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BIOCHEMISTRY OF THE INTERACTION OF BOVINE FACTORS XIa AND IX*

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FOOTNOTES

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This communication is concerned with two of the blood coagulation factors which participate in the intrinsic coagulation pathway, Factor XIa (activated plasma thromboplastin antecedent) and Factor IX (Christmas factro; autoprothrombin II). According to the waterfall or cascade hypothesis for intrinsic coagulation as initially proposed by Davie and Ratnoff (1) and Macfarlane (2) in 1964 and later modified by Davie et al. (3), the factors that we are interested in are thought to participate early in the intrinsic pathway. We are, in particular, interested in the biochemistry of the interaction between Factor XIa and Factor IX which yields an activated enzyme, Factor IXa.

A certain amount of information on the nature of the interaction between Factor XIa and Factor IX has been reported previously, utilizing partially purified preparations of human origin. Ratnoff and Davie (4) were the first to describe the activation of Factor IX by Factor XIa. Kingdon and Davie (5) contributed information regarding the divalent cation requirement, showing that while calcium ions were most effective, strontium, cupric and zinc ions were also effective. These workers also identified human XIa as a "serine" enzyme on the basis of inactivation with diisopropylphosphofluoridate and subsequent isolation of a tetrapeptide, glycyl-aspartyl-phosphoseryl-glycine, from partial acid hydrolysates of the inactivated enzyme (6). In the intervening time from the initial observation by Ratnoff and Daive (4) a number of investigators have contributed further to our understanding of the nature of the interaction between Factor XIa and Factor IX (7-10). Unfortunately our knowledge of the biochemistry of this reaction is still quite rudimentary in nature. We have recently initiated a series of investigations with

the hope of further elucidation of the details of this reaction. The purification and characterization of the proteins involved is a prerequisite for achieving this goal. The large quantity of starting material (blood and/or serum) has precluded further investigation of protein of human origin and thus we have been concerned with fractions of bovine origin.

We shall first be concerned with a description of our efforts to obtain purified preparations of Factor XIa. Figure 1 shows our present purification method which is an adaptation of the previously described method for human XIa (4,6). Our procedure involves collecting bovine blood in glass, harvesting the serum approximately 24 hours later, and adsorbing the serum with barium sulfate. The barium sulfate-adsorbed serum is subjected to a heat treatment to destroy any other coagulation factors present, and then diluted with an acetate buffer (0.15 M, pH 5.2) and applied to a carboxymethyl-cellulose column previsouly equilibrated with the same solvent at room temperature. The column is then washed with the acetate buffer and crude Factor XIa eluated with 0.033 M sodium phosphate, pH 6.8 - 0.5 M NaCl. The effluent fractions are assayed for their ability to accelerate the clotting of plasma in plastic tubes (4,6). Fractions containing activity are pooled and further fractionated by ammonium sulfate precipitation. The fraction precipitating between 35% and 50% saturation contains Factor XIa activity and is utilized for further studies. The assay for Factor XIa (which is also utilized for Factor IXa) is, in fact, a modified partial thromboplastin time using non-contact-activated human plasma as the substrate in plastic tubes (4,6,11). Arbitrary units of Factor XIa activity are obtained utilizing a standard curve. The standard curve is a plot of the clotting time

of several dilutions of a Factor XIa preparation versus the extent of dilution on double logarithm paper. A value of 100 units is arbitrarily assigned to the most active fraction. A typical standard curve for Factor XIa is shown in Fig. 2. By the use of such a standard curve, clotting time can be assigned a value in arbitrary units.

The next step in our purification of bovine Factor XIa involves the gel filtration of the material obtained by ammonium sulfate fractionation on G-200 Sephadex. The results of a typical experiment are shown in Fig. 3. Gel filtration of crude bovine Factor XIa yields two peaks of activity when assayed in the previously described plasma system. Only one of these peaks has the ability to activate Factor IX as shown in Table I. The first peak accelerated plasma clotting, and also activated a component found in the barium sulfate eluate from bovine serum. The second paek, as yet unidentified, accelerated plasma clotting but had no effect on the barium sulfate eluate. Neither the Factor XIa nor the unidentified material clotted fibrinogen in 24 hours. There is a direct relationship between the quantity of Factor XIa as estimated from a standard curve as previously described and the ability to activate crude Factor IX as shown in Fig. 4. The data demonstrate that the amount of Factor IXa generated is directly proportional to the amount of Factor XIa added. Note that this experiment was performed under nearly initial reaction rate conditions, and does not address itself to the question of the amount of product obtained if the reaction had gone to completion.

