THE NATURE OF HYPOTHALAMO-NEUROHYPOPHYSEAL NEUROSECRETION IN THE RAT

A Study by Light- and Electron Microscope Autoradiography

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ABSTRACT

The nature of hypothalamo-neurohypophyseal neurosecretion was examined in the rat by means of intraventricular injections of tritiated amino acids. Quantitation of autoradiographs was used at the light microscope level to study the sites of synthesis of proteins and their time of arrival in the neural lobe. Electron microscope autoradiographs were used to study the labeling of neural lobe tissue. It was concluded that the great majority of the labeled material was translocated inside dense-cored granules and was probably composed mostly of neurophysins.

The effect of ether anesthesia was also examined. It was found to remove the dense cores from about 20% of the granules in the neural lobe tissue, a process accompanied by the loss of most of their labeled material. The mechanism of the ether effect is discussed and compared to the normal secretion process.

INTRODUCTION

In 1951, Bargmann (3) confirmed the neurosecretory nature of the mammalian hypothalamo-neurohypophyseal tract. He proposed that stainable neurosecretory material was elaborated by the neurons of the anterior hypothalamus—chiefly the cells of the supraoptic and paraventricular nuclei. From these nerve cell bodies it subsequently passed down the axons to be stored and finally released into the bloodstream by the neural lobe.

During recent years the material synthesized by the hypothalamic neurosecretory cells has been shown to consist of the active posterior pituitary principles, oxytocin and vasopressin, loosely bound to carrier proteins termed neurophysins (1, 2, 25). A major fraction of the active hormone content of the neural lobe is associated with elementary granules, which can be isolated by differential centrifugation (4, 5, 19). These granules appear in ultrathin sections as membrane-bound vesicles of 100-150 nm diameter enclosing a core of electron-opaque material. Such granules appear within the profiles of neurosecretory axons and are especially numerous in their terminations (6).

When radioisotopically labeled amino acids (7, 8) are introduced into the cerebrospinal fluid of rats, light microscope autoradiography shows that the hypothalamo-neurophypophyseal tract becomes significantly labeled. The subsequent behavior of the incorporated radiochemicals is compatible with the suggestion that substances synthesized in the supraoptic and paraventricular nuclei are accumulated in the neural lobe.

We report here the application of quantitative

light microscope autoradiography, using three different amino acids, to define the kinetics of the arrival of labeled products in the neural lobe. Electron microscope autoradiography was then applied to [^{3}H]cystine-labeled neural lobe tissue to decide if the labeled material which accumulated could be localized more accurately on the ultrastructural level. In addition, electron microscope autoradiography was used to trace the events involved in the depletion of the neurosecretory granules (NSG).

Ether anesthesia is a convenient stimulus for inducing depletion of the neural lobe, since its administration is simple and its effect immediate. In contrast, procedures such as dehydration may take 2-3 days to have significant effect. Within 1 h of the commencement of ether anesthesia, a marked loss of stainable neurosecretory material can be detected by light microscopy (9), and a loss of electron opacity in the cores of the elementary granules may be evident in the electron microscope (10). Ether also causes the release of neurohypophyseal hormones into the bloodstream (11). The experiments were designed to test whether ether anesthesia modified the distribution of radioactivity within the neural lobe, and, if such a change occurred, whether it could be related to the ultrastructural changes ether produces.

EXPERIMENTAL DESIGN

Before embarking upon extensive experiments using electron microscope autoradiography, it was necessary to investigate the following points: (*a*) the value of different amino acids in tracing hypothalamic-neuropypophyseal neurosecretion, (*b*) the most informative time intervals for tissue sampling, (*c*) the most effective route of injection for radiochemicals, and (*d*) the necessary dose levels.

Many neurophysins contain high proportions of sulphur-containing amino acid (12), and both neurohypophyseal peptides contain a cysteine residue (32, 33). Hence the use of [^{3}H]cystine as a label for neurosecretory material was preferable on theoretical grounds. However, it was necessary to demonstrate that satisfactory autoradiographs could indeed be obtained.

The results of labeling with several different amino acids were compared. Groups of rats were injected with [^{3}H]leucine, [^{3}H]proline, or [^{3}H]-cystine. The animals were then killed by decapitation at various intervals after the administration of the tracer from 5 min to 24 h, usually two to five animals per time interval. Tissue blocks containing the hypothalamic nuclei and the pituitary were removed for autoradiographic examination.

Each amino acid was tested by injection at various dose levels via the intraperitoneal, intravenous, and intraventricular routes. By the intravenous and intraperitoneal routes, the doses were varied between 25 and 500 μCi, and by the intraventricular route between 20 and 80 μCi.

MATERIALS AND METHODS

Radiochemicals and Animals

Groups of white laboratory rats of an inbred strain and having a body weight of 200–210 g were injected with one of three labeled amino acids, [^{3}H]-leucine, [^{3}H]proline, or [^{3}H]cystine. L-[4,5-^{3}H]-leucine (sp act 7.6 Ci/mM) and L-[^{3}H]proline (G) (sp act 1.0 Ci/mM) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England, as solutions in distilled water. L-[^{3}H]-cystine of sp act 0.36–0.63 Ci/mM was obtained from Schwartz Bio Research Inc., Orangeburg, N. Y. in vials containing 1 mCi [^{3}H]cystine in 1 ml 0.01 N HCl.

Intraventricular Injection of Radiochemicals

Under light Nembutal anesthesia a short (15 mm) midline incision was made between the eyes and ears, exposing a flat area of skull and revealing the suture lines. The periosteum was gently scraped away from an area lateral to the interofrontal suture and anterior to the frontoparietal suture. A high-speed dental drill with a 1 mm diameter rose-head burr was used to thin the skull in the angle between these two sutures. Bone was removed from a 1 mm diameter circle until the thin layer which remained could be dented by slight pressure. The skin was closed with two sutures, and the animals were kept under normal conditions for 48 h.

For the injections the animals were held in a specially designed stand which kept the head in a horizontal position and allowed anesthesia to be maintained throughout the procedure (13). When the skin sutures were cut, the incision opened under gentle tension. The radiochemicals were dispensed from an "Agla" micrometer syringe (Burroughs & Wellcome Ltd., Beckenham, England) fitted with a 0.5 inch 30G needle. It was held vertically in a three-dimensional micromanipulator (Prior & Co. Ltd., Bishop Stortford, Herts., England).

The tip of the needle was brought into position just above the diaphragm of bone closing the burr hole in the skull. The needle was then wound down 4 mm to a position where the tip should have been in the lateral ventricle. The solutions were injected into the ventricle at the rate of 0.001 ml/5 s. After about half the dose had been administered the needle was withdrawn 1 mm and the rest of the dose dispensed. The maximum vol injected in any experiment was 0.08 ml. After a further 15 s, the needle was withdrawn completely and the skin incision resutured with sterile thread.

