Activation of Venom Proteases and Reversal of Chelating Effects by Sodium Bicarbonate.* (24838)

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Proteolytic activity of rattlesnake venom on gelatin layer of photographic film is activated by mammalian serum and its ash(1). Attempts to achieve the same degree of activation by specific inorganic substance were previously unsuccessful, although salts containing phosphate ion brought about partial activation. In the present work the bicarbonate ion in concentration approximating that in mammalian serum had the same activating effect as whole serum on rattlesnake venom gelatinase. This ion also reverses the effect of chelating agents on both water moccasin and rattlesnake venom gelatinases and increases proteolytic activity of these venoms on urea denatured hemoglobin.

Materials and methods. Venoms of the water moccasin (*Agkistrodon piscivorus* and Eastern diamond back rattlesnake (*Crotalus adamanteus*) were purchased from Ross Allen's Reptile Inst. in dry powdered form. They were dissolved in 0.9% sodium chloride solution and kept in frozen state until immediately before use. Inorganic compounds and chelating agents were of analytical reagent grade. Solutions of sodium bicarbonate were prepared initially in 0.02N concentration and pH adjusted to 7.4 with hydrochloric acid. The photographic film was Eastman's Kodak verichrome panchromatic. Urea denatured hemoglobin was prepared using modification of method by Anson(2). After urea and sodium hydroxide had been added to the dialyzed beef erythrocytes, the mixture was allowed to stand for one hour at room temperature, then dialyzed 24 hours against running tap water and another 24 hours against several baths of distilled water. Dialysis removed all urea and brought the pH from 12.0 to 7.7. Some of the hemoglobin precipitated, but enough remained in solution to maintain a concentration of 0.8 g%. Enough sodium chloride was added to this solution to bring the concentration to 0.9% and hydrochloric acid was then added to bring the pH to 6.8. Sodium bicarbonate, disodium phosphate, and sodium tetraborate (borax) were then added to aliquots of this material as indicated in Table II (0.02N concentration) and pH of all solutions was adjusted to 7.4 ± 0.1 by addition of HCl. Sodium hydroxide was added to a fourth aliquot of hemoglobin (pH 6.8) to bring the pH to 7.4. The digestion of gelatin layer of photographic film was carried out by method previously described(1). Doubling dilutions of the venom were prepared in 0.1 ml quantity with and without various activators and inhibitors in 0.1 ml quantity in concentrations shown in Table I. Two-hundredths (0.02) ml of each solution was placed on ¼ inch strips of film, and after 1 hour incubation at 37°C, the film strips were rinsed in gently flowing tap water. Figures in Table I represent smallest amount of venom in μg required to completely remove gelatin from the nitrocellulose base of film. Although there is an analytical error of 50 to 100%, activation of 2500% is reported. Variations of 100% or less, such as slight decrease in activity of moccasin venom by DHEG (line 11, Table I) are not considered significant. Digestion of hemoglobin was carried out by the method of Anson(2). Venom solutions with various anions were added to urea denatured hemoglobin and incubation carried out at 37°C for 15 minutes. The amount of tyrosine liberated was determined by use of Folin-Ciocalteau reagent. Disodium phosphate, sodium bicarbonate, and sodium tetraborate have a buffering action at pH 7.4 and of these sodium tetraborate is the most effective. The hemoglobin also has some buffering effect. Water moccasin venom toxicity studies were carried out in mice by mixing maximum tolerated dose of various inhibitors with 2-3 LD_{50}'s of venom and inoculating with these solutions intra-

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TABLE I. Effect of Various Substances on Amount of Venom Required for Liquefaction of Gelatin on Film.

	Venom	
	Water moccasin*	Rattlesnake*
Saline control (pH 7.4 with $Na_2B_4O_7$)	2	25
Substances added†		
Human serum	1	1
Acid ash		25
$NaHCO_3$ (.02 M, pH 7.4 with HCl)	1	1
Acidified $NaHCO_3$		25
Na_2HPO_4 (.02 M, pH 7.4 with HCl)		6
Penicillamine (.6-1.0%, pH 7.4 with NaOH)	50	100
Penicillamine (.6-1.0%, pH 7.4 with $NaHCO_3$)	2	12
EDTA (2.5×10^{-3} M, pH 7.4 with NaOH)	33	67
EDTA (2.5×10^{-3} M, pH 7.4 with $NaHCO_3$)	8	17
DHEG (.1 M, pH 7.4 with NaOH)	4	25

* μg of venom in .02 ml required to remove gelatin from film in 1 hr at 37°C and 100% moisture.
† .1 ml of activators and inhibitors in concentrations listed plus .1 ml of venom dilutions in .9% sodium chloride sol.

peritoneally. The effect of these inhibitors on rattlesnake venom was determined by inoculating with the chelating agent intraperitoneally prior to inoculation with the venom. Observations on mortality were made for 48 hours. Acid ash of human serum was prepared by bringing the pH of an aqueous solution of ash to 1.5 with hydrochloric acid, boiling, and readjusting pH to 7.4 with sodium hydroxide. A solution of sodium bicarbonate initially .02N concentration was acidified in like manner with hydrochloric acid and restored to pH 7.4 with sodium hydroxide.

Results. Rattlesnake and water moccasin venom gelatinases in unbuffered saline are not affected by pH changes within range of 5 to 8. Sodium tetraborate has no effect on activity of these enzymes and is therefore the compound used to adjust the pH of saline controls to 7.4.

