HAEMORRHAGIC ACTIVITIES OF HABU SNAKE VENOM, AND THEIR RELATIONS TO LETHAL TOXICITY, PROTEOLYTIC ACTIVITIES AND OTHER PATHOLOGICAL ACTIVITIES

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THE most striking effects produced by the parenteral injection into animals of many snake venoms, especially of Crotalidae venoms, are local, consisting of haemorrhage, necrosis, and oedema (van Heyningen, 1954 ; Slotta, 1955). Mitsuhashi *et al.* (1959) reported that the pathological signs characteristic of Habu (*Trimeresurus flavoviridis*) bite are haemorrhage and necrosis, and that necrosis is severest in areas rich in muscular tissue.

Haemorrhage and necrosis are believed to be due, at least in part, to the action of proteolytic enzymes. Houssay (1930) and others (Kellaway, 1939 ; Zeller, 1948 ; Porges, 1953 ; van Heyningen, 1954 ; Kaiser and Michl, 1958 ; Maeno and Mitsuhashi, 1958) considered that proteolytic enzymes were mainly responsible for haemorrhage and necrosis at the site of the bites, while Slotta (1955) regarded them as being attributable mainly to the joint action of proteases and phosphatases.

The concept generally accepted that the effect of snake venom is manifest either locally or systemically does not hold true, because many venoms, including Habu venom, have both local and systemic elements. For elucidation of the pathological changes in animals caused by snake venom, not only local effects such as haemorrhage, necrosis and oedema but also systemic or lethal effects should be taken into consideration. It has been reported that the lethal toxicity of this venom was independent of such enzyme activities as phospholipase A, phosphodiesterase, 5′-nucleotidase and proteinase (with casein as substrate), which had been claimed to possess toxicological significance (Ohsaka, 1958 and 1960).

Here attention is concentrated on the following two points : firstly to elucidate the chemical nature of the principle responsible for haemorrhage or necrosis and secondly to show whether or not the principle responsible for the local effects also has the lethal toxicity or systemic effect. However, difficulties both in the quantitative estimation of haemorrhagic and necrotic activities and in the strict chemical separation of individual components have hindered solution of these problems.

Recently we have established a quantitative method for estimation of haemorrhagic activity—one of the most striking local actions of Habu venom (Kondo, Kondo, Ikezawa, Murata and Ohsaka, 1960). It consists of intracutaneous injections of venom into the depilated back skin of rabbits, a special device for accurate measurement of haemorrhagic areas and application of the parallel line assay method for the estimation. This has made it possible to isolate and charac-

terize the principle responsible for haemorrhage and to test the relation of haemorrhage to proteolytic activities and other pathological changes.

In the present work the venom of Habu (*Trimeresurus flavoviridis*) was fractionated on a large scale by zone electrophoresis employing starch as a supporting medium in an attempt to elucidate the relationships of haemorrhagic activity to lethal toxicity, proteolytic and other pathological activities.

MATERIALS AND METHODS

Snake venom.—The venom used was a dried and powdered pool (Batch No. 48) collected in Amami Oshima, a southern island of Japan, in 1948 from a species of Habu, *Trimeresurus flavoviridis* (Hallowell). The venom was reconstituted to the desired potency in physiological saline solution immediately before use.

Substrate.—Purified casein was purchased from Merck and Co., Inc., Rahway, N.J., U.S.A.

Experimental animals.—An inbred strain of white mice (general purpose colony, Yoken) of both sexes weighing 14–17 g. were used to determine lethal toxicity. Commercial adult white rabbits of both sexes weighing about 2·5 kg. were employed to determine the haemorrhagic activity.

Estimation of protein.—The protein content of the venom solution was estimated in terms of its ultra-violet absorption at 280 mμ in the 1 cm. cell of a Beckman DU spectrophotometer.

Estimation of proteolytic activity.—The proteolytic activity on casein was estimated by the modified method of Kunitz (1946), as previously described (Ohsaka, 1960). The reaction mixture contained 0·5 ml. of 4 per cent casein dissolved in 0·1 M disodium phosphate solution, 1·0 ml. of enzyme solution and 0·5 ml. of 0·2 M phosphate buffer (pH 7·5). After 10 min. incubation at 35°, the reaction was stopped by the addition of 2·0 ml. of 0·4 M trichloroacetic acid. The extent of digestion was evaluated by determination of the absorption at 280 mμ of the trichloroacetic acid filtrate.