Trials using methylene blue solutions showed that material injected by this technique completely penetrated the ventricular system and the subarachnoid space.

Paraffin Sections

A block of midbrain containing the hypothalamus and the whole pituitary was dissected out into 10% formol calcium and fixed for 24 h. The tissues were dehydrated and embedded in paraffin wax. It was routine to include a piece of liver from a normal untreated animal to be embedded and sectioned alongside each piece of radioactive tissue. The piece of "cold" tissue provided an area over which to estimate the background density of the final autoradiographs. Coronal sections 5–6 μm in thickness were mounted on slides coated with gelatin-chrome alum.

Araldite Sections

Pituitaries were dissected out as rapidly as possible into a drop of 3% glutaraldehyde in phosphate buffer (14). The neural lobe was shelled out from the rest of the gland and cut with a razor blade into two to four pieces which were fixed in 3% glutaraldehyde at 4°C for 4–5 h and then postfixed in 2% OsO_4 for 1 h. After dehydration in graded ethanols the tissue was embedded in Araldite in gelatin capsules. Sections 2 μm thick were cut and mounted on narrow strips of cover slip. Six of these strips were mounted on each slide. The thickness of each Araldite section was measured on a Vickers shearing-type interference microscope using successively, a fringe field and a half shade eyepiece in monochromatic light (546 nm). Any sections which deviated more than 10% from the chosen thickness were discarded.

Light Microscope Autoradiography

Kodak AR10 stripping film was applied (15) to the dewaxed paraffin sections and the dry Araldite sections. Slides were exposed in light-tight boxes containing silica gel at 4°C for periods of 1–3 wk. The autoradiographs were developed in D19 for 6 min at 18°C without agitation, briefly rinsed in distilled water, and fixed in 20% $Na_2S_2O_3$.

Preparations from paraffin sections were stained with new methylene blue and mounted in Polymount. Autoradiographs of Araldite sections were viewed unstained, using either bright-field, incident darkfield or phase-contrast optics.

Visual grain counting was used to quantitate the autoradiographic reaction over paraffin sections. Grains were counted under bright-field conditions using a × 100 oil immersion objective, the area to be counted being delineated by an eyepiece reticule. The background levels of five areas chosen blind over the control section of liver were counted in the same way.

Reflectance photometry, which was used for quantitation of Araldite preparations, was performed with a Leitz Ortholux microscope fitted with a × 60 Pol-opak objective and MPV photometer attachment. The preparations were measured with incident plane polarized light, read through crossed polars, and referred to a fully blackened standard as described previously (30, 31).

Electron Microscope Autoradiography

Several animals were labeled with 50 μCi [^{3}H]-cystine for each chosen sampling time with and without ether treatment. Light microscope autoradiographs were prepared from each of several electron microscope tissue blocks from each animal. The best two or three animals from each treatment and time were chosen from these on the bases of (*a*) quality of tissue fixation, (*b*) the assurance that the tissue was adequately radioactive, and (*c*) that so far as could be seen at light microscope level, the pattern of labeling was "typical."

Ultrathin sections of gray or silver interference color were cut from each chosen block of Araldite-embedded material (two to four blocks per neural lobe). Ribbons of sections were picked up on 200-mesh gold grids coated with collodion. Six to ten grids were prepared for each tissue block. They were then stained with lead citrate (16) for 4 min and coated with a thin layer of carbon (17). Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) was applied as a monogranular layer, using a wire loop (20). The autoradiographs were exposed at 4°C for 10–16 wk and developed at 20°C in D19 for 3 min.

Analysis of Electron Microscope Autoradiographs

COLLECTION OF DATA: A total of 16,000–32,000 μm^2 of section was recorded on micrographs from each neural lobe (180–360 10 inch × 8 inch prints), the micrographs being spread over all of the available grids of sections. This was achieved by a

TABLE I

Complete grain and Circle Analysis of Micrographs Obtained 10 H after Labeling (Untreated Rats)

Item no.	Items	No. of random circles	Actual no. of silver grains		Expected no. of silver grains if random	
1	Axoplasm of neurosecretory axons	4502	175	175	225.3	225.3
2	Synaptic vesicles	12	4		0.6	
3	Empty granules/mitchondria/Axoplasm	2	0		0.1	
4	Empty granules/axoplasm	238	4	12	11.9	13.5
5	Empty granules/NSG/Axoplasm	18	4		0.9	
6	NSG/Synaptic Vesicles	0	0		0	
7	NSG/Axoplasm	1104	282	292	55.3	55.9
8	NSG/Mitochondria/axoplasm	12	10		0.6	
9	Mitochondria	46	0	14	2.3	21.2
10	Mitochondria/axoplasm	218	14		10.9	
11	Axonal membranes	48	0		2.4	
12	Non-neurosecretory axons	71	0		3.5	
13	Non-neurosecretory axons	24	0	1	1.2	17.3
14	Pericytes	98	1		4.9	
15	Pericytes/perivascular space	106	0		5.3	
16	Perivascular space	737	9	9	36.9	36.9
17	Perviascular space/Neurosecretory axons	259	7		12.9	
18	Perivascular space/NSG/Axoplasm	4	0		0.2	
19	Perivascular space/Empty granules/Axoplasm	2	0	7	0.10	17.4
20	Perivascular space/Endothelium	41	0		2.0	
21	Perivascular space/Pituicytes	43	0		2.2	
22	Endothelium	81	0	4	4.1	12.3
23	Blood vessel lumen/endothelium	163	4		8.2	
24	Blood vessel lumen	557	2	2	27.9	27.9
25	Pituicyte cytoplasm	2078	29		104.0	
26	Pituicyte/neurosecretory axon	193	6	37	9.7	133.3
27	Pituicyte nucleus	327	0		16.4	
28	Pituicyte nucleus/cytoplasm	64	2		3.2	
	Total	11048	553		553.0	

systematic sampling procedure. Each micrograph was printed at a final true magnification of 24,000.

Data were collected from each set of micrographs by an objective multistep procedure similar in essentials to that described previously (18, 20).

This consisted of the following steps:

(1) The tissue was divided up into a series of putative sources of radiation termed "primary items" (20). These items were selected (*a*) to be as far as possible features of established significance in cellular activity, and (*b*) to be features presenting at least a proportion of profiles with a smallest diameter exceeding 30 nm, 8.0 mm in this system. (30 nm equals 1.7 × the half distance (HD) value for this autoradiographic system [29].)

