Biochemistry

 Volume 18, Number 22 October 30, 1979

Interaction of Isotopically Labeled and Unlabeled Filipin with Egg Lecithin Vesicles and Rat Erythrocytes†

Patrick M. Kelly,‡ John F. Holland,* and Loran L. Bieber*

ABSTRACT: The binding of [^{3}H]- and [^{14}C]filipin and unlabeled filipin in micromolar concentrations to egg lecithin, egg lecithin–cholesterol vesicles, and rat erythrocytes was investigated. Separation of the free filipin from bound filipin and the vesicles was achieved by ultrafiltration and molecular sieving on Sepharose 4B. Little, if any, filipin bound to egg lecithin vesicles and only 3–6% bound to egg lecithin–cholesterol vesicles when excess cholesterol was present (even though the spectral characteristics were measured in the molecular sieving experiments and they were those of filipin complexed to cholesterol). Molecular sieving experiments demonstrated that at least two species of the filipin–cholesterol complex of different size existed in aqueous solution. Other studies have shown that dispersed egg lecithin can remove cholesterol from preformed filipin–cholesterol complex. Rat erythrocytes were incubated with [^{3}H]filipin and unlabeled filipin in micromolar concentrations, and separation of the free from the membrane-bound filipin was achieved by centrifugation. About 5% of the initial filipin was associated with the cells during cellular lysis. In addition, most of the cholesterol released from erythrocytes occurred with fragmentation of the cells, but some leakage of K^+ occurred immediately after addition of filipin. The data indicate that most of the filipin–cholesterol complex that is formed with membrane cholesterol does not remain associated with the membrane during lysis. The detection of at least two spectrally detectable species of filipin–cholesterol complexes, one much larger than the other, provides a basis for an explanation of the results obtained with lecithin–cholesterol vesicles and rat erythrocytes. The smaller complex could be the water-soluble one that shows a filipin/cholesterol ratio of 1:1, and the larger one could be a complex residing in membranes.

Studies with natural membrane systems such as *Acholeplasma laidlawii* (Weber & Kinsky, 1965; Feingold, 1965), *Saccharomyces cerevisiae* (Lampen et al., 1962), and *Neurospora crassa* (Kinsky, 1962, 1963a,b) provided the initial evidence that polyene antibiotics interacted with membrane sterols. This interaction with sterols seems to be responsible for the observed lytic effects on the various microorganisms and on mammalian erythrocytes. Attempts to elucidate the mechanism(s) of polyene antibiotic interaction with membrane-bound sterols have used both artificial and natural membrane systems [see the recent review of Norman et al. (1976)].

By the use of erythrocytes (Kinsky et al., 1966, 1967a), it was demonstrated that both the antibiotic/erythrocyte ratio and the antibiotic concentration were important for cell lysis. Their electron micrographic investigations (Kinsky et al., 1966, 1967b) showed that after treatment with filipin, liposomes and rat erythrocytes developed "pits" which may have been the result of structural rearrangement of lipids. Further work by Verkleij et al. (1973) and Tillack & Kinsky (1973) confirmed the presence of pits and indicated the existence of protrusions and "doughnut-shaped" structures on the surfaces of erythrocytes and *A. laidlawii* cells which had been treated with filipin and amphotericin B. The pits were apparently not holes through the membrane.

More recent investigations have also exploited the spectral properties of polyene antibiotics (Schroeder et al., 1972, 1973; Bittman et al., 1974a,b; Norman et al., 1972a,b). Norman and co-workers (1972a,b) demonstrated that upon addition of filipin to suspensions of erythrocyte ghosts or *A. laidlawii* membranes, this polyene exhibited the spectral properties characteristic of filipin–cholesterol complexes. In addition, binding experiments with *A. laidlawii* membranes revealed that substantial amounts of various polyene antibiotics were bound only to membranes from organisms grown with sterol (Norman et al., 1972a). In an effort to correlate permeability, micrographic, spectroscopic, and calorimetric data, DeKruijff et al. (1974a,b) and DeKruijff & Demel (1974) published a comprehensive proposal for the mechanisms of membrane interaction for various polyene antibiotics. Of particular interest for this paper was the proposal that filipin formed cholesterol–filipin aggregates ~150–250 Å in size with hydrophobic exteriors that would be formed in the interior region of the membrane. The presence of these aggregates could then cause fragmentation of the membrane and, subsequently, the loss of internal components.

