The interaction of factor Va with phospholipid monolayers was studied using alternating current polarography. For these studies a hanging mercury drop electrode is positioned in contact with the monolayer at the air-monolayer interface. Factor Va introduced into the aqueous subphase beneath the monolayer caused alterations in the capacitance of the circuit. When factor Va was introduced beneath monolayers containing 13–25% mole fraction phosphatidylserine (PS), 87–75% mole fraction phosphatidylcholine (PC), a significant capacitance increase occurred, which is interpreted to be the result of the penetration of the factor Va molecules through the monolayer. No penetration or associated capacitance changes were observed with monolayers composed of pure PC or pure PS.

Polarography experiments were also conducted with the electrode placed in the aqueous subphase to measure the binding of factor Va to pure PS monolayers. This approach has allowed detection of factor Va (at nm concentrations) adsorption to a pure PS monolayer. Thus the membrane adsorption process can be distinguished from factor Va penetration through the monolayer. These results suggest that there are two types of interaction of the factor Va molecule with PS-containing monolayers. The interaction with the PS component of the monolayer is essential for binding the factor Va (at nm concentrations) that occurs by surface adsorption. For mixed PC/PS monolayers, PS adsorption is followed by penetration of the protein through the membrane.

During the expression of the coagulation cascade, soluble plasma zymogens are converted into active enzymes by enzymatic complexes that are located at the surface of activated or damaged cell membranes. In the terminal complex, prothrombin is activated by factor Xa in the presence of factor Va on the membrane surface to form a-thrombin. In in vitro reconstituted systems the activation of prothrombin is dramatically enhanced when anionic phospholipids and Ca²⁺ are present. The interaction of the vitamin K-dependent proteins with membranes is dependent upon ionic strength in the medium, and it is likely that these interactions are, at least in part, driven by electrostatic forces.

The binding of factor Va to the membrane surface is strongly influenced by acidic phospholipids and involves the light chain of the protein (7, 13, 14). The factor Va binding interactions with 75% phosphatidylcholine (PC), 25% phosphatidylserine (PS) vesicles are not Ca²⁺-dependent and show only a limited dependence upon changes in the ionic strength of the media (7, 8, 14). Factor Va binds to phospholipid vesicles composed of 75% PC, 25% PS with a Kₜ of 2.7 nm (8). At 40% PS, 60% PC, the Kₜ increases to 6.7 nm. Thus the interaction of factor Va with PC/PS phospholipid vesicles is subject to a multiplicity of parameters.

Step-flow kinetic analysis of the binding of factor Va to 25% PS, 75% PC vesicles shows that this reaction occurs at a diffusionally controlled rate with an extraordinarily high collisional efficiency (10). The interaction of factor Xa with acidic phospholipid membrane surfaces also occurs at a nearly diffusionally controlled rate and with high collisional efficiency (10, 11). Thus these rate-limiting steps for prothrombinase complex formation are dependent upon protein-membrane interactions rather than the protein-protein interactions that are weak (15) and occur once the constituent proteins have been bound to a membrane.

The factor Va light chain is comprised of three domains labeled A3, C1, and C2 (16–18). The results of photoaffinity-labeling studies are consistent with the conclusion that the factor Va light chain is embedded into the hydrocarbon core of 75% PC, 25% PS phospholipid vesicles (19). Proteolytic activation, or made available by mechanical damage to the cell membrane (2–4). When compared with factor Xa in solution, the activation of prothrombin to a-thrombin by the prothrombinase complex on an appropriate membrane surface is enhanced approximately 300,000-fold (3, 5). Thus, knowledge of the membrane binding properties of the proteins involved in this reaction is essential to our understanding of blood coagulation processes. Equilibrium membrane binding studies (6–8) and rapid reaction kinetics (9–11) have been used to study the binding of each protein involved in the reaction as well as the overall assembly of the prothrombinase complex (10).

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fragmentation studies have indicated that a COOH-terminal peptide region of the A3 domain is protected from enzymatic hydrolysis in the presence of 75% PC, 25% PS vesicles, and the protected peptide after isolation binds to these vesicles, probably through hydrophobic interactions (20).

