Ionophoric Properties of Angiotensin II Peptides. Nuclear Magnetic Resonance Kinetic Studies of the Hormone-Mediated Transport of Manganese Ions across Phosphatidylcholine Bilayers†

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ABSTRACT: The linear peptide hormones angiotensin II and [Asn[1],Val[5]]angiotensin II are found to mediate the transport of Mn(II) ions across phosphatidylcholine bilayers. Nuclear magnetic resonance spectroscopy (NMR) is applied to monitor the rate of transport of Mn(II) ions by measuring the rate of disappearance of the ^{1}H NMR signal of the choline methyl groups of the inner phospholipid layer. This rate of disappearance is analyzed in terms of a pseudo-first-order rate equation for the transport process. The rate of transport of Mn(II) varies linearly with both the concentrations of Mn(II) and angiotensin II (A-II) present, suggesting that the ions are transported in a complex with 1:1 stoichiometry. An analysis of the temperature dependence of the rate of transport yielded an energy of activation of 29 ± 5 kcal/mol and an entropy of activation of 10 eu for the transport process. The activation parameters are discussed in terms of defining the rate-limiting step in the transport process. The pH dependence of the hormone-mediated rate of Mn(II) transport is similar to the pH dependence of the metal complexation process measured in a separate study. The presence of La(III) or Tris decelerates the rate of Mn(II) transport by presumably competing with either Mn(II) or A-II, respectively, in the binding process. On the basis of the results presented here as well as literature data, we suggest that the ionophoric properties of these two hormones may be relevant to understanding the role of metal ions in their physiological activities.

Angiotensin II (A-II) and [Asn[1],Val[5]]angiotensin II (A-II′) are linear octapeptide hormones whose primary structures are

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

angiotensin II

Asn-Arg-Val-Tyr-Val-His-Pro-Phe

[Asn[1],Val[5]]angiotensin II

These hormones exhibit a wide range of physiological activities, including potent pressor and myotropic actions [for reviews, see Schröder & Lübke (1966), Khosla et al. (1973), Marshall et al. (1974), Regoli et al. (1974), and Soffer (1976)]. In vivo and in vitro studies have shown that the contractile responses induced by A-II and A-II′ are affected by the concentration of calcium ions present and/or are accompanied by a change in distribution of the calcium ions in the tissue. The depletion of extracellular calcium ions reduces or eliminates the physiological activities of these two hormones. Ackerley et al. (1978) have shown that the removal of extracellular calcium ions or the addition of verapamil attenuates the A-II-induced contractile response in rabbit aortic strips. Similar results have been reported for rat uterus (Khairallah et al., 1965), rat tail artery (Hinke et al., 1964), descending rat colon (Crocker et al., 1977), guinea pig ileum (Khairallah et al., 1965), and dog mesenteric arteries (Burks et al., 1967). Sato et al. (1978) have reported that the presence of diltiazem, a calcium antagonist, prevented the pressor response of rats to A-II′. In assay systems for pressor action and uterotonic activity, Schaechtelin et al. (1974, 1975) have found that calcium and magnesium ions potentiate the activity of A-II′ but surprisingly not A-II, providing one of the few examples where such a selective interaction has been reported for the angiotensin peptides.

It has also been reported that A-II and A-II′ can effect a translocation of calcium ions in smooth muscle cells. Allman et al. (1971) have shown that A-II can stimulate changes in the extracellular and intracellular concentrations of calcium ions in beef heart mitochondria. An increase in the concentration of calcium ions in the cytoplasma of rabbit aortas was observed with A-II (Van Breemen et al., 1972a,b; Deth & Van Breemen, 1974). In studies performed in microsomes of smooth muscles, A-II was found to increase the release of calcium ions into the intracellular cytoplasma (D'Auria et al., 1972; Baudoin et al., 1972). Bailey & Fenwick (1975) have reported that the injection of A-II′ into intact eels, *Anguilla rostrata*, increased the concentration of calcium ions present, providing an in vivo example of the ability of A-II′ to influence the levels of calcium ions in tissues. It is also worth noting that the presence of 10^{-6} M A-II had a marginal effect on the uptake of calcium ions by the plasma membrane fraction of the rat myometrium (Rangachari et al., 1976).

