J. Membrane Biol. 72, 85–91 (1983)

The Journal of
Membrane Biology

In Vitro Effects of Vitamin D_3 on the Phospholipids of Isolated Renal Brush Border Membranes

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Summary. A model system is described in which cholecalciferol (vitamin D_3) is incorporated into phosphatidylcholine liposomes and then the liposomes are incubated *in vitro* with isolated renal brush border membrane vesicles. The incubation results in an alteration of the phospholipid composition, the fluidity, and the transport properties of the membrane. The findings provide evidence consistent with the hypothesis that vitamin D_3 and metabolites modify membrane structure and function by "liponomic regulation."

Key words membrane structure and function · liposomes · phosphatidylcholine · cholecalciferol · kidney microvillus membrane · electron spin resonance · membrane fluidity

Introduction

There is increasing evidence that vitamin D metabolites act, in part, by mechanisms other than the general scheme involving steroid hormone receptors, genome activation, and synthesis of specific proteins (Norman & Ross, 1979). Support for this concept comes from findings that there is an increase in the phosphorus content of the membrane phospholipids in the intestinal brush border and an alteration in the composition of the fatty acids in phosphatidylcholine following the administration, *in vivo*, of the vitamin to vitamin D-deficient chicks (Goodman, Haussler & Rasmussen, 1972; Max, Goodman & Rasmussen, 1978); and the insertions of the lipophilic polyene, filipin, and the methyl ester of the fatty acid, *cis*-vaccinic acid, into the membrane increase the rate of calcium uptake by membrane vesicles prepared from vitamin D-deficient, but not from vitamin D-repleted, animals (Adams, Wong & Norman, 1970; Rasmussen, Fontaine, Max & Goodman, 1976; Fontaine, Matsumoto, Goodman & Rasmussen, 1981). However, the direct, *in vitro* effect of a vitamin D metabolite on the lipids of isolated membranes has not been reported.

In the present paper, we examined the effects of cholecalciferol (vitamin D_3) on the isolated brush border membrane of the renal proximal tubule. Our approach to this study stems from the observations that liposome-cell interactions alter plasma membrane phospholipid and cholesterol content (Pagano & Weinstein, 1978). In the model system described here cholecalciferol is first incorporated into egg lecithin liposomes and then the liposomes are incubated *in vitro* with isolated brush border membranes. Evidence is reported showing that the presence of the vitamin in the liposome modifies the phospholipid composition, the fluidity, and the transport properties of the membrane.

Materials and Methods

Brush Border Membranes

Rabbit renal proximal tubule brush border membranes were prepared as described previously (Beck & Sacktor, 1978). After isolation the membranes were washed three times in a medium containing (mM): 100 mannitol, 100 KCl, 2.5 $MgCl_2$, and 5 Hepes/Tris buffer, pH 7.5. The quality of the membrane preparations, evaluated by specific activities of enzyme markers, was the same as reported earlier (Beck & Sacktor, 1978).

Liposomes

Egg L-α-lecithin, purchased from Avanti Biochemicals, was used without further purification. In some experiments the lecithin liposomes were labeled with L-α-phosphatidylcholine, di[1-^{14}C]palmitoyl (114 mCi/mmol), purchased from Amersham. Chloroform solutions (0.7 ml) of lecithin (50 mg/ml) were dried under N_2 and then under vacuum for 5 min. Medium (1 ml), the same as that used to suspend the brush border membranes, was added. After shaking vigorously for 1 min a milky suspension was obtained. Then, 6 ml of medium was added and the suspension was sonicated to clarity with pulses (30 sec on, 30 sec off, setting 4, Branson Heat Systems Sonicator) under N_2 in an ice-water bath. After sonication, the preparation was centrifuged at 30,000 × *g* for 1 hr at 0 to 4 °C. Liposomes remaining in the supernatant were used to incubate with brush border membranes. To prepare liposomes containing cholecalciferol or other steroids, the identical procedure was used except that the chloroform solutions were comprised

of the phospholipid and steroid. Essentially 100% of the added vitamin D_3 was incorporated into the liposome. When radioactive cholecalciferol was added, [1α, 2α(n)−^{3}H] vitamin D_3 (16 Ci/mmol) from Amersham was used. Liposomes were used in experiments the same day that they were prepared.

