L. THE ACTION OF SNAKE VENOMS ON SURFACE FILMS.

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THE work of Kyes [1902] and of Kyes and Sachs [1903] showed that the action of snake venom haemolysins is upon the lecithin portion of the cell membrane. Later, Delezenne and Fourneau [1914] found that egg-lecithin undergoes a partial hydrolysis by snake venom, the unsaturated fatty acid residues being specifically removed to form lysolecithin, which contains a single saturated aliphatic hydrocarbon chain. No satisfactory mechanism has been suggested for this specific hydrolysis. A study has been made in the present work of the actions of various snake venoms on unimolecular films of lecithin and other compounds in relation to the processes involved in venom haemolysis.

As opposed to the study of reactions in bulk, the technique of surface films has the advantage that the enzyme substrate can be studied in a definite and reproducible state, that of an oriented monolayer. Chemical reactions occurring therein are observable by the accompanying changes in the surface potential of the film. The surface potentiometer employed has been described elsewhere [Schulman and Rideal, 1931].

Preliminary investigation.

The following venoms were available:

COLUBRIDAE:	Black snake	<i>Pseudechis porphyriacus</i> .
	Copperhead	<i>Denisonia superba</i> .
	Black tiger	<i>Notechis scutatus</i> .
	Cobra	<i>Naia naia</i> .
VIPERIDAE:	Daboia	<i>Vipera elegans</i> .

A film of lecithin was spread on the surface of dilute NaCl solution in the trough of the surface potentiometer and compressed to a fixed reference area per molecule, usually 100 sq. Å. A dilute solution of venom was then injected under the film from the far side of the movable glass barrier enclosing the film. The solution was well mixed and the change of surface potential was measured. In cases where the change was rapid it was found advisable to mix the venom solution in the trough prior to spreading the film to ensure uniformity of the solution. The lecithin film is spread immediately after cleaning the surface, since protein, which is always present with the venom, accumulates at the free liquid surface in the more concentrated solutions.

With a venom concentration of 0.01 % in the underlying solution a rapid fall in surface potential takes place, *i.e.* in the direction of a conversion of lecithin into lysolecithin (*cf.* Fig. 1), in the case of all the venoms except cobra. This anomaly of cobra venom, ascribable to the different nature and greater proportion of associated protein, will be referred to later. The final values of the

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surface potential of a lecithin film on the other four venoms depend on the degree to which the associated protein is adsorbed under a lysolecithin film. Thus injection of venom under a lysolecithin film causes a small rise in potential to a value within about 15 mv. of the final value of lecithin on that solution.

Fig. 1. Surface potentials of lecithin and lysolecithin, 0.9 % NaCl, 18°.

None of the venoms has any effect on a unimolecular film of cholesterol or of protein, and they cause no hydrolysis of tripalmitin, triolein, cerebron or sphingomyelin.

The action of the venom on a lecithin film was found to depend on three main factors:

- (i) The p_H of the underlying solution.
- (ii) The surface concentration of the lecithin film.
- (iii) The venom concentration.

These effects were examined in detail.

(i) *The effect of p_H .* The action of black tiger venom was studied over a p_H range from 4 to 11 using $M/25$ phthalate, phosphate and borate buffers and a fixed venom concentration of 0.001 %. Fig. 2 gives a family of curves showing the change of surface potential (ΔV) with time at various p_H values for a definite initial surface concentration of lecithin ($n = 1.0 \cdot 10^{14}$ mols./sq. cm.). There is no reaction on solutions more acid than p_H 4.8, the velocity at p_H 6.0 is about equal to that at p_H 8.0, while at p_H 10.8 the reaction is again strongly inhibited. The optimum is at about p_H 7.3.

The venom itself is stable in fairly strongly acid media but not in alkaline. After keeping for 3 hours in $N/10$ HCl at room temperature and returning to p_H 7.2 the venom solution shows unimpaired activity. Treatment with $N/10$ NaOH for the same period destroys the venom almost completely as regards its action on a lecithin film. The action is not inhibited in the presence of 0.5 % NaF.

(ii) *Effect of surface concentrations of lecithin.* The rate of attack of a lecithin film is considerably diminished as the number of molecules of lecithin per sq. cm. is increased (Fig. 3). The time for complete hydrolysis of the film increases from 7 to 100 minutes for a doubling of the initial surface concentration of lecithin, from 1.04 to $2.07 \cdot 10^{14}$ mols./sq. cm. It is known that the venom specifically removes the unsaturated hydrocarbon chain from lecithin. A triolein film is not

Fig. 2.