Lethal toxicity.—Lethal toxicities were assayed by intravenous injections of mice with 4–5 doses of each fraction increasing by 1·4-fold steps in volumes of 0·2–0·5 ml. Five animals were injected with each dose. The range of dilutions inoculated covered from 0 to 100 per cent mortality. All deaths during the 4 days following injection were ascribed to venom toxicity, although most of the deaths occurred before 24 hr. The LD_{50} was calculated by the Reed-Muench method. The standard error of the LD_{50} was calculated according to Pizzi (1950), the fiducial limits of which are 30 per cent for a probability of 0·05. The LD_{50} of the crude venom used (Batch No. 48) was 61 μg. $\pm$ 3 μg. as estimated by a statistical analysis of the results from titrations repeated 30 times, carried out in the above-described manner.

Assay of haemorrhagic activity.—The haemorrhagic activity was assayed by the method previously described (Kondo *et al.*, 1960). Series of 4–5 doses of 0.1 ml. of each fraction graded with 3-fold intervals were injected intracutaneously into the depilated back skin of 2–3 rabbits weighing about 2·5 kg. The rabbits were killed by chloroform inhalation 24 hr. later and the skins were removed immediately. The skin was spread and fixed on a glass plate so as not to distort the original size. The cross-diameters of each haemorrhagic spot were measured from the visceral side of the skin through the glass plate and the mean of the 2 diameters was taken as an indicator of the intensity of the response. The results were analysed statistically. As previously described (Kondo *et al.*, 1960) the minimum haemorrhagic dose (MHD) of venom was defined as the least quantity of venom, which caused a haemorrhagic reaction 10 mm. in diameter 24 hr. after intracutaneous injection under the above conditions. The MHD of the crude venom used (Batch No. 48) was approximately 0·20 μg., its fiducial limits being $\pm$25 per cent for a probability of 0·05.

Histological examination.—In each experiment 0·1 ml. series of several doses of several fractions increasing by 3-fold steps were injected intracutaneously into the depilated back skin of one rabbit. The rabbit was killed 24 hr. after injection by chloroform inhalation and blocks of tissue were taken from the skin. These were fixed in Orth's solution and embedded in paraffin. Sections were cut at a thickness of about 3 μ and stained with Carazzi's haematoxylin and eosin.

Zone electrophoresis.—Zone electrophoresis in a column using starch as the supporting medium was carried out in a vertical-type apparatus with a few of the modifications of Flodin and Porath (1954). Commercial potato starch was thoroughly washed and treated with buffer (Ohsaka, 1958). Runs were made in borax-NaOH buffer of pH 9·20 and 0·1 ionic strength in a cold room with an ambient temperature of 2–4°. The column consisted of a plastic tube 120 cm. long and 3·6 cm. in diameter with an outer cooling jacket (diameter 5 cm.) along its whole length. Water from a thermostatic bath circulating through the jacket might have substituted for the cold room treatment. The starch packing was supported by 2 sheets of filter paper (Toyo Roshi No. 26, for the filtration of oils) which were fixed at the base of the column by a close-packed screwed plug. The total length of the packed column was 114·5 cm., the cross-sectional area 10·2 cm.^{2} and the ratio of starch volume/mobile liquid volume 2·0. The hold-up volume was 1164 ml. The sample of crude venom amounting to about 2·0 g. in 20 ml. of physiological saline, was introduced in the starch stratum of about 5·9 cm. in thickness at the height of 87 cm. The anode was connected to the bottom of the packed column through the tube filled up with buffer. A current of 20 mA with a potential drop of about 380 volts was passed for about 75 hr. At the end of the electrophoresis the starch column was eluted with the same buffer at a flow rate of 31·8 ml./hr. and 10·6 ml. fractions were collected by a fraction collector. The amount of protein, the proteolytic activity, the lethal toxicity and the haemorrhagic activity in each fraction was determined.

RESULTS

Evidence for the presence of two haemorrhagic activities and their relations to lethal toxicity

The elution diagram of Habu venom subjected to zone electrophoresis in a packed starch column is presented in Fig. 1 and 2. The electrophoretic conditions are given in Fig. 1 and the arrow represents the place where crude venom was applied. The pattern was reproduced in a duplicate experiment. The protein recovery was 97 per cent. The distribution of haemorrhagic activity, lethal toxicity and proteolytic activity on casein in each fraction were surveyed with the results shown in Fig. 1 and 2. In Table I are recorded the haemorrhagic activity of each fraction and its fiducial limits. The total recovery was 71 per cent (57–89 per cent) for haemorrhagic activity and 71 per cent for lethal toxicity.

