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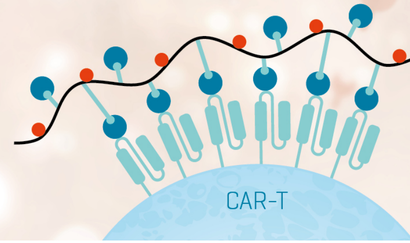

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IN VITRO STUDIES OF PREKALLIKREIN ACTIVATION IN RABBIT BLOOD: THE PARTICIPATION OF RED BLOOD CELLS[1]

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The activation of prekallikrein (PK) and coagulation Factor XII by dextran sulfate (DS) was studied with radioiodinated proteins as tracers in both rabbit plasma as well as rabbit blood. Activation was measured by SDS-PAGE of samples containing ^{125}I-proteins treated with β-mercapethanol (β-ME) at 100°C prior to electrophoresis. The addition of DS (25 to 2500 μg/ml) to rabbit blood resulted in a marked activation of both FXII and RPK so that up to 40 to 50% of the zymogen proteins were found to be activated. In contrast the addition of DS to rabbit plasma resulted in significantly less activation, with a maximum of 10 to 15% of these proteins being activated when DS was added to final concentrations between 25 and 2500 μg/ml. Fractionation of blood into platelet-rich plasma, buffy coat, and a red blood cell-rich fraction and subsequent reconstitution of these cell fractions with platelet-free plasma demonstrated that only the red blood cell fraction (essentially free of platelets and blood leucocytes) restored the activation of RPK to levels observed in whole blood. Prior depletion of blood PMN leukocytes with nitrogen mustard did not decrease the activation of RPK by DS in whole blood. Activation of RPK in blood by DS was shown to be blocked by anti-FXII antibody, suggesting that RPK activation results from the direct action of activated FXII.

The studies demonstrate the participation of red blood cells in the activation of FXII and RPK by a soluble polyanionic molecule and suggest an additional mechanism by which the activation of these proteins is regulated.

The rate of activation of coagulation Factor XII (Hageman factor, FXII)[2] is markedly enhanced by the interaction of FXII, prekallikrein, and high m.w. kininogen with a negatively charged surface. The molecular biochemistry of this process has been explored in *in vitro* studies with purified plasma proteins and genetically deficient human plasmas. This approach has rapidly advanced our understanding of the mechanism of FXII activation (1–6). However, considerably less is known about the biologic effects of activation of Hageman factor *in vivo*. This gap in our knowledge results, in part, from an absence of studies with activators of FXII that can be examined both *in vitro* as well as *in vivo*. Although kaolin and glass are the activating surfaces most commonly employed, it has been shown that sulfated polysaccharides, notably cellulose sulfate (7, 8) and carrageenan (9) apparently enhance FXII activation in plasma.

Most recently Kluft (10) and Amundsen *et al.* (11) have shown that the addition of dextran sulfate (DS) to human plasma activates prekallikrein (PK) by a FXII-dependent mechanism. DS is a highly water soluble material that may be useful for investigation of both the effects of FXII activation in experimental animals as well as a model compound for FXII activation by naturally occurring polyanionic macromolecules.

The studies described here were initiated to evaluate further DS as an activator of FXII and prekallikrein by using radioiodinated proteins and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to quantitate the effect of DS on these proteins in both rabbit plasma as well as rabbit blood. The present communication describes a series of *in vitro* experiments that demonstrate that although the addition of DS to rabbit plasma results in a modest activation of FXII and PK, the presence of the formed blood elements, specifically the red blood cells, markedly enhances this activation. These findings represent a newly described mechanism of FXII and PK activation and suggest that studies with soluble polyanionic macromolecules such as DS will provide new insight into the regulation of the activation of FXII-dependent pathways.

MATERIALS AND METHODS

Proteins. Rabbit prekallikrein (RPK) was purified from rabbit plasma by sequential ion exchange and lectin chromatography and preparative PAGE. The purified protein is a single-chain glycoprotein with an apparent m.w. of 88,000 determined by SDS-PAGE. The purification, characterization, and mechanism of activation of this protein have been recently described (12). Activation of RPK by activated FXII occurs by limited proteolytic cleavage and results in the formation of the enzyme, kallikrein. This protein consists of two disulfide-linked polypeptide chains; the larger or heavy chain has been shown to have an apparent m.w. of 55,000 and bears up to 90% of the ^{125}I after radioiodination. The light chain has an apparent m.w. of 35,000 and has been shown to contain the DFP-sensitive active site (12). Activation, in terms of enzymatic activity, was shown to parallel closely the proteolytic cleavage of RPK (12). Rabbit FXII was prepared from rabbit plasma as described by Wiggins

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[1] This is publication Number 1846 from the Department of Immunopathology, Scripps Clinic and Research Foundation. This work was supported in part by National Institutes of Health Grants AI-15136, AI-07007, and HL-16411.

