REGULATION OF PROTEIN SYNTHESIS IN THE VENOM GLAND OF VIPERID SNAKES

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ABSTRACT

Morphological changes in the venom gland of *V. ammodytes* were studied after the removal of the venom from the gland lumina (milking) It was found that the height of the secretory cells was changed during the secretory cycle. The patterns of the rough endoplasmic reticulum and of the Golgi complex were changed as well Milking induced an increased incorporation of [^{14}C]amino acids into total and venom proteins In *V ammodytes*, during the first day after milking, 25% of the total counts in protein were precipitable by anti-venom serum, while at 8 days, 80% of the proteins synthesized were venom proteins At this stage, the incorporation was 10- and 20-fold that of unmilked glands for total and venom proteins, respectively. Venom was accumulated (secreted) in the gland lumina of *V. ammodytes* at a relatively high rate up to 2 wk after milking and leveled off afterwards. Intact glands and gland slices of *V ammodytes* and *V palaestinae*, taken from snakes a few days after milking, incorporated [^{14}C]amino acids into proteins in vitro at a rate higher than that of unmilked glands. The activity of two exportable enzymes (phosphodiesterase and benzoyl arginyl ethyl esterase) was assayed in gland homogenates of *V. ammodytes*. It was found that 2–3 wk after milking, the intracellular level of these enzymes was up to 2-fold that of unmilked glands.

INTRODUCTION

Venom glands of snakes have a unique structure and properties not found in mammalian salivary glands (Kochva and Gans, 1970) The main portion of the venom gland of viperid snakes is made up of branching tubules arranged around a large central lumen in which considerable quantities of venom may be stored At the so-called resting stage, lumina are filled with venom and the glands have a low secretory epithelium. Kochva (1960) and Rotenberg et al (1971) showed that during the first 2 wk after milking, accumulation of venom (secretion) in gland lumina of *Vipera palaestinae*, proceeds at a relatively high rate and slows down afterwards During the first few days after milking, the secretory cells increase in size and the endoplasmic reticulum is extended (Ben-Shaul et al., 1971). The rate of RNA synthesis is also maximal between the first and the fourth day after milking (Rotenberg et al , 1971).

The present communication describes studies on the incorporation of labeled amino acids into the total proteins and into venom proteins of the venom glands of two viperid snakes Protein synthesis and venom accumulation is compared with morphological changes in the secretory epithelium at the different secretory stages. Some of these results have been previously reported (Ehrenwald [Oron] and Bdolah, 1971).

MATERIALS AND METHODS

Snakes

Specimens of *Vipera ammodytes* (40–100 g) and *V. palaestinae* (400–500 g) were kept at 29°–31°C and

fed as described previously (Sobol-Brown et al., 1971).

Venom was removed from the gland lumina by exerting pressure on the sides of the snake's head, while the fangs were caught with a glass vial (Klauber, 1956; Kochva, in press) This procedure will be referred to as "milking" Milking of venom from only one gland was done under anaesthesia with Fluothane (2-bromo-2-chloro-1:1:1-trifluoroethane, I.C. I., England). Pressure was exerted on one side of the head, special care being exercised to prevent leakage of venom from the other fang.

Light and Electron Microscopy

A small section of one gland of each secretory stage was fixed in Bouin's fluid (Barka, 1963) for a week, embedded in paraffin, and sectioned at 4 μm

For electron microscopy, the glands were fixed for 20 h in 3.5% glutaraldehyde (Ladd Research Industries, Inc , Burlington, Vt.) in 0.1 M cacodylate buffer, pH 7.2, washed in several changes of the same buffer, and postfixed in 2% osmium tetroxide The tissue was embedded in Epon 812 and sections were prepared on an LKB ultramicrotome Sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined in a Jeolco Jem 7 or Jem T-7 electron microscope.

Preparation of Venom Glands for Analysis

Snakes were anaesthetized, milked, decapitated, and the main portions of the glands were removed, without the accessory glands (see Kochva and Gans, 1965). Each gland was cut into 10–15 pieces and rinsed with excess of 0.3 M sucrose solution to ensure complete removal of the venom absorbed onto the surface of the secretory epithelium. 30–100 mg of washed tissue was homogenized with 1 ml of Tris acetate buffer, 0.01 M, pH 7.4 and Triton X-100, 0 1% (vol/vol). Homogenization was for 2 min employing a Teflon glass homogenizer revolving at 800 rpm. The supernatant obtained after centrifugation at 270 g for 5 min was used for enzyme analysis and for isolation of protein.

Preparation of Proteins for Radioactive Measurements

Total proteins (including venom protein and structural protein) were precipitated with 5% trichloroacetic acid (TCA) at 0°C. The precipitate was washed on a fiber glass filter (Gelman Instrument Co., Ann Arbor, Mich.) with 40 ml of cold TCA and dried for 1 h at 110°C. Venom proteins were selectively isolated by first centrifuging the gland homogenate at 200,000 g for 45 min. The supernatant

contained 90% of the TCA precipitable counts after in vivo labeling and 70%–85% after in vitro labeling (see Results) Venom proteins were precipitated from the supernatant with a specific antiserum (see below). The precipitate was washed on a filter with the homogenization buffer and dried as above The filters were counted directly in 5 ml of 2,5-diphenyloxazole, 0.5% (wt/vol), 1,4-bis-(4-methyl-5 phenyloxazole-2-yl) benzene 0.01% (wt/vol) in toluene, with a Packard Tri-Carb Liquid-Scintillation Spectrometer.

Preparation of Immune Serum Against V. Ammodytes Venom

For this work the efficiency of the antiserum will be defined as the percentage of [^{14}C]labeled venom proteins precipitated by this antiserum from the total labeled proteins in the venom (precipitable by 5% TCA). Commercial antisera showed efficiencies of 20%–40% Antisera with higher efficiencies were, therefore, prepared.

Rabbits were immunized once a month intramuscularly with 0.5 mg of dried venom per kg body weight in adjuvant (Difco Labs, Detroit, Mich.). Each rabbit received at least three injections and bleeding was performed 10 days after the last injection. With these antisera, 100–200 μg of venom were precipitated by 0.4 ml of serum in a final volume of 0.6 ml, with 90% efficiency.

