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Mechanics of Venom Expulsion in *Crotalus*, With Special Reference to the Role of the Fang Sheath

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ABSTRACT

A combination of anatomical and experimental preparations were used to explore the function of the venom delivery system in rattlesnakes (*Crotalus*). The distal end of the venom duct is compressed near the point where it empties into the venom chamber, a space surrounding the fang defined by the fang sheath. Within the venom chamber, the inner fang membrane lies obliquely over the base of the fang at least partially occluding the entrance orifice. When the fang is retracted the combination of the compressed venom duct and the spatial position of the inner fang membrane serve to inhibit or block venom flow. As the fang is erected beyond approximately 60° (relative to the roof of the mouth) localized compression of the fang sheath decreases the size of the venom chamber, relieves the compressive force from the venom duct, and displaces the inner fang membrane away from the entrance orifice of the fang. Pressure recordings taken at different locations along the venom delivery system demonstrate that the venom gland produces suction during relaxation of the extrinsic glandular musculature. These findings suggest that the venom delivery system of *Crotalus* is both more flexible and more regulated than previously assumed. Anat Rec 264: 415–426, 2001.

Key words: functional morphology; feeding; snake; Lepidosauria; prey capture

Despite the general recognition of both the potential dangers of snake venom and the ability of (some) snakes to ingest proportionately large meals, many aspects of prey capture and ingestion in snakes remain unclear (Greene, 1997). Earlier descriptive studies (e.g., Albright and Nelson, 1959; Boltt and Ewer, 1964; Frazzetta, 1966) led to the formation of the "pterygoid walk" model for prey ingestion (in which the right and left palato-maxillary arches alternately move through a cycle of protraction and retraction) , a model that has been supported by the limited experimental analyses of snake feeding (e.g., Cundall and Gans, 1979; Cundall, 1983; Kardong and Berkhoudt, 1998). Specialized oral venom delivery systems, although rare in vertebrates, are found in four groups of snakes: the Viperidae (including the crotalines or pit vipers), the Elapidae (cobras and related taxa), the Atractaspidae (the African stiletto snakes), and several "rear-fanged" taxa within the large Colubridae. The evolution of a specialized venom delivery system is generally taken as a key innovation in the behavioral, ecological, and phylogenetic diversity of snakes (e.g., Kardong, 1980; Kardong et al., 1997; Savitzky, 1980). Still, there remains debate over the phylogeny of the snake venom delivery system (e.g., McDowell, 1968, 1986; McCarthy, 1985). Although there has been much speculation about the evolutionary significance of the venom delivery system, and the structural features of the system have been detailed from several snakes (see below), no detailed experimental study has examined the mechanics and regulation of venom ejection in snakes.

The gross and microscopic anatomy of the venom gland, surrounding musculature, primary venom duct, and accessory venom gland have been well documented, par-

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ticularly from crotalines (e.g., Gans and Kochva, 1965; Kochva and Gans, 1966; Mackessy, 1991). Rosenberg (1967) detailed the intraglandular pressure hypothesis to explain venom expulsion from the venom gland. Support for this hypothesis can be found in the few manipulations of this system (Freyvogel and Honegger, 1965; Kardong and Lavin-Murcio, 1993; Rosenberg, 1967), and in a recent functional analysis of venom expulsion in *Crotalus* (Young et al., 2000). The morphological variation and specialization of snake fangs have been described (e.g., Bogert, 1943; Young and Kardong, 1996). The portion of the venom delivery system between the fang and the accessory venom gland, comprised of what is commonly called the secondary venom duct and the fang sheath, is less well known. Beyond some of the classic studies on rattlesnake feeding (e.g., Mitchell, 1861; Wyman, 1861; West, 1898), the morphology of the fang sheath has been described infrequently (e.g., Dullemeijer, 1956; Halstead et al., 1978; Schaefer, 1976) though many points of the anatomy remain contentious, particularly the presence of an open chamber at the base of the fang. Most of the previous studies were concerned with the phenomenon of fang succession. Though Rosenberg (1967) and Kardong and Lavin-Murcio (1993) speculated about the possible role of the fang sheath in influencing venom flow, to date there have been no experimental analyses of this system.

There are several reasons to suspect that the distal portion of the venom delivery system in vipers may function to both impede and facilitate venom flow. Only very rarely will a viperid eject venom without first erecting the fangs; manually extracting venom from a snake while the fangs are in the retracted position (when possible) requires the application of substantial force (Mitchell, 1861; Young, personal observation), and the venom is often discharged through the fang sheath, not the fang. Elongate venom glands, in some cases over 25% of the length of the snake (Dathke, 1990), occur in three major lineages of venomous snakes, having been described from several species of *Atractaspis* (Kochva et al., 1967; Underwood and Kochva, 1993), several species of the viperid *Causus* (Haas, 1952; Radovanovic, 1967), and the elapid *Maticora* (Haas, 1973; Radovanovic, 1935). Such a venom gland would be subjected to extrinsic pressure (and thus venom expulsion) with every undulatory deflection of the body, yet no expulsion of venom has ever been described. These aspects of vipers suggest that some form of anatomical or physiological impediment to venom flow exists beyond just the inactivity of the extrinsic venom gland musculature. Earlier claims (e.g., Mitchell, 1861) of a sphincter in the venom delivery system have proven to be erroneous; to date no apparent morphological or physiological barriers to venom flow have been documented. Severing the primary venom duct of an anesthestized rattlesnake results in a copious discharge of venom (Young, personal observation), suggesting that any barrier must be located in the distal portion of the venom delivery system. This study was undertaken to provide an experimental analysis of this barrier to venom flow.

MATERIALS AND METHODS

Live Animals

Venom flow was examined in six specimens of the western diamondback rattlesnake, *Crotalus atrox* (Snout-Vent Length, SVL = 84–120 cm, mean = 102.3; mass = 220–1,000 gm, mean = 536.7 gm) and one specimen of black-tailed rattlesnake, *C. molossus* (SVL = 100 cm, mass = 460 gm). The *C. atrox* were wild-captured in West Texas, while the *C. molossus* was obtained commercially. At Lafayette, the animals were housed in a specially designed venomous snake room with a temperature range of 26–31°C, a 12:12 light:dark cycle, and water ad lib. The snakes were maintained on a diet of pre-killed mice and rats. To ensure that the snakes had a normal venom supply, no food was given within 14 days of the experiments (Kochva, 1987). Some of the observations reported herein are based on an additional group of 11 adult crotalines used for an earlier study of the venom gland (Young et al., 2000).

