Asynchrony in the Synthesis of Secretory Proteins in the Venom Gland of the Snake *Vipera palaestinae*

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1. Venom of *Vipera palaestinae* was subjected to isoelectrofocusing on polyacrylamide gel. The protein separation profiles were similar for different venom samples; more than 25 protein bands with a wide range of pI values could be demonstrated by this technique.
2. Labelled venom was obtained 8 h after an intracardial injection of [^3H]leucine. The relative radioactivities of four out of 12 main protein bands were significantly different in the venom synthesized during the 2nd day of the venom regeneration cycle as compared with the venom of the 4th day. The comparison was made in venom samples obtained from the two glands of the same snake at two different secretory stages.
3. It is concluded that the asynchronous synthesis of exportable proteins after the initiation of a new venom regeneration cycle is responsible for the non-parallel secretion of some venom proteins by the venom gland of *Vipera palaestinae* during the first few days after milking.

Our present understanding of the synthesis and intracellular transport of proteins in gland cells is largely due to the extensive studies of Palade and his collaborators on the exocrine pancreas (Palade, 1975). These authors found that secretory proteins are synthesized on the rough endoplasmic reticulum and transported via the Golgi complex to membrane-bound secretory vesicles, which are the immediate source of the secretory material. Palade's group also presented evidence in favour of the notion that secretion of the different digestive enzymes in the guinea-pig exocrine pancreas occurs in proportions which are constant with time, showing a pattern of parallel secretion (Tartakoff *et al.*, 1975). This assumption was challenged in a series of studies by Rothman and his collaborators (Rothman, 1975) who claimed that the discharge of some digestive enzymes in the rabbit pancreas under a variety of conditions is of a non-parallel fashion. However, Steer & Glazer (1976), who also worked with rabbit pancreas, found a parallel discharge of the same enzymes. These conflicting results may be partially due to the fact that enzymes in digestive glands are usually stored in the cells as non-active pre-enzymes (zymogens).

In the venom glands of viperid snakes, very little secretion is stored inside the cells; no activation of venom enzymes could be demonstrated in gland homogenates [Shkolnik (Gefen), 1973]. The newly synthesized venom proteins are secreted continuously and accumulated in the gland lumina. Manual extraction of the venom from these lumina (milking) induces an accelerated rate of venom synthesis and

secretion (Oron & Bdolah, 1973; De Lucca *et al.*, 1974; Sobol-Brown *et al.*, 1975). Thus far, all venom antigens tested (four venom antigens) were localized in each of the epithelial cells lining the gland lumina. It may thus be assumed that each of the secretory cells synthesizes and secretes the full spectrum of venom components (Shaham & Kochva, 1969; Sobol-Brown, 1974; Shaham *et al.*, 1974).

Snake venom is composed of a great variety of proteins, which span a broad range of molecular weights, isoelectric points and enzymatic and toxic activities (Kochwa *et al.*, 1960; Jiménez-Porras, 1970). It was therefore assumed that some differential treatment of these secretory proteins may occur, and they could be easily detected in the accumulated venom. Indeed, a non-parallel regeneration of venom components was found in the snake *Bothrops jararaca* after successive milkings (Shenberg *et al.*, 1970). In *Vipera palaestinae* the secretion of three exportable enzymes (L-amino acid oxidase, phosphodiesterase and arginine ester hydrolase) during the first few days after milking showed some fluctuation from linearity (Sobol-Brown *et al.*, 1975). Significantly more L-amino acid oxidase was secreted during the 3rd day after milking, while on the 4th day the secretion of this enzyme slowed down. The secretion of phosphodiesterase was higher than the average on the 4th day. At the same time no significant changes in the intracellular activities of these enzymes could be demonstrated. Since the intracellular pool of venom in the gland is very low, it was suggested that the 'non parallel' accumulation resulted from changes in the rate of synthesis (Sobol-Brown *et al.*, 1975).

This assumption will be tested in the present paper by directly measuring the rate of synthesis of the major venom components in venom glands at two different stages of secretion. A preliminary report of this work has been published (Oron *et al.*, 1977).

