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COMPARISON OF HEMORRHAGIC FACTORS OF THE VENOMS OF *NAJA NAJA*, *AGKISTRODON PISCIVORUS* AND *APIS MELLIFERA**

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Abstract—The hemorrhagic factors from the venoms of *Naja naja*, *Agkistrodon piscivorus* and *Apis mellifera* have been fractionated on Sephadex G 50. Different polypeptides with a molecular weight of less than 10·000 are responsible for the hemorrhagic activity. The hemorrhagic effect observed on canine lung surface caused by bee venom cannot be attributed to phospholipase A activity. Although the snake venom fractions which induced hemorrhages possessed phospholipase A activity, it appears that the enzyme is not responsible for the hemorrhages.

Heparin was found to form an inactive complex with the hemorrhagic factors of *Naja naja* and bee venom, whereas the hemorrhagic activity of *Agkistrodon piscivorus* venom was unaffected by heparin. Protamine could release the hemorrhagic factor in an active form, from the inactive complexes.

Esterase activity of *Agkistrodon piscivorus* venom and *Bothrops jararaca* can be inhibited by Trasylol (R).

BONTA and coworkers[1, 2] reported that oestriol-16-17 disuccinate‡ counteracted the hemorrhages induced by the venom of *Naja naja* (indian cobra) on dog lung surface. This anti-hemorrhagic activity was shown to be related to the vascular permeability effect of oestriol succinate. The hemorrhages caused by *Agkistrodon piscivorus* venom (cotton mouth moccasin snake), could not however, be inhibited by oestriol succinate.[2] The above investigators have further demonstrated the presence of a hemorrhage inducing basic peptide in *Naja naja* venom, which was found to be absent in the venom of *A. piscivorus*.[3]

It was established that different mechanisms are involved in the mode of action of the venoms belonging to two different species, i.e. Elapidae (*N. naja*) and Crotalidae snakes (*A. piscivorus*). In the present communication the results of comparative studies on hemorrhagic factors of these two snake venoms with that of bee venom are described.

MATERIAL AND METHODS

N. naja and *A. piscivorus* venoms were obtained from Miami Serpentarium. Venoms

* Dedicated to Professor Dr E. Auhagen in honour of his 65th birthday.

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‡ Marketed by N. V. Organon, Oss, under the trade name Styptanon(R) (Estriol Succinate).

of *Bothrops jararaca* and of *Apis mellifera* were purchased from Sigma Chemical Co., U.S.A. Thromboliquine(R) was a product of N.V. Organon, Oss and contained 50 mg of heparin per ml (500 U.I./ml). Sephadex G-50 was obtained from Pharmacia, Uppsala, Sweden. Protamine sulphate from Nordisck Insulin Laboratorium, Denmark, and saponin from E. Merck, Darmstadt. Trasylol(R) (Kallikrein Inhibitor crystallized by Schultz, Kraut and Bhargava[4] from bovine parotid glands[23] or lungs[13]), 5000 KIE/ml, was a commercial preparation of Farbenfabriken Bayer Leverkusen (Germany). Benzoyl-1-arginine ethyl ester (BAEE) was purchased from Serva, (Heidelberg, Germany).

Gel filtration on Sephadex G-50. Gel filtration of the venoms was carried out under the following conditions. About 100 mg of the lyophilised venom was dissolved in a small amount of 0·1 N acetic acid and applied on a Sephadex G-50 column (2·5 × 100 cm). Equilibration of the gel and elution were carried out with 0·1 N acetic acid (pH 3·2). Fractions were collected and their optical density was measured at 2800 Å using quartz cuvettes of one centimeter thickness in a Beckman spectrophotometer (Model DB-G).

Hemorrhagic activity of venom solutions or their fractions was determined according to the method of Bonta *et al.*[5] using the canine lung surface as the test organ.

An additional test to check the hemorrhagic activity of bee venom was performed in mice. The various fractions were administered intrathoracically and in addition to the estimation of the survival time of the animals, their lungs were removed, weighed and the occurrence of hemorrhages was noted. It has been shown by previous work[2] that intrathoracical administration of cobra venom to mice increased the weight of the lungs as a result of local blood extravasation. Unless otherwise specified, a dose of 7·5 mg/kg was injected intrathoracically.