† From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824. *Received August 29, 1978.* This work was supported in part by National Institutes of Health Grant No. AI 12187. This is paper no. 8646 from the Michigan Agricultural Experiment Station.

‡ State University of New York College of Technology, Utica, NY 13502.

0006-2960/79/0418-4769$01.00/0

We have prepared radioactive filipin and determined the amount of filipin which remains with lecithin, lecithin–cholesterol vesicles, and erythrocytes after exposure to this antibiotic. It was found that only small amounts of filipin remain bound to the vesicles and to erythrocytes; yet filipin removes cholesterol from both. The experimental findings also indicate that two types of filipin–cholesterol complexes occur.

Materials and Methods

Crude egg lecithin was purified on Bio-Sil A from Bio-Rad (Richmond, CA) with chloroform-methanol (1:1 v/v). Further purification was carried out by silica gel thin-layer chromatography with chloroform–methanol–water (65:35:4 v/v). The purified lecithin was lyophilized from 2-methyl-2-propanol and stored desiccated at −20 °C.

Filipin was isolated from a culture of *Streptomyces filipinesis* which was the generous gift of P. G. Pridham, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL. The crude filipin was purified by the method of Patterson et al. (1979) and was stored lyophilized in the dark at −20 °C.

[^{3}H]- and [^{14}C]filipin was also isolated from culture in which ^{3}H- ^{14}C-labeled sodium acetate from New England Nuclear (Boston, MA) was used as a portion of the carbon source by the method of Schroeder & Bieber (1975). Purification was carried out by silica gel thin-layer chromatography with dichloroethane–methanol (85:15 v/v). The labeled filipin was removed from the silica gel by several extractions with water–ethyl acetate (7:10 v/v). The ethyl acetate was removed in vacuo, and the labeled filipin was lyophilized from 2-methyl-2-propanol and stored in the dark at −20 °C.

[^{14}C]Cholesterol from New England Nuclear and unlabeled cholesterol from Sigma were used without further purification. All other chemicals were of reagent grade.

The buffering system used with the lecithin vesicles was a KCl (0.1 M) in Tris (0.01 M) solution adjusted to pH 7.6 at 25 °C. The buffer system (PBS) for the cells was a NaCl (0.15 M) and Na_2HPO_4 (0.005 M) solution adjusted to a pH of 8.0 at 25 °C (Steck, 1974).

Preparations of Vesicles. Two methods were employed for the preparation of lecithin and lecithin–cholesterol vesicles. Sonicated and unsonicated vesicles were prepared by the method of Huang (1969) and by the method of Batzri & Korn (1973), respectively. After preparation, both vesicle types were subjected to gel filtration on Sepharose 4B and only the fractions near the elution maxima were used for these studies.

Interaction of Filipin with Vesicles. Stock solutions of isotopically labeled filipin were prepared by the dissolution of filipin in the KCl–Tris buffer by stirring at room temperature in the dark overnight. Appropriate amounts of vesicles and filipin were then mixed and allowed to stand in the dark at room temperature for 36 h.

Separation of Unbound Filipin from the Vesicles. The separation of unassociated filipin from the vesicles was accomplished in two ways. In some of the experiments, an Amicon (Lexington, MA) Model 52 ultrafiltration device in conjunction with an Amicon XM-100A filter was used for the separation. In these experiments, the reaction mixtures were diluted to ~30 mL and placed in the filtration device. They were then concentrated to 2 mL, and an additional 28 mL of the KCl–Tris buffer was added to bring the total volume to 30 mL. Again, the mixtures were concentrated. This process was repeated until each reaction mixture had been subjected to five concentrations. The vesicles were then removed from the filtration device, diluted to a known volume, and analyzed. All separations of this type except for some in Table I were carried out at room temperature. In most cases, ~85% of the lecithin was recovered and nearly all of the free filipin passed through the filter during the working process.

