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STUDIES ON THE MECHANISM OF ACTION OF CALCIFEROL

IV. INTERACTION OF THE POLYENE ANTIBIOTIC, FILIPIN, WITH INTESTINAL MUCOSAL MEMBRANES FROM VITAMIN D-TREATED AND VITAMIN D-DEFICIENT CHICKS

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SUMMARY

1. The polyene antibiotic, filipin, has been shown to be a specific tool with which to probe the biochemical function of vitamin D (calciferol)-stimulated calcium translocation, *in vitro*, in the chick intestinal ileum. Filipin treatment, *in vitro*, (concentration about $2 \cdot 10^{-5}$ M) of ileal segments obtained from vitamin D-deficient chicks increased the calcium flux, J_{ms} (flux in the mucosal-to-serosal direction), by 150–250 %. Filipin treatment, *in vitro*, of ileal segments from vitamin D-treated chicks had no effect on calcium flux, J_{ms}. The effect of filipin on calcium was shown to exist only when the antibiotic was administered to the mucosal surface of an ileum. The filipin effect was specific for calcium translocation; little or no effect of filipin was noted on the flux, J_{ms}, of phosphate, sulfate, serine or rubidium. Also the magnitude of the filipin-mediated increase in calcium flux was inversely proportional to the time after vitamin D administration, *in vivo*, to rachitic chicks, so that 60 h after vitamin D there was no filipin effect.

2. Studies carried out on thiourea and water flux, J_{ms}, in response to filipin treatment showed that the increase in specific calcium transport in ileal segments from vitamin D-deficient chicks is not mediated by a general increase in permeability.

3. Filipin was shown to bind to a greater extent and at a faster rate to ileal segments obtained from rachitic chicks than those from vitamin D-treated chicks.

4. Electron micrographic studies showed that after prolonged exposure (80 min) to filipin on the mucosal side, *in vitro*, gross morphological changes could be detected in the microvillar region of the ileal segments obtained from vitamin D-deficient chicks but not in the segments obtained from vitamin D-treated chicks. There were no major morphological changes apparent in either system when ileal segments were exposed to filipin, *in vitro*, for periods equivalent to those used in studying the transport activities (20–40 min).

5. Examination of the time-course of events noted after administration of filipin, *in vitro*, to ileal segments from vitamin D-deficient and vitamin D-treated chicks, supports the hypothesis that a fundamental difference exists in some structural aspect of the intestinal microvillar membranes of these two systems.

INTRODUCTION

The use of polyene antibiotics (filipin, nystatin and amphotericin B) as a tool for studies of natural and artificial membranes has been well documented[1-8]. It has been shown that polyene antibiotics cause a change in permeability in fungi promoting a leakage of important cellular constituents[1] and that none of the antibiotics had any effect on bacterial protoplasts[3]. Polyene antibiotics have also been used in studies of the hemolysis of erythrocytes[5], and have been shown to cause the formation of pits in lecithin–cholesterol dispersions and erythrocyte membranes from rats and humans[6].

Polyene antibiotics such as amphotericin B have been utilized as specific tools in the study of various transport systems. SHARP *et al.*[9] used amphotericin B to study the role of aldosterone in sodium translocation in the toad bladder. LIPPE AND GIORDANA[10] also utilized this antibiotic to study thiourea flux in the small intestine of the turtle. We have reported earlier that the polyene antibiotic, filipin, can be utilized as a specific tool to study the vitamin D-stimulated calcium transport system in the chick intestine, *in vitro*[11].

Evidence presented in this paper substantiates the observations that the effect of the polyene antibiotic, filipin, *in vitro*, shows a remarkable similarity to the response elicited by vitamin D administration, *in vivo*, on the calcium transport system of the vitamin D-deficient chick intestine.

METHODS

Chickens

White leghorn cockerels (H & N of California, Inc.) were used in all experiments. 1-day-old chicks were raised for 3 weeks on a vitamin D-deficient diet whose composition is described elsewhere[12]. All chicks were utilized during the fourth week at which time they had become rachitic (—D), showed a leveled growth rate (100–110 g), and had calcium levels of 6.3 mg/100 ml and bone ash percentages of 28 %. Vitamin D-treated chicks (+D) were prepared by administration of 500–2500 I.U.* (32.5–162 nmoles) of vitamin D_3 dissolved in 0.20 ml of 1,2-propanediol to rachitic chicks at varying time periods before sacrifice. After sacrifice, the chick's intestine was immediately excised, the intestinal contents expressed and the tissue placed in cold 0.15 M NaCl until it was utilized in the appropriate experiment.

