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B ea e a d age: T e e g a d f f -fa ged a e ☆

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Ab ac

A paradoxical task of the venom gland of snakes is the synthesis and storage of an instantly available suite of toxins

agencies. The taxonomy of the Western Rattlesnake (*i idi /o egan*) complex follows that of Ashton and de Queiroz (2001); *C. a o* is monotypic. All snakes were housed in the UNC Animal Facility in accordance with UNC-IACUC protocol no. 9204.1 and ASIH/SSAR guidelines. Venoms were manually extracted from captive animals using standard methods (Mackessy 1988), and lyophilized venom was stored frozen and desiccated until used. Venoms from a subset of *C. a o* specimens from southcentral Texas were collected during surgery to remove the venom glands. Venom was collected at the fang tip on one side of the snake; this venom passes through the entire venom apparatus (designated as ''whole gland'' venom) and is identical to venom collected from snakes as described above. On the contralateral side, venom was collected from the main gland only (designated as ''main only'' venom) after isolation via occlusion of the primary duct, thereby bypassing the primary duct, the accessory gland, the secondary duct and the fang. Both of these venom preparations from Texas *C. a o* were supplied by Bruce Young. The pH of freshly expressed whole venom was measured using a microelectrode and a Beckman pH meter.

2.2. P e a a f e g a d

Venom glands were removed from snakes that had been lightly anesthetized with Halothane or Fluothane and subsequently sacrificed by decapitation. One group of snakes had not been extracted of venom for at least two months and provided ''unextracted venom glands''; these venom glands are not actively synthesizing venom proteins and their epithelial cells are cuboidal. The other group of snakes were extracted of venom as above, sacrificed 4 days after venom extraction, and produced ''extracted venom glands''. These glands had been stimulated to synthesize venom proteins by venom extraction, and their epithelial cells are columnar. The venom glands and tissues were prepared for light microscopy or electron microscopy as previously described (Mackessy 1991). Briefly, samples for electron microscopy were cut into 1 mm blocks while immersed in 100 mM sodium cacodylate buffer (pH 7.2) containing 5% (v/v) glutaraldehyde. Fixation proceeded for three hours, followed by three 10 min washes with sodium cacodylate buffer (100 mM, pH 7.2) containing 10% sucrose. Samples were post-fixed in 1% osmium tetroxide in 100 mM sodium cacodylate buffer (pH 7.2). Specimens for transmission electron microscopy were dehydrated in an ethanol series followed by propylene oxide and then embedded in Epon 812 resin. Thin sections prepared with a diamond knife on a Reichert OM-U2 microtome, lifted onto Formvar-coated grids, stained with uranyl acetate and lead citrate and viewed with a Hitachi 300 (75 kV) transmission electron microscope.

Tissues for histochemistry and light microscopy were preserved in 10% formalin buffered with 100 mM HEPES (pH 7.4), embedded in paraffin and sectioned to 5 m. Because initial observations suggested structural similarities between mitochondria-rich cells of the venom gland and parietal cells of the stomach, rattlesnake and mouse stomach tissues were also prepared for comparative purposes and as a positive control for histochemical staining. Slides were deparaffinized and hydrated (xylene followed by an ethanol series) using standard protocol (e.g., Humason 1972). Aqueous periodic acid-Schiff's reagent (PAS) staining was immediately followed by hematoxylin and then Orange G counterstain (Wheater et al. 1979); this method results in preferential light orange staining of acid-secreting cells such as parietal cells. Tissue samples were also stained with nitro-BT (2,2′-di- -nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene; Nachlas et al. 1957), a method that hasitive

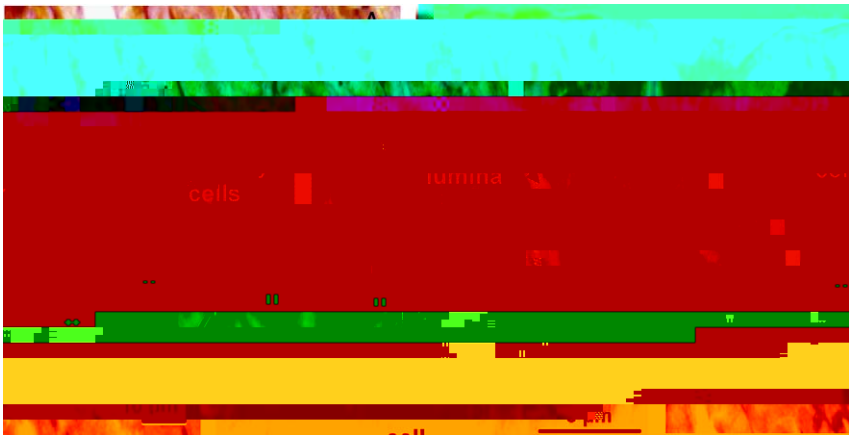
2.4. E e H a a

Purified enzymes from whole venoms of several species were assayed for optimal activity pH. These included thrombin-like and kallikrein-like serine proteases (

protein profile between the two sample types (Fig. 6). Preliminary reversed phase high-pressure liquid chromatography (RP-HPLC) analysis also indicates that

also stain differentially (

venom gland in rattlesnakes is quite similar to the condition in the stomach (i.e., gastric gland) of both the mouse and the rattlesnake, in which the mucus-secreting cells are abundant near the opening of the gastric glands and in the mucosa of the stomach, providing protection against HCl and digestive enzymes (Csendes et al. 1992). The morphology of the accessory gland in rattlesnakes also suggests that substances are contributed to the venom bolus as it passes through the proximal region of this gland, perhaps activating venom components.



