Kirby, E. P., Niewiarowski, S., Stocker, K., Kettner, C., Shaw, E., & Brudzynski, T. M. (1979) *Biochemistry 18*, 3564–3570.
Kornalik, F., & Blombäck, B. (1975) *Thromb. Res. 6*, 53–63.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Li, Z. Y., & Bon, C. (1983) *J. Neurochem. 40*, 338–349.
Mann, K. G., Elion, J., Butkowski, R. J., Downing, M., & Nesheim, M. E. (1981) *Methods Enzymol. 80*, 286–303.
Michl, H. (1954) *Monatsh. Chem. 85*, 1240–1247.
Morita, T., & Iwanaga, S. (1978) *J. Biochem. (Tokyo) 83*, 559–570.
Morita, T., & Iwanaga, S. (1981) *Methods Enzymol. 80*, 303–311.
Morita, T., Iwanaga, S., & Susuki, T. (1976) *J. Biochem. (Tokyo) 79*, 1089–1108.
Nahas, L., Denson, K. W. E., & Mac Farlane, R. G. (1964) *Thromb. Diath. Haemorrh. 12*, 355.
Nahas, L., Kamiguti, A. S., & Barros, M. A. R. (1979) *Thromb. Haemostasis 41*, 314–328.
Niewiarowski, S., Kirby, E. P., & Stocker, K. (1977) *Thromb. Res. 10*, 863–869.
Niewiarowski, S., Kirby, E. P., Brudzynski, T. M., & Stocker, K. (1979) *Biochemistry 18*, 3570–3577.
Owen, W. G., & Jackson, C. M. (1973) *Thromb. Res. 3*, 705–714.
Radvanyi, F., & Bon, C. (1982) *J. Biol. Chem. 257*, 12616–12623.
Rhee, M. J., Morris, S., & Kosow, D. P. (1982) *Biochemistry 21*, 3437–3443.
Stocker, K., & Egberg, N. (1973) *Thromb. Diath. Haemorrh. 1*, 361–370.
Stocker, K., & Barlow, G. H. (1976) *Methods Enzymol. 45*, 214–223.
Tans, G., Govers-Riemslag, J. W. P., van Rijn, J. L. M. L., & Rosing, J. (1985) *J. Biol. Chem. 260*, 9366–9372.
Walker, F. J., Owen, W. G., & Esmon, C. T. (1980) *Biochemistry 19*, 1020–1023.

Blood Coagulation Induced by the Venom of *Bothrops atrox*. 2. Identification, Purification, and Properties of Two Factor X Activators†

Hélène Hofmann‡ and Cassian Bon*
Unité des Venins, Unité associée Institut Pasteur/INSERM (285), Institut Pasteur, 75015 Paris, France
Received February 28, 1986; Revised Manuscript Received August 26, 1986

ABSTRACT: We have characterized and purified the two components of the venom of *Bothrops atrox* that activate the coagulation factor X. Activator 1 and activator 2 were separated by ion-exchange chromatography but otherwise presented similar characteristics. They consist of a heavy polypeptide of M_r 59 000 and either one or two light chains forming a doublet of M_r 14 000–15 000. They are inactive on synthetic substrates and on prothrombin or fibrinogen and thus appear to act specifically on factor X. They are not sensitive to inhibitors of serine proteases or thiol esterases. The activation of factor X is activated by Ca^{2+} ions with a Hill coefficient of 2.4 and is inhibited by Hg^{2+}, Ba^{2+}, and Cd^{2+}. Its pH dependency suggests that the activity depends on the ionization of a group with an apparent pK of 6.9. We studied the cleavage of purified bovine factor X by *B. atrox* activators and compared it to that obtained with the factor X activator from *Vipera russelli* venom. Like the physiological activators, the venom's activators cleave the heavy chain of factor X, producing the activated factor Xaα. They produce however two other cleavages: one near the N-terminal end of the heavy chain of factor X, generating factor Xμ, and a second one located at one extremity of the heavy chain of factor Xaα, generating factor Xaν.

The venom of *Bothrops atrox* contains several procoagulant components that act at various levels of the blood coagulation cascade. The first procoagulant component that was purified from *B. atrox* venom is a fibrinogen clotting enzyme called batroxobin, a serine protease that converts fibrinogen into fibrin (Stocker & Egberg, 1973; Stocker & Barlow, 1976; Holleman & Weiss, 1976). However, at variance with thrombin, batroxobin cleaves only the Aα chains of the fibrinogen molecule, liberating the fibrinopeptide A but not the fibrinopeptide B (Funk et al., 1971). In addition, batroxobin does not share with thrombin the ability to aggregate blood platelets. Subsequently, another serine protease, called thrombocytin, was isolated from the venom of *B. atrox*. It aggregates blood platelets but has limited effects on purified fibrinogen and on factors V and VIII (Niewiarowski et al., 1977, 1979; Kirby et al., 1979). In the preceding paper (Hofmann & Bon, 1987), we reported the purification of a protease from *B. atrox* venom that activates prothrombin by specifically cleaving it into meizothrombin. This prothrombin activator strongly resembles activators previously described in the venoms of *Echis carinatus* and *Dispholidus typus* (Franza et al., 1975; Kornalik & Blombäck, 1975; Morita et al., 1976; Morita & Iwanaga, 1978, 1981; Guillin et al., 1978; Briet et al., 1982; Rhee et al., 1982). The existence in the venom of *B. atrox* of a component that activates factor X has also been reported (Nahas et al., 1964, 1979; Hofmann et al., 1983).

In the preceding paper (Hofmann & Bon, 1987), we described the fractionation of *B. atrox* venom by DEAE-cellulose chromatography and reported that two fractions possess the capacity to activate factor X. In this investigation, we describe a further purification of the two venom components and an-

† This investigation was supported in part by funds from the Ministère de l'Industrie et de la Recherche, the Centre National de la Recherche Scientifique, and the Direction des Recherches, Etudes et Techniques.
‡ Present address: Service de Biochimie, Hôpital Ambroise Paré, 92100 Boulogne, France.

0006-2960/87/0426-0780$01.50/0

alyze their molecular structure, enzymatic properties, and mechanism of action on purified bovine factor X. In particular, we compared the two activators from the venom of *B. atrox* with the activator from the venom of *Vipera russelli* (Williams & Esnouf, 1962; Fujikawa et al., 1972; Furie et al., 1974; Furie & Furie, 1975, 1976; Kisiel et al., 1976; Furukawa et al., 1976; Di Scipio et al., 1977; Morris et al., 1978; Amphlett et al., 1982).

Experimental Procedures

Materials. The materials were those described in the preceding paper (Hofmann & Bon, 1987). The venom from *Vipera russelli* was provided by Kabi-Diagnostica (Amsterdam, The Netherlands). Brain phospholipids (from bovine brain type III) were obtained from Sigma (St. Louis, MO).