In the second of the two separation methods, a Sepharose 4B column was used. The column was 2.5 × 45 cm in size and was conditioned by incubation with an excess of cholesterol–lecithin vesicles. Preconditioning was necessary because of the apparent affinity of Sepharose 4B for lecithin. Fluorescence analysis indicated that there was no affinity of Sepharose for filipin. All separations were carried out at 4 °C with a flow rate of 20 mL/h. Five-milliliter fractions were collected for analysis.

Filipin Solutions Used with Erythrocytes. Fresh filipin stock solutions were prepared before each series of experiments. [^{3}H]Filipin solutions were prepared by dissolving ~200 nmol in 10 mL of PBS and then stirring at room temperature in the dark overnight. Aqueous solutions of unlabeled filipin were prepared in a similar manner.

Preparation of Rat Erythrocytes and Erythrocyte Ghosts. Erythrocytes were prepared as described by Steck (1974). The washed erythrocytes were prepared from heparinized male rat blood immediately before use in the experiments. Stock suspensions of cells were prepared by suspending 2.0 mL of packed cells in 20 mL of PBS buffer.

Experimental Techniques Used with Erythrocytes. Lysing experiments were carried out by the addition of the [^{3}H]filipin or unlabeled filipin stock solutions to 3.0 mL of PBS buffer. Then the appropriate amount of the erythrocyte suspension was added and the solution mixed. After incubation in the dark for the appropriate time, the cells were centrifuged at three-quarter speed for 5 min on an International Equipment Co. Model HN tabletop centrifuge.

Analytical Methods. Phospholipids were extracted from the other cellular materials by the method of Rose & Oklander (1965). The method of Bartlett (1959) for phosphate analysis was used for the determination of lecithin in both the vesicles and cells. Cholesterol was extracted into chloroform and then analyzed. A modified Libermann–Burchard method similar to the one outlined by Chen et al. (1964) was used with the vesicles. The excitation and emission wavelengths were changed to 546 and 595 nm, respectively, to minimize the lecithin background fluorescence. Extracted cellular cholesterol was analyzed by a cholesterol oxidase–diacetyl-2′,7′-dichlorofluorescein method developed in this laboratory. Except for the substrate, the method is similar to the one developed by Heider & Boyett (1978). K^+ was determined by flame photometry on a Corning Model 430 flame photometer. Hemoglobin contents were determined by absorption spectrometry at 550 nm. Assays for isotopically labeled compounds were carried out by placing 100 μL of sample into 10 mL of a toluene–Triton X-100–PPO–dimethyl-POPOP scintillation fluid. The samples were then counted in a Packard Tri-Carb liquid scintillation counter. Radioactive assays of filipin in cells were corrected for hemoglobin quenching. Analysis of unlabeled filipin was carried out fluorometrically with a Turner Model 111 filter fluorometer. The excitation and emission wavelengths were 365 and 486 nm, respectively. The absorbance spectra for filipin and the filipin–cholesterol complex were determined either on a computer-centered spectrofluorometer (Holland et al., 1973) or on a Cary Model 15 spectrophotometer. Criterion for the presence of the filipin–cholesterol complex was based on the ratio of filipin absorbance at 322 and 356 nm (A_{322}/A_{356}) (Norman et al., 1972a).

Table I: Separation of Unbound from Vesicle-Bound [^{3}H]Filipin by Ultrafiltration at Room Temperature and at 4 °C[a]

expt	mole ratio filipin/lecithin initial	mole ratio filipin/lecithin final	% filipin remaining with vesicles	temp (°C)
1	1.1×10^{-2}	5.5×10^{-4}	5.0	25
2	1.1×10^{-2}	6.1×10^{-4}	5.6	4
3	1.1×10^{-2}	5.0×10^{-4}	4.6	25
4	1.1×10^{-2}	4.9×10^{-4}	4.5	4

[a] Experiments 1 and 2 utilized unsonicated egg lecithin vesicles, and experiments 3 and 4 utilized unsonicated egg lecithin–cholesterol vesicles. Initial amounts of lecithin and filipin for the first two experiments were 1.0 μmol and 10.9 nmol, respectively. Initial amounts of lecithin, cholesterol, and filipin for the remaining experiments were 1.0 μmol, 70 nmol, and 10.9 nmol, respectively. The concentration of [^{3}H]filipin during incubation of all samples was 2.5×10^{-6} M. After the samples were incubated at room temperature in the dark for 36 h, any unassociated filipin was removed by ultrafiltration as described under Materials and Methods at the temperature shown above. The samples were then analyzed for filipin and phosphate to determined the final mole ratio.

