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ACTION OF SNAKE VENOM HEMORRHAGIC PRINCIPLES ON ISOLATED GLOMERULAR BASEMENT MEMBRANE*

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SUMMARY

1. The reaction of venom hemorrhagic principles (HR1, HR2a and HR2b) with isolated glomerular basement membrane was studied *in vitro*. For comparison, experiments were also done with a bacterial collagenase which is known to induce the hemorrhage. Upon incubation of the basement membrane preparation with either of the four agents, its supernatant fluid gave positive reactions for proteins (or peptides) and carbohydrates. The optimal pH of the protein-liberating activities of HR1, HR2a and HR2b was around 8.0.

2. The approximate ratio of the proteins or peptides liberated by HR2a, HR2b, HR1 and collagenase, under standard conditions, was 1:1:2:4; and for the carbohydrates liberated 0.5:0.5:2:4, thus suggesting that HR2a or HR2b acted on the basement membrane in a different manner from HR1. This interpretation is supported by comparison between the split-products of the membrane by these principles in gel filtration experiments.

3. Liberation of both proteins and carbohydrates from basement membrane by HR1, HR2a and HR2b was inhibited by EDTA, cysteine, antivenin and anti-hemorrhagic factor from snake serum, all of which inhibit the hemorrhagic activity of these principles. The results suggest that one and the same entity is responsible for both the hemorrhagic and liberating activities in each venom principle.

4. Purified HR1 was resolved partially into four components by polyacrylamide gel electrophoresis. Both liberating and hemorrhagic potencies, as expressed by their specific activities, however, were constant throughout, which further supports the idea that the same factor is responsible for both activities.

5. The hemorrhagic effect of HR1, HR2a, HR2b and collagenase is attributed to enzymatic destruction of the basement membrane with consequent lowering of the stability of the vessel wall.

INTRODUCTION

Hemorrhage is one of the most striking manifestations evoked by the parenteral injection of crotalid and viperid venoms[1–3]. Recently Ohsaka *et al.* succeeded in

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isolating three hemorrhagic principles, HR1[4,5], HR2a and HR2b[5,6], from the venom of *Trimeresurus flavoviridis*, a crotalid. The purified HR1 contained some proteolytic activity on casein while HR2a and HR2b were free of this activity. Collagenolytic activity with native collagen was detected neither in the crude venom[7] nor in the purified hemorrhagic principles[5,6].

The venom hemorrhagic principles act on the microcirculatory system to produce true hemorrhage with no prior increase in vascular permeability[9,10]. The venom principle is distinct in its vascular effects (1) from histamine or bradykinin effects, which produce an increased permeability for albumin but not for erythrocytes; (2) from proteolytic enzyme (trypsin and chymotrypsin) effects, which produce massive leakage of albumin followed by hemorrhage[10].

To understand the mechanism of action of the hemorrhagic principles, it must be clarified biochemically and morphologically how the erythrocytes cross the lining endothelial cells and also the basement membrane[9].

The availability of the highly purified hemorrhagic principles, together with the recent development in isolation and chemical characterization of the glomerular basement membrane[11,12], prompted us to investigate the biochemistry of the *in vitro* action of the venom principles on the basement membrane. For comparison, experiments were also done with a bacterial collagenase known to induce the hemorrhage[10,13,14]. We demonstrated that Folin-positive and anthrone-positive materials were liberated from the basement membrane by the venom principles.

The present paper describes characteristics of the *in vitro* reaction between the venom principles and the basement membrane and some properties of the split-products of the membrane. Our results indicate that the basement membrane is damaged by enzymatic action of the venom principles and that the same entity is responsible for both the disruption of the membrane and the hemorrhagic activity.

A preliminary account of this work has appeared[15,29].

MATERIALS AND METHODS

Snake venom

The venom used (Batch No. 70-B) was a pool of dried venom taken from the specimens of Habu, *Trimeresurus flavoviridis*, collected in the Amami Oshima Islands in 1970 and donated by the Division of Public Health, Kagoshima Prefecture, Japan.

HR1, HR2a and HR2b

The purified HR1 was prepared from the venom of *T. flavoviridis* by Dr T. Omori-Satoh according to the method previously described[4]; purified HR2a and HR2b were prepared by Dr T. Takahashi by the method of Takahashi and Ohsaka[6]. Protein was estimated by measuring absorbance at 280 nm in a 1-cm cell. To convert $A_{280\ nm}$ to mg of venom protein per ml a factor of 1.20 was used.

Enzyme

Purified collagenase containing 600 units/mg [Clostridiopeptidase (EC 3.4.4.19)] was purchased from Worthington Biochemical Corporation, Freehold, N.J., U.S.A.

Antivenin

The antivenin used as a standard (Lot C-9) was prepared at the National Insti-

tute of Health of Japan from the serum of horses hyperimmunized by subcutaneous injections with a crude venom of *T. flavoviridis*. 1 unit of the antivenin neutralized 90 MHD of the preparation of HR1, where MHD is the least quantity of venom causing a hemorrhagic spot of 10 mm in diameter 24 h after intracutaneous injection into rabbits[16].