(2) The datum for each silver grain was collected by circumscribing each with an 8.0 mm diameter circle. For this purpose the center of each grain was taken to be the midpoint of its longest axis. Each grain whose circle lay wholely upon the profile of a primary item was alloted to that item. Grains which lay astride the interface of two items were allotted to a "junctional item" composed of parts of the two primary items. In some cases extensive primary items such as axoplasm (item 1, Table I) are interspersed with small features such as NSG (too small in profile area to qualify as primary items themselves). In these cases, grains which overlapped NSG and axoplasm together were recorded as such. This happening is of the type termed previously a "compound item" (18). In this manner, including primary, junctional, and compound cases, silver grain data were collected into 28 different items (Table I).

(3) The relative effective area of each item was

TABLE II

Observed and Random Silver Grain Distributions on Grouped Items from Neural Lobe Tissue of Untreated Rats

Item* no.	Grouped items	10 h after [^{3}H]cystine: Silver grains observed	Silver grains expected‡	(Observed-expected)^2 / expected	19 h after [^{3}H]cystine: Silver grains observed	Silver grains expected‡	(Observed-expected)^2 / expected
1	(Axoplasm)	175	225.3	11.25	107	170.4	36.60
2–6	(Empty granules/ Axoplasm)	16	13.5	0.46	14	14.6	0.03
7–8	(NSG/Axoplasm)	292	55.9	997.0	156	71.7	99.10
9–10	(Mitochondria)	14	13.2	2.44	19	18.7	0.005
11–15	(Axon membranes)	1	17.3	15.36	1	3.7	1.97
16	(Perivascular space)	9	36.9	21.10	22	24.2	0.20
17–21	(Perivascular space, etc.)	7	17.4	6.21	10	5.6	3.46
22–23	(Blood vessels)	4	12.3	5.60	1	6.0	4.16
24	(Blood vessel contents)	2	27.9	24.04	5	8.9	2.70
25–28	(Pituicytes)	37	133.3	69.50	58	69.2	1.41
		Total circles 11,048 Total grains 553		$\chi^2 = 1150.57$	Total circles 3760 Total grains 393		$\chi^2 = 149.64$

* See Table I.

‡ If randomly dispersed.

measured by applying random arrays of 8.0-mm circles to each micrograph using a transparent overlay screen. Each circle was then allotted to its appropriate item in the list of 28. The number of circles necessary to give a satisfactory relative effective area estimate was determined by the method of progressive means (28). The necessary "minimal sample size" was greatly exceeded for all major items. A total of 3,700–11,000 circles was applied per treatment.

(4) For ease of handling of the grain and the effective area data, and particularly for the application of the chi-square test, the totals for some quantitatively minor items were grouped with those from some other larger items (see Table I). The grouping was done on as rational a biological basis as possible, and the process was performed similarly for each batch of data. Each resultant group of items is referred to in subsequent calculations and discussions by its most characteristic feature in parentheses, e.g., (NSG/axoplasm) which represents a grouping of NSG/axoplasm and NSG/mitochondria/axoplasm (see items 7 and 8 in Table I).

PROCESSING OF GRAIN AND CIRCLE DATA: Grain and circle frequency distributions of the different item groups were compared using the chi-square test (see Tables II, V). Close correspondence between the grain and circle frequency distributions for any given sample (i.e., a low chi-square value) would indicate equal mean grain frequencies over all item groups and hence similar mean radioisotope concentrations in each group. The relative specific activities of the different parts of the tissue and of the different item groups were determined by expressing each grain count and circle count as a percentage and dividing grain figures by the appropriate circle figures. This treatment was appropriate since it was shown (see Results) that the overall tissue radioisotope concentration varied relatively little between the treatments studied. The results were then grouped to give relative specific radioactivities of the various parts of the tissue—(neurosecretory axons), (nonneurosecretory axons), (pituicytes), and (blood vessels) (Figs. 5, 8). The same process was carried out for the various components of the neurosecretory axons (see Figs. 6, 9).

Determination of the Relative Specific Radioactivity of Dense-Cored Granules

Subanalysis of the compound item (NSG/axoplasm) was performed by combining silver grain and effective area data with volume fraction data derived by a point-counting method (20). For this purpose the volume fraction of dense-cored granules and axoplasm in NSG/axoplasm were determined by the superimposition of a 1 cm square lattice and collection of the proportions of points falling on the two features. The specific activities of the dense-cored granules relative to that of the axoplasm was calculated as shown in Tables III and VI.

TABLE III

Calculation of the Relative Specific Radioactivity of Dense-Cored Granules (NSG) in Untreated Rats

		Relative specific activity	Vol. % in item	
			Axoplasm	NSG
10 h after [^{3}H]cystine	Axoplasm	0.49	100.0	0
	NSG/axoplasm	3.33	86.0	14.0
	Relative specific activity:			
	$NSG = \frac{3.33 - (0.86 \times 0.49)}{0.14} = 20.78$			
		Relative specific activity	Vol. % in item	
			Axoplasm	NSG
19 h after [^{3}H]cystine	Axoplasm	0.52	100.0	0
	NSG/axoplasm	2.02	78.3	21.7
	Relative specific activity:			
	$NSG = \frac{2.02 - (0.783 \times 0.52)}{0.217} = 7.43$			

The Ultrastructure of NSG

The proportion of granule profiles which contained dense cores and the proportion which was empty was determined for each tissue sample by taking random counts. This was achieved by systematic placement of a frame to delineate an area on each micrograph. Between 1250 and 1600 profiles were analyzed for each treatment, the counts being spread over 20–24 micrographs in each case.

RESULTS

Light Microscope Autoradiography

Intraperitoneal and intravenous injections of each of the amino acids, even at high dose levels, failed to give sufficient intensity of labeling to permit electron microscope autoradiography, although light microscope autoradiographs could be obtained. However, intraventricular injections proved highly effective with doses >20 μCi, and this was the technique adopted for all subsequent experiments.

In the case of all three amino acids, the hypothalamic nuclei were significantly labeled within 5 min of the intraventricular injection. The intensity of labeling reached a maximum after 1-2 h and then gradually fell away to about 50% of the peak value by 15-20 h (Fig. 1). At 1-2 h after injection, labeling in the neural lobe was only a little above background. Such activity as was detected was associated with pituicytes. However, neural lobe tissue sampled at times between 10 and 20 h always showed greatly increased levels of radioactivity. In tissues fixed 10-20 h after injection, the autoradiographic image overlay material staining intensely with new methylene blue, which substance also corresponded with that stained by Gomori's chrome-alum hematoxylin in serial sections.

Although intraventricular injections of [^{3}H]-leucine and [^{3}H]proline produced generally similar results, significant amounts of radioactivity began to appear in the neural lobe as early as 2 h after administration of [^{3}H]leucine. Despite this difference, all of the amino acids could probably be used successfully for electron microscope autoradiography in this system. However, [^{3}H]cystine gave an autoradiographic image which corresponded more precisely with the staining of neurosecretory material than the other amino acids. Thus these results offer experimental support to the strong theoretical case for the use of [^{3}H]cystine in electron microscope autoradiograph experiments. Bearing in mind the results of these experiments, electron microscope autoradiography was concentrated on neural tissue of rats injected intraventricularly 10 or 19 h previously with 50 μCi of [^{3}H]cystine.