Venom Fractionation and Purification of Factor X Activators. B. atrox venom was first fractionated by ion-exchange chromatography in a DEAE-cellulose column, as previously described (Hofmann & Bon, 1987). Briefly, the venom was dissolved and dialyzed against 10 mM Tris-HCl,[1] pH 7.5, containing 5 mM benzamidine and applied to the DEAE-cellulose column. The elution was achieved with a linear gradient from 0 to 0.2 M NaCl in the same Tris-HCl buffer. Various fractions were pooled according to their coagulant and enzymatic properties. The fractions (fractions V and VI) that activate factor X were applied to Sephadex G-150 columns. The fractions containing factor X activator (fractions V-b and VI-b) were pooled as indicated in Figure 1, and the purification of the two factor X activators was achieved by a second ion-exchange chromatography on DEAE-cellulose columns. The fractions containing factor X activator (fractions V-b-2 and VI-b-2) were pooled as indicated in Figure 2, lyophilized, and stored at −20 °C.

Activation of Purified Factor X. The activation of purified factor X was followed by measuring the amidolytic activity of the factor Xa that was formed. Purified bovine factor X (10–400 μg/mL) was incubated at 25 °C in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, and an appropriate concentration of activator. Aliquots (1–50 μL) were removed at various times, and their amidolytic activity was immediately determined in 950 μL of 200 mM Tris-HCl, pH 8.3, 150 mM NaCl, 10 mM EGTA, and 0.25 mM S-2337 by recording the formation of *p*-nitroaniline.

Enzymatic Assays. Phospholipase activity was determined either by a colorimetric method as described by Bon and Saliou (1983) or by the titrimetric method of Desnuelle et al. (1955) as described by Radvanyi and Bon (1982). Other biochemical methods are described in the preceding paper (Hofmann & Bon, 1987).

Results

Purification of Factor X Activators. The venom of *B. atrox* possesses the ability to activate factor X. The identification and partial purification of the activator involved has been previously reported (Hofmann et al., 1983). The fractionation of *B. atrox* venom by chromatography in a DEAE-cellulose column at pH 7.5 yielded two components that activate human and bovine factor X (fractions V and VI), which eluted at different ionic strengths [Hofmann and Bon (1987), Figure 1]. The factor X activators present in fractions V and VI were further purified by gel filtration chromatography in Sephadex G-150 (Figure 1). In both cases, the factor X activators eluted as single symmetrical peaks (fractions V-b and VI-b) corresponding to proteins with an apparent M_r of 77 000. The elution profiles indicated the presence of numerous other protein components. Fraction VI also contained phospholipase A_2 activity [Hofmann and Bon (1987), Figure 1], which eluted as a dissymmetrical peak, corresponding to proteins of M_r of 15 000–30 000.

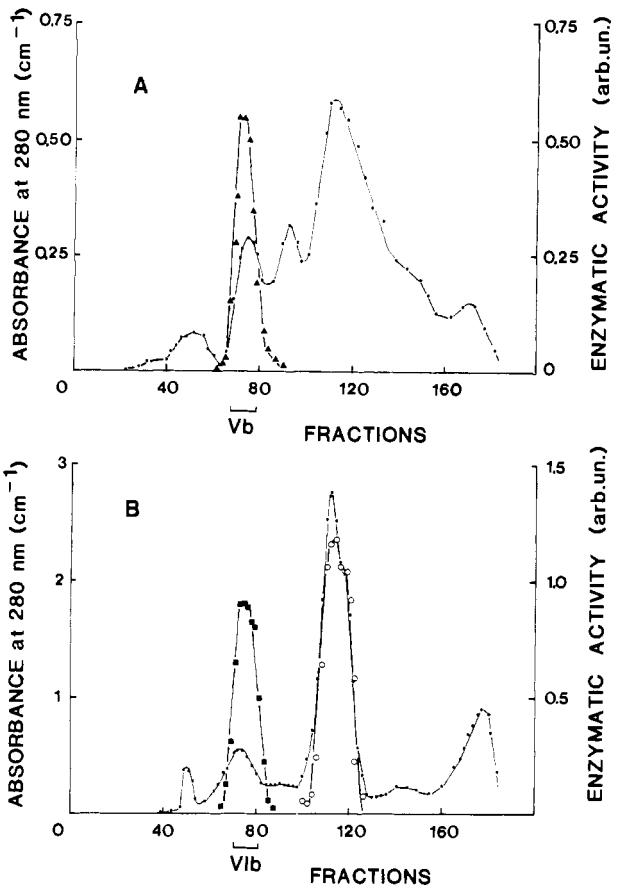


FIGURE 1: Gel filtration of fractions V and VI. Fraction V (143 mg, 12.5 mL) (A) and fraction VI (178 mg, 11 mL) (B) were applied to a Sephadex G-150 column (2.5 × 90 cm) previously equilibrated with 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 5 mM benzamidine. The elution was performed with the same buffer, at a flow rate of 8 mL/h, at 4 °C. The protein concentration was estimated by measuring absorbance at 280 nm (−·−). The activities of factor X activator 1 (▲), factor X activator 2 (■), and phospholipase A_2 (○) were determined as described under Experimental Procedures. The fractions were pooled as indicated in the figure.

A further purification of the two factor X activators of *B. atrox* venom was achieved by submitting fractions V-b and VI-b (Figure 1) to a second chromatography in DEAE-cellulose columns. Panels A and B of Figure 2 indicate that in both cases factor X activators elute as symmetrical peaks at 75 mM NaCl for fraction V-b-2 and 85 mM NaCl for fraction VI-b-2. These values are in good agreement with those previously obtained for the isolation of fractions V and VI: respectively 72 mM and 83 mM [Hofmann and Bon (1987), Figure 1]. The factor X activator that eluted at the lower ionic strength (75 mM NaCl) was called activator 1 (fractions V, V-b, and V-b-2) and that eluting at the higher ionic concentration (85 mM NaCl) was called activator 2 (fractions VI, VI-b, and VI-b-2).

[1] Abbreviations: DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; S-2337, benzoyl-Ile-Glu-Pip-Gly-Arg-*p*-nitroanilide hydrochloride; TAME, *N*-tosylarginine methyl ester; TLCK, *N*-*p*-tosyllysine chloromethyl ketone; TPCK, *N*-*p*-tosylphenylalanine chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