Results

Liposomal Systems

Interaction of Filipin with Vesicles. The interaction at room temperature of [^{3}H]filipin with unsonicated egg lecithin and unsonicated egg lecithin–cholesterol vesicles was initially investigated by using the ultrafiltration method for separations. After interaction, the vesicles were then washed of unassociated filipin at both room temperature and 4 °C. The vesicles at 4 °C should be in the liquid-crystal state and consequently would be expected to trap any incorporated filipin–cholesterol complex. The results in Table I show that vesicles washed at both temperatures contained the same amount of filipin. Vesicles washed at room temperature did not contain less membrane-incorporated filipin–cholesterol complex than those washed at 4 °C.

In other experiments (data not shown) [^{3}H]filipin was allowed to interact with lecithin vesicles of both the unsonicated and sonicated types. The initial amounts of lecithin and [^{3}H]filipin were 2.0 μmol and 1.19 nmol, respectively, for the sonicated type and 2.0 μmol and 1.02 nmol for the unsonicated type. The controls consisted of [^{3}H]filipin in buffer. After separation of the free filipin from the vesicles, the final mole ratio of filipin to lecithin was determined as described under Materials and Methods. The final filipin/lecithin mole ratios were corrected for the filipin controls which amounted to less than 1% of the initial filipin present. Approximately 10% or less of the filipin initially present was associated with both types of lecithin vesicles. On a molecular basis this percentage represents 1 filipin molecule/20 000 molecules of lecithin and indicates that there is little association betweeen filipin and lecithin vesicles.

Interaction of [^{14}C]Filipin with Sonicated and Unsonicated Lecithin–Cholesterol Vesicles. Experiments similar to those described for Table I were done in which [^{14}C]filipin was allowed to interact with unassociated lecithin–cholesterol vesicles. The initial amounts of lecithin, cholesterol, and [^{14}C]filipin were 2.0 μmol, 144 nmol, and 15.8 nmol, respectively. The filipin–lecithin controls contained only [^{14}C]filipin and unsonicated lecithin vesicles. After the separation of unassociated filipin from the vesicles by ultrafiltration, the final mole ratio of filipin to lecithin was determined. The percentages of filipin remaining with the vesicles have been normalized to 100% lecithin recovery because of variations of the actual recoveries which ranged from 33 to 89%. The results in Table II indicate that the percentage of filipin associated with the unsonicated vesicles was ~4% above the controls. Data for the interaction of [^{14}C]filipin with sonicated lecithin–cholesterol vesicles are also shown in Table II. The initial amounts of lecithin, cholesterol, and [^{14}C]filipin were 2.0 μmol, 210 nmol, and 168 nmol, respectively. As with the unsonicated vesicles, the lecithin recoveries (77 → 84%) were normalized to full recovery. Again, the data show that very little of the filipin (<2%) is associated with the vesicles.

Table II: Interaction of [^{14}C]Filipin with Unsonicated and Sonicated Lecithin–Cholesterol Vesicles

expt	mole ratio filipin/lecithin initial	mole ratio filipin/lecithin final	% filipin remaining with vesicles
unsonicated vesicles[a]			
1	7.9×10^{-3}	6.4×10^{-4}	8.1
2	7.9×10^{-3}	7.7×10^{-4}	9.8
3	7.9×10^{-3}	8.3×10^{-4}	10.5
controls	7.9×10^{-3}	5.6×10^{-4}	7.0
sonicated vesicles[b]			
1	8.4×10^{-2}	5.7×10^{-3}	6.8
2	8.4×10^{-2}	5.1×10^{-3}	6.0
3	8.4×10^{-2}	4.9×10^{-3}	5.9
controls	8.4×10^{-2}	4.7×10^{-3}	5.6