By use of intracellular microelectrodes, it was found that a concentration of 10^{-7}–10^{-6} M A-II induced membrane depolarization and brought about a reduction in the resistance of isolated cat supercervical, ciliary, and spinal ganglion cells (Dunn et al., 1978). The presence of Tris[1] abolished this effect. The presence of Tris was also found to inhibit A-II contractile responses in rat portal veins and aortas (Turlaparty et al., 1978), presumably by binding to the extracellular calcium ions. The presence of EDTA or EGTA has been shown to reduce the cation-stimulated binding of A-II′ to rat renal glomeruli (Blanc et al., 1978). These reports clearly indicate that chelators of calcium ions can affect the physiological activity of A-II or A-II′. The addition of other cations can also affect some of the physiological actions of A-II and A-II′. For

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; NMR, nuclear magnetic resonance; EPC, phosphatidylcholine from egg lecithin.

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example, the addition of lanthanum ions to the extracellular medium of rabbit aortas has been shown to decrease the influence of calcium ions (Deth & Van Breemen, 1974). Also, the presence of magnesium or manganous ions has been shown to reduce the stimulation of binding of A-II by calcium ions to rat renal glomeruli (Blanc et al., 1978). This latter report is of particular relevance to the present study since it demonstrates that manganous ions can stimulate the binding of A-II to the renal glomeruli in a fashion similar to magnesium and calcium ions. For all of the three cations the stimulation was found to be pH dependent, having an apparent pK of ~7.2. Also, as pointed out earlier, EDTA and EGTA suppressed this stimulation.

The present study demonstrates that both A-II and A-II′ possess ionophoric properties. Specifically, NMR spectroscopy is applied to monitor the hormone-mediated transport of manganous ions across phospholipid bilayers by measuring the rate of disappearance of the NMR signal of the cholines of the inner phospholipid layer due to the broadening effects of the transported manganous ions. This method has previously been applied to the study of Mn(II) transport mediated by the ionophore X-537A (Degani, 1978; H. Degani, A. McLaughlin, and S. R. Simon, unpublished results). In the following sections we describe the results of similar studies conducted with A-II and A-II′. We report the effects of pH, concentration of the hormone, concentration of Mn(II), the presence of Tris and La(III), and temperature on the rate of Mn(II) transport through phosphatidylcholine vesicles.

Materials and Methods

Human angiotensin II, [Asn^1,Val^5]angiotensin II, angiotensin III, and saralasin were purchased from Peninsula Labs (San Carlos, CA). The hormones were dissolved in ^2H_2O, and their concentrations were determined spectrophotometrically by using a molar absorptivity of 1.4×10^3 at 275 nm or of 675 at 285 nm. Pure phosphatidylcholine (EPC) dissolved in chloroform was purchased from Makor Chemical, Ltd. (Jerusalem, Israel) and was used without further purification.

Preparation of Vesicles. The EPC dissolved in chloroform was evaporated to dryness by a stream of nitrogen and subsequent vacuum pumping. The dry lipid film was resuspended in ^2H_2O and sonicated with a Branson W-375 sonifier for ~12 min at power level 4, in the pulsed mode, 40% fractional power. The sonication chamber was maintained at 4 °C throughout the sonication. The sonicated suspensions were then centrifuged for 30 min at 40000g at 4 °C, and the zone containing clear supernatant was removed and kept refrigerated under nitrogen for subsequent studies. All of the experiments were performed within 48 h of the preparation of the vesicles. This procedure yields a fairly homogenous population of vesicles which have an average diameter of ~250 Å (Degani, 1978; Huang et al., 1974).

Vesicle preparations were analyzed for phospholipid content by ashing and analyzing for liberated phosphate. Lipid concentrations were calculated by employing an average molecular weight of 770.

NMR Measurements. All measurements were performed on a Bruker WH-90 Fourier transform spectrometer equipped with variable temperature accessory. The temperature was determined to ±1 °C from the chemical shift separation of ethylene glycol, and one transient was sufficient to provide spectra with good signal to noise levels (see Figure 1).

Analysis of NMR Kinetic Data. We employed a method for determining the rate of transport of Mn(II) ions which has been described by Degani (1978) and H. Degani, A. McLaughlin, and S. R. Simon (unpublished results). We will briefly outline the method for convenience.