Morphology

Morphological investigations were performed on six adult specimens of *Crotalus atrox* previously preserved in neutral-buffered formalin and maintained in 70% ethanol. The venom duct and fang sheath complex was dissected in four specimens. In the third specimen portions of the fang sheath immediately lateral and medial to the base of the functional fang were removed. This tissue was dehydrated through a graded ethanol series, cleared in Hemo-De (Fisher), then embedded in paraffin. Parasagittal sections were cut at 10 μ and stained with either hematoxylin and eosin (Presnell and Schreibman, 1997), or Weigert's resorcin-fuchsin stain for elastin (Luna, 1960). In the fourth specimen a small radial saw was used to excise blocks of tissue containing the fang and maxilla, fang sheath, and distal venom duct, from both sides of the head. One of these blocks was quick frozen in liquid nitrogen and cleaved with a razor blade to produce a frontal section through the base of the venom fang. The fang sheath of the second block was dissected to expose the relationship between the entrance orifice of the fang and the fang sheath. Both blocks were subsequently dehydrated for seven days in 100% ethanol, critical point dried (Polaron), sputter coated (PS-2, International Scientific Instruments) with 300 Å of gold, and examined at 15 kV under a Super-3A scanning electron microscope (International Scientific Instruments). Additional morphological information was obtained from previously prepared slides of the venom apparatus of *C. atrox* and *C. adamanteus* in the private collection of BAY.

Demarcation of the Venom Chamber

To explore the dimensions of the venom chamber the head was removed from an adult specimen of *Crotalus atrox* that had been previously preserved in neutral-buffered formalin and maintained in 70% ethanol. Leaving the fang sheath intact, two supralabial scales were removed to expose the distal venom duct. The duct was severed immediately distal to the accessory venom gland and the isolated head positioned in normal posture (with the fang ventral). Isolated drops of blue India ink were placed in the exposed lumen of the distal venom duct and were allowed to course through the duct into the venom chamber; as gravity pulled the India ink into the venom chamber additional drops were applied (with no pressure) into the venom duct. When the flow of India ink slowed, the outer epithelium of the fang sheath was dissected away, and the venom chamber and distal venom duct photographed.

Manipulation of Anesthetized Specimens

Three specimens, including the *Crotalus molossus*, were lightly anesthetized through oral exposure to Isoflurane. Once the animal was fully anesthetized, a variety of manipulations were performed. With the fang retracted, manual pressure was applied to the venom gland in an effort to force venom expulsion. A small incision was made in the scalation over the venom gland and a single 0.5 msec duration stimulation was applied to the surface of the compressor glandulae using a S88 stimulator (GRASS Instruments). The fangs were forced into the erect position through manual pressure applied at the compound-pterygoid joint. These manipulations were repeated both with, and without, tactile stimulation (light contact with a wooden applicator) being applied to the roof of the mouth and the fang sheath.

Differential Pressure and Velocity

The differential pressure and velocity experiments were performed on four specimens of *C. atrox*. The specimens were briefly exposed to Isoflurane, weighed on a DS10 digital balance (OHAUS), then manually restrained in a clear plexiglas tube and given an IM injection of 80 mg/kg of a 9:1 mixture of Ketamine Hydrochloride and Acepromazine. Once anesthetized the specimen was placed on a heated surgical table (VSSI) and a clamp positioned along the dorsal and ventral midline of the skull. This clamp held the head in a fixed position with the jaw open, and the fang partially erect (leading edge of the fang sheath approximately 50° relative to the superior margin of the oral cavity). Once the head was secured a 40 cm length of PE tubing was fitted over the end of the fang; the inner diameter of the PE tubing was such that it would form a tight seal with the distal portion of the fang while not occluding the exit orifice. The tubing was attached to a PT300 pressure transducer (GRASS Instruments) and filled with snake ringer's solution (Beyenbach, 1984); the exit port of the pressure transducer was closed and the pressure transducer positioned at a height equal to the venom gland. The pressure transducer was connected to a P122 AC/DC amplifier (GRASS Instruments).

A portion of the venom duct was surgically isolated and fitted with a 1R perivascular flow probe (Transonic Systems, Inc.); the flow probe and venom duct were coated with HR jelly to ensure a good signal. The flow probe was connected to a T106 flow meter (Transonic Systems, Inc.). A bipolar stimulating probe (total contact area approximately 2 × 2 mm) connected to a S88 stimulator (GRASS Instruments) was used to deliver a stimulation to the surface of the dorsomedial portion (Young et al., 2000) of the compressor glandulae. Stimulations applied ranged from 0.5–5 msec, but were held constant throughout each experiment.

The output signals from the T106 flow meter, the P122 amplifier, and the synchronized pulse from the S88 stimulator, were received at a 100B A/D converter (GW Instruments) connected to a G3 computer (Apple, Inc.). The Instrunet data acquisition system (GW Instruments) was used to simultaneously recorded the three signals for 20 sec intervals at a sampling rate of 20 kHz. The SoundScope wave analysis package (GW Instruments) was used to examine and quantify the signals.

After an initial series of recordings (4–5 separate stimulations) the PE tubing was removed from the fang. An incision was made in the distal portion of the venom duct near the accessory venom gland. Through this incision the PE tubing was passed into the exposed proximal end of the venom duct and sutured in place. A second series of stimulations (again consisting of 4–5 separate stimulations) was then applied to the compressor glandulae. Additional implantations of the PE tubing were performed, in a distal-proximal sequence, with multiple stimulations at each site. If the PE tubing came close enough to the flow probe to suggest that either signal could be affected, the flow probe was removed. In two animals the flow probe was surgically attached to the venom duct, and the compressor glandulae stimulated as described above, before any connection of the PE tubing. These records were used, in part, for controls on any possible alterations of the venom flow pattern caused by the presence of the PE tubing and pressure transducer.

Radiopaque Injection

A specimen of *C. atrox* (SVL = 102 cm, mass = 465 gm) was anesthetized through oral exposure to Isoflurane, then injected intramuscularly with 80 mg/kg of a 9:1 mixture of Ketamine Hydrochloride and Acepromazine. Once fully anesthetized, the proximal portion of the venom duct was surgically isolated and severed. Manual pressure was used to evacuate the distal venom duct of venom, with care being taken to avoid tissue damage or the application of pressure to the region of the fang sheath. A small piece of stainless steel wire was attached to the lateral surface of the accessory venom gland using polyacrylamide adhesive (Vetbond). A hypodermic syringe was inserted into the exposed distal end of the venom duct and held in place with suture. The syringe was filled with an iodine-based radiopaque solution (Omnipaque 350, Winthrop Laboratories) that was injected into the venom duct. Pressure was exerted on the syringe until the venom duct was observed to distend slightly, then the location of the radiopaque marker was documented with a radiograph (Quanta III-T radiographic film, Dupont, exposed at 100 MA and 55 KVP for 1.6 MAS).

Fang Cannula and Venom Flow

As described above, pressure recordings were initially taken by placing PE tubing around the distal end of the fang. Using four anesthetized specimens, including the *Crotalus molossus*, we placed PE tubing slightly larger than the maximum diameter of the fang over the distal tip of the fang. By slowly advancing the PE tubing along the length of the fang the membranes of the fang sheath were disrupted. This cannulization of the fang sheath was performed independent of any other stimulation or contact with the venom delivery system.

