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HYDROGEN BONDING OF VITAMIN D AND EGG LECITHIN IN THE SOLID STATE

A POSSIBLE MOLECULAR ROLE FOR VITAMIN D IN CALCIUM BINDING BY MEMBRANES

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SUMMARY

Attenuated total reflectance infrared spectra of films of vitamin D– egg lecithin mixtures show low frequency shifts for the vitamin –OH stretching and the $[PO_2]^-$ stretching absorption of the phospholipid. These shifts maximize at a 2 sterol:1 phospholipid ratio and appear to represent formation of a hydrogen-bonded complex of lecithin with vitamin D. Films of vitamin D trap nonpolar solvents, *e.g.*, carbon tetrachloride, but this phenomenon is not seen in phospholipid–vitamin D mixtures. The results are interpreted in terms of a possible interaction of vitamin D with phospholipids in cell membranes and the potential effects of such an interaction on calcium binding by membrane systems.

INTRODUCTION

Studies of sterol interactions with cell membranes have centered on the postulated interaction of cholesterol with phospholipids[1,2]. There is no direct evidence for such an interaction in cell membranes. However, the fact that cholesterol is a major constituent of many membranes has prompted studies of cholesterol-containing monolayers[3,4] and other model membrane systems[5]. These investigations have given rise to extensive speculation on the probable structure of cholesterol–phospholipid complexes in cellular membranes.

Since vitamin D is a sterol structurally related to cholesterol, it occurred to us that vitamin D phospholipid complexes might form in a manner similar to that proposed for cholesterol. Furthermore, the recent observation by FRASER AND KODICEK[6] that vitamin D can compete with cholesterol for sites on the cholesterol-esterifying enzyme shows that these structural similarities are reflected in biological systems which are fairly specific.

Since we have already shown apparent hydrogen bonding of cholesterol to lecithin by infrared techniques[7], we decided to investigate the possibility of lecithin–vitamin D interactions in the hope of obtaining some new insight into the role of these two lipids in membrane processes such as calcium transport. Data presented in this

paper suggest that vitamin D and lecithin do in fact interact by hydrogen bonding of the sterol hydroxyl to the nonesterified phosphate oxygens of lecithin. The possible importance of this interaction to calcium transport and the action of vitamin D is discussed.

MATERIALS AND METHODS

The techniques and materials used in this work were identical to those reported previously[7]. Attenuated total reflectance spectroscopy of lipid mixture films was employed, using a Wilks model 12 attenuated total reflectance attachment with the Perkin–Elmer 521 infrared spectrophotometer. The sample chamber was continuously flushed with dry air while spectra were being run.

"Mixture films" consist of lipid mixtures dried on the attenuated total reflectance crystal, while "addition films" consist of lipids dried separately on an attenuated total reflectance crystal. Thus, in the latter case, addition spectra are obtained (see ref. 7).

Synthetic dipalmitoyl lecithin was purchased from Mann Research Labs.

Crystalline vitamin D-3 was obtained from Nutritional Biochemical.

RESULTS

The attenuated total reflectance spectrum of vitamin D in the –OH stretching region is shown in Fig. 1. This spectrum may be compared to that obtained earlier with cholesterol[7]. The hydroxyl group participates in intermolecular hydrogen bonding in the solid film. The –OH stretching absorption maximized at about 3350 cm^{-1}, some 50–60 cm^{-1} below the –OH stretching frequency for cholesterol films. Thus, intermolecular association appears to be somewhat stronger for vitamin D than for cholesterol. Also, the –OH band is more symmetrical for the vitamin than for cholesterol.

Fig. 2 indicates that films of mixtures of vitamin D and egg lecithin show increased hydrogen bonding of the –OH group in the vitamin. These data represent 1:1 molar mixtures, and the –OH stretching frequency of the mixture is shifted downfield by approximately 100 cm^{-1}, compared to that of the addition spectrum. The shoulder at 3360–3380 cm^{-1} seen in the mixture spectrum represents bound water in the lecithin[7]. The –OH stretching absorption maximum of the vitamin–phospholipid mixture is very nearly the same as that for cholesterol and lecithin, and in this respect, the interaction appears to be similar for the two sterols.