Antihemorrhagic factor from snake serum

An antihemorrhagic factor was isolated from the serum of *T. flavoviridis* by Dr T. Omori-Satoh by the method of Omori-Satoh *et al.*[8]. 1 unit of the purified factor, as determined relative to the standard antivenin[8], neutralized 90 MHD of the preparation of HR1.

Chemicals

Sephadex G-25 and G-50 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, "Hide Powder Azure" (B-grade) from Calbiochem., Los Angeles, U.S.A., Temed (*N,N,N',N'*-tetramethylenediamine) and cyanogum (acrylamide + *N,N'*-methylenebisacrylamide) from Serva Entwicklungslabor, Heidelberg, Germany. All other chemicals were of analytical reagent grade.

Estimation of protein

Protein was estimated by the method of Lowry *et al.*[17], with bovine serum albumin as standard.

Determination of proteolytic activity

(*a*) *Gelatine plate method.* Gelatinolytic activity was determined on a photographic plate (Kodak Fine Grain Autoradiographic Stripping Plate AR 10, 12.1 cm $\times$ 16.5 cm). 10 μl of 0.05 M barbital buffer (pH 8.0) was added to the plate together with an equal volume of serial dilutions of test sample in 0.85% NaCl. The plate was incubated for a specific period of time (usually 6 h) in a moist chamber maintained at 37 °C. The plate was washed carefully with water 2–3 times to remove the solubilized gelatine film. Great care was taken to avoid the mechanical destruction of the partially solubilized film. Gelatinolytic activity was expressed as the highest dilution of the test sample showing a completely solubilized spot. This method, although semi-quantitative, is sensitive enough to detect minute amounts of proteolytic enzyme. The method was used for screening the activity in eluates from polyacrylamide gel segments.

(*b*) "*Hide Powder Azure*" *method.* Proteolytic activity on "Hide Powder Azure" was assayed by the method of Steyn and Delpierre[18] with some modifications to increase the sensitivity. The 8-ml tube capped with a bulb contained substrate (10 mg) suspended in 1.0 ml of 0.05 M barbital buffer (pH 8.0), 0.5 ml of an HR1 solution and 10 μl of 0.01% merthiolate. After incubation at 37 °C for 6 h or longer with constant shaking, the mixture was diluted with 2.5 ml of water and immediately filtered. The filtrate was read spectrophotometrically at 595 nm against a blank. The intensity of the color released was directly proportional to HR1 concentration in a range tested from 2 to 33 μg per ml reaction mixture; a crude venom at 33 μg/ml completely solubilized the substrate giving $A_{595\ nm}$ of 1.13 in a 1-cm cell. The experimental error of this assay was within $\pm$6.5%.

Determination of hemorrhagic activity

Hemorrhagic activity was determined according to the method of Just *et al.*[10] with the following modifications.

Prior to the application of test samples, a rabbit received intravenously ^{51}Cr-labeled erythrocytes from 16–18 ml blood together with Evans Blue in a dose of 20 mg/kg, but no ^{125}I-labeled albumin. 10 min later test samples, in a volume of 100 μl instead of 25 μl, were injected intracutaneously into the skin of the rabbit. Five injections were made with each sample; ten injections with physiological saline. HR1 in a dose of 3 μg/ml and 5 μg/ml was always injected for reference. The animals were sacrificed 24 h after injection, and the skin was removed; pieces of skin at the site of injections were weighed and measured for radioactivity.

The mean value for cpm/mg, calculated from 10–20 pieces, of uninjected skin was taken as background radioactivity. The hemorrhagic activity was expressed in terms of net counts: net counts = cpm (sample) − [cpm/mg (background) · mg (sample)] and the average net counts from five injections with each sample were calculated.

Isolation of glomerular basement membrane

For each isolation 20–25 male rats, Wistar strain weighing 200–300 g, were anesthetized by intraperitoneal injection with 5-ethyl-5-(1-methylpropyl)-thiobarbituric acid (Inactin®) in a dose of 100 mg per kg body weight, and the animals were bled from the A. abdominalis. Glomeruli were enriched first by the method of Fong and Drummond[19]. Then the basement membranes were isolated by the method of v. Bruchhausen and Merker[20] as modified by Frimmer *et al.*[21]. The basement membrane fraction was washed three times with 20 ml Tyrode solution (pH 7.5), without any glucose, suspended in 20 ml of the same medium and stored at −20 °C until used. The preparations were occasionally examined in the electron-microscope and found to consist of mainly intact basement membranes.

Before use the stock suspension of the basement membrane was treated with an ultrasonicator (Sonifer, Branson, Model 875) for 20 s at 120 kcycles/s, washed twice and suspended in 20 ml of the same medium. Total protein content of the basement membrane, derived from a pair of kidneys, was 1.20 ± 0.52 mg, determined by the method of Lowry *et al.*[17].