Fang Erection and Venom Flow

Two specimens of *C. atrox* were anesthetized as described above and one side of the head placed in a clamp that held the head in a normal horizontal orientation. The tip of the contralateral fang was removed with a high-speed radial saw and a 50 cm segment of PE tubing was fitted over the distal end of the fang. To allow maximal range of motion, the PE tubing made a U-bend in the snake's esophagus and was then connected to a PT300 pressure transducer (GRASS Instruments). With continuous stimulation (1 msec duration, 50 pps) applied to the

dorsomedial surface (Young et al., 2000) of the compressor glandulae (S88 Stimulator, GRASS Instruments), the fang was erected by manual pressure applied to the compound-pterygoid joint. The output of the pressure transducer was amplified (P122 amplifier, GRASS Instruments) before being digitally converted and collected by the data acquisition system, as described above. A digital high speed video camera (Motionscope, Redlake Instruments) was positioned to simultaneously record the rotary motion of the fang relative to the skull and the digital pressure readout on the P122 amplifier. Video images were recorded at 500 fps with a 1/20,000 sec shutter speed. The video image was streamed to a computer (Power Mac 8500, Macintosh) and the angle formed between the ventral margin of the supralabial scales and the leading edge of the fang sheath quantified in successive frames using N.I.H. Image 6.12.

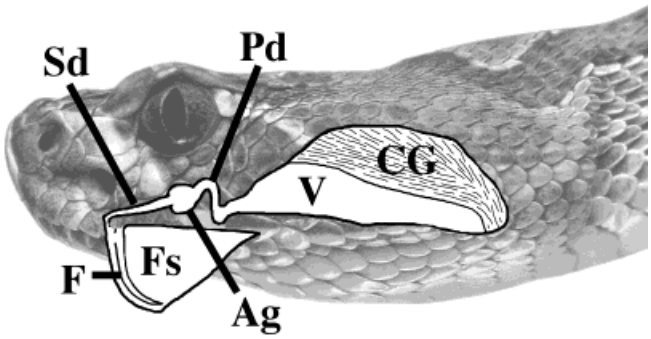

Fig. 1. Generalized depiction of the main components of the venom delivery system in *Crotalus*. Ag, accessory venom gland; CG, Compressor Glandulae; F, fang; Fs, fang sheath; Pd, primary venom duct; Sd, secondary venom duct; V, venom gland.

Sequential Excision of Supportive Elements

Two specimens of *C. atrox* were anesthetized as described above. Once fully anesthetized, the distal tip of the fang was removed with a high speed radial saw, PE tubing was placed over the distal end of the fang, and a pressure transducer coupled to the data acquisition system as detailed above. A small incision was made in the scalation over the compressor glandulae and a stimulating probe used to provide 4–5 individual 5 msec stimuli (D88 stimulator, GRASS Instruments) to the dorsomedial portion of the compressor glandulae (Young et al., 2000). After this, the supportive elements of the venom delivery system were sequentially excised; after each element was excised a series of 4–5 stimulations was applied to the compressor glandulae. The sequence of excision/manipulation was as follows: supralabial scales and adjacent scalation; superficial connective tissue; deep connective tissue of proximal venom duct; proximal venom duct retracted caudally until duct is straight; connective tissue of the distal venom duct up to the surface of the maxilla; distal venom duct isolated from the surface of the maxilla; superficial epithelium of the fang sheath; manual manipulation of the venom chamber.

Internal Displacement of the Inner Fang Membrane

A specimen of *C. atrox* was anesthetized as described above. This specimen was placed in a clamp that held the head in a horizontal orientation, but with the ventral surface up. Suture was used to reflect the lower jaw laterally to expose the fang sheath and oral mucosa. With the exception of a small incision in the scalation over the compressor glandulae, no structural feature on the unclamped side of the head was disturbed. A stimulating probe was used to apply 4–5 individual stimulations (5 msec) to the dorsomedial portion of the compressor glandulae as described above. With the fang still in the retracted position a 10 cm segment of 0.1 mm stainless steel was inserted into the exit orifice of the fang. This stainless steel was used because it was pliable enough to follow contours of the venom canal, yet stiff enough to exert force on the inner fang membrane. Care was taken not to touch the fang sheath while the probe was in the venom canal. With each incremental advance of the probe along the venom canal an additional series of 4–5 stimulations were applied to the compressor glandulae. Advancement of the probe was continued until a distinct release of venom from the exit orifice was observed, at which time the probe was attached to the fang using Polyacrylamide adhesive (Vetbond). The fang sheath was then dissected and photographed to document the position of the probe relative to the fang's entrance orifice, the inner fang membrane, and the venom duct.

RESULTS

Morphology

The venom delivery system of the crotalines examined is composed of several distinct components (Fig. 1). The venom gland, located at the caudolateral margin of the upper jaw is the site of venom synthesis and storage. The connective tissue surrounding the venom gland serves as the origin for the compressor glandulae and the smaller pterygoideus glandulae that is located on the ventromedial surface of the gland. The tubules of the venom gland coalesce to form the primary venom duct that follows a convoluted path, including at least one nearly 180° turn, as it courses anteriorly along the lateral margin of the upper jaw. Near the cranial margin of the orbit the primary venom duct expands slightly to form the accessory venom gland.

The secondary venom duct arises from the cranial surface of the accessory venom gland, courses along the lateral surface of the maxilla, then deflects ventrally along the anterior surface of the maxilla (Fig. 1). The secondary venom duct is bound by connective tissue to the maxilla; however, there is no direct contact between the m. pterygoideus and the secondary venom duct. There is a marked decrease in the diameter of the secondary venom duct as it courses along the anterior surface of the maxilla (Fig. 2A). The secondary venom duct terminates just dorsal to the level at which the fang forms an ankylosis with the maxilla.

The functional fang, any replacement fangs, and the distal end of the maxilla are covered by a drape of dense irregular connective tissue termed the fang sheath (Fig. 1). The fang sheath is a fairly large structure, which includes the folds of the oral mucosa that surround the fang. Internally, the fang sheath forms weak attachments along the surface of the fang, at roughly one-third the length of the fang. These attachments define an anatomical space, herein termed the venom chamber (Fig. 2B,C).

