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HEMOLYSIS BY **CROTALUS HORRIDUS ATRICAUDATUS** VENOM

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Canebrake rattlesnakes *Crotalus horridus articaudatus* (Figure 1) are common to Arkansas. These snakes are infrequently found in timber uplands or wooded hills adjoining streams but usually they are found in swamp or bottom lands (Wright and Wright, 1957). Canebrakes are large, heavy-bodied, poisonous snakes which may reach six feet in length. On their posterior body there is a median series of blotches expanding transversely to connect with a ventrolateral series, forming chevron-shaped bands. On many specimens there is a rich red-brown mid-dorsal stripe about four scales wide (Anderson, 1965).

Minton (1967) and Johnson *et al.* (1968) have described several of the characteristics of *C. h. artricaudatus* venom. In this work hemolytic activity of *C. h. articaudatus* venom is studied. Hemolysis tests with and without lecithin as well as hemolysis tests using isotonic and hypotonic solutions were conducted. Hypotonic lysis tests (Seeman, 1966) are used in attempting to determine the effects of heat labile venom components on hemolysis of human erythrocytes (RBC).

MATERIAL AND METHODS

Specimens of *C. h. articaudatus* were obtained from Max Allen's Zoological Garden, Eldon, Missouri. After extraction, the venom was centrifuged to remove insoluble residues and cellular debris and then lyophilized. L-a-lecithin, Type 11-E from Sigma Chemical Company was used.

Phospholipase A activity was assayed by following the procedures of Brown and Bowles (1966).

Whole venom (50 mg samples) was boiled for 10 min before the supernatants were removed by centrifugation. Disk electrophoresis (Davis, 1964) was used to show the number of heat stabile components in the boiled venom supernatants.

Hemolysis tests using washed RBC were performed in isotonic solutions before tests in hypotonic solutions were conducted. The cells were washed in isotonic saline, 154 mM NaCl in 10 mM sodium phosphate buffer, pH 7. Three ml of isotonic saline containing venom (2.0 mg/ml) and 0.2 ml of RBC (1×10^8 RBC/ml) were incubated for five min at room temperature to determine the presence of direct hemolysis. Indirect hemolysis was indicated by incubating 0.2 ml of RBC in three ml of isotonic saline containing venom (2.0 mg/ml)

plus crude lecithin (1 x 10^{-3} M).

Hypotonic solutions ranging from 22 to 151 mM NaCl in 10mM sodium phosphate buffer, pH 7, were tested to determine the concentration causing 50 percent hemolysis. In these tests a 0.2 ml RBC suspension (1 x 10^8 RBC/ml) was added to a three ml hypotonic solution, mixed, and incubated for five min at room temperature. After incubation, the suspension was centrifuged one min before removing the supernatant. Hemoglobin (Hb) content was used to indicate percent hemolysis. This was measured by recording the optical density at 543 mu ($O.D._{543}$) in a Beckman DB spectrophotometer. The blank was always prepared by replacing the RBC with buffer. All tests were run in triplicate.

Stabilization effects of lecithin on RBC membranes were induced by adding 0.1 ml lecithin solutions to the hypotonic solution causing 50 percent hemolysis. The blank consisted of buffer and lecithin. In other lysis tests, *C. h. atricaudatus* venom was reconstituted in the hypotonic solution (66 mM NaCl), 10 mM sodium phosphate buffer, pH 7) causing 50 percent hemolysis.

Relative hemolysis was obtained by dividing the amount Hb released (during 5 min hypotonic hemolysis) in the presence of venom by the amount Hb released in the absence of venom. A relative hemolysis unit of less than one indicated a nonspecific lysis behavior whereas a relative hemolysis unit of more than one was indicative of specific lysis.

RESULTS AND DISCUSSION

Repeated phospholipase A determinations showed no, or quite low, phospholipase A activity in *C. h. atricaudatus* venom. Similar determinations with other crotalid venoms showed high phospholipase A activities. Phospholipase A determinations for all venoms were conducted simultaneously.

Disk electrophoresis of the boiled venom supernatants showed the presence of one wide disk and one or two small disks. Perhaps one of these protein components was phospholipase A.

RBC inclubated for five min in isotonic saline containing venom (2.0 mg/ml) were not hemolyzed. When RBC were inclubated for five min in isotonic saline containing venom (2.0 mg/ml) and lecithin (1 x 10^{-3} M) hemolysis occurred. Approximately one eighth of the RBC were lyzed.