Recent studies by Ortel and by co-workers (21, 22) using deletion mutation analysis of recombinant human factor Va suggest that the C2 domain of the factor Va light chain is also associated with phospholipid binding. In these studies 100% PS adsorbed to microtiter plates was used to study the binding interaction. This interaction was ionic strength-dependent.

Thus at least two elements of the factor Va light chain are implicated with respect to membrane binding, and both hydrophobic and electrostatic interactions are suggested. In the present study we have employed electrochemical methods to explore the nature of the interaction of Factor Va with phospholipid monolayers. This study provides insight into the contribution of both electrostatic and hydrophobic interactions to this process.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine factor Va was prepared as described previously (23, 24). The extinction coefficient (E\textsubscript{1%}) used to determine protein concentrations was 1.74 (8). The molecular weight of bovine factor Va was taken as 168,000 (1), and the value of the diffusion coefficient D was 4.3 x 10\textsuperscript{-7} cm\textsuperscript{2}s\textsuperscript{-1} (10, 15, 25, 26).

Chromatographically pure egg lecithin (PC) and ox brain phosphatidylethanolamine, grade I, purchased from Lipid Products (Nutfield, United Kingdom) were supplied in chloroform/methanol solution. To prepare membrane monolayers composed of 75% PC, 25% PS, an aliquot of each solution was introduced in a vial, the sample was evaporated under a stream of nitrogen, and the lipid content was determined by weight. The condensed lipid monolayer was formed by spreading a 2-3-fold excess of lipid in a trough (10-30 cm\textsuperscript{2}) over solvent. The excess lipid on the condensed state obtained according to surface pressure measurements (27). The excess lipid formed collapsed structures in equilibrium with the spread monolayer. Control studies demonstrated that the excess lipid had no contribution to the electrochemical measurements.

**Electrochemical Measurements**—The electrochemical studies were carried out at room temperature, in the presence of CaCl\textsubscript{2}, 0.15 M NaCl, 20 mM Hepes pH 7.4 solutions prepared using ultrapure water from a Millipore Super Q system.

Alternating current polarography was carried out as described previously (28) with the polarographic instrument constructed in our laboratory (29) using a Metrohm polarographic cell filled with electrolyte. After air oxygen was displaced by argon bubbling, a condensed lipid monolayer was formed. The working electrode is a hanging mercury drop that was formed on a capillary tip of the electrode device and positioned in contact with the phospholipid monolayer; an Ag/AgCl, saturated KCl electrode was the reference, and a platinum gauge was the counter electrode. Thus, the monolayer transferred on the mercury electrode–water interface stayed in equilibrium with the monolayer reservoir on the argon–water interface. The electrical potential was scanned at a rate of 50 mV/s, and the frequency of the alternating current modulation (10 V) was 80 Hz. The starting potential was chosen so as to be in the stable region of the monolayer, namely, between −500 and −900 mV relative to the 1 M Ag/AgCl electrode. Perturbation of the membrane's integrity by solute macromolecules is evaluated by measuring the alteration of capacitance (C) of the circuit associated with the mercury electrode that is in direct contact with a condensed phospholipid membrane monolayer.

The circuit is designed in such a way that the main component of the circuit capacitance is due to the mercury electrode. Fig. 1 is a representation of the organization of the electrical circuit, with the monolayer in contact with the electrolyte solution on one side (subphase) and with the working electrode on the other side. The capacitance of the circuit depends upon the solute nature and membrane components that are in contact with the electrode, as shown in Fig. 2.

In Fig. 2 are represented plots of the capacitance (C) versus potential (E/V), imposed between the working and reference electrodes, for different species: pure electrolyte (a), condensed protein monolayer (b), and a condensed phospholipid monolayer (c). A small potential between −0.5 and 0.5 V, the lipids are adsorbed at the electrode by their hydrophobic tails (Fig. 1), and the capacitance of the electrode in contact with an intact condensed phospholipid monolayer (Fig. 2, curve c) is low and characteristic of a hydrocarbon layer, one hydrocarbon chain length thick (varying between 1.2 and 1.7 microfarads/cm\textsuperscript{2}). This value is independent of

![Figure 1](https://example.com/fig1.png)

**Fig. 1. Schematic representation of the working surface monolayer polarographic system.** The hanging mercury drop electrode is adjusted to be in contact with phospholipid monolayer that is spread on the surface of the subphase. The monolayer is oriented such that the head groups of the phospholipid are positioned toward the aqueous medium in the subphase, whereas the hydrocarbon tails extend up into gaseous argon atmosphere. The three electrodes are connected to a potentiostat.