The general equation describing diffusion into the internal volume of vesicular membranes is

$$dC_i/dt = k(C_o - C_i) \quad (1)$$

where C_o is the permeant concentration in the extravesicular medium and C_i is the average permeant concentration inside the vesicles. The solution to eq 1 for the boundary conditions existing in our measurements at $t = 0$ and $C_i(0) = 0$ and at $t = \infty$ and $C_i(\infty) = C_o$ is

$$C_i(t) = C_o(1 - e^{-kt}) \quad (2)$$

In our system the permeant is Mn(II) ions. The concentration of these ions in the extravesicular medium is sufficiently high (≥1 mM) to bring about a complete broadening of the resonances of the outer choline groups, leaving only a sharp signal arising from the cholines at the inner lipid layer of the vesicles. Since the vesicles have a relatively small internal volume, transporting a single Mn(II) ion into a vesicle brings about the complete broadening of the inner choline resonances. Thus, the intensity $s(t)$ of the inner choline signal at time t after initiating transport is proportional to the number of vesicles with no Mn(II) ions within and is given by (H. Degani, A. McLaughlin, and S. R. Simon, unpublished results)

$$s(t) = s(0) \exp[-\bar{n}(t)] \quad (3)$$

where $s(0)$ is the signal intensity at $t = 0$ and $\bar{n}(t)$ is the average number of manganese ions per vesicle at time t; $\bar{n}(t)$ is related to the average manganese concentration $C_i(t)$ in the intravesicular medium by the expression

$$\bar{n}(t) = vN_A C_i(t) \quad (4)$$

where v is the internal volume of a vesicle and N_A is Avogadro's number. Substitution of eq 2 and 4 into eq 3 yields the relation

$$s(t) = s(0) \exp[-vN_A C_o(1 - e^{-kt})] \quad (5)$$

In the region where $kt \ll 1$, eq 5 simplifies to

$$s(t) = s(0) \exp(-k't) \quad (6)$$

where

$$k' = vN_A C_o k \quad (7)$$

Note that k' is the probability per unit time for the transport of manganous ions. In the analysis of data a good fit to eq 6 was obtained for all the experiments described below, confirming the validity of its use. The intrinsic rate constant k can be calculated from the observed rate constant k' according to the above relation.

Results and Discussion

Spectra obtained from a typical Mn(II) transport experiment involving A-II are shown in Figure 1. There is a clear decrease observed in the height of the choline signal with time. In contrast, when no A-II was added, the height of the choline signal in the presence of Mn(II) did not decrease even overnight. Thus, we can conclude that A-II mediates the transport of Mn(II) ions across the phosphatidylcholine bilayers of EPC vesicles. Similar results were obtained when the experiment was repeated with A-II′ rather than with A-II. That is, the presence of A-II′ brought about a time-dependent decrease in the intensity of the inner choline signal. Observed rates obtained (see above) for the hormone-mediated transport are given in Table I. The rates of transport for the two hormones are quite similar, suggesting that A-II and A-II′ transport

Table I: Rates of Angiotensin II and [Asn^1,Val^5]angiotensin II Mediates Transport of Mn(II) across Egg Lecithin Vesicles

hormone	T (°C)	p^{2}H	[lipid] (mM)	[Mn(II)] (mM)	agent present	k' (min^{-1})
A-II (0.95 mM)	55	7.84	7.45×10^{-2}	3.96		0.0202
A-II (0.95 mM)	55	7.84	7.45×10^{-2}	3.96	Tris, 18.7 mM	0.0095
A-II (0.570 mM)	53	7.38	5.93×10^{-2}	7.8	La(III), 87 mM	0.0033
A-II′ (1.81 mM)	47	7.30	6.14×10^{-2}	7.8	Tris, 7.5 mM	0.0102

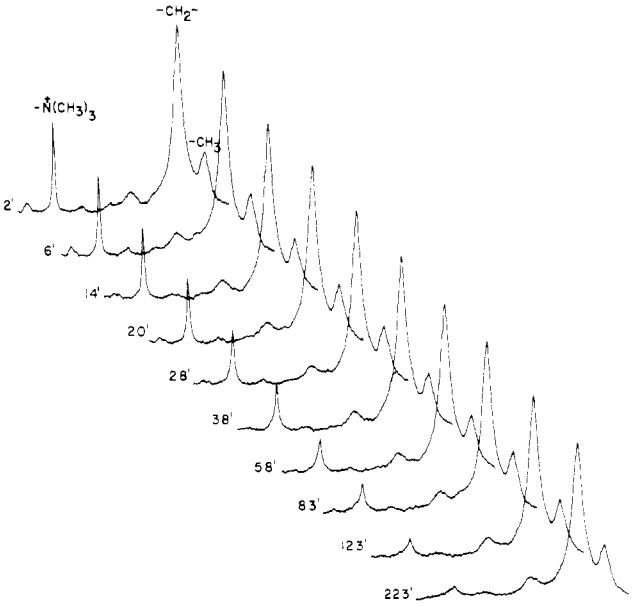

FIGURE 1: ^{1}H NMR spectra of 7.45×10^{-2} M EPC vesicles in ^2H_2O at p^{2}H 7.84 in the presence of 3.96 mM $MnCl_2$ and 0.95 mM A-II at 55 °C.