[a] Initial amounts of lecithin, cholesterol, and filipin were 2.0 μmol, 144 nmol, and 15.8 nmol, respectively. Unsonicated vesicles were mixed with a buffer solution which contained the [^{14}C]-filipin ($\simeq 4 \times 10^{-6}$ M). The filipin controls consisted of only filipin and unsonicated lecithin vesicles. Both the samples and controls were then treated as described for Table I. The percentages of filipin remaining with the vesicles have been normalized for variation in the recovery of PO_4^{-3}. [b] Sonicated lecithin–cholesterol vesicles were mixed with a buffer solution which contained the [^{14}C]filipin ($\simeq 10^{-5}$ M). The initial amounts of lecithin, cholesterol, and filipin were 2.0 μmol, 210 nmol, and 168 nmol, respectively. The filipin controls consisted of only filipin and sonicated lecithin vesicles. The treatment of both the sample and controls is as described for Table I. The percentages of filipin remaining with the vesicles have been normalized for variation in the recovery of PO_4^{-3}.

Separation by Gel Filtration. Because the results obtained with ultrafiltration techniques indicated that little, if any, filipin and filipin–cholesterol complex was associated with vesicles, another method for separating filipin from vesicles was used. Filipin was interacted with lecithin–cholesterol vesicles at room temperature. The unassociated filipin was separated by passage of the sample through a Sepharose 4B column at 4 °C. Since the lecithin should be in a liquid-crystal state at this temperature, any associated filipin should be trapped within the membrane and dissociation should be minimized.

Figure 1 presents the results for two separate experiments which determined the elution patterns for uncomplexed filipin and for sonicated [^{14}C]cholesterol–lecithin vesicles. The sample volumes in both experiments were 19 mL. In the experiment with the vesicles, there was an increase in the mole ratio of cholesterol to lecithin from 0.16 to 0.22 with passage through the column, indicating a column affinity for lecithin. Figure 1B contains the elution pattern for the filipin–[^{14}C]cholesterol complex. The complex was formed by injecting 200 nmol of [^{14}C]cholesterol in 2-propanol into 20 mL of buffer solution which contained 200 nmol of filipin. The final concentration of 2-propanol was 0.3%. The resultant solution was then stirred at room temperature for 2.5 h. At the end of this period, the spectrum of the solution indicated the presence of the filipin–cholesterol complex. The elution

