

The adsorption of coagulation factors onto phospholipids

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The Adsorption of Coagulation Factors onto Phospholipids

Its Role in the Reaction Mechanism of Blood Coagulation

*From the Laboratories of Coagulation Biochemistry and Cardiovascular Biochemical
Research, Departments of Haematology and Cardiology, University Hospital, Leiden,
The Netherlands*

H. C. HEMKER, M. J. P. KAHN* and P. P. DEVILEE



F. K. SCHATTAUER VERLAG · STUTTGART-NEW YORK

It has been shown by many authors (Cole, 1965; Esnouf, 1967; Hanahan, 1965; Papadjopoulos, 1964) that both factor V and factor X_a are adsorbed onto phospholipid micelles from purified preparations. It is probable that this adsorption is an essential feature of the formation of the prothrombin activating enzyme, because the complex of factors V and X_a adsorbed one beside the other on a phospholipid micelle surface is probably the enzymatically active structure (Barton et al., 1967; Hemker et al., 1967).

Phospholipids, however, have been shown to be an essential reactant of the blood coagulation process, not only at the site of formation of prothrombinase but also at the site of interaction of factor IX and factor VIII (Schiffman, 1967).

No certainty has as yet been obtained about the mechanism of the reactions in which factor IX is involved. It was suggested by Biggs et al. (1953, 1958) that this factor was activated subsequent to contact of the plasma with foreign surfaces. Contact product (i.e. the product of factors XII and XI after their activation by foreign surfaces) probably is the entity that brings about this activation (Soulier and Prou-Wartelle, 1960; Waaler, 1969; Ratnoff and Davie, 1962; Schiffmann et al., 1963; Nossel, 1964). The fact that the reaction is inhibited by DFP¹) (Kingdon and Davie, 1965) strongly suggests that contact product exerts an action of limited proteolysis on factor IX and thus activates it. The observations that more factor IX is found in serum than in plasma, and that the activity of factor IX can still be increased by adding contact product to serum, also argue in favour of an activation of this factor in the course of the coagulation process (Sen et al., 1967).

Although no conclusive evidence has as yet been brought forward, the acceptance that factor IX is activated to factor IX_a by contact product seems to be the most rewarding working hypothesis.

In view of the well-known analogies between factor V and factor VIII on the one hand and of factor X and factor IX on the other it might seem worth-while to investigate the possibility that the factors VIII and IX_a form a complex on a phospholipid surface, just as factors V and X_a do, and that the procoagulant function of the anti-haemophilic factors A and B is performed by this complex rather than by a reaction of the cascade type as was suggested by Macfarlane et al. (1964) and Biggs (1965a, b).

*) Present address: Hôpital Universitaire St. Pierre, Service du Centre Albert Hustin, 322 Rue Haute, Bruxelles, Belgique.

1) DFP: diisopropylfluorophosphate.

One experimental approach to test this hypothesis is to show that factors VIII and IX_a are adsorbed onto phospholipid in the same way as are factors V and X_a, and this approach was adopted for the study reported here.

The investigation of the possible adsorption of factors VIII and IX_a seems to be hampered by the difficulty of obtaining these factors in a pure state. We therefore studied the adsorption from media that were not essentially purer than plasma is, i. e. from BaSO₄-adsorbed plasma and from serum. This has the additional advantage that the situations under which the results are obtained closely approximate the physiological conditions. The phenomena observed thus lend themselves easily an assigned role in the physiological mechanism of coagulation.

In practice, this kind of experiment, is apt to be disturbed by formation of a small amount of thrombin from residual amounts of prothrombin in the serum and the adsorbed plasma used, this prothrombin being readily activated when Ca ions and phospholipid are added in the experiment. For this reason we carried out our experiments in the presence of a small amount of hirudin, which specifically neutralized the small amounts of thrombin generated during the experiment (Markward, 1958).