For the isolation of radioactive venom proteins from gland homogenates, a constant amount of nonradioactive carrier venom (150 μg) was added to the homogenate antiserum mixtures. The amount of radioactive proteins precipitated from homogenate by nonimmune serum or by heterogenous antigen and antibody (albumin-antialbumin) was not more than 5% of the total radioactive proteins

In Vitro Experiments

For the experiments with whole intact glands, the snakes were anaesthetized and the primary duct (see Kochva and Gans, 1965) was ligated. The ligated glands were then dissected out and incubated in a synthetic medium. When gland slices were prepared, the snake was first milked and the tissue slices were washed of the venom with 0.3 M sucrose before being transferred into the incubation medium. The medium was a modification of the "Krebs-Ringer bicarbonate" (Cohen, 1957), in which the sodium chloride concentration was 160 mM. It was also supplemented with casein hydrolyzate (BDH Chemicals Ltd., Poole, England), 1 mg per ml; L-tryptophane, 15 μg per ml and D-glucose, 1 mg per ml. Gland slices, about 1 mm thick, or whole intact glands were

incubated in the presence of [14C]protein hydrolyzate[1] (algal, uniformly labeled, The Radiochemical Centre, Amersham, Buckhamshire, England) with a gas phase of 5% CO_2, 95% O_2 At the end of the incubation the tissue was washed with excess of non-radioactive medium on a filter.

Analytical Methods and Statistics

Enzymes were assayed at 37°C with a Gilford model 2000 spectrophotometer Benzoyl arginyl ethyl esterase was assayed according to Schwert and Takenaka (1955) with glycine buffer, 0 05 M, pH 9 5 and N-benzoyl-L-arginyl ethyl ester (Miles-Yeda, Rehovot, Israel) 0 5 mM Phsphodiesterase was assayed according to Razzell and Khorana (1959) with 0.5 mM p-nitrophenyl thymidine-5'-phosphate (Calbiochem, Los Angeles, Calif.) in Tris buffer, 0 05 M, pH 9 0, 1 mM, $CaCl_2$. Protein was estimated according to Lowry et al. (1951) with bovine serum albumin as standard.

The incorporation of labeled amino acids and enzyme activities in the gland at different stages were compared by analysis of variance The differences between the mean values of two different groups were analyzed by the sum of squares simultaneous test procedure (Gabriel, 1964).

RESULTS

Morphological Changes in the Secretory Cells of the Venom Gland of Vipera ammodytes

Morphology of the gland cells of V ammodytes along the secretory cycle is similar to that observed for V. palaestinae (Ben-Shaul et al., 1971). In the unmilked glands, the secretory cells are low and most of them show a cuboidal-to-flat shape (Figs 1, 2) At this stage several electron-opaque vesicles are seen and the Golgi complex is difficult to distinguish. In some cells it is found below or lateral to the nucleus (Fig 3) The RER is arranged in arrays of narrow cisternae around the nucleus and toward the apex of the cell (Fig 2) At 1 day after milking, the secretory cells increase in size (Figs. 1, 4), the membranes of the RER become separated from each other, and the Golgi

complex is easily distinguished Content of the vesicles shows a lower density than in unmilked glands

The highest secretory cells are found at about 1 week after milking (Fig 1) At this stage the expansion of the intracisternal space of the RER becomes conspicuous and a well-developed Golgi complex is evident in the supranuclear region (Fig. 5). The Golgi complex is usually made of a few stacks of saccules, some of them closely associated with large vesicles of low electron opacity (Figs 5, 6) Vesicles of variable density are also found close to the apical membrane

23 days after milking, the cells decrease in size and the RER is again more closely packed Extended cisternae are still seen close to the apex of the cells (Fig. 7)

Incorporation of Labeled Amino Acids into Protein in Glands at Different Stages after Milking

The incorporation of amino acids into protein in the venom gland was studied at different time intervals after injection of labeled amino acids Fig. 8 shows that milking induced an accelerated rate of incorporation in the venom gland. Incorporation into glands 2 or 4 days after milking was significantly higher (P < 0.001) than into unmilked glands, while there was no significant difference between the rate of incorporation in livers of milked and unmilked snakes

In another work done in this laboratory (Sobol-Brown et al, 1971), considerable variability was found among the snakes, while the two glands of the same snake showed no marked differences in enzyme activity In addition, the rate of incorporation of labeled amino acids into protein in the two glands of the same snake in V. ammodytes was also similar. It was, decided therefore, to study the kinetics of protein synthesis in the venom gland by comparison of one gland in a particular secretory stage to the contralateral unmilked gland A group of 28 snakes, kept without food for 70–90 days in order to avoid loss of venom, were used for the following experiment.

It can be seen (Fig. 9) that the amount of labeled protein synthetized by the glands varied at different stages after milking (P < 0.005). This value increased after milking and at 8 days was ten times that of the unmilked gland. At 41 days,

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[1] Amino acid analysis of venom from V palaestinae and V. ammodytes (unpublished) did not show any high concentration of a certain amino acid So far there is no single protein in viperid venom known to contain high concentrations of a single amino acid. A mixture of labeled amino acids was, therefore, preferred over a single one.
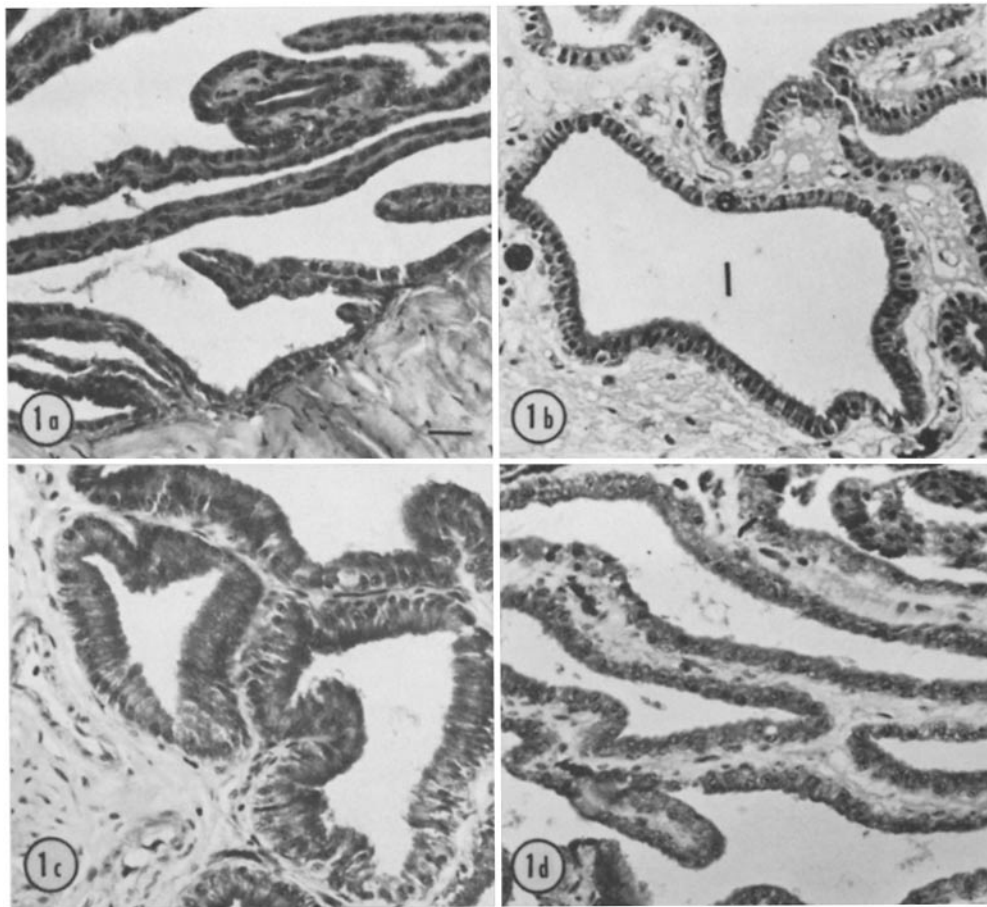
FIGURE 1   Morphological changes in the secretory epithelium of the venom gland of *V. ammodytes* at different stages after milking. Fig 1 a, unmilked gland. Figs. 1 b–1 d, glands after milking: 1 day (1 b), 8 days (1 c), and 22 days (1 d). Note changes in the size of the secretory cells (*e*) during the secretory cycle. Lumen (*l*). Masson's trichrome. Scale, 10 μm. × 570.