Incubation of Membranes with Liposomes

Renal brush border membranes were first pre-incubated with the liposomes and then incubated with other liposomes containing cholecalciferol, other steroids, or liposomes without steroid (control). For the pre-incubation, the membranes (about 10 mg of protein) were mixed with liposomes (about 10 mg of phosphatidylcholine) for 2 hr at 37 °C. The membranes were separated from the liposomes by centrifugation at 30,000 × *g* for 10 min at 4 °C. The process was repeated once. Then, the pretreated membranes were incubated twice with liposomes containing various quantities of cholecalciferol, other steroids, or liposomes without steroid. Each incubation lasted 80 min, at 37 °C. After the last incubation, the membranes were washed twice with the brush border membrane isolation medium and then resuspended in the same medium at a concentration of approximately 10 mg of protein/ml.

Assays

To determine the transfer of labeled phosphatidylcholine and cholecalciferol from liposomes to brush border membranes, the washed membranes were poured onto Millipore filters (0.45 μm) and the filters washed with cold medium. Radioactivity retained on the filters was counted. Phospholipid P was measured by the acid hydrolysis of the brush border membrane or liposome (Umbreit, Burris & Stauffer, 1959) followed by spectrophotometric analysis for inorganic P (Bartlett, 1959). Protein was determined by a standard procedure (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin as the reference protein. All assays were carried out in triplicate. The results were expressed as the mean ± standard deviation.

Transport Measurements

Na^+ gradient-dependent D-glucose uptake by brush border membrane vesicles was measured by a Millipore filtration technique, using 0.45 μm filters, as described previously (Aronson & Sacktor, 1975). The temperature of the uptake medium was 37 °C. The extravesicular medium contained 100 mM mannitol, 100 mM NaCl, 5 mM Tris/Hepes buffer, pH 7.5, and 50 μM D-[^{3}H]glucose. The labeled sugar (30 Ci/mmol) was obtained from New England Nuclear.

Spin Labeling

The electron spin resonance (ESR) label was the stearic acid probe 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl [I(12,3)], obtained from Sylva. The label had the nitroxide attached relatively close (three methylene groups) to the carboxylate end of stearic acid and, thus, probed fluidity near the surface of the lipid bilayer (Hubbell & McConnell, 1971). A 1-mM solution of the probe in ethanol was stored at −60 °C. Aliquots (0.2 μmol) of the solution were transferred to pear-shaped flasks and dried with a stream of dry N_2. Samples of the brush border membrane (about 50 mg of protein and 40 μmol of phospholipid) were added to the flask and vortexed for 5 min at 4 °C (Sauerheber, Gordon, Crosland & Kuwahara, 1977). Studies at various concentrations of probe/membranes indicated that at the concentrations used there were no detectable probe-probe interactions (Sauerheber et al., 1977) that altered the ESR spectrum. The same 200:1 phospholipid/probe molar ratio was used for labeling the liposomes.

Analysis of ESR Spectra

ESR spectra were recorded with a Jeolco JES ME-1 spectrometer equipped with a variable temperature accessory that maintained temperature to ±0.2 °C. An aqueous flat cell with a path length of 0.5 mm was used. Spectra were obtained at 37 °C after the sample reached thermal equilibrium in the cavity. The microwave power was 15 mW.

A typical ESR spectrum of spin-labeled renal brush border membranes is shown in Fig. 1. From such spectra, the hyperfine splittings $T_\parallel$ and $T_\perp$ were calculated (Hubbell & McConnell, 1971; Gaffney & McConnell, 1974). Improved values for $T_\parallel$ were obtained by increasing the spectral gain in the high field and low field regions of the spectrum (Sauerheber et al., 1977). Improved values for $T_\perp$ were obtained by running spectra at ±25 gauss in addition to the usual ±50 gauss that was required to observe the entire spectrum (Sauerheber et al., 1977). Errors in the determination of these parameters were estimated by averaging the values from several spectra of the same sample.

