Characterization of Ribonucleic Acids from the Venom Glands of *Crotalus durissus terrificus* (Ophidia, Reptilia) after Manual Extraction of the Venom

STUDIES ON TEMPLATE ACTIVITY AND BASE COMPOSITION

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RNA synthesis in the venom glands of *Crotalus durissus terrificus* was stimulated by the manual extraction of the venom (milking). RNA was extracted from venom glands activated by milking and fractionated by centrifugation through sucrose density gradients. Template activity for protein synthesis and base composition of the RNA fractions were studied. RNA fractions that sediment between 18S and 4S had the highest template activity. The base composition analysis indicated that the 28S and 18S rRNA have a C+G content of 65.4 and 58% respectively. The 'melting' temperature (T_m) of DNA in 0.15M-NaCl–0.015M-trisodium citrate, pH 7.0, was 85°C, corresponding to a C+G content of 38%. The base ratio of the RNA fractions that showed a high template activity was intermediate between that of rRNA and homologous DNA. The possible role of these fractions in the synthesis of the two main toxins (crotoxin and crotoamine) of the South American rattlesnake's venom is discussed.

The secretory cells of the venom gland of *Crotalus durissus terrificus* have an abundant rough endoplasmic reticulum spread throughout the cytoplasm, and its distribution is comparable with that observed in several mammalian cells involved in protein synthesis for export (Warshawsky *et al.*, 1972, 1973). The product of their secretion has a high content of proteins, and the two main toxins (crotoxin and crotoamine) represent about 60% of the total proteins of the venom (Slotta & Fraenkel-Conrat, 1938; Gonçalves & Arantes, 1957). Because of these characteristics the venom gland of this snake has been selected to study RNA synthesis, particularly of mRNA species (De Lucca & Imaizumi, 1972).

Recent studies from this laboratory showed that manual extraction of the venom (milking) stimulates protein synthesis in the venom gland of the South American rattlesnake (De Lucca *et al.*, 1973). This effect was first observed in the venom gland of the Old World vipers (Oron & Bdolah, 1973). Moreover, it was previously demonstrated that milking stimulates RNA synthesis in the venom gland of *Vipera palaestinae* (Rotenberg *et al.*, 1971). Thus the milked glands of snakes provide a suitable system to detect mRNA species since they are actively synthesizing RNA and proteins.

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In a previous paper (De Lucca & Imaizumi, 1972) RNA synthesis was studied in the rattlesnake's venom gland during the first 8 h after milking. An RNA fraction with a sedimentation coefficient of 10–14S was labelled more rapidly than other classes of RNA. This labelling pattern has been used as one of several criteria to identify the mRNA in eukaryotic cells (Singer & Leder, 1966; Sibatani, 1966). We have therefore investigated whether this 10–14S fraction had other properties attributed to mRNA of animal cells. The experiments were designed to extract the RNA from the milked glands and to determine its base composition and its ability to stimulate the incorporation of amino acids into acid-precipitable material in a cell-free system. These studies were done with RNA fractions obtained by centrifugation through sucrose density gradients.

Experimental

Materials

Chemicals. [5-³H]Uridine (sp. radioactivity 30 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [U-¹⁴C]Lysine (sp. radioactivity 125 mCi/mmol) was obtained from the Commissariat à l'Energie Atomique, Saclay, France. Creatine phosphate, creatine kinase (EC 2.7.3.2) and GSH were from Calbiochem, Los

Angeles, Calif., U.S.A. Pancreatic ribonuclease (EC 2.7.7.16) and GTP (disodium salt) were from Schwarz/Mann, Orangeburg, N.Y., U.S.A. ATP (disodium salt) was from Sigma Chemical Co., St. Louis, Mo., U.S.A. Scintillation chemicals 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)-benzene were from Packard Instrument Co. Inc., La Grange, Ill., U.S.A. Sources of other chemicals are given in a preceding paper (De Lucca & Imaizumi, 1972).

Animals. South American rattlesnakes (*Crotalus durissus terrificus*) of both sexes, weighing 300–400 g, were used. The snakes were starved for at least 1 month to allow the gland to accumulate its venom. They had free access to tap water only.

Methods

Extraction of RNA. The snakes were killed by decapitation 24 h after manual extraction of the venom. The milking was performed by the method of Klauber (1956). The venom glands were quickly removed and the RNA was extracted from the whole cells as previously described (De Lucca & Imaizumi, 1972). The concentration of RNA was determined spectrophotometrically at 260 nm, assuming that 1 E_{260} unit is equivalent to 40 μ g of RNA/ml. Only preparations of RNA with an E_{260}/E_{280} ratio of about 1.9–2.0 were used.