Fifty percent hypotonic hemolysis occurred at 66 mM NaCl in 10mM sodium phosphate buffer, pH 7. Use of varying concentrations

fo lecithin showed that the greatest RBC membrane stabilization against hypotonic lysing occurred at 10^{-3} M final concentration. These stabilizations resulted in a biphasic curve as evidenced by relative hemolysis values of less than one. In this concentration of lecithin (1×10^{-3} M), stabilization persisted even in the presence of *C. h. atricaudatus* venom (Figure 2).

The hypotnic lysis tests indicated that heat labile components in *C. h. atricaudatus* venom have no effect on nonspecific lytic behavior. This non-specific lytic behavior seemingly was the result of low quantities of heat stabile phospholipase A causing the hydrolysis of lecithin to form lysolecithin and fatty acid. Lysolecithin is capable of inducing hemolysis and is also a compound with the capacity to induce RBC membrane stabilization (Seeman, 1966). Crotalid venoms such as *Agkistrodon contortrix mokeson. A. piscivorus leucostoma,* and *C. scutulatus* however, induce a specific lytic behavior in the presence of lecithin. These three venoms also have high phospholipase A activities. Hemolysins with a specific affinity for a membrane component will not cause stabilization whereas non-specific hemolysins will. Thus, hypotonic lysis by these venoms seems to involve other venom components as well as phospholipase A (Byrd and Johnson, unpublished data). From these data, crotalid venoms seem to have varying modes of hemolysis.

SUMMARY

Crotalus horridus atricaudatus venom demonstrated negligible phospholipase A activity. Indirect hemolysis was indicated but no direct hemolysis was observed. Hypotonic lysis tests indicated only non-specific hemolysins in this venom.

ACKNOWLEDGEMENTS

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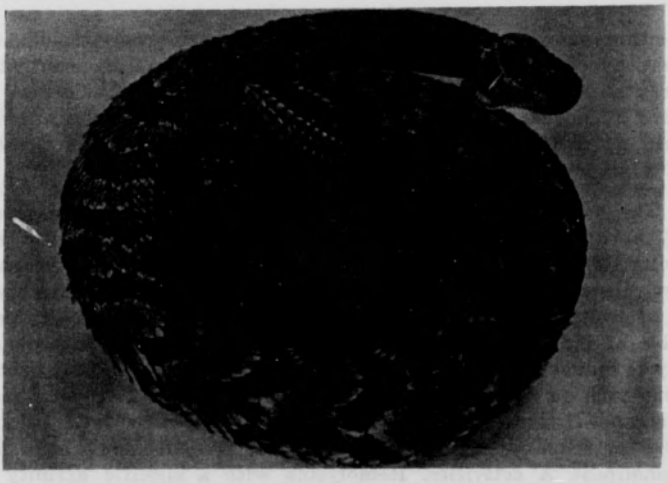

Figure 1. A speciman of a canebrake rattlesnake *Crotalus horridus atricaudatus.*

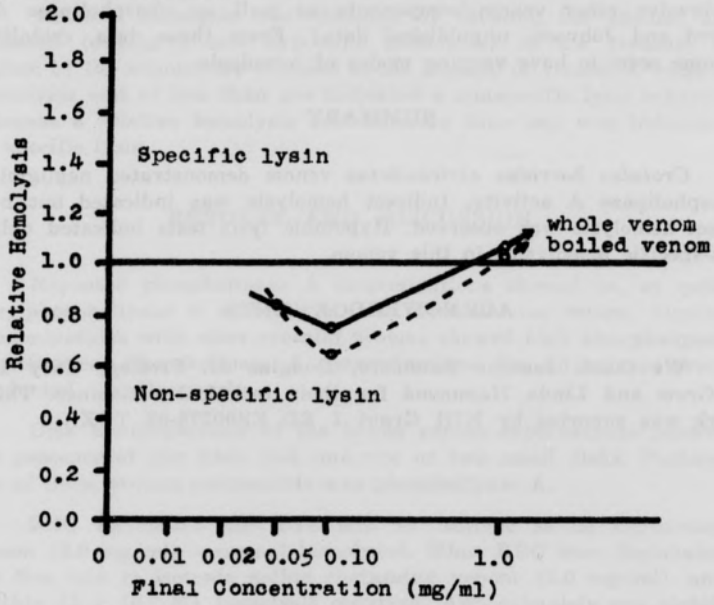

Figure 2. A comparison of effects of *Crotalus horridus atricaudatus* whole venom and *Crotalus horridus atricaudatus* boiled venom supernatant on RBC membrane stabilization in the presence of lecithtin. A relative hemolysis of 1.0 represents a hemolysis of about 50 percent.

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