[2] Abbreviations used in this paper: FXII, coagulation Factor XII; RPK, rabbit prekallikrein; PK, prekallikrein; DS, dextran sulfate; β-ME, β-mercaptoethanol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HN_2, nitrogen mustard; Bz-Pro-Phe-Arg-PNA, benzoyl-prolyl-phenylalanyl-arginyl-*p* NO_2 analide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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and Cochrane[3] and was generously provided for the experiments presented in this paper by Dr. Roger Wiggins (SCRF, La Jolla, California).

Radioiodination of RPK or FXII was performed by a modification of the methods described by McConahey and Dixon (13) by the following procedure: a 0.25-ml aliquot containing 30 μg of protein was combined with 0.025 ml of 0.5 M sodium phosphate, pH 7.5, 500 μCi of high specific activity $Na^{125}I$ (500 mCi/ml; NEN, Boston, Mass.) or $Na^{131}I$ and 0.001 ml of a 1 mg/ml solution of chloramine-T. After 10 min at room temperature this reaction was terminated by the addition of 0.002 ml of a 1 mg/ml of sodium metabisulfite. Before dialysis carrier protein in the form of 100 μg/ml of ovalbumin was added and the resultant solution was dialyzed for 36 hr against sterile 0.9% NaCl with at least four changes of the dialysate to remove unbound $Na^{125}I$. Greater than 95% of the radioactivity was precipitated with 10% trichloroacetic acid at 4°C for the radioiodinated proteins used in this study.

A monospecific IgG fraction of goat anti-rabbit FXII and IgG from nonimmune goat serum was kindly provided by Dr. Roger Wiggins (SCRF, La Jolla, California). The protein concentration of these reagents was determined to be 26 mg/ml for the anti-FXII and 28 mg/ml for the nonimmune serum IgG fraction. The preparation of the IgG fractions was a three step procedure. *Step I:* Solid ammonium sulfate was added to serum reaching a final concentration of 50% and the resultant solution was mixed at 4°C for 4 hr, the precipitate was recovered after centrifugation at 500 × G for 60 min, and the precipitate was washed twice with cold (4°C) 50% ammonium sulfate solution. The washed precipitate was then redissolved in water and dialyzed at 4°C against 0.01 M sodium phosphate, pH 7.7, containing 0.01% sodium azide. *Step II:* The dialyzed protein solution from Step I was then mixed with a slurry of DEAE cellulose (0.01 M sodium phosphate, pH 7.7) in the ratio of 5 g DEAE/g of protein. The resultant solution was gently mixed for 60 min at room temperature, the unbound IgG fraction was collected by vacuum filtration, and the resultant solution was concentrated by 0 to 50% ammonium sulfate precipitation. The protein precipitate was dissolved in water and dialyzed *versus* 0.01 M sodium phosphate, pH 7.7, containing 0.01% sodium azide. *Step III:* The IgG fractions were then absorbed with kaolin to remove traces of goat FXII and FXI. This was accomplished by mixing 2.5 mg of kaolin/ml of IgG fraction, mixing at room temperature for 60 min, and separating the kaolin by centrifugation at 5000 × G for 30 min. This adsorption was repeated four times. The fractions were shown to be free of goat coagulation factors XII and XI as well as prekallikrein (R. Wiggins, personal communication). Specificity of the anti-FXII was demonstrated by Ouchterlony analysis.

Polyacrylamide gel electrophoresis. SDS-PAGE was performed in vertical slab gels with 3-mm thick gels. Gels were prepared according to Laemmli (14) with a 3% stacking gel and a 9% separating gel. Electrophoresis was performed at room temperature with a constant voltage of 20 volts for 18 hr. At the conclusion of the electrophoretic run the gels were fixed and stained with a 0.05% solution of G-250 Coomassie Blue in 7% acetic acid, 5% methanol for 60 min at room temperature. Destaining was accomplished by dialysis of the slab gel against 7% acetic acid, 5% methanol for at least 8 hr. The destained slab gel was dried with a Hoeffer Scientific Instruments slab gel drier (Model SF 540).

Localization of the radioactive protein was accomplished by autoradiography with Kodak XR-1 x-ray film and a Dupont "Par Speed" image intensifying screen. Exposure was performed at −70°C (15). Because the majority of the ^{125}I is localized on the heavy chain, autoradiograms of slab gel containing ^{125}I-RPK will allow visualization of the intact RPK or the heavy chain when samples are treated with reducing agent before electrophoresis. Quantitation of the distribution of radioactivity in the slab gel was accomplished by cutting the gel into 0.5-cm pieces and measuring the radioactivity in each slice. Computer programs were employed to calculate the percentage of the total radioactivity in the peaks observed in the slab gel system.

For some experiments SDS gel electrophoresis was performed with 7% cylindrical gels as previously described (16). The distribution of radioactivity in the SDS gels was measured in 1.1-mm slices.

For analysis of data of the distribution of radioactivity in either slab or cylindrical gels, the radioactivity expressed as cpm/slice was plotted *versus* the normalized gel position where the latter was calculated from the ratio of (gel slice number)/(total number of gel slices). Calculation of the extent of cleavage was either determined from the reduction of the total amount of radioactivity in the precursor RPK molecule (88,000 m.w.) or from the percentage of the radioactivity associated with the heavy chain fragment (55,000 m.w.). Both methods yielded essentially identical findings.

