INACTIVATION OF THROMBOPLASTIN BY COBRA VENOM

by

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It is known that the poisonous secretion of the cobra snake (genus: *naja*) contains a strong anticoagulant. The same secretion also contains lecithinase A which splits off unsaturated fatty acids from phospholipids whereby compounds of the lysolecithin type are formed. The anticoagulant component is largely destroyed by heating for one hour at 70° (A. CALMETTE[1], 1907) while the toxicity and the lecithinase effect are resistent to such heat treatment. For this reason it is unlikely that the anticoagulant effect of cobra venom can be explained on the basis of destruction of the phospholipid component of thromboplastin through the action of lecithinase.

This may be the reason why in earlier investigations on the anticoagulant effect of cobra venom the possibility that an important part of this effect is due to inactivation of thromboplastin has not been taken into consideration.

The present investigations show that destruction of thromboplastin by a thermolabile component is by far the major factor in the inhibition of plasma coagulation by cobra venom, at least within the concentration limits studied.

MATERIALS AND METHODS

Citrate plasma and thromboplastin from chicks were used in these experiments.

Blood was obtained from normal chicks from the carotid artery through a needle and collected in graduated cylinders containing a 3% sodium citrate solution (1 vol. of blood to 2 vols of citrate solution). Centrifugation was carried out at 4500 rpm for 5 minutes.

Thromboplastin was made from brains of vitamin K deficient chicks by grinding with an equal amount of quartz powder and twice the amount of 0.9% NaCl solution. After 5 minutes' centrifuging at 4500 rpm the milky suspension was stored in the frozen state. Several batches were prepared.

Cobra venom in the form of the desiccated secretion was kindly furnished by the *State Serum Institute*. The lethal dose for mice within 7 hours was indicated to be 1 to 5 micrograms.

The *determinations of the clotting times* were carried out in the following way: to 0.6 ml of chicken citrate plasma heated in a water bath at 37° C were added 0.21 ml of a mixture of 0.4 ml thromboplastin, 1.25 ml of 2% $CaCl_2,2H_2O$ and 3.6 ml of 0.9% NaCl, heated in the same way, and the clotting times were determined during regular pendulous movements of the tube in the water bath.

RESULTS

The action of the cobra venom on thromboplastin was studied both with unheated venom and with venom which had been heated in a water bath 72° C for 45 minutes.

Experiments 1 and 2 (Table I) were carried out as follows: a mixture of 0.4 ml thromboplastin, 0.30 mg cobra venom dissolved in RINGER's solution to a volume of 3.6 ml, and 1.25 ml of 2% $CaCl_2,2H_2O$ was kept in water bath at 37° C, and at different times 0.21 ml of this mixture were added to 0.6 ml heated chicken citrate plasma and

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the clotting times determined as previously indicated. In this way the concentration of venom was of 12 μg in each 0.6 ml of plasma. In experiment 3 (Table I) we used a mixture of 0.4 ml thromboplastin, 0.375 mg cobra venom dissolved in 1.8 ml water, 1.8 ml of 1.8% NaCl, and 1.25 ml of 2% $CaCl_2,2H_2O$. In this experiment the concentration of venom was 15 μg in each 0.6 ml plasma.

Table II shows the action of cobra venom on chicken citrate plasma. 0.1 ml of venom solution (in RINGER's solution in experiment 1, and in water in experiment 3) was added to 0.6 ml plasma kept at 37° C. After keeping this mixture at 37° C for the periods of time given in Table II, the thromboplastin–$CaCl_2$–NaCl mixture was added and the clotting times were determined.

