Kinetic Studies of Prothrombin Activation: Effect of Factor V_a and Phospholipids on the Formation of the Enzyme–Substrate Complex†

Jan L. M. L. van Rijn, José W. P. Govers-Riemslag, Robert F. A. Zwaal, and Jan Rosing*

ABSTRACT: The kinetic parameters of bovine prothrombin activation by factor X_a were determined in the absence and presence of factor V_a as a function of the phospholipid concentration and composition. In the absence of factor V_a, the K_m for prothrombin increases proportionally with the phospholipid concentration and correlates well with the affinity of prothrombin for the different membranes. Phospholipid vesicles with a high affinity for prothrombin yield low K_m values compared to membranes with less favorable binding parameters. At limited phospholipid concentrations, the V_{max} of prothrombin activation correlates with the binding affinity of factor X_a for the various phospholipid vesicles. Membranes with a high affinity for factor X_a have high V_{max} values, while for membranes with a low affinity a low V_{max} is observed. Extrapolation of double-reciprocal plots of $1/V_{max}$ vs. 1/[phospholipid] to infinite phospholipid concentrations, a condition at which all factor X_a would participate in prothrombin activation, yields a k_{cat} of 2–4 min^{-1} independent of the type and amount of acidic phospholipid present in the vesicles. Also, in the presence of factor V_a the K_m for prothrombin varies proportionally with the phospholipid concentration. There is, however, no correlation between the binding parameters and the K_m. Factor V_a drastically lowers the K_m for prothrombin for vesicles that have a low affinity for prothrombin. Vesicles composed of 20 mol % phosphatidylglycerol and 80 mol % phosphatidylcholine have a K_m of 0.04 μM when factor V_a is present, compared to 2.2 μM determined in the absence of factor V_a. Vesicles that contain low amounts of phosphatidylserine also have considerably lower K_m values for prothrombin in the presence of factor V_a than in its absence. When factor V_a is present, the V_{max} of prothrombin activation is virtually independent of the affinity of factor X_a for phospholipid. Over a wide range of phospholipid concentrations and compositions, a k_{cat} of 4500 min^{-1} is observed. Our data establish that prothrombin activation by factor X_a in the presence of factor V_a and phospholipid is independent of the prothrombin density at the phospholipid surface. A model in which the enzymatic unit of prothrombinase consists of a three-component complex (factor X_a–factor V_a–phospholipid) with an increased affinity for soluble prothrombin can explain the observed effects of factor V_a on the K_m for prothrombin. Therefore, factor V_a accelerates prothrombin activation by (1) increasing the k_{cat} of prothrombin activation, (2) promoting the binding of factor X_a, and (3) promoting the assembly of the enzyme–substrate complex.

The activation of the blood coagulation factor prothrombin is catalyzed by prothrombinase, an enzymatic complex composed of the serine protease factor X_a and the nonenzymatic cofactors factor V_a, phospholipid, and Ca^{2+} (Suttie & Jackson, 1977). Previous kinetic studies revealed that prothrombin activation by factor X_a alone is an inefficient process with a low V_{max} of thrombin formation and a high K_m for prothrombin (Rosing et al., 1980). Negatively charged phospholipids plus Ca^{2+} stimulate prothrombin activation by lowering the K_m for prothrombin, while factor V_a greatly enhances prothrombin activation by increasing the V_{max} (Nesheim et al., 1979; Rosing et al., 1980).

All protein components of the prothrombinase complex bind to phospholipid. This led to the proposal that phospholipid membranes act as a surface at which the proteins involved in prothrombin activation interact with each other. The increased prothrombin concentration at the phospholipid–water interface explains the observed decrease of the K_m for prothrombin in the presence of phospholipid. This model, in which the K_m is determined by the local density of prothrombin at the membrane surface (Rosing et al., 1980; Nesheim et al., 1981), also explains the increase of the K_m observed at increasing phospholipid concentration.

In an alternative model postulated by Nelsestuen (1978), the prothrombinase complex is viewed as a dissociable three-component enzyme (factor X_a–factor V_a–phospholipid) that acts on soluble prothrombin. The increased affinity for prothrombin is the result of additive free energies of prothrombin–factor V_a and prothrombin–phospholipid interactions occurring at the active site of prothrombinase. Recently, Pusey & Nelsestuen (1983) indeed demonstrated that the K_m for prothrombin is independent of the prothrombin density at the membrane surface when factor V_a is present. However, previous kinetic studies on prothrombin activation in the absence of factor V_a showed that the observed K_m did correlate with the prothrombin density at the phospholipid surface (Rosing et al., 1980).

These observations suggest that factor V_a may alter the mechanism by which prothrombin enters the catalytic site of the prothrombin activating complex. The present report concerns a detailed kinetic analysis of the effects of factor V_a on the kinetic parameters of prothrombinase. Phospholipid vesicles that contain various amounts of different acidic phospholipids were used in order to vary the affinity of the membranes for prothrombin and to test whether a correlation exists between the prothrombin density at the phospholipid surface and the observed K_m for prothrombin. It is shown that in the absence of factor V_a the K_m correlates with the affinity of prothrombin for phospholipid, while in the presence of factor V_a such a correlation does not exist. These findings suggest that the mode of action of phospholipids in prothrombin activation with and without factor V_a cannot be described in one unique model.

Materials and Methods

Reagents. S2238[1] was purchased from AB Kabi Diag-

† From the Department of Biochemistry, Biomedical Center, University of Limburg, Beeldsnijdersdreef 101, 6200 MD Maastricht, The Netherlands. *Received January 4, 1984.* J.L.M.L.v.R. was supported by the Netherlands Organization for the Advancement of Pure Scientific Research (Z.W.O.).

0006-2960/84/0423-4557$01.50/0

nostica; *p*-NPGB was from Nutritional Biochemicals. Ovalbumin, human serum albumin (fatty acid free), soy bean trypsin inhibitor, RVV-X, *Echis carinatus* venom, and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC) were obtained from Sigma. DEAE-Sephadex A-50, QAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-100, and CNBr-activated Sepharose-4B were purchased from Pharmacia. Soy bean trypsin inhibitor was coupled to CNBr-activated Sepharose 4B according to the method of Cuatrecasas (1970). All reagents used were of the highest grade commercially available.