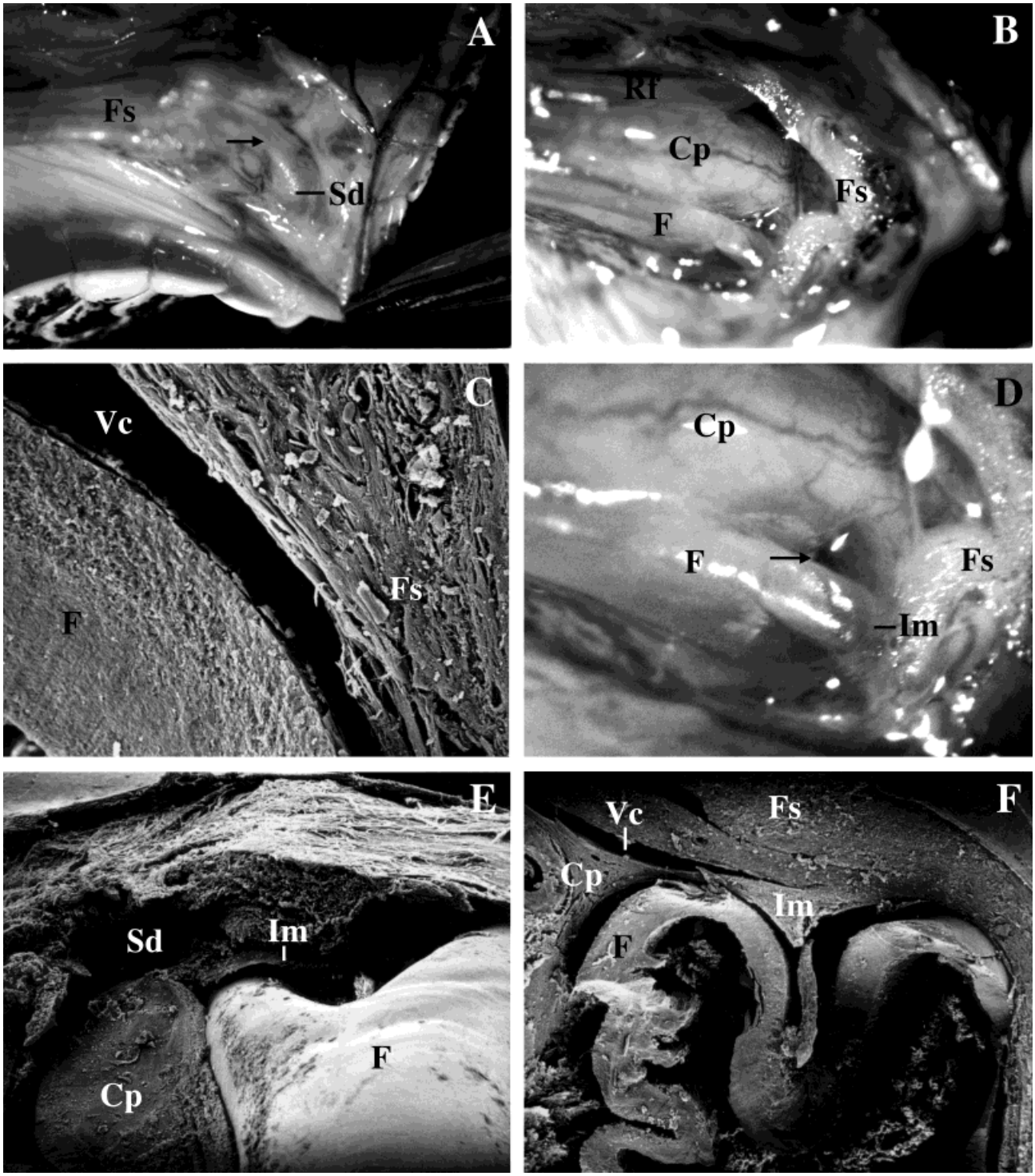

Fig. 2. Morphology of the distal venom delivery system. **A:** ventral view (with cranial to the right) of the left venom delivery system after removal of the oral epithelium, note the distinct decrease in the diameter of the secondary venom duct (arrow) (×1.5). **B:** Ventral view (with cranial to the right) of the right venom chamber after removal of a portion of the fang sheath (×4). **C:** Transverse section through the fang and fang sheath illustrating the natural boundaries of the venom chamber (×225). **D:** Ventral view of the right functional fang (with cranial to the right), note how the adjacent central partition and inner fang membrane obscure the entrance orifice of the fang (arrow) (×10). **E:** Oblique view of the venom chamber, note the spatial relationship between the secondary venom duct and the entrance orifice of the fang (×22). **F:** Transverse section through the entrance orifice of the fang, note how the inner fang membrane penetrates into the entrance orifice (×27). Abbreviations: Cp, central partition; F, fang; Fs, fang sheath; Im, inner fang membrane; Rf, replacement fang; Sd, secondary venom duct; Vc, venom chamber.

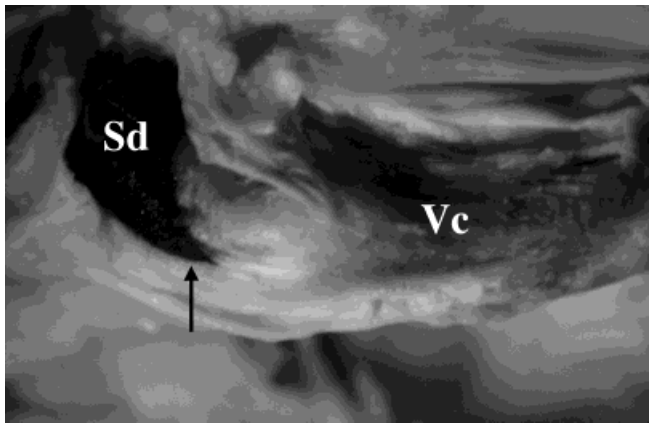

Fig. 3. Lateral view of the left fang sheath showing the dimensions of the venom chamber revealed by introducing India ink into the secondary venom duct (×4). Note how the diameter of the secondary venom duct is greatly reduced as it courses around the base of the fang (arrow). Abbreviations: Sd, secondary venom duct; Vc, venom chamber.

The dorsal, ventral, and lateral borders of the venom chamber are comprised of thicker layers of dense regular connective tissue, with a surface layer of oral mucosa. This connective tissue is orientated predominantly vertically; and although elastic fibers are present in this tissue, most of the fibers are collagenous. The lateral border of the venom chamber contains a thickened pad of connective tissue. A similar pad is located in the proximal portion of the medial border.

Within the venom chamber, positioned between and parallel to the functional fang and any replacement fangs, is a highly vascularized wedge of connective tissue commonly referred to as the central partition (Fig. 2). The central partition closely abuts the medial surface of the functional fang, and attaches to the ventral surface of the maxilla immediately adjacent to the terminal end of the secondary venom duct (Fig. 2E). A second, much thinner, band of connective tissue courses obliquely through the venom chamber, over the proximal surface of the fang (Fig. 2D–F); herein this connective tissue is termed the inner fang membrane. The inner fang membrane is anchored at the anteromedial and lateral borders of the venom chamber. With the fang in the retracted position, the inner fang membrane courses over, and typically projects down into, the entrance orifice of the fang (Fig. 2E,F). As the fang is rotated into the erect position, the inner fang membrane is displaced dorsally away from the entrance orifice and surface of the fang.

Demarcation of the Venom Chamber

Drops of India ink placed into the open lumen of the secondary venom duct slowly flowed the length of the duct and into the venom chamber. The ink was clearly visible through the venom duct and the deep tissues of the fang sheath (Fig. 3). A visual inspection of the flow pattern of the ink reveals a sharp decline in the amount of ink at the distal end of the secondary venom duct, where the secondary venom duct courses along the anterior face of the maxilla (Fig. 3). The India ink flowed through the end of the venom duct and into the venom chamber. Because this ink was not injected under pressure, moving instead only along a slight gravitational gradient and through capillary action, the ability of the ink to fill the venom chamber suggests that this is a real anatomical space. The venom chamber is ovoid shaped; the greatest length, roughly one-third the length of the fang, is on the anterior surface, the lateral and medial portions of the venom chamber are smaller. Judging by the quantity of ink present, the venom chamber expands around the base of the fang, particularly on the medial surface (Fig. 3). This same location frequently bulges during venom milking and injection (Young, personal observation).