We have done preliminary experiments designed to identify functional groups on the Factor XIa molecule important for catalysis. Fig. 5 shows that Factor XIa is inactivated by phenylmethylsulfonyl fluoride suggesting the importance of a seryl residue in the action of bovine

Factor XIa. This is consistent with the previous observations of Kingdon et al. (6) on the sensitivity of purified human Factor XIa to diisopropylphosphofluoridate. Phenylmethylsulfonyl fluoride has been utilized by Lundblad to demonstrate the catalytic importance of a seryl residue in purified bovine thrombin (12). Fig. 6 shows that bovine Factor XIa can also be inhibited by N-acetylimidazole suggesting that tyrosyl residues may also be important in catalysis or substrate binding.

We next turned our efforts to the purification and characterization of bovine Factor IXa. Bovine Factor IXa was prepared by the incubation of Factor XIa and the dialyzed barium sulfate eluate from bovine plasma in the presence of divalent cations at pH 8.0. Portions were removed, diluted in Tris-NaCl, pH 7.5 and assayed as described for Factor XIa. A time course for activation performed in the presence of calcium ions is shown in Fig. 7. Arbitrary units of activity were obtained by reference to a standard curve obtained from the sample at maximum activation. Such a standard curve for crude bovine Factor IXa is shown in Fig. 8. All fractionation procedures to be described employed this evaluation method.

Crude bovine Factor IXa, prepared as described above in the presence of calcium ions, was chromatographed on sulfoethyl-Sephadex C-25. The initial solvent was 0.05 M sodium acetate, pH 5.8. After application of the sample and subsequent washing, the column was developed with a linear gradient to a limit buffer of 0.1 M triethanolamine acetate, pH 7.5 - 1.0 M NaBr. All chromatographic buffers contained 5mM calcium ion. The results of a typical chromatogram are shown in Fig. 9. Considerable purification was obtained during this procedure but the activity peak was definitely asymmetrical. Sulfoethyl-Sephadex C-50 was then

tried with the same solvent system in hopes of obtaining better resolution. The results of such an experiment are shown in Fig. 10. With the use of this medium it is possible to separate two discrete peaks of activity. It was not possible to identify conclusively either of the peaks as another activated clotting Factor as Xa or thrombin. Kingdon and Davie (5) had observed that while calcium ions were most effective in the activation of crude human Factor IX by partially purified human Factor XIa, other divalent cations could also satisfy this requirement; of these, strontium ions were most effective. Somewhat later, Lundblad and Davie reported that the activation of Factor X with the intrinsic system had an obligate requirement for calcium (13). More recent unpublished observations in our laboratory in Chapel Hill suggest that the same is true for Factor X activation in the extrinsic system. We therefore decided to attempt the activation and subsequent chromatography in the presence of strontium to achieve a more specific activation of Factor IX. Figure 11 shows that this is indeed the case, as a single peak of activity was obtained. It would then appear that activation of crude bovine Factor IX by Factor XIa in the presence of strontium is a facile method of obtaining functionally homogeneous Factor IXa.

We have to this point emphasized the presence of divalent cations during our manipulations of Factor IXa. Table II shows that activity is rapidly lost when Factor IXa is dialyzed in the presence of EDTA, a metal chelating agent. Table III shows that this loss of activity can occur in the absence of EDTA and furthermore that partial reactivation can be achieved upon the readdition of calcium ions to a preparation previously treated with EDTA. The role of calcium in the maintenance of the integrity of Factor IXa was first suggested by Bergsagel in 1955 (14).

The metal ion effect suggested a possible function of sulfhydryl groups in Factor IXa activity. Figure 12 demonstrates a curious phenomenon when Factor IXa is incubated with β-mercaptoethanol. Under mild conditions (4° or ambient) an early enhancement of activity is observed followed by a rapid loss of activity whereas at 37°, activity is lost quite rapidly. Figure 13 shows that in the presence of dithiothreitol, activity is lost at all temperatures tested, but the rate of loss increased with increasing temperature. Somer and Castaldi (15) reported similar results upon incubation of their preparation of purified human Factor IXa. Although it appears unlikely that Factor IXa has free sulfydyl groups necessary for the maintenance of biological integrity, these results suggest the presence of a relatively labile disulfide bond or bonds required for the maintenance of native structure.