Electron Microscope Autoradiographs

UNTREATED ANIMALS: It was clear from quantitative light microscope autoradiography

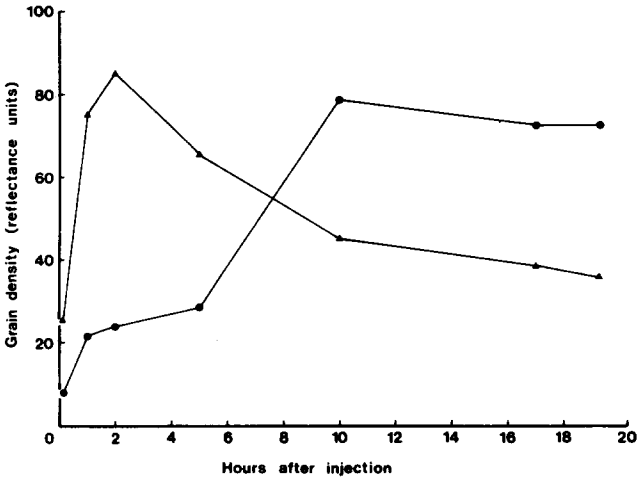

FIGURE 1 The silver grain density of light microscope autoradiographs prepared at various times after the intraventricular injection of [^{3}H]cystine. Closed circles (●) denote neural lobe tissue and closed triangles (▲) paraventricular nucleus. Similar results were obtained for supraoptic nucleus.

that the overall tissue specific radioactivity of the neural lobe was rather similar at 10 h and 19 h after an injection of [^{3}H]cystine into the lateral ventricle. (Reflectance photometry indicates that 19 h samples are up to 20% lower specific activity on average.)

Typical examples of electron microscope autoradiographs are shown in Figs. 2, 3, and 4, and the basic quantitative data in Table II. At both time intervals the grain distribution differed grossly from a random one. Specific activity data for the different parts of the tissue—(neurosecretory axons) (pituicytes) (perivascular spaces), and (blood vessels) (Fig. 5) must be viewed against this background. The data indicate that at both 10 and 19 h after injection the neurosecretory axons have a higher concentration of radioactivity than other parts of the tissue. However, the difference is not as marked at 19 h as at 10 h. Light microscope autoradiographs indicate that pituicyte labeling originates directly from the injection of tritiated cystine rather than by any translocation process. The radioactivity in the neurosecretory axons is of much greater interest here, since this is the site of most of the radioactivity accumulated in the period 5-19 h after the injection—almost certainly by translocation.

Within the neurosecretory axons, at both 10 and 19 h, the item (NSG/axoplasm) carried more silver grains than all the remainder of the axon tissue combined and had a substantially higher specific radioactivity (see Fig. 6). (Empty granules/axoplasm) had a specific activity substantially lower than that of (NSG/axoplasm). The axoplasm itself was also significantly labeled as was the mitochondrial item, but both had much lower activities than the item containing dense-cored granules. The relative specific radioactivity of (NSG/axoplasm) was distinctly lower at 19 h than at 10 h.

More detailed analysis of the compound item (NSG/axoplasm), using the point counting method for determining volume fractions of axoplasm and dense-cored granules in the item, showed that it consisted of more than 78% axoplasm in both the 10 h and the 19 h tissue. A simple calculation (Table III) then permitted an estimate of the relative specific radioactivity of the dense-cored granules. In both the 10 h and the 19 h tissue this figure was very much higher than that of any other item in the neurosecretory axons or indeed in the whole neural lobe tissue. It was noticeable, however, that at 19 h after injection the dense-cored granules were less radioactive than in the 10-h samples. Similar calculations for empty granules yielded relative specific activity figures of about 2.2 for both time intervals. These estimates are very much lower than those for dense-cored granules (20.78 and 7.43 for 10 and 19 h, respectively) but still higher than those of the axoplasm. Thus both dense-cored and empty granules were significantly more radioactive than the axoplasm, although of the two the former were by far the "hottest."