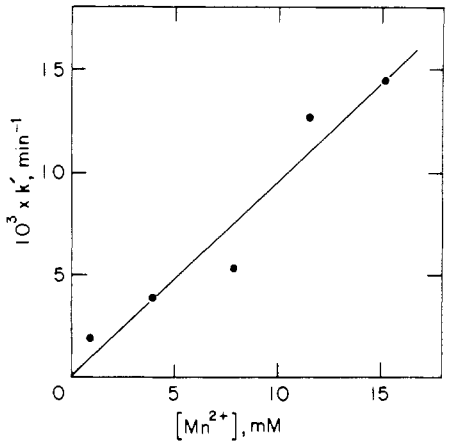

FIGURE 2: Variation of the rate of Mn(II) transport with the concentration of $MnCl_2$ present. The solutions contained 5.93×10^{-2} M EPC vesicles at p^{2}H 7.38 in the presence of 0.570 mM A-II and 7.5 mM Tris at 53 °C.

Mn(II) with similar efficiency. In contrast, when the experiments were carried out with either angiotensin III (Arg-Val-Tyr-Ile-His-Pro-Phe) or saralasin ([Sar^1,Ala^8]A-II) under the same conditions, negligible transport was observed. It should be noted that the rates of transport for A-II and A-II′ are ~2 orders of magnitude smaller than those reported for the X-537A system (H. Degani, A. McLaughlin, and S. R. Simon, unpublished results).

The observed rate constant k' has been determined as a function of both A-II and Mn(II) concentrations. Linear plots of the values of k' obtained at varying Mn(II) and A-II concentrations were obtained (cf. Figures 2 and 3). Plots of log k' against log [Mn(II)] and log[A-II] yielded slopes of 0.9 and

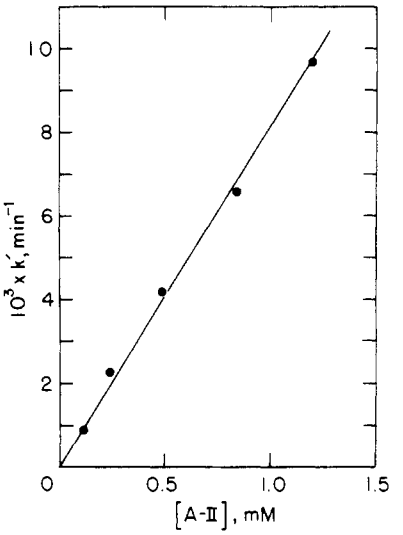

FIGURE 3: Rates of Mn(II) transport at varying A-II concentrations. The solutions contained 6.14×10^{-2} M EPC vesicles at p^{2}H 7.30 in the presence of 3.96 mM $MnCl_2$ and 7.5 mM Tris at 53 °C.

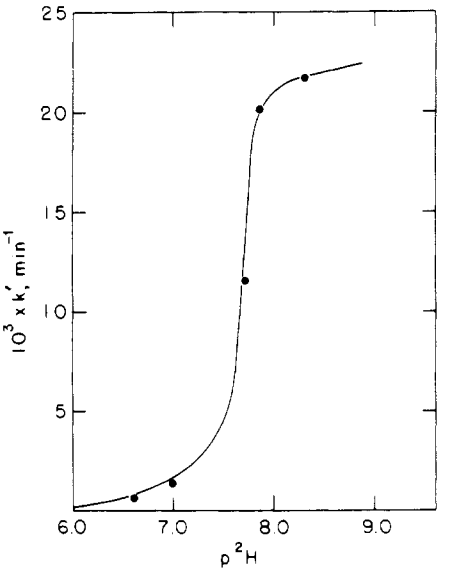

FIGURE 4: p^{2}H dependence of the rate of Mn(II) transport. The solutions contained 7.45×10^{-2} M EPC vesicles in the presence of 3.96 mM $MnCl_2$ and 6.95 mM A-II at 55 °C.