Proteins. Bovine prothrombin and prethrombin 1 were purified according the method of Owen et al. (1974). Thrombin was purified as described earlier (Rosing et al., 1980). Bovine factor X was purified as described by Fujikawa et al. (1972a). RVV-X was purified from the crude venom by the method of Schiffman et al. (1969). Factor X_a was prepared from factor X activated with RVV-X (Fujikawa et al., 1972b). Factor V and factor V_a were obtained as described by Lindhout et al. (1982). Factor X_a, prothrombin, and thrombin were stored at −80 °C in 50 mM Tris-HCl, 175 mM NaCl, and 0.5 mg/mL human serum albumin (pH 7.9). Factor V_a was stored in the same buffer containing 5 mM $CaCl_2$.

Protein Concentrations. The molar concentration of thrombin was determined by active site titration with *p*-NPGB according to Chase & Shaw (1969). Prothrombin and prethrombin 1 concentrations were determined by the same method after complete activation with *E. carinatus* venom. Factor X_a concentrations were determined by active site titration according to Smith (1973). The concentration of factor V_a was determined by kinetic analysis as described by Lindhout et al. (1982).

Phospholipid and Phospholipid Vesicle Preparations. 1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (PS), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (PG), and 1,2-dioleoyl-*sn*-glycero-3-phosphate (PA) were prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC) by enzymatic synthesis as described by Comfurius & Zwaal (1977). Lipid analysis was carried out as described previously (Bevers et al., 1982). Single-bilayer phospholipid vesicles were prepared according to the method of de Kruijff et al. (1975). Phospholipid concentrations were determined by phosphate analysis as described by Böttcher et al. (1961).

Measurement of Rates of Prothrombin Activation. Factor X_a and phospholipids with or without factor V_a were incubated for 5 min at 37 °C in a buffer containing 50 mM Tris, 175 mM NaCl, 5 mM $CaCl_2$, and 0.5 mg/mL human serum albumin at pH 7.9. Prothrombin activation was started by the addition of prewarmed prothrombin in the same buffer. The final concentration of factor X_a in experiments in which factor V_a was absent was 1 nM. In the presence of factor V_a (5 nM), prothrombin activation was carried out at 1 pM factor X_a. After different time intervals, samples were taken from the prothrombin activating mixture and transferred to cuvettes containing the thrombin-specific chromogenic substrate S2238. The cuvettes (thermostated at 37 °C) contained 1 mL of 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, 0.5 mg/mL ovalbumin, and 235 μM S2238. The thrombin concentration was determined by measuring the rates of absorbance change recorded at 405 minus 500 nm on an Aminco DW-2A spectrophotometer in the dual-wavelength mode. The amount of thrombin was calculated from a calibration curve made with known amounts of active site titrated thrombin.

Kinetic Analysis. Michaelis–Menten kinetics was applied to analyze the kinetic data of prothrombin activation. The kinetic parameters were obtained from initial rate measurements. Thrombin formation rates were linear in time, and care was taken that small amounts of substrate were converted during the time course of the experiments. Rates of thrombin formation were calculated and expressed as thrombin formed per minute per factor X_a. K_m and V_{max} were determined by statistical analysis of the Lineweaver–Burk plots as described by Eisenthal & Cornish-Bowden (1974). The Lineweaver–Burk plots were straight lines over a wide range of prothrombin concentrations. No deviation from linearity could be detected with prothrombin concentrations that were more than 5-fold above or below the K_m for prothrombin.

Results and Discussion

Effect of Phospholipid Vesicles Containing Different Polar Head Groups on the Kinetic Parameters of Prothrombin Activation. Previous kinetic studies revealed that negatively charged phospholipids promote factor X_a catalyzed prothrombin activation by lowering the K_m for prothrombin and that the observed K_m increased proportionally with the amount of phospholipid present (Rosing et al., 1980). Two models have been proposed to explain the effects of phospholipid on the K_m for prothrombin.

(1) In the so-called "bound-substrate model" (Rosing et al., 1980; Nesheim et al., 1981), it is suggested that the overall prothrombin density at the phospholipid surface determines the rate of prothrombin activation by phospholipid-bound factor X_a. When phospholipids are present, the prothrombin concentration in a shell surrounding the phospholipid surface greatly exceeds that in free solution, which explains the drastic drop of the K_m for prothrombin. Increasing the phospholipid concentration lowers the prothrombin density at the phospholipid surface with a consequential increase of the observed K_m.

(2) In the so-called "free-substrate model" (Nelsestuen, 1978), the soluble prothrombin concentration directly determines the rate of prothrombin activation by phospholipid-bound factor X_a. The enzymatic unit of the prothrombin activating complex is thought to consist of a dissociable two-component (factor X_a–phospholipid) or three-component (factor X_a–factor V_a–phospholipid) complex that binds prothrombin from solution with increased affinity. The affinity for soluble prothrombin is determined by the additional free energies of prothrombin binding to the individual components of this complex. Increasing the phospholipid concentration lowers the free prothrombin concentration since prothrombin binds to phospholipid outside the enzymatic domain; hence, more prothrombin has to be added to obtain the free prothrombin concentration required for half-saturation of the enzymatic unit. Therefore, also in the free substrate model the K_m for prothrombin increases proportionally with the phospholipid concentration.

The fundamental difference between the two models is that in the bound-substrate model lateral diffusion of prothrombin across the phospholipid surface is an essential step in the formation of the enzyme–substrate complex, while in the free-substrate model the enzyme–substrate complex is formed by direct interaction of prothrombin with the enzymatic unit.

The bound-substrate model predicts that the V_{max} and K_m are directly related to the binding parameters of, respectively,

[1] Abbreviations: PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; PC, phosphatidylcholine; S2238, D-phenylalanyl-L-pipecoloyl-L-arginine-*p*-nitroanilide dihydrochloride; *p*-NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; RVV-X, purified factor X activator from Russel's viper venom; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

Table I: Effect of Factor V_a on Kinetic Parameters of Prothrombin Activation Measured in the Presence of Vesicles Containing Various Acidic Phospholipids[a]

vesicle phospholipid composition	K_m (μM)	V_{max} (II_a min^{-1} X_a^{-1})
−Factor V_a		
PS–PC (25/75, M/M)	0.11	2.56
PA–PC (25/75, M/M)	0.10	2.78
PG–PC (25/75, M/M)	1.81	0.17
+Factor V_a		
PS–PC (25/75, M/M)	0.14	4050
PA–PC (25/75, M/M)	0.11	4184
PG–PC (25/75, M/M)	0.04	3345

[a] Varying amounts of prothrombin were activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL human serum albumin, 50 μM phospholipid, and 1 nM factor X_a and no factor V_a or 1 pM factor X_a and 5 nM factor V_a. Thrombin formation rates and kinetic parameters were determined as described under Materials and Methods.