Reaction of hemorrhagic principles with basement membrane

Basement membrane suspensions were diluted in a Tyrode solution without glucose. The reaction mixture in a microtest tube (Eppendorf) consisted of 0.5 ml of a basement membrane suspension (pH 7.5), containing 650 ± 195 μg membrane protein depending on the preparation, and of 0.2 ml of the sample in physiological saline. The reaction mixtures in duplicate were incubated for 6 h at 37 °C with constant shaking and centrifuged for 5 min at 7000 × *g*. Under standard conditions, a final concentration of 8.57 μg/ml of the sample was used.

Folin-positive material was determined with an 0.1-ml aliquot of the supernatant fluid according to Lowry *et al.*[17]; the quantity of the peptides or proteins liberated was expressed as μg bovine serum albumin equiv. liberated per mg membrane protein or as a percent of the total protein present in the original basement membrane. Anthrone-positive material was also determined with an 0.5-ml aliquot of the supernatant fluid according to Spiro[22]; the quantity of carbohydrates was ex-

pressed as μg glucose equiv. liberated per mg membrane protein. In each determination, duplicated controls containing only hemorrhagic principles or basement membrane suspension were subtracted.

Polyacrylamide gel electrophoresis of HR1

Electrophoresis in 3.375% polyacrylamide gel, pH 8.9 (gel size, 0.55 cm $\times$ 10.2 cm) was performed in the buffer system described by Maurer[23], but both sample and spacer gels were omitted according to Hjertén *et al.*[24]. In order to ensure the complete removal of ammonium persulfate, gels were subjected to electrophoresis for 2 h at 3 mA/gel in two changes of the electrode buffer (pH 8.9). 100 μl of a sample solution containing 100 μg of HR1, added with sucrose was layered onto the top of each gel. Electrophoresis was run with eight gels at 1 mA/gel for 10 min, 2 mA/gel for 10 min, and 3 mA/gel for 260 min. Two gels were stained with 1% amido black 10B in 7% acetic acid and scanned at 578 nm for analysis of the proteins (Eppendorf spectrophotometer connected with Eppendorf compensation recorder, model 4412). The remaining six gels were divided into 2 groups; each gel was cut into 75 sections (1.37-mm wide). Corresponding sections from 3 gels were combined and eluted with 0.7 ml or 0.8 ml of 0.85% NaCl with constant shaking for at least 5 days. Eluates (0.8 ml each) from one group were analysed for protein-liberating activity; those (0.7 ml each) from the other group were tested for both hemorrhagic activity and proteolytic activity with "Hide Powder Azure".

RESULTS AND DISCUSSION

Recent electron-microscopic studies of the vascular endothelial cells treated with venom suggest that the erythrocytes "spurt" through a junction of the endothelial cell lining (Ohsaka, A., Suzuki, K. and Ohashi, M., unpublished). On the other hand, McKay *et al.*[27] reported that the erythrocytes traversed the cytoplasm of the endothelial cells. In either event the basement membrane adjacent to the endothelial cells was destroyed.

We undertook experiments to test the possibility that the venom hemorrhagic principles can act directly on isolated basement membrane, in the hope that it would provide information on the mode of action of venom principles *in vivo*. For comparison, experiments included use of bacterial collagenase (a mixture of several collagenolytic enzymes)[28], which is known to induce the hemorrhage[10,13,14].

We demonstrated that proteins (or peptides) as well as carbohydrates were liberated from the basement membrane by all these venom principles.

Characteristics of the in vitro reaction between venom hemorrhagic principles and basement membrane

Figs 1a and 1b show the time course of protein and carbohydrate liberation, respectively, from the basement membrane by the venom principles and bacterial collagenase at a given concentration. The bacterial collagenase liberated protein as well as carbohydrate at a much greater rate and to a greater extent than did the venom principles. With the collagenase, the liberation reached a plateau following 6 h of incubation. HR2a and HR2b, showing liberation characteristics similar to each other, acted on the membrane at a much slower rate and to a lesser extent than