However, in contrast to the cholesterol–lecithin spectra, several other perturbations of the mixture spectra are obtained with vitamin D. These differences are shown in Fig. 3. For identification of these lecithin bands which are shifted in the presence of vitamin D, attenuated total reflectance spectra of L-α-glycerophosphoryl choline, lysolecithin and lecithin were obtained. These spectra are presented in Table I. From these data and those reported in the literature[8–11,29,30] the band assignments shown in Table II were made. From Fig. 3, then, it is seen that the antisymmetric $[PO_2]^-$ stretching frequency of lecithin (Table II) at 1250 cm^{-1} is shifted downfield by about 20 cm^{-1}. Furthermore, the symmetric $[PO_2]^-$ stretching frequency at 1080 cm^{-1} seen as a separate peak in the addition spectrum now appears as a shoulder

distinctly decreased in intensity relative to the adjacent downfield band (C–O stretching). This obvious downfield shift in the $[PO_2]^-$ stretching absorptions is expected from hydrogen bonding[8,29,30].

TABLE I

INFRARED BANDS (cm^{-1}) IN LECITHIN AND LECITHIN DERIVATIVES

*Lecithin**	L-α-*Glycerophosphoryl choline***	*Lysolecithin****
3375 (H_2O)		
	3300–3100 (weak, broad)	
		3180–3250
3110 (weak)		
2928 (strong)	2928 (very weak)	2928 (strong)
2860 (strong)	2860 (very weak	2860 (strong)
1730 (strong)		1730 (strong)
1475 (shoulder)		1475 (shoulder)
1460 (strong)		1460 (strong)
1410 (weak)		1410 (weak)
1370 (weak)	1340 (weak)	1370 (weak)
1250 (strong)	1195 (strong)	1235 (strong)
1165 (strong)		1170 (medium)
1140 (shoulder)		1140 (weak)
1083 (strong)	1080 (strong)	1083 (strong)
1050 (strong)	1050 (strong)	1050 (strong)
960 (strong)	970 (strong)	960 (strong)
820 (medium)	823 (medium)	820 (medium)

* Solution in carbon tetrachloride dried on KRS-5 crystal.
** $CdCl_2$ salt pressed on KRS-5 crystal.
*** Solution in 2 parts chloroform, 1 part methanol dried on KRS-5 crystal.

The shift in the antisymmetric $[PO_2]^-$ band at 1250 cm^{-1} with increasing vitamin D concentration is easily followed. This shift appears to maximize when the vitamin D:lecithin ratio is somewhere between 2 and 3 to 1, as shown in Fig. 4. On occasion this $[PO_2]^-$ stretching frequency was observed to shift as low as 1225 cm^{-1} with ratios of 2 as well as 3 to 1. These data appear to indicate that more than two vitamin molecules can at least partially interact with lecithin, but it is clear that the large shift occurs between a ratio of 1 and 2 to 1, with minimal further interaction after this stoichiometry is attained. Similar results were obtained for the shift of the –OH stretching frequency (Fig. 4), and while the results are not nearly as definitive as those obtained with cholesterol[7], again a stoichiometry of two vitamin D molecules to one lecithin appears to give maximal interaction.

Fig. 5 shows a third area of the attenuated total reflectance spectrum which is altered by the interaction of lecithin with vitamin D. The intense peaks at 785 cm^{-1} and 760 cm^{-1} in the addition spectra are completely gone in the mixture spectra. However, these peaks do not represent absorption due to the phospholipid or sterol, but represent rather the C–Cl stretching from the solvent, carbon tetrachloride.

It was discovered from this observation that films of vitamin D trap solvent, but that films of egg lecithin do not. Fig. 6 shows that the vitamin D-trapped solvent slowly escapes, and that upon exposure to additional solvent, the 785–760 cm^{-1}