![Figure 2](https://example.com/fig2.png)

**Fig. 2. Representative capacitance versus potential curves for three sorts of systems, where the mercury electrode is in contact with electrolyte (a), condensed protein monolayer (b), and condensed phospholipid monolayer (c) as illustrated in Fig. 1.** Capacitance (C) versus potential diagrams for each of these conditions are illustrated in the accompanying plots. E, electrical potential; µF, microfarads.)
the phospholipid head group composition. In the case of proteins adsorbed at the electrode (Fig. 2, curve b) the capacitance is higher, about 13 microfarads/cm². When a macromolecule is introduced into the aqueous phase (subphase) beneath the monolayer, three types of process may occur: surface adsorption, surface adsorption followed by penetration, or no interaction. The attainment of equilibrium between the bulk and the surface layer was facilitated by gentle stirring of the subphase with a magnetic stirrer. When a protein interacts by penetrating the monolayer, the observed capacitance will increase. A model of this sort of interaction is illustrated in Fig. 3. For a protein P (Fig. 3) that penetrates the interface between the condensed monolayer and the solution, the circuit will exhibit properties that correspond to electrical condensers in parallel, with contributions from both the protein P and monolayer L. The surface layer capacitance (C) will increase, and C will be a function of the amount of protein penetrating across the membrane and follows the equation.

\[ C = C_L + \theta (C_P - C_L) \]  

(Eq. 1)

where \( C_L \) and \( C_P \) are the capacitance of the intact condensed lipid and protein monolayers, respectively, and \( \theta \) is the fraction of lipid monolayer that has been penetrated by the protein.

For a protein that adsorbs at the condensed lipid monolayer, illustrated by molecule A of Fig. 3, the capacitance of the system would correspond to that of two condensers, the membrane and the protein, which are arrayed in series. In this case one would potentially observe, if anything, a decrease in capacitance. Thus the monolayer polarographic technique allows for the evaluation of the manner in which a protein that binds to a membrane interacts to perturb the membrane, either through simple adsorption or by penetrating across the monolayer.

In addition, if a chemical redox reaction occurs as a consequence of the solute coming into contact with the mercury electrode, there would be a reduction peak at the potential associated with this reaction, such as for S-S bond reduction that occurs at about -0.7 V (28, 30). Previous studies using prothrombin and prothrombin fragment I have been used to test these theoretical interactions (31). A differentiation between surface adsorption and surface penetration as inferred by the combined use of complementary methods of measuring surface interaction has been made using the accumulation of radioactivity (32) to assess binding (adsorption and penetration) and electrochemistry that evaluates penetration. Studies of prothrombin fragment I as a protein-membrane model system indicate binding but not penetration at concentrations below 0.8 \( \mu \)M. At higher concentrations penetration was inferred by electrochemical measurements.

Further direct evidence of monolayer penetration has been reported by electrochemical reduction of S-S in prothrombin fragment I on the surface of the Hg electrode under conditions under which penetration was inferred. It should not be concluded however that S-S reduction is a necessary consequence of monolayer penetration since such a process ultimately depends upon intimate contact between the S-S bonds and the Hg electrode. The latter is dependent upon the accessibility of the S-S bond and via à via, the protein structure itself.

**RESULTS**

**Effect of Factor Va on the Capacitance of Phospholipid Monolayers—**Fig. 4 illustrates the influence of factor Va on the electrical properties of the monolayer-electrode system. The capacitance change of a condensed monolayer (C) is plotted versus potential (E) for a 13% PS, 87% PC monolayer, exposed to factor Va at two concentrations, 0.3 and 1.0 \( \mu \)g/ml (1.8-6 nM). Curve a corresponds to the phospholipid monolayer. At both factor Va concentrations (curves b and c), an increase in capacitance is observed as a function of factor Va concentration. This observation leads to the conclusion that the factor Va molecules are penetrating the monolayer. The absence of a disulfide bond (S-S) reduction peak in Fig. 4 indicates that the region in contact with the electrode does not contain an S-S bond accessible to the electrode.