0.85, respectively. Thus, we can conclude that the complex involved in the transport process has a 1:1 stoichiometry. This conclusion is supported by the finding that A-II forms 1:1 complexes with Ca(II) and several of the trivalent lanthanide ions in solution (Lenkinski et al., 1978; R. E. Lenkinski and R. L. Stephens, unpublished results).

The rate of Mn(II) transport in the presence of A-II is pH dependent (cf. Figure 4), having an apparent pK_a of ~7.7 in ^2H_2O. In solution, the metal binding process has also been found to be strongly dependent on pH, having an apparent pK_a of 7.3 in ^2H_2O (R. E. Lenkinski and R. L. Stephens, unpublished results). This pH dependence of metal binding probably

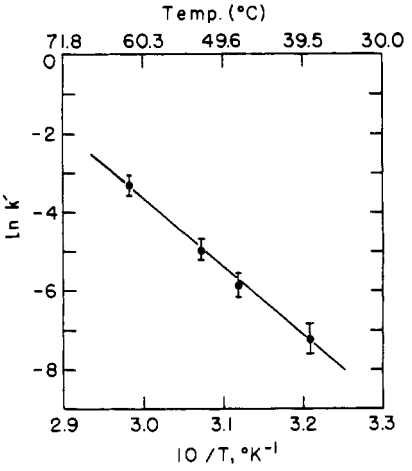

FIGURE 5: An Arrhenius plot of the rates of Mn(II) transport obtained at different temperatures. The solutions contained 4.1×10^{-2} M EPC vesicles at p^{2}H 7.1 in the presence of 1 mM $MnCl_2$ and 0.2 mM A-II.

arises from a change in the conformation of the A-II complex which occurs when the terminal amino group becomes protonated. This interpretation is supported by the fact that the terminal amine has a pK_a of 7.6 in ^2H_2O (Juliano et al., 1979) and by the observation that the angiotensin II peptides undergo a significant conformational change at this pH (Glickson et al., 1974).

The rate of A-II-mediated transport was found to vary significantly with temperature. An Arrhenius plot (see Figure 5) was constructed from rates measured between 30 and 65 °C. The energy of activation for the transport process is 29 ± 5 kcal/mol with an entropy of activation of ~10 eu. These activation parameters are similar to the ones reported for the X-537A system (Degani, 1968) and those reported for the valinomycin–Rb(I) system (Benz & Lauger, 1976). By consideration of the thermodynamic parameters of the various steps involved in the transport mechanism, it becomes clear that the rate of diffusion of the metal complex through the membrane is most probably the rate-determining step in the transport process. Metal complexation is usually accompanied by a smaller energy of activation and a negative entropy of activation arising from a net decrease in solvation of the metal complex as compared with the separate ions.

The effects of Tris and La(III) on the rate of A-II-mediated Mn(II) transport are evident from the data given in Table I. The presence of Tris decreases the rate of transport by a factor of ~520. Since this agent is known to bind to metal ions, we suggest that this decrease is a reflection of the lower concentration of the A-II complex of Mn(II) brought about by the presence of Tris. Lanthanum ions are known to bind to A-II directly (R. E. Lenkinski and R. L. Stephens, unpublished results). Presumably, the presence of these ions also brings about a decrease in the rate of transport by competing with Mn(II) in binding to A-II.

Conclusions

In the preceding sections we have presented results that indicate that both A-II and A-II′ can mediate the transport of Mn(II) ions across the lipid membranes of a model system (EPC vesicles). The species involved in the transport process probably involves a complex which has a 1:1 stoichiometry of metal ion and hormone. This conclusion is supported by the observations that the rate of transport depends linearly on both the concentration of Mn(II) as well as that of hormone. Also, the pH profile of the rate constant is similar to that observed for the metal complexation in solution. By analogy with other ionophores (e.g., X-537A and A-23187) which have been found to transport both Mn(II) and Ca(II) ions, we suggest that angiotensin II may transport Ca(II) as well as other cations across membrane bilayers (Degani & Friedman, 1974).