A preliminary report of these studies has already been published (Hemker and Kahn, 1967). Since then, Hougie, Biggs and Denson (1967) have published the report of a study based on gelfiltration techniques, showing that factor VIII and factor IX_a are bound to phospholipids. The possibility of a complex of the factors IX_a and VIII appears also as a suggestion in a review article of Esnouf and Macfarlane (1968). The subject is treated in extenso in the Ph. D thesis of one of the authors (Kahn, 1970).

Materials and Methods

Standard normal plasma was a pooled plasma from 31 healthy normals (15 males, 16 females, mean age around 30 years) spun platelet free (20 min, 20,000 g, 4° C) and stored at -20° C in 1 ml portions. Before use as a reference plasma, 1 µg/ml of hirudin was added.

Bovine and human oxalated plasma adsorbed with BaSO₄ according to Biggs and Macfarlane (1962); final concentration of oxalate: 10 mM.

Bovine and human serum oxalated after coagulation, prepared according to Biggs and Macfarlane (1962); final concentration of oxalate: 10 mM.

Phospholipid suspension. A 25 mg/ml stock suspension of Inosithin (Associated Concentrates U.S.A.) is made by homogenizing the material in veronal-acetate buffer (pH 7.35). The stock suspension is conserved in small portions at -20° C and diluted before use to the desired concentration. The stock solution is never frozen and thawed more than once. Chemical analysis of the Inosithin (kindly performed by Dr. G. J. M. Hooghwinkel, Lab. of Medical Chemistry, University Hospital, Leiden) showed that the relative distribution of the phospholipids in the Inosithin was 1.5% phosphatidylic acid; 41.4 %phosphatidylethanolamine; 23.3% phosphatidylcholine; 33.8% phosphatidyl inositol. The mixture contained furthermore 20.7 mg cholesterol per gram, 120 mg galactolipids per gram and 49 mg hexose per gram as a non-lipid contaminant.

Hirudin (Sigma U.S.A.). A 1 mg/ml stock solution in veronal-acetate buffer (pH 7.35) is stored at -20° C and diluted before use.

The factors V, X, VIII and IX were determined according to Veltkamp e. a. (1968). Care was taken to add hirudin to all samples tested, so as to obtain the same final concentration of this substance in all tests.

The reaction mixture in which the adsorption was studied consisted of:

- 0.9 ml adsorbed plasma or serum,
- 0.4 ml Inosithin suspension (1.5 mg/ml),
- 0.4 ml CaCl₂ solution of the desired concentration,
- 0.1 ml Hirudin solution (18 µg/ml).

For the calculation of the concentration of Ca ions in the final reaction mixture, the Ca⁺⁺ bound by the residual free oxalate in the plasma or serum was taken into account.

The reaction mixture was incubated at 37° C for 9 min. Half of the mixture was then kept at 4° C after addition of 0.1 ml veronal-acetate buffer (pH 7.35). The other half was centrifuged at 100,000 g for 35 min at 4° C. The supernatant was decanted and 0.1 ml of Inosithin suspension added to restore the original concentration of phospholipid. The barely visible or indistinguishable sediment is resuspended in veronal-acetate buffer containing the same concentration of hirudin and CaCl₂ as the original incubation mixture.

The uncentrifuged part of the incubation mixture, the supernatant after centrifugation as well as the resuspended sediment were tested for the coagulation factors V, X, VIII and IX each in at least three dilutions. From the coagulation times obtained the coagulation factor concentrations were inferred by comparison with a log-log standard curve from normal plasma in the usual way (Biggs and Macfarlane, 1962). The percentage adsorbed onto the sediment was calculated from these data. The coagulation factor concentration in supernatant and resuspended sediment also can be expressed as a percentage of the concentration in the original incubation medium directly by comparing with a reference curve prepared from the original incubation medium. As the reference curves from normal plasma and from the incubation mixtures were parallel in the concentration range used both procedures yielded identical results. Phospholipid concentrations are checked by measuring the ether-extractable phosphorus according to Bereblum & Chain (1938), after degradation of the sample according to Le Page (1957). Ca⁺⁺ concentrations were assessed in an atomic adsorption spectrophotometer, (Klein, 1967).