Finally we evaluated our purified preparations of Factor IXa with respect to their ability to interact with Factor VIII. These results are shown in Table IV. These experiments were performed basically as previously described by Lundblad and Davie (11). The formation of an activated product between Factor IXa and Factor VIII requires the presence of both calcium ions and phospholipid as shown previously by several groups of investigators (11, 16).

For the third phase of this communication, we would like to report briefly on our efforts to obtain purified preparations of bovine Factor IX. Our assay for Factor IX involves the use of kaolin-activated canine Factor IX-deficient plasma as the substrate (17). A standard curve for this system is shown in Fig. 14.

Our initial experiments were based on the use of citrate buffers. Several groups of investigators had successfully utilized such buffer

systems for the purification of Factor IX (15,18). The first step in our procedure involved the gel filtration of the barium sulfate eluate from bovine plasma on G-200 Sephadex in 0.01 M sodium citrate, pH 7.0. The results of such an experiment are shown in Fig. 15. The fractions containing Factor IX activity were then applied to a DEAE-Sephadex A-50 column previously equilibrated with the citrate buffer. A concave gradient to a limit buffer 0.5 M sodium citrate was used to develop the column. The results of this experiment are shown in Fig. 16. Considerable resoltuion of the Factor IX activity was achieved but the use of citrate buffer allowed the spontaneous development of both plasma clotting activity and fibrinogen-clotting activity which appeared earlier during the chromotography. We subjected the Factor IX obtained in this manner to further study. The chromatography of Factor IXa prepared from purified Factor IX on sulfoethyl-Sephadex C-50 is shown in Fig. 17. Only one peak of activity was observed. The protein concentration found in the experiment did not permit further characterization of this material. Figure 18 shows the chromatographic fractionation of material obtained in a similar manner again on sulfoethyl-Sephadex C-50. Again, note the single peak of activity. Purified Factor IX was also chromatographed in this system and would appear to be slightly more retarded (and hence more basic) than the activated form; the position of the center of the Factor IX proenzyme peak is indicated by an arrow.

The mechanism by which Factor IX is converted to Factor IXa likely involves proteolysis (19). The present results suggest that the conversion of Factor IX to IXa involves the removal of an acidic peptide or peptides; support for this concept must, however, await further studies. Schiffman and coworkers have previously noted changes in the

electrophoretic mobility of human Factor IXa compared to Factor IX (20). The change in gel filtration behavior reported by Kingdon is consistent with a change in molecular weight from 90,000 for Factor IX to approximately 55,000 for Factor IXa (19). Similar changes in molecular size during the activation of Factor IX have been subsequently reported by Østerud (21) and Aronson et al. (22); these latter workers used sodium dodecyl sulfate electrophoresis rather than gel filtration. These independent observations strengthen the view that Factor IX is reduced in size during activation.

The last experiments that we wish to describe concern some preliminary studies on alternative purification procedures for bovine Factor IX. We decided to avoid citrate in order to prevent the activation previously discussed. Figure 19 shows the gel filtration of crude bovine plasma Factor IX on G-200 Sephadex in 0.1 M sodium phosphate, pH 7.0, while Fig. 20 shows a similar fractionation of crude bovine serum Factor IX Note the apparent heterogeneity of the serum preparation. Note also that in this buffer system we obtain much better resolution of Factor IX from void volume material than we did using citrate buffer. Both of the crude preparations were rigorously treated with PMSF prior to gel filtration. Table V shows that these fractions all possess precursor IX activity; the serum fractions may contain quantities of activated Factor IX activity as well. Using this phosphate buffer system, there was no evolution of plasma-clotting or fibrinogen-clotting activity during purification.

The current evidence seems to indicate that Factor XIa converts Factor IX to Factor IXa by partial proteolysis. Firmer conclusions and further information regarding the chemistry of each of these entities must await their purification in milligram amounts.

