INTRACELLULAR TRANSPORT OF PROTEINS

IN ACTIVE AND RESTING SECRETORY CELLS OF

THE VENOM GLAND OF *VIPERA PALAESTINAE*

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ABSTRACT

The intracellular transport of venom proteins has been studied in active and resting venom glands of the snake *Vipera palaestinae* by electron microscope radioautography after an intra-arterial injection of [³H]leucine. In the active gland, most of the label is initially (10 min) found over the RER. By 30 min, the relative grain density of the Golgi complex reaches its maximum, with concomitant increase in the labeling of the condensing vacuoles. Later on, a steep increase in radioactivity of the secretory granules is observed. At 3 h, these granules, which comprise about 2% of the cell volume, contain 22% of the total grains. At the following hour, their labeling declines and at the same time the radioactivity of the secreted venom is increased. It is concluded that, in the active cell, venom proteins are transported via the Golgi apparatus into membrane-bounded granules which are the immediate source of the secreted venom. An alternative pathway, which involves the RER cisternae as a storage compartment, seems unlikely, since incorporated label does not accumulate in this compartment after prolonged postpulse intervals. The route of intracellular transport of proteins in the resting glands is similar to that of the active ones, but the rate of synthesis and transport is much slower. The present results and earlier data, thus, show that the increase in the rate of secretion after initiation of a new venom regeneration cycle is the result of accelerated rates of both synthesis and transport.

KEY WORDS   snake · venom gland · venom secretion · intracellular transport

The main portion of the venom gland of viperid snakes is made up of repeatedly branching tubules lined by a typical secretory epithelium (13). At the so-called resting stage, the lumina of the tubuli are filled with venom, and the secretory cells are cuboidal to flat. Activation of the venom synthetic apparatus by removal of the venom from the gland lumina (milking) induces increased rates of incorporation of labeled precursors into RNA and protein (8, 18, 22). At the same time, the structure of the secretory cell undergoes considerable changes (3, 18, 31). The active secretory cell assumes a columnar shape, and a well-developed rough endoplasmic reticulum (RER), typical of active secretory cells, appears together with a prominent Golgi apparatus. However, a relatively small number of vesicles containing venom components are found in the cell (26), and most of the cell volume is occupied by the distended RER.

The unique ultrastructural features of this secretory cell of the viperid venom gland, together with

the fact that secretory products are stored mainly extracellularly in extended lumina (29), raised the question whether the intracellular pathway(s) of exportable proteins in this gland is similar to the well-known transport in mammalian digestive glands (20).

Results of a radioautographic study by Warshawsky et al. (31) with the venom gland of a crotalid snake suggest that venom proteins which are synthesized on the RER are transported via the Golgi complex to secretory vesicles. This study, however, was carried out with glands at a single secretory stage, and no quantitative data were presented.

The present study was undertaken to verify the intracellular pathway(s) of secretory proteins in the venom gland of *Vipera palaestinae* on a quantitative basis. Our results show that the sequence of events in the intracellular transport of protein in the viper's venom gland is similar to that described in mammalian systems, but the rates of intracellular transport and secretion are changed during different stages of the venom regeneration cycle. Some of these results have been previously reported (19).

MATERIALS AND METHODS

Snakes

Specimens of *Vipera palaestinae* (150–200 g) were kept at 29°–31°C. One gland of each snake was milked 4 days before the experiment and the contralateral one was left unmilked. It has been previously shown that milking one gland does not affect the contralateral gland (18).

Labeling Procedure

L-[4,5-^{3}H]leucine (specific radioactivity 33 Ci/mmol, Nuclear Research Center, Negev, Israel) in 0.15 M NaCl was injected into the blood system of each snake, which had been previously cannulated as follows: The snake was anesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (Fluothane) and a polyethylene catheter (0.8 mm OD) was inserted into the right systemic arch (for anatomical details, see reference 11). The whole operation, including the recovery from the anesthesia, took about 40 min. The snakes were kept for an additional 3 h at 30°C before the administration of the radioactive precursor.

With this technique a relatively short pulse of [^{3}H]leucine in the blood could be achieved (Fig. 1), and the experiment could be carried out with a conscious, unrestrained active snake at 30°C. These measures were essential to obtain accurate kinetic data with an ectothermic (poikilothermic) animal.

A total of 25 snakes were labeled by this procedure; for radioautographic analysis, nine snakes were injected with 5–7 mCi [^{3}H]leucine and sacrificed at 10, 20, 30, 60, 80, 135, 170, 240, and 390 min after administration of the precursor.

Radioautography
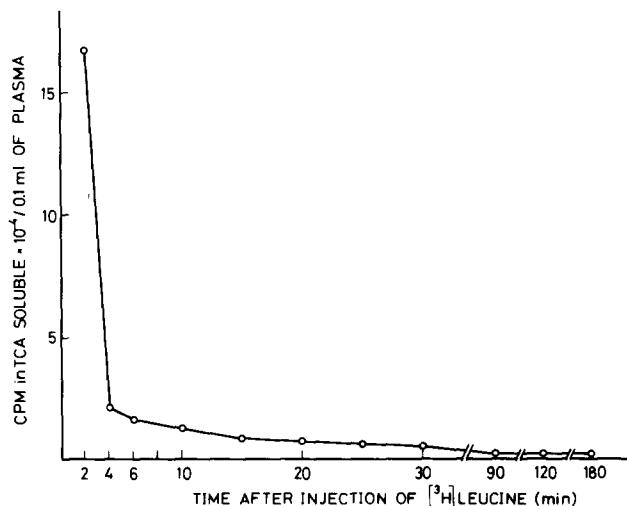
A transverse slice from the main part of each gland

FIGURE 1 Decline of radioactivity in the blood after injection of [^{3}H]leucine to a cannulated snake. 50 μCi of [^{3}H]leucine were injected to a cannulated snake at 30°C (see Materials and Methods). Blood sampling was performed through the cannula at various times after the injection, and the radioactivity in the TCA soluble fraction of the plasma was determined.

was fixed in 4% formaldehyde[1] in cacodylate-HCl buffer, 0.25 M, pH 7.2, for 20 h at 4°C. During this time the fixative was changed several times to wash out unincorporated label. The tissue was then washed with several changes of cacodylate buffer, postfixed with 2% OsO_4 and stained (in block) with 0.5% uranyl acetate in 30% ethanol for 2 h. It was dehydrated in ethanol and embedded in Epon.

For electron microscopy, pale gold sections were cut, collected on 200-mesh copper grids (Formvar and carbon-coated) and covered with an Ilford L-4 photographic emulsion (Ilford Ltd., Ilford, Essex, England), with the method of Caro and van Tubergen (6). The preparations were exposed in light-tight boxes at 4°C with Drierite for 3-4 mo in the case of active glands (4 days after milking) and for 4-9 mo for resting glands (unmilked). In only four out of nine snakes, labeling of the resting glands was high enough for a reliable radioautographic analysis. Development was done with the Phenidone developer (Geigy Chemical Corp., Ardsley, N. Y.) (15) for 1 min at 18°C and fixation with Kodak F-24 fixer 4 min at 20°C. Sections were then stained with lead citrate (30) for 10-20 min and examined in Jeolco Jem T-7 electron microscope.

