Synthesis of Ribonucleic Acid in the Venom Gland of *Crotalus durissus terrificus* (Ophidia, Reptilia) after Manual Extraction of the Venom

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1. The incorporation of [5-³H]uridine into RNA of the venom gland of *Crotalus durissus terrificus* was studied after manual extraction ('milking') of the venom. The labelled precursor was injected immediately after milking. 2. The RNA was extracted 1, 2, 4, 6 and 8 h after injection of the label and analysed by sucrose-density-gradient centrifugation. 3. The sedimentation analysis showed that 18S rRNA synthesis is higher than 28S rRNA at all time-intervals. The specific radioactivities of both ribosomal components did not reach a plateau even at 8 h after injection. 4. An RNA fraction was detected sedimenting between 18S rRNA and 4S tRNA and was called the 10–14S fraction. The specific radioactivity was always higher than that of both classes of rRNA and reached the maximum value at 6 h of labelling. 5. The incorporation of the precursor was also studied by radioautography, which helped to elucidate the intracellular origin of the RNA analysed by sucrose-density-gradient centrifugation.

The metabolism of RNA has been extensively studied in mammalian cells as well as in bacterial systems. However, little is known about the RNA synthesis in Reptilia. The literature about this subject is mainly concerned with the venom gland of snakes (Sato *et al.*, 1969; Rotenberg *et al.*, 1971).

Systems that synthesize large amounts of specific proteins have been selected for the study of the synthesis of mRNA (Singer & Leder, 1966). This is the case with the venom glands of snakes, which are specialized secretory tissues. Their secretion contains several groups of proteins (toxins, enzymes and others), which represent about 90% of the dry weight of the venom (Gosh & Sarkar, 1956). The toxins crotoxin and crotamin constitute about 60% of the total proteins of the venom secreted by the South American rattlesnake *Crotalus durissus terrificus* (Slotta & Fraenkel-Conrat, 1938; Gonçalves & Arantes, 1957), and both toxins have been partially characterized by chemical and physicochemical methods (Fraenkel-Conrat & Singer, 1956; Gonçalves, 1956).

The secretory cycle of the venom gland of *Crotalus durissus terrificus* has been investigated by morphological and radioautographic methods (Warshawsky *et al.*, 1972). All the information below refers to investigations carried out after manual extraction of the venom ('milking'). Preliminary electron-micro-

scopic radioautographic studies have indicated that shortly after injection of [³H]tyrosine there is an abundant incorporation of this amino acid in the ribosomes attached to the membranes of the endoplasmic reticulum of the rattlesnake venom gland. The rough endoplasmic reticulum of the secretory cells is abundant and is distributed throughout their cytoplasm. After milking the rough endoplasmic reticulum of these cells undergoes marked morphological changes during the secretory cycle of the gland. These morphological transformations may be related to changes in RNA metabolism. Rotenberg *et al.* (1971) observed that milking of the venom gland of *Vipera palaestinae* stimulated synthesis of RNA.

In the present paper we describe studies on the incorporation of [5-³H]uridine into RNA of the previously milked venom gland of *Crotalus durissus terrificus*. The incorporation of [5-³H]uridine was followed from 1 to 8 h after injection of the label. The RNA was extracted and analysed by sucrose-density-gradient centrifugation. The incorporation of the labelled precursor was also studied by radioautographic methods.

Experimental

Materials

Chemicals. [5-³H]Uridine (sp. radioactivity 14.3 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Phenol was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.,

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and distilled immediately before use. Sodium dodecyl sulphate was from Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A., and purified according to Schmidt (1957). Bentonite was from Sigma Chemical Co., St. Louis, Mo., U.S.A., and purified according to Fraenkel-Conrat *et al.* (1961). The scintillants, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from Packard Instrument Co. Inc., La Grange, Ill., U.S.A. All other chemicals were of reagent-grade purity obtained from commercial sources.

Snakes. Local South American rattlesnakes (*Crotalus durissus terrificus*) of both sexes, 200–400 g body wt., were used. The animals were provided only with water and were starved for at least 2 weeks to allow the gland to accumulate its venom.