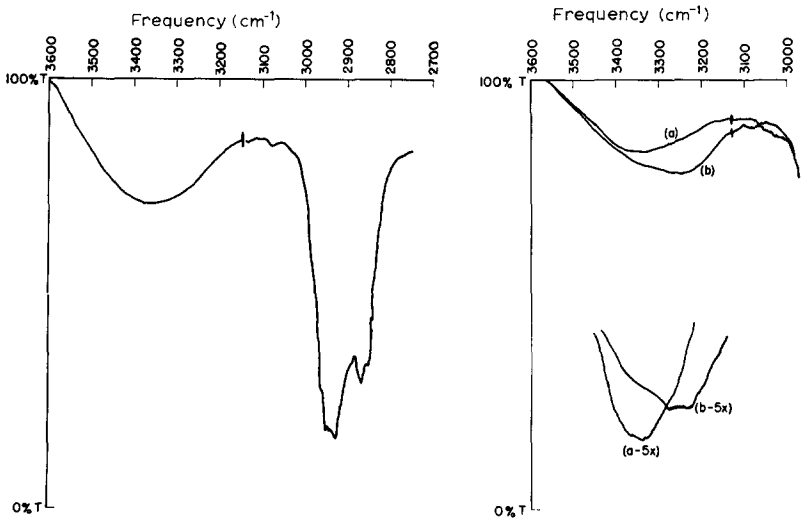

Fig. 1. Attenuated total reflectance infrared spectrum of vitamin D_3 in the –OH, C–H stretching region. 0.2 ml of a carbon tetrachloride solution of vitamin D (4 o mg/ml) was dried on a KRS-5 attenuated total reflectance crystal (Wilks).

Fig 2. Interaction of vitamin D_3 with egg lecithin evidenced by low-frequency shift of –OH stretching in the mixture spectrum (b). Mixture spectrum obtained from 1 1 molar mixture of vitamin D and lecithin in carbon tetrachloride. Addition spectrum (a) obtained as described in[7]. The 5 × spectra represent 5-fold amplification.

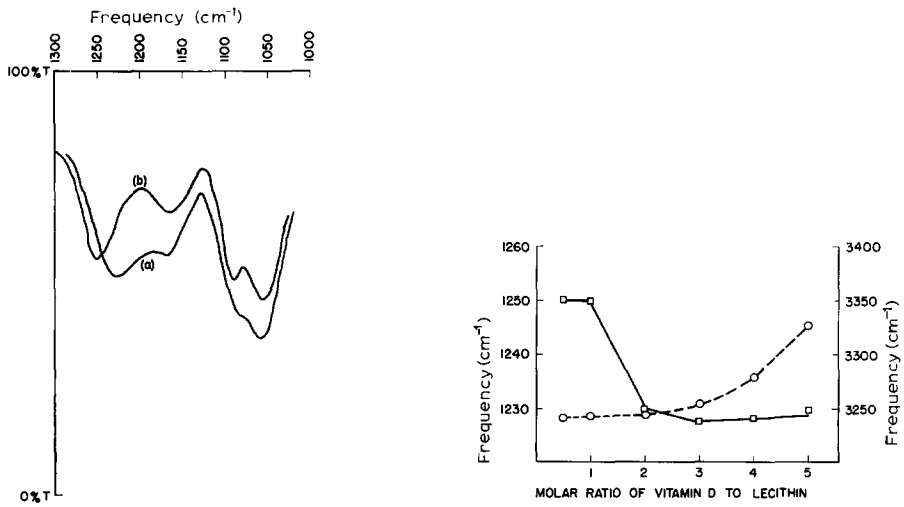

Fig. 3. Low-frequency shift in $[PO_2]^-$ stretching frequencies of lecithin in mixtures with vitamin D a. Mixture spectrum. b Addition spectrum. Spectra were obtained as described before. Mixture spectrum (a) is of a 2·1 (vitamin D: lecithin) molar mixture.

Fig. 4. Stoichiometry of vitamin D–lecithin interaction. Frequency of maximal absorption is plotted *versus* ratio of the two lipids. O----O, –OH stretching; □—□, $[PO_2]^-$ stretching (1250–1225 cm^{-1}).

doublet reappears. Thus, Fig. 5 shows that lecithin interaction with vitamin D eliminates this solvent trapping capability.

Table III presents data on the effects of polar lipids other than lecithin on the sterol –OH stretching frequency and on the solvent trapping. The –OH frequency

TABLE II

LECITHIN GROUP FREQUENCY ASSIGNMENTS

Group	*Mode*	$\bar{\nu}$ (cm^{-1})	*Ref.*
–O–H (water)	Stretch	3375	
–C–H	Stretch	3010	11, 8
H–C(–)–H	Symmetric stretch	2928	11, 8
H–C(–)–H	Antisymmetric stretch	2860	11, 8
C(–)=O	Stretch	1730	11, 8
H–C(–)–H	Bend	1370	11, 8
$[PO_2]^-$	Antisymmetric stretch	1250	10, 11
C–O–C	Stretch	1165	Table I
$[PO_2]^-$	Symmetric stretch	1080	10
[C–O]P	Stretch	1050	10
N–[C–C]O	Stretch	970	10
[P–O]C	Stretch	820	10