the incorporation of [^{14}C]amino acids into protein was similar to that of unmilked glands. A similar pattern was found for the synthesis of venom proteins (Figs. 9, 10). During the period between the third day and the 22nd day after milking, the percent counts found in venom proteins was significantly higher (P < 0.05) than during the first day At 8 days, the amount of labeled venom synthesized by the gland was 20 times that of the unmilked gland. At this stage, 80% of the total proteins synthesized were venom proteins, while during the first day after milking only 25% of the label was found in venom proteins (Fig. 9). It is also shown (Fig. 10) that the rate of venom secretion (accumulation of venom in gland lumina)

proceeded at a relatively high rate up to 2 wk after milking and decreased at the later stages

In Vitro Protein Synthesis in the Venom Gland

Whole glands with ligated ducts, in which the pressure exerted by the accumulated venom was sustained, were incubated in a synthetic medium in the presence of labeled amino acids. The rate of protein synthesis in this system was not linear for more than 30 min, probably because of a slow diffusion of substrates and oxygen through the rather thick capsule of the venom gland. As shown in Table I, the amount of labeled proteins found
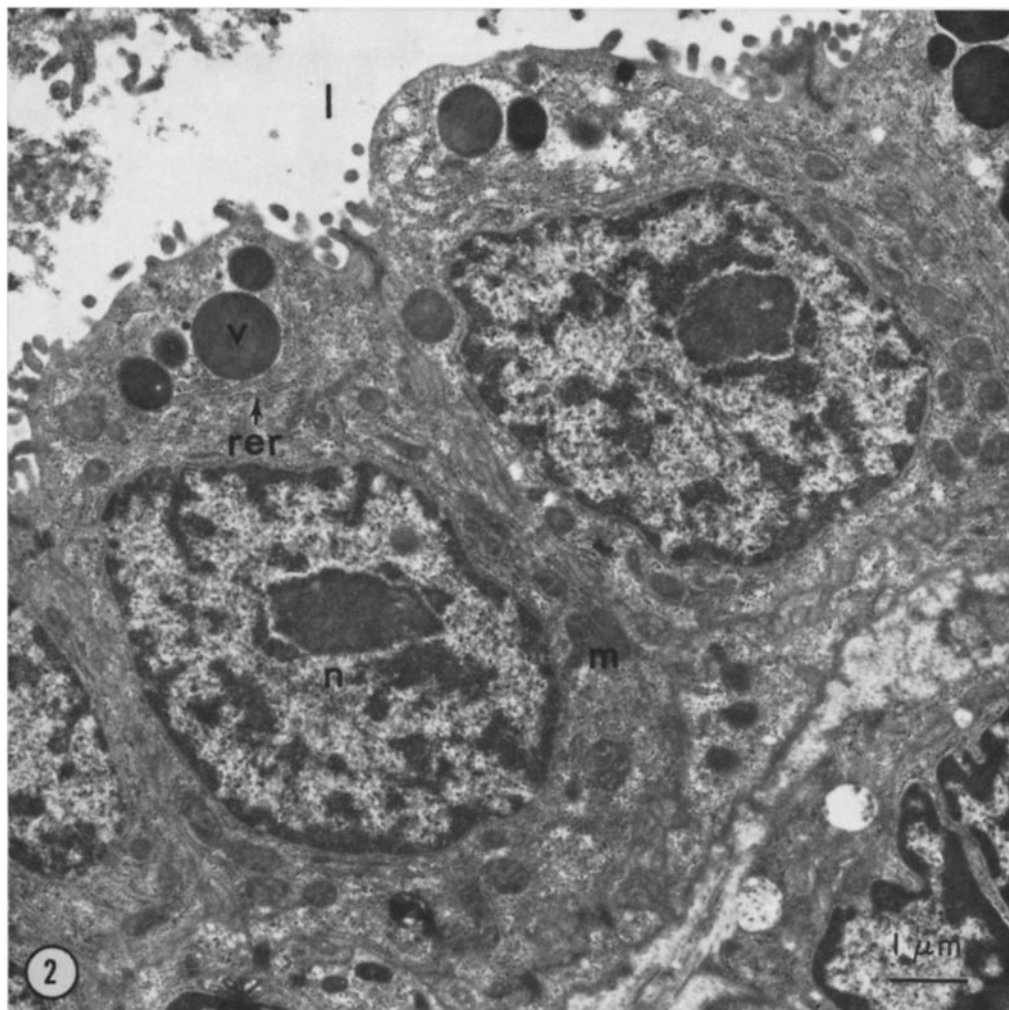
FIGURE 2 Secretory cells of an unmilked gland. Note the electron-opaque vesicles (v) and the packed pattern of the endoplasmic reticulum (rer) Nucleus (n), mitochodrion (m), lumen (l) × 10,200.

in milked glands of both *V ammodytes* and *V. palaestinae* was three to four times higher than that in unmilked glands Also the labeling of venom proteins in milked glands was two to three times that of unmilked glands. About 30% of the total counts in protein was found in venom proteins

Venom gland slices incubated in the same medium incorporated labeled amino acids into protein at a linear rate for at least 1 h (Fig 11) Isolation of venom proteins showed that venom synthesis was also linear at the same time. Leaking of protein from the slices to the medium did not exceed 10% of the total gland protein and loss of radioactive proteins to the medium during the incubation period was negligible Histological

inspection of the slices after 1 h of incubation did not show any extensive damage to the tissue.