Methods

Isotopic labelling. [5-³H]Uridine was injected intraperitoneally (20 μ Ci/100 g body wt.) immediately after milking. The snakes were milked as described by Klauber (1956). The animals were kept in an incubator at 28°C for at least 1 day before the injection of the radioactive compound, and were removed only a few minutes for the milking. Immediately after the injections of [5-³H]uridine they were kept again in the incubator until they were killed. The snakes were killed by decapitation 1, 2, 4, 6 or 8 h after the injection of the radioisotope and two animals were used for each time-interval. In cases when the venom was cloudy the sample was rejected.

Extraction of RNA. The RNA was extracted from whole cells by modifying the cold-phenol procedure described by Cartouzou *et al.* (1968). The venom glands were dissected out as soon as possible after death and all the following steps were done at 0–4°C. The dissected glands (each 100–120 mg fresh wt.) were washed with frog Ringer solution (6.5 g of NaCl, 0.14 g of KCl, 0.12 g of CaCl₂ and 0.20 g of MgSO₄ in 1 litre of distilled water) to remove the residual venom. They were then homogenized in 25 vol. of 0.02 M-tris-HCl buffer, pH 7.4, containing 1% sodium dodecyl sulphate and 0.1% bentonite in a Potter-Elvehjem homogenizer. The deproteinization of the homogenate was done by the addition of an equal volume of 86% (w/v) phenol containing 1% sodium dodecyl sulphate and 0.1% bentonite. The mixture was mechanically shaken during 30 min and then centrifuged at 10000g for 10 min. The phenol phase was discarded and the phenol treatment was repeated twice on the combined aqueous phase and interphase. The phenol treatment was repeated once more, only the aqueous phase being used. An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added to the resultant aqueous phase of the phenol treatment. The mixture was shaken for 15 min and centrifuged at 10000g for 10 min. This treatment was

repeated until the complete disappearance of the interphase; this step is a modification of the cold-phenol procedure described by Cartouzou *et al.* (1968), introduced to complete the deproteinization (Sevag *et al.*, 1938) and also to remove the phenol (Gross *et al.*, 1965).

The RNA was precipitated by the addition to the final aqueous phase of solid NaCl, to a final concentration of 0.1 M, plus 2 vol. of cold aq. 96% ethanol. After being left for 18 h at –15°C the precipitated RNA was collected by centrifugation at 2000g for 15 min, washed twice with aq. 80% ethanol and dissolved in 0.02 M-tris-HCl buffer, pH 7.6, containing 0.01 M-NaCl and 0.001 M-EDTA. U.v. measurements were made in a Zeiss model PMQ II spectrophotometer. An E_{260} of 1.000 for a 1 cm light-path was equivalent to 40 μ g of RNA/ml. The yield was 3.7 mg/g of fresh venom gland. The possibility of contamination by protein and phenol was checked by examination of the E_{260}/E_{280} ratio (Rousseau & Crabbé, 1968) and the E_{220}/E_{260} ratio (Gross *et al.*, 1965) respectively. The test described by Burton (1956) indicated a negligible contamination by DNA. Final RNA solutions were stored at –20°C until further use.

Sucrose-density-gradient centrifugation. RNA samples were diluted to final concentration 2 mg/ml and 0.2 ml was layered on linear 5–20% (w/v) sucrose gradients. The centrifugation was for 4 h at 39000 rev./min at 6°C in the SW-39 rotor of a Spinco model L ultracentrifuge. At the end of the run, 13-drop (about 0.2 ml) fractions were collected by puncturing the bottom of the tubes with a hypodermic needle. A sample (0.05 ml) of each fraction was diluted to 1.0 ml with water and the E_{260} was read in a Zeiss model PMQ II spectrophotometer. The remaining 0.15 ml was used to measure radioactivity.

Measurement of radioactivity. The RNA was precipitated with 5% (w/v) trichloroacetic acid with 1 mg of bovine serum albumin as carrier. After being left for 12 h at 4°C the precipitates were collected at 2000g for 10 min at 4°C and washed twice with 5% (w/v) trichloroacetic acid and dissolved in 0.5 ml of Hyamine hydroxide (1 M solution in methanol) at 55°C for 30 min. The solutions were transferred to counting vials containing 10 ml of scintillation liquid [5.0 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene]. The radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer with an efficiency of 40% for ³H and the results were corrected for background radioactivity.