TABLE I

EFFECT OF COBRA VENOM ON CHICKEN THROMBOPLASTIN

	Duration of incubation of thromboplastin with cobra venom at 37° C minutes	Clotting time in minutes obtained with incubated thromboplastin	
		A. Venom previously unheated	B. Venom previously heated for 45 minutes at 72° C
Exp. 1*	1	1[29]	
	5	2[56]	
	10	8[00]	
	15	11[07]	
	30	>44	
Exp. 2**	1	3[42]	
	1[30]		1[20]
	5		1[46]
	5[30]	>90	
	10	>90	2[10]
	15		2[20]
	30		4[07]
Exp. 3***	1	1[55]	1[12]
	5	3[38]	1[35]
	10	6[03]	1[56]
	15	8[42]	2[07]
	45		2[46]

* The normal clotting time found with the chicken citrate plasma and thromboplastin used was 0[33] min.

** The normal clotting time found with the chicken citrate plasma and thromboplastin used was 0[38] min.

*** The normal clotting time found with the chicken citrate plasma and thromboplastin used was 0[33] min.

Table I shows that the clotting times were greatly prolonged when the thromboplastin was incubated with the venom at 37° C. On the other hand, when normal thromboplastin was added immediately after the addition of the venom-treated thromboplastin, the clotting time was nearly normal. This table also shows that when

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the venom had been heated at 72° C for 45 minutes, it lost most of its ability to destroy thromboplastin.

Table II shows that by incubating the chicken plasma with cobra venom at 37° C the clotting time was somewhat prolonged, but to a much lesser extent than when the venom acted upon thromboplastin.

TABLE II

EFFECT OF PREVIOUSLY UNHEATED COBRA VENOM ON CHICKEN CITRATE PLASMA

	Chicken citrate plasma ml	Cobra venom added μg	Duration of incubation of plasma with venom at 37° C minutes	Clotting time obtained after addition of thromboplastin minutes
Exp. 1*	0.6	0		0^{23}
	0.6	12	10	0^{35}
	0.6	12	16	0^{36}
Exp. 3**	0.6	0		0^{33}
	0.6	15	1	0^{54}
	0.6	15	5	0^{55}
	0.6	15	10	1^{02}
	0.6	15	15	1^{06}

* The same chicken citrate plasma and thromboplastin were used as in Exp. 1, Table I.
** The same chicken citrate plasma and thromboplastin were used as in Exp. 3, Table I.

From these results (Fig. 1) it is concluded that the inactivation or destruction of thromboplastin is an essential factor in the anticoagulant effect of cobra venom.

The lecithinase component of cobra venom is not likely to be responsible for the rapid destruction of thromboplastin, since lecithinase is thermostable (FLEXNER AND NOGUCHI[2], 1902). This was further established when we determined the activity of lecithinase (by the method described by SUMNER AND SOMERS[3], 1943) in untreated and heated solutions of cobra venom (72° C 45 min). The lecithinase activity was found to be the same before and after heating.

In order to study whether the residual, slow action of the heated venom on thromboplastin might be due to lecithinase, we tried to purify the venom by a method similar to that used by SLOTTA AND FRAENKEL-CONRAT[4] (1938), for the purification of rattlesnake (*Crotalus*) venom.

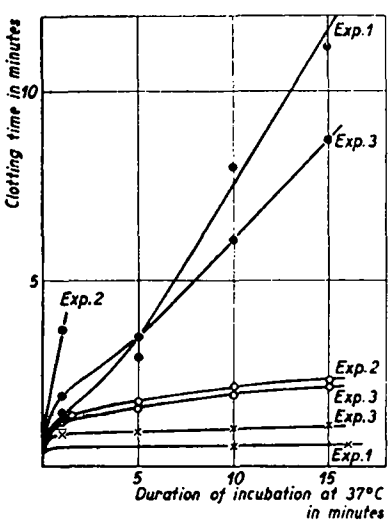

Fig. 1. Effect of cobra venom on chicken thromboplastin and citrate plasma. —•—•— Chicken thromboplastin + venom previously unheated; —o—o— Chicken thromboplastin + venom previously heated for 45 min at 72° C; —×—×— Chicken citrate plasma + venom previously unheated

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After heating the venom on a water bath at 72° C for 45 minutes and removal of the precipitate by centrifugation the solution was fractionated with $(NH_4)_2SO_4$.

