Biochem. J. (1971) **121**, 609–612
Printed in Great Britain

Studies on Ribonucleic Acid Synthesis in the Venom Glands of *Vipera palaestinae* (Ophidia, Reptilia)

By D. ROTENBERG, E. S. BAMBERGER and E. KOCHVA
Departments of Biochemistry and Zoology, Tel-Aviv University, Tel-Aviv, Israel

(*Received* 1 *September* 1970)

RNA metabolism in the venom glands of *Vipera palaestinae* was studied at different stages after manual extraction of the venom (milking). The rate of ^{32}P incorporation into gland RNA was found to be maximal at 1–4 days after milking in correlation with the height of the secretory epithelium. Venom production attained a maximum only after 8–16 days, in parallel with the accumulation of stable species of cellular RNA.

Some properties of the venom glands of snakes make them suitable for the study of exocrine secretion: these glands have a long cycle of venom regeneration, compared with other exocrine glands such as the pancreas, mammary or salivary glands (Jamieson & Palade, 1967; Tucker, 1966; Amsterdam, Ohad & Schramm, 1969), and renewal of venom synthesis may be initiated by manual milking without the use of stimulatory drugs, the action of which is poorly understood. Further, the secretory cycle of the venom glands is accompanied by distinct morphological changes of the cells involved in secretion (Kochva & Gans, 1970).

It has been demonstrated histochemically (Kochva & Gans, 1967) that the tall columnar secretory cells that line the tubules of the venom gland are rich in ribonuclease-sensitive material found in the apical half of the cells. In a gland filled with secretion, the secretory epithelium of which is low (cuboidal to flat) and apparently inactive, the amount of this ribonucleoprotein material decreases.

F. L. De Lucca & J. M. Gonçalves (personal communication to E.S.B.) found that 22h after an intraperitoneal injection of ^{32}P, the isotope was incorporated into an RNA fraction of *Crotalus durissus terrificus* venom glands located between the 18S and 4S fractions. After 17 days the label was detected in the two rRNA fractions 28S and 18S. In a preliminary experiment with *Laticauda*, Sato, Yoshida, Abe & Tamiya (1969) found that the venom glands of this snake injected with [^{32}P]phosphase or [^{14}C]orotic acid show a homogeneous labelling of all RNA fractions. The milking schedule of the snakes used for the experiments was not stated, however.

In this paper, results are presented on the correlation between RNA metabolism and the production of venom at the different secretory stages of the gland, starting with a gland depleted of venom, through intermediate stages and up to the quiescent state of a gland filled with secretion. A preliminary report of this study has been published (Bamberger, Rotenberg, Sharf & Kochva, 1967).

MATERIALS AND METHODS

Snakes. Specimens of *Vipera palaestinae* Werner 1938 were kept in separate cages in a temperature-controlled room at about 30°C. The snakes were provided with water at all times and were offered one white mouse a week. A detailed record of feeding and venom extraction was available for each snake used in the study.

Venom extraction. The vipers were milked in the manner described by Klauber (1956) and Kochva (1960), by exerting pressure on the sides of the head; practically all venom was extracted from the gland lumina and only about 5% appears to remain adsorbed on to the luminal surfaces of the cells. This additional venom could be estimated by washing the gland sections and determining the protein content of the rinse.

Isotopic labelling. RNA synthesis in the venom gland *in vivo* was followed by the incorporation of ^{32}P into gland RNA. Carrier-free $H_3{}^{32}PO_4$ (The Radiochemical Centre, Amersham, Bucks., U.K. and Nuclear Research Center, Negev, Israel) was first boiled with 0.1M-HCl (for the hydrolysis of polyphosphates possibly present in the preparation) and then neutralized with NaOH; 0.7mCi of ^{32}P/100g body wt. was injected intraperitoneally into snakes whose venom glands were at different secretory stages.

Isolation and analysis of RNA. The snakes were decapitated and immediately thereafter the two venom glands and the liver were removed, frozen, and pulverized in solid CO_2. RNA was isolated by a modified phenol–sodium dodecyl sulphate–bentonite (pH9.5) procedure as described by Click & Hackett (1966). The extraction media contained, for each pair of glands: 10ml of glycine buffer (0.1M-sodium glycinate, pH9.5, 0.1M-NaCl and 0.1M-EDTA), 20ml of phenol saturated with water,

10% (w/v) sodium dodecyl sulphate and 5% purified bentonite (Fraenkel-Conrat, Singer & Tsugita, 1961). The pulverized tissue was extracted for 10–15min at 4°C and the aqueous phase was separated by centrifugation at 12000*g* for 10min. The phenol phase was re-extracted with glycine buffer and the first and second aqueous phases were combined and again deproteinized with phenol. The aqueous phase was then washed three times with 3 vol. of diethyl ether and RNA was precipitated with 2vol. of aq. 96% (w/v) ethanol (−20°C). After standing overnight at −20°C the precipitate was collected by centrifugation at 25000*g* for 15min and dissolved in glycine buffer. The highest contents of protein and DNA in the preparation were 8 and 4% respectively.