ETHER-TREATED ANIMALS: The administra-

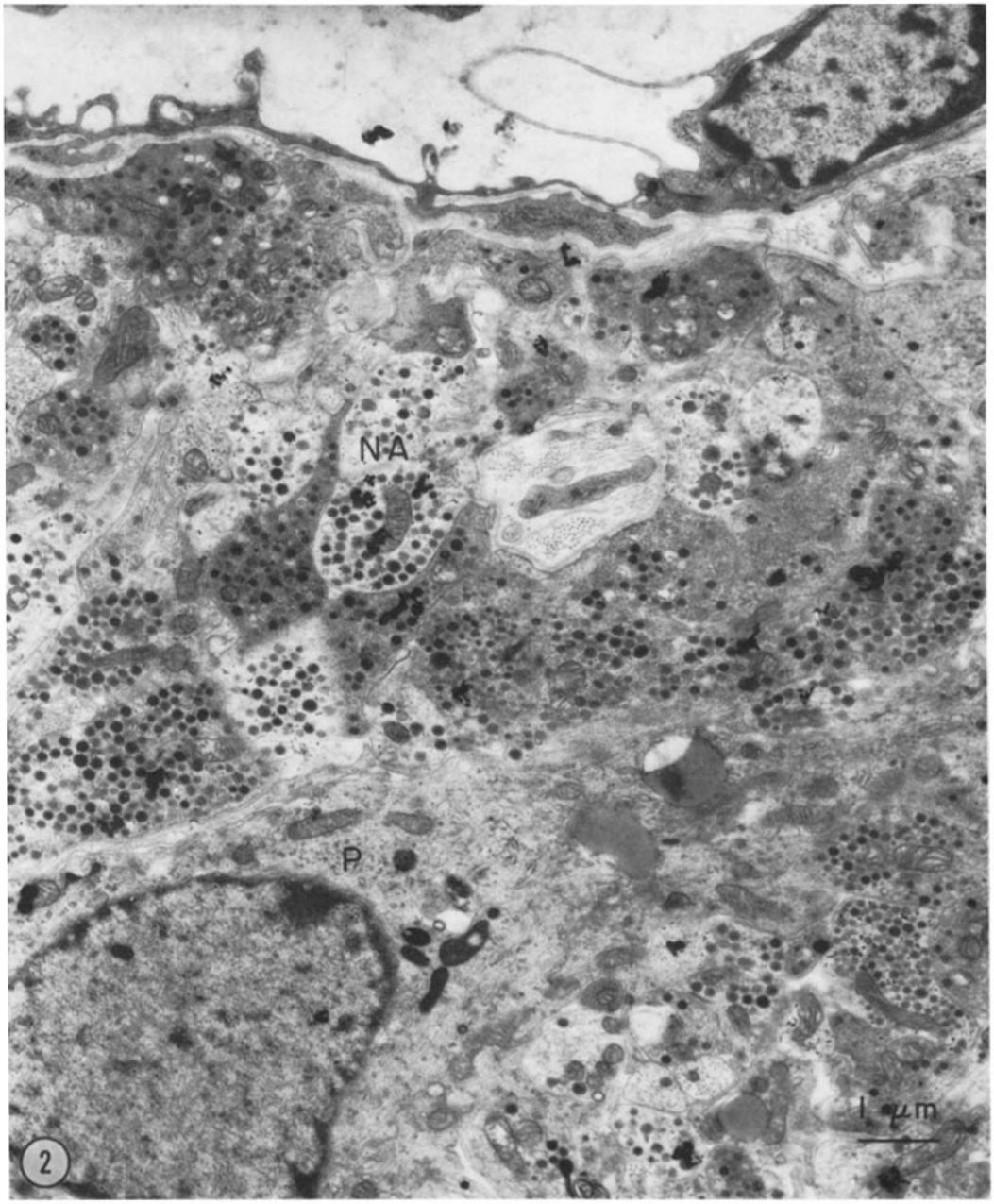

FIGURE 2 Electron microscope autoradiograph of neural lobe tissue 10 h after the administration of [^{3}H]cystine into the lateral ventricle. Note the main tissue components: neurosecretory axons (*NA*) containing many dense-cored granules, pituicytes (*P*), and capillaries. Silver grains are present overlying neurosecretory axons and pituicyte substance. × 11,200.

tion of ether for 1 h was found to have on average a minor (10-20%) depletory effect on the level of radioactive proteins in the neural lobe. Precise quantitation was not possible due to the variation between individual experiments (each of these involved a separately injected animal). However, an effect on the ultrastructure of the neural lobe tissue was seen. In particular, an increase in the

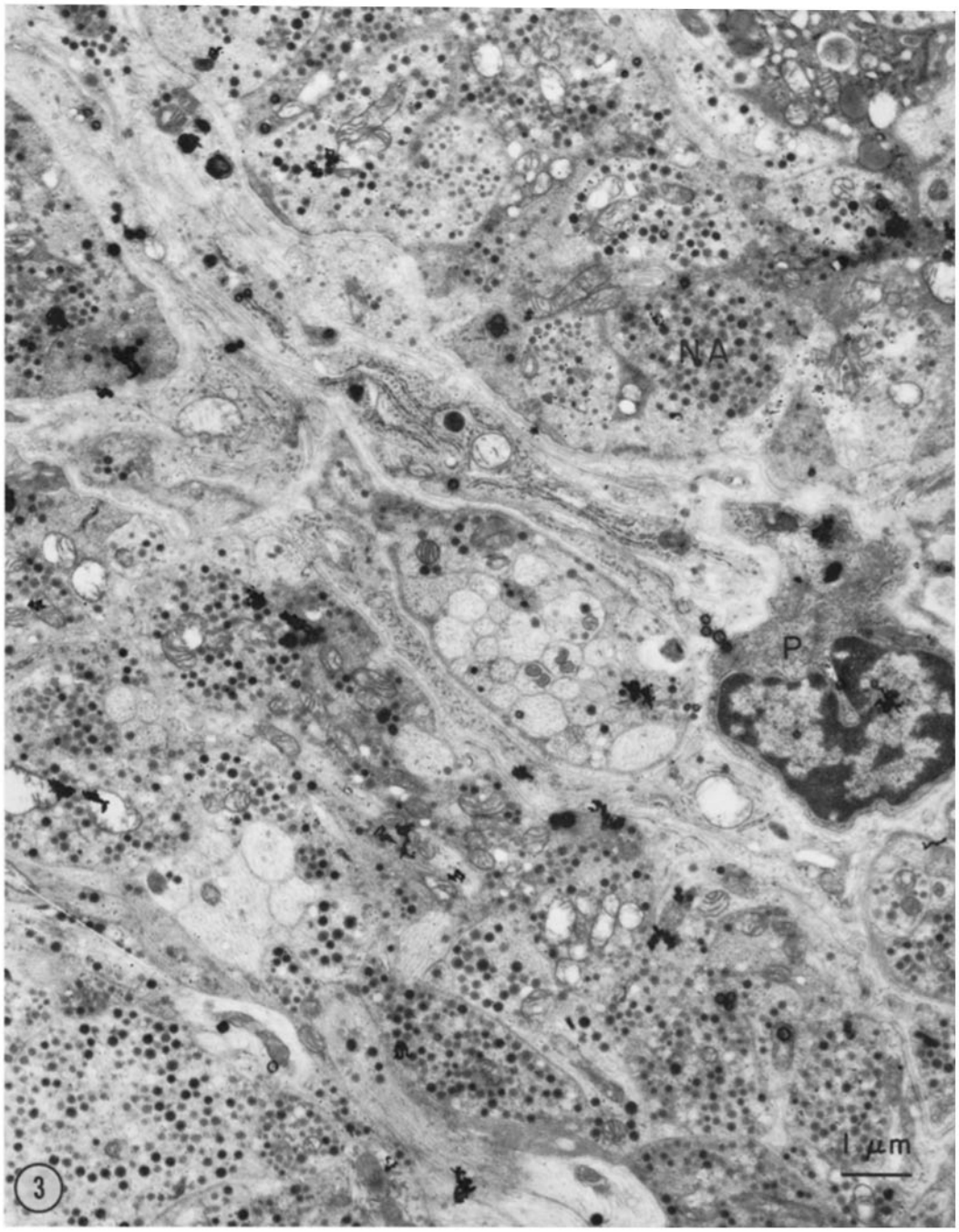

FIGURE 3 Electron microscope autoradiograph of neural lobe tissue 19 h after the administration of [^{3}H]cystine into the lateral ventricle showing the general appearance of the preparations. Silver grains are present overlying neurosecretory axons (*NA*) and pituicytes (*P*). × 10,400.