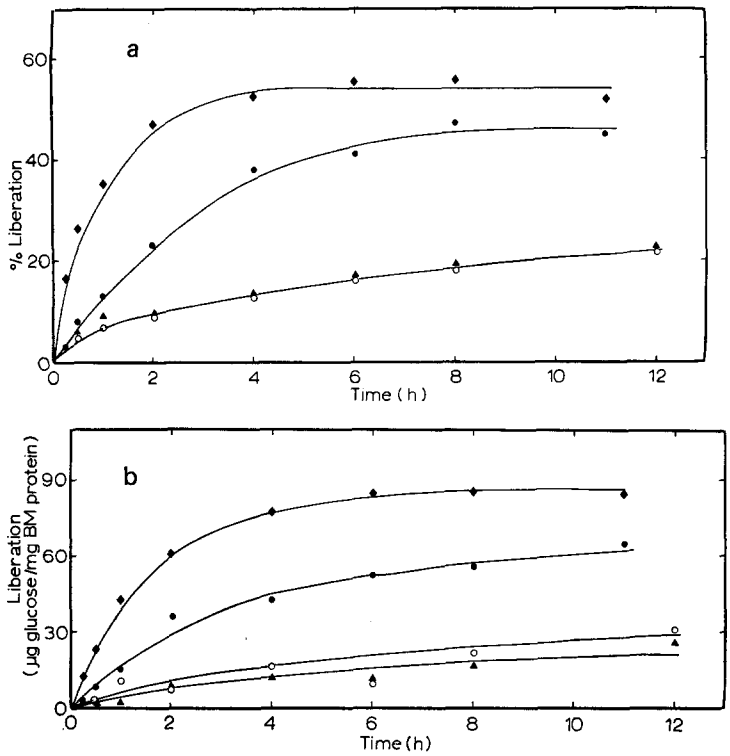

Fig. 1. Time course of liberation of Folin-positive (a) and anthrone-positive (b) material from basement membrane exposed to hemorrhagic principles. A mixture containing 0.5 ml of a basement membrane suspension at pH 7.5 and 0.2 ml of a solution of each hemorrhagic principle at a final concentration of 8.57 μg/ml was incubated at 37 °C. ●—●, HR1; ○—○, HR2a; ▲—▲, HR2b; ◆—◆, collagenase.

did collagenase; the liberation of neither proteins nor carbohydrates reached a plateau in 12 h. HR1 showed liberation characteristics intermediate between collagenase and HR2a or HR2b. HR1 effect did not reach a plateau within 12 h.

Since the liberation of split-product of basement membrane by collagenase reached a maximum in 6 h and the liberation by venom principles went on rather slowly after this period, the incubation of basement membrane with hemorrhagic principles at the specified concentration (8.57 μg/ml) for 6 h at 37 °C was designated as standard conditions for preparation of the split-products.

Table I shows the mean values of digestion by individual hemorrhagic principles of basement membranes from different batches. The approximate ratio of proteins liberated by different hemorrhagic principles (HR2a:HR2b:HR1:collagenase) was 1:1:2:4; but the ratio of carbohydrates liberated (HR1:collagenase) was 1:2. Liberation of carbohydrates by HR2a and HR2b was at its minimum level of detection. It is evident, however, that carbohydrates liberated by these principles were less than half (roughly one fourth) of the quantity liberated by HR1. These results suggest that HR2a or HR2b acted on the basement membrane by a mode of action different than HR1. These results suggest that HR2a or HR2b acted on the basement membrane by a mode of action different than HR1.

TABLE I

MEAN EXTENT OF DIGESTION OF VARIOUS PREPARATIONS OF BASEMENT MEMBRANE AFTER EXPOSURE TO EACH HEMORRHAGIC PRINCIPLE UNDER THE STANDARD CONDITIONS

The average protein content of basement membrane in a reaction mixture was 650 μg $\pm$ 195 μg (n = 22). Extent of digestion was expressed in terms of Folin-positive and anthrone-positive materials liberated.

	Material liberated per mg basement membrane protein	
	μg protein (bovine serum albumin equiv.)	*μg carbohydrate (glucose equiv.)*
HR1	326 $\pm$ 80 (n = 10)	43.4 $\pm$ 8.7 (n = 15)
HR2a	172 $\pm$ 53 (n = 13)	8.6* $\pm$ 4.4 (n = 11)
HR2b	181 $\pm$ 44 (n = 13)	9.5* $\pm$ 2.6 (n = 11)
Collagenase	615 $\pm$ 110 (n = 11)	88.1 $\pm$ 13.9 (n = 7)

* Just on the level of detection.