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LEGENDS FOR FIGURES

Fig. 1. The purification of bovine Factor XIa. The details for this flow sheet are given in the text.

Fig. 2. A standard curve for bovine Factor XIa. Various dilutions of a Factor XIa preparation are assayed for the ability to accelerate the partial thromboplastin time of normal human plasma in plastic tubes. The above graph was obtained by plotting the logarithm of the clotting time (in seconds) against the logarithm of the extent of dilution. A value of 100 units is arbitrarily assigned to the most potent dilution.

Fig. 3. The gel filtration of crude bovine Factor XIa. The ammonium sulfate precipitate containing Factor XIa activity (prepared as described in the text from 5 - 10 liters of serum) was dialyzed overnight vs. 0.15 M sodium acetate, pH 5.2 and then applied to a G-200 Sephadex column (2.5 x 58 cm) previously equilibrated with the acetate buffer. The column was eluted at a flow rate of 18 ml/hour at ambient temperature and the effluent fraction assayed for protein concentration (A_{280}; solid line) or clot-promoting activity (open circles). Units of activity were obtained by reference to a standard curve as described in Fig. 2. The ability to activate Factor IX (solid circles) was determined as described under Table I.

Fig. 4. The relationship between Factor XIa activity and Factor IX activating activity. Factor IXa activity was determined by reference to the standard curve described in Fig. 2 and Factor IX activating activity determined as described under Table I.

Fig. 5. The inactivation of bovine Factor XIa by phenylmethylsulfonyl fluoride. Bovine Factor XIa was prepared by the ultrafiltration on Amicon diaflo membrane #PM-10 of Factor XIa obtained by gel filtration as described in Fig. 3. The retentate was dialyzed against 0.02 M sodium phosphate, pH 7.5. The bovine Factor XIa (0.5 ml) was added to 0.4 ml 0.1 M sodium phosphate, pH 7.5 and 0.1 ml of either 20 mM PMSF (Δ - Δ) or 10 mM PMSF (○ - ○) in methyl alcohol added to initiate the reaction. Portions (0.1 ml) were removed and assayed for Factor XIa activity in normal plasma as described in the text.

Fig. 6. The inactivation of bovine Factor XIa by N-acetylimidazole. Bovine Factor XIa was prepared as described under Fig. 5. A solution containing bovine Factor XIa (0.5 ml) and 0.1 M sodium phosphate, pH 7.5 (0.4 ml) was added to a tube containing N-acetylimidazole evaporated from a dry benzene solution. The amount of N-acetylimidazole is sufficient to give a final concentration of 1.8×10^{-3} M. The remainder of the reaction was performed as described under Fig. 5.

Fig. 7. The activation of crude bovine Factor IX by bovine Factor XIa. The cruder bovine Factor IX (0.6 ml) was mixed with 0.2 ml Tris, pH 8.0 and 0.1 ml 0.05 M $CaCl_2$ and 0.1 ml bovine Factor XIa added to initiate the reaction. Portions (0.1 ml) were removed at the indicated time intervals, diluted with 0.9 ml 0.06 M Tris, pH 7.5--0.09 M NaCl and assayed as described in the text against normal human plasma in plastic tubes. The arbitrary units of Factor IXa activated were determined from reference to a standard curve as described in Fig. 8.

Fig. 8. A standard curve for bovine Factor IXa. Various dilutions of a Factor IX preparation were assayed for their ability to accelerate the partial thromboplastin time of normal human plasma in plastic tubes. The above graph was obtained by the manipulation described under Fig. 2.

Fig. 9. The chromatography of crude bovine Factor IXa on sulfoethyl-Sephadex C-25. Crude bovine Factor IXa was prepared from 300 mg of lyophilized barium sulfate plasma eluate as described under Fig. 7. This was dialyzed against 0.05 M sodium acetate, pH 5.8--0.05 M $CaCl_2$ and applied to a column (1.5 x 28 cm) of sulfoethyl-Sephadex C-25 previously equilibrated with the acetate-calcium buffer. The column was developed with a linear gradient to a limit buffer of 0.1 M triethanolamine acetate, pH 7.5--1.0 M NaBr--0.005 M $CaCl_2$ at a flow rate of 60 ml/hour. Fractions (5 ml) were automatically collected and assayed for protein concentration (A_{280}, solid line) and Factor IXa activity (open circles). Arbitrary units of activity were obtained by reference to a standard curve as described in Fig. 8.