Co-sedimentation of the venom-gland RNA with ³H-labelled RNA from *Escherichia coli*. Tritiated *E. coli* RNA was extracted by the cold-phenol method as described before. Sedimentation values of 16S and 23S were assumed for *E. coli* rRNA (Braun *et al.*,

1966). Venom-gland RNA was mixed with *E. coli* RNA (specific radioactivity 50c.p.m./ μ g) and the mixture was layered on a linear 5–20% (w/v) sucrose gradient, centrifuged and the fractions were collected in the usual manner. U.v. and radioactivity measurements were performed as described above.

Radioautographic analysis. The snakes were injected intraperitoneally with [^3H]uridine (1 μ Ci/g body wt.) immediately after milking and were killed 2h, 22h or 8 days after injection. The venom gland, liver and pancreas of each animal were removed and fixed in Carnoy's fluid (Barka & Anderson, 1963) for 24h. Paraffin sections (5 μ m thick) were processed for radioautography in aq. 50% Ilford Nuclear Research Emulsion type K-5. After a suitable exposure time the radioautographs were developed in D-19b (Kodak), stained with Haematoxylin and Eosin and examined in the light-microscope.

Results

Sedimentation coefficients of the venom-gland RNA

The two classes of rRNA of the venom gland sediment faster than those of *E. coli* (Fig. 1). The sedimentation coefficient is 28S for the major ribosomal component and 18S for the minor. However, the radioactivity of tRNA (bacterial) was coincident with the extinction peak of tRNA of the venom

Fig. 1. Co-sedimentation of venom-gland RNA with labelled *E. coli* RNA

Venom-gland RNA was mixed with ^3H -labelled *E. coli* RNA and the mixture was layered on a linear 5–20% (w/v) sucrose density gradient. The centrifugation was for 4h at 39000 rev./min in the SW-39 rotor of a Spinco model L ultracentrifuge. The measurements of extinction and radioactivity were performed as described in the text. ●, E_{260} ; ○, radioactivity.

gland. The values 28S, 18S and 4S were also observed in liver and pancreas of the South American rattlesnake.

Pattern of RNA synthesis in the venom gland after milking

[^3H]Uridine was injected intraperitoneally to study the early events of RNA synthesis after the manual extraction of the venom. To verify if this route of injection was efficient samples of blood were collected at different time-intervals. The blood was allowed to clot and the radioactivity was measured in the serum (Fig. 2).

At 1h after the injection of the label the radioactivity in serum reached its maximum value. The rapid fall observed thereafter is probably due to the uptake of the labelled precursor by the different tissues including the venom gland. Therefore the study of the kinetics of the incorporation in this tissue was started 1h after the injection of the radioisotope.

The RNA was extracted from the whole cells 1, 2,

Fig. 2. Radioactivity in serum plotted against time

The snakes were killed by decapitation and the blood was immediately collected in centrifuge tubes. The blood was left to clot at room temperature and the samples of serum (20 μ l) were applied to Whatman 3 MM filter-paper discs (25mm diam.). The discs were air-dried and transferred to counting vials. Radioactivity was measured as described in the Experimental section. Each point is the average of three determinations.

Fig. 3. Sucrose-density-gradient sedimentation profile of RNA extracted 1h after injection of $[5\text{-}^3\text{H}]\text{uridine}$

The RNA was extracted from the venom gland by the cold-phenol procedure 1h after injection of the label and was layered on linear 5–20% (w/v) sucrose gradient. The centrifugation was for 4h at 39000 rev./min in the SW-39 rotor of a Spinco model L ultracentrifuge. The measurements of extinction and radioactivity were performed as described in the text. ●, E_{260} ; ○, radioactivity; ■, specific radioactivity.

4, 6 and 8h after injection of $[5\text{-}^3\text{H}]\text{uridine}$ and analysed by sucrose-density-gradient centrifugation. Fig. 3 shows the sedimentation profile of RNA extracted from venom gland 1h after the injection of the label.

The radioactivity (c.p.m./fraction) has a poly-disperse distribution and this profile was very similar to those reported for different organisms (Hiatt, 1962; Mach & Vassali, 1965; Casola & Agranoff, 1968). However, in terms of specific radioactivity (expressed as c.p.m./mg of RNA) there were three peaks in the region higher than 28S and one peak located between the 18S rRNA and 4S tRNA.