Experimental Results

From serum, factors IX_a and X_a are adsorbed onto Inosithin. The adsorption is dependent upon the presence of Ca⁺⁺ ions. From BaSO₄-adsorbed plasma, factors V and VIII are adsorbed by Inosithin. The adsorption is prevented by an excess of Ca⁺⁺ ions (Table 1). It was observed that at Ca⁺⁺ concentrations that are physiological or known to be optimal for the blood coagulation process (~5 mM), all 4 factors are adsorbed. This phenomenon is seen not only in human but also in bovine material (Table 2). Under conditions where adsorption takes place, the activity that disappeared from the supernatant is found again qualitatively though not quantitatively in the sediment. When no adsorption can take place due to unfavourable conditions of Ca⁺⁺ concentration, the activity in the resuspended sediments is negligible (Table 3).

Table 3 also shows that the adsorption onto Inosithin is accompanied by an appreciable over-all loss of activity. The activities of the supernatant fluid and the

Table 1. Adsorption of Human Coagulation Factors onto Phospholipids.

	Ca ⁺⁺ (mM)	Factor V		Factor VIII		Factor IX		Factor X	
		C	A	C	A	C	A	C	A
Uncentrifuged sample	0	71.0	—	83.0	—	124.0	—	143.0	—
Supernatant	0	26.6	58	57.8	30	122.5	1	135.3	5
Supernatant	5	49.7	30	62.7	24	112.4	9	130.1	9
Supernatant	40	63.7	10	82.2	1	111.5	10	125.6	12
Supernatant	90	~71.0	<0.5	~83.0	<0.5	96.7	22	91.0	36

In the columns indicated C the concentration in the supernatant fluid is given in % of that of a standard normal human plasma. In the columns marked A the amount of the clotting factor adsorbed is given as calculated from the relevant figure sub C and the concentration in the uncentrifuged sample, it is expressed as a percentage of the total amount present. The values represent the means of 4 different experiments. In each experiment each coagulation factor determination was carried out 8 times. The percentage adsorbed is calculated as the amount that disappeared from the supernatant fluid.

Table 2. Adsorption of Bovine Coagulation Factors onto Phospholipids.

	Ca ⁺⁺ (mM)	Factor V		Factor VIII		Factor IX		Factor X	
		C	A	C	A	C	A	C	A
Uncentrifuged sample	0	85.0	—	164.9	—	48.0	—	15.0	—
Supernatant	0	33.8	60	66.9	58	34.9	27	9.3	38
Supernatant	5	47.4	44	75.6	52	29.6	38	9.3	38
Supernatant	40	84.2	1	93.8	41	26.2	45	6.0	60
Supernatant	90	84.1	1	160.0	3	15.4	68	3.6	76

As in Table 1.

Table 3. Recovery of Human Coagulation Factor Activity after Adsorption onto Phospholipid.

	Conc. Ca mM	% in sup.	% in sed.	Sum	Loss
Factor V	1	20	8	28	72
	100	100	<1	100	0
Factor VIII	1	50	36	86	14
	100	96	<1	96	4
Factor X	1	78	<1	78	22
	100	21	3	24	76
Factor IX	1	66	2	68	32
	100	12	15	27	73

Each value is the mean of 3 different experiments. In each experiment each coagulation factor determination was carried out eight times. All concentrations are expressed as a percentage of the uncentrifuged control.

Table 4. Activity of the Resuspended Sediment after Adsorption from Deficient Plasmas.