Plasma samples to be electrophoresed in the SDS gel systems were diluted 1:5 with the sample buffer containing 1% β-mercaptoethanol and were brought to 100°C for 15 min before electrophoresis. Usually 0.05-ml samples containing approximately 20,000 to 50,000 cpm total radioactivity were applied to the polyacrylamide gels and greater than 80% of the radioactivity was recovered when the distribution of radioactivity in either the slab or cylindrical gel was quantitated.

Blood. Blood was collected from the medial ear artery of New Zealand White rabbits that had been fasted for 48 to 60 hr before blood collection. The animals had free access to water during this time. Unless otherwise specified the blood was collected in plastic tubes and anticoagulated with 2 units/ml of heparin (Liquamin-Sodium, 5000 USP units/ml, Organon Inc., West Orange, N. J.). Platelet-free plasma was prepared by centrifugation of anticoagulated blood in an Eppendorf Model 3200 centrifuge at 12,000 × G for 5 min.

Experimental protocols. DS (Lot 1359, Pharmacia Chemical Co., average m.w. of 500,000) solutions were prepared in sterile 0.9% NaCl immediately before each experiment. All reactions were carried out in a final volume of 0.075 ml in 1.5 ml conical plastic tubes (Sarstadt No. 609). Each reaction mixture was prepared with the following reagents added sequentially:

	Protocol I	*Volume (ml)*
a)	blood or platelet-free plasma	0.050
b)	^{125}I-RPK (1.0 μg)	0.010
c)	saline	0.005
d)	DS or saline	0.01

(The appropriate concentrated stock of DS was used to achieve the desired concentration and was usually added in no more than 0.005 ml).

When the effect of anti-FXII antibody was tested the following reaction mixture was used:

Protocol II	*Volume (ml)*
Blood	0.05
^{125}I-RPK (1.0 μg)	0.01
DS (5 mg/ml)	0.003
Anti-FXII or a nonimmune serum IgG fraction	0.012

[3] Wiggins, R. C. and C. G. Cochrane. Rabbit Hageman factor. Activation of Factor XI and prekallikrein by α-HF$_a$. The role of a negatively charged surface. Submitted for publication.

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The amount of anti-FXII employed here was shown to reduce the FXII activity of normal rabbit plasma greater than 98% by clotting assays (R. Wiggins, personal communication). The reaction was initiated by immersion of the tube into a 37°C shaking water bath and after 30 min at this temperature platelet free-plasma was recovered from samples containing whole blood by a 2-min centrifugation at 12,000 × G. Greater than 90% of the ^{125}I-protein added to blood was recovered in the platelet-free plasma from incubation mixtures with or without added DS. Aliquots of plasma were diluted into the appropriate SDS buffer and subjected to SDS-PAGE. This standard procedure was followed in all experiments unless otherwise noted.

Fractionation of blood for reconstitution experiments. Blood was collected in plastic 40-ml tubes from the medial ear artery of fasted rabbits and anticoagulated with 2 units heparin/ml of blood. The blood was centrifuged at 25°C in a IEC-PR6 centrifuge (rotor No. 253) for 15 min, 1400 rpm. The following fractions were then removed: a) the platelet-rich plasma; b) the buffy coat; c) the upper one-half of the red blood cell-rich layer was discarded, and d) the lower one-half of the red blood cell-rich layer was saved. A portion of the platelet-rich plasma was centrifuged at 12,000 × G for 15 min to prepare platelet-free plasma. For some experiments the buffy-coat from 5 ml of blood was recombined with different volumes of platelet-free plasma and the lower one-half of the red cell-rich fraction was recombined with the appropriate volume of platelet-free plasma to achieve the desired hematocrit.

The presence of platelets in each blood fraction was determined by measuring the levels of ^{51}Cr-platelets (17) added to the starting blood. Blood PMN leukocytes and mononuclear cells were quantitated by counting the number of cells in each fraction and the red blood cell content was determined by measuring the hematocrit with an International microcapillary centrifuge, (Model MB) and Van-Lab capillary tubes.

Hydrolysis of Bz-pro-phe-arg-pNA. Bz-pro-phe-arg-pNA (Pentapharm A. G., Basel, Switzerland) in a 1-mM solution was prepared in 0.01, HEPES, 0.15 M NaCl, pH 7.5. To study the hydrolysis of this compound by blood or plasma exposed to different amounts of DS we scaled up the reaction mixture of Protocol I (see above) 20-fold and added DS to a final concentration of either 50 to 200 μg/ml. Blood or plasma mixtures were maintained at 37°C for 1, 3, 5, or 10 min and at each time point 200 μl were removed, added to an 0.5-ml microfuge tube, and centrifuged for 1 min at 12,000 × G in a Brinkmann Model 3200 centrifuge. After centrifugation a 50-μl plasma sample was removed, added to 0.95 ml of the Bz-pro-phe-arg-pNA, and the change in absorbance at 405 min was measured in a Gilford Automatic recording spectrophotometer. Blood or plasma samples *without* added DS processed in the same way *did not* demonstrate any hydrolysis of the tripeptide substrate.