F g. 10. Light micrographs of histochemical demonstration of acid-secreting cells in *C. i idi i idi* stomach tissues (A) and *C. i idi i idi* main venom gland (B). Tissues were stained with Orange G, and acid-secreting cells stain light orange. (A) Cross-section of rattlesnake stomach through numerous gastric glands showing mixed function chief/parietal cells lining the lumina. (B) Sagittal section of main venom gland epithelium showing differential staining of a mitochondria-rich cell; the recessed apex is apparent.

The cell morphology of the main gland also shows similarities to that of the mammalian stomach, particularly with respect to the mitochondria-rich cells of the venom gland and the parietal cells of the mammalian gastric glands. Because of these similarities, we hypothesized by analogy that the mitochondria-rich cells of venom glands might participate in the acidification of contents of the venom gland lumen, like the parietal cells for the stomach contents. Histochemical staining of the main venom gland confirmed that the mitochondria-rich cells share acidic properties with the parietal cells of the stomach, and they stain very differently from the much more numerous serous secretory cells of the rest of the gland epithelium. This evidence strongly suggests that the mitochondria-rich cells secrete acidic materials into the glandular lumen, and this proposal is supported by our measurements of pH of freshly expressed venom (5.4). Additionally, all purified venom enzymes assayed have pH optima of approximately 7.5–9.5 and are inactive (or nearly so) at the pH of stored venom. As noted previously, mitochondria-rich cells comprise only about 2% of the secretory epithelium cell population (Mackessy 1991); this lower density of acid-producing cells relative to that of the mammalian stomach is also reflected by the higher pH of the luminal contents of the venom gland as well as by mammalian stomach acid concentrations that are 3–4 orders of magnitude *highe* than those of the venom gland (e.g., Guyton and Hall 2000).

Therefore, we propose that the role of the mitochondria-rich cells in the venom glands is to acidify the venom gland luminal contents, and that the morphological and functional similarities between the parietal cell of the mammalian stomach and the mitochondria-rich cell of the snake venom gland illustrate yet another link between venom production and food digestion. The acidification of venom likely occurs in concert with the release of newly synthesized and exocytosed venom, because mitochondria-rich cells cycle in height with secretory epithelial cells and the canaliculae of mitochondria-rich cells are apparent only during venom protein synthesis, which follows extraction of venom. After synthesis in the rough endoplasmic reticulum of the secretory cells, venom proteins are contained within large intracellular granules which appear to rupture into the main lumen upon release from the cell (Mackessy 1991); rate of acid secretion by mitochondria-rich cells is thus predicted to peak at this time. Mitochondria-rich cells may also have a limited role in water resorption (e.g., Warshawsky et al. 1973), but we believe that the primary role is to acidify stored venom and inhibit venom enzymes. It remains unclear whether free acid (such as HCl) is released from mitochondria-rich cells, and the mechanism of transport to the lumen is not known, but acidification may involve release of citrate via specific transporters. Citrate ($pKa_3 = 5.19$), which could buffer secreted venom at the acidic pH observed (pH 5.4), is an abundant component of many snake venoms (Francis et al. 1992; Odell et al. 1998) and can inhibit many enzymes via metal ion chelation (Francis and Kaiser 1993). However, at the basic pH levels of optimum enzyme activity, citrate, even at very high concentrations, does not show significant inhibition of the major metalloprotease (Cvo Protease V) of *C. ◐. ◐ egan* venom (Mackessy 1996). Storage of venom enzymes at an acidic pH is necessary for inhibition of enzyme activities, and upon injection into prey tissues,

spontaneous activation occurs due to the pH of recipient tissues (~7.2–7.4).

Acidification of stored venom by the mitochondria-rich cells is a primary mechanism that allows storage of potentially dangerous and unstable venom components in an inactive state that is readily and instantaneously reversed upon injection, permitting long-term storage and on-demand deployment of a potent biological weapon. However, inhibition by low pH alone is apparently insufficient to protect venom components from autolysis, and additional protective mechanisms exist. Several tripeptide inhibitors of venom metalloproteases are present in many rattlesnake venoms (Munekiyo and Mackessy 2005), and during storage in the venom gland these peptides stabilize and inhibit the abundant venom proteins that might otherwise catalyze autolytic degradation of venom components. Organic acid inhibitors, such as citrate, also appear to have a role in stabilizing venom (Fenton et al. 1995; Francis et al. 1992; Odell et al. 1998), and zymogen activation of at least one venom metalloprotease is required for activity (Grams et al. 1993). These additional redundant mechanisms allow safe venom storage, and upon injection, the venom bolus encounters an environment where pH favors high enzyme activity, peptide inhibitors

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Ac edg e

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