Ca ion conc. (mM)	1	90
	Coagulation time (sec)	
Factor V-def. plasma	87	87
normal plasma	47	85
Factor VIII-def. plasma	287	290
normal plasma	178	282
Factor X-def. plasma	302	308
normal plasma	298	189
Factor IX-def. plasma	246	241
normal plasma	236	159

The values are the means of 3 experiments (each determination carried out 8 times), and give the coagulation times in a test specific for the factor in which the material was deficient. The starting material was normal or deficient adsorbed plasma for factors V or VIII and normal or deficient serum for factors X and IX.

sediment do not add up to the original activity; no conditions could be found (by varying pH, temperature, ionic strength etc.) under which adsorption was not accompanied by inactivation.

When adsorption experiments are carried out with specifically deficient starting materials, the factor absent in the incubation mixture is specifically lacking in the resuspended pellet (Table 4); this indicated that no artifact is simulating the activities observed.

When adsorption from the supernatant is prevented because of an improper Ca^{++} concentration, the activity is not found in the sediment, as can be seen from the coagulation times obtained with the normal control plasmas in Table 4.

Centrifugation in the presence of an Inosithin suspension thus seems to bring about a partition of the coagulation factors between sediment and supernatant fluid that, in view of its dependence upon Ca^{++} ions and its behaviour in specifically deficient systems, must be considered to be due to adsorption of the coagulation factors onto the lipid material. For factors V and X_a this confirms earlier results (Hanahan, 1965; Cole, 1965; Esnouf, 1965); for factors VIII and IX_a , it represents a new finding.

The possibility that the adsorption observed is due to an a-specific adsorption of proteins onto lipids is excluded by the results shown in Table 5. Although plasma and serum proteins are readily adsorbed, this adsorption does not show the Ca^{++} dependence characteristic of the coagulation factors. In serum there is no Ca^{++} dependency at all, whereas factors X_a and IX_a are adsorbed more when more Ca^{++} is added. In adsorbed plasma the adsorption of proteins is enhanced by Ca^{++} , whereas factors V and VIII are adsorbed less when the Ca^{++} concentration is increased, so here the over-all adsorption of proteins is opposite to the adsorption of the relevant coagulation factors. The phenomenon of Ca^{++} dependent adsorption of plasma proteins onto phospholipids remains to be investigated.

Table 5. Adsorption of U. V. Adsorbed Material from Human Plasma and Serum onto Phospholipid.

Ca concentration (mM)	Percentage E 280 adsorbed	
	from $\text{Ba}(\text{SO}_4)_2$ adsorbed plasma	from serum
0	5	22
40	18	23
90	22	22

Adsorption at 280 $m\mu$ was estimated in samples of AlOH_3 adsorbed normal plasma and serum in a Zeiss PMQ II spectrophotometer. Phospholipid and Ca was added, and centrifugation was carried out as indicated under methods. E 280 was again measured in the supernatant. The amount of U. V. adsorbant material thus removed was expressed as a percentage of the amount originally present.

The values represent the means of 8 experiments. Heparin (1 $\mu\text{g}/\text{ml}$) was added to the plasma.

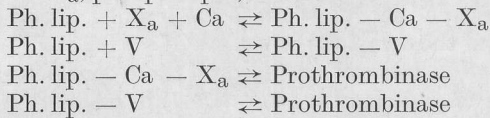
Discussion

After Macfarlane (1964) and a short time later Davie and Ratnoff (1964) proposed the cascade theory as a reaction mechanism for the blood coagulation process, two divergent lines developed. The school of Macfarlane (Jobin and Esnouf, 1967; Hemker et al., 1967), favoured a modification of the original cascade, in which activated factor X (factor X_a) provided the active centre for the conversion of prothrombin into thrombin, but was only able to do so when adsorbed onto a phospholipid micelle next

to factor V. The Ratnoff group maintained the original cascade scheme, stating that factor V is activated by factor X_a , and that factor V_a converts prothrombin into thrombin (Breckenridge, 1965; 1966). Most authors agree that the complex theory is the most probable one. The arguments leading to this opinion are of two kinds:

a) Factors V and X_a are dependent by phospholipids in a way that is specifically dependent upon the Ca^{++} ion concentration (Hanahan, 1965; Cole, 1965; Esnouf, 1967). Adsorption of factor X_a is improved by increasing the Ca^{++} ion concentration whereas adsorption of factor V decreases with Ca^{++} ion concentration. At the Ca^{++} concentration that is optimal for blood coagulation, both factors are readily adsorbed. When the two different proteins are adsorbed onto different phospholipids, their coagulation promoting activity is negligible compared to the situation when the same amount of these proteins is adsorbed onto the same micelle (Cole, 1965).