It is evident in Fig. 1 that the venom contained at least 2 haemorrhagic fractions. For the sake of convenience, the larger peak was designated HR1 and the smaller one HR2. Both peaks were located apart from each other on the starch column under the conditions used here : HR1 peak was found in fraction No. 22 and HR2 peak in fraction No. 14. The recovery was 46 per cent for HR1 and 25 per cent for HR2. Calculating from Table I the specific activity expressed as its ratio to that of the crude venom was 1·4 for HR1 and 3·2 for HR2 peak.

The difference between HR1 and HR2 in the electrophoretic mobility was further confirmed by subsequent electrophoresis when the 2 fractions were introduced separately into parallel troughs of the same size (Ohsaka *et al.*, 1960).

Fig. 1 also demonstrates that HR2 can be separated from the main lethal toxicity peak (Fractions No. 17–26) while it is overlapping the minor part of lethal toxicity (Fractions No. 11–16). Therefore, it is evident that HR2 is unrelated to, at least the main part of, lethal toxicity.

As for HR1, Fig. 1 proves that the peak is overlapping the main lethal toxicity peak.

Relations of haemorrhagic activity to proteolytic activity on casein

Fig. 2 shows that this venom contained at least 5 electrophoretic components

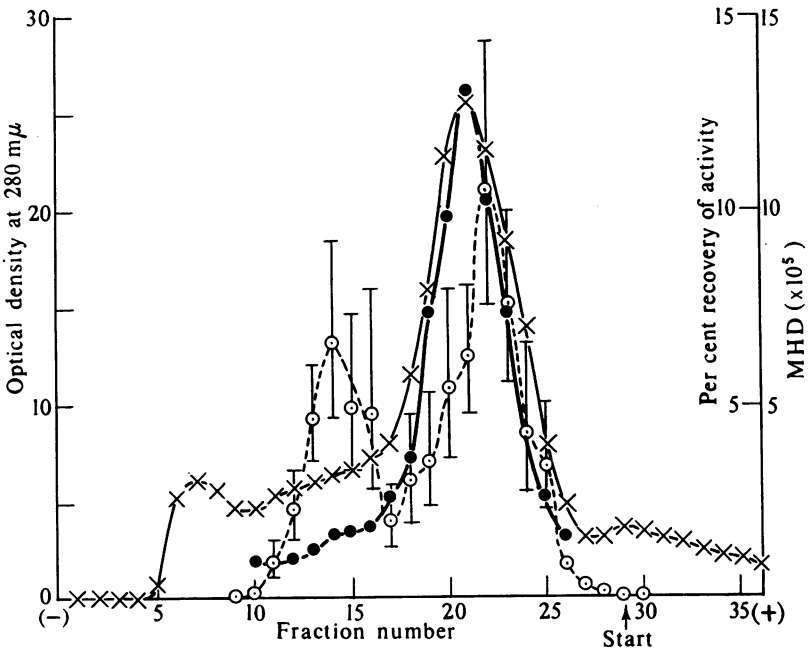


FIG. 1.—Distribution of protein (×————×), haemorrhagic activity (○- - - - -○) and lethal toxicity (●————●) in eluate from column after zone electrophoresis. A quantity of 2·0 g. of Habu venom was subjected to electrophoresis in a column of 3·6 × 114·5 cm. packed with starch. The run was made in borax-NaOH buffer of pH 9·20 and 0·1 ionic strength in a cold room (2–4°). A current of 20 mA with a potential difference of 380 volts was passed for about 75 hr. 10·6 ml. fractions were collected.

having proteolytic activity on casein (Ohsaka, 1960). The total recovery of proteolytic activity was 100 per cent with casein. The possibility that the 5 electrophoretic components represent distinct enzymes was investigated and reported separately (Ohsaka, 1960); where it was shown that these components included at least 3 distinct enzymes.

It is evident that both HR1 and HR2 were separable from the peak of proteolytic activity on casein as found in fraction No. 17. Distribution of proteolytic activity on " azocoll " lacked a peak in fraction No. 22 (Ohsaka, 1960). Therefore, HR1 must be independent of proteolytic activity on " azocoll " (Oakley, Warrack and van Heyningen, 1946). As can also be seen in Fig. 2, however, HR1 and HR2 peaks coincided with those of proteolytic activity on casein as were found in fraction No. 22 and fraction No. 14, respectively, suggesting the possibility that haemorrhagic activity might be correlated with the proteolytic activity on casein.