Fig. 3.

Fig. 2. Influence of p_H on attack of lecithin films by black tiger snake venom, 20° .

Fig. 3. Effect of compression of lecithin film on attack by black tiger venom (0.001 %).

$$\left. \begin{array}{l} A \quad n = 2.11 \cdot 10^{14} \text{ mols./sq. cm.} \\ B \quad n = 1.57 \cdot 10^{14} \text{ mols./sq. cm.} \\ C \quad n = 1.27 \cdot 10^{14} \text{ mols./sq. cm.} \\ D \quad n = 1.04 \cdot 10^{14} \text{ mols./sq. cm.} \end{array} \right\} p_H \text{ 7.2, } 17^\circ$$

hydrolysed even very slowly by venom, the action of which cannot therefore be concerned only with a coupling with the unsaturated group in lecithin. It must also couple with some other point in the lecithin molecule. Compression of the lecithin will alter the spacing of the essential points of attachment, and at the higher compressions the double bonds will be removed from the aqueous surface, as observed in the case of oleic acid [Hughes and Rideal, 1933]. It may thus be suggested that the lecithinase embodies also a spacing of two active groups

which coincide with a similar spacing of two active groups in the distended lecithin molecule for the maximum probability of reaction.

(iii) *Effect of venom concentration.* Fig. 4 shows the rate of attack of a lecithin film from a fixed initial surface potential of 280 mv. by varying concentrations of copperhead venom, in $M/30$ phosphate buffer at p_H 7.2.

It is seen that at concentrations higher than about 10^{-4} g. venom 100 ml. (1 part in a million), the reaction is of zero order and complete in 5 minutes at 20° . Below this concentration and down to the lowest concentration used, 1 part in 40 millions, the rate of reaction falls off sharply with the venom concentration till at $2.5 \cdot 10^{-6}$ % the half life is about 1 hour. It is impossible to give quantitative values for the velocity constants at these low concentrations owing to the varying amounts of protein in the solution and the possible adsorption of the venom on the glass vessel as well as at the surface of the film.

It must be remembered that the values of the venom concentration refer to dry weight of crude venom scale, and that the fraction of this which is pure active principle is unknown.

Attack of lecithin/cholesterol films by venom.

The rate of attack by black tiger venom of a film containing 20 % cholesterol molecules is the same as that for lecithin alone at the same area per molecule. In a 50 % mixture the velocity is not appreciably diminished, but in an 80 % cholesterol film no reaction is observed over a period of hours. It appears that cholesterol has no specific inhibitory power in this reaction other than that due to its causing a general contraction of the lecithin film as the proportion of lecithin is decreased. As shown in Fig. 3, a similar inhibition is observed by mere compression of a pure lecithin film.

Correlation of haemolytic action of venoms with their action on lecithin films.

The following experiments were carried out to ascertain how far the observed changes brought about in a lecithin film by venom were ascribable to the haemolysin of the venom.

Washed guinea-pig cells were used throughout. In Table I a comparison is shown of the effect of black snake venom in haemolysis and on the surface potential of a lecithin film. The venom solution was used (a) unboiled, (b) boiled 1 hour in saline, (c) unboiled but filtered through a Seitz filter.

Table I.

1 ml. 5 % cells, 1 ml. venom solution (1 mg./ml. in saline) at 37° .			
	Unboiled unfiltered	Boiled 1 hr. unfiltered	Unboiled filtered
Haemolysis	C.H. 20 mins.	C.H. 20 mins.	N.H. 20 mins. C.H. 40 mins.
Film activity: fall of ΔV in 10 mins.	46 mv.	41 mv.	22 mv.
C.H. = Complete haemolysis.		N.H. = No haemolysis.	

These figures demonstrate a definite relationship between hydrolysis of a lecithin film and haemolysis, by black snake venom, and further show the remarkable stability of the venom to prolonged boiling in saline, a point which is discussed more fully later.