Phospholipase A activity was determined by the method of Doizaki.[6]

Estimation of hemolysis was done according to the following procedure: One ml of the test solution (20 μg/ml) was incubated for 15 min at 37° with 1 ml of a suspension of three times washed fresh guinea-pig erythrocytes (6% v/v) in 0·01 M phosphate saline buffer of pH 7·4. The incubation mixture was centrifuged at 5000 r.p.m. and 0·2 ml of the clear supernatant was mixed with 5·0 ml of Drabkin's reagent (supplied by the French National Transfusion Centre, Paris) to convert the released hemoglobin into cyanmetheglobin, which was estimated colorimetrically at 5400 Å wavelength. The results were compared with the total hemolysis produced by 1 mg of saponin under identical conditions, which was considered 100 per cent. The results are expressed in terms of percentage hemolysis produced by 10 μg of material tested per ml of final mixture.

Hydrolysis of BAEE by venoms and inhibition by Trasylol(R) were studied under the conditions described by Kraut and Bhargava.[7]

The release of Slow Reacting Substance C (SRS-C) by the venoms and their fractions was detected and compared to that of crude *Naja nigricollis* venom as described by Vargaftig, Miranda and Lacoume.[8] Briefly, the venom samples were incubated with a 40 per cent emulsion of fresh egg yolk at 37° for 30 min. At the end of this period the incubates were injected intravenously (0·4 ml/kg) to a pentobarbital anaesthetized rabbit and the blood pressure recorded from the carotid artery with a Statham transducer connected to an M2 Devices recorder. Phospholipase A containing material was thus detected by the release of a hypotensive factor (SRS-C) from the phospholipids of egg yolk.

RESULTS

Gel filtration of venoms on Sephadex G-50

(a) Naja naja. Fractionation of *N. naja* venom on Sephadex G-50 column resulted in four peaks as observed when the optical density values at 2800 Å were plotted against the collected fractions (Fig. 1).

The fractions belonging to each peak were pooled together and lyophilized. The distribution of the material over the four peaks was: 4·5 mg in the first, 3·0 mg in the second, 59·2 mg in the third and 3·2 in the fourth peak. Hereafter material of these 4 peaks would be designated as IN, IIN, IIIN an IVN respectively. The hemorrhagic activity was demonstrated only in peak IIIN (see Fig. 1), which was the only fraction containing substantial amounts of phospholipase A and which released in our experimental conditions, the hypotensive principle(s) from egg yolk (Table 1).

The hemolytic activity was found in both fractions IIN and IIIN. Hemolytic factors were thus separated from phospholipase A[9] and hemorrhagic activity was demonstrated not to be exclusively dependent on hemolytic factors, as fraction IIN was hemolytic but not hemorrhagic (Table 1).

(b) Agkistrodon piscivorus. Four main peaks (Fraction I (tubes 26–40), II (41–61), III (62–105) and IV (106–136) were obtained from *A. piscivorus* venom when fractionated on Sephadex G-50 column.

TABLE 1. COMPARISON OF PHOSPHOLIPASE A, HEMORRHAGIC, HEMOLYTIC AND ESTERASE ACTIVITIES OF *N. NAJA*, *A. PISCIVORUS* AND BEE VENOMS AND THEIR VARIOUS FRACTIONS

Material tested	Phospholipase activity in turbidimetric units* per μg	SRS-C‡ release	Hemorrhagic activity tested on dog lung surface	% hemolysis by 10 μg of venom	Esterase activity on BAEE
Naja Naja					
Crude venom	2·89	+	+++	4·5	−−
G 50 IN	0·00	0	−−	0·0	−−
G 50 IIN	0·00	0	−−	6·4	−−
G 50 IIIN	1·68	+	+++	4·5	−−
G 50 IVN	? †	?†	? †	? †	? †
Agkistrodon piscivorus					
Crude venom	10·80	+	+++	3·3	+++
G 50 IAP	0·00	0	+	0·0	+++
G 50 IIAP	0·00	0	−−	0·0	++
G 50 IIIAP	8·72	+	+++	0·0	−−
G 50 IVAP	? †	?†	? †	? †	? †
Bee venom					
Crude venom	9·95	+	+++	97·0	−−
G 50 I	? †	?†	−−	? †	−−
G 50 II	18·88	+	−−	0·0	−−
G 50 IIIA	0·00	0	+++	98·1	−−
G 50 IIIB	0·00	0	+++	77·5	−−
G 50 IV	? †	?†	? †	? †	? †

* 0·001 shift of optical density per minute.