b) Kinetic studies of the prothrombinase generation in mixtures of factor V, factor X_a , phospholipid, and Ca^{++} ions can be explained by reaction schemes of the type:

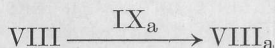


but not by reactions of the cascade type (Hemker et al., 1964; 1967 a, b; Degeller, 1968).

The experimental results supporting the cascade theory (Breckenridge, 1965, 1966; Ratnoff, 1965) can be explained in terms of the "complex theory" if it is taken into account, that factor V can be modified by thrombin as well as by Russell's viper venom into a more active form or even must be acted upon by thrombin to show any activity (Newcomb and Hoshida, 1965; Prentice and Ratnoff, 1969).

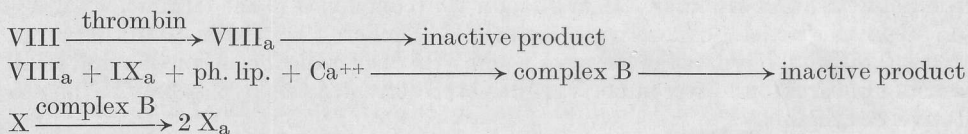
From the experiments reported here, we can conclude that factors VIII and IX_a adsorb onto phospholipid in a way fully analogous to the adsorption of factors V and X_a . In combination with the well known analogies between factor V and factor VIII on the one hand, and factor X and factor IX on the other, this strongly suggests that the way in which factors IX and VIII act, may be analogous to the behaviour of factors V and X, i.e. that factors VIII and IX_a form a complex on a phospholipid micelle. This would provide a satisfactory explanation of the fact that Ca^{++} ions and phospholipids are mandatory reactants of the coagulation process at this stage (Schiffmann et al., 1967).

The assumption that the interaction between factors VIII and IX_a is of the cascade type (Macfarlane et al., 1964; Biggs et al., 1965) i.e.:



is based on kinetic experiments. In our opinion, however, the results of these experiments are not incompatible with a reaction scheme of the "complex" type.

Any scheme must, of course, include the role of thrombin as an activator of factor VIII, as recognized by Biggs et al. (1965a, b) as well as by Rapaport et al. (1965). This activation, however, is not a unique feature of the "cascade" type of reaction scheme. It can be included very well in a scheme of the "complex" type also, which then would read:



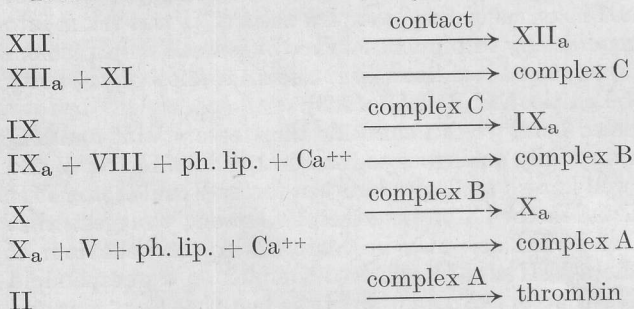
In this reaction scheme factors VIII and IX take an essentially equal place in the formation of complex B. The argument, which leads Macfarlane et al. (1964) to suggest that these factors have an essentially unequal function, as would be necessary for the original cascade scheme, is based on a quantitative difference rather than on a qualitative one. It states that factor-IX preparations have to be diluted much more than factor-VIII preparation to obtain the same kind of variations in the concentration of factor-X activator.