Fig. 4 shows the sedimentation profile of RNA extracted 2 and 4h after the injection of the labelled precursor. This sedimentation profile indicates that even at 2h of incorporation there was a large amount of heterogeneously sedimenting radioactivity throughout the gradient. With a longer labelling time

(4h) the radioactivity and the extinction became nearly coincident.

The peak between 18S and 4S, referred to as the 10–14S fraction, is still present at these time-intervals. It is notable that the specific radioactivity of this fraction increased with pulse-labelling time. However, this was not observed with the peaks that appeared in the region higher than 28S of the sucrose gradient. Moreover, the number and sedimentation coefficients of these peaks were not reproducible. An accurate interpretation of these findings was not possible with the results of these experiments. Perhaps a correct approach would be to study the incorporation of the label into the nuclear fraction, as this fraction usually contains the high-molecular-weight RNA (Georgiev, 1967).

Fig. 5 shows the sedimentation profile of RNA extracted 6 and 8h after the injection of $[5\text{-}^3\text{H}]\text{uridine}$. The radioactivity and the extinction peaks were coincident. However, a heterogeneous material was still found to be present in the region higher than 28S of the sucrose gradient, in terms of c.p.m./fraction. The 10–14S fraction was observed at both labelling times.

Specific radioactivities of the 18S and 28S rRNA computed from data of Figs. 3, 4 and 5 are presented in Fig. 6. These experiments indicated that the specific radioactivity of the 18S component was higher than that of 28S at all time-intervals. It is notable that even at 8h of incorporation the value of the specific radioactivity had not reached a plateau. Similar results have been obtained in rat liver (Trakatellis *et al.*, 1964) and in rat and pig bones (Steinberg & Nichols, 1967).

However, the 10–14S fraction reached a plateau at 6h after injection of the precursor and there was little change in specific radioactivity thereafter (Fig. 7). The values of specific radioactivity of the 10–14S fraction presented in Fig. 7 were also computed from the data of Figs. 3, 4 and 5.

The specific radioactivity of this fraction was always higher than that of 18S and 28S ribosomal components. These findings indicated that the 10–14S fraction is rapidly labelled compared with rRNA of the venom gland, and has kinetics of labelling different from that of the bulk of cellular RNA. Thus this fraction has one of the several properties which have been used to characterize the mRNA in animal cells (Singer & Leder, 1966).

Radioautographic analysis of the incorporation of $[5\text{-}^3\text{H}]\text{uridine}$ into venom gland, liver and pancreas

The present results refer to snakes killed 2h, 22h or 8 days after the injection of the label. The incorporation of $[5\text{-}^3\text{H}]\text{uridine}$ into the venom gland was higher than in liver and pancreas at all time-intervals. However, in Plate 1 only the results obtained with

EXPLANATION OF PLATE I

Radioautographs of paraffin sections (5 μ m thick) of the rattlesnake venom gland

(a) 2h, (b) 22h and (c) 8 days after the injection of [5-³H]uridine. Arrows indicate strong nucleolar labelling in (b). Details of the technique are given in the Experimental section. The bar represents 5 μ m.

Fig. 4. Sucrose-density-gradient sedimentation profile of RNA extracted 2h (a) or 4h (b) after injection of $[5\text{-}^3\text{H}]\text{uridine}$

The RNA was extracted from the venom gland by the cold-phenol procedure 2 or 4h after injection of the label and analysed as described in Fig. 3. ●, E_{260} ; ○, radioactivity; ■, specific radioactivity.

the venom gland are presented. The radioautographs obtained from the animal killed 2h after injection of the radioisotope show that the label was present over the nuclei of the secretory cells of the venom gland. A few silver grains were also observed over the cytoplasm of these cells. However, these grains are not significantly increased above the background. At 22h after injection silver grains were found both over the nucleus and cytoplasm of the secretory cells. A very strong radioautographic reaction was present over the nucleoli of these cells whereas the grain density over the nucleoplasm was weak. The silver grains were distributed throughout the cytoplasm. At 8 days after the injection of the radioisotope labelling of the cytoplasm has increased. The nucleolar reaction observed at 22h of incorporation is still present at 8 days after injection but was not so heavy as at the early time-interval.