For light microscope radioautography, 0.5-1.0-μm thick Epon sections affixed to glass slides were processed as for electron microscope radioautography, but a D-19 (Kodak) developer was used. The sections were then poststained with 1% toluidine blue in 0.1 N NaOH.

During fixation and subsequent preparation for electron microscopy, radioactivity leakage from the tissue was monitored by following the radioactive proteins which leaked into the fixative and washing and dehydration fluids. The acid-insoluble radioactivity in these fluids did not exceed 5% of the total radioactivity in protein in the tissue before fixation.

Quantitative analysis of the distribution of radioactivity was carried out on electron micrographs at a final magnification of 20,000. About 30 cells (derived from different trans-sections of the block) were analyzed at each time interval. The silver grain counts for each cell component were expressed as percentage of the total counts scored over all cell components. The center of each silver grain was used for localization. Morphometric measurements were carried out on the same micrographs, and the area occupied by each cell component was determined as percent of the total area using the point counting method (33). Relative grain density was obtained by relating the percentage of grains to the percentage of area (24).

Chemical and Radioactive Measurements

A portion of each of the ^{3}H-labeled glands was thoroughly rinsed with 0.3 M sucrose to remove any absorbed venom and homogenized in Tris acetate, 0.01

M, pH 7.2, with 0.1% Triton X-100. Total proteins were precipitated with 10% trichloroacetic acid (TCA) and venom proteins by immunoprecipitation techniques as previously described (18). Protein was estimated according to Lowry et al. (16) with bovine serum albumin as standard.

RESULTS

Morphological Observation of the Secretory Cells

The fine structure of the secretory cells of the venom gland of V. palaestinae was studied by Ben-Shaul et al. (3). The unusual structure of this cell is further illustrated in the present study, using morphometric parameters. In unmilked (resting) glands, the secretory cells have a cuboidal to flat shape (Fig. 3 c–d, 9, and 10). The membranes of the RER are closely packed and occupy most of the cell volume (82%), while the secretory granules account for only 2.7% of the total cell volume (Table I). In glands 4 days after milking (active glands; see Rotenberg et al. [22]), the secretory cells have a tall columnar shape (Figs. 2 and 3 a–b). The RER, including the intracisternal space, becomes most conspicuous at this stage and occupies most of the cell volume (86%) (Table I). A well-developed Golgi apparatus is evident in the supranuclear region; some large vesicles (designated here as condensing vacuoles, Fig. 5) are usually associated with the Golgi saccules. The vesicles found close to the apical membrane usually have a more dense and homogeneous content (secretory granules). The condensing vacuoles and the secretory granules together account, in this cell, for only 4% of the total, while the volume fraction of secretory granules in mammalian digestive glands is much greater (Table I).

Incorporation of [^{3}H]Leucine into Active and Resting Glands

Nine cannulated snakes with one gland at the active stage (4 days after milking) and the second one at the resting stage were labeled (see Materials and Methods) with [^{3}H]leucine and sacrificed at different time intervals after administration of the precursor. Incorporation into proteins increased rapidly during the first 10 min after the administration of the radioactive precursor, and a plateau was achieved at 20-30 min postpulse. 75 ± 18% of the total labeled proteins in homogenates of active glands were precipitable by venom antiserum, while in homogenates of resting glands

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[1] This fixative is known to bind only negligible amounts of unincorporated [^{3}H]leucine (1, 7).
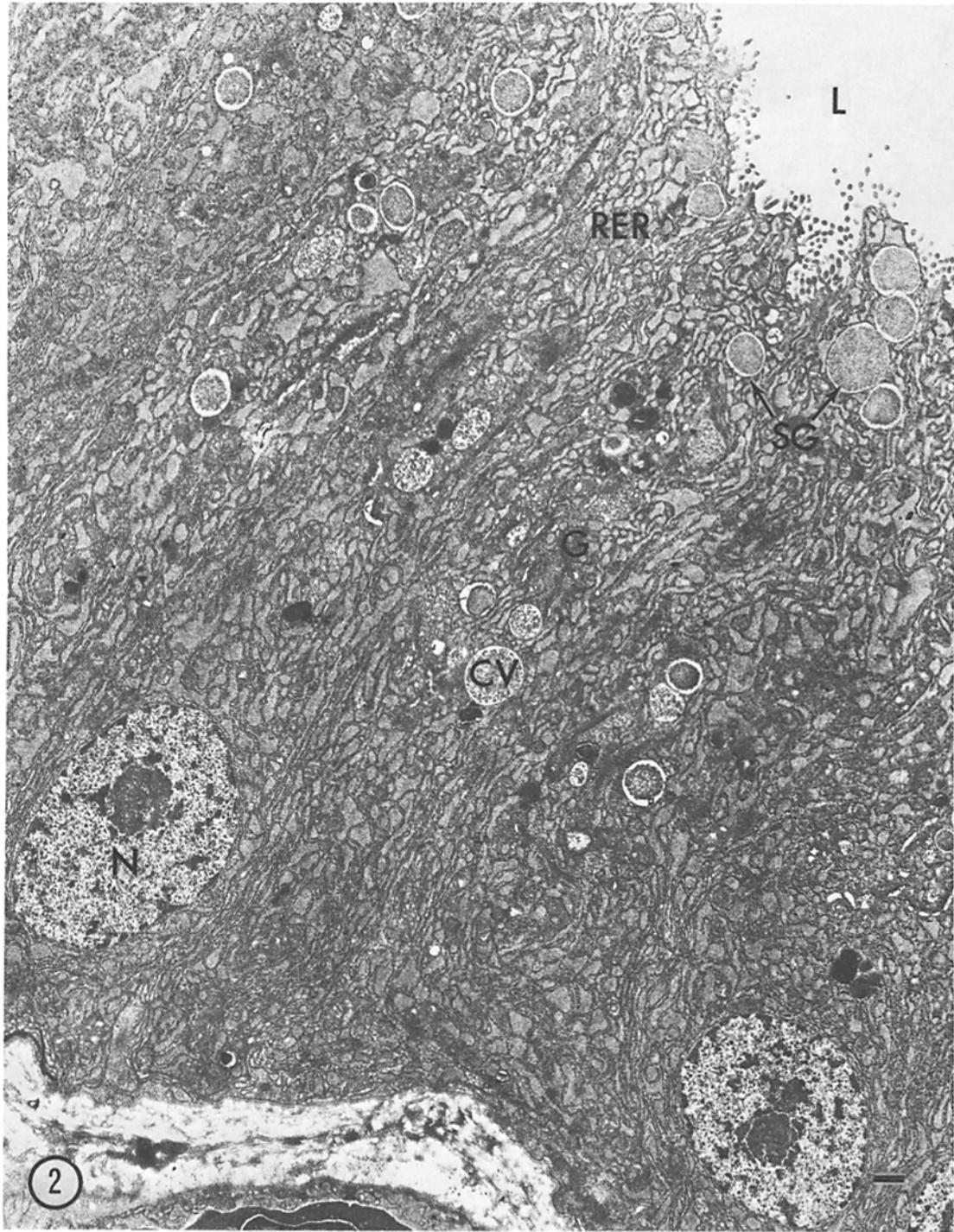
Figure 2 Electron micrograph of secretory cells of *V. palaestinae* 4 days after milking. Note the typical columnar shape with distended endoplasmic reticulum (*RER*). Nucleus (*N*), Golgi complex (*G*), condensing vacuoles (*CV*), secretory granules (*SG*), lumen (*L*). Initial fixation in 3% glutaraldehyde — 0.1 M Na cacodylate (pH 7.2). Bar, 1 μm. × 4,800.