The RNA was analysed on a linear gradient of sucrose (28ml of 2–20%, w/v) dissolved in glycine buffer, pH9.5. The samples were sedimented for 16h at 25000 rev./min in a Spinco SW 25.1 rotor at 4°C. At the end of the run, the bottom of each gradient tube was punctured and successive 1ml fractions were collected. The extinction at 260nm was determined in a Zeiss spectrophotometer. Total radioactivity in each fraction was determined after the addition of 0.1mg of carrier yeast RNA and cold trichloroacetic acid to a final concentration of 10% (w/v). The samples were collected on Sartorius membrane filters (0.6μm) which were washed with 5% trichloroacetic acid, followed by 95% (v/v) ethanol and then dried at 100°C. The samples were placed in scintillation vials containing 12ml of scintillation fluid consisting of 4g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)-thiophen/l of toluene and the radioactivities were counted in a Packard Tri-Carb liquid-scintillation spectrometer. Machine efficiency for ^{32}P was 100% and the background was 15 c.p.m.

Total RNA was derived from the total E_{260} of the sucrose-density-gradient profiles. The specific radioactivity was calculated by dividing the radioactivity of each tube by its E_{260} reading.

Sedimentation rates of snake RNA. The sedimentation coefficients of snake venom-gland material were estimated from the linear plot of the sedimentation coefficients of *E. coli* RNA against the peak tube number of each class of molecules (from the top of the gradient). The sedimentation coefficients of *E. coli* RNA (23.5, 16.5 and 3.8S) were taken from published data obtained in the

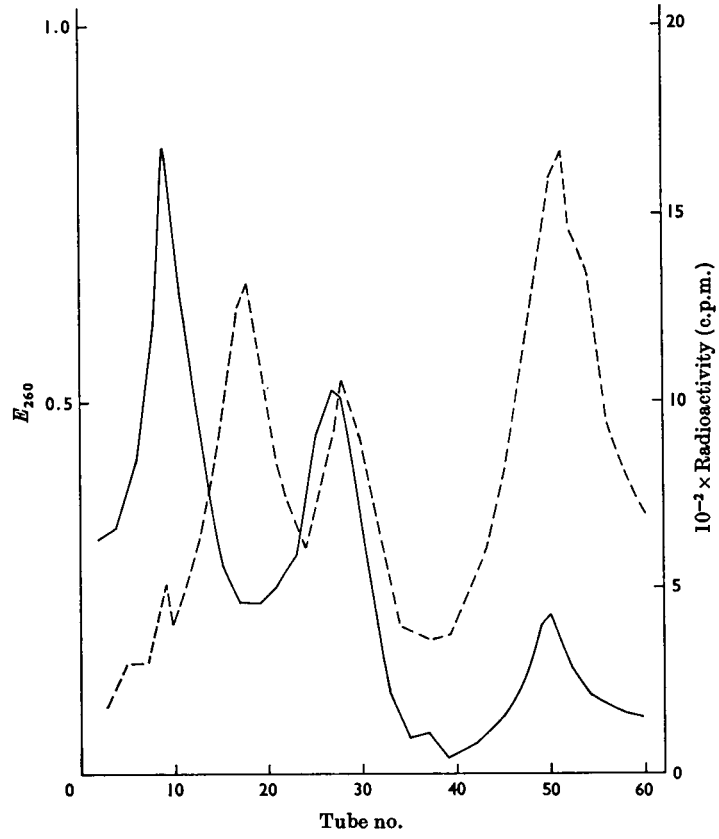
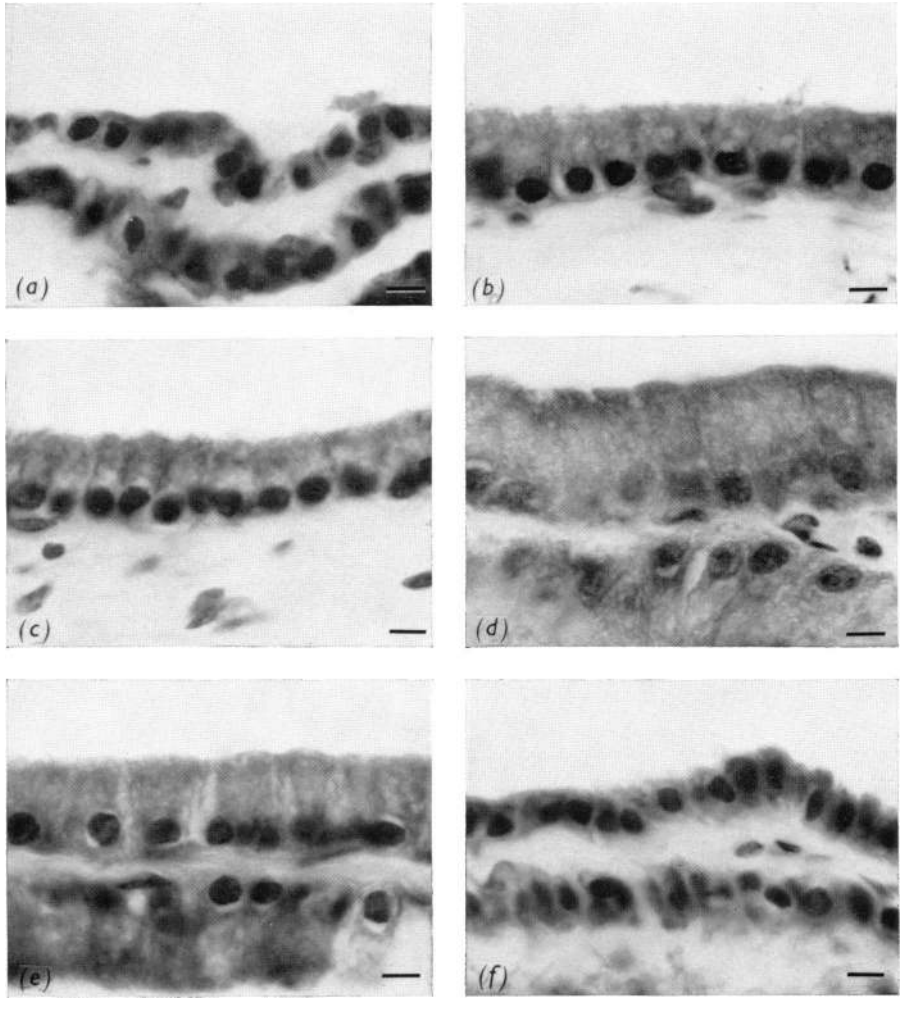