It was again found in the slices system that the rate of incorporation of labeled amino acid into total and venom proteins during 30 min in glands of *V ammodytes*, 8 days after milking is considerably higher than in unmilked glands (Table II)

Changes in the Level of Exportable Enzymes in the Venom Gland Cells at Different Stages of the Secretory Cycle

Sobol-Brown et al (1971) followed the accumulation of three enzymes in the gland lumina of *V.*
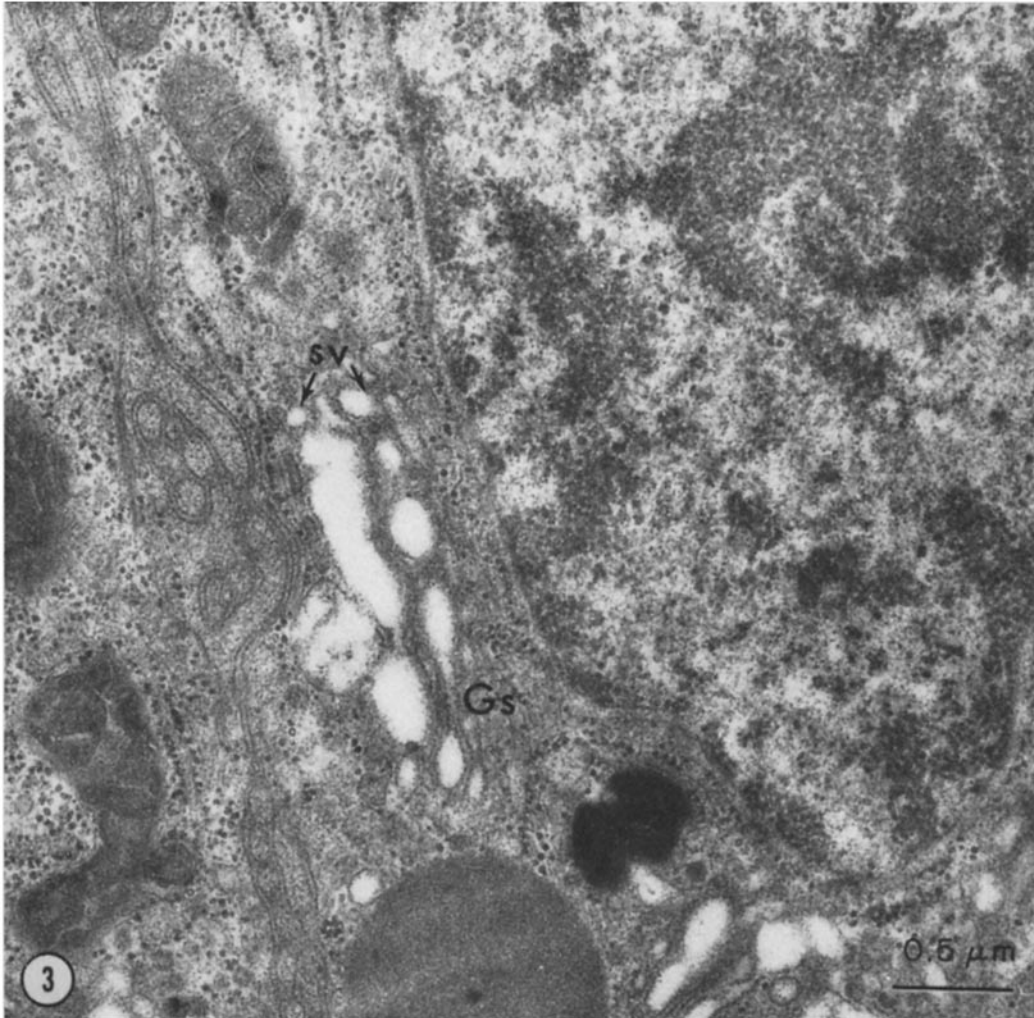
FIGURE 3   Golgi zone in a secretory cell of an unmilked gland. Saccules of the Golgi apparatus (*Gs*) and small smooth vesicles (*sv*) are located at the basal part of the cell. × 32,000.

palaestinae and demonstrated the activities of these exportable enzymes in the gland cells using histochemical methods The same enzymes were found in the venom and venom gland of *V. ammodytes*. The activities of phosphodiesterase (PDE) and of benzoyl arginyl ethyl esterase (BAEE) were measured in gland homogenates at different intervals after milking. The activity of PDE did not vary significantly (P < 0 10) when compared to unmilked glands during the secretory cycle. However, the highest average activity was attained at 3 wk after milking (Fig. 12) Significant differences in BAEE activity (P < 0.005) were found at different secretory stages 2 wk after

milking, the activity of BAEE was twice as high as that of unmilked glands and was significantly different (P < 0.05) from the activities found in glands during the first week after milking. On the first day after milking, activities of both enzymes were lower than in unmilked glands. The total protein contents of gland homogenates was higher (not significant P < 0.10) in glands 2 and 3 wk after milking.