factor X_a and prothrombin for phospholipid. Vesicles with increased affinity for factor X_a bind more factor X_a; hence, more factor X_a will participate in prothrombin activation and higher V_{max} values will be observed. Vesicles with an increased affinity for prothrombin will yield lower K_m values since less prothrombin is required to obtain the prothrombin density at the phospholipid surface at which half of the phospholipid-bound factor X_a is saturated. In order to test these predictions, we determined kinetic parameters of prothrombin activation for vesicles that contain negatively charged phospholipids with different polar head groups (PS, PA, PG), which are known to have different binding parameters for factor X_a and prothrombin (Nelsestuen & Broderius, 1977). Table I shows that, in the absence of factor V_a, both the V_{max} and the K_m depend on the type of acidic phospholipid present in the phospholipid vesicles. PS and PA, which at the experimental pH have two negative charges in their head group (PS also contains one positive charge), give considerably higher V_{max} and lower K_m values than PG, which contains only one negative charge. The observed kinetic parameters relate very well to previously reported binding parameters. Membranes that contain PS and PA have a higher affinity for prothrombin and factor X_a than those containing PG. Table I also reveals that the kinetic parameters, determined in the presence of factor V_a, do not reflect the differences in binding parameters for factor X_a and prothrombin. When factor V_a is part of the prothrombin activating complex, the V_{max} is hardly affected by the type of acidic phospholipid present in the membrane bilayer. This can be attributed to the fact that factor V_a promotes the binding of all added factor X_a to phospholipid vesicles, since rates of prothrombin activation in our experiments were optimized with respect to the amount of factor V_a present. Moreover, the K_m does not relate to the binding parameters of prothrombin for the phospholipid vesicles per se. Vesicles containing PG, which have the lowest affinity for prothrombin, have the most favorable K_m for prothrombin. Therefore, we have to conclude that, in the presence of PG-containing vesicles and factor V_a, the K_m for prothrombin is not determined by the prothrombin density at the membrane surface.

Kinetic Parameters of Prothrombin Activation for Vesicles Containing Various Mole Percentages of Acidic Phospholipid. The question arises whether the effects of factor V_a on the kinetic parameters of prothrombin activation (especially on the K_m) are a unique feature of PG-containing vesicles or they must be attributed to the low affinity of these vesicles for prothrombin and factor X_a. To this end, we determined the K_m and V_{max} for vesicles that contain various mole percentages

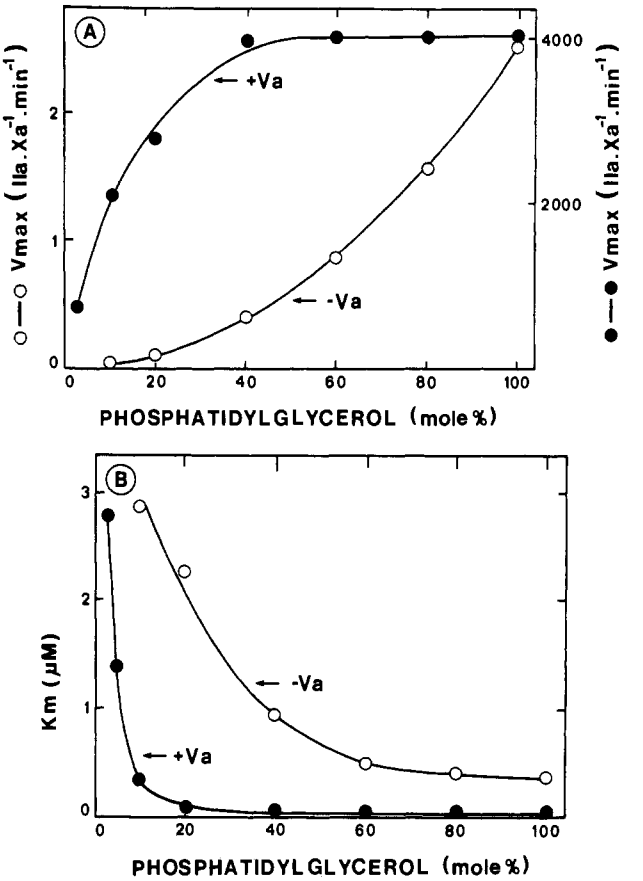

FIGURE 1: Effect of the mole percentage of PG in PG–PC vesicles on the kinetic parameters of prothrombin activation. Various amounts of prothrombin were activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL human serum albumin, 50 μM phospholipid, and 1 nM factor X_a and no factor V_a (○) or 1 pM factor X_a and 5 nM factor V_a (●). The mole percentage of PG present in the phospholipid vesicles is indicated in the figure. Thrombin formation rates, V_{max} (A), and K_m (B) were determined as described under Materials and Methods.

of the acidic phospholipids PG and PS. Lowering the mole percentage of acidic phospholipid in these vesicles results in a weaker binding of factor X_a and prothrombin (Nelsestuen & Broderius 1977).

Figure 1A shows the V_{max} values determined for vesicles that contain various mole percentages PG. Without factor V_a, there is a considerable increase of the V_{max} when the mole percentage PG in the vesicles is increased. In the presence of factor V_a, the V_{max} is much less dependent on the PG content of the vesicles. At more than 40 mol % PG, a maximal value is observed, which suggests that factor V_a is able to promote the binding of all added factor X_a to the phospholipid vesicles.

The K_m values obtained for vesicles with different mole percentages of PG are presented in Figure 1B. Without factor V_a, the K_m increases when the mole percentage of PG is lowered. The increase of the K_m for prothrombin runs parallel with the increase of the dissociation constant of the prothrombin–phospholipid complex. Two striking differences are observed, however, when factor V_a is present: (1) in the presence of factor V_a, the K_m for prothrombin is 10–40-fold lower than in its absence, and (2) in the presence of factor V_a, the K_m is independent of the binding affinity of prothrombin for the phospholipid vesicles between 20 and 100 mol % PG.