Chemicals and radioisotopes

A modified Krebs–Ringer bicarbonate solution was utilized in all transport experiments and tissue incubations. The solution was 0.123 M NaCl, 0.026 $NaHCO_3$, 0.005 M KCl and 0.02 M glucose. The buffer contained no phosphate except as indicated in specific experiments. The pH was adjusted to pH 7.4 after saturation with O_2–CO_2 (95:5, by vol.).

Various radioisotopes were utilized in the transport experiments. The radioisotopes were $^{45}Ca^{2+}$ (11.1 mC/mg), [^{14}C]thiourea (2.69 mC/mmole), $^{35}SO_4^{2-}$ (carrier

* One international unit, I.U., or 0.025 μg is equivalent to 0.063 nmole of ergocalciferol (vitamin D_2) or 0.065 nmole of cholecalciferol (vitamin D_3). Ergocalciferol and cholecalciferol have an equal biological potency in the rat; in the chick cholecalciferol is approx. 10 times more active than ergocalciferol. The minimum daily physiological requirement for cholecalciferol in the chick is 5–10 I.U. (ref. 12).

free), $^{90}Sr^{2+}$ (carrier free), $^{86}Rb^{+}$ (0.65 mC/mg), and $^{3}H_2O$ (25 mC/ml) from New England Nuclear; $^{32}P_i$ (carrier free) from International Chemical and Nuclear Corporation; and generally labeled DL-[^{3}H]serine (1.4 mC/mmole) from Amersham-Searle Company.

Filipin (batch No. 8393-DEG-11-8, 96 % pure) was a generous gift of Dr. G. Whitfield of the Upjohn Company, Kalamazoo, Mich. All other chemicals employed were reagent grade.

Transport methods

For transport studies, a cylindrical ileal segment, 12 cm in length, was mounted on the large-scale glass apparatus as described by FORTE *et al.*[15] or ileal tissue was mounted on the modified[15] Ussing short circuit type apparatus[16] exactly as described elsewhere[14]. The surface area of the tissue orifice through which the transport occurred, was 0.60 cm^2 in the Ussing short circuit type apparatus. The polyene antibiotic, filipin, was added at the desired concentration* in 0.10–0.20 ml of dimethyl sulfoxide or ethanol. Equivalent amounts of only solvent were always added to control incubations.

Radioactivity and flux determination

Radioactive samples were determined by planchet counting or liquid scintillation counting as described previously[14]. The rate of transport or flux, J_{ms} (flux in the mucosal-to-serosal direction), was determined by employing a linear regression analysis[17] on the rate of appearance of isotope as previously described[14]. The rate of transport or flux, (J_{ms}), was normally expressed as nmoles/h per segment.

Filipin binding to ileal segments of intestine

Binding of filipin to an everted segment of ileum from vitamin D-deficient and vitamin D-treated chicks was performed exactly as described by ADAMS *et al.*[11]. Filipin absorbance was monitored at 356 nm in a Beckman-DB spectrophotometer and the amount of filipin bound was determined from the difference between initial and final absorbance.

Electron microscopy

Ileal segments were mounted in the Ussing short circuit apparatus and incubated in the Krebs–Ringer buffer as described for the transport experiments. Filipin was added (final concentration 10 μg/ml) to the mucosal surface and the incubation was allowed to run the prescribed time. Filipin-treated segments were then removed from the transport apparatus and fixed for 1 h at 4° in 2.5 % phosphate buffered (pH 7.0) glutaraldehyde and post-fixed in 1 % phosphate buffered (pH 7.0) osmium tetraoxide. The fixed samples were imbedded in maraglas after dehydration in acetone. Thin sections were cut on a Porter-Blum MT 2 ultra-microtome and picked up on uncoated 400-mesh copper grids. All sections were stained on the grids with uranyl acetate followed by lead citrate[18]. The sections were studied with a Philips 300 electron microscope.

* Filipin concentrations are not normally reported in moles/l since the batch of filipin utilized was not of a homogenous molecular weight. However, 10 μg/ml of filipin is about $17 \cdot 10^{-6}$ M.