The material precipitated between 45% and 62% saturation with $(NH_4)_2SO_4$ contained 3 to 4 times as much lecithinase as the unpurified venom. The action of the various cobra venom solutions on thromboplastin is presented in Table III and Fig. 2. In all the experiments the concentration of venom was the same. The mixture of

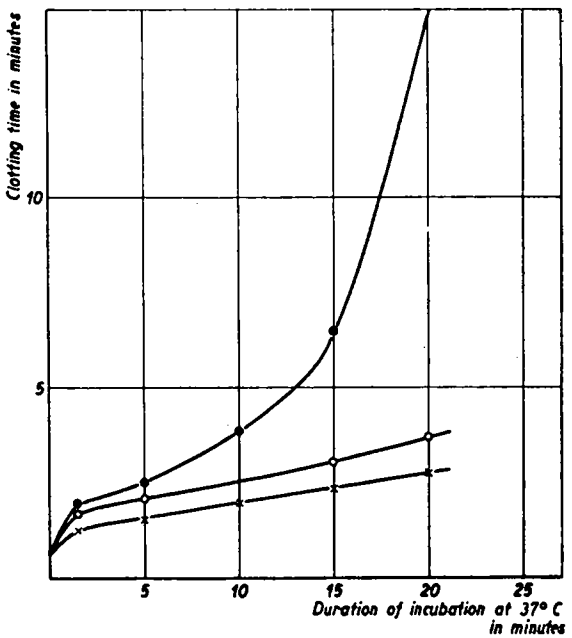

Fig. 2. Effect of previously unheated, heated and purified venom on chicken thromboplastin. —•—•— unheated venom; —×—×— heated venom; —o—o— purified venom

TABLE III

EFFECT OF PREVIOUSLY UNHEATED, HEATED (72° C, 45 MIN) AND PURIFIED COBRA VENOM ON CHICKEN THROMBOPLASTIN

Duration of incubation at 37° C minutes	Clotting time in minutes* obtained with incubated thromboplastin		
	A. Unheated venom	B. Heated venom	C. Purified venom
1^{30}	1^{56}		1^{40}
1^{35}		1^{16}	
5	2^{29}	1^{33}	2^{05}
10	3^{50}	1^{58}	
15	6^{25}	2^{23}	3^{02}
20		2^{45}	3^{38}
24	>20		

* The normal clotting time found with the chicken citrate plasma and thromboplastin used was 0^{34} min.

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thromboplastin–cobra venom–NaCl–$CaCl_2$ was as follows: 0.4 ml thromboplastin, 1.8 ml of a 20 mg% aqueous solution of cobra venom, 1.8 ml of 1.8% NaCl, and 1.25 ml of 2% $CaCl_2$, $2H_2O$.

The results indicate that the action of the purified venom on the thromboplastin

TABLE IV

EFFECT OF THE PRESENCE OF ANTISERUM DURING THE INCUBATION OF THROMBOPLASTIN WITH COBRA VENOM

Duration of incubation at 37° C minutes	Clotting time in minutes* obtained with incubated thromboplastin		
	Thromboplastin–venom mixture without antiserum**	Thromboplastin–venom mixture 0.5 ml antiserum/ 1 mg cobra venom***	Thromboplastin–venom mixture 1 ml antiserum/ 1 mg cobra venom****
1	2^{50}		
6	4^{51}		
15		3^{30}	1^{00}
16	7^{30}		
30		6^{24}	1^{16}
46			1^{34}

* The normal clotting time found with the chicken citrate plasma and thromboplastin used was 0^{36} min.