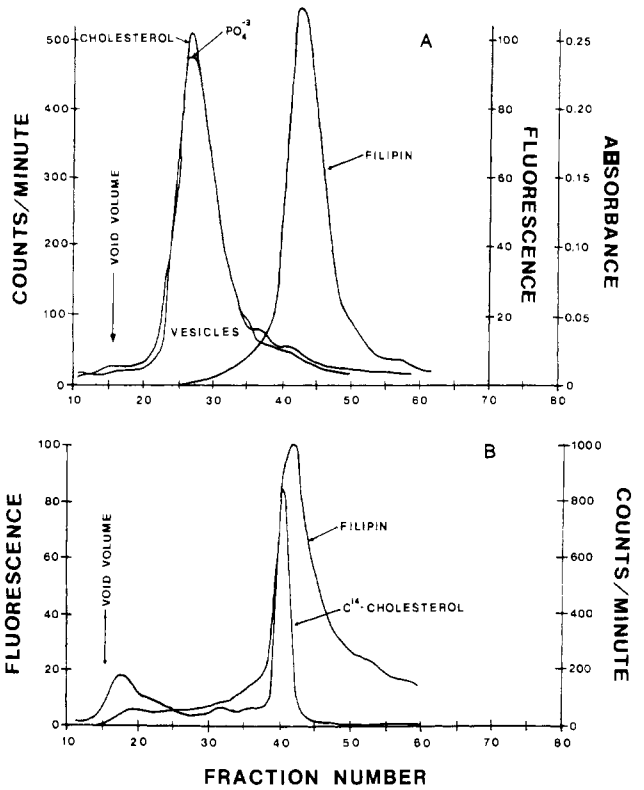

FIGURE 1: Elution patterns for sonicated lecithin–cholesterol vesicles and for free filipin and the filipin–cholesterol complex on Sepharose 4B. A composite of two separate experiments where either 175 nmol of filipin or 2.0 μmol of lipid phosphate and 320 nmol of cholesterol in the form of sonicated lecithin–cholesterol vesicles in ~19 mL of buffer were placed on a 2.5 × 45 cm column. The separations were carried out at 4 °C with a flow rate of 20 mL/h. Five-milliliter fractions were collected and analyzed. Filipin was monitored by fluorescence excited at 365 nm and detected at 486 nm. Lecithin was analyzed as PO_4^{-3}. Cholesterol was monitored by scintillation counting of the ^{14}C isotope. (B) A solution (20 mL) in which 200 nmol of filipin and 200 nmol of [^{14}C]cholesterol were allowed to interact for 2.5 h to form the complex. The chromatographic details are the same as those described in (A).

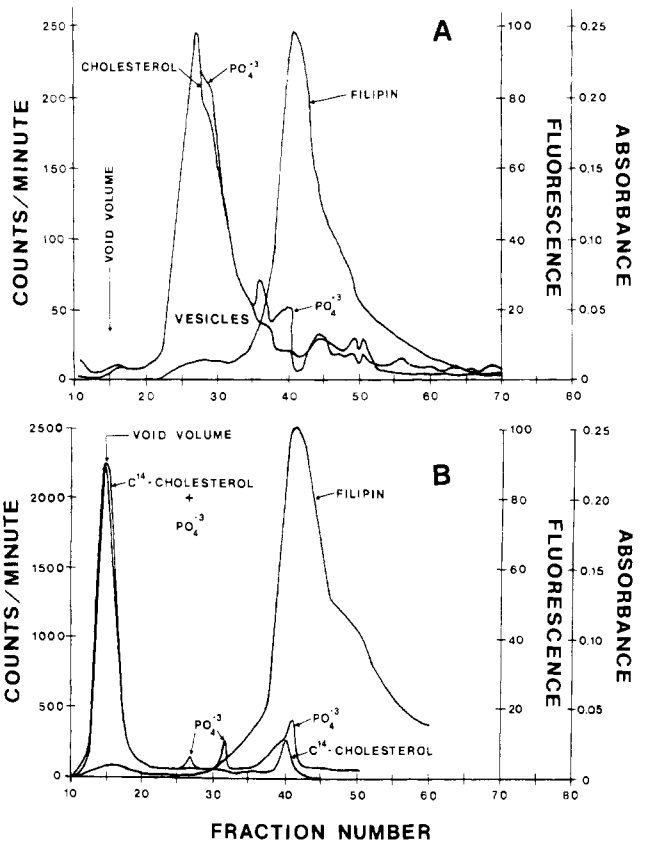

FIGURE 2: Elution pattern on Sepharose 4B for filipin with lecithin–cholesterol vesicles (A) and for the filipin–cholesterol complex with lecithin vesicles (B). Details for the experiments were as described in Figure 1. (A) The sample was prepared by mixing 155 nmol of filipin with [^{14}C]cholesterol–lecithin vesicles which were comprised of 2.0 μmol of lecithin and 330 nmol of cholesterol. The sample (19 mL) was incubated at room temperature in the dark for 36 h before it was placed on the column. (B) 20 mL of sample was prepared by the combination of 200 nmol of the filipin–cholesterol complex as described in (A) with 8–10 μmol of dispersed lecithin in 20 mL of buffer. The mixture was allowed to incubate for 36 h in the dark at room temperature.

pattern in Figure 1B indicates that some degree of separation of the complex from the unbound filipin species can be achieved with Sepharose 4B. Spectral analysis of fractions 18 and 40 revealed that the filipin present in these fractions was in the complexed form (i.e., bound to sterol), while that in fraction 44 was the free species. It is apparent from the elution pattern that at least two different forms of the cholesterol–filipin complex are present. The one in fraction 40 appears to be quite similar in size to the free filipin species and probably represents the simplest complex form. However, the complex at fraction 18 is much larger in size and is almost totally excluded from the gel material. Spectroscopically, it appears to be identical with the complex at fraction 40.