RESULTS

Effect of filipin on calcium transport in chick ileums, in vitro

Studies were made *in vitro* on the effects of filipin on calcium flux, J_{ms}, in both the vitamin D-treated and vitamin D-deficient ileums. Table I shows the results of the effect of filipin (10 μg/ml) on *in vitro* calcium flux as measured in the glass apparatus. In both experiments filipin mediated a marked increase in the calcium flux when added to the mucosal surface of the deficient chick ileum. Under the same conditions ileal segments from vitamin D-treated chicks show no appreciable response to filipin treatment. In experiments previously reported[11], we have established that a maximal response to filipin is obtained over the range of 5–10 μg/ml, and that no further response is obtainable at higher concentrations. No effects on calcium flux have been recorded when the antibiotic was administered to the solution bathing only the serosal surface.

TABLE I

EFFECT OF FILIPIN ON CALCIUM FLUX *in vitro*

The experiments were performed, *in vitro*, in the glass apparatus with an initial calcium concentration of 0.5 mM. Flux determinations were made with 40 μC of $^{45}Ca^{2+}$ added to the mucosal side. Filipin dissolved in 0.10 ml of ethanol was added to a final concentration of 10 μg/ml on the mucosal side. Chicks termed + D received 3 oral doses of 2500 I.U. (162 nmoles) of vitamin D_3 72, 48 and 24 h before sacrifice.

Vitamin D status	*Expt. 1*			*Expt. 2*		
	Calcium flux (J_{ms}) (nmoles/h)		*Increase (%)*	*Calcium flux (J_{ms}) (nmoles/h)*		*Increase (%)*
	− Filipin	*+ Filipin*		*− Filipin*	*+ Filipin*	
− D	202 ± 42 (5)	367 ± 80 (5)	82	100 ± 7 (3)	227 ± 40 (8)	127
+ D	459 ± 58 (15)	473 ± 27 (4)	3	—	—	—

Specificity of the filipin effect for calcium translocation

The effect of filipin on the transport activities of several other ions and compounds was monitored. The results are presented in Table II. Serine, SO_4^{2-}, Rb^+, P_i, thiourea and water show only a slight increase in flux rate due to filipin administration. P_i flux in the presence of calcium showed the same degree of increase as the increase in calcium flux. This may indicate that P_i is transferred as a counter-ion to calcium. Strontium, a cation closely related to calcium, showed a marked flux increase due to filipin treatment. The high specificity of filipin treatment on transport activities in the deficient chick ileum supports the contention that filipin is a specific tool with which to probe the vitamin D-mediated calcium translocation system.

Effect of filipin and vitamin D on calcium translocation

The effect of filipin on calcium flux (J_{ms}) was shown to be abolished after oral administration of vitamin D_3 to a rachitic chick (Table I). Accordingly a study of the length of time required after vitamin D administration to abolish the *in vitro* filipin effect on $^{45}Ca^{2+}$ flux is presented in Table III. It can be seen that there is a gradual decrease in the filipin effect on calcium flux with time, and that at 40 h after vitamin D treatment, the filipin effect is only 30 % of the effect observed in the vitamin D-

TABLE II

THE EFFECT OF FILIPIN ON THE ILEAL FLUX, *in vitro*, OF VARIOUS IONS AND COMPOUNDS IN THE VITAMIN D-DEFICIENT CHICK INTESTINE

Experiments were performed, *in vitro*, in the Ussing type apparatus unless otherwise indicated. The initial concentration of all ions and compounds tested was 0.10 mM except Rb^+ (0.2 mM) and water. The initial flux determination was made for each isotope for a 20-min period following the initial preincubation period as described by ADAMS AND NORMAN[14]. Filipin, in 0.10 ml ethanol was then added to the mucosal compartment to a final concentration of 10 μg/ml. After a 20-min lag the flux determination was made in the presence of filipin. The filipin effect was then measured as the ratio of the flux rate due to filipin over the initial flux rate.

Substance transported	*Number of determinations*	*Initial flux (J_{ms}) (nmoles/h per segment)*	*Ratio of flux rates (J_{ms}) (+filipin/−filipin)*
Ca^{2+}	4	1.02	2.91 ± 0.65
P_i (in the presence of 0.1 mM Ca^{2+}	3	0.32	2.50 ± 0.59
Sr^{2+}	6	32.8 ± 3.8 *	2.00 ± 0.0
P_i	3	1.41	1.53 ± 0.34
Serine	8	—	1.40 ± 0.28
SO_4^{2-}	4	—	1.19 ± 0.30
Rb^+	6	80.7 ± 22 *	1.17 ± 0.0
Thiourea	4	83.0 ± 14	1.13 ± 0.07
Water	4	180 ± 40	1.08 ± 0.04

* Sr^{2+} and Rb^+ were run separately in the glass apparatus.