Relations of muscle degenerating effect to haemorrhagic activity and proteolytic activity on casein

Fig. 3 and 4 show the typical histopathological changes produced in the back skin of rabbits 24 hr. after intracutaneous injection of various amounts of crude Habu venom.

TABLE I.—*Haemorrhagic Activity of Each Fraction Separated by Starch Column Electrophoresis*

Electrophoretic run is the same as described in Fig. 1. Fraction No. in this Table is also the same as in Fig. 1. Haemorrhagic activity is expressed by MHD units. Specific activity of haemorrhagic activity is expressed by MHD per μg. protein. Fiducial limits of each estimation are also shown.

Fraction No.	Number of spots observed	Specific activity (MHD/μg protein)	Activity recovered and its fiducial limits (10^5 MHD/fraction)
Starting material (crude venom 2·0 g.)	30	5·00	100 (80–125)
9	4	0·00735	0·00213 (0·00127–0·00358)
10	4	0·358	0·105 (0·0625–0·176)
11	4	2·50	0·877 (0·522–1·48)
12	7	6·25	2·25 (1·51–3·32)
13	16	11·8	4·61 (3·54–6·00)
14	9	16·1	6·54 (4·64–9·22)
15	8	11·5	4·89 (3·26–7·33)
16	4	9·90	4·73 (2·81–7·94)
17	8	3.73	1·96 (1·30–2·94)
18	5	4·00	3·01 (1·90–4·76)
19	8	3·33	3·51 (2·34–5·27)
20	7	3·57	5·38 (3·64–7·96)
21	17	3·70	6·20 (4·77–8·06)
22	10	6·90	10·5 (7·58–14·4)
23	11	6·25	7·55 (5·56–10·0)
24	6	4·61	4·22 (2·72–6·53)
25	7	6·54	3·38 (2·28–5·00)
26	18	2·57	0·815 (0·632–1·05)
27	3	1·37	0·275 (0·154–0·490)
28	4	0·524	0·105 (0·0625–0·176)
29	2	0·125	0·0304 (0·0145–0·0638)
30	2	0·0752	0·0180 (0·00857–0·0378)
		Sum	71·0 (56·8–88·7)

Fig. 3 shows a typical picture observed when 30 μg. of crude venom was injected. Haemorrhage and infiltration by polymorphonuclear leucocytes were usually observed in the corium. Accompanying muscular degeneration in the muscular layer was observed and in most instances, infiltration by polymorphonuclear leucocytes. Haemorrhage appeared in the muscular layer to an extent which depended upon the amount of venom injected, as exemplified in Fig. 4 where a larger amount of venom was inoculated. In short, haemorrhage in the corium and waxy degeneration in the muscular layer were usually observed. Muscular degeneration proceeded to necrosis or death of the muscle fibres in its severer forms (Fig. 4). These appeared to be the main characteristics of histopathological changes caused by Habu venom.

In order to examine the relationship of the muscle degeneration effect, which leads to necrosis in its severer forms, to haemorrhagic activity and proteolytic activity on casein, histopathological changes evoked in the rabbit skin by intracutaneous injection of several fractions were studied comparatively. These results are summarized in Table II.

It is apparent that an amount as small as 1–0·1 μg. of crude venom could cause haemorrhage (Fig. 5) whereas more than 3–10 μg. was needed to cause muscular degeneration (Fig. 6). The specific haemorrhagic activity in fractions