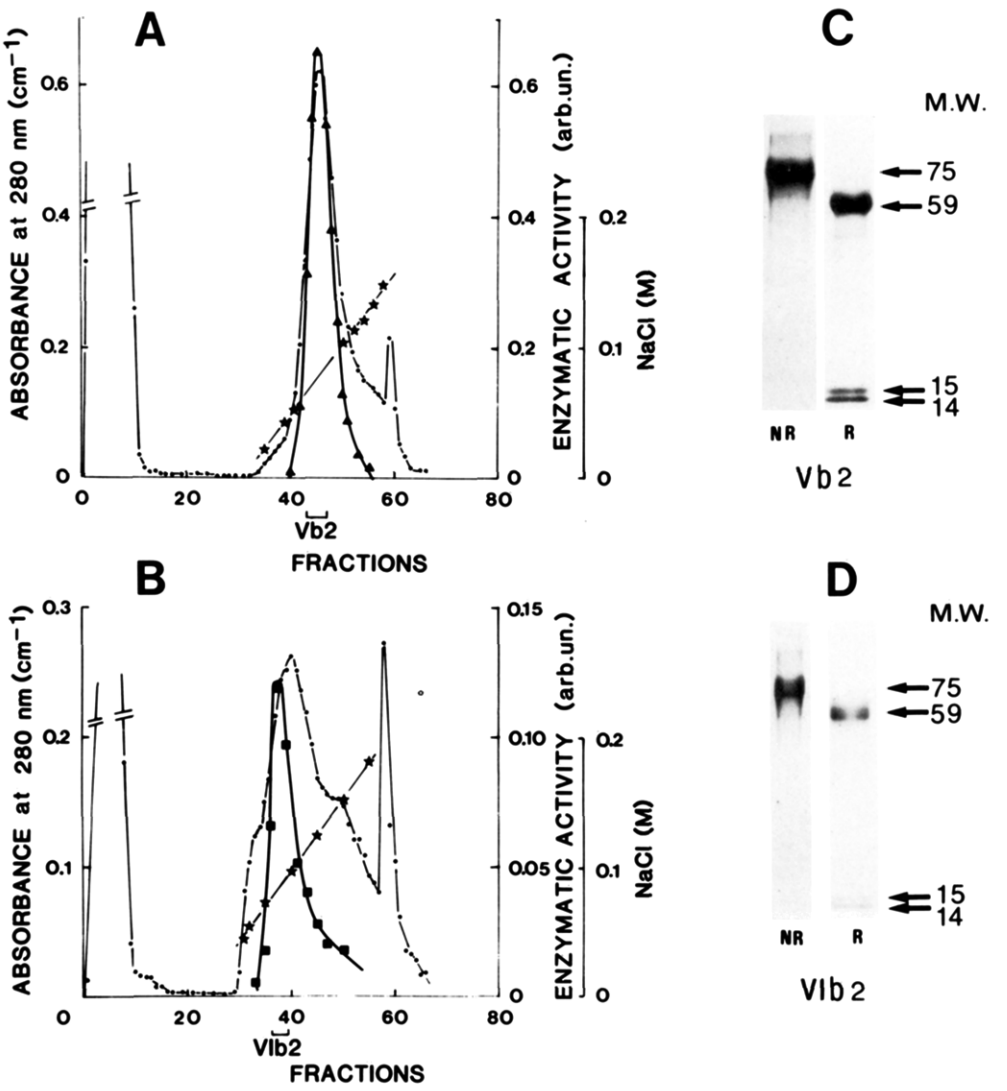


FIGURE 2: Ion-exchange chromatography of fractions V-b and VI-b. Aliquots of 4 mg of fractions V-b (A) and VI-b (B) were dialyzed against large volumes of 10 mM Tris-HCl, pH 7.5, containing 5 mM benzamidine and applied to a DEAE-cellulose column (0.5 × 5 cm) previously equilibrated at 4 °C with the same Tris-HCl buffer without benzamidine. The elution was carried out at a flow rate of 8 mL/h with 150 mL of the same buffer without benzamidine and then with a linear NaCl gradient from 0 to 150 mM (A) or from 50 to 200 mM (B) buffered with 10 mM Tris-HCl, pH 7.5 (2 × 40 mL). The protein concentration (–·–) and the activity of factor X activators 1 (▲) and 2 (■) were assayed as described in Figure 1. The salt concentration (★) was determined by conductimetry. The polypeptide composition of fractions V-b-2 (C) and VI-b-2 (D) was analyzed by electrophoresis in polyacrylamide gels (12%) in the presence of SDS (4%) and in the presence (R) or in the absence (NR) of β-mercaptoethanol (2%).

Fractions V-b-2 and VI-b-2 were analyzed by electrophoresis in polyacrylamide gels in the presence of SDS, under reducing conditions. In both cases fractions V-b-2 and VI-b-2, which correspond to the purified factor X activators, contained a heavy polypeptide of apparent M_r 59 000 and a light doublet of apparent M_r 14 000–15 000. In the absence of reducing agent, polyacrylamide gel electrophoresis showed in both cases a single diffuse band corresponding to an apparent M_r of 75 000. These observations indicate that factor X activators 1 and 2 of *B. atrox* venom were purified to homogeneity and that they are both constituted of one heavy polypeptide of M_r 59 000 linked by a disulfide bridge(s) to one or two light chains forming a doublet of M_r 14 000–15 000.

Table I shows that the specific activities of the purified *B. atrox* venom activators 1 and 2 were roughly similar and that they were more than 30-fold higher than that of the crude venom. This indicates that activators 1 and 2 are relatively minor components of the venom: taken together they represent about 5% of the venom proteins. In addition, their recovery after purification was low, about 8% (5.2% for activator 1 and 2.5% for activator 2). This is due to the fact that extensive inactivation occurred during the second DEAE-cellulose chromatography step because of the extreme protein dilution and also because of the fact that an substantial amount of activity was discarded in fractions IV and VII during the fractionation procedure [Hofmann and Bon (1987), Figure 1].

Enzymatic Properties of Factor X Activators from B. atrox Venom. In a preliminary study (Hofmann et al., 1983), we identified a nonhomogeneous fraction of *B. atrox* venom (fraction III-2) that activated human factor X. We studied the kinetics of activation of purified human factor X by measuring the amidolytic and the coagulant activities of activated factor X (factor Xa) that was formed, as a function of time. When a low concentration of purified venom activator was incubated with an excess of factor X, both amidolytic and coagulant activities were generated simultaneously and without any latency, in a calcium-dependent manner (Hofmann et al., 1983). In this investigation, we made the same observations with the purified activators 1 and 2 (fractions V-b-2 and VI-b-2) (results not shown). The activation proceeded linearly during the first minutes and later reached a plateau corre-

Table I: Specific Activity and Yield of Factor X Activators at Different Stages of Purification[a]

fraction	protein mg	protein %	activation of factor X nmol/min	activation of factor X %	sp act. [nmol/(min·mg)]	purification factor
Factor X Activator 1						
venom	2830	100	5295	100	1.87	1
V (DEAE-cellulose)	143	5	438	8.2	3.06	1.6
V-b (Sephadex G-150)	8.2	0.3	422	7.9	51	27.3
V-b-2 (DEAE-cellulose)	3.5	0.1	273	5.2	78	42
Factor X Activator 2						
venom	2830	100	5295	100	1.87	1
VI (DEAE-cellulose)	178	6.2	1550	29	8.69	4.6
VI-b (Sephadex G-150)	13.7	0.5	744	14	54	29
VI-b-2 (DEAE-cellulose)	2.1	0.07	131	2.5	62	33

[a] The activity of factor X activators was determined, as described under Experimental Procedures, by their ability to activate purified bovine factor X. It is expressed in nanomoles of factor X converted to factor Xa per minute. The protein concentration was estimated from the absorbance at 280 nm, assuming $A_{280}(1\%) = 10$.