TABLE III

THE DECREASE IN FILIPIN RESPONSE *in vitro* AS A FUNCTION OF THE LENGTH OF DIETARY VITAMIN D TREATMENT

The effect of filipin on ileal calcium flux, J_{ms}, *in vitro*, was measured at varying times after vitamin D_3 administration, *in vivo*. An initial calcium flux determination was made for 30 min following a preincubation period as described by ADAMS AND NORMAN[14]. Filipin was then added to the mucosal compartment in 0.10 ml of dimethyl sulfoxide, to a final concentration of 10 μg/ml. After a 20-min lag, the calcium flux in the presence of filipin was measured for a 30-min period. The filipin effect was then measured as the ratio of the flux rate due to filipin over the initial flux rate in the absence of filipin. The vitamin D effect at each time interval was calculated from the initial flux rate, as compared with the initial flux rate for tissue from a rachitic chick (0 h). The initial calcium concentration was 0.10 mM. Chicks termed + D were given a single dose of 500 I.U. (32.5 nmoles) vitamin D_3 intracardially at varying times before death.

Time after vitamin D_3 (h)	*Effect on calcium flux (J_{ms})*		*% Effect of*	
	Filipin effect	*Vitamin D effect*	*Filipin*	*Vitamin D_3*
0	2.91 ± 0.65 (4)	1.00 ± 0.49 (4)	100	0
5	2.62 ± 0.54 (4)	1.01 ± 0.44 (4)	85	1
13.5	2.52 ± 1.10 (4)	1.27 ± 0.48 (4)	80	27
28	2.08 ± 0.11 (3)	2.01 ± 0.70 (3)	57	101
40	1.56 ± 0.41 (4)	2.49 ± 0.95 (4)	29	149

deficient ileum. Table III also shows that at 40 h the calcium flux due to vitamin D administration is enhanced 150 % over the flux rate noted in the rachitic ileum, *in vitro*. These results indicate that calcium transport can be stimulated in the rachitic chick's ileum, by either the administration of filipin, *in vitro*, or vitamin D, *in vivo*. In other experiments[14] we have noted that while actinomycin D given 2 h before vitamin D can block the increased transport of calcium measured, *in vitro*, at 52 h, that actinomycin D treatment, *in vivo*, does not block the effect of filipin treatment, *in vitro*. Thus as long as the tissue has no components of vitamin D-mediated calcium transport, it is capable of responding to filipin.

Filipin interaction with the mucosal surface

In Fig. 1 is shown the rate and extent of filipin binding, *in vitro*, to everted intact ileal segments obtained from vitamin D-treated and vitamin D-deficient chicks.

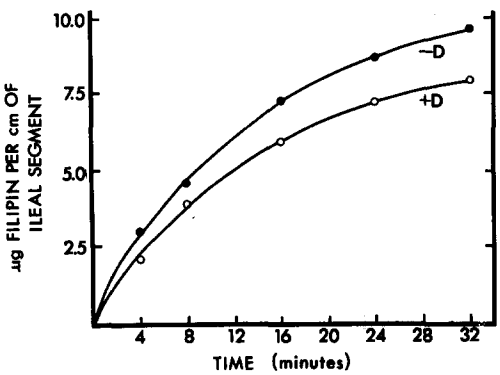

Fig. 1. The binding of filipin, *in vitro*, to 8-cm everted intestinal ileal segments from vitamin D-treated and vitamin D-deficient chicks. Ileal segments termed + D were excised from vitamin D-treated chicks which had received a 500 I.U. (32.5 nmoles) oral dose of vitamin D_3 36 h before sacrifice. Filipin binding was monitored as described by ADAMS *et al.*[11]. The figure represents the average of 4 similar experiments.

The results indicate that both the rate of binding of filipin and the amount bound is higher for the deficient chick intestinal ileum. This observation may indicate that the vitamin D-treated intestine is in some way protected from interaction with the antibiotic or that the membrane organization of the ileal segment from the vitamin D-treated chick is not as favorable for interaction with filipin.