The motional freedom of the noncovalently bound stearic acid probe, which reflected the fluidity of the lipid bilayer, was estimated from the calculated order parameter *S* (Seelig, 1970; Hubbell & McConnell, 1971; Sauerheber et al., 1977):

$$S=\frac{(T_\parallel - T_\perp)a_n}{(T_{zz}-T_{xx})a_n'}.$$

Values for hyperfine splittings T_{zz} and T_{xx} were obtained from the completely immobilized probe incorporated into host crystals as substitutional impurities (Seelig, 1970). The constant a_n represented the isotropic coupling constant in the crystal and was equal to 1/3 $(T_{xx}+T_{yy}+T_{zz})$. The constant a_n' represented the isotropic constant in the membrane and was equal to 1/3 $(T_\parallel+2T_\perp)$. The ratio a_n/a_n' corrected for differences in polarity between the membrane and the crystal. In the calcula-

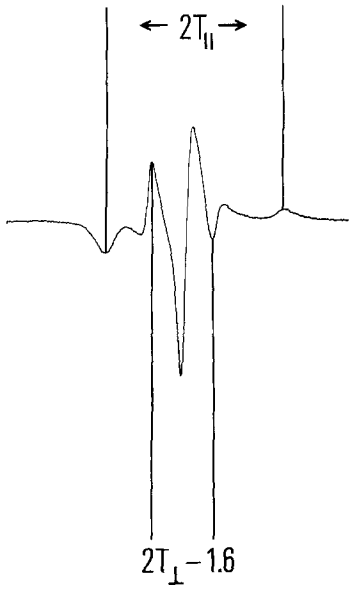

Fig. 1. A typical electron spin resonance spectrum of spin labeled renal brush border membranes. From such spectra, the hyperfine splittings $T_\parallel$ and $T_\perp$ were calculated as described in the text

tions reported in this paper the value for T_{xx} and T_{yy} was 6.1 gauss and the value for T_{zz} was 32.4 gauss. An increase in the order parameter S was indicative of a decrease in fluidity.

Results

Effect of Vitamin D_3 on the Transfer of Phosphatidylcholine Between Liposomes and Brush Border Membranes

The transfer of radioactivity from lecithin liposomes labeled with L-α-phosphatidylcholine, di[1-^{14}C]palmitoyl, to renal brush border membranes is illustrated in Fig. 2. Some radioactivity was detected in the membranes after incubations with liposomes containing either cholecalciferol or 7-dehydrocholesterol. When the steroid/phospholipid ratio in the liposome was relatively small, incorporation of radioactivity was low. With cholecalciferol-containing liposomes, however, the transfer of label into the membrane was increased threefold when the vitamin D_3/lecithin ratio in the liposome approached unity. In contrast, with 7-dehydrocholesterol-containing liposomes, the incorporation of radioactivity was not enhanced as the steroid/phospholipid ratio increased; in fact, radioactivity in the membrane appeared to decrease as the ratio approached unity.

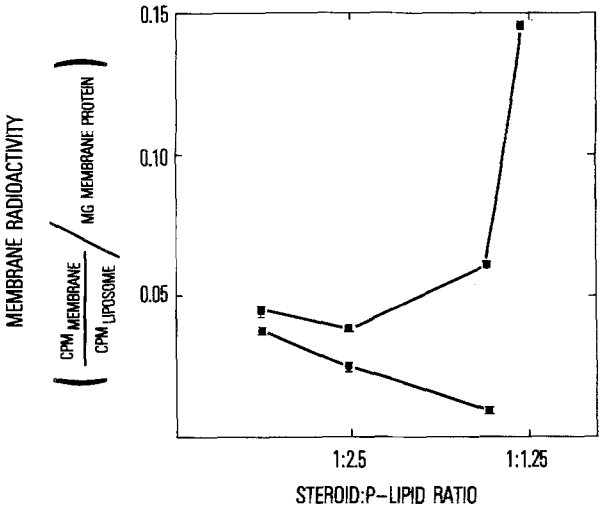

Fig. 2. The transfer of radioactivity from lecithin liposomes labeled with L-α-phosphatidylcholine, di[1-^{14}C]palmitoyl, to renal brush border membranes as a function of the steroid/P-lipid ratio in the liposome. (■) cholecalciferol, (●) 7-dehydrocholesterol