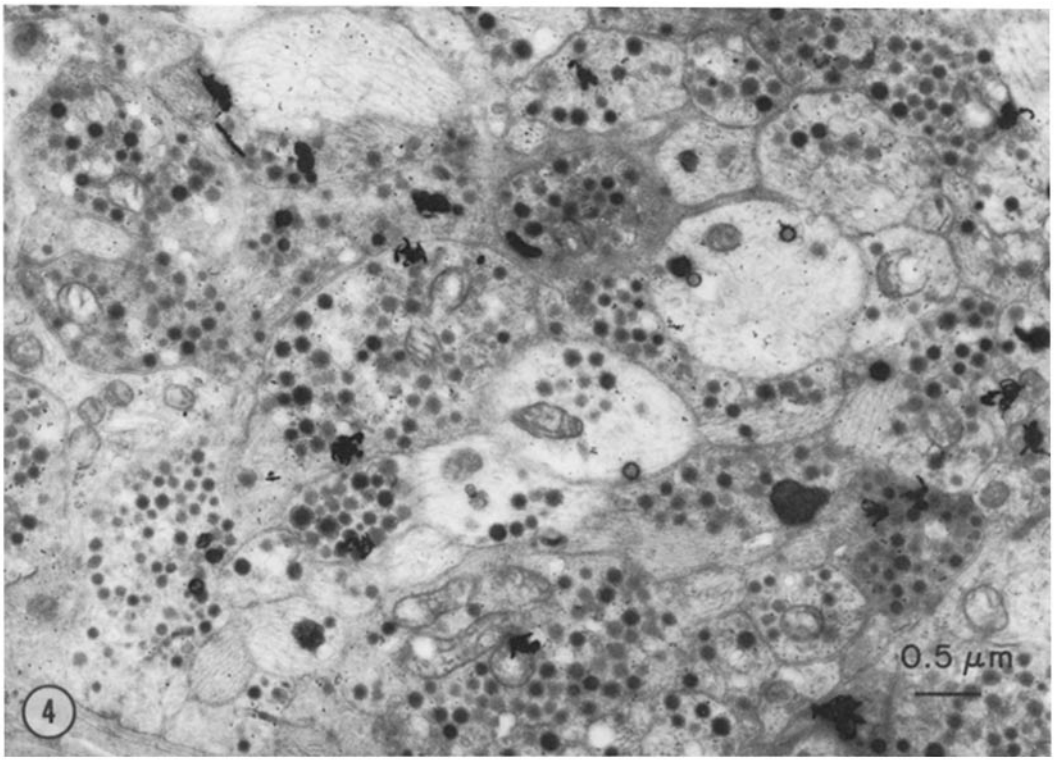

FIGURE 4 Electron microscope autoradiograph of neurosecretory axons from 10 h control neural lobe tissue showing the presence of numerous dense-cored granules and smaller numbers of empty granules. × 16,500.

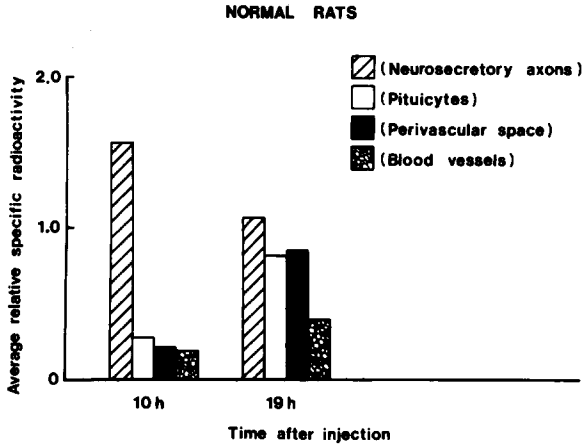

FIGURE 5 Average relative specific radioactivities of the components of neural lobe tissue in untreated rats. The data were generated by dividing the mean silver grain density over each component by the relative effective area of that component. (neurosecretory axons) items 1–15; (pituicytes), items 25–28; (perivascular space), items 16–21; (blood vessels), items 22–24.

occurrence of empty granules was apparent (see Fig. 7). Quantitative estimates of the volume fractions of dense-cored and empty granules by point counting confirmed these impressions (Table IV). In both 10 h and 19 h tissue the volume fraction of empty granules increased at least three fold from values of 15.3% and 6.0% of total granule volume (at 10 and 19 h, respectively) to

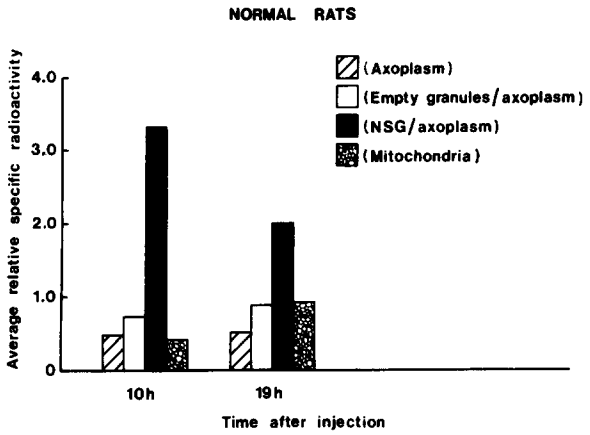

FIGURE 6 Average relative specific radioactivities of the components making up neurosecretory axons in untreated rats. The data were generated by dividing the mean silver grain density over each component by the relative effective area of that component. (Axoplasm), item 1; (empty granules/axoplasm), items 2-6; (NSG/axoplasm), items 7-8; (mitochondria), items 9-10.

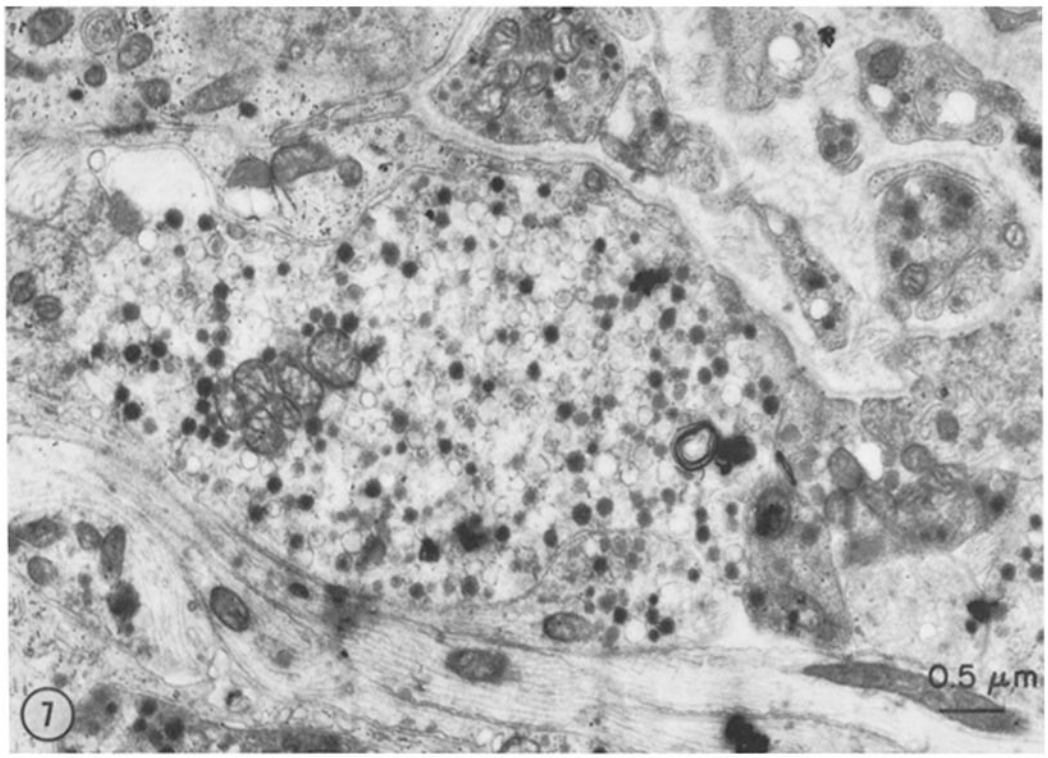

FIGURE 7 Electron microscope autoradiograph of neurosecretory axons from neural lobe tissue of a rat anesthetized with ether 10 h after administration of [^{3}H]cystine. Note the presence of numerous empty granules and also dense-cored granules. × 16,500.

