, E., Endocrinology *66* (1960) 741-762.
11 Castel, M., Gen. comp. Endocr. *22* (1974) 336-337.
12 Lescure, H., and Nordmann, J.J., Neuroscience *5* (1980) 651-659.
13 Nordmann, J.J., and Morris, J.F., Nature *261* (1976) 723-725.
14 Theodosis, D.T., Dreifuss, J.J., Harris, M.C., and Orci, L., J. Cell Biol. *70* (1967) 294-303.
15 Nordmann, J.J., Dreifuss, J.J., Baker, P.F., Ravazzola, M., Malaisse-Lagae, F., and Orci, L., Nature *250* (1974) 155-157.
16 Gratzl, M., Torp-Pedersen, C., Dartt, D., Treiman, M., and Thorn, N.A., Hoppe-Seylers Z. physiol. Chem. *361* (1980) 1615-1628.
17 Herzog, V., and Fahimi, H.D., Analyt. Biochem. *55* (1973) 554-562.
18 Russell, J.T., and Thorn, N.A., Acta endocr. *76* (1973) 471-487.
19 Weinstein, H., Berne, R.M., and Sachs, H., Endocrinology *66* (1960) 712-718.

0014-4754/83/091007-03$1.50 + 0.20/0