Fig. 1. Derivation of sedimentation coefficients of RNA from venom glands of *Vipera palaestinae*. Venom-gland RNA (25 E_{260} units) was mixed with *E. coli* ^{32}P-labelled RNA (3 E_{260} units, containing 24000 c.p.m.). The mixture was layered over a linear 2–20% (w/v) sucrose density gradient and centrifuged and analysed as described in the text. ——, E_{260}; – – – –, radioactivity.



EXPLANATION OF PLATE 1

Secretory epithelium of venom glands at different stages after milking. Scale: 5μm; (*a*) 6h; (*b*) 1 day; (*c*) 2 days; (*d*) 4 days; (*e*) 16 days; (*f*) 60 days.

model E ultracentrifuge (Click & Tint, 1967). Direct comparisons of bacterial and snake rRNA were made by mixing the two RNA preparations and analysing them independently (^{32}P and E_{260}) on sucrose density gradients. *Escherichia coli* strain 0-86 RNA was labelled by growing the bacteria for 24h in 600ml of Tryptose–phosphate broth (2.6%) containing 2mCi of ^{32}P. Bacterial RNA was extracted by the method of Littauer & Eisenberg (1959). The specific radioactivity of the bacterial RNA was 8000 c.p.m./E_{260} unit. To 25 E_{260} units of gland RNA or liver RNA, 3 E_{260} units of ^{32}P-labelled bacterial RNA were added, and the mixtures were layered on a 2–20% (w/v) sucrose density gradient. The samples were sedimented for 16h at 25000 rev./min in a Spinco SW 25.1 rotor at 4°C. After centrifugation 60 samples were collected for E_{260} measurements and radioactivity counting, as described above.

Histology. A small section of one or two venom glands per secretory stage was fixed in formalin, embedded in paraffin and 8μm serial sections were prepared. The heights of 80 cells taken at random from each gland were measured.

RESULTS

Sedimentation coefficients of venom-gland and liver rRNA. As shown in Fig. 1 the sedimentation properties of rRNA from bacteria and snakes were different. The larger rRNA component from *E. coli* migrated more slowly than the corresponding rRNA peak from snake venom glands. The sedimentation coefficients of gland RNA were 28.8S, 17.0S and 3.8S for the heavy and light rRNA and for tRNA respectively. The values for RNA extracted from snake liver were similar.