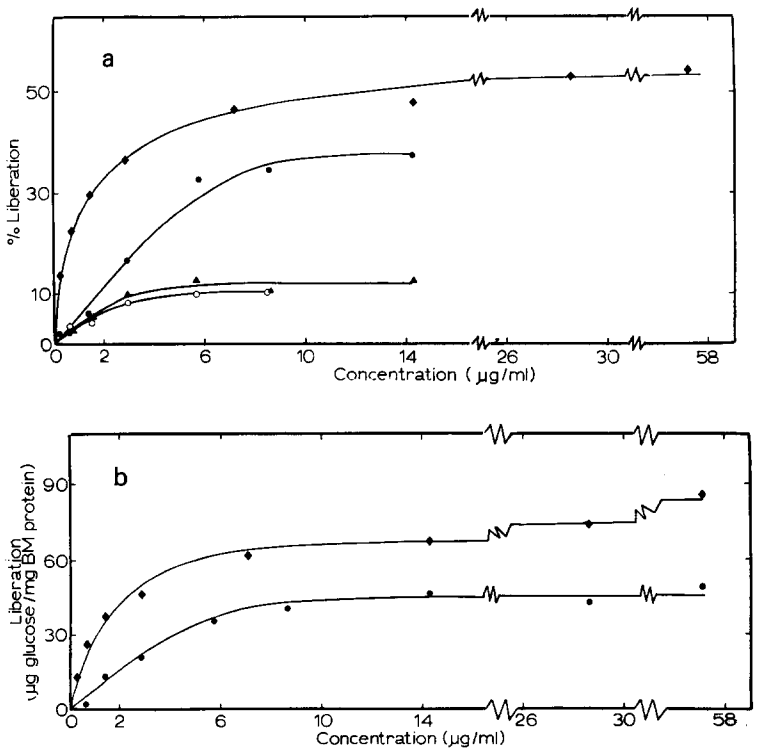

Fig. 2. Effects of varying concentrations of hemorrhagic principles on liberation of Folin-positive (a) and anthrone-positive (b) materials from basement membrane. The basement membrane was incubated with each hemorrhagic principle at the indicated final concentrations for 6 h at 37 °C. ●—●, HR1; ○—○, HR2a; ▲—▲, HR2b; ◆—◆, collagenase.

Effects of varying concentrations of hemorrhagic principles on the liberation of proteins and carbohydrates are shown in Fig. 2a and 2b, respectively. As expected (see Fig. 1) the maximum extent of liberation was different with different agents. With all agents the maximum liberation was attained by about 6 μg/ml. The quantity

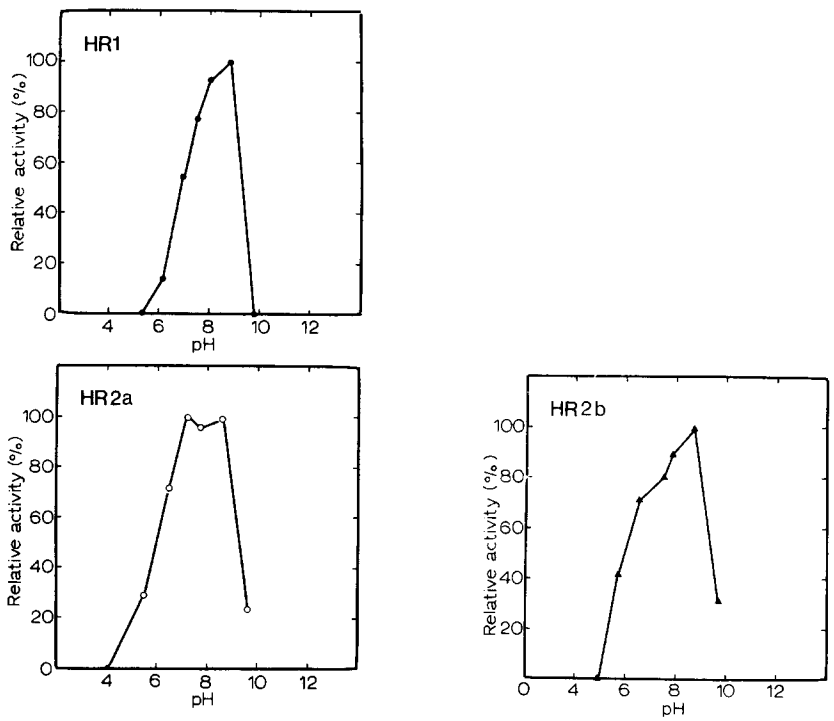

Fig. 3. pH dependence of the liberation of Folin-positive material from basement membrane exposed to venom hemorrhagic principles. HR1, HR2a and HR2b were allowed to react with the basement membrane under the standard conditions except that the pH value of the reaction mixture was varied by the addition of diluted solutions of either HCl of NaOH.

of carbohydrate liberated by HR2a or HR2b in a concentration range from 0.5 to 14 μg/ml, was just detectable and subject to much variation, and, therefore, results are not shown in Fig. 2b.

The pH dependence of liberation of proteins from basement membrane exposed to the venom hemorrhagic principles is relatively steep (Fig. 3). Optimal pH was around 8.0 for all the principles; at pH values lower than 5.0 and higher than 9.0, none of the principles acted on the basement membrane. This optimum pH is in agreement with the reported pH stability of the hemorrhagic activity possessed by these principles[4,6].

Previous reports have demonstrated that EDTA[4,6], cysteine[4,6], antivenin[25] and antihemorrhagic factor from snake serum[8] inhibit the hemorrhagic activity of the venom hemorrhagic principles. It was of interest, therefore, to study whether or not these hemorrhagic inhibitors also inhibit the *in vitro* reaction between the venom principles and the basement membrane.

TABLE II

EFFECT OF HEMORRHAGIC INHIBITORS ON THE LIBERATION OF FOLIN-POSITIVE (F) AND ANTHRONE-POSITIVE (A) MATERIALS FROM BASEMENT MEMBRANE EXPOSED TO HEMORRHAGIC PRINCIPLES

Each hemorrhagic principle was allowed to react with the basement membrane under the standard conditions except that the reaction mixture contained a given inhibitor at the indicated final concentration.