Manipulation of Anesthetized Specimens

In anesthetized crotalines the fang can be manually erected by applying force at the compound-pterygoid joint. This simple manipulation was performed on numerous snakes, and never resulted in the release of venom. If the supralabial scalation is removed and the fangs manually erected, physical displacement of the primary venom duct is observed. The convolutions that characterize the primary venom duct of crotalines disappear, or are greatly reduced, as the venom delivery system is stretched during fang erection. With the fang in the retracted position venom can be expelled through direct manual pressure on the venom gland. It takes a substantial amount of pressure, however, to produce venom expulsion in this manner, and in some specimens it proved impossible. Venom released in these manipulations appeared to emerge from the fang sheath, rather than the exit orifice of the fang. Surface stimulations of the compressor glandulae, with the fang in the retracted position, never resulted in the release of venom. Tactile stimulation of the oral epithelium had no apparent effect on venom discharge. On one occasion a significant quantity of venom was expelled with the fang in the retracted position through a combination of tactile stimulation of the fang sheath and electrical stimulation of the compressor glandulae. Severing the primary venom duct resulted in significant release of venom, even without tactile or electrical stimulation of the venom gland.

Differential Pressure and Velocity

Pressure tracings recorded from a semi-erect fang after electrical stimulation of the dorsomedial portion of the compressor glandulae have a characteristic pattern. The pressure values recorded are very low (typically below 0.5 mm Hg). The initial pressure rise occurs slightly after the stimulation is applied (i.e., there is a normal latency period) and the initial pressure peak decreases sharply as would be expected from a twitch contraction (Fig. 4). The pressure decreases below the resting level (suction or negative pressure is recorded) then increases again well after the presumed cessation of muscle contraction (Fig. 4). Venom pressure recorded directly from the primary venom duct is substantially higher than that recorded from the fang (Fig. 5) and generally decreased slightly along the length of the primary venom duct. These pressures differed from those obtained from the fang in having less pulsatility, but did include a distinct wave of negative pressure (Fig. 6A). Transonic flow probes were used as a control for the influence of the pressure recording apparatus. Flow tracings recorded from the primary venom duct with the fang retracted and venom delivery system unimpeded by the pressure recording apparatus consistently

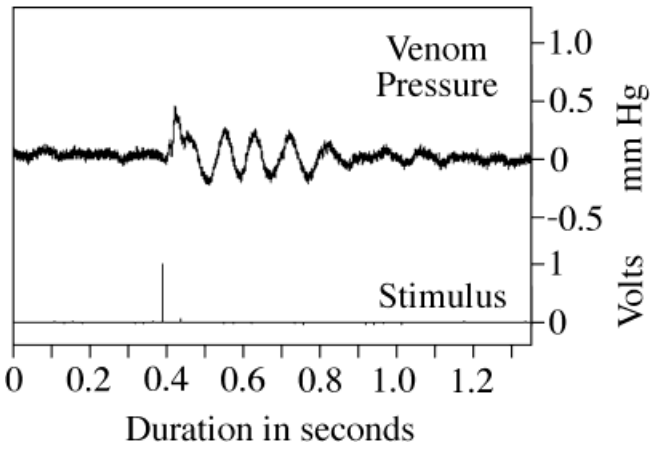

Fig. 4. Venom pressure tracing (upper tracing) recorded from the distal end of the fang after electrical stimulation of the compressor glandulae (stimulus marker bottom tracing). Note the pulsatility of the pressure wave and the presence of negative pressures.

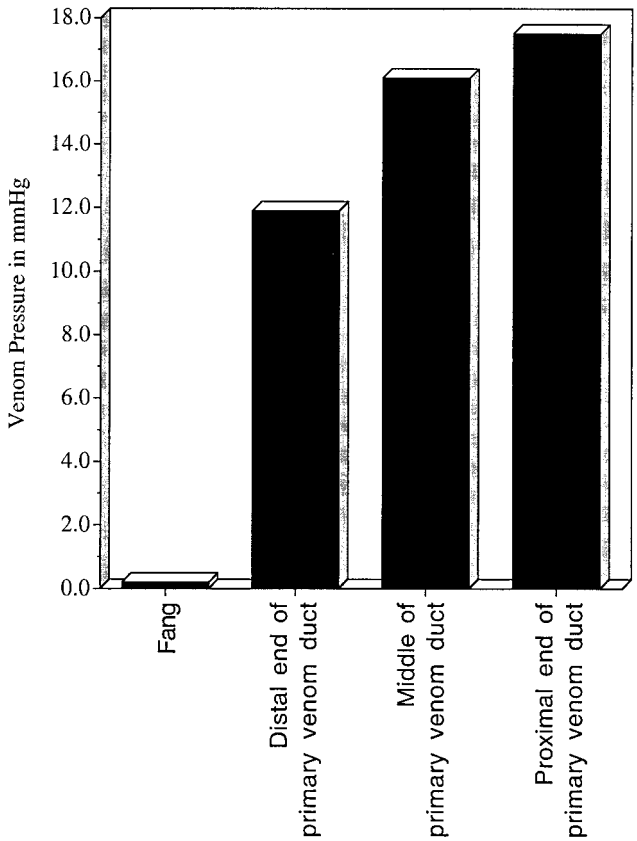

Fig. 5. Differential venom pressure recordings taken from the same side of a single specimen. Values given are the average of five recordings (in each case the SE was less than 0.5 mm Hg). Note the slight decrease in venom pressure along the length of the proximal portion of the venom delivery system, and the marked decrease in pressure recordings obtained from the fang.

showed a period of retrograde venom flow immediately after the initial anterograde pulse (Fig. 6B).

Radiopaque Injection

The radiopaque iodine solution was injected into the primary venom duct with enough force to produce distention of the distal portion of the venom delivery system. Despite this pressure, no iodine was observed in the fang sheath or the exit orifice of the fang. The radiograph taken while the venom duct was still pressurized clearly shows that the radiopaque marker entered the secondary venom duct, but did not extend into the venom chamber (Fig. 7).

Fang Cannula and Venom Flow

Insertion of PE tubing into the fang sheath, around the shaft of the fang, produced consistent results. Once the PE tubing approached the base of the fang there was a sudden release of venom. This venom flowed out of the fang sheath, around the outer surface of the PE tubing. It appeared that little, if any, venom was actually released through the venom canal of the fang.

Fang Erection and Venom Flow

The simultaneous recordings of venom pressure and fang position (via high speed digital video), taken during continued stimulation of the compressor glandulae and manual fang erection, revealed a fairly consistent pattern. The rise in venom pressure was always abrupt and began at approximately 60° of fang rotation (Fig. 8). The venom pressure remained elevated through subsequent fang rotation beyond 60°, but was decreased sharply by fang retraction (data not shown).

Sequential Excision of Supporting Elements

Most of the steps in the sequential excision of connective tissue and other supportive structures contacting the venom delivery system, as well as manipulation of the venom delivery system itself, had no impact on venom flow through the fang or fang sheath. Even when the distal end of the secondary venom duct was isolated from the maxilla, contraction of the compressor glandulae did not result in venom flow. Only when the connective tissue of the venom chamber was manipulated did venom freely flow from the fang during contraction of the compressor glandulae.