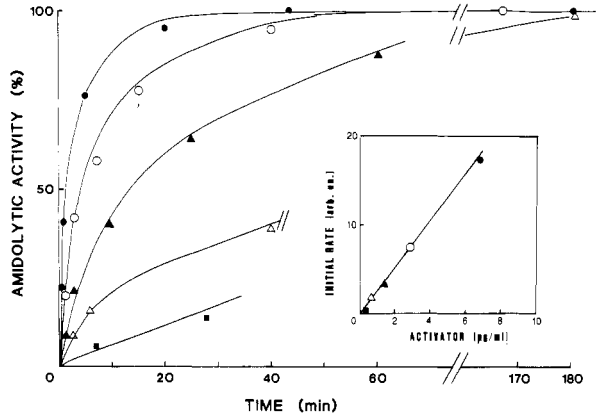


FIGURE 3: Effect of concentration of venom activator on rate of activation of factor X. Purified bovine factor X (15 μg/mL, 0.27 μM) was incubated at 25 °C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 5 mM $CaCl_2$. At zero time, various concentrations [0.34 (■), 0.68 (△), 1.37 (▲), 2.74 (○), and 6.9 (●) μg/mL] of partially purified activator 1 from *B. atrox* venom (fraction V-b) were added. At the indicated times, aliquots were removed, and their amidolytic activity was immediately determined with the chromogenic substrate S-2337, as indicated under Experimental Procedures. Amidolytic activities were expressed as percent of the maximal activity. The insert represents the initial rate of activation of factor X (expressed in arbitrary units) as a function of the concentration of factor X activator 1).

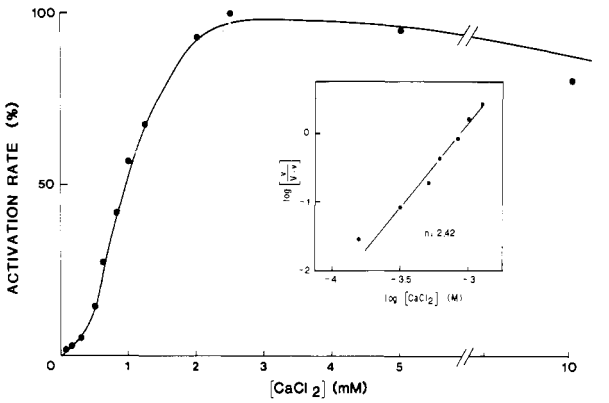


FIGURE 4: Effect of calcium ions on initial rate of activation of factor X by *B. atrox* activators. Purified bovine factor X (16 μg/mL, 0.29 μM) was incubated at 25 °C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and the indicated concentration of $CaCl_2$. At zero time, 4 μg/mL (less than 50 nM) of partially purified activator 1 (fraction V-b) was added. Aliquots were removed at various times, and their amidolytic activity was immediately determined with the chromogenic substrate S-2337. The initial rate of factor X activation (v) was determined as illustrated in Figure 3; it was expressed as percent of its maximal value (V) and represented as a function of $CaCl_2$ concentration. The insert is the Hill representation of the same data: the logarithm of $v/(V-v)$ is plotted as a function of the logarithm of $CaCl_2$ concentration.

sponding to the conversion of the totality of factor X into factor Xa (see below). Figure 3 shows that the initial rate of activation of factor X by partially purified activator 1 (fraction V-b) was proportional to the concentration of the venom activator. Similar results were obtained with activator 2. These observations indicate that the venom activators convert factor X into either factor Xa or an intermediate having the same amidolytic and coagulant activities. The activation of factor X by the *B. atrox* activators did not result from an autocatalytic process, since the rate was not significantly decreased in the presence of a concentration of benzamidine (1.3 mM) inhibiting 97% of the amidolytic activity of factor Xa. Activators 1 and 2 from the venom of *B. atrox* did not possess any hydrolytic activity on synthetic substrates such as TAME, S-2238, and S-2337. In addition, these activators appear to be specific for factor X since they did not act on fibrinogen or prothrombin.

The activation of factor X by activators from *B. atrox* venom required the presence of calcium ions. The optimal concentrations were above 2 mM. In addition, the curve representing the initial rate of activation as a function of calcium concentration (Figure 4) is sigmoidal, with a Hill coefficient of 2.4. This indicates a cooperative effect of the calcium ions. We examined the effect of other divalents ions. Some ions, Hg^{2+}, Ba^{2+}, and Cd^{2+}, did not replace Ca^{2+} for the activation of factor X: no amidolytic activity was generated in the absence of calcium and in the presence of a high concentration (12 mM) of these ions (Table II). Furthermore, Hg^{2+} and Ba^{2+} inhibited the activation observed in the presence of Ca^{2+} with a half-effect in the range of 0.1 and 1 mM respectively. On the contrary, other divalents ions, Mg^{2+}, Mn^{2+}, and Sr^{2+}, allowed the activation of factor X by the venom activators 1 and 2. However, the rate of activation obtained in the presence of a high concentration (12 mM) of these ions was less than 20% of that obtained in the presence of 3 mM $CaCl_2$ (Table II). $SrCl_2$ and $MnCl_2$ appeared to be partial inhibitors in the presence of $CaCl_2$, but $MgCl_2$ was found to increase the rate of activation determined in the presence of a suboptimal concentration of $CaCl_2$ (Table II). We examined the effect of pH on the rate of activation of factor X by the activators from *B. atrox* venom. In the case of activator 1, maximal activity was observed between pH 7.5 and pH 9.0. The variation of the rate of activation as a

Table II: Effects of Divalent Ions on Activation of Factor X by Purified *B. atrox* Venom Activators[a]

divalent ion	activation rate (%) without Ca^{2+}	activation rate (%) with Ca^{2+} (1 mM)
none	0	68
Mg^{2+}	21	84
Mn^{2+}	15	18
Sr^{2+}	15	39
Cd^{2+}	1.5	24
Ba^{2+}	0	2
Hg^{2+}	0.4	1

[a] Purified bovine factor X (21 μg/mL, 0.38 μM) was incubated at 30 °C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl in the presence of 12 mM of the tested divalents ions and in the presence or in the absence of 1 mM $CaCl_2$. Then the activation of factor X was initiated by adding a fixed concentration of purified activator 1 (fraction V-b-2) (0.7 μg/ml, 8.9 nM), and the initial rate of activation was determined as described under Experimental Procedures. The values are expressed as percent of the maximal rate of activation determined in optimal conditions (5 mM $CaCl_2$ and no other divalent ions) and with the same concentrations of factor X and venom activator.