Discussion

Sedimentation properties of the venom-gland RNA

The results reported in the present paper show that the cold-phenol method applied in the thyroid of

sheep (Cartouzou *et al.*, 1968) is also suitable to extract high-molecular-weight RNA of the rattlesnake venom gland. The sedimentation coefficients of the major and the minor ribosomal components are 28S and 18S respectively, and these values are in agreement with those observed for rRNA of vertebrate animals (Spirin, 1966).

Ribosomal RNA synthesis after milking

Electron-microscopic studies of the venom gland of the rattlesnake (H. Warshawsky, A. Haddad, R. P. Gonçalves, V. Valeri & F. L. De Lucca, unpublished work) indicate that newly formed rough endoplasmic reticulum was present after milking. This morphological finding could be an explanation for the continued rise in the rRNA synthesis observed in the tissue after manual extraction of the venom. In fact, rough endoplasmic reticulum begins to increase a few hours after milking and this suggests the synthesis of new ribosomes.

It is of interest that 18S rRNA synthesis was greater than that observed for 28S rRNA. Trakatellis *et al.* (1964) also observed this difference in rat liver

Fig. 5. Sucrose-density-gradient sedimentation profile of RNA extracted 6h (a) or 8h (b) after injection of $[5\text{-}^3\text{H}]\text{uridine}$

The RNA was extracted from the venom gland by the cold-phenol procedure 6 or 8h after injection of the label and analysed as described in Fig. 3. ●, E_{260} ; ○, radioactivity; ■, specific radioactivity.

Fig. 6. Specific radioactivities of 18S and 28S rRNA plotted against pulse-labelling time

The specific radioactivities of the major and minor ribosomal components presented in this graph are the values at the respective extinction peaks. These values were computed from the data of Figs. 3, 4 and 5.

and they suggested that this occurs because the sedimentation pattern of the 18S component overlaps with that of mRNA. These authors demonstrated that true values of specific radioactivities for 18S and 28S rRNA are approximately equal. Probably, the same phenomenon occurs in the venom gland and the detection of the rapidly labelled fraction with a sedimentation coefficient about 10–14S strongly supports this. It is also possible that more of the cytoplasmic than nuclear RNA was extracted (Girard *et al.*, 1964; Saponara & Enger, 1966). This finding is also true for rattlesnake liver (M. T. Imaizumi & F. L. De Lucca, unpublished work).

Biological significance of the presence of 10–14S fraction in the venom gland

In micro-organisms and animal cells mRNA is more rapidly labelled than other classes of RNA (Singer & Leder, 1966). Our results clearly indicate that the 10–14S fraction is labelled more rapidly than 18S and 28S rRNA. This fraction may represent mRNA species that code for the proteins of the rattlesnake's venom. Because these studies were done with active glands, it is reasonable to assume that the venom gland is predominantly making the proteins which constitute its secretion. Recent studies

Fig. 7. Specific radioactivities of 10–14S fraction plotted against pulse-labelling time

The values of the specific radioactivity of 10–14S fraction used in this figure represent the maximum value obtained for each time of incorporation. These values were computed from the data of Figs. 3, 4 and 5.

on incorporation of [³H]tyrosine into the venom gland after milking (Warszawsky *et al.*, 1972) showed that with a 30min pulse this amino acid is strongly incorporated into the ribosomes of the secretory cells. Unless that tissue has stable mRNA species, the venom production implies that new mRNA molecules are synthesized.

The molecular weight of the 10–14S fraction is in accordance with the molecular weight of the main toxin of the South American rattlesnake venom (crotoxin, mol.wt. 30000), but this component has recently been shown to be heterogeneous (Habermann & Rübsamen, 1971). Preliminary results indicate that the 10–14S fraction has a higher template activity than the other classes of RNA of the venom gland.

Radioautographic analysis

Radioautographic analysis showed that the labelled RNA extracted from whole cells, at least until 2h after the injection of the label, was located in the nucleus. The strong labelling of the nucleoli of the secretory cells at 22h may be related to the synthesis of rRNA precursors for new ribosomes. The in-

corporation of the label in the venom gland was higher than in liver and pancreas of the same animal at all time-intervals. This is strong evidence that RNA synthesis in the milked venom gland was greater than in the two other organs studied. Probably this difference is due to the manual extraction of the venom (milking).

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