The capacitance variation at -0.5 V, where the monolayer is the most stable, was selected to evaluate the effect of the protein concentration and the lipid composition upon circuit capacitance. In Fig. 5 are represented plots of the variation of

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**Fig. 3. Model of interface: a schematic representation of the microscopic region of the membrane in contact with the mercury electrode. Two sorts of interactions are illustrated. Protein A is adsorbed on the head groups of the phospholipid monolayer, whereas protein P has interacted to penetrate through the hydrocarbon layer and might be in intimate contact with the mercury electrode. Protein P is also represented as having an accessible disulfide bond that is reduced at redox potential of approximately -0.7 V by the addition of two electrons. The lower part of the diagram illustrates the equivalent electrical capacitance circuit for two types of protein interactions. Protein adsorption (A) corresponds to condensers in series, whereas protein penetration (P) represents a circuit for condensers in parallel.**

**Fig. 4. Capacitance versus electrical potential curves for the phospholipid monolayer (curve a) and for two different concentrations of factor Va: 1.8 nM (curve b) and 6 nM (curve c). \( \mu \)F, microfarad(s). sat., saturated.**
processes are functions of the phospholipid concentration and chemical methods described.

The latter is taken to be related to the maximal level of protein-protein complex formation, the condensation of the prothrombin substrate on the phospholipid vesicle, and the subsequent expression of hydrolytic activity. All of these processes are functions of the phospholipid concentration and composition. At 100% PC and nanomolar concentrations of factor Va and factor Xa, enzyme complex formation does not occur to any significant extent. Thus the explanation for the lack of prothrombinase activity at 100% PC appears to be obvious. The unusual dependence on PS content, however, cannot be explained by binding occupancy alone, since factor Va occupancy actually increases with increasing PS up to 40% PS, 60% PC. However, when one compares the data of Rosing et al. (4) with data in the present paper (Fig. 6), it appears that the degree of penetration of factor Va into the membrane is changing in a fashion similar to that observed for the activity changes over the interval of PS composition evaluated. Thus an additional complexity exists in the formation of the catalyst, which is associated with the phenomenon of membrane penetration by factor Va.

Since some controversy has existed regarding the influence of Ca$^{2+}$ on factor Va binding, we evaluated the influence of Ca$^{2+}$ ions on capacitance. This experiment was performed by adding EDTA at the point of maximal observed capacitance change for monolayers of different PC/PS composition. The addition of chelator produced less than 10% change in capacitance in every instance. The chelator EDTA removes Ca$^{2+}$ and dissociates factor Va heavy and light chains (33, 34). Since the light chain contains the lipid binding segment of factor Va, it has been well established that this subunit remains bound to the membrane (7). Since little capacitance change was observed, we may conclude that Ca$^{2+}$ is not essential for penetration of factor Va into the monolayer.

Factor Va Adsorption to Monolayers Composed of 100% PC or 100% PS—In cases where there is no capacitance change associated with penetration, it is still possible to distinguish whether there is protein-membrane adsorption. We have evaluated protein-membrane adsorption by using another aspect of the electrochemical method. In this instance, the procedure can be used to determine the concentration of a protein in the bulk solution. For this experiment the mercury drop is introduced into the bulk solution beneath the monolayer (35). The solute adsorption rate can then be used to assess the concentration of bulk solute. Thus by deduction from what was added, the fraction of solute bound to the monolayer can be estimated.

As illustrated in Fig. 2, when a macromolecule is adsorbed on the electrode there is a decrease of the capacitance relative to the pure electrolyte. In this instance, the capacitance change with time is a function of the rate of adsorption of the macromolecule. Thus the rate of capacitance change is proportional to the rate of adsorption, which in turn is a function of the macromolecular concentration in solution, when the adsorption is diffusionally controlled, in the linear part of the curve.