DISCUSSION

Morphological changes in the secretory cells of the venom gland after expulsion of venom were already noticed by Velikii (1890) in *V. ammodytes*.
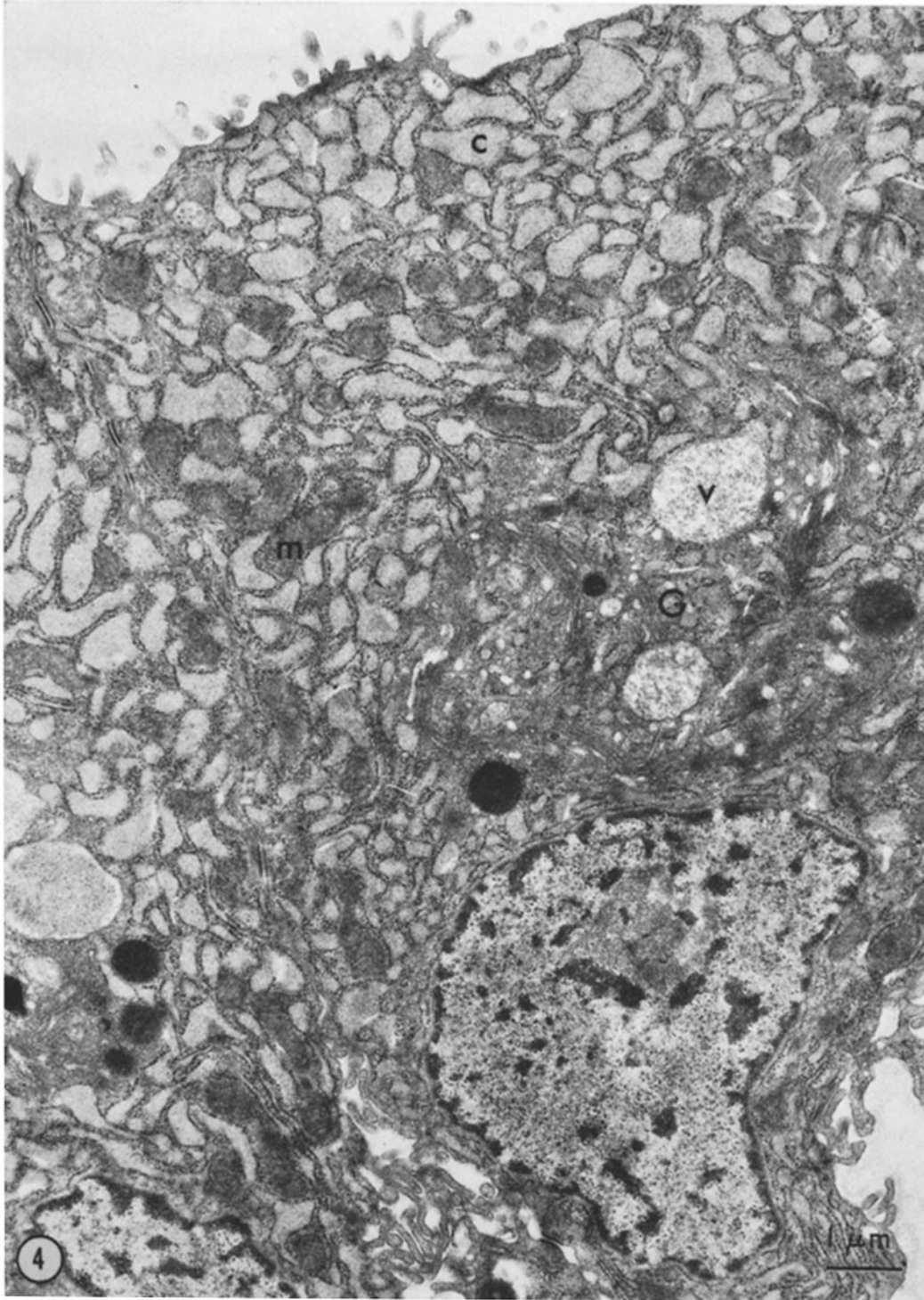
FIGURE 4 1 day after milking. Note the lower density of the vesicles (v) and the extended intracisternal space (c) of the RER. Golgi zone (G), mitochondrion (m). × 11,000.
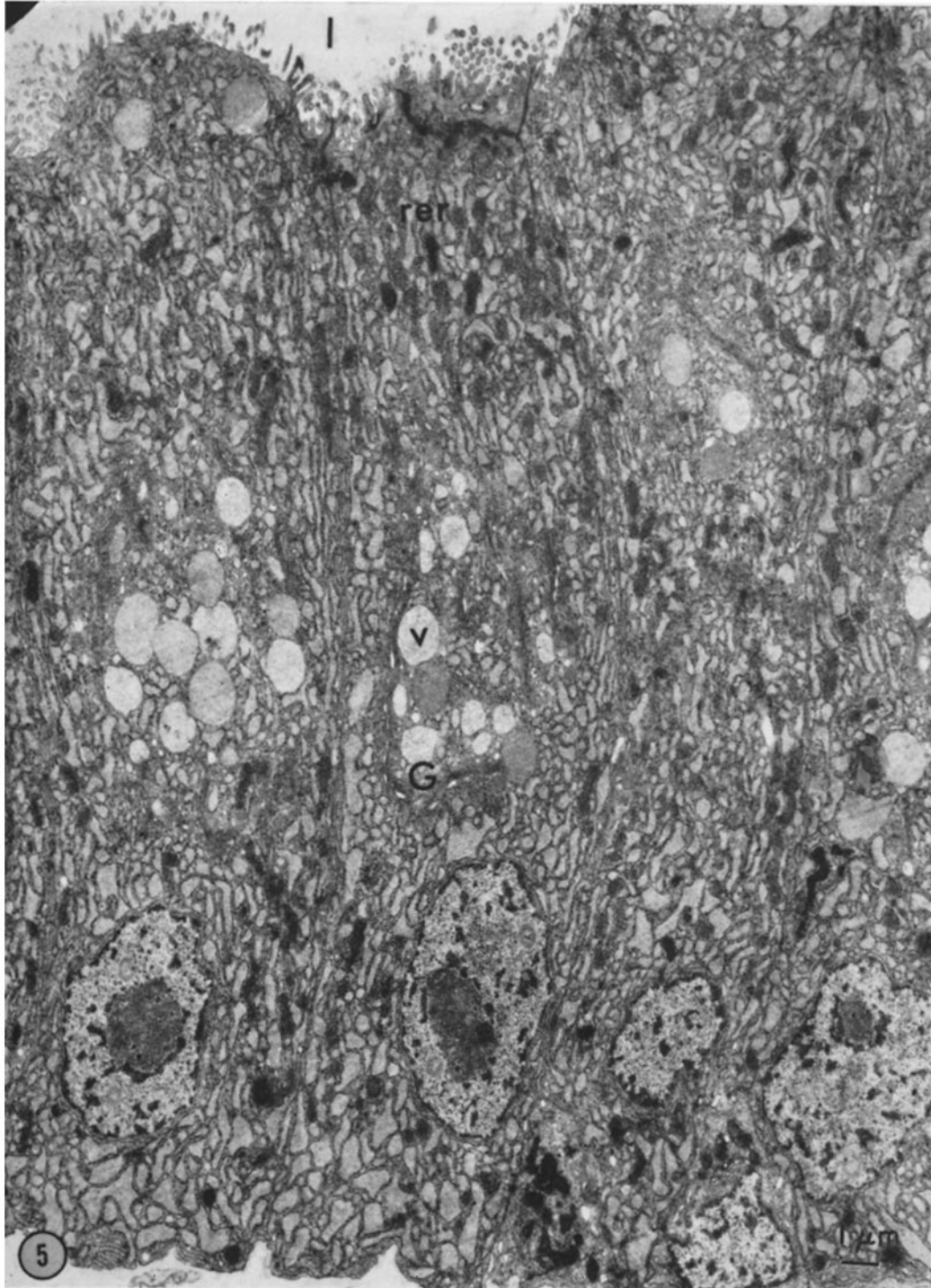
FIGURE 5   8 days after milking. Cells with a typical columnar shape, a well-developed Golgi complex (*G*), and distended endoplasmic reticulum (*rer*). Vesicles (*v*) are located at the Golgi zone and close to the apical membrane. Lumen (*l*). × 5400.
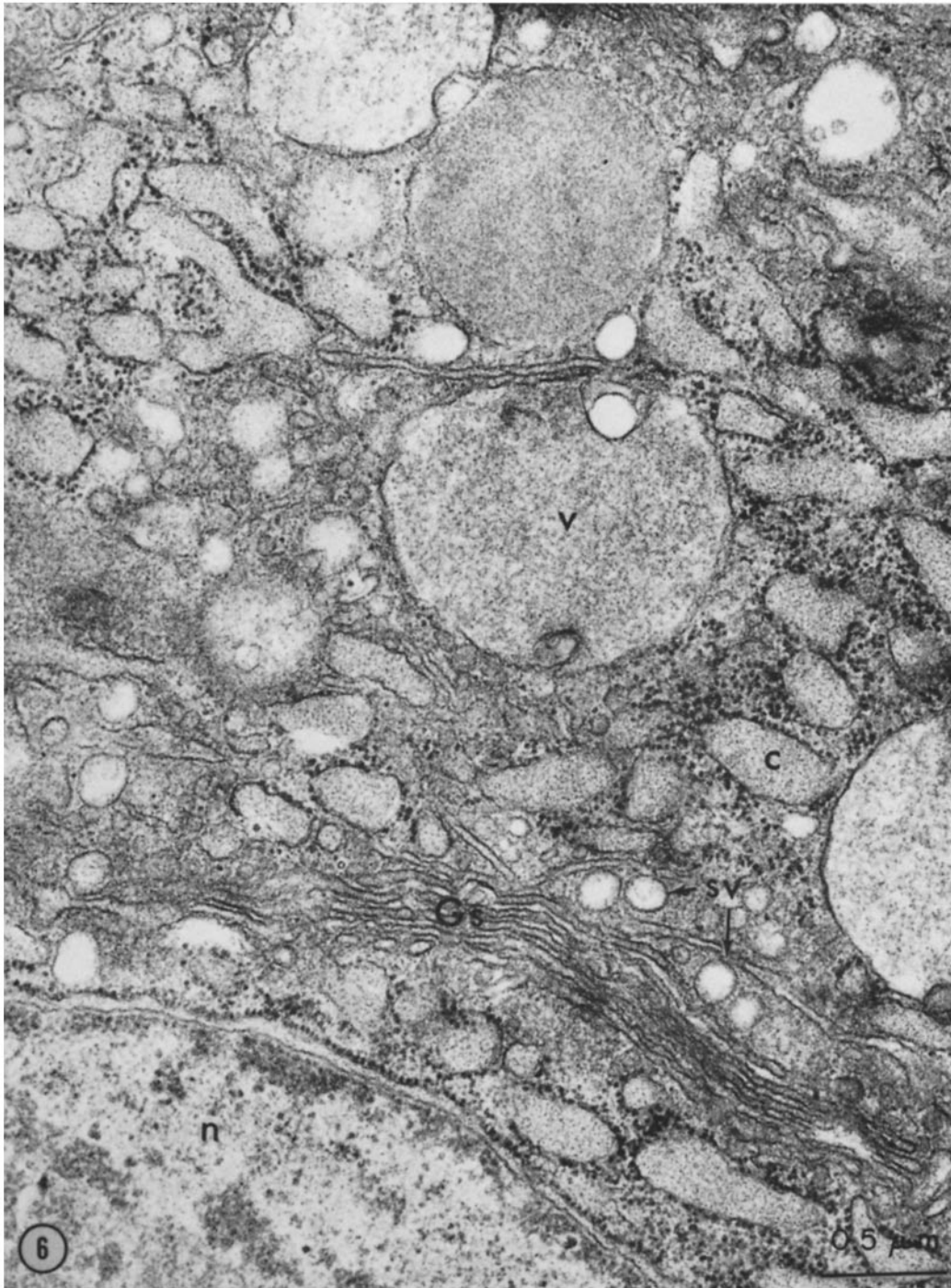
FIGURE 6 Golgi zone in a secretory cell 8 days after milking. A stack of Golgi saccules (*Gs*) and numerous small smooth vesicles (*sv*) are located above the nucleus (*n*). Large vesicles (*v*), extended cisternae of RER (*c*). × 36,000.
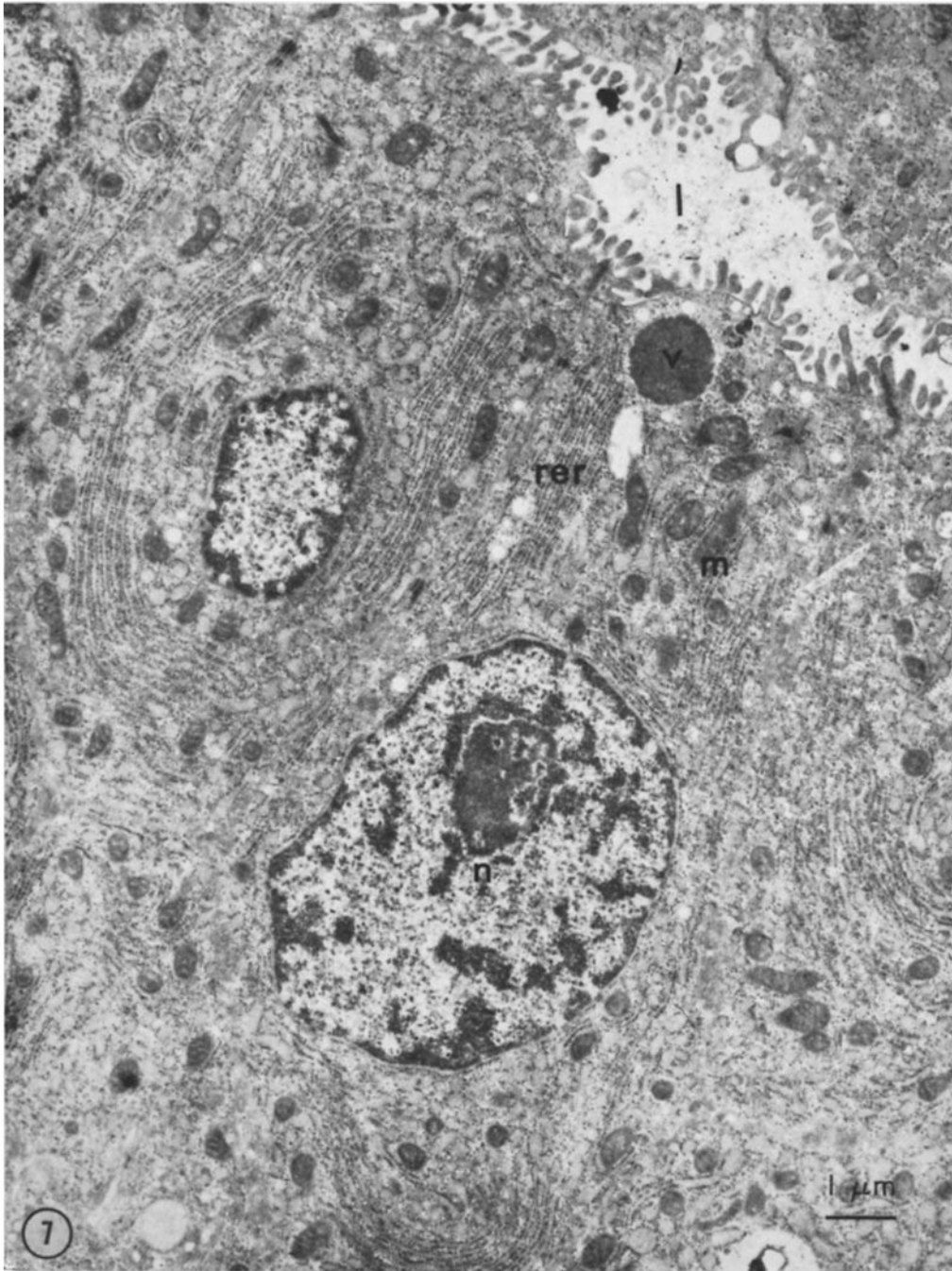
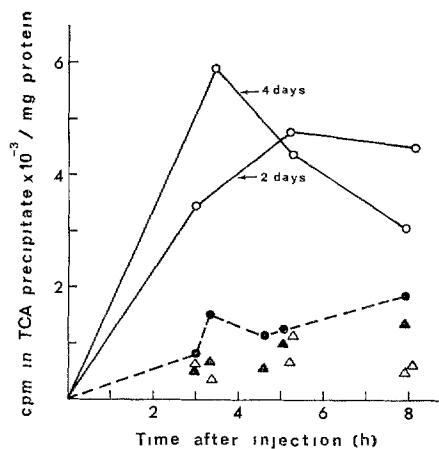FIGURE 7 23 days after milking. RER (*rer*), vesicle (*v*), mitochondrion (*m*), lumen (*l*). × 9800.