Materials and Methods

Animals

Specimens of *Vipera palaestinae* weighing 200–300 g were kept and fed as previously described (Sobol-Brown *et al.*, 1975). Venom was removed from the gland by manual extraction (milking) (Klauber, 1956; Kochva, 1978) and each gland was milked into a separate vial (Oron & Bdolah, 1973).

Enzyme assays

Assay of L-amino acid oxidase (EC 1.4.3.2) was performed with L-leucine as substrate (Shaham & Bdolah, 1973) and of arginine ester hydrolase with α -N-benzoyl-L-arginine ethyl ester (Miles-Yeda,

Rehovoth, Israel) by the method of Schwert & Takenaka (1955). A unit of L-amino acid oxidase is defined as the amount of enzyme required to oxidize 1 μ mol of L-leucine/min and of arginine ester hydrolase as the amount of enzyme required to produce 1 μ mol of product/min.

Isoelectrofocusing

Isoelectrofocusing on gels was carried out essentially by the method of Catsimpoolas (1968) in 7.5% (w/v) acrylamide (Fluka A.G., Buchs, Switzerland) with 1% ampholytes (LKB-Producter AB, Bromma, Sweden) at a pH of 3–10. Protein samples (50–200 μ g) were mixed into the monomer solution and photopolymerized in a 0.7 cm $\times$ 10 cm tube with riboflavin. The anode chamber contained 1% (w/v) phosphoric acid and the cathode chamber 1% (w/v) NaOH. The electrofocusing was carried out for 18 h at 20 V/cm and at 4°C. The voltage was then raised to 30 V/cm for 2 h. The gels were washed with several changes of 10% (w/v) trichloroacetic acid, and were

Fig. 1. Gel isoelectrofocusing of *V. palaestinae* venom

Samples of 50–200 μ g of venom proteins were subjected to isoelectrofocusing on polyacrylamide gels at a pH range of 10 (top of gel) to 3. Staining of protein and L-amino acid oxidase was performed as described in the Materials and Methods section. (a), (b), Separation profiles of venom samples milked from the right and left gland of the same snake; (c)–(f), venom samples from different snakes. Gels (a)–(f) were stained for protein; gels (g) and (h) were a duplicate run of the same venom, (g) stained for L-amino acid oxidase (LAO) and (h) for protein. Protein bands are marked as described in Table 1.

then stained for protein with 0.01% (w/v) Coomassie Brilliant Blue (Sigma Chemical Co., St. Louis, MO, U.S.A.) in ethanol/acetic acid/water (20:15:60, by vol.) at 25°C for 18h. Background destaining was accomplished by washing the gels with the same solvent for 2h. Scanning of gels was performed at 560nm with a Gilford spectrophotometer (model 2400) equipped with a linear transport device (model 2410).

Histochemical staining of the gels for L-amino acid oxidase (Avrameas & Uriel, 1965) was carried out at 37°C upon completion of the run without any prior fixation. Gels were stained for phosphodiesterase (EC 3.1.4.1) by the method of Sierakowska & Shugar (1963).

Arginine ester hydrolase activity in the gels was determined by the biochemical method. Gel slices (1–3mm) were extracted by repeated (three times) freezing and thawing in 0.3ml of water. L-Amino acid oxidase activity was determined in extracts of gel slices after incubation in 0.2ml of 0.01M-Tris/acetate buffer, pH7.2, for 18h at 4°C (0.05M-Tris was adjusted to pH7.2 with acetic acid and diluted to 0.01M).

Labelling of venom proteins

Snakes were kept at 30°C and were not fed 60 days before the experiment so as to ensure maximum

accumulation of venom in the glands. In order to avoid individual variation, one gland (right or left) of each snake was milked 4 days before the experiment, whereas the contralateral one was milked 2 days later (cf. Oron & Bdolah, 1973). Each snake was injected intracardially with 1mCi of L-[4,5-³H]leucine (Nuclear Research Center, Negev, Israel, sp. radioactivity 33Ci/mmol) in 1ml of 0.15M-NaCl. Then 8h later, the two glands were milked into separate vials. The labelled venom was diluted in 1ml of 0.01M-Tris/acetate buffer, pH7.2, and further dialysed against 0.002M of the same buffer at 4°C for 18h.