Fig. 10. The chromatography of crude bovine Factor IXa on sulfoethyl-Sephadex C-50. Except for the replacement of the C-50 derivative for the C-25 derivative, the experiment was performed as described under Fig. 9.

Fig. 11. The chromatography of crude bovine Factor IXa on sulfoethyl-Sephadex C-50 in the presence of strontium. The experiment was performed as described under Figures 9 and 10 except that calcium was replaced by strontium at a concentration of 0.005 M in both the activation and chromatographic steps.

Fig. 12. The effect of β-mercaptoethanol on bovine Factor XIa. Bovine Factor IXa was prepared by chromatography as described in Fig. 10 and dialyzed against 0.05 M Tris, pH 7.5--0.005 M $CaCl_2$. β-mercaptoethanol was added to give a final concentration of 1 mM and reaction allowed to proceed at the indicated temperature. Portions (0.1 ml) were removed at the indicated time intervals and assayed for ability to clot plasma as described in the text. Arbitrary units of activity were obtained by reference to a standard curve as described in Fig. 8.

Fig. 13. The effect of dithiothreitol on bovine Factor IXa activity. The experiment was performed as described under Fig. 12 except that β-mercaptoethanol was replaced by dithiothreitol at a final concentration of 1 mM.

Fig. 14. A standard curve for bovine Factor IX. This graph was prepared as described under Figures 2 and 8 except that bovine Factor IX was used as the sample with kaolin-activated canine Factor IX-deficient plasma as the substrate.

Fig. 15. The gel filtration of crude bovine Factor IX. Lyophilized bovine plasma barium sulfate eluate (500 mg) was dissolved in 5 ml 0.01 M sodium citrate, pH 7.0. This was then applied to a column (2.5 x 58 cm) of G-200 Sephadex previously equilibrated with the citrate buffer. The flow rate was 18 ml/hour and effluent fractions were assayed for protein concentration (A_{280}, solid line) or Factor IX activity (open circles) as described in the text.

Fig. 16. The chromatography of bovine Factor IX on DEAE-Sephadex A-50. The effluent fractions from gel filtration (see Fig. 15) containing Factor IX activity were pooled and applied to a column (2.5 x 25 cm) of DEAE-Sephadex A-50 previously equilibrated with 0.01 M sodium citrate, pH 7.0. The column was developed with a concave gradient to a limit buffer of 0.5 M sodium citrate, pH 7.0 at a flow rate of 60 ml per hour. Effluent fractions (5 ml) were assayed for protein concentration (A_{280}, solid line) or Factor IX activity (shaded area) as described in the text.

Fig. 17. The chromatography of bovine Factor IXa prepared from purified Factor IX on sulfoethyl-Sephadex C-50. Bovine Factor IXa was prepared as described under Fig. 7 using Factor IX obtained from chromatography on DEAE-Sephadex A-50 as described in Fig. 16. The Factor IX was dialyzed against 0.05 M Tris, pH 7.5 prior to the activation step. The chromatography was achieved as described in Fig. 10. Protein concentration (A_{280}, solid line) and Factor IXa activity (open circles) were determined as described in the text.

Fig. 18. Chromatographic comparison of bovine Factor IXa and IX. This experiment was performed as described in Fig. 17. In the indicated experiments Factor IX was chromatographed in the precursor form.

Fig. 19. The gel filtration of crude bovine plasma Factor IX. Lyophilized bovine plasma barium sulfate eluate (500 mg) was dissolved in 5 ml 0.1 M Tris, pH 8.0 and 25 mg phenylmethylsulfonyl fluoride in 1.0 ml MeOH added and the solution stirred for 30 minutes at room temperature. A second portion of phenylmethylsulfonyl fluoride was added and the solution stirred for an additional 30 minutes at room temperature. This mixture was then centrifuged and the supernatant fraction applied to a column (2.5 x 58 cm) of G-200 Sephadex previously equilibrated with 0.1 M sodium phosphate, pH 7.0, at room temperature. The column was eluted at a flow rate of 18 ml/hour and fractions (3.0 ml) collected and assayed for protein concentration (A_{280}, solid line) and Factor IX activity (open circles) as described in the text.