The experimental results obtained from binding studies and the increase in calcium flux upon the administration of filipin to the deficient chick's ileum suggest that the mucosal microvillar membrane of the intestinal epithelial cell may be an important rate-limiting obstacle in the calcium transport system.

Cytological examination of the filipin effect

Since it has been established that filipin interacts with membranes[1–6], we found it necessary to examine with the electron microscope the microvillar regions of ileal segments from vitamin D-deficient and vitamin D-treated chicks which had been treated with filipin. As seen in Fig. 2 the morphological integrity of the vitamin D-treated intestinal microvillar membrane is preserved after a 40- or 80-min incubation with 10 μg/ml of filipin as described in MATERIALS AND METHODS. Fig. 3 shows the

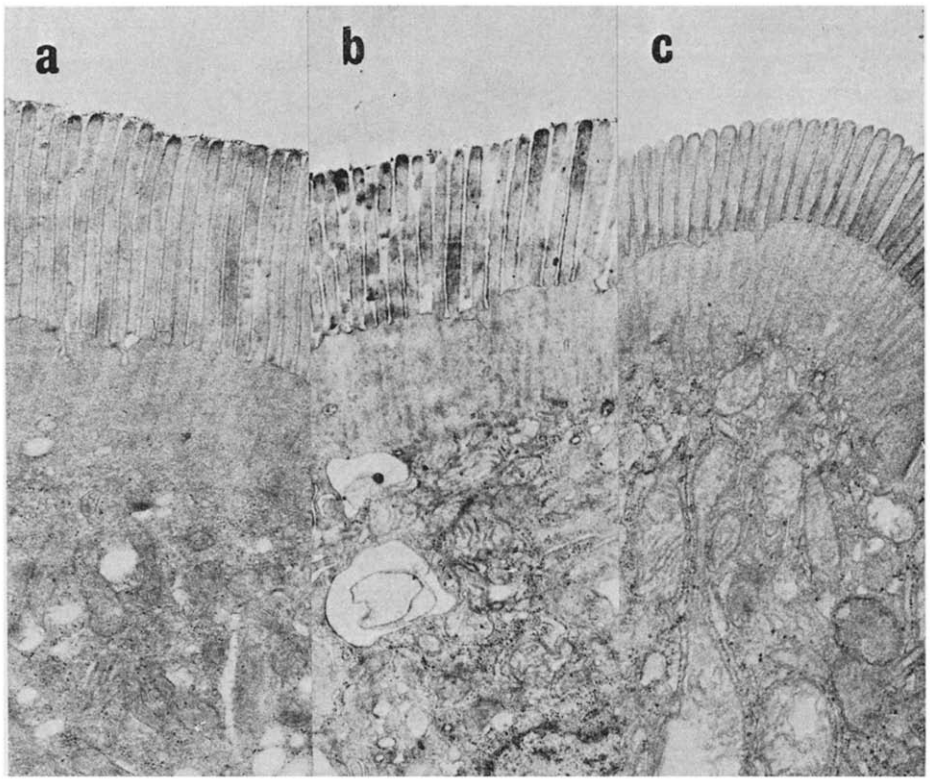

Fig. 2. Electron micrographs of the effect of filipin, *in vitro*, on the ileal microvillar region of intestinal segments obtained from vitamin D-treated chicks. Ileal segments were mounted in the Ussing type apparatus and incubated in the modified Krebs–Ringer solution as described under MATERIALS AND METHODS. Filipin was added in 0.10 ml ethanol to a concentration of 10 μg/ml to the solution bathing the mucosal surface at the start of the incubation. After the incubation, the ileal segments were removed and prepared for examination with the electron microscope, as described under MATERIALS AND METHODS. Vitamin D-treated chicks received 3 oral, 2500-I.U. doses of vitamin D_3 in 0.2 ml of 1,2-propanediol at 72, 48, and 24 h before sacrifice. a. Microvillar surface after zero time incubation. b. Microvillar surface after 40-min incubation with filipin. c. Microvillar surface after 80-min incubation with filipin. Magnification 16 000 × for all micrographs.

effects on the vitamin D-deficient chick ileum when it was exposed to filipin under the identical conditions mentioned for the vitamin D-treated system. At 40 min, the microvillar membrane surface of the deficient chick still shows the same degree of integrity as the vitamin D-treated tissue; while at the later time period (80 min) it can be noted that filipin causes a gross morphological alteration of the −D ileal microvillar regions. These results correlate well with the transport data which are presented in Tables I, II and III. The importance of these findings becomes evident in that at the 40-min time interval after filipin administration both the vitamin D-deficient and vitamin D-treated tissues show no great morphological dissimilarity in response to filipin. Therefore the transport data which were collected during a a 20–40-min exposure to filipin are not just a reflection of some drastic difference in intestinal morphology.