TABLE I

Volume Fractions of Various Organelles of the Secretory Cell of the Venom Gland

Cell component	Venom gland		Parotid gland*	Exocrine pan-creas‡
	4 days after milking	Unmilked		
	$\bar{M} \pm SD$	$\bar{M} \pm SD$	$\bar{M} \pm SE$	$\bar{M}$
Nuclei	6.2 ± 2.9	10.4 ± 4.3	9.5 ± 0.2	
Secretory granules	1.8 ± 1.4	2.7 ± 1.8	36.3 ± 0.3	16.1
Condensing vacuoles	2.3 ± 0.9	2.5 ± 1.6	—	1.4
Golgi complex	3.6 ± 1.3	2.9 ± 1.6	—	6.3
RER (membranes + cisternae)	86.1 ± 3.8	81.5 ± 3.2	—	59.0

Computations of volume fractions of each of the cell compartments were analyzed by the point counting method (33). Measurements were made on prints with final magnification of 20,000 with a transparent overlay carrying a quadratic lattice with 0.5-cm spacing. The volume fraction values are expressed in the table as percent of total. The results are the mean ± standard deviation ($\bar{M} \pm$ SD) of nine glands (170 cells) for milked and five glands (230 cells) for unmilked glands.

* Bedi et al. (2).
‡ Berg et al. (4).

$52 \pm 10\%$ of the total labeled proteins could be precipitated by the antiserum. It can, thus, be assumed that, at least at the active stage, most of the labeled proteins are exportable. To check further the activity of the milked glands, the incorporation (count per milligram protein) into venom protein was compared between milked and unmilked glands, before the appearance of labeled venom (not later than 2.5 h postpulse). It was found to be 5.3 ± 1.8 times higher in glands 4 days after milking as compared with the resting glands (unmilked for 40–50 days) (cf. references 8 and 18).

Radioautographs of thick Epon sections of the same blocks prepared for electron microscopy are presented in Fig. 3. It can be seen that silver grains are distributed over the entire population of secretory cells of the labeled glands. 4 h after the injection of [³H]leucine, venom in the lumen of the active gland is heavily labeled (Fig. 3 b), while in resting glands the grain density over the venom in the lumen is very low, even at 6.5 h (Fig. 3 d).

Intracellular Transport and Secretion in Active Glands

Electron microscope radioautography of venom glands from snakes 10 and 20 min after labeling shows that about 85% of the silver grains in the secretory cells are found over the RER (Fig. 4, Table II). Later on, a progressive decrease of label associated with the RER is evident, and at 6.5 h about 50% of the total grains in the cells are localized over the RER (Table II). It should be noted that at this time interval more than 50% of the labeled protein has already been secreted (Fig. 8). Therefore, the relative amount of labeled protein in the RER compartment accounts for only about 25% of the total labeled protein synthesized by the gland. The Golgi complex shows a low but distinctive peak of grain density at 30 min after the injection (Fig. 8). There is a concomitant increase in the grain density of the condensing vacuoles associated with the Golgi saccules (Figs. 5 and 6). At this time, very few silver grains can be found over the secretory granules located closer to the apical membrane (Table II). A steep (15-fold) increase in the relative grain density of these granules is observed between 1 and 2 h after the injection (Fig. 8). At 3 h, these granules, which comprise less than 2% of the cell volume (Table I), contain 23% of the total silver grains (Table II, Fig. 7). 3–4 h after administration of the radioactive precursor, a decline in the labeling of the granules is observed, and at the same time an increase in secretion of radioactive venom is evident (Fig. 8). It is thus concluded that also in the venom gland the secretory granules are the immediate source of the secreted venom.

Intracellular Transport and Secretion in Resting Glands

In the unmilked glands the incorporation was generally low, and radioautographic analysis could not be carried out before 30 min after the injec-
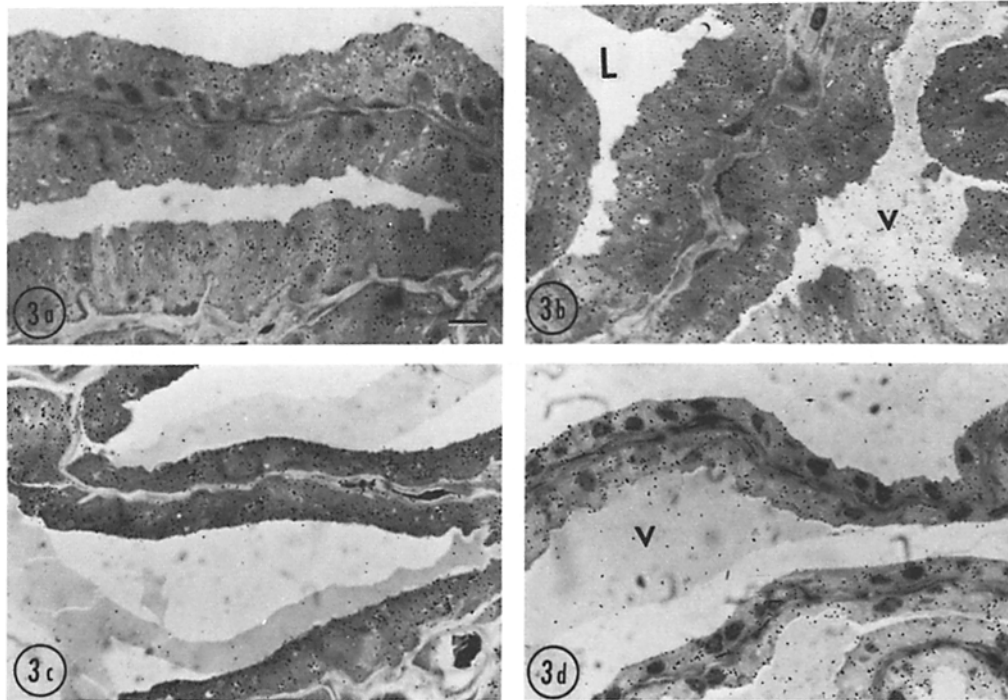
FIGURE 3   Light microscope radioautographs of secretory epithelium of venom glands. (a and b) Glands 4 days after milking. (c and d) Unmilked glands. Snakes were sacrificed 1 h after injection of [³H]leucine (Fig. 3 a and c), 4 h (Fig. 3 b) or at 6.5 h (Fig. 3 d). Note the columnar shape of the cells in the milked glands and the cuboidal shape of cells of resting glands. Epon sections (0.5-1.0 µm) stained with 1% toluidine blue. Lumen (L), venom in the lumen (v). Bar, 10 µm. × 500.