Fig. 7 illustrates the temporal dependence of system capacitance for different solutes. For pure electrolyte little or no change in capacitance is observed with time. When a macromolecular solute (factor Va) is added to the bulk, a progressive decrease in capacitance is observed. The slope ($P$) of the linear part of the capacitance/time curve is proportional to the concentration of the specific macromolecular solute (extreme purity of the electrochemical system is fundamental to these studies). Extrapolation of the initial linear part to constant capacitance yields the maximum surface concentration of factor Va ($\Gamma_{\text{factor Va}}^{\text{(max)}}$), which can be calculated as described previously (35) and which allows estimation of the value of $C_v$ for a truly packed layer of adsorbed molecules. This maximum is attributed to a completely protein-covered electrode that gives $C_v$. Indeed, the experimentally determined $\Gamma_{\text{factor Va}}^{\text{(max)}}$ ($\approx 10^{-12}$ mol/cm$^2$) corresponds to the value obtained for a capacitance (at equilibrium) as a function of factor Va concentration for phospholipid monolayers of different compositions. The capacitance varies with both protein concentration and phospholipid composition. No capacitance change is observed for 100% phosphatidylcholine (open circles) even at the highest protein concentrations used (~30 nM) or for 100% phosphatidylserine (closed squares). For both PS/PC mixtures a cooperative interaction appears to be involved, as indicated by the sigmoidal nature of the plots. Above a certain protein concentration saturation of the process occurs as there is almost no further change in capacitance with increasing protein concentration, and a plateau is reached. For membranes composed of 13% PS, 87% PC (filled circles), there is a dramatic increase of capacitance with factor Va concentration. At 25% PS, 75% PC (open squares) an increase in capacitance is observed, but the resulting maximum is less than that observed at 13% PS, 87% PC. Thus only for mixtures of PC and PS was a capacitance change observed, and the largest increase in capacitance was observed at the lower mole fraction PS. Consequently, (as inferred from the increase in capacitance) the penetration of factor Va into a monolayer containing 25% PS is quantitatively less than into a monolayer containing 13% PS. This result is somewhat surprising since the $K_d$ for factor Va binding to single compartment phospholipid vesicles is nearly independent of lipid composition between 15 and 40% PS (8). The present observations (Fig. 5) suggest that binding and penetration are separate processes that can be distinguished using the electrochemical methods described.

Fig. 6 represents the influence of the phospholipid composition on the "penetration" of factor Va into the monolayer. The latter is taken to be related to the maximal level of capacitance in Fig. 5. Factor Va penetration is not observed in the absence of PS but increases with increasing mole fraction PS and then declines. These data are reminiscent of the dependence of prothrombinase activity observed under similar conditions of phospholipid composition (4).

Prothrombinase formation involves a number of processes including factor Xa and factor Va membrane binding, subsequent protein-protein complex formation, the condensation of the prothrombin substrate on the phospholipid vesicle, and the subsequent expression of hydrolytic activity. All of these processes are functions of the phospholipid concentration and composition. Above a certain concentration of factor Va, enzyme complex formation does not occur to any significant extent. Thus the explanation for the lack of prothrombinase activity at 100% PC appears to be obvious. The unusual dependence on PS content, however, cannot be explained by binding occupancy alone, since factor Va occupancy actually increases with increasing PS up to 40% PS, 60% PC. However, when one compares the data of Rosing et al. (4) with data in the present paper (Fig. 6), it appears that the degree of penetration of factor Va into the membrane is changing in a fashion similar to that observed for the activity changes over the interval of PS composition evaluated. Thus an additional complexity exists in the formation of the catalyst, which is associated with the phenomenon of membrane penetration by factor Va.
condensed layer of molecules taking in account the cross-sectional area of factor Va (36). These data can be used to construct a standard curve from which one can estimate the concentration of factor Va in bulk solution.

In the presence of 100% PC, the concentration of factor Va in the subphase is equivalent to that added (4.2 μg/ml). This observation leads to the conclusion that no protein is bound to the PC monolayer at the factor Va concentrations used. In the presence of 100% PS, the concentration of factor Va remaining in the bulk after interaction is reduced from 4.4 to 3.7 μg/ml. This result is consistent with the conclusion that the monolayer in this instance bound one factor Va molecule for every 107 PS molecules. Thus whereas factor Va binds to pure PS monolayers, it does not penetrate these monolayers (Fig. 5).