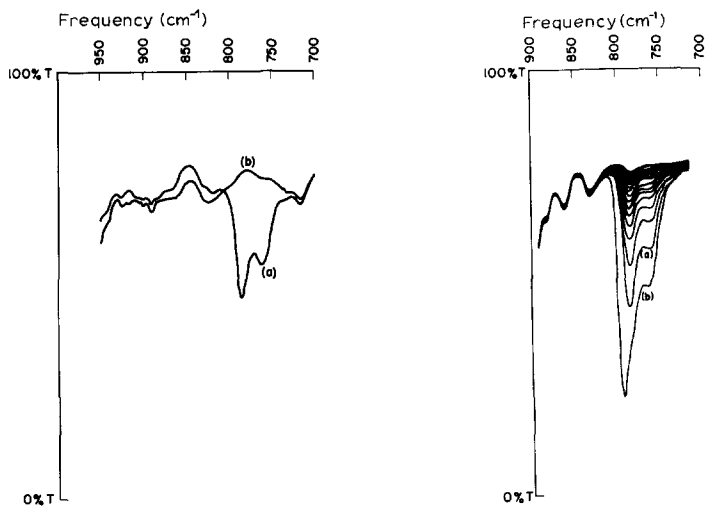

Fig 5. Effect of lecithin on solvent trapping by vitamin D. The 785-cm^{-1} and 769-cm^{-1} absorptions are due to C–Cl stretching in carbon tetrachloride (ref. 8 and Fig. 6). a. Addition spectrum. b. Mixture spectrum.

Fig. 6. Release of solvent trapped by vitamin D with time (a) and reappearance of 785–760 doublet upon addition of solvent (b). Unlabeled spectra are the series starting with (a). The spectra were recycled every 5 min as shown. For (b), one drop of carbon tetrachloride was added to the lipid film and the surface was flushed with N_2 before running the spectrum.

results are identical to those obtained with cholesterol[7]. Furthermore, with the exception of dipalmitoyl lecithin, the ability of the vitamin to trap solvent can be seen to correlate with interaction of the lipids with the vitamin D hydroxyl group. Except for the saturated lecithin, lipids which produce an –OH shift eliminate the solvent trapping effect while those which do not alter the –OH stretching have no effect on solvent trapping.

TABLE III

VITAMIN D –OH STRETCHING FREQUENCY IN LIPID MIXTURES

Lipid	*–OH maximum* (cm^{-1})	*Solvent trapped*
Egg lecithin	3260–3240	No
Lysolecithin	3260–3230	No
Cetyl trimethyl ammonium bromide	3310	No
Cetyl trimethyl ammonium chloride	3280	No
Tristearin	3350	Yes
Dicetyl phosphate	3350	Yes
Dipalmitoyl lecithin (synthetic)	3250	Yes

In the course of these studies we observed that while egg lecithin trapped no solvent, synthetic dipalmitoyl lecithin was even more effective than vitamin D at retarding solvent loss. Thus, this capability to trap solvent molecules is related to the unsaturation in the hydrocarbon side chains of the phospholipids. Furthermore, there is some structural difference between vitamin D and cholesterol which enables the vitamin to trap solvent. These results are helpful in interpretation of the molecular organization of the lipid molecules in the solid film.

DISCUSSION

The concomitant shifts of the –OH and $[PO_2]^-$ stretching frequencies suggest direct interaction between the two groups. This interaction is apparently hydrogen bonding of the vitamin to the non-esterified phosphate oxygens in lecithin. Maximum interaction appears to occur at a 2 sterol: 1 phospholipid ratio, as with cholesterol. This stoichiometry suggests that each vitamin D molecule hydrogen bonds with one of the phosphate oxygens in lecithin. The data in Table I are essentially identical to those obtained with cholesterol, and the conclusions concerning possible interaction with the choline nitrogen are therefore the same[7].

The magnitude of the –OH shift for vitamin D is no greater than that for cholesterol, even though the free phosphate oxygens are involved in the former and not in the latter. Since the net negative charge on the free phosphate oxygens would be expected to be greater than that on the phosphate ester oxygens, this result suggests that the O–H···O bond distance in the vitamin D system is somewhat greater than that in the cholesterol system. Although data of this sort are difficult to quantitate, one can estimate that the O–H···O bond distance must be of the order of 2.65–2.80 Å for both systems[12], with the vitamin–lecithin system approximating the higher value, and the cholesterol–lecithin nearer to the lower value.