A more complete set of experiments was carried out using four varieties of venom: copperhead, daboia, cobra and black tiger, while a definite p_H was main-

tained with isotonic phosphate p_H 7.4. Control experiments on the isotonic buffers employed showed that these had no effect on the fragility of the cells. The system for haemolysis was made up as follows: 1 ml. of venom solution in isotonic saline (1 mg. per ml.), 1 ml. isotonic phosphate buffer and 0.5 ml. of 5 % washed guinea-pig erythrocytes. The venom solution was used (a) unboiled, (b) boiled 10 minutes, (c) boiled 60 minutes, the heating being carried out at p_H 7.4. It was immediately noticed that the cobra venom solution on heating behaved very differently from the other venoms in giving a bulky yellowish white precipitate, whereas the other solutions only showed a slight opalescence even after 1 hour's boiling. This and other anomalies of cobra will be discussed later.

The main results were, in the first place, that ability to hydrolyse a lecithin film does not necessarily imply haemolytic power on red cells of any given species. Thus daboia gave no haemolysis, black tiger only a trace after 16 hours, while copperhead and cobra as well as black snake were strongly haemolytic, showing complete haemolysis in 2 hours with a concentration of 0.2 mg./ml.

Secondly, after 10 minutes' boiling at p_H 7.4 no haemolysis was detectable with any of the solutions. This result in comparison with the results shown in Table I indicates that the heat stability of the venom depends on the p_H of the solution. This question was investigated more closely. It was found for copperhead and cobra that 15 minutes' boiling at p_H 5.9 (phosphate) has no effect on copperhead and only a slight effect on cobra, while apparently complete destruction ensues at p_H 7.0 (phosphate) and at p_H 9.0 (borate) (Table II).

Table II.

1 drop 50 % cells, 1 ml. venom solution (1 mg./ml. in isotonic buffer) incubated at 37°.
for 2 hrs. then at room temperature.

	p_H	5.9 phosphate	7.0 phosphate	9.0 borate
Cobra				
Unboiled		C.H. 3-3½ hrs.	C.H. 3-3½ hrs.	C.H. 1½ hrs.
Boiled 15 mins.		P.H. 3-3½ hrs.	N.H.	N.H.
Copperhead				
Unboiled		C.H. 1½ hrs.	C.H. 1½ hrs.	P.H. 3-3½ hrs.
Boiled 15 mins.		C.H. 1½ hrs.	N.H.	N.H.

C.H. = Complete haemolysis. P.H. = Partial haemolysis. N.H. = No haemolysis in 20 hrs.

This sharp effect of p_H on heat stability has not previously been examined and may account for varied reports as to the influence of heat on different venoms [cf. Phisalix, 1922].

The experiments with surface films of lecithin revealed further that where complete destruction of the venom is registered with regard to haemolysis, an appreciable quantity of lecithinase may still be present. It has been already mentioned that a concentration of venom as low as $2.5 \cdot 10^{-6}$ % is detectable by means of surface potentials, while a concentration of $1.5 \cdot 10^{-3}$ % venom only produces complete haemolysis in 16 hours (cf. Table III and Fig. 4).

Table III. *Effect of venom concentration on haemolysis by copperhead.*

		Conditions as in Table II, p_H 7.0 phosphate.						
% venom ...	10^{-1}	$5 \cdot 10^{-2}$	$2.5 \cdot 10^{-2}$	$1.2 \cdot 10^{-2}$	$6.2 \cdot 10^{-3}$	$3.1 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$	$7.5 \cdot 10^{-4}$
Time 1½ hrs.	4	3	3	3	3	1	—	—
3½ hrs.	4	4	4	4	4	4	1	—
16 hrs.	4	4	4	4	4	4	4	3

(4 represents complete haemolysis.)

It is seen that at a concentration of $7.5 \cdot 10^{-4}$ % venom haemolysis is not yet complete even in 16 hours, whereas a lecithin film is completely transformed in 3 minutes by the same venom concentration. The method of surface potentials provides therefore a very much more sensitive check on the presence of a lecithinase haemolysin.

Fig. 4. Attack of lecithin films by copperhead venom, p_H 7.2, 20° .

Venom concentrations: $A \ 5 \times 10^{-4}$. $B \ 1 \times 10^{-4}$. $C \ 5 \times 10^{-5}$.
 $D \ 1 \times 10^{-5}$. $E \ 5 \times 10^{-6}$. $F \ 2.5 \times 10^{-6}$. } g. %.

The surface activities of venom solutions boiled at various p_H values were compared with the activity of the same venom on progressive dilution and the results are summarised in Table IV. The quantity of undecomposed venom is estimated from the rate of attack on a lecithin film.