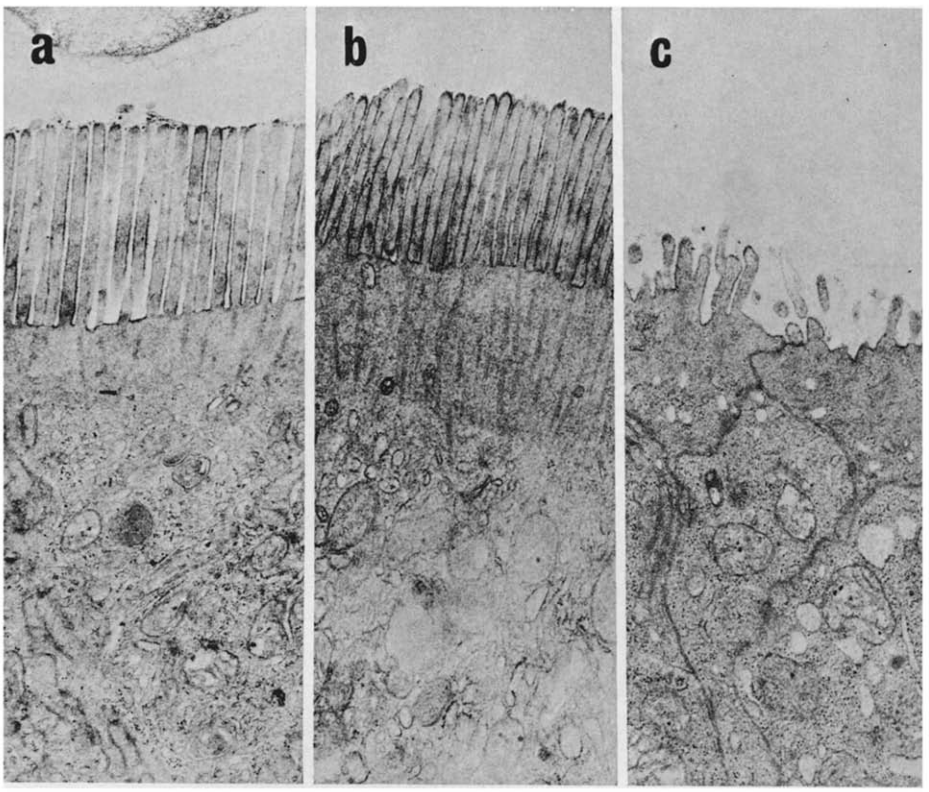

Fig. 3. Electron micrographs of the effect of filipin, *in vitro*, on the ileal microvillar region of intestinal segments obtained from vitamin D-deficient chicks. Ileal segments were treated exactly as described in the legend to Fig. 2. a. Microvillar surface after zero time incubation. b. Microvillar surface after 40-min incubation with filipin. c. Microvillar surface after 80-min incubation with filipin. Magnification 16000 × for all micrographs.

Effect of filipin on the passive transport of thiourea and water

In order to ascertain that the increase in calcium flux, J_{ms}, in the deficient chick ileum was not just a reflection of osmotic effects due to passive transport processes, the transport of thiourea and water was studied in the filipin-treated systems in ileal segments from vitamin D-deficient and vitamin D-treated chicks. Table IV shows results obtained for the translocation of calcium, thiourea and water, *in vitro*, as a function of the time of filipin exposure in ileal segments obtained from vitamin D-deficient and vitamin D-treated chicks. In the deficient chick ileum, calcium flux is greatly enhanced at both time periods by filipin treatment while thiourea and water show little increase in flux at the 40-min interval, but show approx. a 2-fold increase after a 100-min incubation with filipin. This latter increase may be due to the loss of membrane integrity that was shown in Fig. 3 after an 80-min incubation with filipin. The vitamin D-treated tissues show no appreciable increase in flux rates for any of the three compounds tested at either of the two time periods. From these results it can be ascertained that the specific increase in calcium flux (Table II) caused by filipin is not due to an increase in a passive diffusional process. These results also substantiate the view that at the 40-min period after filipin administration, the