TABLE II

Distribution of Radioautographic Grains over the Secretory Cell Components of the Active Gland

Cell component	Time								
	10	20	30	60	80	135	170	240	390
					min				
RER	87.8	85.4	77.8	56.4	66.7	62.0	64.9	66.1	53.2
Golgi complex	6.2	6.0	10.4	7.7	13.7	7.1	—*	4.9	1.7
Condensing vacuoles	1.4	1.7	3.9	30.1	13.9	17.5	10.8	9.5	6.5
Secretory granules	0.6	0.2	0.7	1.3	3.2	10.6	22.3	16.1	37.8
Nuclei	3.2	3.8	5.2	3.2	1.5	2.5	2.0	2.4	0.4
Mitochondria	0.8	2.0	2.0	1.3	1.0	0.3	—*	1.0	0.4
Total grains counted	1,713	835	891	1,355	704	671	1,059	627	231

Nine cannulated snakes were injected each with 5-7 mCi [³H]leucine (see Materials and Methods). The results are expressed as the percent of total of silver grains found over each organelle. About 30 cells were analyzed in each time interval.
* These compartments were not analyzed at 170 min.

tion of [³H]leucine. At this time-point, 75% of the total silver grains were associated with the RER. Later on, a progressive decrease in the labeling of this compartment was evident, and at 6.5 h only 26% of the grains were found over the RER (Table III). Following the kinetics of labeling as depicted by the grain density curves, a decline in the labeling of the Golgi complex is evi-
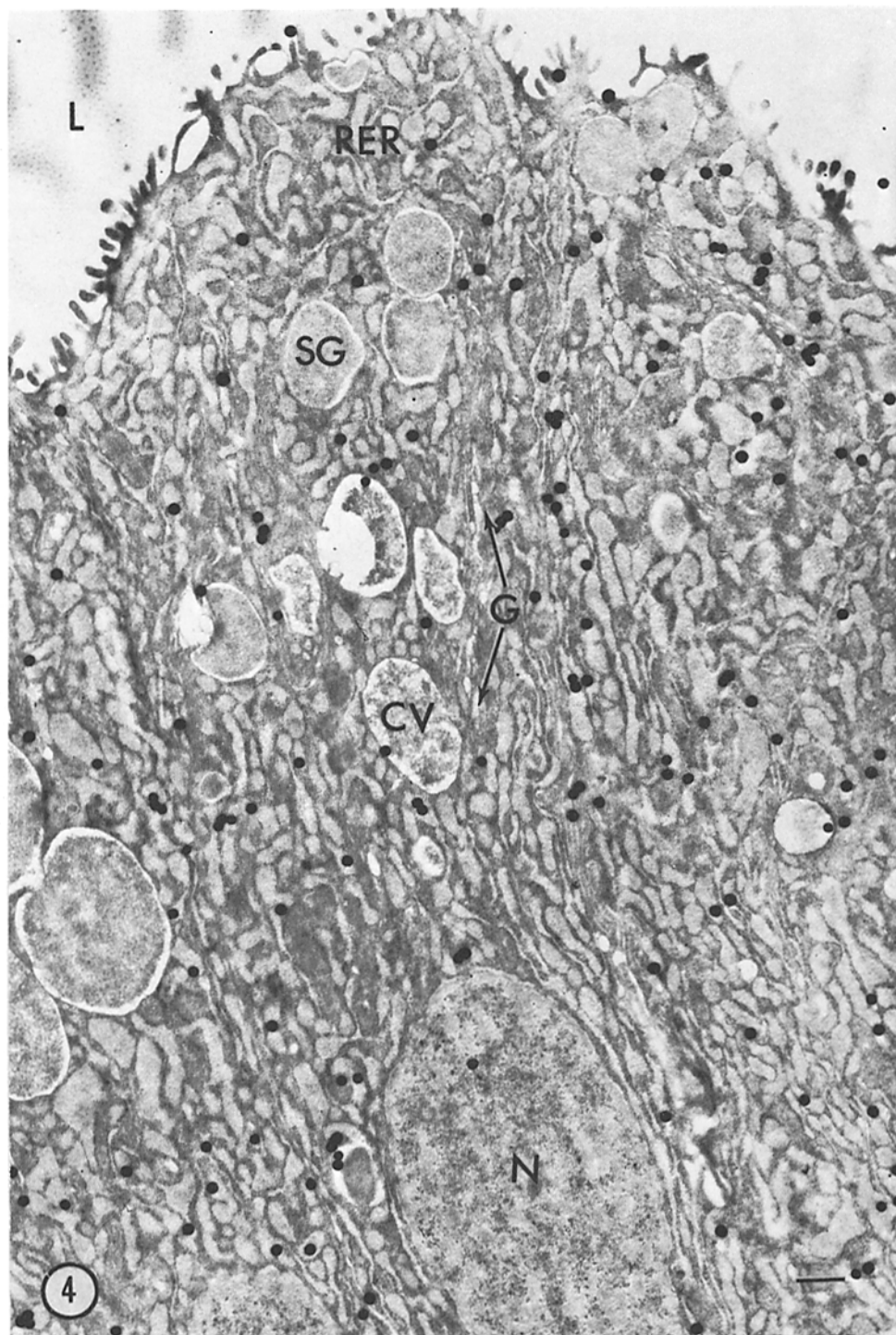
FIGURE 4 Radioautograph of an active secretory cell in the venom gland 10 min after injection of [³H]leucine. Silver grains are located almost exclusively over the RER. Nucleus (N), Golgi complex (G), condensing vacuoles (CV), secretory granules (SG), lumen (L). Fixation with formaldehyde. Bar, 1 μm. × 7,000.
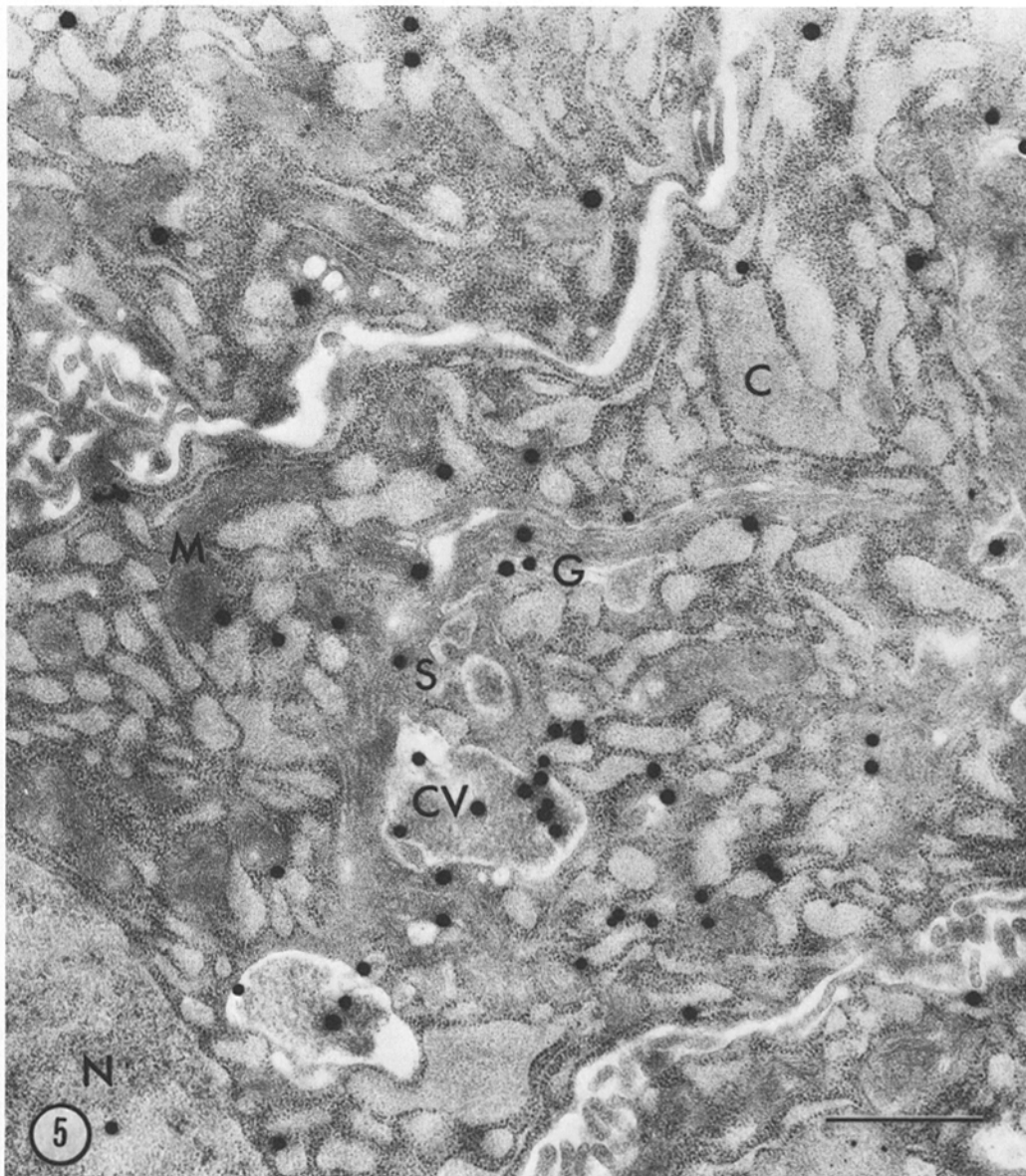
FIGURE 5 Radioautograph of supranuclear region of an active secretory cell 30 min after injection. Silver grains are associated with the membrane of the RER, are found over the Golgi complex (G), and are located also in the condensing vacuoles (CV). Nucleus (N), peripheral vesicles of the Golgi apparatus (SV), mitochondrion (M), cisternae of the RER (C). Bar, 1 μm. × 21,000.