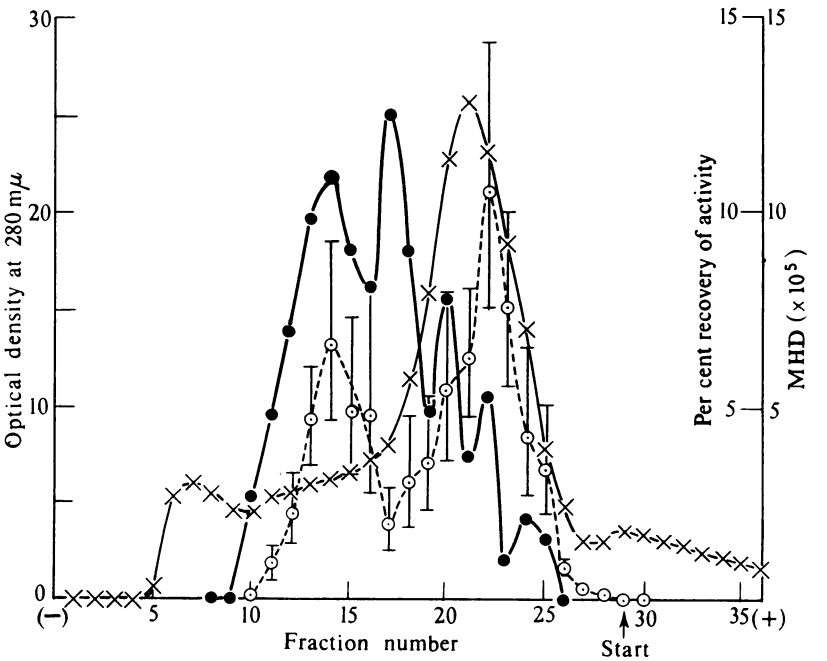


FIG. 2.—Distribution of protein (×————×), haemorrhagic activity (○- - - - -○) and proteolytic activity on casein (●————●) in eluate from column in the same electrophoretic run as in Fig. 1.

13 and 14 were 2·4 times and 3·2 times as large as in crude venom respectively. Nevertheless, they showed rather weaker muscle degenerating effects than crude venom (Fig. 7 and 8). Therefore, it is suggested that the muscle degeneration effect did not run parallel to haemorrhagic activity.

Table II also shows that the specific proteolytic activity on casein in fraction 13 was 5-fold and in fraction 14 5·4-fold larger than in crude venom. Yet, they showed rather weaker muscle degeneration effects (Fig. 7 and 8). On the other hand, despite the absence of detectable proteolytic activity on casein, fraction 26 showed very much the same muscle degeneration effect as crude venom (Fig. 9). This observation strongly suggested the absence of a parallel between the muscle degeneration effect and the proteolytic activity on casein.

DISCUSSION

The new quantitative method for estimating haemorrhagic activity proposed by us (Kondo *et al.*, 1960), coupled with electrophoretic fractionation on a large scale in a packed starch column, of Habu snake venom made it possible to study the principle responsible for haemorrhage.

The data presented (Fig. 1) indicate that at least 2 haemorrhagic fractions, different in electrophoretic mobility, were present in this venom (HR1 and HR2). Difference in mobility between HR1 and HR2 was confirmed by repeated electrophoresis (Ohsaka *et al.*, 1960).

TABLE II.—*Summary of Histopathological Changes Evoked in the Rabbit Skin by Intracutaneous Injection of Crude Venom as well as Several Fractions Separated by Starch Column Electrophoresis*

Electrophoretic run is the same as described in Fig. 1.

Amount of injection (μg. protein)	Histopathological changes									
	Crude venom (Pr = 1, Hr = 1)				Fraction No. 13 (Pr = 5·0, Hr = 2·4)		Fraction No. 14 (Pr = 5·4, Hr = 3·2)		Fraction No. 26 (Pr = 0, Hr = 0·5)	
	Exp. No. 1		Exp. No. 2		Exp. No. 1		Exp. No. 2		Exp. No. 1	
	H	MD	H	MD	H	MD	H	MD	H	MD
300 μg.	..	..	+	+	+	+	+	+	+	+
100	+	+	+	+	+	±	+	+	+	+
30	+	+	+	+	+	±	+	+	+	+
10	+	+	+	+	+	−	+	±	+	±
3	+	−	+	+	+	−	+	−	+	−
1	+	−	+	−	+	−	+	−	..	..
0·3	±	−	+	−	+	−	+	−	..	..
0·1	−	−	+	±	−	−	+	−	..	..
0·03	−	−	−	−	−	−	+	−	..	..

Pr : Specific activity of proteolytic activity on casein.
Hr : Specific activity of haemorrhagic activity.
H : Haemorrhage in histopathological picture.
MD : Muscular degeneration or necrosis of muscle fibres in histopathological picture.
+ : Presence of waxy degeneration or necrosis of muscle fibres ,or presence of haemorrhage.
± : Doubtful degeneration of a few muscle fibres or doubtful haemorrhage in the whole section of the examined tissue of the skin.
− : Without degeneration of muscle fibres or without haemorrhage.

EXPLANATION OF PLATES

Figs. 3–9 are all of the same magnification (×85).

FIG. 3.—Section of the rabbit skin inoculated with venom (1) : From Expt. 2 (crude—30 μg.). Muscle fibres show waxy degneration (MD) with infiltration of polymorphonuclear leucocytes (L) around them. Haemorrhage (H) is seen between muscle fibres. (H and E.) ×85.