Inhibitor added	*HR1*		*HR2a*		*HR2b*		*Collagenase*	
	*F**	*A***	*F*	*A*	*F*	*A*	*F*	*A*
None	32	53	16	7	17	9	64	90
EDTA, 0.1 mM	5	13	0	0	0	0	18	—
1.0 mM	0	0	0	0	0	0	27	—
10.0 mM	0	0	0	0	0	0	21	—
Cysteine, 0.02 mM	8.4	15.5	8.4	7	9.5	8	61	114
0.2 mM	0.3	1.6	0	1.2	0.7	0.4	22	22
2.0 mM	0.8	0	0	0.8	0.8	0	8	0.9
Antivenin, 50 units/ml	0	9.6	0	0	0	0.4	—	—
100 units/ml	0	1.3	0	0	0	1.7	—	—
Antihemorrhagic factor from snake serum, 12 units/ml	3	0.7	1	0.5	0.3	1.4	75	108

* Proteins (bovine serum albumin equiv.) liberated in % of total basement membrane protein.
** μg carbohydrates (glucose equiv.) liberated/mg basement membrane protein.

As shown in Table II, the liberation of both proteins and carbohydrates from basement membrane by all the venom principles was inhibited almost completely by 1.0 mM of EDTA, 0.2 mM of cysteine, 50 units/ml of antivenin and 12 units/ml of antihemorrhagic factor. The concentrations of the inhibitors required to abolish the liberating activity *in vitro* were similar for inhibiting the hemorrhagic activity on animal skin (see Materials and Methods)[4,6].

The results suggest that one and the same entity is responsible for both hemorrhagic and liberating activities possessed by individual venom principles.

It might be noted in Table II that the liberating activity of collagenase was inhibited by cysteine whereas no inhibition was produced by antihemorrhagic factor; some inhibition of protein liberation was caused by EDTA without showing any concentration dependence. This enzyme is known to be inhibited by EDTA[26] and cysteine[26].

Relationship of liberating activity to hemorrhagic and proteolytic activities possessed by the purified preparation of HR1

In spite of its high degree of homogeneity proved by several criteria[4,5], the purified HR1 was partially resolved into four components by polyacrylamide gel electrophoresis (Fig. 4, lower). We attempted to establish the relationship of liberating activity to hemorrhagic and proteolytic activities in the preparation of HR1. We demonstrated that both liberating and hemorrhagic activities, as expressed by their

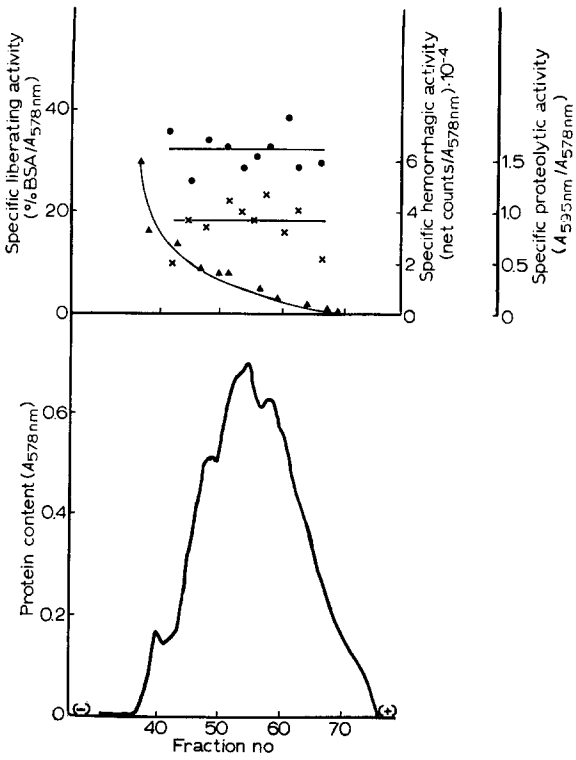

Fig. 4. Polyacrylamide gel electrophoresis of HR1 and the relationship of liberating activity with hemorrhagic and proteolytic activities. The lower figure shows the distribution of protein (A_{578nm}) in disc gel; the upper figure shows the distribution in eluates from disc gel of protein liberating activity (●—●), hemorrhagic activity (×—×), and proteolytic activity on "Hide Powder Azure" (▲—▲) in terms of their specific activity. For further details, see the text.

specific activities were constant throughout, while specific proteolytic activity in the gel decreased with increasing migration distance toward anode (Fig. 4, upper).

These results provided additional evidence in support of the suggestion that the same entity is responsible for both liberating and hemorrhagic activities. Apparently, the proteolytic activity on "Hide Powder Azure" is attributable at least partially to another entity which has nothing to do with liberating and hemorrhagic activities.