dent already after the earliest time interval analyzed (Fig. 11). At this time (30 min), the relative grain density of the condensing vacuoles is already four times that of random distribution, while no grains are observed in the secretory granules (Table III, Fig. 9). At the longer time intervals, labeling of secretory granules increases. About 50% of the grains in the cell are associated with these

granules at 6.5-h post-labeling (Table III, Fig. 10), and no decline in the radioactivity of the secretory granules in cells of the resting glands was observed at the time-points analyzed. It should also be noted that only about 10% of the radioactive proteins have been secreted by this time in the resting gland (Fig. 11), while in the active glands secretion of radioactive venom starts much
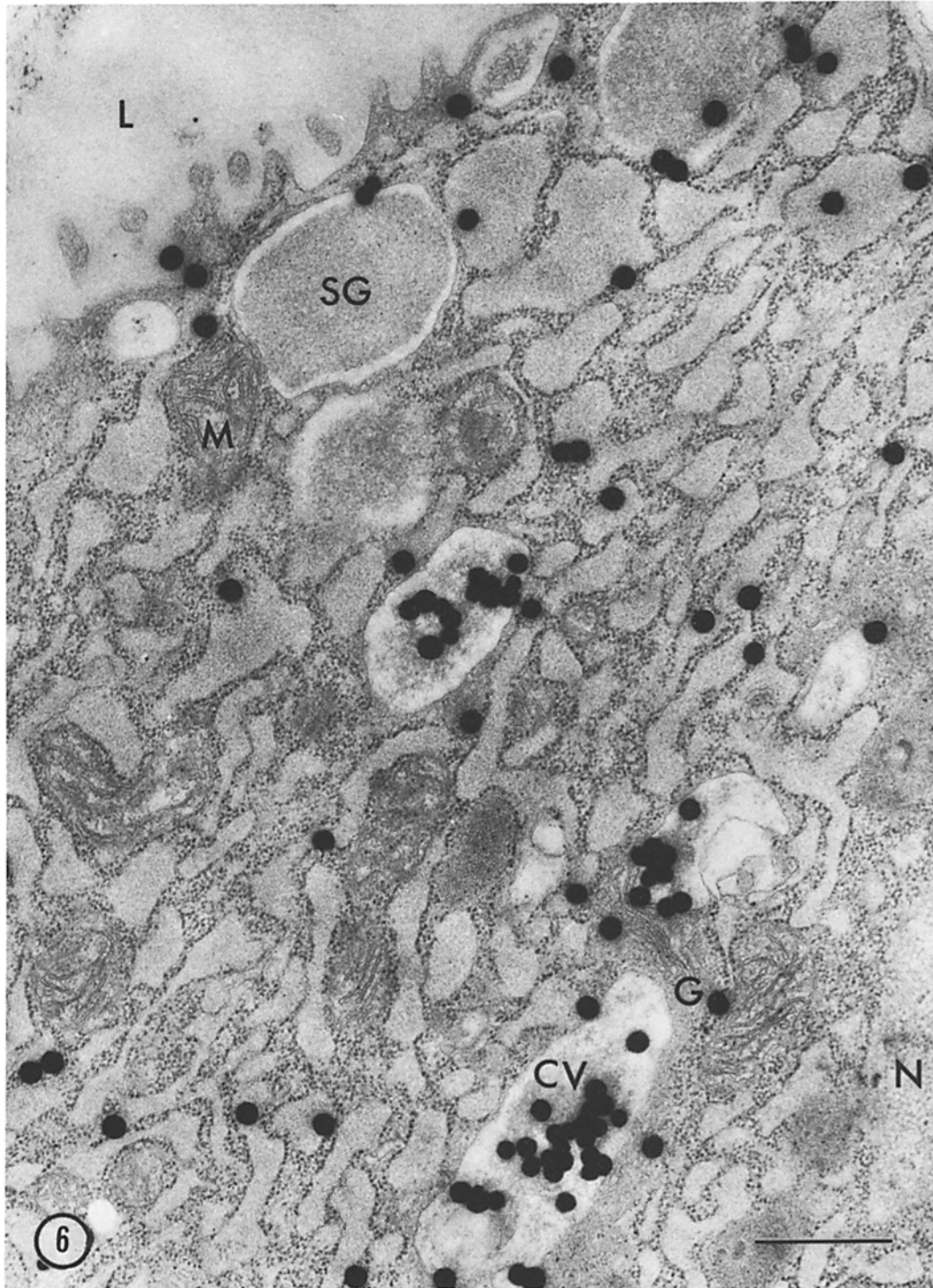
FIGURE 6 Radioautograph of an active secretory cell 60-min post labeling. Condensing vacuoles (*CV*) with a loosely packed content are highly labeled while the secretory granules (*SG*) located at the apex of the cell are still devoid of label. Nucleus (*N*), Golgi complex (*G*), mitochondrion (*M*), and lumen (*L*). Bar, 1 μm. × 24,000.
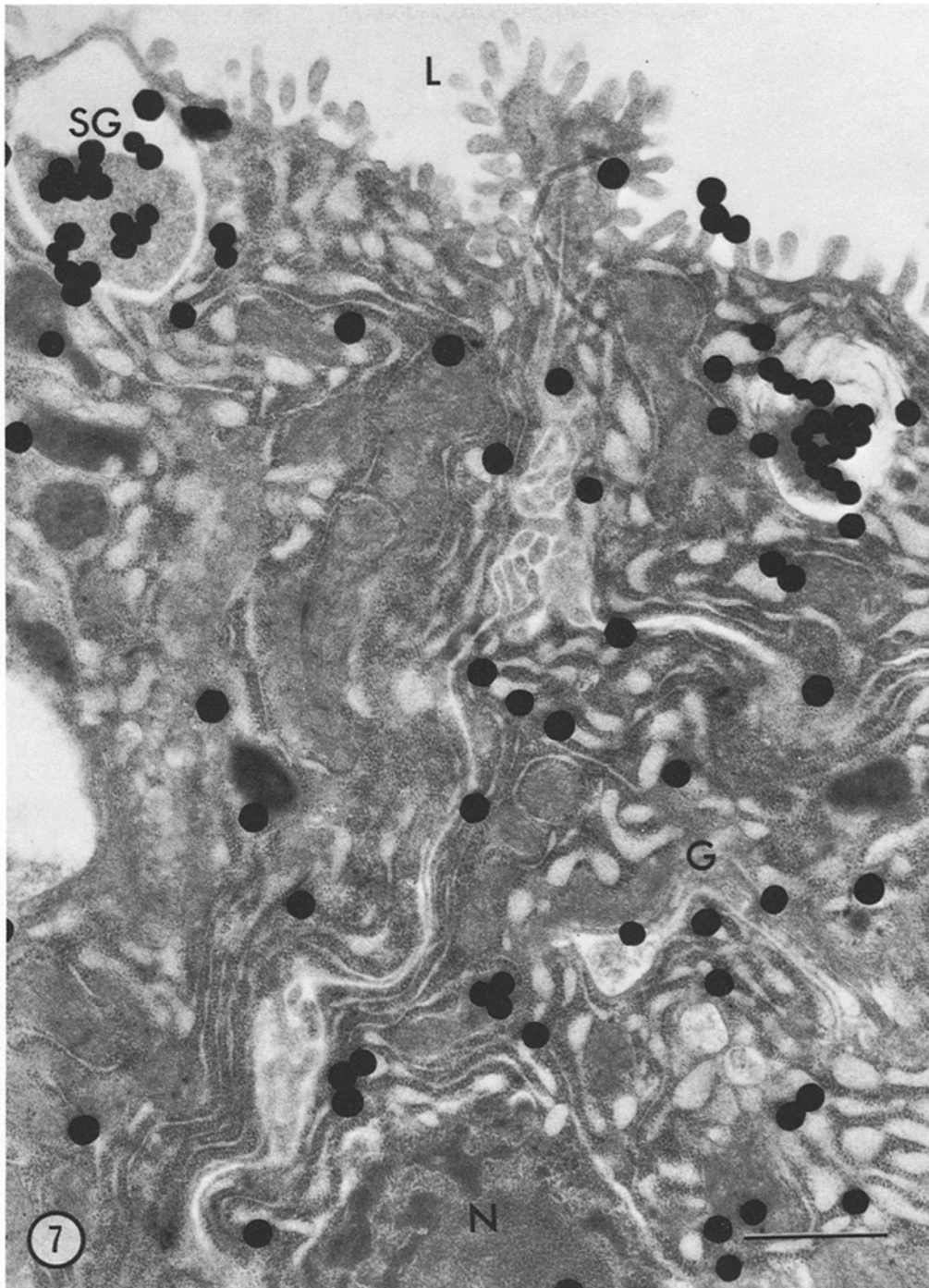