Miscellaneous procedures. Protein was measured by a colorimetric procedure (18). Nitrogen mustard (HN_2) injection was employed to deplete circulating polymorphonuclear (PMN) leukocyte levels to less than 5% of normal level. To do this we injected 3.5 mg of Mustargen (HN_2) (lot 1340T, Merck Sharp and Dohme, West Point, Pa.) i.v. into 3.5-kg rabbits. Blood samples were drawn 72 hr later for the experiment. Control animals received 3.5 ml of saline used as a diluent for the HN_2.

RESULTS

Activation of PK in the presence of DS. The ability of DS to activate PK was first examined by using ^{125}I-RPK added to either rabbit blood or rabbit plasma anticoagulated with heparin. Activation of RPK by activated FXII has previously been shown to occur by limited proteolytic cleavage of the parent 88,000 precursor, resulting in 55,000 and 35,000 m.w. fragments joined by disulfide linkage (12). Thus, activation may be quantitated by SDS-PAGE of ^{125}I-proteins electrophoresed under reducing conditions.

DS (200 μg/ml) was added to either rabbit blood or plasma containing ^{125}I-RPK and the resultant solutions were maintained at 37°C for 30 min. After removal of the formed blood elements the plasma samples containing the ^{125}I-RPK were electrophoresed in an SDS slab gel system (see *Methods*). Upon completion of electrophoresis the localization of the radioactivity was determined by autoradiography. The results of this experiment are shown in Figure 1 where it is shown that ^{125}I-RPK exposed to rabbit blood for 30 min remains at its native m.w. of 88,000 (panel a) whereas addition of 200 μg/ml of DS resulted in cleavage of the parent molecule so that the radiolabeled heavy chain (m.w. = 55,000) was detected after electrophoresis (panel c). In contrast, the addition of 200 μg/ml of DS to rabbit plasma containing ^{125}I-RPK did not alter the electrophoretic mobility of the ^{125}I-RPK, suggesting that no activation had occurred (panel b).

In order to quantitate the extent of the cleavage, the slab gels were sliced into 0.5-cm pieces, the radioactivity in each slice was measured, and the data were plotted as cpm/slice *versus* normalized gel position. Results obtained from the previous experiment are shown in Figure 2. For this experiment the extent of activation was calculated by determining the reduction of radioactivity associated with the 88,000 m.w. RPK peak in the activated samples. The data of Figure 2 indicated that approximately 45% of the RPK in whole blood was activated by DS under these conditions whereas no cleavage was detected in normal rabbit plasma.

Effect of anticoagulant. The previous observations resulted from experiments in which the blood and plasma were anticoagulated with heparin. We next sought to determine if the same phenomenon could be demonstrated when other anticoagulants were employed. To do this we collected rabbit blood using 0.38% citrate, 0.02% EDTA, or 2 units/ml heparin as anticoagulants, prepared platelet-free plasma, and compared the ability of three different concentrations of DS (10, 100, and 2500 μg/ml) to activate RPK in the blood and plasma samples. The result of SDS slab gel electrophoresis of the blood samples are

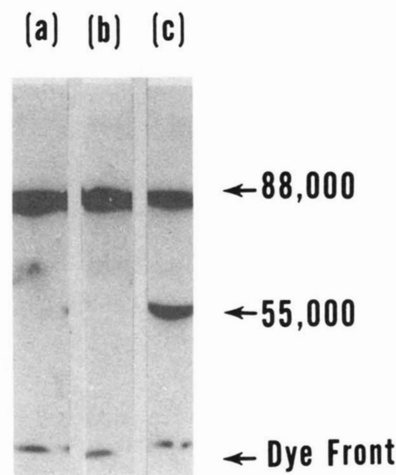

Figure 1. Autoradiographic analysis of SDS-PAGE of ^{125}I-RPK in rabbit blood and plasma after exposure to 200 μg/ml DS for 30 min at 37°C: *panel a,* ^{125}I-RPK + rabbit blood; *b,* ^{125}I-RPK + rabbit plasma + 200 μg/ml DS; and c, ^{125}I-RPK + rabbit blood + 200 μg/ml DS. The dried slab gels were exposed to x-ray film at −70°C as described in *Materials and Methods.*

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shown in Table I. These data show that over the range of DS concentrations examined, cleavage of ^{125}I-RPK was detected, irrespective of the anticoagulant employed. In no case was any cleavage of ^{125}I-RPK in plasma detected for the three different anticoagulants. These findings clearly show that the DS activation of RPK in blood is not dependent upon the anticoagulant employed even when the DS concentration was varied 250-fold.