Determination of radioactivity

Radioactive venom solution (50–100μl) was mixed with 3ml of Insta-Gel (Packard Inst., IL, U.S.A.) and counted in mini-scintillation vials. Gels were sliced (1–2mm) with a gel slicer (Miles-Yeda), or manually after staining for L-amino acid oxidase and protein. Each slice was incubated in a mini-vial with 0.2ml of 35% (w/v) H₂O₂ at 60°C for 48h, 3ml of Insta-Gel was then added and radioactivity counting was performed in a Packard Tri-Carb liquid-scintillation spectrometer (model 3370). Correction for counting efficiency (10–15% for ³H) was made by using an external standard.

Fig. 2. Localization of L-amino acid oxidase and arginine ester hydrolase on gels after isoelectrofocusing of venom. Identical venom samples were run in triplicate on polyacrylamide gels. One gel was stained with Coomassie Brilliant Blue and scanned at 560nm (----). Protein peaks corresponding to the bands in Fig. 1 are numbered accordingly. The other two gels were frozen with solid CO₂, sliced with a gel slicer (1–2mm) and the activities of L-amino acid oxidase (○) or arginine ester hydrolase (●) were assayed in extracts of the gel slices from the alkaline toward the acidic end.

Results

Fractionation and partial characterization of venom proteins

The separation of the venom of *Vipera palaestinae* by isoelectrofocusing with a wide pH range (3–10) is illustrated in Fig. 1. More than 25 protein bands could be demonstrated by this technique. The protein

separation profiles of venom samples milked from the two glands of the same snake were similar (Figs. 1a and 1b), but in venom samples obtained from different snakes quantitative differences were evident (Figs. 1c–1f). Several bands of L-amino acid oxidase with a pI range of 4.6–5.0 could be demonstrated (Fig. 1, Table 1) (cf. Hays & Wellner, 1969; Shaham & Bdolah, 1973). Arginine ester hydrolase, for which

Table 1. *Distribution of radioactivity between the different venom components in venom milked from glands 2 and 4 days after milking*

Labelled venom samples were obtained as described in the text and were subjected to isoelectrofocusing in gels. On completion of the run, the gels were first incubated in the histochemical medium for L-amino acid oxidase and the stained bands were cut out (designated as LAO bands in Fig. 1), while the other protein bands were sectioned manually after staining with Coomassie Brilliant Blue. Radioactivity (quoted as the mean with the range in parentheses) was determined as described in the Materials and Methods section. Only bands containing more than 1% of the total radioactivity were analysed (marked as bands 1–11 in Fig. 1) and their relative radioactivities (% of total) were computed. The radioactivity found in these bands accounts for about 80% of total radioactivity in the gel. The ratios were obtained within each animal (expressed as mean $\pm$ S.E.M.). The significance of the deviation of the ratios from unity was assessed by the two-way analysis of variance (pair comparisons) as described in the text. Venom samples of control snakes, in which both glands were at the same stage, were processed as above. The 11 protein bands were analysed in at least four snakes (two at 2 days after milking and two at 4 days; LAO bands were analysed in four snakes at 4 days and three at 2 days). None of the ratios of relative radioactivities of the control group deviates significantly from unity. Abbreviation: n.s., not significant.