† Fraction to less for estimation.

‡ Presence(+) or absence (0) of a hypotensive factor in the incubates of egg yolk and the various venoms or their fractions at a concentration of 10 μg/ml, after 30 min at 37° as described.[8]

The lyophilized samples of the combined fractions belonging to these peaks are designated as fractions IAP, IIAP, IIIAP, IVAP. The major portion of the hemorrhagic activity was found to be associated with fraction IIIAP. It may be recalled that in the case of *N. naja* venom also a corresponding combined fraction (IIIN) contained almost the entire hemorrhagic activity.

Obviously hemorrhagic factors of these two venoms possess similar molecular sizes. Phospholipase A activity was found concentrated only in fraction IIIAP, which released the hypotensive principle, SRS-C, from egg yolk (Table 1).

A clear cut differentiation between hemolytic and hemorrhagic activities was observed in this case, as neither crude *A. piscivorus* venom nor any of its fractions induced significant hemolysis, despite the intensive hemorrhagic activity of fraction IIIAP and *A. piscivorus* crude venom. In contrast to IN, IAP fraction showed a slight hemorrhagic effect, which could however, only be observed 2 hr after its application on the lung surface.

(c) *Bee venom.* Jentsch and Habermann[10] fractionated bee venom on a 300 cm long Sephadex G-50 column while purifying melittin. Recently Shipmann and Cole[11] tried to reproduce the experiments of these investigators and have encountered difficulty in maintaining a uniform elution speed with such a long column. We, therefore, used a relatively short Sephadex column (2·5 × 100 cm); 0·1 N acetic acid instead of ammonium formate was used as eluant. Our elution-pattern is apparently similar (see Fig. 3) to that obtained by Jentsch and Habermann.[10] We were however, able to resolve partially the melittin peak (peak III) into two components (IIIA and IIIB).

The fractions of peak IIIA produced hemorrhages more rapidly and the intensity of the hemorrhages was appreciably more than that produced by IIIB fractions (see Table 2).

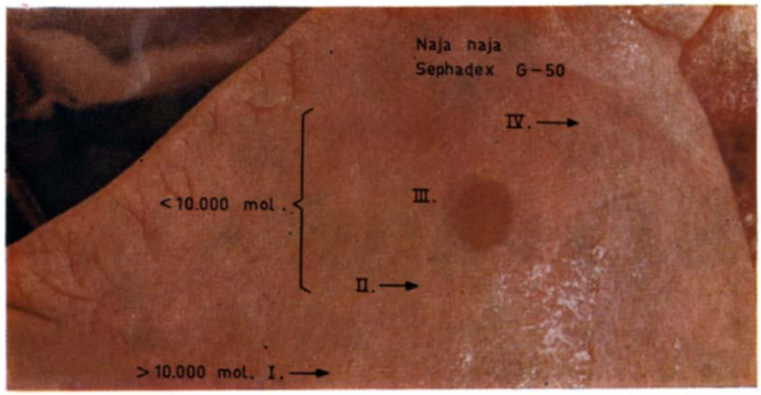
TABLE 2. ESTIMATION OF HEMORRHAGIC ACTIVITY OF BEE VENOM AND ITS FRACTIONS (4 mg/ml) ON CANINE LUNG SURFACE

Material tested	Time lapse*	Intensity†
Bee venom		
Crude	0·0	22·0
G-50-I	Inactive	00·0
G-50-II	Inactive	00·0
G-50-IIIA	0·0	22·0
G-50-IIIB	3·0	13·5
G-50-IV	Inactive	00·0

* Time elapsed in minutes between removal of filter paper disc (soaked in venom solutions) from lung surface and appearance of hemorrhages.

† An arbitrary scale of 0 to 3 was used for measuring intensity of hemorrhages every minute after removal of the filter paper discs during 10 min so that a maximum score of 33 could be achieved theoretically.