FIGURE 7   Radioautograph of an active secretory cell 3 h after injection of [³H]leucine. Note the high concentration of silver grains over the secretory granules (*SG*). Nucleus (*N*), Golgi complex (*G*), and lumen (*L*). Bar, 1 μm. × 21,000.
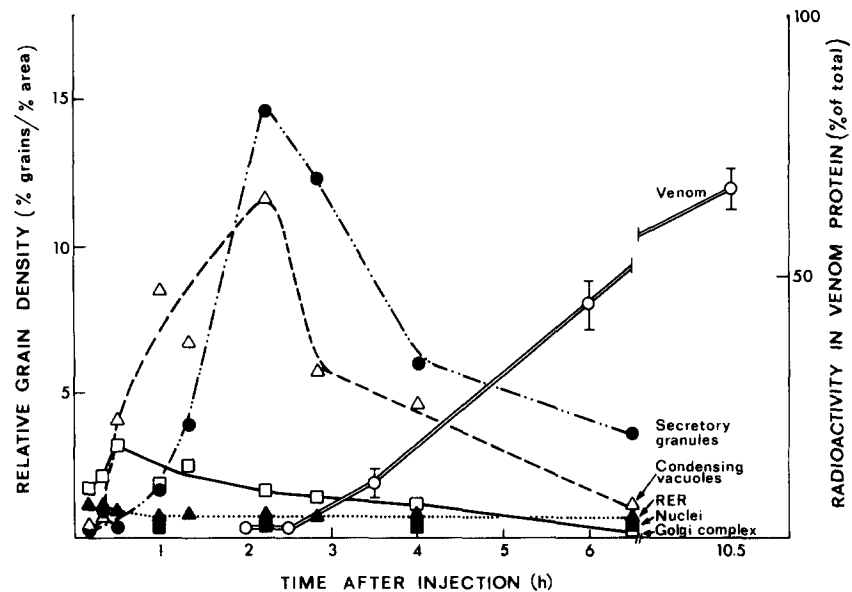
FIGURE 8   Kinetics of labeling of the various cell organelles and the venom in the active secretory cell after administration of [³H]leucine. The radioautographic data are from the same experiment described in Table II. Labeling of the venom was followed in a separate experiment. Cannulated snakes with milked and unmilked glands (see Materials and Methods) were injected with 1 mCi of [³H]leucine each. Venom was milked at various time intervals after the injection, and glands were removed and homogenized. Radioactivity in the venom (○) is expressed as percentage of total radioactivity in proteins of the venom and gland homogenate. Values of each time are the mean of two to three glands ± SD (bars). Relative grain densities are calculated as follows: (% grains/% area) × (100 − % of ³H-proteins secreted)/100. The values, thus, represent concentration of radioactive proteins in each cell organelle as percentage of the total labeled proteins.