Band no. (pI value in parentheses)	Number of snakes	Relative radioactivity (% of total)		Ratio (2 days/4 days)	Significance of deviations of the ratios from unity	Ratio of relative radioactivity left gland/ right gland in control snakes
		2 days after milking	4 days after milking			
1 (10.4)	8	12.8 (6–20)	12.8 (11–15)	0.99 $\pm$ 0.13	n.s.	1.18 $\pm$ 0.09 (4)
2 (10.0)	8	20.8 (15–39)	24.6 (16–44)	0.91 $\pm$ 0.01	$P < 0.05$	1.22 $\pm$ 0.11 (4)
3 (7.3)	8	6.7 (1–13)	8.0 (1–13)	0.92 $\pm$ 0.07	n.s.	1.00 $\pm$ 0.06 (5)
4 (7.2)	5	4.4 (2–12)	4.2 (1–11)	1.23 $\pm$ 0.18	n.s.	1.02 $\pm$ 0.05 (4)
5 (6.9)	7	17.0 (16–24)	18.1 (16–26)	0.97 $\pm$ 0.03	n.s.	0.99 $\pm$ 0.06 (5)
6 (6.6)	8	2.3 (1–6)	1.2 (1–4)	1.50 $\pm$ 0.25	n.s.	0.95 $\pm$ 0.08 (5)
7 (6.3)	8	4.4 (2–11)	4.2 (3–6)	1.14 $\pm$ 0.16	n.s.	1.06 $\pm$ 0.10 (5)
LAO (4.6–5.0)	10	9.8 (6–18)	6.9 (3–12)	1.48 $\pm$ 0.13	$P < 0.01$	1.06 $\pm$ 0.06 (7)
8 (4.2)	5	2.2 (2–4)	1.7 (1–2)	1.48 $\pm$ 0.12	$P < 0.05$	1.12 $\pm$ 0.11 (2)
9 (3.4)	8	11.8 (8–19)	11.2 (8–17)	1.17 $\pm$ 0.07	$P < 0.05$	0.95 $\pm$ 0.09 (5)
10 (3.0)	7	3.8 (2–9)	3.2 (2–7)	1.34 $\pm$ 0.15	n.s.	1.21 $\pm$ 0.11 (4)
11 (2.8)	7	3.7 (2–7)	3.6 (2–9)	1.19 $\pm$ 0.07	n.s.	1.19 $\pm$ 0.18 (5)

Fig. 3. Distribution of radioactivity and protein on gels after isoelectrofocusing of radioactive labelled venom ^3H -labelled venom was obtained from a snake 4 days after milking. Dialysed venom was subjected to gel-isoelectrofocusing. The gel was stained for protein, scanned at 560nm and then frozen and sliced (as in Fig. 1). Radioactivity in each slice (1–2mm width) was determined as described in the Materials and Methods section, with an overall recovery of radioactivity of 80–90%. pH measurements were carried out in aqueous extracts of frozen gel sections of a blank gel. ----, A_{560} ; ○, pH; ●, ^3H radioactivity.

no histochemical method is available, was measured in gel extracts and showed two peaks at pI values 4.0 and 4.6 (Fig. 2). Multiple molecular forms of phosphodiesterase with a very wide range of pI values were evident (cf. Levy & Bdolah, 1976). None of these bands coincided with any single major protein band. Two protein bands with pI values 10.0 and 3.4 (band nos. 2 and 9 in Fig. 1 respectively) have the same pI values as 'A' and 'D' components of the venom neurotoxic fraction (Ovadia *et al.*, 1977).

Incorporation of [^3H]leucine into venom components in glands 2 and 4 days after milking

The protein separation pattern and the distribution of radioactivity after isoelectrofocusing of a venom sample milked from a snake after injection of [^3H]leucine is depicted in Fig. 3. It can be seen that most proteins are labelled, but incorporation into some peaks is quite low, whereas others show a much higher specific radioactivity. To overcome the quantitative variability in the composition of venom obtained from different snakes (Fig. 1), radioactivities of the main venom proteins were compared in venom samples milked from the two glands of the same snake.

A group of ten snakes in which one gland was at the stage of 2 days and the other one 4 days after milking was injected with [^3H]leucine as described in

the Materials and Methods section. The labelled venom was milked separately from each gland 8 h later. At this time labelled secretory proteins have already been secreted into the gland lumen (U. Oron & A. Bdolah, unpublished work). Venom samples were subjected to isoelectrofocusing on polyacrylamide gel, and the relative radioactivity (% of total) of 12 protein bands was calculated (Table 1). In venom samples milked from different snakes a considerable variability in the relative radioactivity of some homologous bands was found (e.g. bands 3, 4, 6, 10, Table 1), whereas other bands, such as the components of the neurotoxic fraction, were considerably less variable (bands 2 and 9, Table 1).