As there was no sharp separation between peaks IIIA and IIIB, it cannot, at this stage, be ascertained which of the two fractions would contain melittin after complete separation, despite the fact that fraction IIIA was found to be more hemolytic and more toxic than IIIB. After administration of fraction IIIA the survival time of the



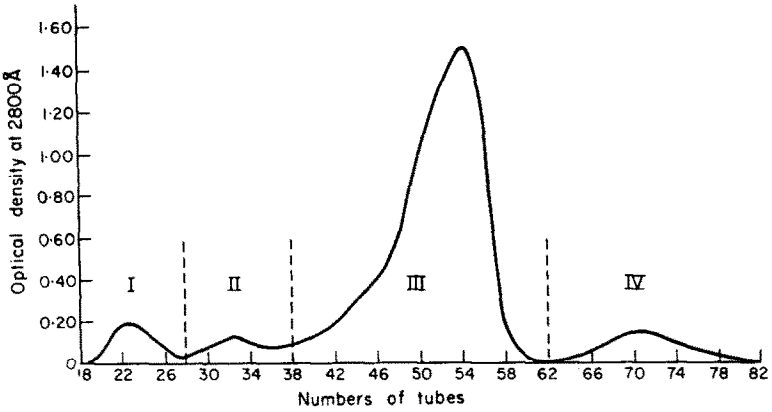
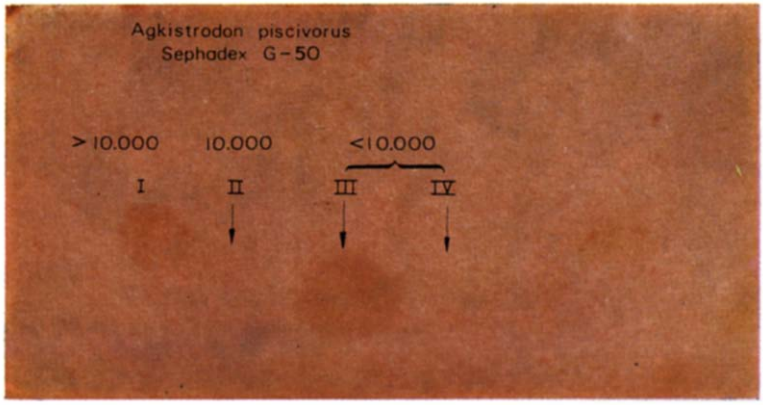


FIG. 1. Elution diagram obtained from gel filtration of *Naja naja* venom on Sephadex G-50; Eluant 0·1 N acetic acid, ———— optical density at 2800 Å. above: Effect of *Naja naja* venom and its fractions on canine lung surface. Concentrations 2 mg/ml.



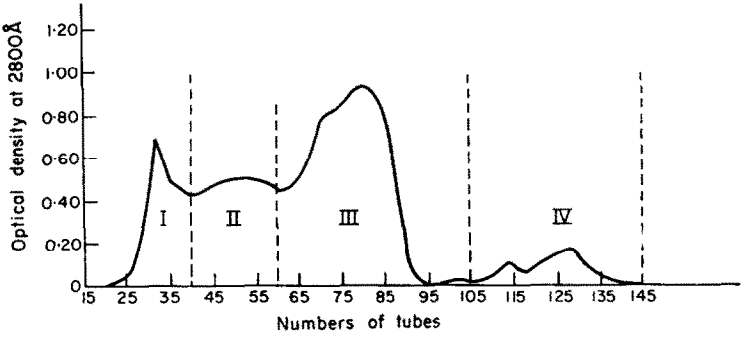
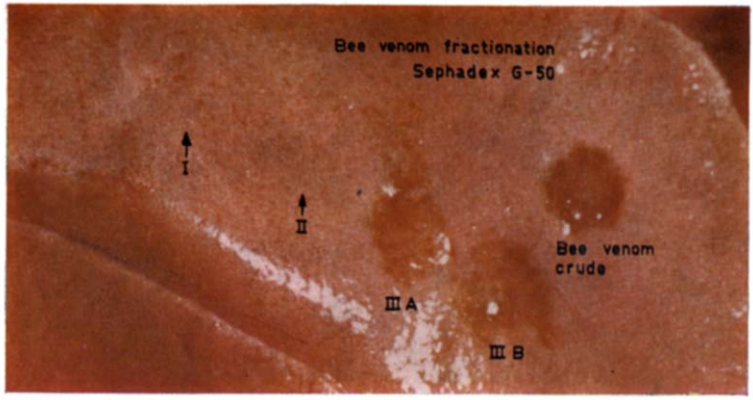