Comparison of ^{32}P incorporation into RNA of a resting and an active venom gland. The incorporation of ^{32}P into the venom-gland RNA was studied in two extreme stages of venom secretion: (*a*) in a resting gland with a flat secretory epithelium and with lumina filled with venom; (*b*) in a presumably stimulated gland, just after milking. For (*a*) (resting gland) three adult snakes were kept without food for 24 days to avoid loss of venom. The snakes were anaesthetized with Fluothane (I.C.I.) and ^{32}P was injected intraperitoneally. At 3, 6 and 24h after the ^{32}P injection the snakes were killed and RNA was extracted from the glands and from the liver. For (*b*) (active gland) the snakes were milked and immediately injected with the radioisotope.

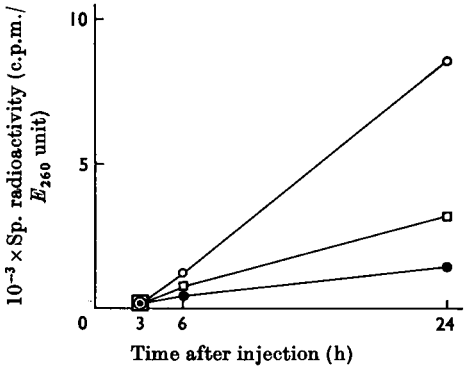


Fig. 2. Kinetics of labelling of total RNA from liver (□) and from venom glands of milked (○) and unmilked (●) snakes. The specific radioactivities were calculated from the c.p.m./E_{260} ratio in the sucrose-density-gradient-sedimentation profiles.

In the resting gland ^{32}P incorporation was detected in the three species of RNA only 24h after injection. In the active gland, however, some ^{32}P was equally distributed between the three RNA peaks already 6h after injection. At 24h the difference between the active and resting glands was more evident.

It is quite obvious (Fig. 2) that there was a lag of at least 3h in the incorporation of ^{32}P into the gland RNA, which might be a result of the slow spreading of ^{32}P from the injection site or of the 'recovery' of the glands after the pressure imposed during milking. In the following experiments we chose the period of 6h between injection and killing of the snake.

Rate of RNA synthesis in the gland at different secretory stages. A group of 23 snakes was kept without food for 60 days and then milked for removal of all available venom. At 1, 2, 4, 8, 16, 32 and 60 days after milking, two to four snakes were injected with ^{32}P (0.7mCi/100g body wt.). At 6h after ^{32}P injection the snakes were milked and then decapitated, the venom glands were removed, and RNA was extracted and separated on a sucrose density gradient as described in the Materials and Methods section. The results of this experiment (Fig. 3) show that the rate of RNA synthesis reached a maximum value between day 1 and day 4 after milking. Thereafter and up to day 60 the rate of ^{32}P incorporation into RNA showed a marked decrease (Fig. 3*a*). The venom production during the same period (Fig. 3*b*) shows a biphasic curve; during the first days after milking there was a fast rate of venom synthesis (as calculated per mg of gland tissue), which levelled off after 16 days. The total amount of extractable RNA was highest on day 8 (Fig. 3*c*).

Cell height measurements. The bulk of the venom originates in the main, posterior part of the venom gland. This part of the gland is made of branching tubules lined with an epithelium of several cell types (Ben-Shaul, Lifshitz & Kochva, 1971). One cell type predominates and is involved in the main secretory activity. The height of these cells was measured in sections taken from the same glands used for the kinetic measurements.