The ability of lipids to trap solvent appears to be due to the molecular architecture in the hydrocarbon portion of the lipids, as evidenced by the difference between saturated and the unsaturated lecithin. This property of the system is most easily interpreted if one postulates that the lipids are organized in spherical or quasi-spherical micelles in solution, as illustrated in Fig. 7. This suggestion is not new, and molecular-weight determinations for lecithin are known to give values suggesting extensive

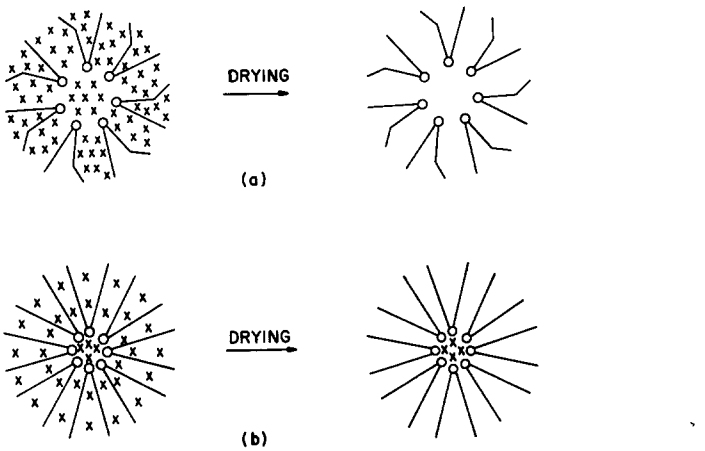

Fig 7 Diagrammatic illustration of solvent molecules (x) trapped by saturated lecithin (b) and failure of egg (unsaturated) lecithin to trap solvent. The unsaturation in egg lecithin (a) produces "kinks" in the side chains and this reduces close packing of the micelles, forming a more porous ionic barrier than that formed by the saturated lecithin.

association even at high dilution[13,14]. Our suggestion is that during solvent removal and film formation on the attenuated total reflectance crystal, this micellar structure is retained. Solvent molecules may then be encapsulated within the micelles because of their inability to penetrate the spherical ionic barrier formed in the micelle. This is a process analogous to the well-known retardation of evaporation of water by a lipid monolayer. Such encapsulation would be expected to be very effective if the distance between the polar portions of adjacent molecules are on the same order of magnitude as the molecular diameter of the solvent molecules. Forces tending to separate the polar ends of the lipids would reduce the solvent trapping. It is well known from monolayer studies that unsaturation in the side chain of phospholipids considerably reduces the close packing of the molecules[15–17]. Egg lecithin contains significant amounts of unsaturated hydrocarbon chain. Furthermore, surface potential studies show that unsaturated lecithin does not interact as strongly with divalent cations as does the saturated lecithin[4]. The interpretation in Fig. 7 shows diagramatically how these facts may explain the solvent-trapping capabilities of the two lecithins studied here.

It is thus suggested that the molecules essentially retain the micellar organization as they are dried on the attenuated total reflectance crystal. If this is correct, it implies that in the dried film the micelles pack together "back to back" with the hydrocarbon chains of one micelle approaching and packing together with those of another. The net molecular organization achieved by this process would resemble the postulated organization for lipids in cell membranes, that of the lipid bilayer. Therefore, the observation of solvent trapping in the dried film of lipids gives us some infor-

mation about the organization of the molecules in the film and reinforces the belief that their organization in some respects resembles that of cellular membranes.

For rationalization of the solvent-trapping capability of vitamin D relative to that of cholesterol, we may turn to molecular models. Fig. 8 shows space-filling models of vitamin D and cholesterol. The most predominant feature which distinguishes the two is the decrease in bulk in the vitamin molecule at the polar end. In fact, the vitamin may be considered to be a slightly bent "wedge" with the hydroxyl group at the point of the wedge.

Fig 8 CPK (Ealing) space-filling models of vitamin D (right) and cholesterol (left) showing the greater bulk of cholesterol at the polar end of the molecule. The bend in the vitamin D molecules arises because of the extension of the conjugated π system equatorial to the chair form of ring C.