** Mixture of: 0.4 ml thromboplastin + 0.4 ml of 100 mg% aqueous solution of cobra venom + 3.2 ml of 0.9% NaCl + 1.25 ml of 2% $CaCl_2$,$2H_2O$

*** Mixture of: 0.4 ml thromboplastin + 0.2 ml antiserum + 0.4 ml of 100 mg% aqueous solution of cobra venom + 3.0 ml of 0.9% NaCl + 1.25 ml of 2% $CaCl_2$,$2H_2O$

**** Mixture of 0.4 ml thromboplastin + 0.4 ml antiserum + 0.4 ml of 100 mg% aqueous solution of cobra venom + 2.8 ml of 0.9% NaCl + 1.25 ml of 2% $CaCl_2$,$2H_2O$

TABLE V

EFFECT OF COBRA VENOM ON THROMBIN

Duration of incubation at 37° C	Clotting time in minutes* obtained with incubated thrombin		
	Thrombin solution** without venom	0.25 mg cobra venom/ 1 mg thrombin***	1 mg cobra venom/ 1 mg thrombin****
4 min	0^{28}	0^{28}	
6 min			0^{34}
16 min		0^{28}	
36 min		0^{27}	
18 hours	0^{43}	1^{10}	2^{05}

* To 0.6 ml chicken citrate plasma heated in a water bath at 37° C were added 0.21 ml of the thrombin solution (or mixture) heated in the same way, and the clotting times were determined.

** 200 mg% aqueous solution of thrombin

*** 1.0 ml of 400 mg% aqueous solution of thrombin + 1.0 ml of 100 mg% aqueous solution of cobra venom

**** 1.0 ml of 400 mg% aqueous solution of thrombin + 1.0 ml of 400 mg% aqueous solution of cobra venom

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was stronger than that of the heated venom, but not proportional to the ratio between the lecithinase content of the two preparations.

In another experiment we studied the influence of antiserum (sérum antivenimeux obtained from the INSTITUT PASTEUR, Paris, through the State Serum Institute in Copenhagen) on the destruction of thromboplastin by cobra venom. It was found that if antiserum is added to a thromboplastin solution before the addition of cobra venom, only a very slight decrease of thromboplastin activity occurs as a result of the incubation with venom. These findings are presented in Table IV and Fig. 3.

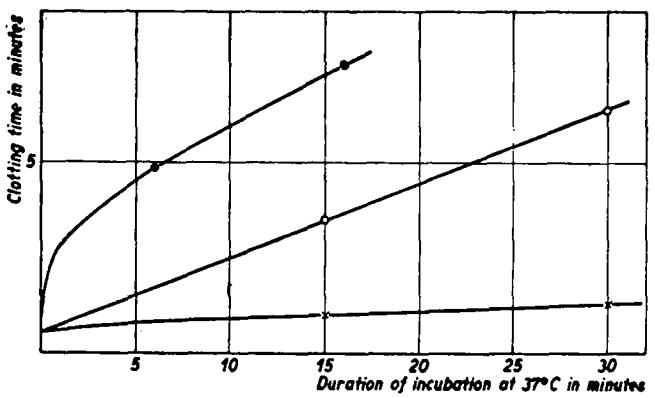

Fig. 3. Effect of the presence of antiserum during the incubation of thromboplastin with cobra venom.
—•—•— without antiserum; —o—o— 0.5 ml antiserum/1 mg cobra venom;
—×—×— 1 ml antiserum/1 mg cobra venom

TABLE VI

EFFECT OF THE PRESENCE OF COBRA VENOM ON THE INCUBATION OF THROMBIN WITH ANTISERUM

Duration of incubation at 37° C	Clotting time in minutes* obtained with incubated thrombin		
	Thrombin**	Thrombin, antiserum***	Thrombin, antiserum, cobra venom****
5 min	0[31]	0[37]	0[39]
15 min	0[30]		0[45]
17 min		0[43]	
45 min	0[31]	0[45]	0[46]
20 hours	0[30]	>6 hours	between 3½ and 4½ hours

* The clotting times were determined as explained in footnote 1, Table V
** 200 mg% aqueous solution of thrombin
*** 0.5 ml of 800 mg% aqueous solution of thrombin + 1.0 ml antiserum + 0.5 ml H_2O
**** 0.5 ml of 800 mg% aqueous solution of thrombin + 1.0 ml antiserum + 0.25 ml of 400 mg% aqueous solution of cobra venom + 0.25 ml H_2O

On the other hand, when antiserum was added to the already incubated mixture of thromboplastin, a marked decrease of thromboplastic effect was found. It should be mentioned—*en passant*—that the antiserum itself had some thromboplastin effect.