FIG. 2. Elution diagram obtained from gel filtration of *Agkistrodon piscivorus venom* on Sephadex G-50; Eluant 0·1 N acetic acid; ——— optical density at 2800 Å; above: Effect of *Agkistrodon piscivorus venom* and its fractions on canine lung surface. Concentration 2 mg/ml.



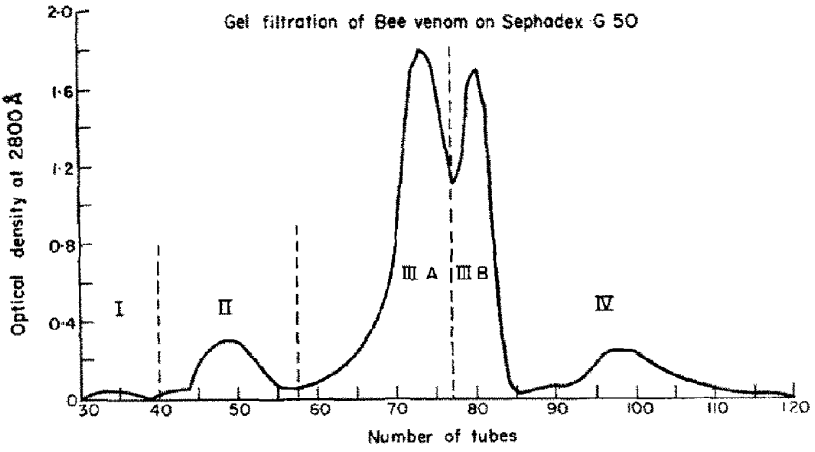


FIG. 3. Gel filtration of *Apis mellifera* (bee) on Sephadex G-50 using 0·1 N acetic acid as eluant, ———— optical density at 2800 Å. above, The effect of bee venom and its fractions on canine lung surface. Concentration of venom 2 mg/ml.

TABLE 3. EFFECT OF ADMINISTRATION* OF BEE VENOM AND ITS FRACTION (7·5 mg/kg) ON LUNG WEIGHT AND SURVIVAL TIME OF MICE

Materials	Lung weight	Lung index	Surviving time	Remarks
Bee venom crude	346 ± 7·7	1·03 ± 0·02	9′ 27″ ± 39″	All mice died within 10 min
Bee venom G-50 I	too little to be tested			
Bee venom G-50 II	274 ± 19·9	1·06 ± 0·09	—	1 mouse died after 1 hr 9 mice were sacrificed after 1·5 hr by ether
Bee Venom G-50 III	299 ± 8·3	1·03 ± 0·02	9′ 50″ ± 35″	All mice died within 10 min
Bee Venom G-50 IIIB	315 ± 9·7	1·13 ± 0·02	111′ 36″ ± 6′ 25″	2 mice did not die even after 2 hr They were sacrificed after 2 hr by ether
Bee Venom G-50 IV	174 ± 4·8	0·62 ± 0·01	—	None of the mice died They were sacrificed after 1·5 hr by ether The lungs showed no hemorrhages

* 0·1 ml of the solution per 10 g body weight of mouse was injected intrathoracically.
† A group of ten mice was used to test the toxicity of one sample.

animals was of 9 min, which is the same as obtained by injecting the same amount of crude venom (7·5 mg/kg) (Table 3). In the case of fraction IIIB it was of 111 min, although the lung weight increased equally in both cases (Table 2).

Determination of phospholipase A activity showed that fractions which possessed hemorrhagic and hemolytic activities did not contain phospholipase A and vice-versa (Table 1). Fractions IIIA and IIIB induce hemorrhages and hemolysis, whereas Fraction II presented a high phospholipase A activity (Table 1). These findings are in agreement with our other results that only fractions of peak II (which contains phospholipase A) were able to release hypotensive factors from egg yolk whereas that of peak III did not display this activity (Fig. 4).