FIGURE 8 Kinetics of incorporation of [^{14}C]amino acids into protein in venom glands and liver Snakes ($V.$ $ammodytes$, 40 g), never milked in captivity were kept without food for 60–90 days in order to avoid loss of venom. Water was offered once a week Nine snakes were milked 2 or 4 days before the experiment Snakes were anaesthetized, each injected intraperitoneally with 2 5 μCi [^{14}C]protein hydrolyzate in saline, and were sacrificed at a different time interval after injection. Venom glands were milked before removal of the glands and proteins were precipitated from the gland homogenates and from the venom. The amount of labeled protein synthesized by the gland is the sum of radioactivity found in TCA precipitates of the homogenate and of the venom. Each dot represents the result of one snake, except for snake 2 days after milking in which the experimental points are the average of two snakes O, milked glands, ●, unmilked glands; △. liver from milked snakes, ▲, liver from unmilked snakes

Rotenberg et al (1971) showed in V $palaestinae$ that the secretory cells in active stages were significantly higher than in the resting stage Similar changes were observed in the present work in $V.$ $ammodytes.$

The fine structure of the secretory cell also undergoes changes during the secretory cycle A prominent change is apparent in the pattern of the RER.

In the secretory cell of the unmilked gland the reticulum is made of closely packed membranes and after milking it forms extended cisternae It seems that at about 1 wk after milking, when the synthetic activity is maximal, the total amount of RER per cell is the highest. This estimation is compatible with the results of Rotenberg et al (1971) who found in $V.$ $palaestinae$ the highest RNA concentration at this stage.

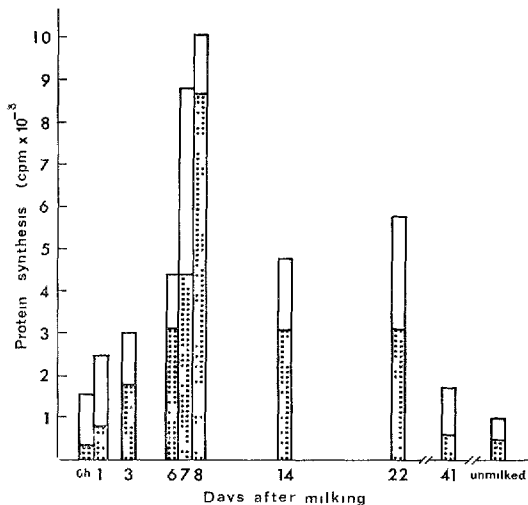Secretion of venom at the active stages is much

FIGURE 9 Incorporation of [^{14}C]amino acids into total and venom proteins as a function of time after milking. Snakes ($V.$ $ammodytes$) were kept without food for 70–80 days One gland was milked while the contralateral gland remained unmilked. (The milked gland, right or left, was selected at random) Each snake received 2.5 μCi [^{14}C]protein hydrolyzate as described under Fig 8 and was sacrificed 6 h after the injection The synthesis of protein (columns) was calculated as in Fig 8 Values were corrected according to the deviation of the control unmilked glands. The values for venom synthesis (shaded area) are the sum of the radioactivity in the homogenate-antiserum precipitate plus the radioactivity in TCA precipitate of the milked venom.

faster than at the resting stage The secretory cell after milking should, therefore, have the suitable apparatus for an accelerated intracellular transport Indeed the Golgi complex, which is hardly distinguished in cells of the unmilked gland increase in size after milking and, especially at 8 days, a well developed complex is apparent. A few large vesicles of low density are usually found in close association with the Golgi saccules These vesicles are highly reminiscent of the condensing vacuoles found in the mammalian exocrine pancreas (Jamieson and Palade, 1971). Warshawsky et al. (1972) in a recent autoradiographic study with the venom gland of $Crotalus$ $durissus$ $terrificus$ suggested that the "membrane bound granules" of the Golgi region are prosecretory granules These authors also reported the existence of intracisternal granules in the RER, to which they assigned a storage function. Such granules were not observed in viperid snakes.

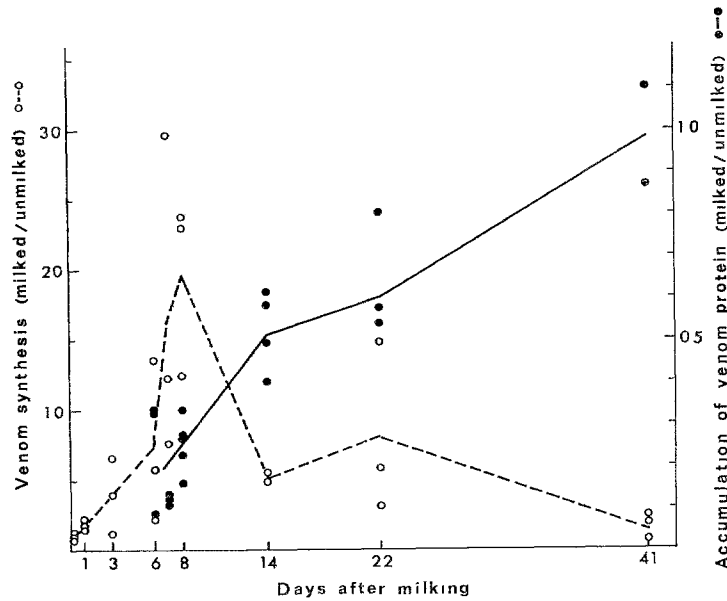It should be mentioned that the main storage of

FIGURE 10 Venom synthesis and venom accumulation in the gland lumina as a function of time after milking. The results presented in this figure are from the same experiment as Fig. 9 Venom synthesis was calculated as described in Fig. 9. Venom accumulation is expressed as the ratio between the amount (protein) of venom obtained from the milked gland and the unmilked gland. O, venom synthesis; ●, venom accumulation.