Activation of FXII and RPK in blood and plasma: concentration dependence. In the previous experiment activation of RPK in blood was detected only at 100 μg/ml of DS when 10, 100, 2500 μg/ml of DS were employed. We next sought to determine accurately the concentration dependence of DS activation of FXII and RPK in both blood as well as plasma. Concentrations of DS between 25 and 2500 μg/ml were added to blood or plasma and after 30 min at 37°C analysis of the individual reaction mixtures was performed by SDS-PAGE in a slab gel system. The results of these studies are shown in Figure 3. The blood and plasma were obtained from a single animal although the data shown for HF or RPK represents findings made with different rabbits.

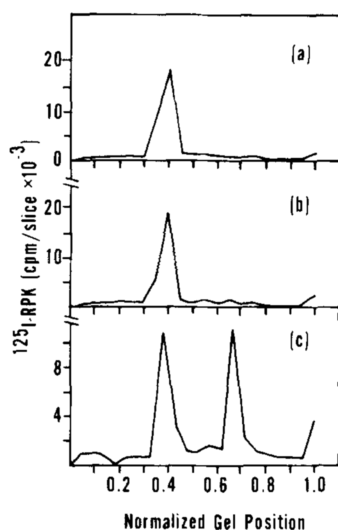

Figure 2. The distribution of ^{125}I-RPK in the SDS polyacrylamide slab gels determined after cutting the dried slab gels into 0.5-cm segments and counting each segment. The experimental conditions are as described for Figure 1 and the tracking dye has a normalized gel position of 1.0. The extent of cleavage of ^{125}I-RPK shown in *panel c* was calculated to be 45%.

TABLE I

The effect of anticoagulation on activation of RPK in blood and plasma

Anticoagulant	DS	Cleavage of ^{125}I-RPK[a]	
		Blood	Plasma
	μg/ml	%	
0.38% citrate	10	0	0
	100	26	2.8
	2500	2.4	0
2 units heparin/ml	10	0	0
	100	26	0
	2500	1.3	0
0.02% EDTA	10	0	0
	100	20	0
	2500	2.5	0

[a] The per cent cleavage was determined by the reduction in the peak of ^{125}I-RPK produced by the addition of DS.

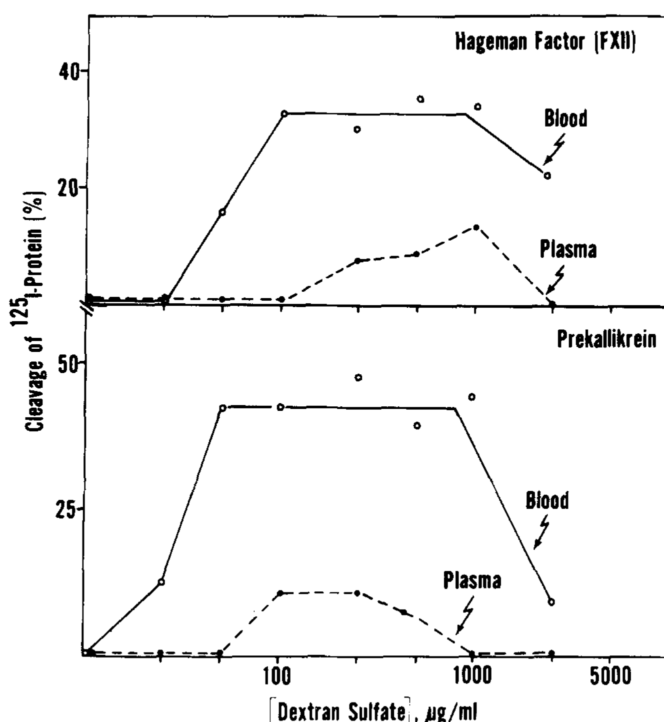

Figure 3. The effect of varying the DS concentration on FXII and RPK activation in rabbit blood or plasma. Data for FXII is shown in the upper panel whereas data for RPK is shown in the lower panel. Each reaction mixture was maintained at 37°C for 30 min before electrophoresis in an SDS-polyacrylamide slab gel system.

These data demonstrate that optimal activation of FXII and RPK is detected in blood by using 50 to 100 μg/ml of DS with apparent inhibition of this process occurring at DS concentrations of greater than 1000 μg/ml. The amount of both FXII and RPK activated in blood is at least 3-fold greater than that observed in plasma over the entire DS concentration range studied. We also determined that the enhanced activation observed in blood could not be explained by a difference in the rate of activation since maintenance of DS (100 to 1000 μg/ml) in blood or plasma for up to 2 hr did not result in any increased activation of either FXII or RPK from that observed in 30-min incubation.

In order to determine if the cleavage observed in blood after the addition of DS resulted in the production of active enzymes we examined the capacity of DS-treated blood or plasma to hydrolyze the kallikrein-specific substrate Bz-pro-phe-arg-pNA. The protocol for this experiment is described in detail in *Methods.* As shown in Figure 4 plasma recovered from *DS-treated blood* hydrolyzed the tripeptide whereas we failed to demonstrate the hydrolysis of this substrate when samples from *DS-treated plasma* were examined. Thus, the cleavage of the radiolabeled RPK is associated with the production of enzymatically active kallikrein. The decrease in activity during the 10-min interval studied presumably reflects the inhibition of kallikrein by plasma protease inhibitors.