Although it is not known whether vitamin D or cholesterol form micelles in organic solvents, the fact that the vitamin traps solvent in dried films suggests that this may be the case. One would expect that a wedge-shaped molecule would be capable of forming a tightly packed quasi-spherical micelle, with the polar groups inward and in close proximity to one another. On the other hand, the more bulky polar end of cholesterol will prevent close approach of the hydroxyls to one another. Thus, the vitamin may form a micelle with an effective polar barrier to solvent loss. Since the –OH stretching absorption of the vitamin maximizes some 50–60 cm^{-1} below that of cholesterol (Fig. 1), the data appear to support the above idea. The loss of the solvent-trapping capability in mixtures of egg lecithin and vitamin D thus suggests that the vitamin D micelles do not form, which is expected if the phospholipid and sterol are aligned "side by side" in a mixed micelle as suggested earlier[7].

The postulated arrangement of the vitamin and phospholipid is shown diagrammatically in Fig. 9. The vitamin D molecules aligned parallel to the lecithin hydrocarbon chains pack in such a way as to insert the hydroxyl groups into an area no more than 2.8 Å from the phosphate oxygens. This arrangement has the capacity for at least two sterols per lecithin.

Why vitamin D interacts with the free phosphate oxygens, while cholesterol does not, may be presumed to be the result of the molecular dimensions and shape of the sterols. Perhaps because of the greater bulk of the polar end of cholesterol, this

sterol simply cannot fit into the space available in the matrix area of the free phosphate oxygens.

In any case, it is clear that vitamin D can and does influence the $[PO_2]^-$ group of lecithin when the molecules are aligned as they might be in a cellular membrane. The physiological significance, if any, of this fact is not known. However, the apparent similarity of the experimental system to postulated cell membrane structure prompts some speculation. It should first be noted that it is unlikely, although not impossible, that the present system reflects a molecular model for the so-called "physiological" effect of vitamin D in curing rickets and maintaining normal blood calcium levels.

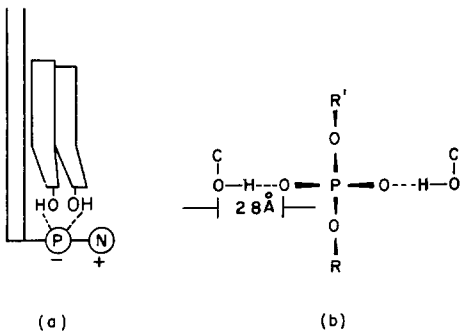

Fig. 9. Proposed arrangement of lecithin–vitamin D complex. a. A 2:1 complex, showing hydrogen bonding of sterol to the lecithin phosphate. b "End-on" view of the phosphate oxygen–hydroxyl interaction

NORMAN AND DE LUCA[27] discovered, and BLUNT *et al.*[26] identified, a new chemical form of vitamin D, 25-hydroxycalciferol, which is faster acting than the parent vitamin[18], and which apparently represents an active metabolite. Further, a general interaction of vitamin D with lecithin in cell membranes is too gross a system to provide a satisfactory rationale for the response of animals to doses of less than a μg of vitamin D.

However, the present system may have relevance to the well-known toxic effects of pharmacologic doses of vitamin D[19,20]. It has been suggested frequently that phospholipids bind calcium and other divalent cations at the surface of cell membranes. It is believed that while normal serum calcium levels are of the order of 2.5 mM, intracellular free calcium concentrations are much lower than this. This appears to be the result of the fact that cellular membranes as well as proteins and other macromolecular constituents extensively complex intracellular calcium[21,22]. The interaction of vitamin D with phospholipids in the manner suggested in Fig. 9 would have the effect of displacing ions bound to phospholipids by partial neutralization of the negative oxygen through the –OH dipole. The net result in a cell membrane would be reduced binding of divalent cations such as calcium. In the presence of excess vitamin D, such an effect could produce a significant alternation in calcium binding by the cell membranes. It has been known for some time that large amounts of vitamin D do alter calcium binding by subcellular organelles. For example, mitochondria treated with the sterol are caused to release bound calcium sooner than the control mitochondria[23]. In addition, vitamin D-treated microsomes bind less calcium than untreated microsomes[24]. Further, in addition to the metabolite of vitamin D, large amounts of unmetabolized vitamin are always found in the cellular membranes when

rats are given large doses of the sterol[28]. This is evidence that the presence of vitamin D at concentrations greatly in excess of those required for "normal" action can reduce the binding of calcium by membranous systems. ZULL *et al.*[25] concluded earlier that these effects of the vitamin were nonphysiological and at best represented a model system for the primary action of the vitamin. Whether reduced uptake of calcium by cellular membranes *in vivo* brought about by pharmacological levels of vitamin D is related to the toxic action of the vitamin can only be speculated upon at the present. In any case, it is clear that the results presented here do suggest a mechanism whereby vitamin D may alter calcium binding by membrane systems.