Essentially the same effects on the kinetic parameters are found when PS is utilized as acidic phospholipid. Unfortunately, the mole percentage of PS is limited to 40% since at higher percentages the vesicles tend to aggregate due to the

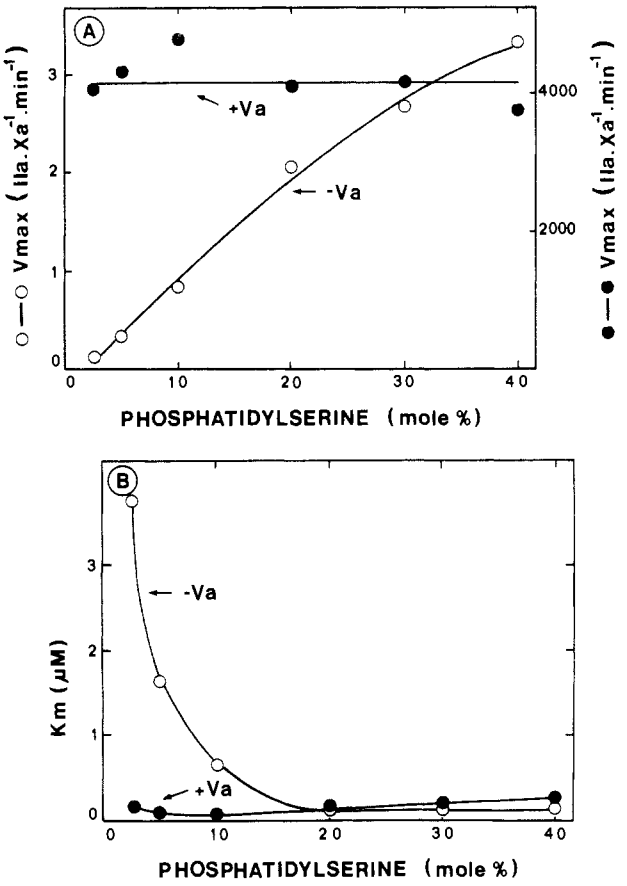

FIGURE 2: Effect of the mole percentage of PS in PS–PC vesicles on the kinetic parameters of prothrombin activation. The experimental conditions are described in the legend of Figure 1. Panel A represents the effect of mole percentage of PS on the V_{max} and Panel B the effect of mole percentage of PS on the K_m [(○) no factor V_a present; (●) 5 nM factor V_a present].

presence of $CaCl_2$. Figure 2A shows that in the absence of factor V_a there is a continuous increase of the V_{max} when the mole percentage of PS in the phospholipid vesicles is increased from 2.5 to 40%. With factor V_a, the V_{max} is independent of the mole fraction of PS, which again indicates that under these conditions all factor X_a molecules are bound to phospholipid and participate in prothrombin activation. Figure 2B reveals that in the absence of factor V_a the K_m for prothrombin increases when the binding affinity of prothrombin for the vesicles is decreased. Factor V_a has minor effects on the K_m of prothrombin for vesicles containing between 20 and 40 mol % PS. At amounts of PS below 20 mol %, factor V_a seems to compensate for the reduced affinity of prothrombin for phospholipid; much lower K_m values are observed in the presence of factor V_a than in its absence.

It is even questionable whether in the presence of factor V_a prothrombin–phospholipid interactions still contribute to the formation of the enzyme–substrate complex. Since the phospholipid binding capacity of prothrombin resides in its fragment 1 region, it is interesting to compare prothrombin and prethrombin 1 (which lacks fragment 1) as substrates for prothrombinase. Table II gives the kinetic parameters for prethrombin 1 activation, determined in the presence of factor V_a and vesicles containing either PS, PA, or PG as acidic phospholipid. For all vesicles, the K_m values for prethrombin 1 are 100–200-fold higher than those for prothrombin (cf. Table I). These experiments actually exclude a variant of the free-substrate model in which prothrombin–phospholipid interactions do not contribute to the formation of the enzyme–substrate complex. In such a model, the K_m for prothrombin

Table II: Kinetic Parameters of Prethrombin 1 Activation[a]

vesicle phospholipid composition	K_m (μM)	V_{max} (II_a min^{-1} X_a^{-1})
PS–PC (25/75, M/M)	11.3	1711
PA–PC (25/75, M/M)	12.1	1563
PG–PC (25/75, M/M)	8.1	1352

[a] Various amounts of prethrombin 1 were activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL human serum albumin, 50 μM phospholipid, 1 pM factor X_a, and 5 nM factor V_a. Measurement of thrombin formation rates and determination of kinetic parameters were carried out as described under Materials and Methods.

would only depend on its affinities for factor X_a and factor V_a. The large difference between the K_m for prothrombin and prethrombin 1 strongly suggests that prothrombin–phospholipid interactions are essential for the binding of prothrombin to the prothrombinase complex. We have as yet no explanation for the fact that the V_{max} of prethrombin 1 activation is 2–3-fold lower than the V_{max} for prothrombin activation. The important information contained in the experiments reported in this section is that the kinetic parameters of prothrombin activation in the presence of factor V_a and phospholipid have no relation to the prothrombin density at the phospholipid membrane.

Effect of Phospholipid Concentration on the K_m for Prothrombin. Previous kinetic studies of prothrombin activation carried out in our laboratory revealed that the K_m for prothrombin has to be regarded as an apparent K_m (K_m^{app}) since it increases at increasing phospholipid concentrations (Rosing et al., 1980). For both the free-substrate and the bound-substrate models, an equation can be derived that relates the observed K_m with the phospholipid concentration and the binding parameters of prothrombin for the phospholipid vesicles present in the kinetic experiment. The total prothrombin concentration required to attain the observed K_m equals the sum of the concentrations of free and phospholipid-bound prothrombin (eq 1). Since the concentration of factor X_a

$$K_m^{app} = [PT_{free}] + [PT_{bound}] \quad (1)$$

present in our experiments is far below the concentration of binding sites ($[S_{total}]$) for vitamin K dependent coagulation factors provided by the phospholipid vesicles

$$K_d = [PT_{free}]([S_{total}] - [PT_{bound}])/[PT_{bound}] \quad (2)$$

where K_d is the dissociation constant of the prothrombin–phospholipd complex. Defining q as the maximal amount of prothrombin that can bind to phospholipid (mol/mol) gives

$$[S_{total}] = q[\text{phospholipid}] \quad (3)$$

Substitution of eq 3 into eq 2, followed by rearrangement of eq 2, yields

$$[PT_{bound}] = \frac{q[\text{phospholipid}]}{K_d[PT_{free}]^{-1} + 1} \quad (4)$$

Substituting eq 4 into eq 1 gives the relation between K_m and the phospholipid concentration (eq 5). This equation is ap-

$$K_m^{app} = [PT_{free}] + \frac{q[\text{phospholipid}]}{K_d[PT_{free}]^{-1} + 1} \quad (5)$$

plicable to both the free-substrate and the bound-substrate models. In the free substrate model, the free prothrombin concentration ($[PT_{free}]$) at the observed K_m^{app} is constant and independent of the amount of phospholipid present. Since also

