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Received November 26, 1962. P.S.E.B.M., 1963, v112.

Detoxification of Snake Venoms and Venom Fractions by Formaldehyde.* (28113)

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Previous reports from this institution have described the separation of Near Eastern snake venoms into fractions with various toxic, biochemical and antigenic properties (1,2,3). The venom of *Vipera palestinae* was shown to be composed of 2 main toxic fractions, a neurotoxic and a hemorrhagic, whereas *Echis colorata* venom which contains two different hemorrhagins, was found to be devoid of neurotoxic activity. Recent investigation of the venom of two other snakes, *Walterinnesia aegyptia* and *Pseudocerastes fieldii*, showed them to be mainly neurotoxic (4).

Production of potent antivenins is an elaborate and time-consuming procedure and often the antisera obtained are not satisfactory as in the case of *Vipera palestinae* in which antivenin was devoid of antineurotoxic activity (5). Detoxification of snake venoms by formaldehyde treatment has been attempted in the past(6,7). In the search for more effective methods of preparation of antisera against the venoms of Near Eastern snakes we have investigated the effect of formaldehyde treatment on their toxicity and immunogenic activity.

Materials and methods. Venoms and venom fractions. Venoms of *Vipera palestinae* and *Echis colorata* were obtained according to methods described previously(2,3), dried from the frozen state and stored at −20°C until use. Fractionation was carried out by chromatography on DEAE cellulose. Venom fractions consisted of pools of tubes at the peaks of toxic activity. LD_{50}'s of venoms and venom fractions were determined

* Aided by research grant from Nat. Inst. Health.

† Submitted in partial fulfillment by C. Moroz-Perlmutter for Ph.D. degree from Hebrew University.

by intraperitoneal injection of half log dilutions into 20 g white mice and calculated according to the method of Reed and Muench (8). *Formaldehyde treatment.* U.S.P. formaldehyde (40%) was diluted in distilled water and added to the venom or fraction to the desired concentration. Concentrations of formaldehyde referred to are final concentrations. Addition of formaldehyde resulted in an immediate decrease in the pH of the neutral venom solutions. Required changes in the pH were made by the addition of 0.2N NaOH. Incubation with formaldehyde was carried out at 37°C. *Antisera.* Antisera were prepared in rabbits by weekly injections of detoxified venom and venom fractions in amounts corresponding to 100-150 mouse LD_{50} each, with addition of complete Freund's adjuvant(9). Prior to injection, they were dialyzed against 20 volumes of phosphate buffered saline. Injections were made intramuscularly over a period of 6 weeks; 10 days after the last injection, the animals were bled from the heart. The neutralizing power of the antisera was determined by protection tests in mice as described previously(5).

Results. Action of formaldehyde on Vipera palestinae *venom.* 10 LD_{50} of whole *Vipera palestinae* venom were incubated with various concentrations of formaldehyde ranging from 1:500 to 1:4000. Samples drawn on days 2, 5, 9 and 12 were titrated, both undiluted and diluted 1:10, by intraperitoneal injection of 5 mice each. No significant diminution in toxicity was found even with the highest formaldehyde concentration and the longest period of incubation. However, these mice revealed signs of intoxication similar to those seen following injection of the separated neurotoxic fraction. In contrast to the controls, these mice showed no hemorrhages at autopsy. This observation led us to investigate the effect of formaldehyde on the 2 toxic components of *Vipera palestinae* venom, the neurotoxic and the hemorrhagic separately. The results obtained indicated clearly that the hemorrhagic fraction, even up to 100 LD_{50}, was detoxified by 1:500 formaldehyde already after 6 days of incubation, while the neurotoxin was unaffected. Lower formaldehyde concentrations, however, did not result in complete detoxification. Incubation of 100 LD_{50} with formaldehyde 1:1000 and 1:2000 caused a steep initial drop in toxicity but no further decrease in toxicity was obtained when treatment was prolonged up to 18 days.