FIG. 4. Release of hypotensive principle from egg yolk by phospholipase A containing fraction of the bee venom. All legends refer to the incubates of egg yolk emulsion with the venom or its various fractions at a concentration of 10 μg/ml.
1. Control test with *Naja nigricollis*; 2. Crude bee venom; 3. Fraction II of bee venom (phospholipase A containing); 4. Fraction III of bee venom.

Behaviour of heparin with the venoms and their fractions

(a) Naja naja *and bee venom.* Bonta *et al.*[3] found that the hemorrhagic activity of *N. naja* venom depends on the presence of a heparin precipitable factor, whereas that of *A. piscivorus* does not. Kraut and Bhargava[12, 13] have reported that addition of heparin to an aqueous solution of bovine lung Kallikrein-Inhibitor (Trasylol(R)) leads to the precipitation of the polypeptide as a Trasylol(R)-heparin symplex, which redissolves in the presence of an excess of heparin, due to its increased solubility in higher salt concentrations. A similar phenomenon occurred when an excess of heparin was added to solutions of the two snake venoms and of bee venom. Addition of one or two drops of heparin to the test solution (10 mg of *N. naja*, bee venom or its fraction III dissolved in one ml of distilled water), followed by centrifugation provided a precipitate; the supernatant did not contain appreciable hemorrhagic activity. When an excess of heparin was added to the venom solution, so as to redissolve the hemorrhagic factor–heparin symplex, the resulting solutions were found to be inactive on the lung surface. However, addition of a few drops of protamine solution (50 mg/ml) restored completely the hemorrhagic activity of the supernatant.

(b) Agkistrodon piscivorus. Identical experiments were carried out with *A. piscivorus* venom, which gave a heavy precipitate after addition of a drop of heparin solution. However, after centrifugation, the hemorrhagic activity was completely recovered in the supernatant. Further addition of heparin dissolved the precipitate, but the venom remained hemorrhagic as before.

Esterase and hemorrhagic activity

With a view to examine whether hemorrhagic activity has any correlation with esterase activity, we have estimated the BAEE hydrolysing capacity of venoms and their fractions. The results indicated that the bee venom possessed no, whereas *N. naja* venom showed negligible and *A. piscivorus* venom exhibited significant esterase activity[14] under our test conditions.[7] The activity of 10 μg of this venom was found to be equivalent to 5 μg of bovine crystalline trypsin or 0·5 Kallikrein Unit (K.E.). Esterolytic activity of this venom could be inhibited by a high concentration of Trasylol(R). About

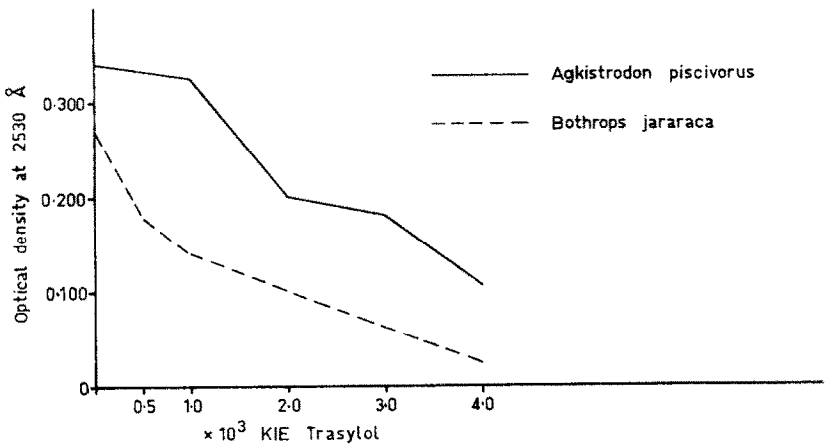


FIG. 5. Comparison of esterase activity (BAEE) of 10 μg of *Agkistrodon piscivorus* venom and *Bothrops jararaca* venom and effect of Trasylol(R) on their enzymatic activity.