TABLE IV

EFFECT OF FILIPIN ON CALCIUM, THIOUREA AND WATER FLUX AS A FUNCTION OF FILIPIN INCUBATION TIME

Experiments were run in the Ussing apparatus with a calcium concentration of $1.0 \cdot 10^{-4}$ M. Thiourea fluxes were determined with an initial thiourea concentration of $2.0 \cdot 10^{-4}$ M, and 25 μC of ^3H_2O were used for water flux experiments. Chicks termed + D received one oral dose of 500 I.U. (32.5 nmoles) vitamin D_3 60 h before death in the calcium flux experiment. + D chicks utilized for the thiourea and water flux experiments received 2 oral 500-I.U. (32.5 nmoles) doses of vitamin D_3 at 72 and 36 h before death. Initial flux rates were determined, and then filipin was added to the mucosal solution in 0.2 ml of ethanol to a final concentration of 10 μg/ml. After a 20-min lag, a second rate was determined in the presence of filipin (40-min filipin effect); then after a 40-min incubation a third rate was determined again in the presence of filipin (100-min filipin effect). All rates were determined by taking 5 samplings at 5-min intervals. For each experiment the initial rate in the absence of filipin (0 time) was set to 1.0 and then the relative rate after 40 min and 100 min filipin incubation calculated.

Vitamin D status	*Incubation time in filipin (min)*	*Increase in flux ratio (J_{ms}) due to filipin treatment*		
		Calcium	*Thiourea*	*Water**
− D	0	1.0	1.0	1.0
	40	2.48 ± 0.56 (4)	1.27 ± 0.23 (4)	0.98
	100	5.26 ± 1.45 (4)	1.77 ± 0.14 (4)	1.94
+ D	0	1.0	1.0	1.0
	40	1.18 ± 0.02 (3)	1.00 ± 0.08 (4)	0.92
	100	1.23 ± 0.04 (3)	1.41 ± 0.29 (4)	1.37

* Mean of two samples.

transport observed, *in vitro*, is not due to an artifact produced by a grossly altered intestinal mucosal membrane but rather that there is a basic difference in the mucosal microvillar membranes of vitamin D-deficient and vitamin D-treated chicks.

DISCUSSION

The polyene antibiotic, filipin, has been shown to interact with the mucosal membrane of the rachitic chick intestine *in vitro*. The observed effects were those of an increase in calcium flux, J_{ms}, and the initiation of gross morphological alterations of the microvillar membrane after prolonged exposure to filipin. Intestinal microvillar membranes from vitamin D-treated chicks showed none of these responses when treated with filipin, *in vitro*.

The interaction of polyene antibiotics with membranes and specifically with membrane sterols has previously been studied[1-8,18-21]. It is generally agreed that a prerequisite for filipin interaction with either artificial or natural membranes is the presence of sterol[7,22-24]. From the observed higher rate and extent of filipin binding to the vitamin D-deficient chick ileum (Fig. 1), it seems possible that a basic difference in sterol composition might exist between the intestines obtained from vitamin D-deficient and vitamin D-treated chicks. ADAMS *et al.*[11] found that the microvillar membranes of vitamin D-treated chick intestines contained 40% more cholesterol than the vitamin D-deficient chicks' microvillar membranes, which is in direct contradiction to the sterol hypothesis for filipin action. This might indicate that the vitamin

D-treated chick's microvillar membrane may have a protective coat of some kind which inhibits filipin interaction. It is known[25–27] that a "fuzzy coat" or glycocalyx, composed of acid mucopolysaccharide, is attached to and covers the external surface of the intestinal microvillar membranes. A vitamin D-induced calcium-binding protein has been isolated and characterized by WASSERMAN *et al.*[28] and WASSERMAN AND TAYLOR[29]. It has been shown to localize at either the glycocalyx surface coat or the microvillar membrane[30].