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The effect of cobra venom on thrombin was also studied. In this experiment "Thrombin Topical", *Parke Davis*, was used. It was found that small amounts of cobra venom had no inhibiting effect on thrombin within relatively short periods of time. With larger amounts of venom and longer periods of time (18 hours) a slight inhibiting action of the venom on thrombin was found. The results are presented in Table V.

In a final experiment (Table VI) we studied the effect of the presence of cobra venom on the incubation of thrombin with antiserum (at 37° C). The antiserum seemed to have some effect on the thrombin irrespective of whether the venom was present or not.

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SUMMARY

This paper reports some studies on the anticoagulant effect of cobra venom on chicken blood. It was found that the venom has a strong inactivating or destructive action on thromboplastin, and it is concluded that the destruction of thromboplastin is an essential factor in the anticoagulant effect of cobra venom. The action of the venom on the plasma was found to be much smaller.

The strong thromboplastin inactivating component of the venom is destroyed by heating at 72° C during 45 min. Under certain conditions it is counteracted by antiserum.

After heating a solution of the venom at 72° C for 45 minutes a residual, slow antithromboplastin effect could be demonstrated.

RÉSUMÉ

Dans ce mémoire nous rapportons quelques études concernant l'effet anticoagulant excercé par le venin de cobra sur le sang de poulet. Nous avons trouvé que le venin désactive ou détruit fortement la thromboplastine et nous en concluons que la destruction de la thromboplastine est un facteur essentiel de l'effet anticoagulant du venin de cobra. L'action du venin sur le plasma s'est montrée beaucoup plus faible.

La composante du venin qui désactive fortement la thromboplastine est détruite par chauffage à 72° C pendant 45 minutes. Sous certaines conditions cette désactivation est contrecarrée par l'antisérum.

Nous avons montré qu'un effet antithromboplastine lent subsiste après chauffage du venin à 72° C pendant 45 minutes.

ZUSAMMENFASSUNG

Wir berichten über einige Studien die den hemmenden Effekt des Cobragiftes auf die Gerinnung des Hühnerblutes betreffen. Wir fanden, dass das Gift auf Thromboplastin eine starke Inaktivierung oder Zerstörung ausübt und schliessen daraus, dass die Zerstörung des Thromboplastins ein wesentlicher Faktor des Antikoagulations-Effektes des Cobragiftes ist. Die Wirkung des Giftes auf Plasma war viel geringer.

Die stark Thromboplastin desaktivierende Komponente des Giftes wird durch Erwärmen auf 72° C während 45 Minuten zerstört. Unter gewissen Bedingungen wird sie durch Antiserum gehemmt.

Nach dem Erwärmen einer Lösung des Giftes auf 72° C während 45 Minuten konnte noch ein langsamer Antithromboplastin-Effekt festgestellt werden.

REFERENCES

[1] A. CALMETTE, *Les venins, les animaux venimeux et la sérothérapie antivenimeuse*, Paris 1907.
[2] S. FLEXNER AND H. NOGUCHI, *J. Exptl Med.*, 6 (1902) 277.
[3] JAMES B. SUMNER AND G. FRED. SOMERS, *Chemistry and Methods of Enzymes*, New York 1943.
[4] K. H. SLOTTA AND H. L. FRAENKEL-CONRAT, *Ber.*, 71 (1938) 1076.

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