Mechanism of the enhanced activation of FXII and RPK in blood. The previous experiments demonstrated that DS-induced activation of FXII and RPK was minimal in plasma but was significantly increased in the presence of the formed elements of blood. Thus, we next sought to identify the blood cell(s) that participate in FXII and RPK activation. Several

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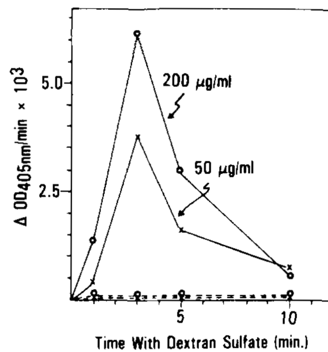

Figure 4. A comparison of the ability of DS-activated blood and plasma to hydrolyze Bx-Pro-Phe-Arg-PNA. Results obtained with plasma prepared from DS-treated blood are shown as (×——×) for 50 μg DS/ml and (○——○) for 200 μg DS/ml; whereas results obtained with DS-treated plasma are given by (×-----×) for 50 μg DS/ml and (○-----○) for 200 μg DS/ml. The details of this experiment are given in *Methods.*

TABLE II

Cell content of fractionated blood

Sample	PMN Leukocytes	Mononuclear Cells	^{51}Cr Platelets Starting level	Hematocrit
	cells/mm^3	*cells/mm*^{3}	%	%
Starting blood	4,650	5,000	100	42
Platelet-rich plasma			31	
Buffy coat	13,000	38,000	324	
Red blood cell-rich fraction[a]	100	700	4	37

[a] The lower one-half of the red blood cell-rich fraction was reconstituted with platelet-free plasma to achieve a final hematocrit of 37%.

different experimental approaches were utilized to investigate the question.

In the first approach we fractionated blood by centrifugation into four fractions; platelet-free plasma, platelet-rich plasma, buffy coat, and red blood cell-rich (lower one-half) fractions (see *Methods* for procedures used). The buffy coat and red cell-rich fractions were reconstituted with platelet-free plasma. For this experiment ^{51}Cr-platelets were added to the starting blood sample to quantitate the number of platelets in each fraction. The concentration of platelets, leukocytes, and the hematocrit were determined for the various reconstituted fractions. These data are presented in Table II. Each cell fraction was then examined for the capacity to activate ^{125}I-RPK after increasing amounts (50, 100, 250, and 500 μg/ml) of DS were added. The results of these experiments are shown in Figure 4 where the activation of RPK in platelet-rich plasma, buffy-coat, and a red blood cell-rich fraction was compared with that observed in the starting blood sample. These data show that neither the buffy coat fraction or the platelet-rich plasma supported the DS-induced activation of RPK to the extent observed with whole blood. In contrast, the red blood cell fraction that contained less than 10% of the blood leukocytes and 4% of the blood platelets demonstrated DS-induced activation of RPK to the same levels observed in whole blood in the experiment. The extent of activation of RPK by DS in platelet-free plasma was indistinguishable from that shown in Figure 5 for both buffy coat as well as platelet-rich plasma. These data suggest that the red blood cells are the essential formed blood elements participating in the DS-induced activation of RPK. In separate experiments not shown here we also failed to observe any activation of RPK when DS was added to buffy coat reconstituted with platelet-free plasma to either normal blood leukocyte levels or one-fifth of the normal blood leukocyte levels.

We next sought to establish the concentration dependence for red blood cells in the DS-induced activation of RPK. To accomplish this we prepared a red blood cell-rich fraction (lower one-half) by centrifugation (see *Methods*) and reconstituted these cells with graded volumes of platelet-free plasma to achieve final hematocrits between 40 and 10%. Activation of RPK was determined after a 30-min incubation with 200 μg/ml DS at 37°C. The results of these experiments are shown in Table III where data for activation of RPK in whole blood, the red blood cell-rich fractions, and platelet-free plasma are shown. These data further support the role of the red blood cell and demonstrate that RPK activation with DS is detected with a hematocrit as low as 20%.

We also employed nitrogen mustard (HN_2) injection to reduce the levels of circulating PMN cells to less than 5% of normal. Blood was obtained from an HN_2-treated rabbit and a control rabbit injected with saline and these samples were compared for the ability to support DS-induced activation of RPK. The results of experiments performed with 50, 100, 250 and 500 μg/ml of DS are shown in Figure 6. The extent of activation of RPK was indistinguishable in the control and PMN depleted blood. These data demonstrate that the presence of the PMN is not required for the DS-induced activation of RPK. In the HN_2 treated animals the concentration of blood mononuclear cells was also reduced to 60% of normal blood concentration.