Fig. 20. The gel filtration of crude bovine serum Factor IX. This experiment was performed as described under Fig. 19 with the plasma eluate replaced by an equivalent amount (on the basis of weight) of serum eluate.

TABLE I

GEL FILTRATION OF CRUDE BOVINE FACTOR XIa

Sample[a]	Plasma C.T.[b] (seconds)	+ Serum Eluate[c] 0'	5'
First Peak (XIa)	124.4/147.1	29.7/2816	24.0/24.7
Second Peak (?)	136.5/147.6	33.5/35.1	36.4/33.6
Blank[d]	321.5/385.7	32.4/33.5	31.1/36.4

[a]The samples were obtained from the gel filtration of crude bovine Factor XIa as described in Fig. 3.

[b]The effect of the various samples on the partial thromboplastin time of normal human plasma in plastic tubes. The samples were diluted 1:10 in 0.06 M Tris--0.09 M NaCl prior to assay.

[c]A portion (0.1 ml) was added to a reaction mixture containing 0.5 ml bovine serum $BaSO_4$ eluate (1/10 volume) previously dialyzed against 0.05 M Tris, pH 7.5, 0.3 ml 0.05 M Tris, pH 7.5 and 0.1 ml 0.05 M $CaCl_2$. Portions of this reaction mixture were removed at the indicated time, diluted 1:10 in the Tris-NaCl buffer and assayed against normal plasma.

[d]The sample was replaced by 0.06 M Tris, pH 7.5--0.09 M NaCl.

TABLE II

METAL IONS AND FACTOR IXa

Dialysis Conditions[a]	C.T.[b] (seconds)
+ Ca^{2+}	117
+ Sr^{2+}	160
+ EDTA	300 +

[a]The preparations were dialyzed against 0.05 M imidazole, pH 6.0 containing the indicated substance at a concentration of 0.085 M for 16 hours at 4°.

[b]The ability of the dialyzed preparation to accelerate the partial thromboplastin time of normal human plasma in plastic tubes.

TABLE III

EFFECT OF METAL IONS ON BOVINE FACTOR IXa

DIALYSIS MEDIUM[a]	C.T. (Seconds)
+ Ca^{2+}	31.0/31.1
+ EDTA	210.5/211.1
No Additions	127.7/126.9

[a]Buffer = 0.05 M imidazole, pH 6.0 containing indicated additive at 0.005 M.

[b]Partial thromboplastin time in normal plasma (see Table I).

Partial Reactivation of EDTA-Treated Sample with 0.1 M $CaCl_2$		
	C.T. (Seconds)	C.T. (Average)
0'	204.7/206.3	205.5
60'	193.5/123.9	158.7

TABLE IV

ABILITY OF PURIFIED BOVINE IXa TO REACT WITH FACTOR VIII

Conditions[a]	Clotting Times (Seconds)[b]	
	0'	5'
IXa, VIII, Ca^{2+}, Phospholipid	63.5	46.6
IXa, VIII, Ca^{2+},	89.0	91.4
IXa, VIII, , Phospholipid	90.9	86.2

[a]The complete reaction mixture contains 0.1 ml Factor IXa, 0.5 ml bovine Factor VIII, 0.2 ml 0.1 M Tris, pH 7.0, 0.1 ml Centrolex P (0.1%) and 0.1 ml 0.05 M $CaCl_2$. In the indicated experiments the volume of the detailed component was replaced with an equal volume of Tris buffer.

[b]The partial thromboplastin time of normal human plasma in plastic tubes (see text for details).

TABLE V

BOVINE FACTOR IX PURIFICATION - GEL FILTRATION

Sample[a]	IX-Deficient Plasma C.T. (Seconds)[b]	+ Factor XIa[c]			
		0'	5'	10'	20'
Plasma IX	21.0/21.1	61.1	51.4	46.0	44.2
Serum IX - First Peak	16.2/16.6	66.8	54.4	49.6	46.4
Serum IX - Second Peak	15.4/15.6	47.0	38.9	38.7	35.4

[a]Fractions from gel filtration of barium sulfate eluates as described in Figures 19 and 20.

[b]Data obtained using kaolin-activated canine Factor IX-deficient plasma (see text for further details).

[c]These activations were performed as described in Fig. 7.