Sucrose-density-gradient centrifugation. Total RNA was fractionated by centrifugation on linear 5–20% (w/v) sucrose gradients as reported by De Lucca & Imaizumi (1972).

Preparation of microsomal fractions. Microsomal fractions were prepared from rat liver. The animals were killed by decapitation, the livers were removed and rapidly placed on ice. The subsequent procedures were based on those of Gelboin (1964) and were all carried out at 0–4°C. The microsomal fraction was collected by centrifugation at 105 000 g_{av} for 60 min in a Spinco model L ultracentrifuge. The pellets were resuspended in 0.25 M-sucrose solution (1.4 ml/g wet wt. of liver) by homogenizing with a hand-held Teflon-glass homogenizer. The resulting supernatant and the microsomal fractions were kept on ice until required.

Cell-free amino acid-incorporation system. The complete incubation medium used was essentially that described by Gelboin (1964). The medium contained, in a total volume of 1.7 ml (final concns.) 12 mM-potassium phosphate, pH 7.4, 3 mM-ATP, 0.3 mM-GTP, 0.6 mM-MgCl₂, 24 mM-phosphocreatine, 0.25 mg of creatine kinase, 6 mM-GSH, 90 mM-sucrose, 0.3 μ Ci of [¹⁴C]lysine (sp. radioactivity 125 mCi/mmol), microsomal fractions and supernatant equivalent to 200 mg and 67 mg of rat liver respectively. The system was preincubated in the absence of the labelled amino acid at 37°C for

20 min in all experiments. This time of preincubation was considered sufficient to deplete the system of endogenous mRNA (Gelboin, 1964). Additional 24 mM-phosphocreatine, 0.25 mg of creatine kinase, 0.3 μ Ci of [¹⁴C]lysine and various amounts of the venom-gland RNA were then added. Unless otherwise stated, 0.1 ml of the RNA fraction collected from the sucrose gradients was added to the cell-free system. Incubations were done in duplicate at 37°C for 20 min. The reaction was stopped by the addition of an equal volume of ice-cold 10% (w/v) trichloroacetic acid containing 0.2% of unlabelled lysine, followed by heating at 90°C for 10 min. After being left on ice for 15 min the precipitated proteins were collected on Millipore filters (HA; pore size 0.45 μ m). The filters were washed with 20 ml of ice-cold 5% trichloroacetic acid, dried at 60°C for 30 min and 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 a litre of toluene]. The radioactivity was measured in a Packard Tri-Carb liquid-scintillation counter (efficiency 70%). The blank consisted of a similar incubation medium in which the reaction was terminated immediately after the addition of the RNA. The results were expressed in terms of pmol of [¹⁴C]lysine incorporated/mg of added RNA.

Base composition of RNA. This determination was carried out by the method of Katz & Comb (1963).

Base composition of DNA. DNA was extracted from the rattlesnake liver by the method of Marmur (1961). DNA was dissolved in 0.15 M-NaCl–0.015 M-trisodium citrate, pH 7.0, and the 'melting' temperature (T_m) was determined (Mandel & Marmur, 1968). The E_{260} values were corrected for solvent expansion. The C+G content was calculated from the T_m value by using the following equation: $C+G = (T_m - 69.3) \times 2.44$ (Mandel & Marmur, 1968).

Radioautography. The left gland only was milked in the snakes used in these experiments, and the right gland was the control. [³H]Uridine (2 μ Ci/g body wt.) was injected intraperitoneally 2 days after milking. The animals were killed 24 h after injection of the radioisotope and the venom glands were removed and fixed in Bouin's fluid for 24 h (Barka & Anderson, 1963). Paraffin sections (5 μ m) pre-stained with Haematoxylin and Eosin were processed for radioautography by using Kodak Track Emulsion type NTB-2. The exposure time was 35 days. The radioautographs were developed in D-170 (Kodak), fixed in 24% (w/v) sodium thiosulphate and examined with the light-microscope (Kopriwa & Leblond, 1962).