It has been reported that the pH of formaldehyde-venom mixtures markedly affects the detoxification(6,7). Since the addition of 1:500 formaldehyde to *Vipera palestinae* venom, caused a decrease in pH to 5.5, detoxification at pH 7.4 and 9.2 respectively was investigated after incubation for 6 days. The hemorrhagic fraction was detoxified at a wide pH range, from 5.5 to 9.2. On the other hand, both the neurotoxic fraction and the whole venom underwent detoxification at alkaline pH only. The failure to detoxify whole venom is presumably due to presence of neurotoxin.

Action of formaldehyde on Echis colorata *venom.* Various formaldehyde concentrations were applied to whole venom as well as to each of the 2 main hemorrhagic fractions(3). At acidic pH no decrease in the toxicity of whole venom was obtained, even of minimal amounts, and at the highest formaldehyde concentration used (1:500), for as long as 14 days of treatment. Neither did alkalinization up to pH 9.0 lead to detoxification. However, the 2 hemorrhagic fractions were each separately found to be susceptible to the action of formaldehyde. The effect of formaldehyde on the hemorrhagic fraction, first eluted from the column(3), was difficult to interpret owing to its low mouse toxicity; when 3 LD_{50} were treated with 1:500 formaldehyde, complete detoxification was observed after 3 days. A more pronounced effect was observed with the second hemorrhagic fraction, 1:500 and 1:1000 formaldehyde causing complete detoxification of 10 LD_{50} in 6 days.

Immunogenicity of formaldehyde-treated Vipera palestinae *venom.* To find out whether formaldehyde detoxification of *Vipera palestinae* venom and its fractions influences their antigenicity, detoxified venom and separated fractions were used to immunize rabbits. For each, favorable conditions for detoxification were chosen, *i.e.,* the largest amount of LD_{50} and formaldehyde at a final

TABLE I. Neutralization of Hemorrhagin of *Vipera palestinae* Venom by Rabbit Antiserum Prepared Against Hemorrhagic Fraction Treated with Formaldehyde at pH 5.5.

No. LD_{50} used	Amt of antiserum used, ml			
	.1	.2	.5	—
1	—	—	—	2/5
2	0/5	0/5	0/5	5/5
5	4/5*	1/5*	0/5	5/5
10	5/5	5/5	2/5*	5/5

* Delayed deaths.

concentration of 1:1000 were employed; the pH of the solution was 5.5. Samples drawn and tested for toxicity at 3-day intervals indicated that complete detoxification was achieved in 12 days. The 12-day samples were used to immunize rabbits. Various amounts of pooled serum obtained from several rabbits, immunized with the detoxified fraction, were tested for their ability to neutralize varying amounts of the untreated hemorrhagic fraction. Table I shows details of such an experiment. 0.1 ml and 0.5 ml of antiserum neutralized completely 2 and 5 LD_{50} of the hemorrhagic fraction, respectively, whereas 10 LD_{50} were only partially neutralized by 0.5 ml antiserum. It is estimated that 1 ml of this antiserum will neutralize 20 to 25 LD_{50}.

Table II shows the antigenicity of *Vipera palestinae* venom and its neurotoxic fractions which, in order to detoxify the neurotoxin, were treated with formaldehyde at alkaline pH prior to immunization. The antigenicity of untreated whole venom and neurotoxin is added for comparison. Antiserum obtained following immunization with the neurotoxic fraction was tested for neutralization of neurotoxin only, whereas that obtained after immunization with whole venom was tested for its power to neutralize whole venom and each of its toxic fractions. Aliquots of 50 LD_{50} of whole venom and of neurotoxic fraction were treated with formaldehyde 1:500 for a period of 6 days at pH 7.4, conditions found favorable for complete detoxification. Two findings were outstanding. First, detoxified whole venom and detoxified neurotoxic fraction were devoid of immunogenic activity. Secondly, the antiserum obtained following immunization with the detoxified whole venom was also devoid of anti-hemorrhagic activity.