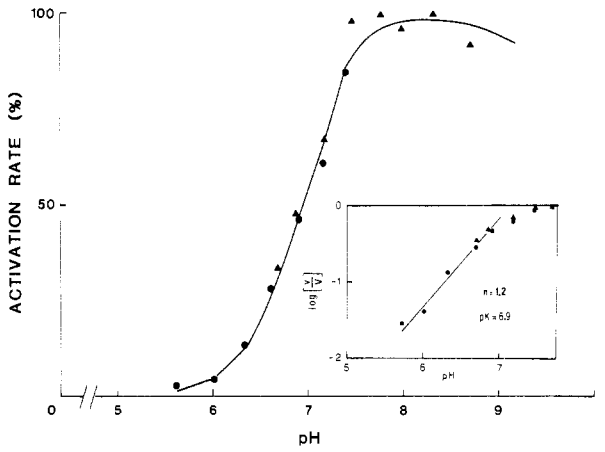


FIGURE 5: pH dependence of initial rate of activation of factor X by *B. atrox* activators. A fixed concentration of purified bovine factor X (11 μg/mL, 0.2 μM) was activated at various pH values by a fixed concentration of partially purified activator 1 (0.6 μg/mL; less than 10 nM) (fraction V-b). We determined the initial rate of activation by measuring the amidolytic activity that was generated. The activation buffer was 20 mM sodium cacodylate (●) or Tris-HCl (▲) containing 150 mM NaCl and 5 mM $CaCl_2$. The figure represents the initial rate of activation (v) expressed as percent of its maximal value (V) as a function of pH. The insert represents the logarithm of this value as a function of pH.

function of pH suggests that it depends on the protonation of an ionizable group having a pK of 6.9 (Figure 5). A similar result was obtained with activator 2 (results not shown).

The reagents of serine proteases, DFP, PMSF, TLCK, and TPCK, failed to inhibit the factor X activators from *B. atrox* venom, even at a high concentration (1 mM) and for long periods of time (over 20 h) (Table III). *p*-Bromophenacyl bromide as well as the reagents of thiol groups [iodoacetic acid, iodoacetamide, and *p*-(chloromercuri)benzoate] were also inactive under similar experimental conditions. On the other hand, the *B. atrox* factor X activators 1 and 2 were rapidly and irreversibly inactivated by compounds that reduce disulfide bridges, such as cysteine and DTT (Table III). These results indicate that factor X activators 1 and 2 are neither serine proteases nor thioenzymes. Although they may be metalloenzymes, we failed to demonstrate this hypothesis because factor X requires calcium ions to be activated.

Cleavage of Factor X by Activators 1 and 2 from the Venom of B. atrox. Catalytic cleavage of purified bovine factor X by purified activators 1 and 2 and by the activator of *V. russelli*

Table III: Effect of Various Reagents on Activity of *B. atrox* Factor X Activators[a]

reagent	concn (mM)	incubation time (h)	activation rate
		0	100
		20	90
DFP	25	20	90
PMSF	0.7	20	100
TLCK	3.6	20	96
TPCK	0.7	20	91
iodoacetic acid	3.6	18	84
iodoacetamide	3.6	18	100
p-(chloromercuri)benzoate	0.36	18	95
p-bromophenacyl bromide	0.9	2	77
L-cysteine	3.7	0.5	33
DTT	3.7	0.25	17
		0.75	3.6

[a] Partially purified factor X activator 1 from *B. atrox* venom (fraction V-b; 43 μg/mL) was incubated at 30 °C for the indicated periods of time in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 5 mM $CaCl_2$ with or without (control) the indicated concentrations of the various reagents. Then the initial rate of activation of purified bovine factor X by treated or untreated (control) activator 1 was determined as described under Experimental Procedures. In each case, the initial rate of activation is expressed as percent of the control value. Similar results were obtained in the case of activator 2.

venom was analyzed by polyacrylamide gel electrophoresis in the presence of SDS, in parallel with the appearance of the amidolytic activity. In order to minimize the cleavages caused by activated factor X, the analyses were performed in the presence of 5 mM benzamidine, which efficiently inhibits factor Xa activity.

With the three activators we obtained the same plateau, indicating that the factor Xa that was generated possessed the same specific activity and that this activity was not modified by further cleavages (Figure 6A). We adjusted the concentrations of the different venom activators to obtain similar activation kinetics in order to compare the order of appearance of the proteolytic cleavages. Factor X consists of two polypeptide chains of apparent M_r 25 000 and 50 000, linked by a disulfide bridge. With the three venom activators, the heavy chain disappeared concomitantly with the appearance of a polypeptide of apparent M_r 36 000. This cleavage coincided with the generation of the amidolytic activity that characterizes factor Xa (Figure 6A). The light chain was not modified. Therefore, activators 1 (Figure 6C) and 2 (Figure 6D) act like the activator from *V. russelli* venom (Figure 6B), cleaving the heavy chain of factor X and generating similar or identical activated factor X. The activator from *V. russelli* venom is known to cleave factor X at the same site as physiological activators (position α in the Scheme I, between Arg_{51} and Ile_{52}), and this corresponds to the formation of factor X$a\alpha$ (Fujikawa et al., 1972, 1974; Furie et al., 1974; Jesty et al., 1974, 1975; Di Scipio et al., 1977).

Contrary to the activator from *V. russelli*, the *B. atrox* activators 1 and 2 produced an additional transient polypeptide of apparent M_r 48 000 (Figure 6C,D). The fact that the activation product was factor X$a\alpha$ in all cases, at the end of the activation process, indicates that the additional cleavage observed with activators 1 and 2 occurred at the NH_2-terminal part of the factor X heavy chain (position μ in Scheme I).

It has been previously reported that bovine factor X$a\alpha$ is autoconverted without any loss of activity into factor X$a\beta$, with the concomitant release of a small molecular weight polypeptide corresponding to the COOH-terminal part of the heavy chain (Jesty et al., 1974, 1975; Fujikawa et al., 1975). This conversion is calcium-dependent and considerably accelerated by phospholipids (Figure 7). In the case of the factor X$a\alpha$