Table III: Effect of Mole Percentage of PS on the Intrinsic K_m for Prothrombin and the Slope of Plots of K_m^{app} vs. [Phospholipid][a]

PS (mol %)	−factor V_a		+factor V_a	
	slope (M/M)	intrinsic K_m (μM)	slope (M/M)	intrinsic K_m (μM)
2.5	7.32×10^{-3}	3.35	[b]	0.145
5	5.50×10^{-3}	1.30	0.07×10^{-3}	0.072
10	4.55×10^{-3}	0.325	0.25×10^{-3}	0.055
20	2.47×10^{-3}	0.023	1.35×10^{-3}	0.034
30	2.46×10^{-3}	<0.01[c]	4.18×10^{-3}	0.027
40	2.67×10^{-3}	<0.01[c]	5.28×10^{-3}	0.030

[a] Intrinsic K_m values and the slope of plots of K_m^{app} vs. [phospholipid] for vesicles containing different mole percentages of PS were calculated from the data presented in Figure 3. The intrinsic K_m for vesicles containing 20, 30, and 40 mol % PS was calculated for K_m vs. [phospholipid] plots by using a phospholipid concentration between 1 and 25 μM (data not shown). [b] It was not possible to determine an accurate value for the slope. [c] To determine accurate values for the intrinsic K_m, phospholipid concentrations below 1 μM were necessary, which gave prothrombin activation rates that were too low to be measured.

K_d and q are constants for a particular kind of phospholipid, the K_m^{app} will be linearly dependent on the phospholipid concentration. In the bound-substrate model, the K_m^{app} is attained at a particular prothrombin density at the phospholipid surface. The latter model is defined in such a way that this prothrombin density is independent of the phospholipid concentration and composition. Since the required prothrombin density for a certain kind of phospholipid is always reached at the same free prothrombin concentration, $[PT_{free}]$ at K_m^{app} is constant, and a plot of K_m^{app} vs. the concentration of phospholipid is also a straight line in the bound-substrate model. Differences exist between the intercept at the ordinate and the slope of the line when eq 5 is applied to the free-substrate or the bound-substrate model. The intercept at the ordinate is actually the K_m that would be measured at infinitely low phospholipid concentration. We will call this value the intrinsic K_m of the prothrombinase complex. In the free-substrate model, the intrinsic K_m is the real K_m for prothrombin since no excess of phospholipid is present outside the enzymatic domain that can bind prothrombin and lower the free prothrombin concentration that interacts with the enzymatic complex. In the bound-substrate model, $[PT_{free}]$, that is, the intrinsic K_m, is the free prothrombin concentration required to obtain the prothrombin density at the phospholipid surface at which half of the phospholipid-bound factor X_a is involved in prothrombin activation. In this model, the intrinsic K_m directly relates to the binding parameters of prothrombin for the kind of phospholipid vesicles used in the kinetic experiment. Membranes with less favorable binding parameters for prothrombin will have higher intrinsic K_m values, since more free prothrombin has to be present to obtain the required prothrombin density at the phospholipid surface.

The slopes of the K_m^{app} vs. [phospholipid] plots contain important information. The slope, $q/(K_d[PT_{free}]^{-1} + 1)$, equals $[PT_{bound}]/[phospholipid]$ (eq 4), which is actually the prothrombin density at the phospholipid surface required at K_m^{app}. In the bound-substrate model this prothrombin density is defined to be a constant, which is independent of the phospholipid concentration and composition. When this model is applicable to prothrombin activation, plots of K_m^{app} vs. [phospholipid] will yield a set of parallel lines for phospholipid vesicles with different binding parameters for prothrombin. In the free-substrate model there are no restraints on the prothrombin density at the membrane surface at K_m^{app}. The slope of the K_m^{app} vs. [phospholipid] plots is therefore in this model a function of the binding parameters of prothrombin and the intrinsic K_m ($[PT_{free}]$). Phospholipid vesicles with favorable binding parameters for prothrombin (high q and low K_d) will produce steep lines in the K_m vs. [phospholipid] plot.

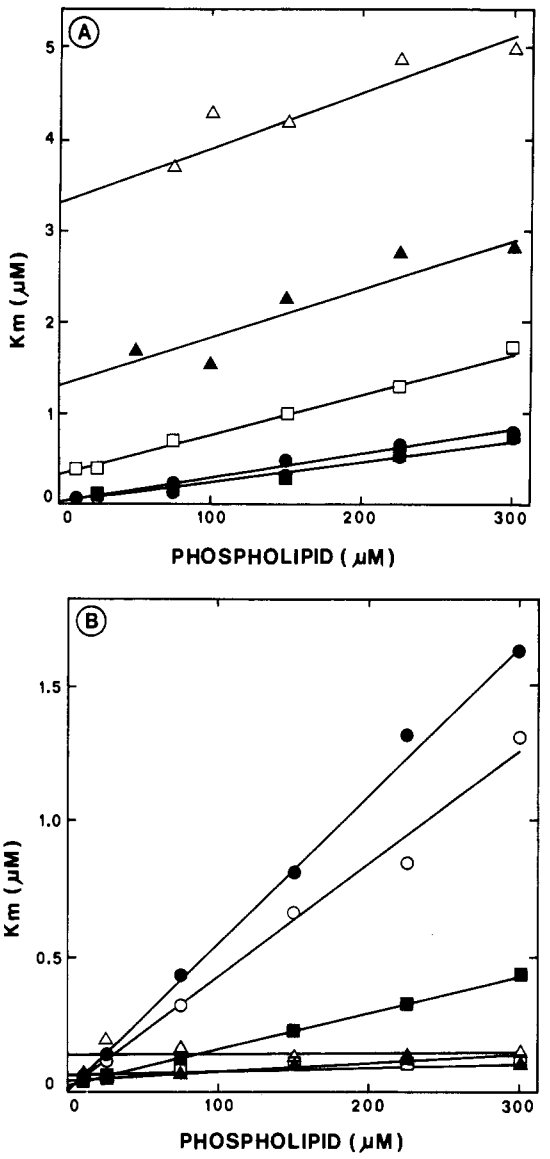

FIGURE 3: Effect of the phospholipid concentration on the K_m of prothrombin for phospholipid vesicles containing various mole percentages of PS. Various amounts of prothrombin were activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL human serum albumin, amounts of phospholipid indicated in the figure, and 1 nM factor X_a and no factor V_a (A) or 1 pM factor X_a and 5 nM factor V_a (B). The mole percentage of PS present in the vesicles was 2.5 (△), 5 (▲), 10 (□), 20 (■), 30 (○), or 40% (●). Thrombin formation rates and the kinetic parameters were determined as described under Materials and Methods.