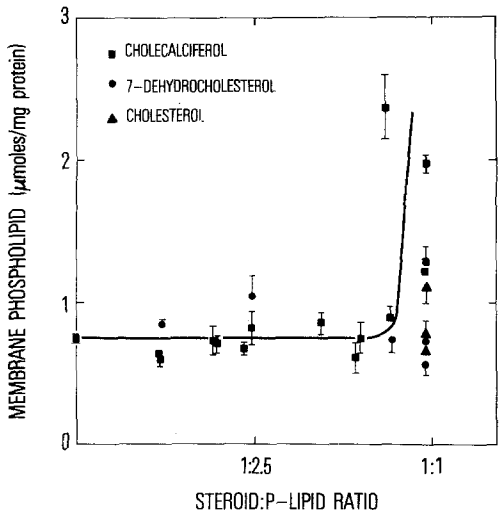

Fig. 3. The net transfer of phospholipid from lecithin liposomes to renal brush border membranes as a function of the steroid/P-lipid ratio in the liposome

The increased incorporation of radioactivity into the membrane phospholipid from the lecithin liposomes suggested a vitamin D_3-induced stimulation of exchange and/or of the net transfer of phosphatidylcholine. The experiments shown in Fig. 3 indicated that the total phospholipid content of the brush border membrane was increased about twofold when the membranes were incubated with cholecalciferol-containing lecithin liposomes. Again, the molar ratio of steroid/phospholipid was of critical importance, because net transfer was found only when the ratio was approximately 1:1. This effect was specific for vitamin D_3 and not observed when the liposomes contained 7-dehydrocholesterol or cholesterol, at comparable relative concentrations.

In several parallel experiments it was found that when the phospholipid content of the brush border membrane was increased there was a corresponding loss in phosphatidylcholine from the cholecalciferol-containing liposomes. Table 1 shows that the lecithin contents of liposomes were not remarkedly altered when membranes were incubated with liposomes containing either no chole-

Table 1. Cholecalciferol-dependent decrease in liposomal phospholipid (P-lipid) after incubation of liposomes with brush border membranes

Cholecalciferol (steroid/P-lipid ratio)	P-Lipid (mM)	
	Before incubation	After incubation
control	8.5±0.8	7.1±0.5
1:3.2	8.5±0.8	6.9±0.5
1:1.8	9.2±1.0	7.4±0.9
1:1.25	7.2±0.8	2.8±0.4

Phosphatidylcholine liposomes containing vitamin D_3 at the indicated steroid/P-lipid ratio were incubated with brush border membranes as described in the text. P-lipid was determined in the liposome-containing supernatant before and after incubations.

calciferol or relatively low quantities of cholecalciferol. However, when the vitamin D_3 content of the liposome was increased so that the steroid/phospholipid ratio in the liposome was 1:1.25, the phosphatidylcholine in the liposome was significantly decreased.

The Transfer of Cholecalciferol from Liposome to Brush Border Membrane

Table 2 shows that [^{3}H]cholecalciferol incorporated into liposomes could be transferred to brush border membranes. As was the case with the transfer of phosphatidylcholine from liposome to membrane, the amount of vitamin D_3 found in the membrane depended on the stoichiometric relationship between cholecalciferol and phospholipid in the liposome. In a series of experiments in which the steroid/phospholipid ratios were 1:3.2, 1:1.6, and 1:1.4, 95, 210, and 844 nmol of cholecalciferol were transferred per mg of membrane protein, respectively. These values represented 10.8, 12.7 and 46.2%, respectively, of the total cholecalciferol originally incorporated into the liposomes.

Table 2. Transfer of cholecalciferol from liposomes to brush border membranes

Stereoid/P-lipid ratio	Membrane cholecalciferol (nmol/mg protein)
1:3.2	95± 3
1:1.6	210±23
1:1.4	844±16

Table 3. Fluidity of lecithin liposomes and liposomes containing cholecalciferol or 7-dehydrocholesterol labeled with the stearic acid spin label I (12,3)

Preparation	Fluidity (order parameter *S*)
liposomes	0.533±0.004
liposomes + cholecalciferol[a]	0.579±0.004
liposomes + 7-dehydrocholesterol[a]	0.595±0.008

[a] Steroid/P-lipid ratio was approximately 1:1.

Table 4. Fluidity of renal brush border membranes labeled with the stearic acid spin label I (12,3)

Membrane preparation	Fluidity (order parameter *S*)
Untreated	0.627±0.006
Incubated with buffer	0.635±0.013
Incubated with liposomes	0.609±0.008
Incubated with liposomes + 7-dehydrocholesterol[a]	0.638
Incubated with liposomes + cholecalciferol[a]	0.580±0.006

[a] Steroid/P-lipid ratio was approximately 1:1.