46.9% and 50.6%, respectively). The total granule volume was unaffected by ether treatment. Counts on the proportions of dense-cored and empty granule profiles also indicated a considerable increase in the proportion of empty granules. Ether anesthesia, therefore, quite clearly causes a significant loss of granule cores. This observation correlates with a decrease in the amount of stain-

able neurosecretory material in the neural lobe (9) and the appearance of increased amounts of vasopressin in the blood stream (11).

The autoradiographs of the ether-treated rats were analyzed in the same manner as those from untreated rats (see Table V). The overall grain distributions differed markedly from randomness at both 10 and 19 h. As in the case of untreated rats, the neurosecretory axons contained the great majority of the radioactivity present in the neural lobes (Fig. 8), while within neurosecretory axons the item (NSG/axoplasm) was again pre-eminent as a source of silver grains (Fig. 9). Its relative specific radioactivity was much higher than that of all other items in tissue ether-treated 10 or 19 h after labeling. However, the specific activity of (NSG/axoplasm) was lower at 19 h than at 10 h. Again, as in the untreated animals, significant (but lower) levels of radioactivity occurred in all the other parts of the neurosecretory axons. Calculations of the relative specific radioactivity of the dense-cored granules (Table VI) again indicated that the granules contained much higher concentrations of tritium than any other part of the axons, although the granules were hotter in 10 h than in 19 h tissue (24.17 against 13.49). The empty granules, by similar calculations, showed relative specific activities of 2.5 in the 10 h specimens and 1.4 in the 19 h material.

Thus it seems clear that the effect of ether anesthesia is to convert a significant fraction of the dense-cored granules into empty ones. This process is accompanied by a loss of radioactively labeled material, presumably neurophysins and attached hormones. However, no evidence relating to the destination of this released material has been adduced in this study.

TABLE IV

The Effect of Ether-Treatment on the Ultrastructure of Neurosecretory Granules

	Untreated		Ether treated	
	10 h	19 h	10 h	19 h
Total granule vol. as % neurosecretory axon	15.0	21.6	19.2	23.7
% granule vol. occurring as empty granules	15.3	6.0	46.9	50.6
% granule No. occurring as empty granules	9.31	7.07	32.48	24.79

TABLE V

Observed and Random Silver Grain Distributions on Grouped Items from Neural Lobe Tissue of Ether-Treated Rats

		10 h after [^{3}H]cystine			19 h after [^{3}H]cystine		
Item* no.	Grouped items	Silver grains observed	Silver grains expected‡	$\frac{(\text{Observed-expected})^2}{\text{expected}}$	Silver grains observed	Silver grains expected‡	$\frac{(\text{Observed-expected})^2}{\text{expected}}$
1	(Axoplasm)	104	138.8	8.70	81	92.4	1.41
2–6	(Empty granules/axoplasm)	45	46.4	0.04	15	24.7	3.81
7–8	(NSG/axoplasm)	141	33.0	354.00	113	32.1	204.00
9–10	(Mitochondria)	24	19.9	0.85	11	9.8	0.15
11–15	(Axon membranes)	3	10.0	4.90	2	5.6	2.32
16	(Perivascular space)	30	30.7	0.02	6	18.1	8.09
17–21	(Perivascular space, etc.)	10	14.6	1.45	4	5.6	0.46
22–23	(Blood vessels)	9	8.9	0.001	1	7.5	5.63
24	(Blood vessel contents)	5	9.0	1.78	9	18.4	7.06
25–28	(Pituicytes)	23	82.7	43.10	13	40.8	18.90
		Total circles 7486 Total grains 394		$\chi^2 = 414.84$	Total circles 3867 Total grains 255		$\chi^2 = 251.83$

* See Table 1.

‡ If randomly dispersed.

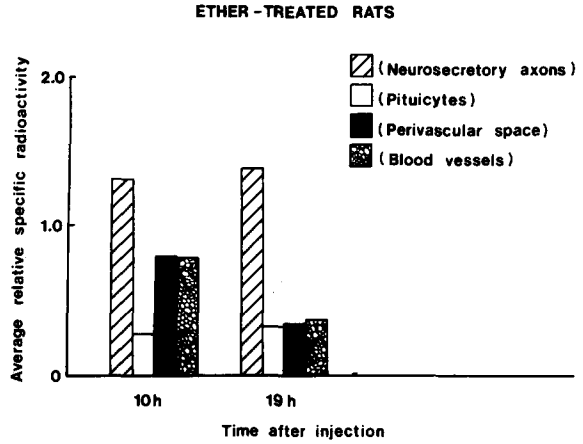

FIGURE 8 Average relative specific radioactivities of the components of neural lobe tissue in ether-treated rats. The data were generated by dividing the mean silver grain density over each component by the relative effective area of that component. (Neurosecretory axons), items 1–15; (pituicytes), items 25–28; (perivascular space), items 16–21; (blood vessels), items 22–24.

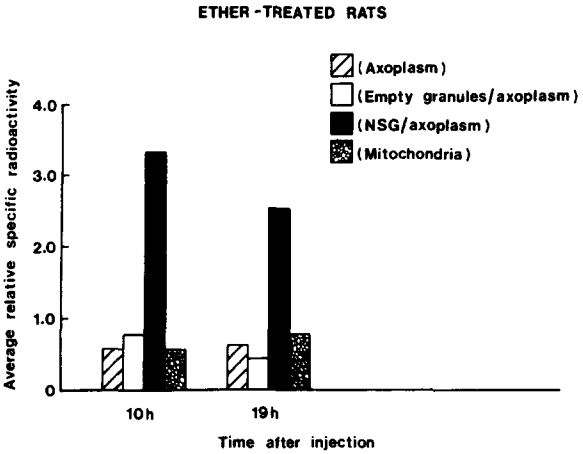

FIGURE 9 Average relative specific radioactivities of the components making up neurosecretory axons in ether-treated rats. The data were generated by dividing the mean silver grain density over each component by the relative effective area of that component. (Axoplasm), item 1; (empty granules/axoplasm), items 2–6; (NSG/axoplasm), items 7–8; (mitochondria), items 9–10.