Figure 3 shows the effect of increasing phospholipid concentrations on the K_m for prothrombin measured in the absence and presence of factor V_a. In this experiment, phospholipid vesicles containing different mole percentages of PS were used. The plots obtained in the absence and presence of factor V_a are distinctly different. The slopes of the lines vary in the absence of factor V_a 2–3-fold and in the presence of factor V_a 70-fold for vesicles with mole percentages of PS between 2.5 and 40%. Table III summarizes the intrinsic K_m values and the slopes calculated from the data presented in Figure 3. In order to determine accurate intrinsic K_m values for 20, 30, and 40 mol % PS, a separate experiment was carried out at phospholipid concentrations between 1 and 25 μM (data not shown). In the absence of factor V_a, the intrinsic K_m shows

Table IV: Prothrombin Binding Parameters Derived from Plots of K_m vs. Phospholipid Concentration for Prothrombin Activation in the Presence of Factor V_a

PS (mol %)[a]	K_d (μM)	sites/ phospholipid (M/M)	K_d (μM)[b]	sites/ phospholipid (M/M)[b]
5	5.1	0.005	5.5	0.004
10	1.5	0.007	1.6	0.007
20	0.3	0.012	0.3	0.011
30	0.1	0.013	0.1	0.012
40	0.05	0.013	0.1	0.013

[a] PS at 2.5 mol % is omitted since no direct binding data were available. [b] From Nelsestuen & Broderius (1977).

a wide variation for vesicles that contain different amounts of PS. At 2.5 mol % PS, the intrinsic K_m is 3.35 μM, while at 40 mol % PS a value below 0.01 μM is calculated (the intrinsic K_m was actually too low to be accurately measured). When factor V_a is present, there is much less variation of the intrinsic K_m. For vesicles with amounts of PS between 10 and 40 mol %, the intrinsic K_m is almost constant (0.055–0.030 μM). From the different effects of PS on the slope of the K_m^{app} vs. [phospholipid] plot and on the intrinsic K_m, it is obvious that the mode of action of phospholipids on prothrombin activation in the absence and presence of factor V_a cannot be described in one unique model.

The wide variation of slopes of K_m^{app} vs. [phospholipid] plots determined in the presence of factor V_a (Figure 3B) indicates that only the free-substrate model can adequately describe the observed phenomena. In that case, the slopes of the lines should correlate with known prothrombin binding parameters for the phospholipid membranes used in this experiment. Parameter fitting of the lines according to eq 5 indeed yields values for K_d and prothrombin binding sites per phospholipid (M/M) that fulfill the requirements of slope and intercept and that are in close agreement with prothrombin binding parameters for these membranes reported by Nelsestuen & Broderius (1977). Table IV summarizes the binding parameters for prothrombin obtained by parameter fitting and those reported in the literature. The variation of the intrinsic K_m (real K_m for prothrombinase) with the mole percentage of PS observed in the presence of factor V_a is also adequately explained in the free-substrate model, since there is no correlation between the intrinsic K_m and the binding parameters of prothrombin for vesicles with varying mole percentage of PS. In the free-substrate model, the intrinsic K_m is determined not only by the free energy of prothrombin–phospholipid interaction but also by the free energies of prothrombin–factor V_a and prothrombin–factor X_a interactions. Since information about the quantitative contribution of each of these interactions is lacking, no prediction of the actual value of the intrinsic K_m can be made.

The data obtained in the absence of factor V_a are qualitatively better explained in the bound-substrate model. The intrinsic K_m varies in parallel with the dissociation constants reported for the prothrombin–phospholipid complex (Nelsestuen & Broderius, 1977), and the slopes of the plots of K_m^{app} vs. [phospholipid] (although they should be parallel in the bound-substrate model) vary considerably less (2–3-fold) in the absence of factor V_a than in its presence. It is impossible, however, to obtain by parameter fitting binding constants for prothrombin that are comparable to those determined in direct binding studies and at the same time meet the requirements of slope and intercept of the experimentally observed K_m^{app} vs. [phospholipid] plot. This is caused by the low value of the intrinsic K_m, determined at the higher mole percentages of PS. Intrinsic K_m's below 0.01 μM (Table III) cannot be reconciled

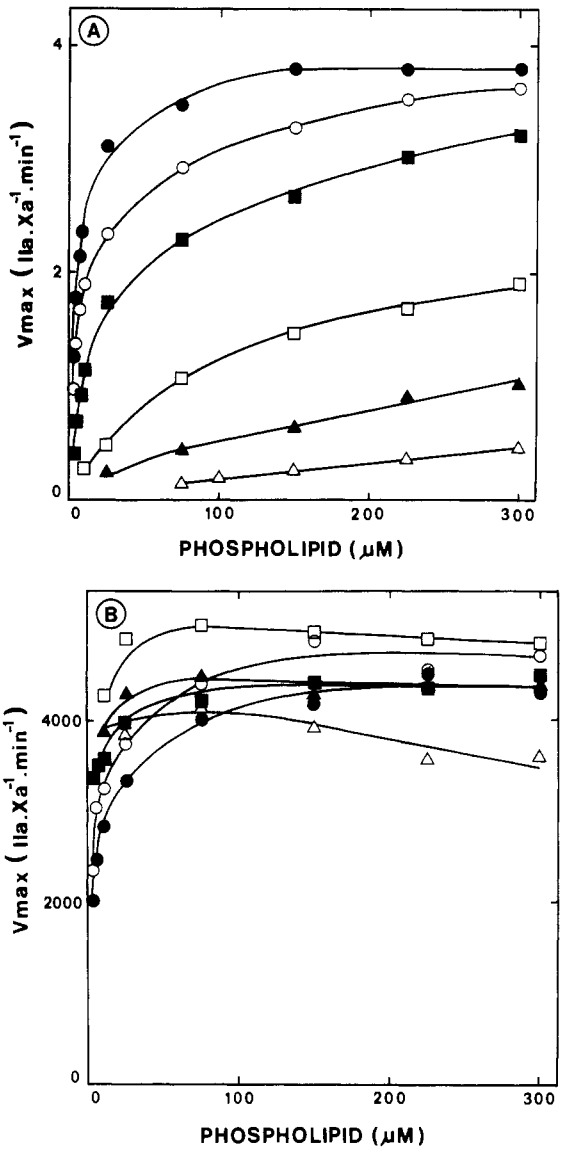

FIGURE 4: Effect of the phospholipid concentration on the V_{max} of prothrombin activation for phospholipid vesicles containing various mole percentages of PS. The experimental conditions are described in the legend of Figure 3. (A) No factor V_a present; (B) plus 5 nM factor V_a. The mole percentage of PS present in the vesicles was 2.5 (△), 5 (▲), 10 (□), 20 (■), 30 (○), or 40% (●).

with either of the two models and known parameters of prothrombin binding to phospholipid.