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REFERENCES

1 J. B. FINEAN, in J. A V BUTLER AND H. E HUXLEY, *Progress in Biophysics and Molecular Biology*, Vol. 16, Pergamon Press, New York, 1966.
2 F A. VANDENHEUVEL, *J. Am Oil Chemists Soc.*, 40 (1963) 455.
3 L DEBERNARD, *Bull. Soc. Chim. Biol*, 40 (1958) 161
4 D. O SHAH AND J. H SCHULMAN, *J. Lipid Res.*, 8 (1967) 215.
5 D. CHAPMAN AND S. A. PENKETT, *Nature*, 211 (1966) 1304.
6 D. R. FRASER AND E. KODICEK, *Biochem. J.*, 109 (1968) 457.
7 J. E ZULL, S. GREANOFF AND H. K ADAM, *Biochemistry*, 7 (1968) 4172.
8 L. J. BELLAMY, *The Infrared Spectra of Complex Molecules*, Wiley, New York, 1959, p 13.
9 T. SHIMANOUCHI, M TSUBOI AND Y. KYOGOKN, in J. DUCHESNE, *Structure and Properties of Biomolecules and Biological Systems*, Interscience, New York, 1963, p 34.
10 M. J. TSUBOI, *Polymer Sci , Part C, Polymer Symp. Conf. on Vibrational Spectra of High Polymers*, Interscience, New York, 1964, p. 125.
11 D. CHAPMAN, *The Structure of Lipids*, Wiley, New York, 1965, p. 110.
12 G C. PIMENTEL AND A L. MCCLELLAN, *The Hydrogen Bond*, Reinhold, New York, 1960, p. 87
13 P. H. ELWORTHY, *J Chem Soc.*, 813 (1959) 1951.
14 D. G DERVICHIAN, *Progr. Biophys Mol Biol.*, 14 (1964) 263.
15 L L M. VAN DEENEN, U. M T. HOUTSMULLER, G. H DE HAAS AND E MULDER, *J. Pharm Pharmacol*, 14 (1962) 429
16 D. G. DERVICHIAN AND L DEBERNARD, *Bull. Soc. Chim Biol.*, 37 (1955) 943.
17 R. A DEMEL, L L M. VAN DEENEN AND B. A. PETHICA, *Biochim. Biophys. Acta*, 135 (1967) 11.
18 J. LUND AND H F. DELUCA, *J. Lipid Res.*, 7 (1966) 739
19 C J. HARRIS AND J. R. M INNES, *Biochem. J*, 25 (1931) 367.
20 R. NICHOLAYSEN AND N EEG-LARSEN, *Vitamins and Hormones*, Vol 11, Academic Press, New York, 1963, p. 29.
21 W. HOHMAN AND H. SCHRAER, *Federation Proc*, 24 (1965) 709
22 S WALLACH, D L REIZENSTEIN AND J. V BELLAVIA, *J Gen Physiol.*, 49 (1966) 743.
23 G. ENGSTROM AND H F. DELUCA, *Biochemistry*, 3 (1964) 203
24 A W. SNELLGROVE AND H. F. DELUCA, *Arch. Biochem Biophys.*, 111 (1965) 23
25 J. E. ZULL, E. CZARNOWSKA-MISZTAL AND H. F. DELUCA, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 177
26 J W. BLUNT, H. F. DELUCA AND J. K SCHNOES, *Biochemistry*, 7 (1968) 3317.
27 H. W NORMAN AND H F DELUCA, *Arch Biochem. Biophys*, 107 (1964) 69.
28 A W NORMAN AND H. F. DELUCA, *Biochemistry*, 2 (1963) 1160
29 L. J BELLAMY, *The Infrared Spectra of Complex Molecules*, Wiley, New York, 1959, p 178
30 L J BELLAMY, *The Infrared Spectra of Complex Molecules*, Wiley, New York, 1959, p 311.