Activation of RPK by DS: dependence on FXII. The previous

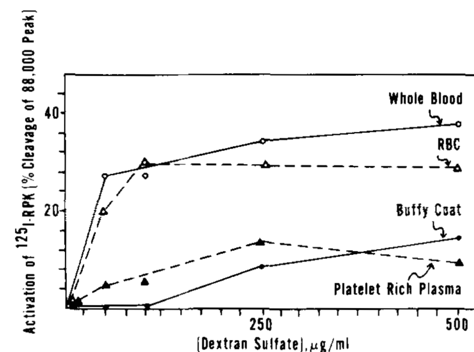

Figure 5. Activation of ^{125}I-RPK by DS in whole blood compared with that obtained in a red blood cell-rich fraction, platelet-rich fraction and buffy-coat fraction added to platelet-free plasma. Analysis of reaction mixtures was performed by SDS-PAGE in a slab gel system as described in *Methods.*

TABLE III

The effect of decreasing RBC concentration on DS-induced activation of RPK[a]

Sample[b]	Hematocrit	Cleavage of ^{125}I-RPK[c]
	%	%
Whole blood	37	54
Red blood cell fraction	40	60
Red blood cell fraction	30	37
Red blood cell fraction	20	14
Red blood cell fraction	10	Undetectable
Platelet-free plasma	0	Undetectable

[a] Activation was performed with a final concentration of 200 μg/ml DS, 30 min at 37°C.

[b] The red blood cell-rich fraction was prepared as described in *Materials and Methods,* and platelet-free plasma was added back to achieve the range of hematocrits shown in this table.

[c] Cleavage of ^{125}I-RPK was calculated from the reduction in the radioactivity associated with the 88,000 m.w. peak.

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experiments demonstrated that the enhanced activation in blood of RPK by DS requires the presence of the red blood cell. We next sought to determine if the participation of FXII was required for the DS-induced activation of RPK. To do this we utilized an IgG fraction of a monospecific goat antiserum to rabbit FXII. Experiments were performed with whole blood or the red cell-rich fraction of whole blood prepared by centrifugation as described in *Methods*. The red cell-rich fraction for this experiment, reconstituted with platelet-free plasma, had a hematocrit of 44%, contained no mononuclear cells or platelets, and was found to have 6% of the PMN leukocytes present in the starting blood. Aliquots of whole blood or the red cell-rich fractions containing ^{125}I-RPK and ^{131}I-RHF were combined with an IgG fraction of either goat anti-FXII or nonimmune goat serum for 30 min at 37°C. After 30 min DS was added to a final concentration of 200 μg/ml and the resultant solutions were maintained at 37°C for an additional 30 min. Plasma was separated from the formed blood elements by centrifugation at 12000 × G and an aliquot of plasma was diluted with SDS containing β-ME, brought to 100°C for 15 min, and electrophoresed in 7% cylindrical polyacrylamide gels containing SDS. The gels were sliced and the distribution of ^{125}I-RPK and ^{131}I-RHF was determined. The results obtained from SDS-PAGE of plasma prepared from blood with ^{125}I-RPK are summarized in Table IV. These data show that anti-FXII inhibits the DS-induced cleavage of both FXII and RPK in whole blood as well as the red blood cell-rich fraction.

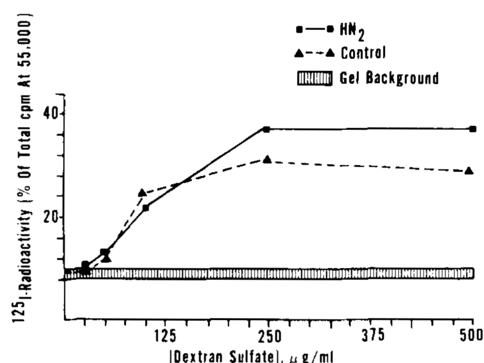

Figure 6. The effect of HN_2 depletion of PMN leukocytes on DS activation of RPK in rabbit blood. HN_2 treatment reduced the PMN leukocyte levels to below 95% of normal. Activation was performed with different DS concentrations for 30 min at 37°C, and the results were analyzed by SDS-PAGE in a slab gel system. For this experiment the activation of RPK was determined from the amount of radioactivity associated with the 55,000 m.w. heavy chain.

TABLE IV

The effect of anti-FXII on DS activation of FXII and RPK in whole blood[a]

	Anti-FXII	Non-immune IgG	% Inhibition of Cleavage	
			FXII	RPK
Whole blood	−	−	0	0
	+	−	93	100
	−	+	0	<10
Red blood cell-rich fraction[b]	−	−	0	0
	+	−	100	100
	−	+	9	0

[a] Whole blood or the red blood cell-rich fraction was exposed to 200 μg/ml of DS for 30 min at 37°C.

[b] The preparation and characterization of the red blood cell-rich fraction is described in the text.

DISCUSSION

In the present report we have demonstrated that the addition of DS to normal rabbit plasma results in the modest activation of coagulation FXII and PK. Significantly, the presence of the formed blood elements, notably the red blood cells, increased by at least 3-fold the amount of FXII and PK activated by DS. These findings demonstrate, for the first time, a role for blood cells in the activation of FXII by polyanionic substances under conditions that closely resemble the intravascular milieu.