FIG. 4.—Section of the rabbit skin inoculated with venom (2) : From Expt. 3 (crude—300 μg.). Almost all muscle fibres show the necrosis and dissolution. Diffuse infiltration of polymorphonuclear leucocytes (L) and remarkable haemorrhage (H) are found.

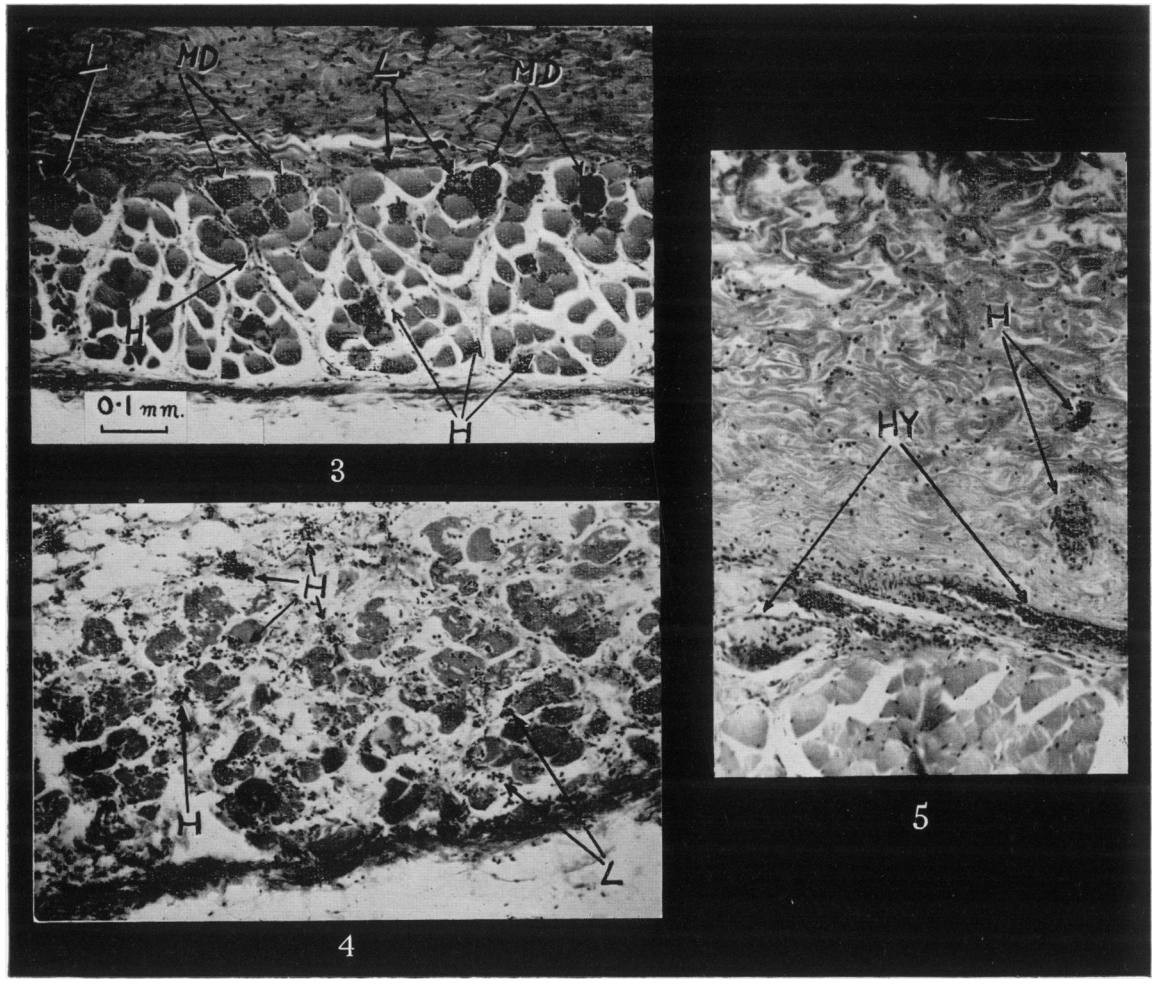
FIG. 5.—Section of the rabbit skin inoculated with venom (3) : From Expt. 2 (crude—0·1 μg.). Haemorrhage (H) is found in the corium and remarkable hyperaemia (HY) in the profound layer of the corium above the muscular layer. No muscle fibres show degeneration.