Gross comparison of the split-products obtained with different hemorrhagic principles

Figs 5–8 show the elution profiles from a Sephadex G-50 column of the split-products of basement membrane incubated with the venom hemorrhagic principles and bacterial collagenase under the standard conditions.

The profiles show apparent difference with HR1, HR2 (a or b) and collagenase; a major part of the split-product with HR1 was of high molecular weight (Fig. 5), whereas much of the product with HR2a (Fig. 6) or HR2b (Fig. 7) consisted of low-molecular weight substances. The product with collagenase was of another type, containing both high- and low-molecular weight substances at a ratio of roughly 1:2

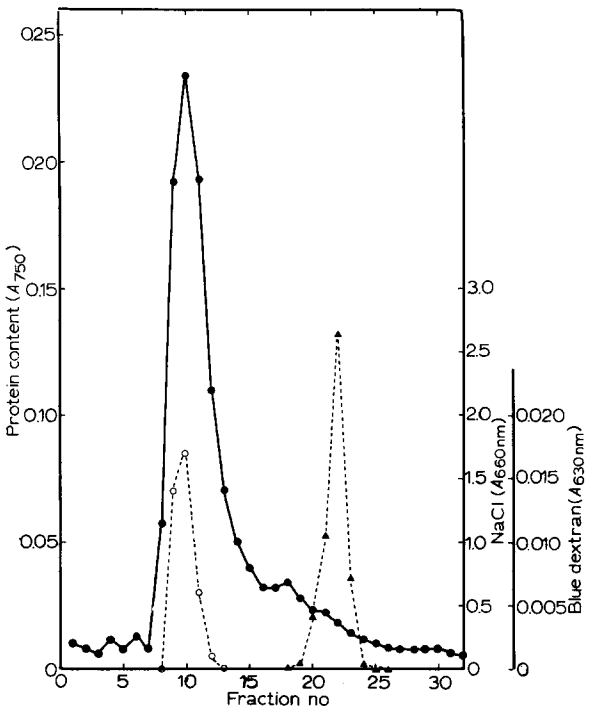

Fig. 5. Gel filtration on Sephadex G-50 of the split-product of basement membrane exposed to HR1. The supernatant fluid from a reaction mixture, prepared under the standard conditions, of HR1 (8.57 μg/ml) and basement membrane was lyophilized; the lyophilized material dissolved in water to a volume one-fifth that of the original supernatant fluid. 200 μl of this solution containing 416 μg bovine serum albumin equiv. was placed on a column (0.6 cm × 17 cm) of Sephadex G-50 (fine) equilibrated with 0.5% (v/v) acetic acid. The column was eluted with the same medium. 300 μl-fractions were collected at a flow rate of 5–6 ml/h. Protein content (●—●) was determined according to Lowry *et al.*[17]. Prior to the sample run, the void volume had been determined with blue dextran (○---○) as a marker, and the emergence of NaCl (▲---▲) had been detected with $AgNO_3$ solution.

(Fig. 8). The basement membrane alone was incubated and the supernatant fluid, after concentration, was also subjected to gel filtration; no appreciable amount of Folin-positive material was eluted from the gel.

As already mentioned (Table I), the liberation of Folin-positive material from basement membrane with HR2a or HR2b was about one-half that with HR1 and one-fourth that with collagenase. A major part of the split-product with HR2a or HR2b consisted, nevertheless, of low-molecular weight substances. The results can be interpreted to indicate that the mode of action of HR2a or HR2b on basement membrane is different from that of HR1 or bacterial collagenase.

Enzymatic nature of the action of venom hemorrhagic principles

On the basis of the kinetic properties of the *in vitro* reactions of the venom principles on basement membrane (Figs 1–3) and of the observed presence of different molecular-weight substances in the split-products by these principles (Figs 5–8), we

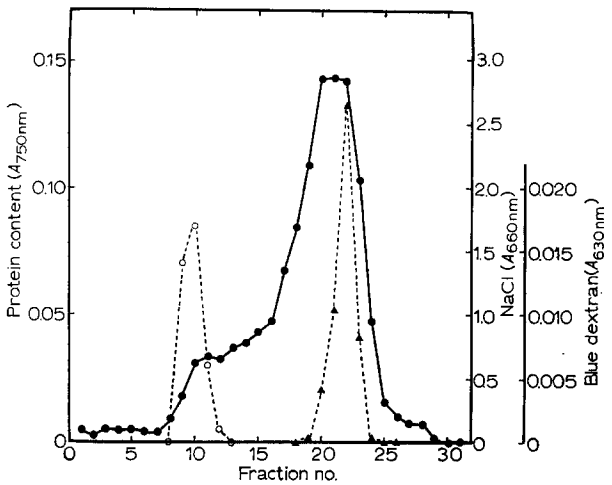

Fig. 6. Gel filtration on Sephadex G-50 of the split-product of basement membrane exposed to HR2a. The supernatant fluid from a reaction mixture, prepared under the standard conditions, of HR2a (8.57 μg/ml) and basement membrane was lyophilized; the lyophilized material dissolved in water to a volume one-sixteenth that of the original supernatant fluid. 200 μl of this solution containing 760 μg bovine serum albumin equiv. was submitted to gel filtration under the same conditions as in Fig. 5.