**DISCUSSION**

The data represented in this study show that factor Va, at nanomolar concentrations, interacts with monolayers that contain phosphatidylserine. For membrane preparations that contain phosphatidylcholine and phosphatidylserine, factor Va increases circuit capacitance in a concentration-dependent fashion. This indicates that factor Va penetrates or distorts the membrane to produce the equivalent of a condenser in parallel. These data lead to the conclusion that factor Va molecule penetrates the hydrocarbon phase of the monolayer.

The capacitance change observed with monolayers composed of 13–25% PS, 87–75% PC is not substantially altered by the addition of EDTA; this observation is consistent with previous conclusions reached with bilayer vesicles and platelets that the interaction of factor Va with membranes is independent of Ca²⁺ and that the EDTA-dissociated light chain remains associated with membrane. These data also indicate that the interaction of factor Va with the monolayer system is analogous to the interaction of factor Va with membrane bilayers presented as vesicles.

The capacitance change associated with factor Va-monolayer penetration appears to be a cooperative process. This, together with the combined binding and penetration data collected using monolayer and subphase capacitance measurements for factor Va interaction with phospholipid monolayers, suggests that at least two types of binding interactions are associated with factor Va-membrane interaction. Phosphatidylserine is essential for all binding interactions at the factor Va concentrations (nanomolars) studied. The binding of factor Va to monolayers composed of 100% PS is not associated with a monolayer capacitance change. From this observation we infer that factor Va does not penetrate the
phosphatidylserine-water interface. However factor Va is depleted from the subphase, and hence we may conclude that factor Va is adsorbed to monolayers composed of 100% PS.

At the protein concentrations used in the present study (nanomolars) no binding, either associated with penetration or adsorption, was observed for membranes that were composed of pure PC. This observation is consistent with other studies that showed that PS in membrane vesicles is an essential requirement for the binding of factor Va to membranes at nanomolar concentrations of the protein. Although PS as shown from the present experiment is not sufficient to promote penetration of factor Va through the membrane, it appears that phosphatidylserine is required to permit adsorption to the monolayer. The influence of membrane composition on penetration appears to be related with prothrombinase activity.

There are conflicting reports in the literature regarding the contributions of hydrophobic and electrostatic interactions toward the overall stabilization of the factor Va-phospholipid complex (7, 14). These have been distinguished by the effect of ionic strength on binding. The ionic strength dependence for interaction has been observed using nonequilibrium binding techniques and low factor Va concentrations at which substantial penetration, as deduced from electrochemical measurements, is not occurring. In comparison, ionic strength effects have not been observed for measurements of factor Va binding conducted at the higher factor Va concentrations required for equilibrium binding techniques. These higher concentrations are those associated in the present study with membrane penetration. These observations, when coupled with stop-flow kinetic measurements that indicate that factor Va membranes binding interactions with 75% PC, 25% PS occur at diffusionally controlled rates, suggest that the interaction of the factor Va molecule with PC/PS-containing membranes initially involves adsorption through a charge-dependent process followed by the rearrangement and penetration of the membrane process that depends upon the adsorbed lipid's position within the PS membrane.

It is intriguing to evaluate the present data in light of reports regarding the contributions of various regions of factor Va light chain to membrane binding. The data accrued using 75% PC, 25% PS vesicles, the natural protein, and proteolytic fragmentation studies suggest a hydrophobic binding contribution from the A3 domain of the light chain. The data obtained by deletion mutation studies on recombinant factor Va expressed in vitro have identified a membrane binding site in the C2 domain using 100% PS and nonequilibrium plate-binding techniques (21). In these studies the C2 domain of factor Va interacts in an ionic strength-dependent fashion with a pure PS membrane preparation. Since penetration of factor Va into the hydrocarbon layer is dependent upon other phospholipids present in addition to PS, one might speculate that the A3 domain is responsible for the nonionic strength-dependent hydrophobic binding, whereas the C2 domain is the site of PS dependent on electrostatic interaction. One might envision the mechanism of factor Va binding to PC/PS membranes involving an initial interaction between factor Va molecules and a PS-rich region of the membrane that occurs by virtue of adsorption of factor Va to the PS component through electrostatic interactions. Following adsorption, rearrangement of the membrane and the protein-membrane complex leads to penetration and tight binding. As a consequence of these latter interactions, a lipophilic photochemical reagent soluble in the hydrocarbon phase is able to label the factor Va (19) light chain only, whereas factor Va is interacting with the membrane.

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