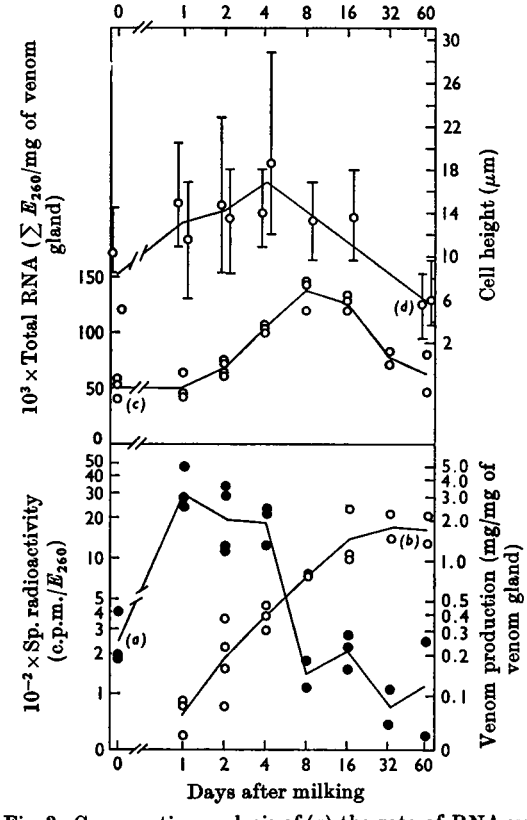


Fig. 3. Comparative analysis of (*a*) the rate of RNA synthesis as measured by ^{32}P incorporation into RNA during a 6h period, (*b*) the accumulation of venom, (*c*) the change in total RNA and (*d*) the change in the height of the secretory epithelium in the venom glands at different intervals after milking. Each dot represents the results obtained from one snake. The vertical bars in (*d*) indicate the range.

In the available material, the average height of the cells was 13 μm at 1 day and 17 μm at 4 days after milking, with some cells reaching a maximum height of 30 μm at the latter stage. After day 4 the height of the cells decreased to about 6 μm in the glands of snakes that had not been milked for 60 days (Fig. 3*d* and Plate 1). It appears that the same cells are involved in a new secretory cycle after venom extraction, since a very low rate of cell division was observed in the gland epithelium.

Secretion stage of the venom glands after a single bite. In a preliminary experiment with three snakes forced to bite a plastic lid, it was found that ^{32}P incorporation after 6h was in correlation with the amount of venom delivered during the bite. The snake that ejected the greatest amount of venom (270mg) showed the highest specific radioactivity (500 c.p.m./E_{260} unit) and also the highest secretory epithelium (about 15 μm).

DISCUSSION

In the mammary glands, Tucker (1966) found that the extent of ^{32}P incorporation into total RNA of the milked glands was considerably higher than in the unmilked glands. A similar phenomenon was demonstrated for *Vipera palaestinae*, where the removal of the venom from the venom glands activates the secretory epithelium and results in a high rate of RNA synthesis at 1–4 days after milking. During the same time, the secretory cells increase in height, with no evidence of any significant cell multiplication. These processes clearly precede accumulation of RNA in the cells and of venom in the gland lumina; both total RNA and venom production attain a maximum at 8–16 days after milking, when cell size and RNA synthesis are already decreasing.

It is speculated that the earliest event in the renewal of venom production is the biosynthesis of some specific informational RNA species in correlation with the increase in cell height up to day 4 after milking. This is followed by accumulation of stable, presumably ribosomal, RNA, which seems to be a necessary step in the regeneration of venom by the venom glands.

We thank Dr S. Yankofsky and Dr B. Moav for comments on the manuscript, Dr M. B. Brown and Dr. D. Wool for help with the statistics, Miss L. Widzer for the cell measurements, Mrs M. Sharabani for technical assistance, Mr R. Kalef for the drawings and Mr Y. Cohen for the photographs.

REFERENCES

Amsterdam, A., Ohad, I. & Schramm, M. (1969). *J. Cell Biol.* **41**, 753.

Bamberger, E., Rotenberg, D., Sharf, Y. & Kochva, E. (1967). *Israel J. Chem.* **5**, 116p.

Ben-Shaul, Y., Lifshitz, Sh. & Kochva, E. (1971). In *Toxins of Animal and Plant Origin.* Ed. by de Vries, A. & Kochva, E. New York and London: Gordon and Breach (in the Press).

Click, R. E. & Hackett, D. P. (1966). *Biochim. biophys. Acta*, **129**, 74.

Click, R. E. & Tint, B. L. (1967). *J. molec. Biol.* **25**, 111.

Fraenkel-Conrat, H., Singer, B. & Tsugita, A. (1961). *Virology*, **14**, 54.

Jamieson, J. D. & Palade, G. E. (1967). *J. Cell Biol.* **34**, 577.

Klauber, L. M. (1956). *Rattlesnakes.* Berkeley: University of California Press.

Kochva, E. (1960). *Am. J. trop. Med. Hyg.* **9**, 381.

Kochva, E. & Gans, C. (1967). In *Animal Toxins*, p. 195. Ed. by Russell, F. E. & Saunders, P. R. Oxford: Pergamon Press Ltd.

Kochva, E. & Gans, C. (1970). *Clin. Toxicol.* **3**, 363.

Littauer, U. Z. & Eisenberg, H. (1959). *Biochim. biophys. Acta*, **32**, 320.

Sato, S., Yoshida, H., Abe, H. & Tamiya, N. (1969). *Biochem. J.* **115**, 85.

Tucker, H. A. (1966). *Am. J. Physiol.* **210**, 1209.