DISCUSSION

The results of the experiments using light microscope autoradiography are consistent with the hypothesis that substances of a peptide nature are synthesized in the hypothalamic nuclei and thereafter translocated to the neural lobe where they accumulate. The labeled substances correspond in site and behavior with neurosecretory material as demonstrated with stains such as chrome-alum hematoxylin and performic acid-Alcian blue. Electron microscope autoradiographs indicate clearly that the labeled material accumulating in the neural lobe 5-20 h after intraventricular injection of [^{3}H]cystine is concentrated in the neurosecretory axons. This observation is in accord with light microscope observations on paraffin sections of the same tissue in which the silver grains lie predominantly over stainable neurosecretory material.

For any particular combination of radioisotope, section thickness, carbon layer thickness, and emulsion crystal size (and assuming a packed

TABLE VI

Calculation of the Relative Specific Radioactivity of Dense-Cored Granules (NSG) in Ether-Treated Rats

		Relative specific activity	Vol. % in item	
			Axoplasm	NSG
10 h after [^{3}H]cystine	Axoplasm	0.58	100.0	0
	NSG/Axoplasm	3.34	88.3	11.7
	Relative specific activity:			
	$NSG = \frac{3.34 - (0.883 \times 0.58)}{0.117} = 24.17$			
		Relative specific activity	Vol. % in item	
			Axoplasm	NSG
19 h after [^{3}H]cystine	Axoplasm	0.62	100.0	0
	NSG/Axoplasm	2.55	85.0	15.0
	Relative specific activity:			
	$NSG = \frac{2.55 - (0.85 \times 0.62)}{0.15} = 13.49$			

monocrystalline layer of emulsion), the resolution is a constant (29). The resolution comprises a measure of the lateral spread of the autoradiographic image. Experimentally, values for the resolution for various combinations of emulsion and section thickness have been determined using radioactive line sources (29). The image spread thus observed is recorded as a HD value, i.e., the distance from the radioactive line within which half the silver grains fall. The degree of image spread around a particular radioactive source depends on both the shape and the size of the source. In the case of extensive sources, most of the silver grains form within the profiles of the source. In the case of small sources, the contrary applies, i.e., most of the silver grains that arise lie outside the outline of the particular profile from which they originated and hence may overlie profiles of another type of source. In such a case the silver grains are said to be "cross-fired." Dense-cored granules are so small that they give rise almost exclusively to cross-fired grains. Thus in this sense the resolution of the system is not fine enough to permit the visualization of the autoradiographic images of individual granules. However, the ability of an autoradiographic system to discriminate label in different organelle types (18, 20) depends to a large extent on the pattern of organelles in the tissue concerned. Since the granules occur mostly in clusters, many silver grains originating from one granule, although laterally displaced, may well form overlying another granule(s) of the same type. This system has, therefore, considerable ability to localize radioactivity to clusters of granules (interspersed of course with axoplasm)—the item in the analysis termed NSG/axoplasm. The more refined analysis of this compound item (18, 20) by the method suggested by Williams (1969) indicates quite clearly that it is the dense-cored granules that contain the great bulk of the radioactivity. This calculation assumes that the specific radioactivity of the axoplasm in the neurosecretory axons is fairly even throughout the neural lobe, and in particular that it is not especially hot in the vicinity of the granule masses. While it is unlikely that the axoplasmic activity is entirely uniform, the assumption appears for the present to be a reasonable one. On this basis, it seems that a preponderance of the radioactive material is transported packaged in granules. The evidence derived here for the transport of labeled peptides and/or proteins in NSG fits well with evidence obtained by isolation of the granules in an ultracentrifuge (4, 5, 19).

The relative specific radioactivity calculations for the NSG are likely to be underestimates, since a proportion of the silver grains originating in the granules is probably cross-fired far enough to fall on axoplasm, blood vessels, or pituicytes. However, cross-fire from dense-cored granules cannot explain all of the axoplasmic silver grains. Many

of them are so distant from granule profiles (or indeed any other source) that axoplasmic labeling has to be acknowledged. This labeling presumably represents the results of axoplasmic flow of non-neurosecretory axonal proteins towards the neural lobe.

Studies in several species (21) have indicated that the neurohypophyseal hormones oxytocin and vasopressin are borne on carrier proteins inside NSG. The neurophysins have mol wt of about 20,000 daltons and hence contain many more amino acid residues than the hormones themselves. The neurophysin peptide chains include leucine, proline, and cysteine residues (12). It is thus highly likely on quantitative grounds that the bulk of the NSG label on the autoradiographs represents neurophysins. Norström et al. (22) have labeled neural lobe tissue in a manner similar to that reported here but with [^{35}S]cysteine. They recovered 80% of the label in identifiable neurophysin (as determined by microimmunodiffusion and microelectrophoresis). The neurophysins are of sufficient molecular weight to permit efficient fixation with glutaraldehyde. The hormones, being quite strongly bound, are probably also retained in the fixed tissue either by actual chemical bonding or merely by being enclosed in a meshwork of fixed proteins (23). No truly relevant data is available on this point. It is possible, therefore, that a minority of the granule radioactivity represents labeled vasopressin and oxytocin.

A reported effect of ether anesthesia, observed by electron microscopy, is the loss of the dense cores from a proportion of the NSG stored within the neural lobe (10). In our study the effect was quantitated, and it was found that about 20% of the granules lost their cores during 1 h of ether anesthesia. Stainable neurosecretory material was also reduced in amount—as determined by scanning microdensitometry (24). In the untreated animals NSG devoid of dense cores, (about 8% of the total granules) had a much lower relative specific activity than dense-cored granules. Ether treatment appeared to convert dense-cored to empty granules, a process accompanied by a loss of radioactivity. The empty vesicles thus formed had a similar relative specific radioactivity to the same organelles in untreated tissue. Thus the great majority (but not all) of the radioactivity borne in labeled NSG appears to be in the core substance. Ether treatment causes the release of detectable amounts of hormones into the blood stream (11) although there is relatively little decrease in the levels of stored hormones (10). The experiments reported here correlate well with these reports.

Although the ether effect reported here is in certain respects similar to the neurophysin-releasing effect of Ca^{++} ions (27), this action of ether is probably not an enhancement of the normal physiological process of secretion. The normal process is widely believed to be an exocytosis (25, 26), whereas ether treatment leaves the granule membranes in position. It appears likely that the action of ether is mediated by a solubilizing effect on cellular membranes. The process perhaps involves solvation and extraction of some lipid component(s), thereby effecting an increase in granule and probably also neurolemmal permeability. Further experiments involving the isolation of granules from normal and ether-treated rats will be necessary to test this postulate.

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