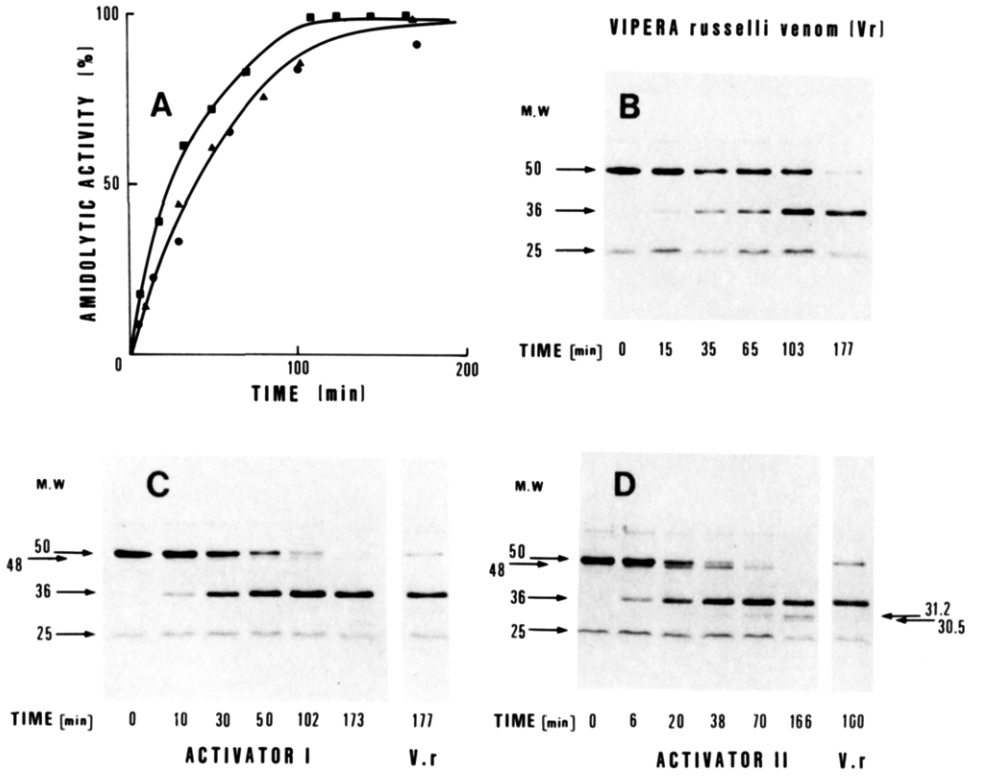
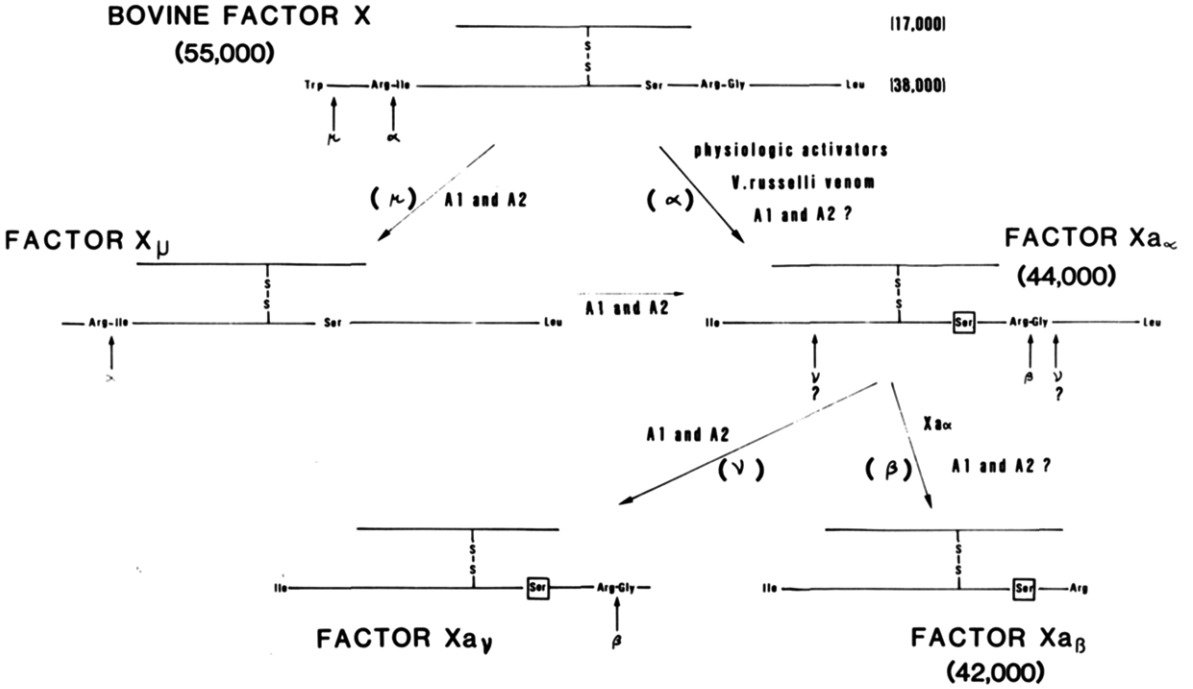


FIGURE 6: Cleavage of bovine factor X by *B. atrox* activators 1 and 2 in the presence of benzamidine: Analysis by electrophoresis in polyacrylamide gels. Purified bovine factor X (400 μg/mL, 7.2 μM) was incubated at 25 °C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 5 mM benzamidine, and 5.5 mM $CaCl_2$ in the presence of purified *B. atrox* activator 1 (fraction V-b-2) (▲) (3.8 μg/mL, 50 nM) or activator 2 (fraction VI-b-2) (■) (10 μg/mL, 130 nM) or of a partially purified *V. russelli* activator (●) (1.3 μg/mL). (A) Aliquots were removed at the indicated times; their amidolytic activity was immediately determined with S-2337. The amidolytic activity was expressed as percent of its maximal value. (B–D) Aliquots were removed at the indicated times and immediately frozen at −18 °C after addition of 13 mM EGTA. After lyophilization, their peptide composition was analyzed by electrophoresis in polyacrylamide gels (12%) in the presence of SDS (4%) and a reducing agent (2% β-mercaptoethanol). The apparent molecular weights indicated in the figure were determined in parallel experiments.

Scheme I: Cleavage of Bovine Factor X[a]



[a] Cleavages of bovine factor X occurring during activation by the activators from the venoms of *B. atrox* and *V. russelli*. The serine residue of the active site is enclosed in a square when the activation peptide has been proved to possess an amidolytic activity.

generated by the *B. atrox* activators 1 and 2, we observed the same conversion of the heavy chain (M_r 36 000) into the heavy chain of factor Xaβ, (M_r 30 500), in the presence of phospholipids and calcium ions (Figure 7B,C). In the absence of phospholipids and in the presence of the *B. atrox* activators, we observed an additional cleavage of the heavy chain of factor Xaα, corresponding to the formation of a polypeptide of apparent M_r 31 200 (Figure 7B). This additional cleavage did