The ratio of relative radioactivity (2 days versus 4 days) was calculated for each pair of homologous bands of venom samples milked from the two glands of the same snake. Deviation of this value from unity would imply asynchrony in the synthesis of the protein(s) of the band analysed.

Two-way analysis of variance (Sokal & Rohlf, 1969) carried out on the relative radioactivities of the pairs of homologous bands from the different snakes showed significant deviations from unity for the ratio of four out of 12 protein bands (Table 1). In two of these bands (the bands of L-amino acid oxidase and band no. 8) the relative radioactivities were about 50% higher in venom obtained from glands 2 days

after milking than those of 4 days. In venom samples obtained from control snakes, in which both glands were at the same stage (2 days or 4 days after milking), ratios of relative radioactivities for homologous bands did not deviate significantly from unity (Table 1).

Discussion

Close to 30 protein bands could be demonstrated in the venom by isoelectrofocusing on gels. The separation profiles of the venom samples derived from different snakes are qualitatively similar, although some bands seem to contain different amounts of stainable material. This variability is also apparent by the wide range of relative radioactivities of homologous protein bands in different venom samples. It is of interest to note that the relative radioactivity of the two neurotoxic bands in venom samples from different snakes is much less variable. These components form one of the major toxic principles of the venom and are essential for the immobilization of the prey (Ovadia *et al.*, 1977); it could therefore be expected that their amount in the venom should be relatively stable, even in a genetically non-homogenous population of venomous snakes.

As mentioned in the introduction, in the guinea-pig pancreas, the secretory process of the exportable proteins is synchronous from the Golgi stage on (Tartakoff *et al.*, 1975). However, parallelism in the early steps of protein synthesis has not yet been proven (see discussion by Tartakoff *et al.*, 1975). In fact there are reports in the literature that suggest that the labelling *in vivo* of ribonuclease (Keller *et al.*, 1961) and lipase (Christophe *et al.*, 1973) by radioactive amino acids proceeds at lower rates than that of other secretory proteins.

In the pancreas a long-term dietary adaptation has been shown to induce changes in the rates of synthesis of the digestive enzymes (Desnuelle *et al.*, 1962; Christophe *et al.*, 1971). Dagorn *et al.* (1977) have reported on non-parallelism in rates of synthesis and secretion of two enzymes by the pancreas after intragastric administration of oleic acid. However, no analogy can be found between this dietary regulation and the induction of venom synthesis by milking of unfed snakes.

In the present work the synthesis of venom proteins after initiation of a new venom regeneration cycle was studied in *V. palaestinae* after administration of [³H]leucine. To avoid the influence of individual variability, the contralateral glands of a snake at different stages of secretion were compared. Since the comparison was made between homologous protein bands, the difference in leucine content of various venom proteins did not affect the results. In addition,

it has been previously found (Allon, 1973) that the amino acid pool of the venom glands has not been changed significantly during the secretory cycle. Thus it may be assumed that the specific radioactivity of the precursor is similar in both glands.

The present results show that the relative radioactivities of several venom proteins from glands 2 days after milking are significantly different from those obtained for the homologous bands of venom milked from the contralateral glands at 4 days. Of special significance is the difference found for L-amino acid oxidase, which accounts for about 5% of the total venom protein (Shaham & Bdolah, 1973). The bands stained for this enzyme in venom synthesized during the 2nd day showed a relative radioactivity about 50% higher as compared with the homologous bands of the 4th day venom.

These results, together with the fact that the small intracellular pool of secretory enzymes remains practically unchanged at the pertinent intervals (Sobol-Brown *et al.*, 1975), clearly indicate that asynchrony in the synthesis of exportable proteins at the first few days after activation of the venom synthetic apparatus is responsible for the previously reported non-parallel accumulation of enzymes in the venom of *V. palaestinae*.

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