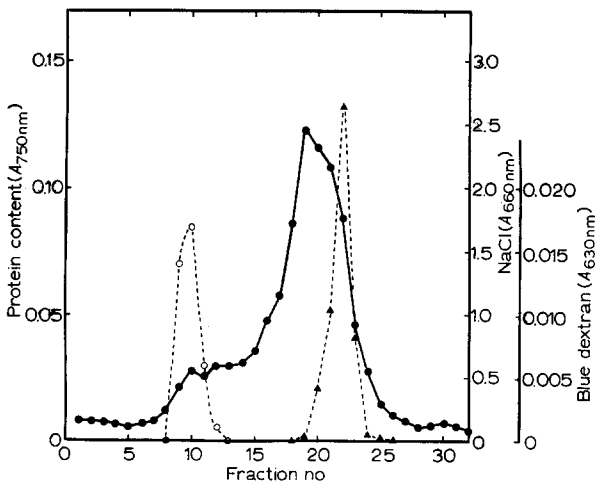

Fig. 7. Gel filtration on Sephadex G-50 of split-product of basement membrane exposed to HR2b. The supernatant fluid from a reaction mixture, prepared under the standard conditions, of HR2b (8.57 μg/ml) and basement membrane was concentrated as described in Fig. 6. 200 μl of this concentrated solution containing 732 μg bovine serum albumin equiv. was submitted to gel filtration under the same conditions as in Fig. 5.

conclude that the basement membrane is damaged by enzymatic action of the venom principles. The difference in ratio of quantity of proteins to carbohydrates liberated (Table I), suggests that HR2a or HR2b acts on the membrane by a mode of action different from HR1. Gross comparison of molecular weights among the split-products of the membrane with these principles (Figs 5–8) favours our interpretation.

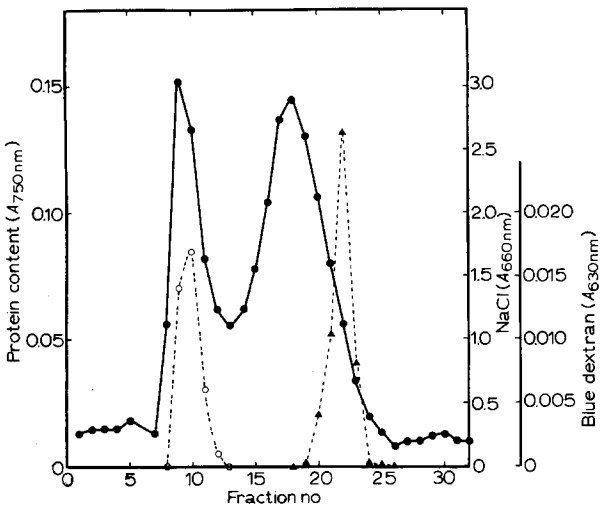

Fig. 8. Gel filtration on Sephadex G-50 of the split-product of basement membrane exposed to bacterial collagenase. The supernatant fluid from a reaction mixture, prepared under the standard conditions, of collagenase (8.57 μg/ml) and basement membrane was lyophilized; the lyophilized material dissolved in water to a volume three-tenths that of the original supernatant fluid. 200 μl of this solution containing 480 μg bovine serum albumin equiv. was submitted to gel filtration under the same conditions as in Fig. 5.

The present studies demonstrated that the proteolytic activity on "Hide Powder Azure" possessed by HR1 failed to correlate with the protein-liberating and hemorrhagic activities of this principle (Fig. 4). It has also been suggested[4] that the factors in HR1 responsible for hemorrhagic activity and for proteolytic activity on casein are not identical. It was previously demonstrated that HR2a and HR2b were free of caseinolytic activity[5,6]; and that collagenolytic activity with native collagen was detected neither in the crude venom[7] nor in the purified HR2a and HR2b[5,6]. These facts, however, do not necessarily imply that the hemorrhagic principles are not proteolytic enzymes.

Since the basement membrane consists of mainly collagen and non-collagen proteins bound to carbohydrates[11,12], the results herein reported suggest that the venom hemorrhagic principles may be proteolytic enzymes with highly selective specificity, including collagenases of unknown types.

Experiments are in progress to characterize precisely the enzymatic nature of the venom hemorrhagic principles by biochemical analyses of the split-products of the basement membrane.

The present findings strongly support our working hypothesis[10] that the hemorrhage is attributed to damage of the stabilizing apparatus of the microvessels, *e.g.* basement membranes and surrounding fibrils. The data available are consistent with our interpretation that the erythrocytes "ooze" out through the endothelial cell junctions, possibly opened after the basement membrane has been damaged by the enzymatic action of the venom hemorrhagic principles.

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