Table IV.

Effect of boiling on the lecithinase content of copperhead venom. Initial weight of venom 10^{-3} g.

p_H ...	5.9	6.5	7.2	9.0
Boiled for 15 mins.	10^{-3}	10^{-3}	$ca. 10^{-5}$	nil g. venom remaining
Boiled for 60 mins.	10^{-3}	$ca. 10^{-4}$	$ca. 10^{-6}$	„ „ „

The active principle of the venom is therefore rapidly decomposed on the alkaline side of a sharp p_H limit of 6.5–7.0 and boiling for 15 minutes at p_H 7.2 will inactivate the venom as far as haemolytic experiments can decide, by reducing the venom concentration a hundredfold, although this small quantity, 10^{-5} g., is still readily detected by the method of surface potentials. The complete loss of surface activity as well as haemolytic activity on boiling at p_H 9.0 seems convincing evidence of the close relation of the two effects.

An attempt was made to examine the influence of p_H on the rate of venom haemolysis. It is probably impossible in some cases to separate the effect of p_H from that of the buffering ion. For cobra and copperhead venoms the rate of attack was about the same in two isotonic phosphate buffers at p_H 5.9 and 7.0, but whereas cobra is considerably more active at p_H 9.0 (borate), copperhead is considerably less so (cf. Table II). This effect of p_H on copperhead haemolysis supports the results of Holden [1934] that rabbit cells are haemolysed more rapidly at p_H 5.6 than at p_H 8.0.

The anomalies of cobra venom.

Three different specimens of cobra venom were examined, and the behaviour was found to be uniformly different from that of the other venoms studied. At a concentration of 0.01 % and down to 0.0001 % cobra venom gives no detectable hydrolysis of a lecithin film, but on diluting to 0.00005 % (1 part in $2 \cdot 10^6$) a slow reaction takes place as in the case of the other venoms. The inactivity of cobra venom in higher concentration is possibly related to the protective action of excess of this particular venom on the haemolysis of erythrocytes [Kellaway and Williams, 1933]. This inhibition occurs to a negligible extent with the Australian venoms. It is possible that there is a marked preferential adsorption of the protein portion of cobra venom on a lecithin film, and at a concentration sufficient to form a complete layer of protein at the surface the lecithinase is prevented from reaching the lecithin. It is indeed found that the presence of such a quantity of cobra venom will almost completely inhibit the attack of a lecithin film by other normally active venoms such as black tiger or daboia. Again, addition of an equal weight of egg albumin to black tiger venom reduces its rate of attack on a lecithin film tenfold.

Another anomaly of cobra venom has recently been stressed by McFarlane and Barnett [1934], namely its anticoagulant action on plasma, as opposed to the strong coagulant action of Russell's viper (daboia) venom and certain other venoms. It is significant that attempts to separate the toxins from the coagulant principle in these cases have so far failed. There is thus a possibility of a close relation between the coagulant and the lecithinase.

Pre-haemolytic swelling.

In cases of venom haemolysis a large pre-haemolytic swelling of the erythrocytes is usually observed. If the first stage of haemolysis is indeed the liberation of lysolecithin in the lipoidal surface layer of the cell membrane one would anticipate a considerable increase in area since the area per hydrocarbon chain in lysolecithin films is nearly double that obtaining in a lecithin film at the same state of compression. The more expanded nature of the film thus formed would be accompanied by an increased fragility and permeability.

SUMMARY.

1. The physico-chemical properties of snake venom have been examined through its reaction with unimolecular films, particularly lecithin films. Five varieties of venom were studied: black snake, black tiger, copperhead, daboia and cobra.
2. The rate of hydrolysis of a unimolecular film of lecithin to lysolecithin by venom lecithinase is dependent on p_H , surface concentration of lecithin molecules and the venom concentration. The p_H optimum is about 7.3. Compression of the lecithin molecules greatly decreases the rate of hydrolysis. Venom concentrations as low as 1 part in 40 millions are detected by the method of surface potentials.
3. The lecithinase is stable to prolonged boiling at p_H 5.9, but is rapidly destroyed on boiling in solutions more alkaline than p_H 7.0.
4. Haemolysis conducted concurrently with experiments on surface films shows a direct relation between haemolysis and lecithinase content as measured by rate of attack on a lecithin film.
5. Anomalies of cobra venom are discussed.

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