In other experiments, filipin was allowed to interact with sonicated [^{14}C]cholesterol–lecithin vesicles. Preparation of the mixture was carried out by the combination in 19 mL of buffer of 155 nmol of filipin with vesicles which were comprised of 2.0 μmol of lecithin and 330 nmol of [^{14}C]cholesterol. The mixture was then incubated at room temperature in the dark for 36 h to allow interaction. At the end of this period, the absorbance spectrum indicated that the filipin was in the complexed form and, subsequently, the mixture (19 mL) was placed on the Sepharose 4B column and eluted with buffer (see Figure 2A). Upon elution, only one-third of the lecithin and one-third of the [^{14}C]cholesterol were recovered with no change in the mole ratio of the constituents. However, only ~6% of the eluted filipin is associated with the intact vesicles.

The fate of the filipin–cholesterol complex after interaction with a suspension of lecithin is shown in Figure 2B. Approximately 200 nmol of the complex was prepared as outlined for Figure 2A. The presence of the complex was verified spectroscopically, and 8–10 μmol of dispersed lecithin was mixed with the complex. The mixture (20 mL) was incubated as usual for 36 h and then placed on the Sepharose 4B column and eluted with buffer. The resultant pattern indicates that very little of the simple complex is present. In addition, most of the recovered cholesterol (about two-thirds of the initial cholesterol) appears in the void volume with the dispersed lecithin, while only a small amount of the total filipin (≃2%) is associated with these large particles.

Erythrocyte Systems

Interaction of Filipin with Erythrocytes. Since the investigations with lecithin vesicles indicated that very little, if any, filipin–cholesterol complex associates with the vesicles, similar experiments were performed with a cholesterol-containing natural membrane system, namely, erythrocytes. The leakage of hemoglobin from rat erythrocytes upon interaction with filipin as a function of time is shown in Figure 3A. The shape of the curve is typical and very similar to those obtained by Kinsky and co-workers (Kinsky et al., 1962; Kinsky, 1962). In addition, the lysing time was found to be