Results and Discussion

The radioautographic results of [³H]uridine incorporation into RNA of the venom gland are

EXPLANATION OF PLATE I

Radioautographs of the venom glands 24 h after injection of [5-³H]uridine

(a) Unmilked gland; (b) milked gland. The radioisotope was injected 2 days after milking. For further details see the Experimental section. The bars represent 10 μ m.

shown in Plate 1. The milked-gland RNA was labelled, whereas the number of silver grains in the RNA of the unmilked gland was close to background. These results clearly demonstrate that milking stimulates RNA synthesis in the venom gland of *Crotalus*. The milked gland should therefore be a good source of mRNA species, and consequently all experiments described here were performed with RNA extracted from these activated glands.

Fig. 1. Effect of venom-gland RNA on [^{14}C]lysine incorporation by the rat cell-free system

The composition of the cell-free system and the conditions of incubation are given in the Experimental section. Values represent the means of two experiments.

Fig. 2. Time-course of [^{14}C]lysine incorporation directed by the venom-gland RNA

The composition of the cell-free system and the conditions of incubation are given in the Experimental section. Samples ($100\mu\text{l}$) were periodically taken from the incubation medium and applied to Whatman 3MM filter-paper discs. The discs were air-dried and transferred to counting vials. Values represent the means of two experiments.

Fig. 1 shows that the incorporation of [^{14}C]lysine by the rat cell-free system was proportional to the amount of venom-gland RNA added, and this response was linear at least up to $160\mu\text{g}$. The time-course of amino acid incorporation indicates that the reaction reached a plateau after approx. 20min of incubation (Fig. 2). The incorporating activity is stimulated by synthetic polyribonucleotide (Gelboin, 1964), and the results described here show that the rat cell-free system is also able to respond to the addition of natural and heterologous RNA. This finding is in agreement with other reports (Stavnezer & Huang, 1971; Mathews *et al.*, 1971, 1972; Berns *et al.*, 1972), indicating that the factors required for the translation of mRNA *in vitro* are not species-specific.

To determine the template activity of the RNA fractions, the sedimentation profile was arbitrarily divided into six groups (Fig. 3). The experiments were done by using only one RNA fraction of the different groups at each time, with various amounts of RNA ($30\text{--}70\mu\text{g}$) to assure a linear response. The highest template activity was always obtained with the fractions of groups V and the result of a typical experiment is presented in Fig. 3. This activity was measured by adding 0.1 ml of the RNA fraction to the cell-free system. It was observed that the variation in the amount of sucrose present in the RNA samples

Fig. 3. Template activity of venom-gland RNA fractions

Total RNA was obtained from the milked glands and fractionated by centrifugation on a linear 5–20% (w/v) sucrose gradient at 39000 rev./min for 4h. The sedimentation profile was arbitrarily divided into six groups and the template activity of the RNA fractions was measured as described in the Experimental section. ○, E_{260} ; ●, [^{14}C]lysine incorporated (pmol/mg of added RNA).

was not critical to the incorporating activity. It was also found that the [^{14}C]lysine incorporation directed by the venom-gland RNA is inhibited by the addition of ribonuclease to the cell-free system (Table 1).

The base-composition analysis of the six groups of RNA (Table 2) indicated that the C+G contents of 28S and 18S rRNA of this reptile are 65.4% and 58.0% respectively. These values are similar to those described for rRNA of mammalian cells (Munro, 1964; Darnell, 1968) and are in agreement with those reported in the rattlesnake liver (Giorgini & De Lucca, 1973). It is noteworthy that the RNA fractions of group V have the lowest C+G composition and the highest A content. Recent studies showed that mRNA of eukaryotic cells contains polyadenylate sequences (Lim & Canellakis, 1970; Burr & Lingrel, 1971; Perry *et al.*, 1972; Reed & Wintersberger, 1973; Hunt, 1973). The high A content of group V could be explained by the presence of RNA fractions rich in poly(A), but this needs further investigation.

The T_m value of DNA extracted from the liver of this reptile is 85°C (Fig. 4), corresponding to a C+G content of 38%. This value is considered low when compared with that found in amphibians (Decroly *et al.*, 1964). Table 3 shows the base ratio of DNA and that of the different groups of RNA separated by

sucrose-density-gradient centrifugation. From these results it was concluded that none of the six groups is DNA-like. Group V, which contains the rapidly labelled 10–14S fraction (De Lucca & Imaizumi, 1972), presented a base-ratio value intermediate between those described for the rRNA and the homologous DNA. This finding has also been reported in other animal cells (Kitazume *et al.*, 1962; Kitazume & Yčas, 1963; Decroly *et al.*, 1964) but the criterion of the base-ratio equivalence used in identifying an RNA fraction as mRNA has some limitations [see Sibatani (1966) and Singer & Leder (1966)].