Effects of Vitamin D_3 on the Fluidity of Phosphatidylcholine Liposomes and Brush Border Membranes

The stearic acid spin label I(12,3) was incorporated into liposomes and brush border membranes. It was calculated from ESR spectra of the liposomes that cholecalciferol increased the order parameter *S*, indicative of a decrease in the fluidity of the liposomes (Table 3). The effect of 7-dehydrocholesterol on the order parameter *S* of the liposome was somewhat greater. These results were consistent with previous findings showing that cholesterol as well as many other steroids decrease the fluidity of phospholipid liposomes when the liposomes were in a liquid crystalline state (Hsia, Long, Hruska & Gresser, 1972; Demel & DeKruyff, 1976).

Table 4 shows the effects of various incubations on the order parameter *S* of brush border membranes. Incubation of the membranes with buffer medium had no effect, indicating that the long-term incubation at 37 °C did not grossly damage the membrane. Incubation of the membranes with phosphatidylcholine liposomes that did not contain steroid decreased the order parameter of the membranes from 0.635 ± 0.013 to 0.609 ± 0.008, i.e. increased the fluidity of the membranes. This type of behavior would be expected if the endogenous cholesterol of the membrane was removed by the incubation of the membranes with steroid-free liposomes (Bruckdorfer, Demel, DeGier & Van Deenen, 1969). When liposomes containing 7-dehydrocholesterol were incubated with membranes the order parameter of the membranes was found to be 0.638. This increased order parameter relative to an order parameter of 0.609 with membranes incubated with control liposomes indicated that the fluidity of the membranes decreased. In contrast, when liposomes containing cholecalciferol were incubated with membranes the order parameter decreased to a value of 0.580 ± 0.006, indicating an increase in the fluidity of the membrane.

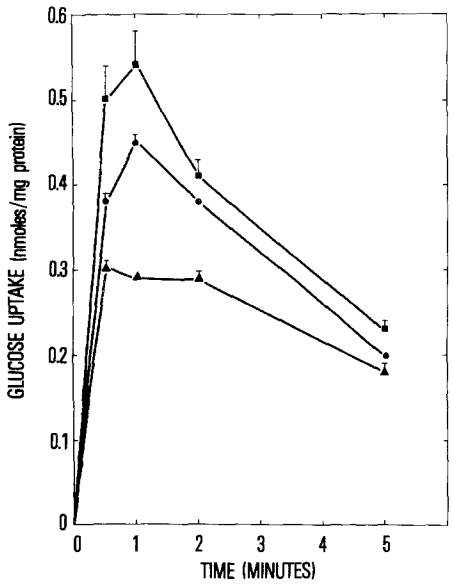

Fig. 4. Na^+ gradient-dependent D-glucose uptakes by membrane vesicles incubated previously with liposomes without cholecalciferol (■) or liposomes containing vitamin D_3, 1:4.12 (●) and 1:1.25 (▲) steroid/phospholipid ratios

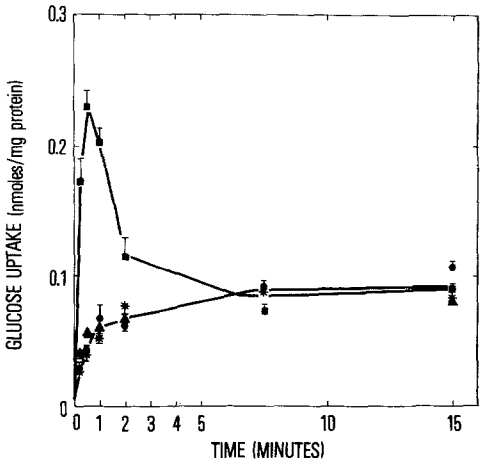

Fig. 5. D-Glucose uptakes by membrane vesicles treated with liposomes which did not contain cholecalciferol (●, *) or with liposomes which contained vitamin D_3, 1:1.25 molar ratio of steroid/phospholipid (▲, ●). Uptakes were measured either in the presence of a Na^+ gradient, 100 mM KCl_{in} and 100 mM $NaCl_{out}$ (■, ▲), or in the presence of Na^+ but with the absence of a gradient, 100 mM $NaCl_{in}$ and 100 mM $NaCl_{out}$ (*, ●)