As shown in line 1 of Table I, 25 μg of rattlesnake venom in borax buffered saline are required to liquefy gelatin whereas only 1 μg is required if sodium bicarbonate is added. This 25-fold activation is equal to that of human serum and its ash and is 4 times greater than that of sodium phosphate. Acid ash of human serum and acidified sodium bicarbonate have no activating action (Table I). Ethylenediamine-tetraacetic acid (EDTA) and penicillamine are effective inhibitors of water moccasin venom gelatinase when the pH of these compounds is brought to 7.4 with sodium hydroxide. When sodium bicarbonate is used to adjust the pH, the inhibitory action of these compounds is diminished. Similar results were obtained using these chelating agents on rattlesnake venom although the inhibitory effect of EDTA is slight. The iron chelating agent dihydroxyethyglycine (DHEG) has no significant effect on venom proteolysis at pH 7.4

When urea denatured hemoglobin is used as substrate (Table II), 1 mg of water moccasin venom without sodium bicarbonate will liberate 3.8-4.0 millimoles of tyrosine X 10^{-4} in 15 minutes at 37°C and with bicarbonate the amount is increased to 6.4 mM X 10^{-4}, an increase of 70%. Two mg of rattlesnake venom liberate 0.9 to 1.0 mM of tyrosine X 10^{-4} in the absence of sodium bicarbonate and 2 mM X 10^{-4} with bicarbonate, an increase of 100%.

EDTA inhibits water moccasin venom pro-

TABLE II. Effects of Various Inorganic Compounds on Digestion of Hemoglobin* by Pit Viper Venom.

Venom		Tyrosine liberated‡			
Water moccasin, mg†	Rattlesnake, mg†	$NaHCO_3$§	$NaHPO_4$§	$Na_2B_4O_7$§ .10 H_2O	NaOH‖
1		6.4	3.9	4.0	3.8
1 + EDTA¶ (pH 7.4 with NaOH)		2.4	1.6	1.6	1.6
1 + EDTA¶ (pH 7.4 with $NaHCO_3$)		2.2			
	2	2.0	.9	.95	1.0

* 5 cc of .8 g % in physiological saline. † Quantity contained in 1 cc. ‡ Millimoles of tyrosine X 10^{-4}/15 min. at 37°C. § Solution originally .02 N adjusted to pH 7.4 with HCl. ‖ Added to all solutions to bring pH to 7.4. ¶ .9 ml of 10^{-3} M conc.

teolysis of urea denatured hemoglobin as it did the liquefaction of gelatin. Sodium bicarbonate in presence of this compound increases tyrosine liberated from 1.6 to 2.4 mM X 10^{-4}, which is an increase of 50%. EDTA brought to pH 7.4 with sodium bicarbonate is at least as active an inhibitor as when sodium hydroxide is used to adjust the pH. Activity of either rattlesnake or water moccasin venom proteases on denatured hemoglobin is approximately the same in presence of either sodium borate, sodium phosphate, or sodium hydroxide when the pH of these solutions is adjusted to 7.4. Although penicillamine and EDTA effectively inhibit the proteolytic action of these venoms, they have no significant effect on toxicity of these venoms in mice.

Discussion. Results reported herewith indicate strongly that activation of venom proteases and reversal of chelating effects by mammalian serum and its ash are due largely to their content of bicarbonate ions. Salts of calcium, magnesium, manganese, cobalt, potassium, ammonium, lithium, iron and copper fail to bring about activation of rattlesnake venom in the absence of bicarbonate ion. Deutsch and Diniz(3), who previously described the action of EDTA on venom proteases, report that inhibition is not reversed by the chlorides of strontium, cobalt, barium, manganese, cadmium, magnesium, zinc, and calcium and nitrates of copper and cobalt in a concentration of 1 X 10^{-2} M.

The possibility of a contaminant being responsible for activation has been considered although only analytical reagent grade chemicals were used. Inactivity of acid ash and acidified sodium bicarbonate, however, is strong evidence for participation of the bicarbonate ion. Total ionic strength, pH, and temperature of all reactions have been carefully controlled.

Activity of EDTA and penicillamine still points in the direction of a cation necessary for venom proteolysis. Reversal of this effect by the bicarbonate ion may be due to indirect inhibition of ability of these compounds to chelate a metallic ion. A close correlation between inhibition of venom proteases and venom toxicity in mice by various serum preparations has been reported(1). The reversal effect of sodium bicarbonate may explain why EDTA and penicillamine are effective proteases inhibitors but ineffective in preventing the lethal effects of the venom in mice.

When fully activated, water moccasin venom is only 6 times as effective as rattlesnake venom on urea denatured hemoglobin with urea removed by dialysis. In previously reported work(1,3) using the same substrate in presence of urea, water moccasin venom was 30 times more effective than rattlesnake venom. They may point to a more rapid denaturation of rattlesnake venom than of water moccasin venom by urea.

Summary. 1. The bicarbonate ion increases proteolytic activity of rattlesnake venom on gelatin layer of photographic film and of both rattlesnake and water moccasin venom on urea denatured hemoglobin with urea removed. 2. The bicarbonate ion also interferes with inhibition of these proteases by EDTA and penicillamine. 3. EDTA and penicillamine have no significant effect on mouse toxicity of these venoms possibly because of bicarbonate content of body fluids.

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