The evidence for the participation of red blood cells was obtained from several different experimental observations. First we observed that the enhanced activation of both FXII and PK in blood *versus* plasma occurred over a large concentration range of DS with optimal activation in whole blood occurring with as little as 50 μg/ml of DS. These results suggest that the enhanced activation cannot be explained by a different concentration dependence on DS for FXII and RPK activation in blood or plasma.

Next we showed that the addition of a red blood cell-rich concentrate to platelet-free plasma effectively reconstituted the DS-induced activation of PK. It is important to note that for the experiments shown here the red blood cell fraction contained 4% of the normal blood platelets and less than 10% of blood leukocytes with essentially normal red blood cell content. Despite this marked depletion of blood leukocytes and platelets the extent of activation of RPK by DS was similar to that observed in whole blood when four different DS concentrations were tested. Significantly, the platelet-rich plasma and buffy coat fractions were not effective in supporting the DS-induced activation. In addition, depletion of greater than 95% of PMN leukocytes from peripheral blood by prior treatment with HN_2 did not prevent the DS-induced activation of PK.

Lastly, we showed that the activation of PK by DS was similar in blood anticoagulated with EDTA, sodium citrate, or heparin. These data suggest that the presence of divalent ions is not required for activation. The lack of requirement for external divalent cations, e.g., Mg^{++} or Ca^{++}, suggests that DS *does not* induce the noncytolytic release of a protease, capable of FXII activation, from secretory cells such as PMN leukocytes, platelets, or monocytes. In general, release reactions from these cells have been shown to require external divalent cations, especially calcium (19). However, we cannot exclude the possibility of release of cellular enzymes by a lytic mechanism, although we consider this possibility unlikely. In this regard we have shown that DS-induced activation of PK is similar if not identical when blood from normocomplementemic and C3-depleted (cobra venom factor treated) rabbits was compared (R. Ulevitch, unpublished data). Thus, the anticomplementary activity of DS (20, 21) appears to be unrelated to the activation of FXII.

The activation of PK by DS in whole blood or in a red blood cell-rich fraction was shown to be dependent upon FXII. The presence of an IgG fraction of anti-FXII abrogated the cleavage of RPK produced by DS whereas an IgG preparation from nonimmune serum had no effect. These data exclude the possibility that PK activation occurs as a result of DS-induced release of cellular enzymes capable of directly activating PK.

The cleavage pattern of ^{125}I-RPK observed in the present experiments is indistinguishable from that obtained with trypsin, $FXII_a$, or in kaolin-activated rabbit plasma containing ^{125}I-RPK (12). Although not presented in the present paper we have obtained evidence that the addition of DS to rabbit blood results in the cleavage of high m.w. kininogen, a natural substrate for kallikrein. We have also observed that the addition of DS to rabbit blood results in the formation of an amidolytic

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enzyme capable of hydrolyzing the kallikrein-specific tripeptide substrate benzoyl-prolylphenylalanylarginyl-p-NO_2 analide (12). These data suggest that the cleavage of RPK induced by DS results in the conversion of the zymogen RPK to an enzymatically active protease, kallikrein. In contrast, preliminary studies with ^{125}I-rabbit FXI failed to demonstrate significant activation of FXI in blood or plasma after the addition of DS. Thus, it would appear that DS promotes the formation of active FXII with marked PK activating activity but with minimal procoagulant activity. The molecular details of FXII activation are currently under study and will be described elsewhere.

A possible linkage between cells and the activation of FXII has been suggested by several other studies, although in contrast to our findings the proposed mechanisms would require activation of the cell and release and/or generation of a proteolytic enzyme. For example, Walsh (22) first suggested that human platelets may participate in the activation of the intrinsic coagulation pathway. Recently, Walsh and Griffin (23) reported in abstract form that washed human platelets treated with ADP or collagen promoted the activation of FXII. Although these studies suggested that the platelets might participate in FXII activation, other investigators have challenged the proposed role of the platelet in FXII activation (24). Most recently Lipscomb and Walsh (25) have reported that human platelets contain an activatable membrane-bound FXI-like activity apparently distinct from plasma FXI. However, in the present studies we showed that the platelet was not essential or sufficient for the DS-induced activation.

The recent report of Newball *et al.* (26) has described a kallikrein-like enzyme released from IgE sensitized human basophils after challenge with antigen. This enzyme, in addition to its activity on plasma kininogen apparently can activate FXII.

The mechanism by which DS and the red blood cell interact to enhance FXII and RPK activation is unknown. Preliminary findings suggest that a complex between DS and the red cell is formed, providing an appropriate surface for FXII activation. The molecular mechanism of the observations described here is currently under study. However, this study suggests that utilization of soluble polyanions such as DS may provide new insight into pathways of FXII activation, which may play a role in both the maintenance of normal hemostasis as well as providing a means of FXII and PK activation during inflammatory injury.

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