FIG. 6.—Section of the rabbit skin inoculated with venom (4) : From Expt. 2 (crude—3 μg.). A few muscle fibres show waxy degeneration (MD) with infiltration of polymorphonuclear leucocytes (L) in and around them. Haemorrhage (H) is seen between muscle fibres.

FIG. 7.—Section of the rabbit skin inoculated with venom (5) : From Expt. 1 (Fraction No. 13—100 μg.).
No muscle fibres show degeneration. But remarkable haemorrhage (H) between muscle fibres is found.

FIG. 8.—Section of the rabbit skin inoculated with venom (6) : From Expt. 2 (Fraction No. 14—10 μg.).
No muscle fibres show degeneration. But haemorrhage (H) between them is found.

FIG. 9.—Section of the rabbit skin inoculated with venom (7) : From Expt. 1 (Fraction No. 26—30 μg.).
A few muscle fibres show waxy degeneration (MD) with infiltration of polymorphonuclear leucocytes (L) around them. Haemorrhage (H) is seen between muscle fibres.



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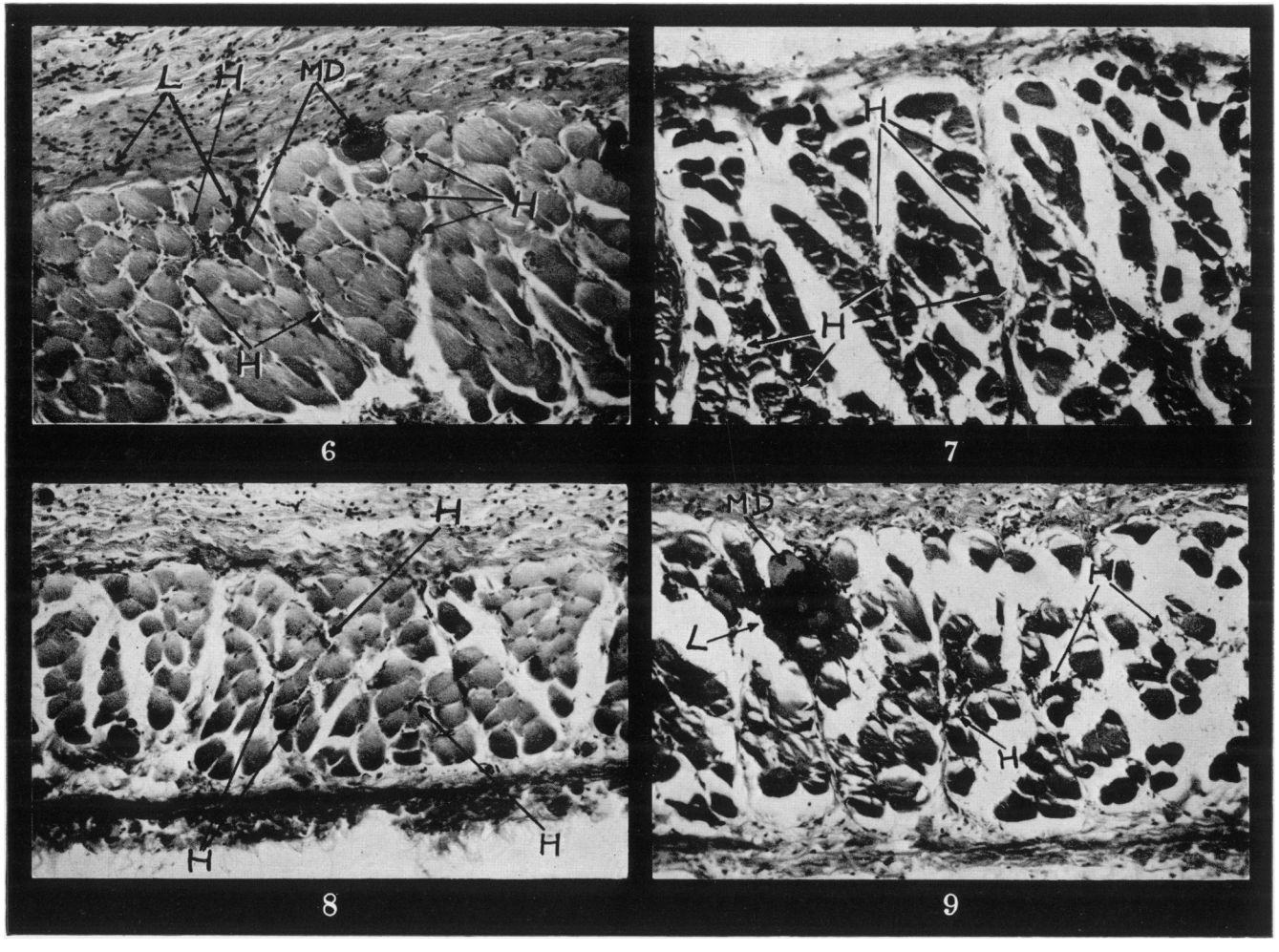