Internal Displacement of the Inner Fang Membrane

Insertion of the stainless steel probe into the venom canal of the retracted fang, concurrent with continued stimulation of the compressor glandulae, resulted in two stages of venom flow. The initial stage was a low pressure discharge of a small quantity of venom. Later dissection revealed that this stage of discharge occurred when the probe either penetrated or displaced the inner fang membrane (Fig. 9). Through additional manipulation it was possible to work the probe retrograde through the secondary venom duct. As the probe passed a short distance into the secondary venom duct the second stage of venom release occurred. In contrast to the first stage, the second stage of venom flow was marked by a significant volume of venom that appeared to be moving under pressure, rather than simply seeping. In both stages of venom flow, the venom appeared to move exclusively through the venom canal of the fang, rather than through the fang sheath.

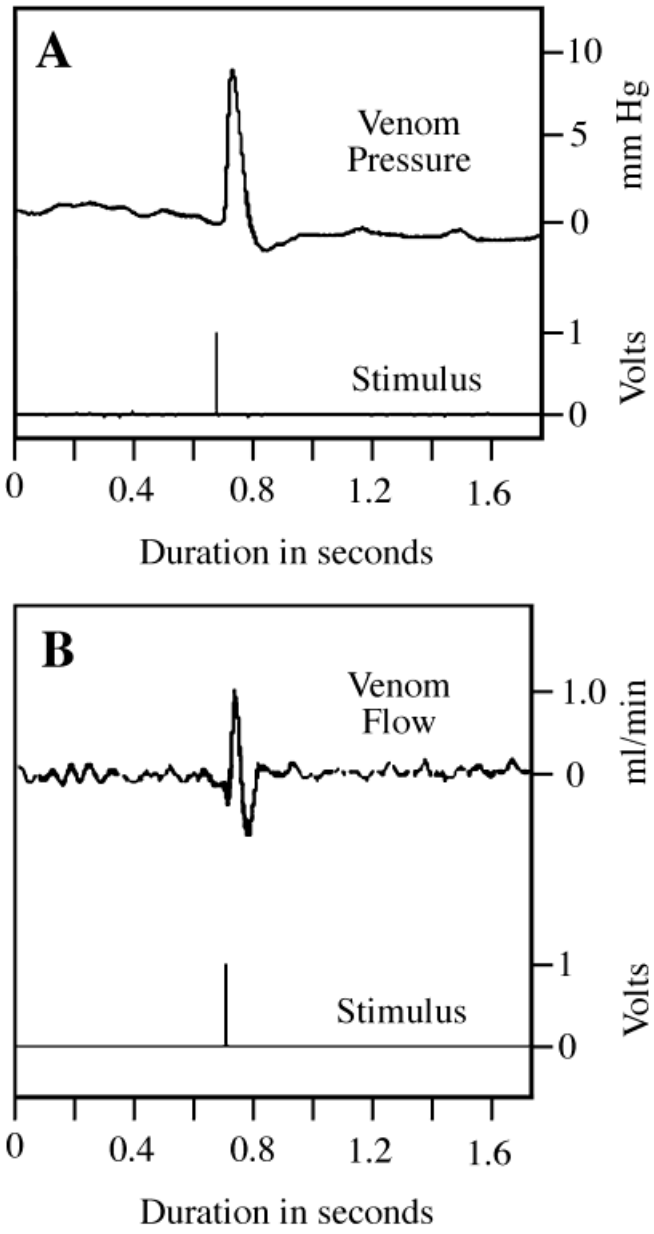

Fig. 6. Analysis of venom pressure and flow in the primary venom duct. **A:** Venom pressure recording (top trace) obtained during surface stimulation of the compressor glandulae (bottom trace). Note the marked increase in pressure compared to Figure 4, the overall lack of pulsatility, and the presence of a small pulse of negative pressure. **B:** Venom flow data (top trace) recorded during stimulation of the compressor glandulae (bottom trace). Note the presence of a distinct period of retrograde flow in this system.

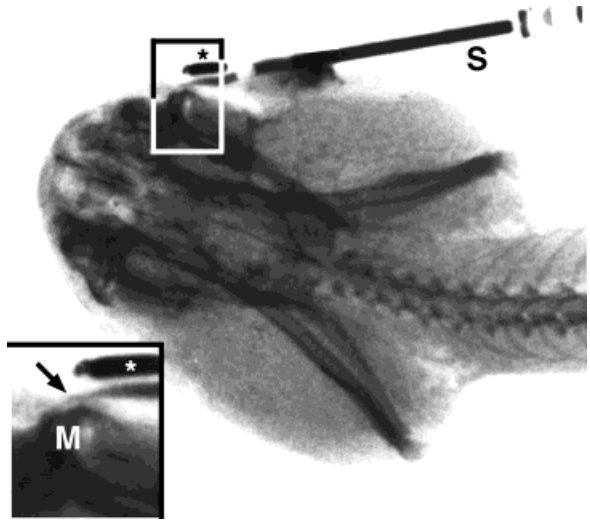

Fig. 7. Radiograph of the distal venom delivery system after the introduction of a radiopaque marker into the primary venom duct. A stainless steel marker (asterisk) indicates the location of the accessory venom gland. Note that the radiopaque solution entered the secondary venom duct then stopped along the surface of the maxilla without expanding in the venom chamber (inset). M, maxilla; S, syringe.

DISCUSSION

The combination of experimental results suggest that the regulation of venom flow in the distal portion of the venom delivery system is controlled by four factors: a restriction in the secondary venom duct along the anterior surface of the maxilla, the volume of the venom chamber, the position of the inner fang membrane, and suction produced in the venom gland. The first three of these factors are related to the erectile mechanics of the rattlesnake fang, which produces rotation of the fang in both the parasagittal and transverse planes (Mitchell, 1861; Zamudio et al., 2000). This rotation of the maxilla causes differential tension, and displacement, of the surrounding connective tissue, particularly on the anterior and posterior surfaces of the fang.

An anatomical restriction in the secondary venom duct was evident during dissections (Fig. 2A), non-pressurized India ink marker (Fig. 3) and the pressurized injection of radiopaque marker (Fig. 7). An anatomical restriction to venom flow at this site would also explain the difficulty in manually expelling venom from snakes while the fangs were retracted, the venom pressure differential recorded between the fang tip and primary venom duct (Fig. 5), the rapid rise in venom pressure as the fang sheath distorts during fang erection (Fig. 8), and the marked release in venom when a probe is inserted into the distal end of the secondary venom duct. Previous workers (e.g., Mitchell, 1861) had speculated that this portion of the venom delivery system could function to impede the flow of venom, but no previous evidence has been offered to support this claim.