Effect of the Vitamin D_3-Dependent Alteration in Membrane Lipid on the Transport Properties of the Membrane Vesicles

The experiments described above demonstrated that the incorporation of cholecalciferol into phosphatidylcholine liposomes and the subsequent incubation of these liposomes with brush border membranes resulted in a change in membrane lipid composition and fluidity. These findings prompted us to study whether these modifications of the membrane would be concomitant with an altered membrane transport function. To answer this question, brush border membranes were incubated with liposomes, containing cholecalciferol or devoid of vitamin D_3. Then we examined a well-characterized transport system in these membranes, the Na^+ gradient-dependent D-glucose uptake system.

Figure 4 shows D-glucose uptakes by membrane vesicles incubated previously with liposomes without cholecalciferol (control) or liposomes containing vitamin D_3 (1:1.25 and 1:4.12 steroid/phospholipid ratios). Control membranes exhibited the typical Na^+ gradient-dependent D-glucose uptake pattern with time of incubation, showing an "overshoot" peak at 1 min (Aronson & Sacktor, 1975). The uptake of the sugar with membranes incubated with the control lecithin liposomes was not significantly different from the uptake found with membranes incubated with buffer alone (*not illustrated*). However, with membrane vesicles, which were previously incubated with liposomes containing cholecalciferol, sugar uptakes were decreased. The magnitude of the decrement was dependent on the steroid/phospholipid ratio in the liposome, inhibition being greater the more cholecalciferol relative to phospholipid. When the ratio was 1:1.25, Na^+ gradient-dependent D-glucose uptake was strongly inhibited. When the ratio was 1:4.12, only a relatively modest inhibition was found. As noted in Fig. 3, however, treating membranes with liposomes containing this ratio of steroid/phospholipid did not measurably augment the phosphatidylcholine content of the membrane. Perhaps this might suggest that transport was more sensitive than phospholipid determination as an indicator of an alteration in the brush border membrane.

Figure 5 shows that inhibition of D-glucose uptake by membrane vesicles previously incubated with liposomes containing cholecalciferol was found only when the uptake of the sugar was carried out in the presence of a Na^+ gradient, extravesicular $[Na^+]$ > intravesicular $[Na^+]$. In the absence of a Na^+ gradient, extravesicular $[Na^+]$ = intravesicular $[Na^+]$, membrane vesicles previously incubated with control liposomes or vitamin D_3-containing liposomes had essentially identical D-glucose uptakes. Uptakes at 15 min, the equilibrium uptake (Aronson & Sacktor, 1975), were the same in all experiments. These findings indicated that the D-glucose carrier, *per se,* was probably not affected by treatment of membranes

with steroid-containing liposomes. Rather, the results suggested that inhibition of Na^+ gradient-dependent D-glucose uptake was due to the rapid dissipation of the Na^+ gradient.

Discussion

The present results demonstrated that cholecalciferol, but not 7-dehydrocholesterol, when incorporated into phosphatidylcholine liposomes, stimulated the interaction of phospholipid from liposomes with isolated renal proximal tubule brush border membranes. This was evident from the findings that the transfer of L-α-phosphatidylcholine di[1-^{14}C]palmitoyl from liposome to membrane was enhanced threefold, the total phospholipid content of the brush border membrane was increased about twofold, and there was a corresponding loss in liposomal lecithin. These results could be explained by a vitamin D_3-dependent enhancement in exchange and/or net transfer of phospholipid from liposome to brush border membrane. The cholecalciferol-induced transfer might represent the translocation of phosphatidylcholine from liposome to membrane, the fusion of the liposome with the membrane, and/or the tight adsorption (i.e. resistant to washings with an aqueous medium) of the liposome to the membrane.