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It is an interesting problem whether or not the principle responsible for haemorrhage can also have the lethal toxicity. Fig. 1 shows that HR2 can be separated from the major part of lethal toxicity (fractions 17–26) while overlapping the minor part of lethal toxicity (fractions 11–16). Therefore, it is evident that HR2 has no relation with, at least the main part of, lethal toxicity. On the other hand, the HR1 peak is overlapping the main lethal toxicity peak (Fig. 1). However, a small difference in mobility between the HR1 and the main lethal toxicity peaks (Fig. 1) led us to entertain an idea that both might be separate entitites. Further work is required to establish the relationship of haemorrhagic activity to lethal toxicity.

Houssay (1930) and others (Kellaway, 1939 ; Zeller, 1948 ; Porges, 1953 ; van Heyningen, 1954 ; Slotta, 1955 ; Kaiser and Michl, 1958) considered that haemorrhage is due, at least in part, to the action of proteolytic enzymes. Recently Maeno and Mitsuhashi (1958) proposed that a proteinase (substrate casein) partially purified from Habu venom was the only factor responsible for haemorrhagic necrosis. Their claim, however, was not based on any quantitative evidence. To elucidate the relationship of haemorrhagic activity to proteolytic activity on casein, experiments were carried out on a quantitative basis. The results testified that both HR1 and HR2 could be regarded as being different from the proteolytic activity peak with casein as found in fraction 17 (Fig. 2). HR1 can be separated from the proteolytic activity on " azocoll ". However, the peaks HR1 and HR2 coincided with those (fractions 22 and 14) of proteolytic activity on casein (Fig. 2). It appears that a correlation exists between haemorrhagic activity and proteolytic activity on casein. However, it seemed that repeated electrophoresis could further fractionate HR2 into 2 components, one of which was separable from the proteolytic activity (Ohsaka *et al.*, 1960). Further studies are necessary to examine the possible correlation between haemorrhagic and proteolytic activities.

Histological observations indicated that the easily discernible pathological changes produced in the back skin of rabbits by intracutaneous injection of crude Habu venom were haemorrhage in the corium and muscular degeneration in the muscular layer. Muscular degeneration proceeded to necrosis or death of the muscle fibre in its severer forms.

It is of great interest whether or not the same principle is responsible for necrosis and haemorrhage. However, little is known concerning this point. Kaiser and Michl (1958) considered that venom causes severe necrosis at the site of the bite as a result of its proteolytic action on tissue and furthermore proteolytic enzymes, when very active, cause dissolution of small blood vessels, resulting in haemorrhage. In other words, they insinuated that one and the same principle was responsible for necrosis and haemorrhage. However, histopathological observations presented here indicated that the muscle degeneration effect, whose severer forms would be manifested as necrosis, did not run parallel to haemorrhagic activity (Table II).

Necrosis is also believed to be due, at least in part, to the action of proteolytic enzymes (Houssay, 1930 ; Kellaway, 1939 ; Zeller, 1948 ; Porges, 1953 ; van Heyningen, 1954 ; Slotta, 1955 ; Kaiser and Michl, 1958 ; Maeno and Mitsuhashi, 1958). However, Table II also showed that the muscle degeneration effect did not run parallel to proteolytic activity on casein.

Further purification of haemorrhagic and lethal principles are being continued.

SUMMARY

The venom of *Trimeresurus flavoviridis* (Habu), a crotalid, was fractionated on a large scale by zone electrophoresis in a packed starch column. Haemorrhagic activity, lethal toxicity and proteolytic activity in each fraction were determined quantitatively.

It was found that at least 2 haemorrhagic principles, designated as HR1 and HR2, were involved in this venom. HR2 could be separated from the main part of lethal toxicity. Both HR1 and HR2 were associated with proteolytic activity on casein.

Histopathological observations indicated that the muscle degeneration effect was related neither to haemorrhagic activity nor to proteolytic activity on casein, as far as herein tested.

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