Several lines of evidence support the presence of a functional venom chamber immediately around the proximal end of the fang and bordered by the fang sheath. This chamber was demarcated by the India ink (Fig. 3), and manipulation of the chamber produced venom pressure at the fang tip during the sequential excision and manipulation experiments. Disruption of the venom chamber (as occurred during fang cannulization) resulted in venom release through the fang sheath. The structural features of the fang sheath suggest that the venom chamber is a purely passive structure that serves as a conduit between the distal end of the secondary venom duct and the entrance orifice of the fang. Although the dimensions of the venom chamber are likely to be reduced during fang erec-

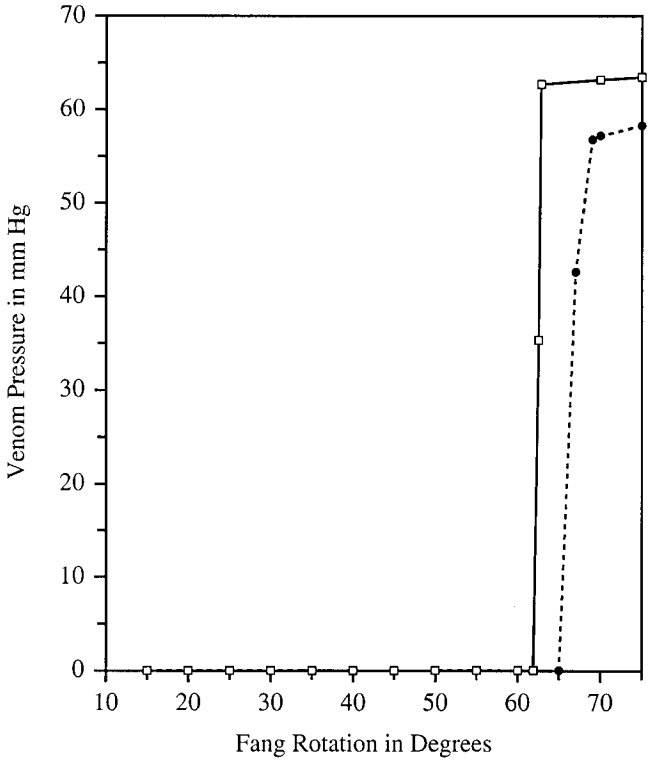

Fig. 8. Venom pressure (recorded from the distal end of the fang) during manual erection of the fang with continual stimulation of the compressor glandulae. Two traces shown are from opposite sides of the same snake. Note the abrupt rise in venom pressure when the fang reaches approximately 60° rotation. Fang rotation was measured between the ventral surface of the supralabial scales and the leading edge of the fang sheath.

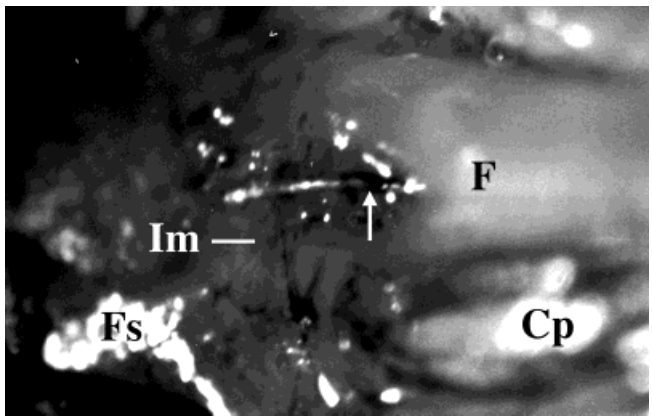

Fig. 9. Ventral view of the left venom delivery system after removal of the fang sheath (cranial is to the left) (×10). By threading a stainless steel probe (arrow) through the venom canal, the inner fang membrane could be displaced resulting in venom flow. Abbreviations: CP, central partition; F, fang; Fs, fang sheath; Im, inner fang membrane.

tion, and even more so during fang penetration, there is no structural indication that *Crotalus* could pump venom from the venom chamber.

When the fang is in the retracted position the inner fang membrane covers, and penetrates into, the entrance orifice (Fig. 2C–E). In this position the inner fang membrane appears to function as a physical block against fluid entering the venom canal of the fang. The role of the inner fang membrane as a venom plug is supported by the differential pressure recorded at the retracted fang tip and the proximal venom duct (Fig. 5), the absence of venom flow when the secondary venom duct is isolated from the maxilla (indicating that restricted flow in this region is not the sole inhibitor of venom flow), and the release of venom from the venom chamber when a probe is passed through the inner fang sheath of a retracted fang (Fig. 9). Deflections of the inner fang membrane, produced by a pressure increase in the venom chamber, could account for the consistent shape of the pressure tracings recorded from the fang tip (Fig. 4).

All of the venom pressure and flow tracings recorded included a negative, or retrograde, component (Figs. 4,6). The presence of a retrograde component in the flow probe data recorded when the fang was free of PE tubing (Fig. 6B) indicates that these values are not artifactual. Pressure tracings from the primary venom duct (Fig. 6A) or venom gland (data not shown), include a negative pressure component, indicating that the venom gland is the source of this pressure. Rosenberg (1967) detailed the intraglandular pressure hypothesis that argues that external muscle forces increase the fluid pressure within the venom gland and cause venom to flow out the venom duct; this link between muscle contractile force and venom flow was recently demonstrated experimentally (Young et al., 2000). The negative pressure and flow obtained in this study appear to arise from expansion of the venom gland after contraction of the compressor glandulae or pterygoideus glandulae. Similar negative pressures and pulsatility are evident in the pressure and flow recordings presented by Young et al. (2000).

Retrograde venom movement is a natural consequence of the morphology and emptying mechanism of the venom gland. This retrograde flow may function to pull venom out of the venom canal of the fang, thereby preventing crystallization of the venom and possible occlusion of the venom canal. The positive venom pressure is greater than the negative, yielding a net expulsion of venom into the prey harasser. In cases where the duration of fang penetration exceeds the duration of activity in the extrinsic venom gland musculature, the negative pressure could withdraw venom other fluids from the other organism. This would be particularly likely if the local site of injection has a high resistance to venom impregnation (Kardong and Young, 1991).

Taken collectively, the results presented herein suggest a relatively simple mechanism for the regulation of venom flow. In the retracted state the distal segment of the secondary venom duct is compressed, the venom chamber (that may hold residual venom from the last expulsion) is at its greatest linear dimensions and volume, and the inner fang membrane is occluding the entrance orifice of the fang (Fig. 10A). As the palatomaxillary arch is protracted and the fang erected (see Boltt and Ewer, 1964), the connective tissue around the maxilla is subject to differential tension and displacement: this tension and displacement is magnified upon fang penetration. The displacement of the maxilla, and the forces acting on the fang sheath during fang penetration, have three effects: 1) a reduction in the compression on the secondary venom duct (caused by displacement of the maxilla relative to the