The effect of vitamin D_3 in facilitating the interaction between liposome and membrane was crucially dependent on the ratio of cholecalciferol to phosphatidylcholine in the liposome, the effect being most pronounced when the two lipids were in approximately equimolar proportions. In an earlier report on the properties of aqueous dispersions of phosphatidylcholine and steroid, a somewhat analogous observation was made (Bruckdorfer, Edwards & Green, 1968). Very little lipid could be extracted with diethyl ether or chloroform until the molar ratio of phospholipid to steroid was 1:1; then, nearly all the steroid and 60% of the phosphatidylcholine could be extracted. It was proposed that there existed a very fine balance between hydrophilic and hydrophobic parts of the phosphatidylcholine molecule at a neural pH and the affinity for water became sufficiently low when the steroid content of the liposome was raised above a certain level, to allow extraction by the organic solvents (Bruckdorfer et al., 1968). A similar mechanism might explain the present results. When the cholecalciferol content in the lecithin liposome reached a critical level and the two lipids became "close-packed" (Vilallonga, Altschul & Fernandez, 1967), phosphatidylcholine and, indeed, vitamin D_3 itself, was transferred and dissolved into the lipid matrix of the brush border membrane.

ESR measurements revealed that 7-dehydrocholesterol decreased the fluidity of the lecithin liposomes and the brush border membranes. This would be in accord with results reported previously on the effects of cholesterol and various steroids on the fluidity of different kinds of phospholipid liposomes and membrane preparations (Hsia et al., 1972; Vanderkooi, Fischkoff, Chance & Cooper, 1974; Demel & DeKruyff, 1976). On the other hand, the findings that cholecalciferol effected the fluidity of the liposomes and the renal membranes oppositely were especially noteworthy. Vitamin D_3, like the steroids, decreased the fluidity of the phosphatidylcholine liposome, but increased the fluidity of the brush border membrane. The increase in the phosphatidylcholine content of the brush border membranes incubated with cholecalciferol-containing liposomes could provide a possible explanation for this increased membrane fluidity. It was found previously that the fluidity of biological membranes varied inversely with the ratio of endogenous cholesterol to phospholipid (Vanderkooi et al., 1974; Araki & Rifkind, 1980, 1981) and the association of phospholipids with protein in a membrane decreased the fluidity of the membrane (Marsh et al., 1982). The cholecalciferol-mediated transfer of phosphatidylcholine from liposomes to brush border membranes would, therefore, increase the fluidity of the membrane both by decreasing the proportion of membrane phospholipid associated with the membrane protein and by lowering the endogenous cholesterol to phospholipid ratio. Vitamin D_3 was also transferred from liposomes to brush border membranes during the incubation. However, because the transfer of lecithin always coincided with the transfer of vitamin D_3, no direct effect of cholecalciferol on membrane fluidity could be ascertained in the present experiments.

Brush border membranes, previously incubated with cholecalciferol-containing lecithin liposomes, presented a decreased Na^+ gradient-dependent D-glucose uptake. This effect could be attributed to either a direct action on the Na^+-D-glucose carrier or to an indirect effect in which leak pathways for Na^+ were increased and this resulted in dissipation of the Na^+ gradient driving force for sugar uptake. The finding that D-glucose transport was not decreased when the membrane vesicles were pre-equilibrated with Na^+, suggested that the latter alternative was the probable mechanism. Whether this effect of cholecalciferol in altering Na^+ flux represented a physiological action of the

vitamin remains to be established. It should be noted that treatment of D-deficient chicks with 1-hydroxycholecalciferol *in vivo* did not alter Na^+-dependent D-glucose accumulation in vesicles isolated from the chick intestine (Rasmussen et al., 1979). On the other hand, it was reported that steroids (aldosterone) exerted major effects on membrane lipids in toad bladder cells and did stimulate Na^+ transport (Goodman, Wong & Rasmussen, 1975; Lein, Goodman & Rasmussen, 1975).

In conclusion, we have found that when vitamin D_3 was first incorporated into phosphatidylcholine liposomes and then the liposomes were incubated *in vitro* with isolated renal brush border membrane vesicles that the phospholipid composition, the fluidity, and the transport properties of the membrane were modified. These *in vitro* effects would be consistent with the report that 1-hydroxycholecalciferol given *in vivo* to vitamin D-deficient chicks increased the content of lipid phosphorus per mg of intestinal microvillus membrane protein and specifically altered the fatty acid profile in the phosphatidylcholine fraction (Max et al., 1978). Although it is recognized that the present study describes the action of vitamin D_3 in a model system, the findings do provide additional evidence consistent with the hypothesis that vitamin D_3 and metabolites modify membrane structure and function by "liponomic regulation" (Fontaine et al., 1981).

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Received 18 May 1982