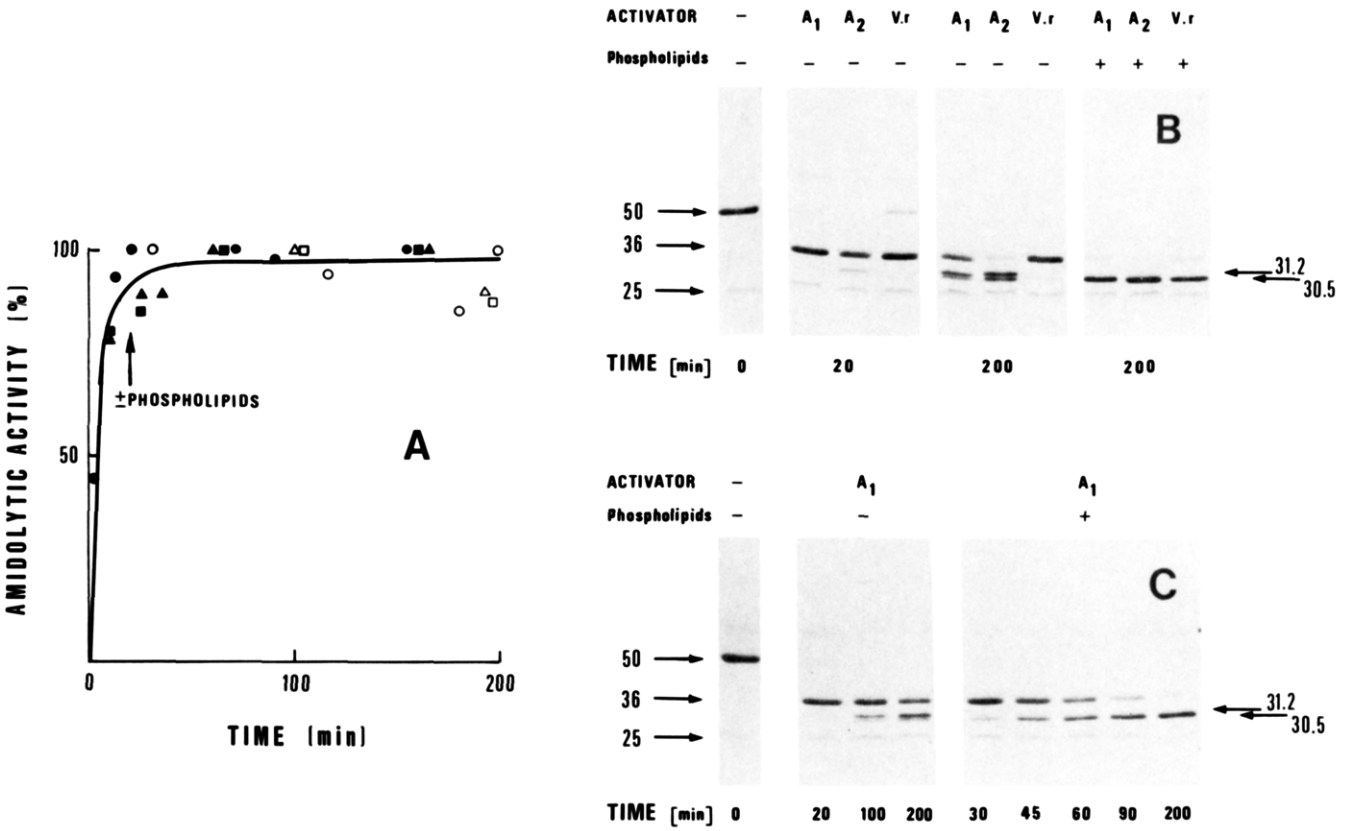


FIGURE 7: Effect of phospholipids on autocleavage of factor Xaα generated by venom activators: Analysis by electrophoresis in polyacrylamide gels. Purified bovine factor X (400 μg/mL, 7.2 μM) was incubated at 25 °C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 5 mM benzamidine, and 5.5 mM $CaCl_2$ in the presence of *B. atrox* activator 1 (fraction V-b-2) (▲) (45 μg/mL, 0.59 μM), *B. atrox* activator 2 (fraction VI-b-2) (■) (61 μg/mL, 0.8 μM), or *V. russelli* activator (●) (41 μg/mL). After 20 min, brain phospholipids (0.58 mg/mL) were added to the incubation medium (△, □, ○). (A) Aliquots were removed at the indicated times; their amidolytic activity was immediately determined with S-2337. The amidolytic activity was expressed as percent of its maximal value. (B and C) Aliquots were removed at the indicated times and immediately frozen at −18 °C after addition of 13 mM EGTA. After lyophilization, their polypeptide composition was analyzed by gel electrophoresis as described in Figure 6.

not reduce the amidolytic activity of factor Xaα (Figure 7A). It was not due to an autocleavage of factor Xaα, but it was catalyzed by activators 1 and 2 (Figure 7B,C) but not by the activator from *V. russelli* venom (Figure 7B). We called factor Xaν the new molecular form of factor Xa (Scheme I).

Discussion

The fractionation of the venom of *B. atrox* by ion-exchange chromatography on DEAE-cellulose showed that it contains several components that act at various levels of the blood coagulation cascade. One fraction (fraction II) was found to specifically activate prothrombin, and two fractions (fractions V and VI) were found to activate purified bovine factor X (Hofmann & Bon, 1987). In this investigation, we studied the two activators of factor X, which were purified to homogeneity by gel filtration in Sephadex G-150 and by a second ion-exchange chromatography on a DEAE-cellulose column.

By gel filtration, the two factor X activators eluted with the same apparent M_r of 77 000. Analyses by gel electrophoresis revealed that both activators were obtained in an homogeneous form and are constituted of one heavy chain of apparent M_r 59 000 and one light doublet of M_r 14 000–15 000 linked by one (or several) disulfide bridge(s). It is not possible at the moment to decide whether each heavy chain is associated with one of the two light chains or with both light chains. The two factor X activators of *B. atrox* venom eluted from the DEAE-cellulose column at different ionic strengths. These observations suggest that the two activators, which differ only by their isoelectric point, may be isoforms of the same protein. The factor X activator (Hofmann et al., 1983) identified and partially purified from the venom of *B. atrox* was probably a mixture of the two molecular forms described here. In fact, in the preliminary analysis a single ion-exchange chromatography was used, and factor X activity was eluted before application of the salt gradient. The molecular structure of factor X activators from the venom of *B. atrox* appears to resemble that of the factor X activator from *V. russelli* venom. There is however some disagreement in the literature about the structure of this activator: Furie and Furie (1975) found a single polypeptide of apparent M_r 60 000, whereas Furukawa et al. (1976) and Kisiel et al. (1976) proposed a heavy chain of M_r 60 000 linked by disulfide bridge(s) to one or two light chains of M_r 10 000–20 000. A similar structure has also been proposed by Amphlett et al. (1982) for the three molecular forms isolated by ion-exchange chromatography, which differ only by their content in sialic acid.

The activity of factor X activators from the venom of *B. atrox* is calcium-dependent. The effect of divalent ions on the activation of factor X by the components of *B. atrox* venom resembles that reported for the activator from *V. russelli* venom: Ca^{2+} is needed, its effect is cooperative with a Hill coefficient of 4.3 (Lindhout et al., 1978), and Mn^{2+} and Mg^{2+} are partial activators (Bajaj et al., 1977; Morris et al., 1978). According to Furie and Furie (1975), 2 mol of Ca^{2+} binds to 1 mol of bovine factor X in a cooperative manner. This interaction induces a conformational change of factor X that allows its activation by the *V. russelli* activator (Nelsestuen et al., 1976). It is likely that a similar mechanism also applies in the case of the activators from *B. atrox* venom. The factor X activators of *B. atrox* venom are insensitive to reagents of

serine and thiol proteases. They may therefore be metalloproteases, although we failed to demonstrate it since factor X itself must bind calcium ions to be activated. With the exception of the electric charge, the two factor X activators of the venom of *B. atrox* were found to have the same molecular structure, to have the same enzymatic properties, and to catalyze the same cleavages of the peptide bonds of factor X molecule at the same rate. Therefore, they may be considered as isoforms of the same enzyme. In addition, the factor X activators of *B. atrox* venom closely resemble the factor X activator from the venom of *V. russelli*. They have a similar molecular structure and similar enzymatic properties and mechanism of action. However, the *B. atrox* activators cleave the heavy chain of factor X at two additional positions that have not been observed with the *V. russelli* activator.