Effect of Phospholipid Concentration on the V_{max} of Prothrombin Activation. In both the free-substrate and the bound-substrate models, prothrombin activation is catalyzed by phospholipid-bound factor X_a. Figure 4A shows that without factor V_a the V_{max} is dependent on both the phospholipid concentration and the amount of PS present in the membranes. Increasing the phospholipid concentration or the mole percentage PS results in an increase of the V_{max} of prothrombin activation. This is explained by the fact that more factor X_a is bound to the phospholipid vesicles and, hence, more factor X_a participates in prothrombin activation. Double-reciprocal plots of $1/V_{max}$ vs. 1/[phospholipid] are straight lines (Figure 5), which upon extrapolation to infinite phospholipid concentration yield V_{max} values between 2 and 4 mol of thrombin formed min^{-1} (mol of factor $X_a)^{-1}$ independent of the amount of PS in the membranes. This shows that the catalytic activity of phospholipid-bound factor X_a is hardly affected by the phospholipid composition of the vesicles. In view of the fact that all added factor X_a participates in pro-

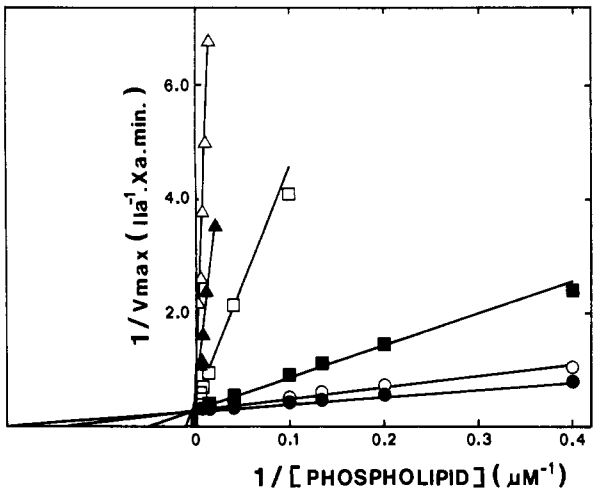

FIGURE 5: Double-reciprocal plots of $1/V_{max}$ against 1/[phospholipid] for vesicles containing various mole percentages of PS. The experimental conditions for prothrombin activation in the absence of factor V_a are described in the legend of Figure 3. The mole percentage of PS present in the vesicles was 2.5 (△), 5 (▲), 10 (□), 20 (■), 30 (○), or 40% (●).

thrombin activation, the V_{max} at infinite phospholipid concentration is actually the k_{cat} of prothrombin activation.

Figure 4B shows that there is no correlation between the V_{max} and the affinity of factor X_a for phospholipid when factor V_a is present. Only minor changes of V_{max} are observed for membranes containing between 2.5 and 40 mol % PS in a phospholipid concentration range of 10–300 μM. Apparently, factor V_a promotes the affinity of factor X_a for phospholipid to such an extent that almost all factor X_a molecules are bound to phospholipid and participate in prothrombin activation. Factor V_a increases the k_{cat} of prothrombin activation 3 orders of magnitude. In the absence of factor V_a, the k_{cat} of prothrombin activation is 2–4 min^{-1} while in the presence of factor V_a values of 4500 min^{-1} are observed.

Conclusions

In this paper we presented an extensive study of the effects of different phospholipids on the kinetic parameters of prothrombin activation in order to discriminate between the two models (i.e., the bound-substrate model and the free-substrate model) that have been proposed to explain the mode of action of phospholipids in prothrombin activation.

The effects of phospholipids on the K_m for prothrombin determined in the absence of factor V_a cannot be satisfactorily explained in either of the two proposed models. The K_m for prothrombin measured in the absence of factor V_a varies in parallel with the binding affinity of prothrombin for phospholipid (membranes with a low affinity have a high K_m, while for membranes with a high affinity a low K_m is measured). Neither in the bound-substrate model nor in the free-substrate model is it possible, however, to correlate the observed K_m on a quantitative basis with known binding parameters of prothrombin for phospholipid.

The mode of action of phospholipid in prothrombin activation in the presence of factor V_a can be adequately explained in the free-substrate model for the following reasons: (1) Factor V_a causes a considerable decrease of the K_m for prothrombin when phospholipid vesicles with a low affinity for prothrombin are part of prothrombinase. (2) There is no relation between the observed K_m and the prothrombin density at the membrane surface when prothrombin is activated in the presence of factor V_a and phospholipid vesicles with a low affinity for prothrombin. (3) The slopes of K_m^{app} vs. [phospholipid] plots vary considerably for membranes with different affinities for prothrombin, which is one of the predictions of the free-substrate model. (4) The experimentally obtained K_m^{app} vs. [phospholipid] plots can be theoretically fitted with binding parameters for prothrombin reported in the literature.

Our experimental data for prothrombin activation in the presence of factor V_a support the proposal of Nelsestuen (1978) that prothrombinase is a dissociable three-component enzyme (factor X_a–factor V_a–phospholipid) that converts soluble prothrombin; the observed increase of affinity for prothrombin, measured as a decrease of the K_m, is the result of prothrombin–factor V_a and prothrombin–phospholipid interactions that occur near the active site of prothrombinase.