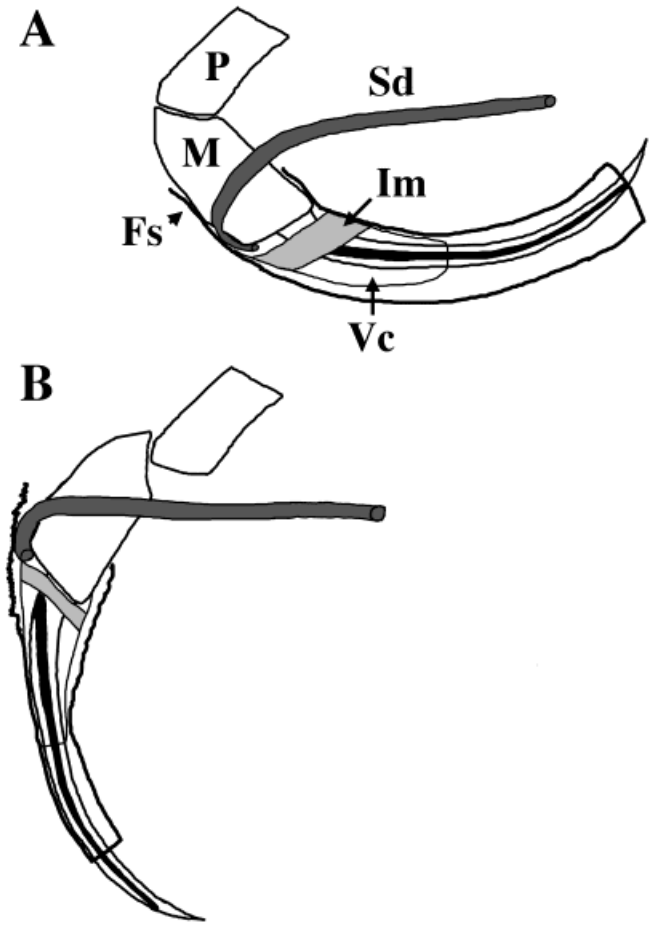

Fig. 10. Summary model for the function of the distal venom delivery system in *Crotalus*. **A:** With the fang in the retracted position the distal end of the secondary venom duct (Sd) is compressed, the venom chamber (Vc) is relatively large, and the inner fang membrane (Im) courses over the entrance orifice of the fang. **B:** As the Maxilla (M) rotates about the Prefrontal (P) causing fang erection, and the fang penetrates the target, the fang sheath (Fs) is thrown into pleats that reduces the compression on the secondary venom duct and the dimensions of the venom chamber, and the inner fang membrane is physically displaced from the entrance orifice thus allowing venom to enter the fang.

remainder of the secondary venom duct); 2) a reduction in the size of the venom chamber, particularly in the long axis, (caused by compression of the fang sheath); and 3) a dorsal displacement of the inner fang membrane such that the entrance orifice of the fang is no longer occluded (Fig. 10B).

Few previous workers have examined the fang sheath in terms of a possible role in the regulation of venom flow. Mitchell (1861) proposed that compression of the distal venom duct by the overlying fang sheath could serve to prevent venom flow. The potential for the fang sheath to occlude the entrance orifice of the fang, as proposed herein in the form of the inner fang membrane, has not been previously described. Halstead et al. (1978) include a micrograph of the fang sheath of *Lapemis hardwicki* including what appears to be the inner fang membrane. Though the language is ambiguous, Johnston (quoted in Mitchell, 1861) appears to describe the inner fang membrane when detailing the relationship of the fang to the "gums."

Numerous models have been proposed for the mechanics of venom flow between the distal end of the secondary venom duct and the entrance orifice of the fang. Earlier works claimed that the venom duct anchored to the entrance orifice (Aerskin, 1702), or was directed by muscle fibers within the fang sheath (West, 1895); neither of these claims have been supported by later anatomical investigations. One of the oldest, and most frequently cited, hypotheses argues that during fang erection the distal end of the venom duct is displaced by the activity of the m. pterygoideus such that it forms a direct physical seal with the entrance orifice of the fang (e.g., Dullemeijer, 1956; Kardong and Lavin-Murcio, 1993; Mitchell, 1861). There are several challenges to this hypothesis, including the following: 1) several workers have relied on a presumed direct connection between the m. pterygoideus and the fang sheath or secondary venom duct to achieve this seal, but no such connection was observed in *Crotalus*; 2) the venom duct extends distally to a level equal to the entrance orifice (Fig. 2E) that minimizes the potential for displacement during fang rotation; 3) the venom duct, like the fang itself, is securely attached to the maxilla and capable of little independent movement; 4) the distal end of the venom duct has an ovoid shape in cross-section, whereas the entrance orifice is larger and has more of a V-shape (Fig. 2E); these spatial differences make it difficult to envision a pressure-tight seal forming between the two; 5) numerous species, including both solenoglyphs and proteroglyphs, have been described as having two transiently functional fangs on one maxilla (e.g., Bogert, 1943; Edmund, 1969; Klauber, 1956), meaning that the single venom duct would have to form physical seals with two spatially separate entrance orifices; 6) during the regular periodic fang replacement, the new fang "migrates" to its spot on the lateral surface of the maxilla; if the venom duct is attached to the entrance orifice indirectly by the m. pterygoideus or directly by compression of the fang sheath during the strike, it is difficult to see how this system could account for changing position of the fang during fang replacement; and last, 7) during manual erection of the fangs, with no stimulation of the pterygoideus or tactile stimulation of the fang sheath, we recorded pressurized flow of venom through the fang with no evidence of flow through the fang sheath (Fig. 8). To date, no experimental evidence has been put forth to support a direct apposition between the distal end of the venom duct and the entrance orifice of the fang.

In addition to the anatomical evidence (Fig. 2), the presence and function of a venom chamber is supported by several lines of evidence including the demarcation of the venom chamber with India ink (Fig. 3), the cannulization of the fang, and the internal displacement of the inner fang membrane. Previous workers have speculated about the possibility of venom pooling (e.g., Kochva, 1958; Schaefer, 1976; Takacs, 1986) but provided no experimental evidence for the presence of a venom chamber. Work is currently underway to determine the prevalence of the mechanism described in this study, where the expelled venom enters a chamber defined by the fang sheath before passing through the entrance orifice, among solenoglyphs and proteroglyphs.

This project was undertaken in an effort to clarify the role of the distal portion of the venom delivery system in regulating venom expulsion. The results show that such a regulation does take place, and that it may be purely

mechanical in nature. Although our findings do not preclude additional physiological controls, the four factors identified herein as potentially influencing venom flow; compression of the distal end of the secondary venom duct, the size of the venom chamber, the position of the inner fang membrane, and suction produced by the venom gland, appear adequate to explain several previous observations regarding venomous snakes. For example, compression of the secondary venom duct and the presence of an inner fang membrane could prevent venom discharge during locomotion in snakes with elongate venom glands. Similarly, because the compression of the secondary venom duct and the occluding inner fang membrane are only relieved during fang erection, these structures explain not only why manual expulsion of venom from a snake with retracted fangs is so difficult, but why the venom often flows from the fang sheath (the seal of the venom chamber failing before that of the inner fang sheath). The results of the current study indicate that venom flow through the distal portion of the venom delivery system is influenced by the degree of fang erection, at least until a threshold of approximately 60° degrees is reached (Fig. 8).

A complete understanding of the mechanics of venom expulsion will require integrating data on the contractile activity of the compressor glandulae with quantitative venom flow profiles. In this way the relationships between muscle contraction, venom pressure, and venom expulsion can be explored. Feedback loops, in which elevated venom pressure due to peripheral resistance from the target tissue, or venom pooling in the venom chamber, influences the contractile activity of the compressor glandulae, could play a significant role in determining the duration and quantity of venom flow.

The results of the current study, when combined with the earlier findings of compartmentalization and the potential for fine control of venom expulsion in the compressor glandulae (Young et al., 2000), indicate that the venom delivery system in rattlesnakes is more complex, and has more potential for regulation, than previously suggested. What remains to be explored is whether this complexity and regulation is significant to the behavioral ecology of rattlesnakes, as has been suggested from studies of venom metering (e.g., Hayes et al., 1995), and whether the venom delivery system in other lineages of snakes includes similar functional specializations.

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