TABLE III

Distribution of Radioautographic Grains over the Secretory Cell Components of the Resting Gland

| Cell component	Time			
	30	60	240	390
	min			
RER	75.1	61.0	48.3	26.3
Golgi complex	7.7	15.0	6.7	2.3
Condensing vacuoles	8.7	15.0	19.3	19.0
Secretory granules	0.0	0.0	21.7	48.8
Nuclei	7.2	1.0	4.0	1.1
Mitochondria	—*	—	—	2.3
Total grains counted	375	120	327	647

Distribution of silver grains was analyzed in unmilked glands of four snakes used in the experiment described in Table II.

* Grains were not counted over the mitochondria of glands 30, 60 & 240 min after the injection.

earlier and by 6.5 h more than 50% of the labeled protein has already been secreted (Fig. 8).

DISCUSSION

The ultrastructural features of the secretory cell of the viperid's venom gland are assessed in this work by quantitative parameters. This cell, like many other glandular cells, has an RER and a Golgi apparatus characteristic for most secretory cells. However, while in mammalian digestive glands the secretory granules comprise up to about 35% of the cell volume, only 4% of the venom gland cell is occupied by secretory granules and condensing vacuoles (Table I). The cells of seminal vesicles and of the prostate are somewhat similar to the cells of the venom gland. In these glands, secretory vesicles account for only 1-2% of the cell volume and, as in the venom gland, most of the secretory products accumulate in rather large lumina (9, 10, 21). A similar pattern
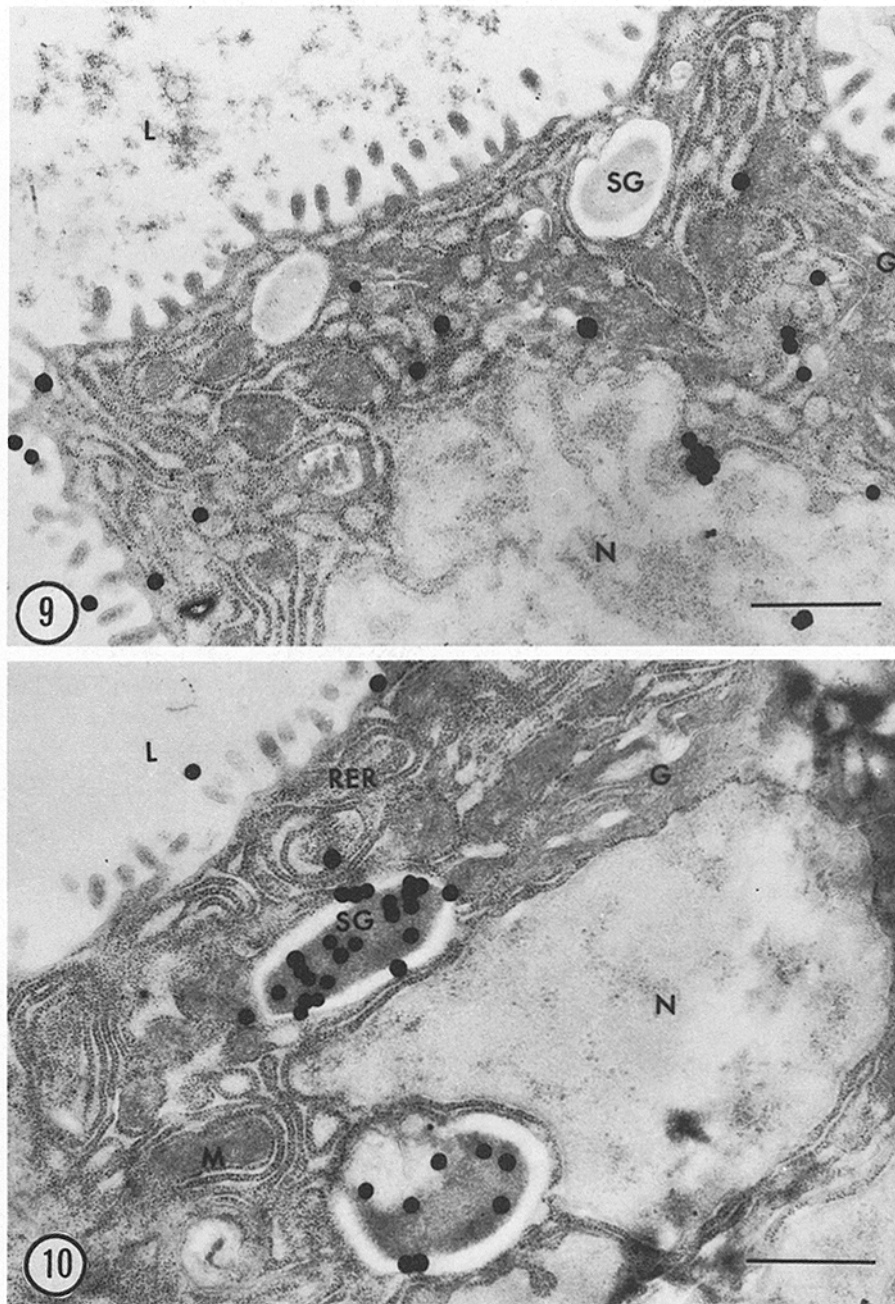
FIGURES 9-10 Radioautographs of secretory cells from unmilked glands 1 h (Fig. 9) and 6.5 h (Fig. 10) after administration of the labeled precursor. Note the flat shape of the cells and the packed pattern of the endoplasmic reticulum (*RER*). 1 h after injection grains are seen only over the *RER*, while at 6.5 h grains are located mainly over the secretory granules (*SG*). Nucleus (*N*), Golgi complex (*G*), mitochondrion (*M*), and lumen (*L*). Bar, 1 μm. × 17,000.
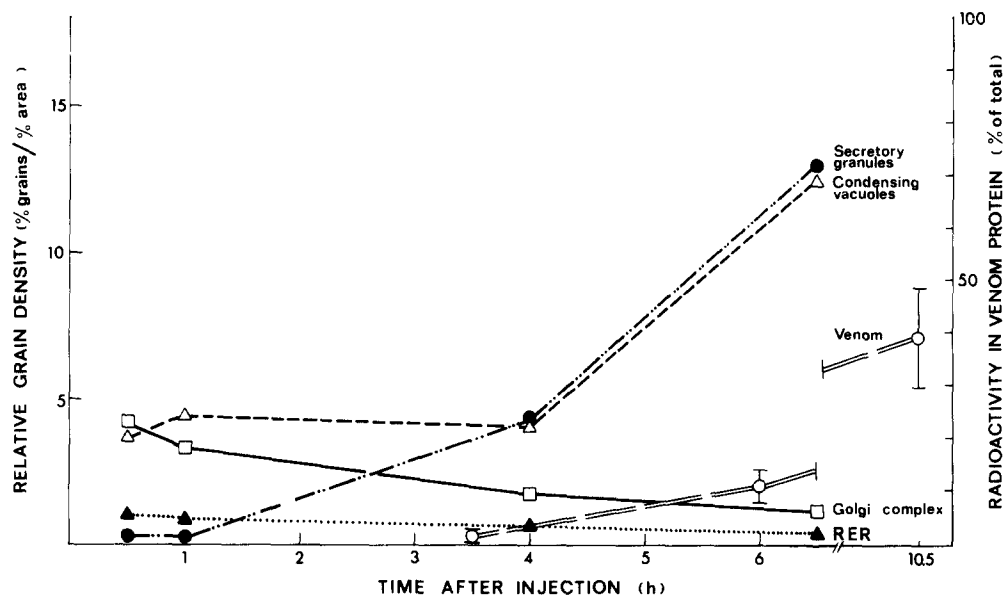
FIGURE 11 Kinetics of labeling of various cell organelles and venom in the resting secretory cell. Radioautographic analysis and venom secretion were followed in the unmilked glands of the same snakes used in the experiments described in Fig. 8.