In our opinion, however, this argument cannot be advanced as long as the binding constants of the two factors to phospholipid are unknown. Moreover, we do not know what the relation is between real concentration expressed in number of molecules per unit volume and relative concentration expressed as per cent of the activity of normal plasma. This makes it extremely difficult to judge the meaning of qualitative variations of a reaction - product as a function of varying concentrations of the reactant.

Therefore, we think, the experiments of Macfarlane et al., are compatible with the "complex theory" as well as with the "cascade theory" whereas the binding experiments reported in this article strongly favour the complex theory.

Before trying to give a reaction scheme of blood clotting based on these considerations, we want to draw attention to an article of Haanen et al. (1967) in which it is made probable that the activator activity, that converts factor IX into factor IX_a is a complex XII_a and XI rather than factor XI_a.

If this conclusion is correct, an elegant scheme of thrombin generation develops:



This is essentially an enzyme cascade as originally proposed by Macfarlane, but now consisting of 4 steps rather than 7 steps. In general terms its function as a biological amplifier will be the same.

The fact that convincing evidence has been provided that factor VIII in the form in which it circulates in plasma must be acted upon by thrombin before it can take part in the reactions leading to thrombin generation (Rapaport, 1965) seems to invalidate all previously proposed clotting cascades. In presence of minute amounts of injured tissue this condition can be overcome because the extrinsic pathway constitutes a short circuit of thrombin generation. The first thrombin generated can thus serve to render factor VIII in a form suitable for reaction in the intrinsic pathway.

Under circumstances in which the presence of even minute amounts of contamination by wounded tissue seems to be ruled out, blood still clots. A possible explanation for this phenomenon may lie in the observation that the active product generated by contact activation of factor XII and factor XI (complex C in the terminology introduced in this article) can activate factor VII (Soulier, 1960; Altman and Hemker, 1968). This would bypass the factors IX and VIII during the formation of a first small amount of thrombin. After factor VIII has been activated this bypass may be quantitatively negligible.

Summary

It is shown that factors VIII and IX react with phospholipid in a way fully analogous to the reaction of factors V and X. From this it is concluded that a complex of factors VIII and IX adsorbed onto a phospholipid micelle may be the entity that activates factor X in the intrinsic pathway. The implications of this finding for the concepts of the reaction mechanism of blood coagulation are discussed. It is shown that instead of the original 7-step cascade of Macfarlane, a 4-step cascade consisting partly of complex enzymes is more probable.

Résumé

On démontre que les facteurs VIII et IX réagissent avec le phospholipide d'une façon tout à fait analogue à la réaction des facteurs V et X. On admet qu'un complexe des facteurs VIII et IX adsorbé sur une micelle de phospholipide pourrait être l'entité qui active le facteur X dans le système intrinsèque. On discute les implications de ce résultat sur les théories concernant le mécanisme de coagulation. On montre qu'au lieu de la cascade à sept étages de MacFarlane, une cascade en 4 étages, constituée en partie par des complexes d'enzymes est plus probable.

Zusammenfassung

Es wird gezeigt, daß die Faktoren VIII und IX mit Phospholipiden in völlig analoger Weise reagieren wie die Faktoren V und X. Es wird daraus geschlossen, daß ein Komplex der Faktoren VIII und IX, adsorbiert an Phospholipidmicelle, jener Komplex sein könnte, welcher den Faktor X im endogenen System aktiviert. Die Konsequenzen dieses Befundes für das Konzept des Reaktionsmechanismus der Blutgerinnung werden diskutiert. Es wird gezeigt, daß anstelle des ursprünglichen Siebenstufenkaskaden-Schemas von Macfarlane eine Vierstufenkaskade, welche z. T. aus komplexen Enzymen besteht, größere Wahrscheinlichkeit besitzt.

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Note added in proof

Sommer & Castaldi recently reported experiments which they interpret as being in contradiction with our conclusions. In our opinion an alternative explanation of their experiments is possible.

Østerud & Rapaport describe in a recent article experiments of a more direct nature that confirm our conclusions.

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