From its effect on the K_m, it is obvious that factor V_a promotes the binding of prothrombin to the prothrombinase complex. This can be accomplished by (1) a direct interaction of prothrombin with factor V_a, which provides additional free energy for the binding of prothrombin to the enzymatic complex, and/or (2) a factor V_a induced rearrangement of phospholipid molecules in the enzymatic domain that results in the creation of a better phospholipid surface for prothrombin binding. The first possibility is supported by direct binding studies (van de Waart et al., 1983) in which it was shown that factor V_a promotes the binding of prothrombin to negatively charged phospholipids. The formation of a prothrombin–factor V_a complex with a 1:1 stoichiometry is indicative for direct prothrombin–factor V_a interaction. Insufficient quantitative data prevent, however, direct correlation of the effect of factor V_a on prothrombin binding to phospholipids with its effect on K_m^{app}. With respect to the second possibility, Mayer & Nelsestuen (1983) reported that factor V_a is able to cause lateral phase separation (clustering) of acidic phospholipids in membranes and suggested that this lateral phase separation may be an important process in the formation of the prothrombinase complex. Whether this clustering of acidic phospholipid molecules by factor V_a also promotes prothrombin binding to the membrane is, however, unknown. At present, we cannot draw conclusions with regard to the two possibilities mentioned above.

It is to be expected that the intrinsic K_m should continuously decrease when the mole percentage of acidic phospholipid in membranes is increased since this would result in an increased free energy of prothrombin–phospholipid interaction. However, at mole percentages of PS exceeding 5%, the intrinsic K_m is virtually constant (Table III), although direct binding experiments (Nelsestuen & Broderius, 1977) revealed that the affinity of the membranes for prothrombin still increases. The intrinsic K_m for prothrombinase in the presence of factor V_a and membranes containing a high mole percentage of PS is even higher than the intrinsic K_m measured in the absence of factor V_a, which seems to contradict the proposal that factor V_a promotes the affinity of the prothrombinase complex for prothrombin. These phenomena can be explained when the rate constants of prothrombinase are considered. From the intrinsic K_m and the k_{cat}, a k_{cat}/K_m of 2.5×10^9 M^{-1} s^{-1} is calculated for a prothrombinase complex consisting of factors X_a and V_a and phospholipids that contain 5 or more mol % PS. This indicates that prothrombinase under these conditions is a diffusion-controlled process in which a further increase of the binding affinity of phospholipids does not further change the kinetic parameters because of diffusion limitation. Hence, the intrinsic K_m will become constant for vesicles that contain 5 or more mol % PS. When factor V_a is absent, k_{cat}/K_m is 3 orders of magnitude lower, which indicates that prothrombin activation under these conditions is not diffusion limited. This

can explain why the intrinsic K_m values decrease below those observed in the presence of factor V_a. The rate constant of prothrombinase of 2.5×10^9 M^{-1} s^{-1} exceeds the rate constant for association of prothrombin with a single binding site on a PS-containing membrane ($k_1 = 3 \times 10^7$ M^{-1} s^{-1}) reported by Wei et al. (1982). This rate constant, which was determined in the absence of factor V_a, is not compatible with the rate constant of prothrombinase with factor V_a. To account for the rate constant of prothrombin activation, factor V_a has to increase the association rate constant of prothrombin binding to the complex.

In conclusion, factor V_a has three important effects in prothrombinase: (1) it accelerates prothrombin activation by increasing the k_{cat} more than 1000-fold; (2) it promotes the binding of factor X_a to phospholipids and therefore increases the amount of factor X_a that participates in prothrombin activation; (3) it promotes the affinity of prothrombinase for its substrate prothrombin on membranes that have a low binding affinity for prothrombin. The latter effects presumably have physiological importance. It has been proposed that activated blood platelets provide the phospholipid surface at which prothrombin activation takes place. Activated platelets expose relatively small amounts of acidic phospholipids (Bevers et al., 1982). On platelet membrane areas with low PS density, factor V_a will have an important role in the binding of prothrombin and factor X_a and the subsequent formation of the enzyme–substrate complex.

Acknowledgments

We thank Prof. Dr. H. C. Hemker, Dr. T. Lindhout, Dr. P. van de Waart, and Dr. G. Tans for many stimulating discussions. Part of the chromogenic substrates used in this study was kindly donated by AB Kabi Diagnostica.

Registry No. PS, 70614-14-1; PG, 61617-08-1; PC, 4235-95-4; factor V_a, 65522-14-7; factor X_a, 9002-05-5; prothrombin, 9001-26-7; prothrombinase, 72162-96-0.

References

Bevers, E. M., Comfurius, P., van Rijn, J. L. M. L., Hemker, H. C., & Zwaal, R. F. A. (1982) *Eur. J. Biochem. 122*, 429.

Böttcher, C. J. F., van Gent, C. M., & Pries, C. (1961) *Anal. Chim. Acta 24*, 203.

Chase, T., & Shaw, E. (1969) *Biochemistry 8*, 2212.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta. 488*, 36.

Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059.

de Kruijff, B., Cullis, P. R., & Radda, G. K. (1975) *Biochim. Biophys. Acta 406*, 6.

Eisenthal, R., & Cornish-Bowden, A. (1974) *Biochem. J. 139*, 715.

Fujikawa, K., Legaz, M. E., & Davie, E. W. (1972a) *Biochemistry 11*, 4882.

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

Lindhout, T., Govers-Riemslag, J. W. P., Van de Waart, P., Hemker, H. C., & Rosing, J. (1982) *Biochemistry 21*, 5494.

Mayer, L. D., & Nelsestuen, G. L. (1983) *Biochim. Biophys. Acta 734*, 48.

Nelsestuen, G. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 2621.

Nelsestuen, G. L., & Broderius, M. (1977) *Biochemistry 16*, 4172.

Nesheim, M. E., Taswell, J. B., & Mann, K. G. (1979) *J. Biol. Chem. 254*, 10952.

Nesheim, M. E., Eid, S., & Mann, K. G. (1981) *J. Biol. Chem. 256*, 9874.

Owen, W. G., Esmon, C. T., & Jackson, C. H. (1974) *J. Biol. Chem. 249*, 594.

Pusey, M. L., & Nelsestuen, G. L. (1983) *Biochem. Biophys. Res. Commun. 114*, 526.

Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A., & Hemker, H. C. (1980) *J. Biol. Chem. 255*, 274.

Schiffman, S., Theodor, J., & Rapaport, S. J. (1969) *Biochemistry 8*, 1397.

Smith, R. L. (1973) *J. Biol. Chem. 248*, 2418.

Suttie, J. W., & Jackson, C. M. (1977) *Physiol. Rev. 57*, 1.

van de Waart, P., Hemker, H. C., & Lindhout, T. (1984) *Biochemistry 23*, 2838.

Wei, G. J., Bloomfield, V. A., Resnick, R. M., & Nelsestuen, G. L. (1982) *Biochemistry 21*, 1949.