TABLE I

Protein Synthesis by Intact Venom Glands Incubated in Synthetic Medium

| Snake | Incubation time | Incorporation of [^{14}C]-amino acids into protein (milked/unmilked) | |
		Total proteins	Venom proteins
	(mın)		
V ammodytes	60	3 0	2.1
	120	3.0	3.4
V palaestınae	60	4 2	—
	60	3.7	—

Snakes were kept without food for 60–90 days. Milked and unmilked glands were from the same snake (see Fig. 9). Whole intact glands were removed after ligation (see Materials and Methods) and incubated in the same vessel at 30°C The experiments with V. ammodytes were done with glands 7 days after milking with 3 ml of medium containing 0.5 μCi [^{14}C]protein hydrolyzate. Glands 3 days after milking were used in the case of V. palaestınae (active gland, according to Rotenberg et al., 1971). Incubation was in 10 ml of medium containing 3 μCi of [^{14}C]protein hydrolyzate

secretion in venom glands is in the extensive lumina In contradistinction to mammalian salivary gland in which secretion is accumulated mainly or exclusively in intracellular granules (Schramm, 1967), only a few intracellular inclusions are apparent in venom glands of viperids (see also, Ben-Shaul et al 1971) The maximal intracellular storage of the two exportable enzymes checked in this study was never more than twice the value in inactive glands.

Synthesis and secretion in the venom glands can be initiated by the removal of venom from gland lumina without the use of drugs. After this induction, there is a comparatively long cycle of venom regeneration in comparison with mammalian salivary glands and pancreas (cf. Junqueira and Hirsch, 1956, Amsterdam et al , 1969).

It is shown in the present work that the incorporation of labeled amino acids into protein increased after milking. During the first day after milking, most of the proteins synthesized are not precipitable by antiserum against venom and are probably structural proteins which are needed for the building of the synthetic apparatus When the synthetic capacity of the cells is maximal (8 days

after milking) most of the proteins synthesized are venom proteins

The control mechanisms operating in the synthesis and secretion in the venom glands are not
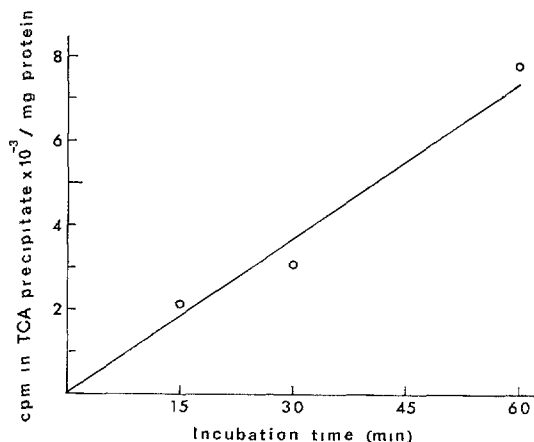

Figure 11   Kinetics of incorporation of [¹⁴C]amino acids into protein in gland slices   Glands from six snakes V. ammodytes), 2 days after milking, were sliced, washed, and transferred to three Erlenmeyer flasks. Incubation was at 37°C for the time indicated in 2 ml of medium containing 1 μCi [¹⁴C]protein hydrolyzate.

well understood. Wolter (1924) proposed that during the resting stage, tubules are filled with secretion which "presses the cells and inhibits new synthesis of venom" Peakall (1969) showed that synthesis of silk in a spider could be initiated by emptying the silk gland and demonstrated the existence of special receptor cells "sensitive to the amount of silk stored in the gland"

Despite the fact that no receptor cells were

TABLE II

Protein   Synthesis   by   Slices   of   Venom   Glands Incubated in Synthetic Medium

| Experiment | Incorporation of [¹⁴C]amino acids into protein (milked/unmilked) | |
	Total proteins	Venom proteins
I	7 5	7.0
II	3 0	1 6
III	1.6	1.8
IV	1 5	1.6

One gland from each snake (V   ammodytes) was milked 8 days previously while the other gland was unmilked. Slices were incubated in 3 ml of medium with 1.7 μCi [¹⁴C]protein hydrolyzate   Incubation was at 30°C for 30 min.
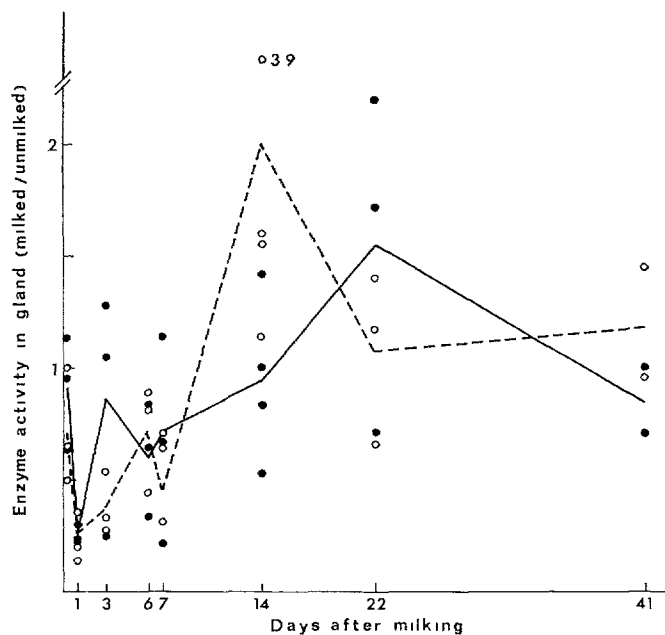

Figure 12   Activities of exportable enzymes in gland cells as a function of time after milking. Glands were cut into small pieces, rinsed to remove venom, homogenized, and further treated as described under Materials and Methods. The results presented in this figure are from the same experiment as those presented in Figs. 9 and 10. Each dot is the result of one snake. O, BAEE; ●, PDE.

found in the venom gland, it might be speculated that the release of pressure after milking induces an increased rate of protein synthesis, which is relatively low immediately after milking and attains its maximum value only 8 days later. The experiments with gland slices incubated for 0.5 h corroborate this assumption by showing that, despite the release of pressure, the recently milked (sliced) glands are less active than the glands at 8 days after milking.

Although the influence of chemical stimuli cannot be ruled out at present, it is obvious that the amount of venom present in the lumen regulates the synthetic activity of the secretory epithelium.

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