is also found in the thyroid gland, where secretion is stored mainly in the follicular lumen (34).

In the present study, the intracellular transport of labeled proteins was followed in the venom gland after administration of [³H]leucine into the blood system. A relatively short pulse labeling was thus obtained, and most of the proteins synthesized in the active gland were venom proteins.

The wavelike passage of radioactive proteins through the intracellular compartments is readily observed from quantitative analysis of the radioautographs. At the short intervals after labeling of the active secretory cells, most of the silver grains are located over the RER. 1 h after the injection, increase in concentration of label over the condensing vacuoles is evident, while the secretory granules are still poorly labeled. These granules show the highest grain density 2-3 h after the injection, and later, when a decline in their labeling is observed, secretion of radioactive venom increases.

It can thus be concluded that the transport of venom proteins in the active secretory cells of viperid venom gland follows the same pathway described in the exocrine pancreas (20): RER → Golgi complex → condensing vacuoles → mature secretory granules.

However, in view of the fact that the total population of secretory vesicles in the venom

gland (including the condensing vacuoles) accounts for only 4% of the cell volume, while most of its volume is occupied by the RER, other intracellular pathway(s) should be considered. An alternative pathway might involve the accumulation of venom proteins in the extended cisternae of the RER and direct secretion from this pool into the lumen (cf. Rothman [23]).

Warshawsky et al. (31), working with the Brazilian rattlesnake (*Crotalus d. terrificus*), found in the secretory cells of its venom gland dense intracisternal granules in the RER. It was stated by these authors: "These granules . . . become more frequently labeled . . . after the main mass of labeled secretion has passed. . . ." Such granules have never been observed in the venom glands of viperid snakes (3, 18). In the present study, the label never becomes concentrated (grain/volume) in regions occupied by the RER at time-points after initial synthesis. Thus, direct secretion from the intracisternal space in the secretory cells of the viperid venom glands seems unlikely.

In nature, viperid venom glands are usually filled with venom, and most of the secretory cells are at the resting stage (14). In order to study the intracellular transport at this stage, unmilked labeled glands from the same snakes, in which the contralateral glands were at the active stage, were analyzed by radioautography. It was found that

the intracellular transport in these glands follows the same route as in the active ones, but in a much slower rate. Thus, in milked glands radioactive proteins are observed in the lumen 2-3 h after labeling, while in the unmilked gland they are found in the venom only 4-5 h after the [³H]leucine injection.

Control of secretory processes may involve regulation of synthesis, intracellular transport and of discharge. It is generally accepted today that various secretagogues affect primarily the discharge step, and their mode of action has been extensively studied (17, 25). The effects of secretory stimuli on synthesis and transport are not well understood (28, 32). Most studies on the exocrine pancreas failed to show influence of secretagogues on the rate of intracellular transport (see reference 12 and 27). Only recently, Bieger et al. (5) showed that prolonged infusion of rats with caerulein led to change in rate of transition of secretory proteins through the cell.

In the venom glands of Viperidae, it has been shown that milking is followed by an increased rate of incorporation of labeled amino acids into protein (8, 18). It is assumed that the specific radioactivities of the precursors were similar in the active and resting glands, since no significant change has been found in the size of the intracellular pool of amino acids during the secretory cycle of the venom gland.² We therefore believe that activation of the gland by milking induces an accelerated rate of venom synthesis.

So far, there is no evidence as to a control mechanism involved in the discharge of proteins by the venom gland cells. Venom produced by the gland is accumulated in the gland lumina, while no substantial changes in the intracellular concentration of exportable enzymes have been found (29).

It is thus concluded that in viperid venom glands the increased rate of secretion after initiation of a new venom regeneration cycle is a result of both an increased rate of venom synthesis and an accelerated rate of transition of secretory proteins through the cell.

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² Unpublished results from this laboratory.

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