When injected intravenously into mice, the procoagulant fractions of the venom of *B. atrox* account for only 12% of the lethal potency of the crude venom. In addition, the procoagulant fractions do not seem to act synergistically with other toxic components of the venom, since the toxicity of the total venom can be quantitatively accounted for by the toxicity of its fractions. However, disorders of the blood coagulation are one of the features of the symptomatology to man by *B. atrox*, and this venom has long been recognized to contain a fibrinogen clotting enzyme, batroxobin (Stocker & Egberg, 1973; Stocker & Barlow, 1976; Holleman & Weiss, 1976). The presence in the venom of two activators of factor X and one prothrombin activator, as reported in our investigations, would produce localized or disseminated intravascular coagulations difficult to see, as they are often overpowered by a massive systemic activation of the fibrinolytic system.

Acknowledgments

We gratefully acknowledge Dr. B. Boris Vargaftig and Professor Paul Giraudet for their continuous interest in this work, for encouragements, and for improvement of the manuscript. We are indebted to Dr. François Radvanyi for helpful discussions. We thank Bernard Saliou for his help with venom fractionation.

Registry No. Blood coagulation factor Xa, 9002-05-5; blood coagulation factor Xaα, 81811-43-0; blood coagulation factor Xμ, 105881-85-4; blood coagulation factor Xaν, 105881-86-5; blood coagulation factor Xaβ, 73613-00-0; calcium, 7440-70-2; *Bothrops atrox* blood coagulation factor X activator, 106039-45-6.

References

Amphlett, G. W., Byrne, R., & Castellino, F. J. (1982) *Biochemistry 21*, 125–132.

Bajaj, S. P., Byrne, R., Nowak, T., & Castellino, F. J. (1977) *J. Biol. Chem. 252*, 4758–4761.

Bon, C., & Saliou, B. (1983) *Toxicon 21*, 681–698.

Briet, E., Noyes, C. M., Robert, H. R., & Griffith, M. J. (1982) *Thromb. Res. 27*, 591–600.

Desnuelle, P., Constantin, M. J., & Baldy, J. (1955) *Bull. Soc. Chim. Biol. 37*, 285–290.

Di Scipio, R. G., Hermodson, M. A., & Davie, E. W. (1977) *Biochemistry 16*, 5253–5260.

Franza, R. B., Jr., Aronson, D. L., & Finlayson, J. S. (1975) *J. Biol. Chem. 250*, 7057–7067.

Fujikawa, K., Legaz, M. E., & Davie, E. W. (1972) *Biochemistry 11*, 4892–4899.

Fujikawa, K., Coan, M. H., Legaz, M. E., & Davie, E. W. (1974) *Biochemistry 13*, 5290–5299.

Fujikawa, K., Titani, K., & Davie, E. W. (1975) *Biochemistry 14*, 3359–3363.

Funk, C., Gmur, J., Herold, R., & Staub, P. W. (1971) *Br. J. Haematol. 21*, 43–52.

Furie, B. C., & Furie, B. (1975) *J. Biol. Chem. 250*, 601–608.

Furie, B., & Furie, B. C. (1976) *J. Biol. Chem. 251*, 6807–6814.

Furie, B. C., Furie, B., Gottlieb, A. J., & Williams, W. J. (1974) *Biochim. Biophys. Acta 365*, 121–132.

Furukawa, Y., Matsunaga, Y., & Hayashi, K. (1976) *Biochim. Biophys. Acta 453*, 48–61.

Guillin, M. C., Bezeaud, A., & Menache, D. (1978) *Biochim. Biophys. Acta 537*, 160–168.

Hofmann, H., & Bon, C. (1987) *Biochemistry* (preceding paper in this issue).

Hofmann, H., Dumarey, C., & Bon, C. (1983) *Biochimie 65*, 201–210.

Holleman, W. H., & Weiss, L. J. (1976) *J. Biol. Chem. 251*, 1663–1669.

Jesty, J., Spencer, A. K., & Nemerson, Y. (1974) *J. Biol. Chem. 249*, 5614–5622.

Jesty, J., Spencer, A. K., Nakashima, Y., Nemerson, Y., & Konigsberg, W. (1975) *J. Biol. Chem. 250*, 4497–4504.

Kirby, E. P., Niewiarowski, S., Stocker, K., Kettner, C., Shaw, E., & Brudzynski, T. M. (1979) *Biochemistry 18*, 3564–3570.

Kisiel, W., Hermodson, M. A., & Davie, E. W. (1976) *Biochemistry 15*, 4901–4906.

Kornalik, F., & Blombäck, B. (1975) *Thromb. Res. 6*, 53–63.

Lindhout, M. J., Kop-Klassen, B. H. M., & Hemker, H. C. (1978) *Biochim. Biophys. Acta 533*, 327–341.

Morita, T., & Iwanaga, S. (1978) *J. Biochem. (Tokyo) 83*, 559–570.

Morita, T., & Iwanaga, S. (1981) *Methods Enzymol. 80*, 303–311.

Morita, T., Iwanaga, S., & Susuki, T. (1976) *J. Biochem. (Tokyo) 79*, 1089–1108.

Morris, S., Robey, F. A., & Kosow, D. P. (1978) *J. Biol. Chem. 253*, 4604–4608.

Nahas, L., Denson, K. W. E., & Mac Farlane, R. G. (1964) *Thromb. Diath. Haemorrh. 12*, 355.

Nahas, L., Kamigut, A. S., & Barros, M. A. R. (1979) *Thromb. Haemostasis 41*, 314–328.

Nelsestuen, G. L., Broderius, M., & Martin, G. (1976) *J. Biol. Chem. 251*, 6886–6893.

Niewiarowski, S., Kirby, E. P., & Stocker, K. (1977) *Thromb. Res. 10*, 863–869.

Niewiarowski, S., Kirby, E. P., Brudzynski, T. M., & Stocker, K. (1979) *Biochemistry 18*, 3570–3577.

Radvanyi, F., & Bon, C. (1982) *J. Biol. Chem. 257*, 12616–12623.

Rhee, M. J., Morris, S., & Kosow, D. P. (1982) *Biochemistry 21*, 3437–3443.

Stocker, K., & Egberg, N. (1973) *Thromb. Diath. Haemorrh. 1*, 361–370.

Stocker, K., & Barlow, G. H. (1976) *Methods Enzymol. 45*, 214–223.

Williams, W. J., & Esnouf, M. P. (1962) *Biochem. J. 84*, 52–62.