400 Kallikrein-Inhibitor Units (KIE) were required to inhibit the activity of 1 μg of this venom. *Bothrop jararaca* venom, which releases kinins as *A. piscivorus*, also requires high concentration of Trasylol(R) to be inhibited by it (300 KIE for 1 μg of venom). Obviously the esterolytic enzyme of these snake venoms are not identical with kallikrein or trypsin as only one KIE is required to inhibit the enzymatic activity of one kallikrein unit or about 1 μg of crystalline trypsin.[7] The fact that our purified fraction IIIAP did not hydrolyse BAEE but still possessed significant hemorrhagic activity proved further that the two activities are due to different factors (Table 1).

DISCUSSION

Although enzymes, specially proteases are held responsible for the hemorrhagic effect of venoms,[15–18] we have found that the hemorrhagic activity of the venoms in the low molecular weight fractions, which are expected to be less than 10,000 as they are retarded by Sephadex G-50. It appears reasonable to assume that the hemorrhagic effect is not due to proteases, which are usually proteins of a molecular weight higher than 20,000 with the exception of a protease isolated from fish[19] (*Astacus fluviatilis*) and from the larva of *Vespa orientalis*.[20]

This observation confirms the statement of Bonta and coworkers,[3] who showed that proteases, [i.e. pancreatic kallikrein, trypsin] are devoid of hemorrhagic activity on the lung surface. The hemorrhagic fractions from *N. naja* and *A. piscivorus* venoms were also found to contain non negligible amounts of phospholipase A to which the hemorrhagia might be attributed by either a direct or an indirect action. However, the hemorrhagic activity observed does not seem to be related to this enzyme, as in the case of bee venom we have been able to clearly isolate the two activities in separate fractions. Also according to Bonta *et al.*,[3] purified phospholipase A does not produce appreciable hemorrhages on the canine lung surface.

As revealed from gel filtration experiments, all the three venoms contain polypeptides of relatively low molecular weight which are responsible for producing hemorrhages. The observation that the heparin treatment results in precipitation of hemorrhagic factor from *N. naja* venom but not from *A. piscivorus* venom indicates that these two active principles are not identical as far as their affinity to heparin is concerned. Additional arguments that these hemorrhagic factors are not identical are: firstly oestriol succinate is only able to inhibit hemorrhagic activity of *N. naja* and not that of *A. piscivorus* venom and secondly the bleeding produced by *N. naja* on the dog lung surface are of diffuse character, whereas those caused by *A. piscivorus* venom are petechial as seen by the unaided eye.

In the case of bee venom the hemorrhagic activity was localized in the fraction III, apparently the same from which Jentsch *et al.*[10] have also purified melittin. However, more investigations are needed to ascertain whether the hemorrhagic factor is identical with melittin or not. Further fractionation of bee venom resulted in a partial enrichment of the hemorrhagic activity and in a very marked concentration of toxicity in fraction IIIA, whereas fraction IIIB remained less toxic. Since the separation of hemolytic activity between these two fractions was not very sharp, it is not possible to ascertain which of them was contaminated with melittin.

The hemorrhagic factor (IIIAP) differs from the kinin releasing principle purified from *A. piscivorus* venom by Rocha e Silva *et al.*[21] which also hydrolyses BAEE. The factor obtained by latter is free from hemolytic activity, whereas our fraction IIIAP

is free from esterase, as evidenced by (see Table 1) its inability to hydrolyse BAEE, although the crude *A. piscivorus* venom certainly possessed significant esterase activity against this substrate.

The observation that Trasylol(R) inhibits the esterase activity but not the hemorrhagic activity of *A. piscivorus* venom[14] further supports the fact that the esterasic or kinin releasing factors are not responsible for inducing hemorrhages. A purified kinin releasing factor from *B. jararaca*,* when assayed on the lung surface at a concentration of 4 mg/ml, did not display any hemorrhagic activity, despite the fact that crude venom was very active already at a concentration of 1 mg/ml.[22]

Equally high concentrations of Trasylol(R) were required to inhibit the esterasic activity of *A. piscivorus* and *B. jararaca* venoms, that it may be concluded that BAEE hydrolysing enzymes of both venoms exhibit similarities. They are, however, certainly different from pancreatic kallikrein or trypsin, despite the fact that both these venoms release kinins.

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