It has also been noted that the filipin effect on the promotion of increased calcium flux, *in vitro*, is only manifested when added to the mucosal surface of the rachitic chick intestine[31]. Similar results were noted by LIPPE *et al.*[32] who found that amphotericin B was able to increase thiourea permeability in the turtle, *Testudo hermanii*, jejunum and large intestine only when it was applied to the solution bathing the mucosal surface of the tissue. Also no effect of polyenes was observed when they were added to the serosal surface of the bladder of the marine toad, *Bufo marinus*[33]. However, LIPPE *et al.*[32] concluded that the lack of an effect of the polyenes at the serosal side was due to the presence of a protective coat of proteins. They demonstrated that thiourea flux, J_{ms}, in the turtle large intestine could be increased by amphotericin B when the serosal side had been pretreated with proteolytic enzymes. Similarly the concept of the existence of a protective coat may also apply to the ileal microvillar membranes from vitamin D-treated chicks; the glycocalyx or Wasserman's calcium-binding protein may be capable of functioning in this manner. Thus whether an ileal microvillar membrane can respond to filipin treatment may be determined by either the relative amount of cholesterol directly exposed on the mucosal membrane surface or by the extent to which this cholesterol is protected or covered by the glycocalyx or calcium-binding protein.

The experimental evidence presented shows that the filipin-stimulated increase in calcium flux, J_{ms}, in the vitamin D-deficient ileum is: (a) specific for calcium translocation (Table II), (b) sensitive to vitamin D treatment, *in vivo*, (Table III), and (c) is not the result of some gross morphological alteration (Figs. 2 and 3) of the microvillar membrane which results in a general increase in permeability (Table IV), (at the time of studying the transport activities of the filipin-treated tissue (40 min).

ADAMS *et al.*[11] studied further the filipin-stimulated calcium transport system in the vitamin D-deficient ileum, *in vitro*, and found that it had several common features with the vitamin D-stimulated calcium transport system in chick ileum. Both calcium transport systems were shown to be (i) an active transport process, (ii) specific for calcium, (iii) cold temperature sensitive, and (iv) sensitive to *N*-ethylmaleimide. The untreated transport systems showed none of these features.

Prolonged incubation (80 min) of the −D ileum with filipin showed a gross morphological alteration of the mucosal microvillar membrane which was not evident in the vitamin D-treated chick when examined by electron microscopy (Figs. 2 and 3). Although it is not precisely clear as to the details of the steps involved in producing this morphological change, it does appear as if a large proportion of the microvilli have been lost.

Presented in Table V is a time-course of events noted after administration, *in vitro*, of filipin to ileal segments from vitamin D-deficient and vitamin D-treated chicks. After 80 min incubation of the mucosal surface with 10 μg/ml filipin there is a gross morphological alteration of the −D mucosal tissue and no effect noted in

TABLE V

SUMMARY OF FILIPIN EFFECTS IN INTESTINAL MUCOSA

Length of time of filipin treatment (10 μg/ml), in vitro (min)	*Observed consequences*
80	Gross morphological alterations of only − D mucosal tissue; no effect on + D tissue
40	Small morphological alterations on only − D mucosal tissue
15–30	Increase in calcium flux in only − D mucosal tissue (no effect on Rb^+, SO_4^{2-}, water or thiourea flux)
1–4	Filipin binds to intact mucosal tissue more rapidly in − D than + D
0	Suggest possible difference in mucosal membrane structure between + D and − D intestine

the + D mucosal tissue. After incubation in the filipin solution for 20–40 min there is no real visible morphological alteration in either of the mucosal tissues. However, there is observed a marked increase in calcium flux, J_{ms}, in the − D mucosal tissue which is specific for calcium and is not the result of a general increase in permeability. It was also demonstrated that within 1–4 min of placing mucosal tissue in a solution containing filipin, that the filipin bound more rapidly and to a greater extent to the ileal segments from rachitic chicks as compared to ileal segments from vitamin D-treated chicks. Extrapolation of this time study to zero time tends to support the hypothesis that the intestinal microvillar membranes of the vitamin D-deficient and vitamin D-treated chicks are different. One prime manifestation of this difference is the presence of a highly competent active transport process for calcium in the + D system. Biochemical evidence presented by ADAMS *et al.*[11,34] and MARTIN AND DELUCA[35] suggests that the microvillar membrane is the site of the rate-limiting step in the vitamin D-stimulated calcium transport system in the rachitic chick.

It has been reported that vitamin D is first metabolized to a series of metabolites and that one of these metabolites mediates the specific increase in calcium translocation through a series of genetically related events[36–38]. It is possible that this genetically controlled vitamin D-mediated activity may be the result of some alteration of the intestinal microvillar membrane of the rachitic chick. Further studies now in progress are directed toward identification and isolation of the vitamin D-dependent factor(s) present in the mucosal microvillar membrane.

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