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Interaction of Prothrombin and Its Fragments with Monolayers Containing Phosphatidylserine. 1. Binding of Prothrombin and Its Fragment I to Phosphatidylserine-Containing Monolayers†

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ABSTRACT: The adsorption isotherms of prothrombin and its fragment I on phosphatidylserine monolayers and on mixed monolayers of phosphatidylcholine and phosphatidylserine were determined by measuring surface radioactivity emanating from the tritium-labeled adsorbed proteins at 0.1 N NaCl and between 0 and 10 mM Ca^{2+}. The proteins were adsorbed from very dilute solutions, about 10 times more than in previous investigations on bilayer vesicles. The binding constants as obtained from the Scatchard plots were between 3×10^6 and 3×10^8 mol/L, depending on the experimental conditions. These values are between 2 and 50 times larger, respectively, than the binding constants obtained on bilayer vesicles. Prothrombin adsorbs appreciably also in the absence of Ca^{2+}. The significance of these results is discussed.

The importance of the vitamin K dependent γ-carboxyglutamic acid in the binding of prothrombin and factor Xa to phosphatidylserine-containing biological membranes and the blood clotting reactions has been well established (Nelsestuen, 1978; Esmon et al., 1975). Ca^{2+} is essential for the binding of these proteins to the negatively charged catalytic lipid surface, even though it can be to some extent replaced by other divalent ions (Nelsestuen & Lim, 1977; Nelsestuen et al., 1976; Furie et al., 1976).

The equilibria involved in the binding reaction are considered to be as shown in Scheme I (Nelsestuen & Lim, 1977), where Pt is the protein, Pt* is the protein after Ca^{2+}-induced conformational change, PL is the phospholipid, and i, j, and m are the molecularities of Ca^{2+} in each reaction. Most of the binding studies have been carried out on liposomes or bilayer vesicles. Quasielastic light scattering (Nelsestuen & Lim, 1977) and fluorescence quenching (Nelsestuen et al., 1976; Prendergast & Mann, 1977) were among the methods used for the investigation of the conformational relation of these proteins to their interaction with Ca^{2+} and with phospholipids. There is evidence that Ca^{2+} and phospholipid induce a conformational change in fragment I, but hardly any in prothrombin (Nelsestuen, 1976).

In a previous publication (Lecompte & Miller, 1980), we reported on the interaction of prothrombin with phosphatidylserine monolayers. The stoichiometry of the interaction was inferred from the measured surface radioactivity of ^3H-labeled prothrombin and of ^{45}Ca, while structural parameters were inferred from the electrical capacitance of the monolayer.

Scheme I

$$\left.\begin{array}{l} Pt + i\,Ca \overset{1}{\rightleftarrows} PtCa_i \overset{2}{\rightleftarrows} Pt^*Ca_i \\ \qquad\qquad\qquad\qquad + \\ PL + j\,Ca \overset{3}{\rightleftarrows} PL\cdot Ca_j \end{array}\right\} + m\,Ca \overset{4}{\rightleftarrows} Pt^*PL\cdot Ca_{(i+l+m)}$$

In the first paper of this series we wish to describe the adsorption isotherms of prothrombin and of its fragment I on the monolayer of phosphatidylserine mixed with phosphatidylcholine at different ratios. The binding constants and stoichiometry, as well as the Ca^{2+} requirement as inferred from these measurements, will be compared with the results obtained with lipid dispersions.

Materials and Methods

The phospholipids (egg lecithin and ox brain phosphatidylserine, purchased from Lipid Products, Nutfield, England) were supplied in chloroform–methanol solution. For spreading, samples were evaporated in a stream of nitrogen, the lipid content was determined by weight, samples were dissolved in hexane, and the desired compositions were obtained by mixing the hexane solutions.

A two- to threefold excess of the lipid was spread over an aqueous phase containing 0.1 M NaCl and 10^{-3} M Tris at pH 7.8. The excess assured a fully compressed monolayer in equilibrium with the collapsed excess lipid layers.

Human prothrombin and its fragment I were purified by R. Benarous and J. Elion, according to Mann (1976).

Radioactive ^3H-labeled prothrombin and fragment I were prepared by oxidizing their sialic acid with sodium metaperiodate and then by reducing the obtained aldehyde with sodium [^3H]borohydride (Butkowski et al., 1974). The radioactive [^3H]borohydride was purchased from the Radiochemical Center, Amersham, England.

For determination of adsorption onto the lipid monolayer, between 10 and 100 μL of radioactive protein solution was injected into 15 mL of buffer solution underneath the spread

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