In summary, the results reported here together with previous work (De Lucca & Imaizumi, 1972)

Fig. 4. Thermal-denaturation profile of rattlesnake DNA

DNA was extracted from the liver and dissolved in 0.15M-NaCl-0.015M-trisodium citrate, pH 7.0. For details see the Experimental section. Values represent the means for two determinations.

Table 1. Effect of ribonuclease on [^{14}C]lysine incorporation directed by the venom-gland RNA

The composition of the cell-free system and the conditions of incubation were as described in the Experimental section. Samples (30 µg) of RNA obtained from the same fraction of group V were added to each assay mixture. The results are the means of duplicate experiments.

Addition to medium	[^{14}C]Lysine incorporated (pmol/mg of RNA)	Activity (%)
Control	75	100
Ribonuclease (30 µg)	3	4

Table 2. Base composition of the six groups of RNA obtained by centrifugation through sucrose density gradients

The sedimentation profile was arbitrarily divided into six groups as shown in Fig. 3. The RNA fractions of each group were pooled and precipitated with 3 vol. of ethanol at -15°C for 12 h. The base-composition analysis was done by the method of Katz & Comb (1963). The results are the means of duplicate determinations.

Group of RNA	Sedimentation coefficient	Content (mol/100 mol)				
		U	A	C	G	C+G
I	> 28S	20.1	13.4	29.1	37.4	66.5
II	28S	20.0	14.6	28.2	37.2	65.4
III	28–18S	23.5	15.9	25.7	34.9	60.6
IV	18S	24.4	17.6	25.6	32.4	58.0
V	18S–5S	26.5	19.0	23.2	31.3	54.5
VI	5–4S	26.6	14.1	26.4	32.9	59.3

Table 3. Base ratio of DNA and the six groups of RNA obtained by centrifugation through sucrose density gradients

The A+U and C+G contents of the six groups of RNA were calculated from the information in Table 2. The C+G content of the DNA extracted from rattlesnake liver was determined from the T_m value (Fig. 4) by the method of Mandel & Marmur (1968).

	Group of RNA						DNA
	I	II	III	IV	V	VI	
Sedimentation coefficient	>28S	28S	28–18S	18S	18–5S	5–4S	–
$\frac{A+U(T)}{C+G}$	0.50	0.53	0.65	0.72	0.83	0.68	1.63

show that group V has RNA fractions with the following characteristics: (1) they are rapidly labelled compared with rRNA, (2) they have the highest template activity, and (3) they have a base composition intermediate between rRNA and homologous DNA. It is noteworthy that all these characteristics have been attributed to mRNA of eukaryotic cells (Singer & Leder, 1966; Sibatani, 1966). Since RNA was extracted from the glands activated by milking, it is possible that these fractions represent mRNA species involved in the synthesis of the venom toxins. The milked venom glands resemble in some aspects the mammary glands during lactation (Greenbaum & Slater, 1957; Sirakov *et al.*, 1968). It was found that lactating glands mainly synthesized a 15S fraction of high template activity, and it was suggested that the synthesis of this fraction is connected with the production of casein and lactoglobulin (Sirakov *et al.*, 1968). It should be emphasized that the molecular weight of the RNA fractions present in group V is in accordance with the information required for the synthesis of crotoxin and crotoamine (Fraenkel-Conrat & Singer, 1956; Gonçalves & Giglio, 1964). The synthesis of a specific protein has been reported as the best criterion for identification of the mRNA of animal cells (Singer & Leder, 1966). Thus the final proof that group V contains the mRNA of crotoxin and crotoamine should be the synthesis *in vitro* of these toxins directed by the fractions from this group. The fact that both toxins have pharmacological properties (Gonçalves, 1956; Moussatché *et al.*, 1956; Cheymol *et al.*, 1972; Vital Brazil, 1972) would facilitate their detection *in vitro*.

It was recently reported that the venom glands of *Vipera ammodytes* during the first day after milking make predominantly structural proteins (Oron & Bdolah, 1973). On the other hand, in the maximal active stage (eighth day) the venom proteins represent about 80% of the proteins synthesized by the milked glands. Since in rattlesnake the maximal active stage in protein synthesis occurs at 4 days after milking (De Lucca *et al.*, 1973), it is reasonable to assume that the glands in this stage should be a good source of mRNA species involved in the synthesis of crotoxin

and crotoamine. Thus the milked glands of *Crotalus* constitute a promising system to study mRNA in eukaryotic cells.

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