Discussion. The results presented may help clarify the complex problem inherent in the detoxification by formaldehyde of snake venoms possessing different toxic components. The hemorrhagin of *Vipera palestinae* is easily detoxified by formaldehyde at a wide pH range from 5.5 to 9.2. The immunogenicity of the detoxified hemorrhagin, however, depends on the pH at which the formaldehyde treatment is carried out, its antigenic power being preserved at acidic pH only. *Vipera palestinae* neurotoxin, on the other hand, has a different pH "optimum" of detoxification, losing its toxicity only at pH above 7.0. However, similarly to the untreated neurotoxic fraction, the detoxified neurotoxin even when large amounts of the fraction are used for immunization, is not antigenic. Treatment of whole venom with formaldehyde at a pH which detoxified both neurotoxic and hemorrhagic components, not only is ineffective in the production of anti-neurotoxin, but also abolishes the immunogenic activity of the hemorrhagic component.

The mechanism of formaldehyde inactivation of *Echis colorata* venom seems also to be complex. Its two hemorrhagic fractions un-

TABLE II. Immunogenicity of Detoxified *Vipera palestinae* and Neurotoxic Fraction.

Venom or fraction	Treatment prior to immunization			Neutralizing power of 1 ml antiserum	
	Formaldehyde concentration	pH	Days	Against	LD_{50} neutralized
Whole	1:500	7.4	6	Whole venom	6
				Hemorrhagic fraction	5
				Neurotoxic fraction	2
	Untreated			Hemorrhagic fraction	30
Neurotoxic	1:500	7.4	6	Neurotoxic fraction	2–6
	Untreated			" "	6

dergo partial detoxification, while the whole venom is not affected. This may point to the presence in this venom of an additional, as yet not defined, toxic component.

Resistance of *Vipera palestinae* neurotoxin to formaldehyde treatment at low pH and the lack of antigenicity of both untreated and detoxified fraction may represent a general characteristic of snake venom neurotoxin. Also *Walterinnesia aegyptia* and *Pseudocerastes fieldii* venoms, the toxicity of which is due to neurotoxin mainly(4), are equally insusceptible to the detoxifying action of formaldehyde(10). This, together with the stability of all these neurotoxins to heat and treatment with HCl(10) raises the question of their chemical nature. The above properties of the neurotoxins of these venoms would seem to indicate that they are non-protein in nature and of low molecular weight, similarly to neurotoxins of other snake venoms(11,12, 13,14) and of scorpion venoms(15).

Summary. The effect of formaldehyde on the toxicity of *Vipera palestinae* and *Echis colorata* and their chromatographic fractions was investigated. The detoxification was found to be pH dependent. *Vipera palestinae* hemorrhagin was detoxified over a wide pH range, from 5.5 to 9.2, but its antigenicity was preserved at acidic pH only. The neurotoxin of *Vipera palestinae* was detoxified by formaldehyde at alkaline pH only. The detoxified neurotoxin was devoid of immunogenic activity. Treatment of *Echis colorata* venom with formaldehyde over a wide pH range did not result in complete detoxification although its separated hemorrhagins were detoxified.

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Received November 30, 1962. P.S.E.B.M., 1963, v112.

Effect of Deuterium Oxide on Lipid Components of L-5178Y Cells.* (28114)

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The growth characteristics of various lines of cells in tissue culture medium containing deuterium oxide have been described(1,2). Our interest has centered on the observed increase in sudanophilic material in the cells grown in D_2O. Preliminary experiments(3) carried out with cells maintained in deuterium oxide medium for 3 to 5 days indicated that the major increase in cellular lipids was due to increased glyceride content. The cells used for these analyses were in the stationary phase of growth, a time when certain strains

* This work was supported in part by grants from Nat. Science Foundation and Nat. Inst. Health.