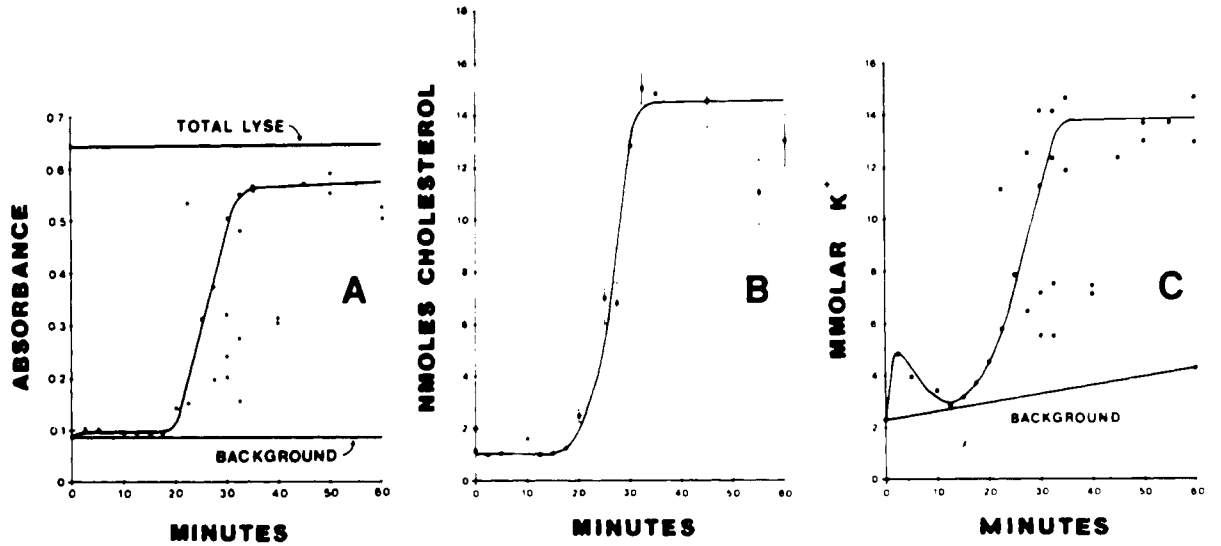

FIGURE 3: Time course of filipin-induced lysis of rat erythrocytes. (A) 350 μL of a 1 × 10^{-5} M filipin solution was added to 2.55 mL of PBS buffer with mixing. Subsequently, 100 μL of a stock solution of erythrocytes (9.1 μL of packed cells) was added and the suspension incubated for the desired time at room temperature in the dark. The cells were then spun down as described under Materials and Methods and the supernatants subjected to analysis at A_{550}. (B) Time course of cholesterol appearance in the supernatants. (C) Release of K^+ into the supernatant fluid for the experiment in (A).

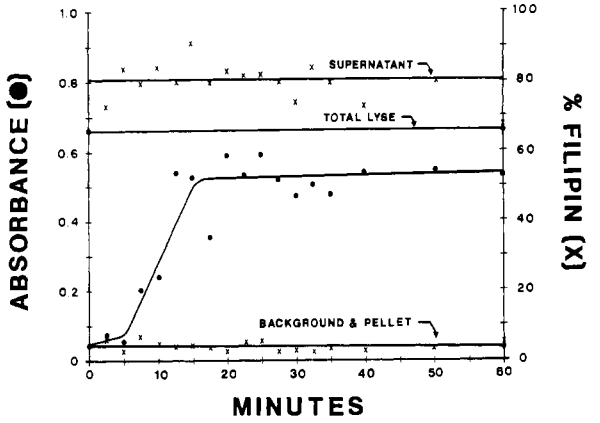

FIGURE 4: Time course for the binding of filipin to erythrocytes. 125 μL of a buffer solution which contained ~2.5 nmol of [^{3}H]filipin was added to 2.78 mL of PBS buffer. The 100 μL of a stock solution of erythrocytes (9.1 μL of packed cells) was added and the suspension incubated at room temperature in the dark for the desired times. After centrifugation, both the cells and supernatants were analyzed for [^{3}H]filipin. The term background refers to the absorbance of hemoglobin (●) as a function of time with no filipin present. Pellet refers to the percent of filipin (×) associated with the pellet as a function of time.

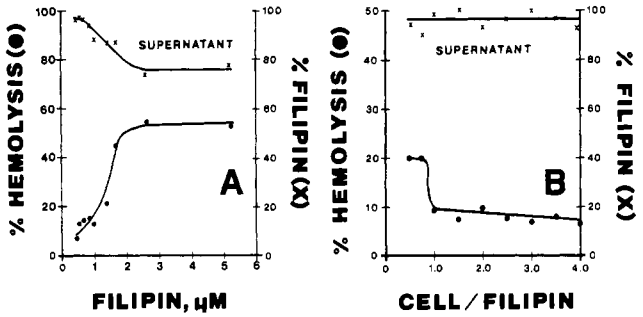

FIGURE 5: Effect of filipin concentration on the binding of filipin to erythrocytes. (A) The samples were prepared by mixing 150 μL of a stock [^{3}H]filipin solution (1.3 nmol) with the appropriate amount of PBS buffer, and 100 μL of the stock cell solution (9.1 μL of packed cells) was then added with mixing. All samples were incubated in the dark at room temperature for 75 min before centrifugation. (B) The filipin concentration was held constant at 0.53 μM and the amount of cells varied. Samples (3.0 mL) were prepared by mixing 100 μL of the filipin stock (1.6 nmol) with an appropriate amount of PBS buffer. Then aliquots of the cell suspension were added, and the sample was mixed [i.e., a sample with a cell/filipin ratio of 3.0 would have 300 μL of cell suspension (27 μL of packed cells) and 100 μL of filipin (1.6 nmol) in a total sample volume of 3.0 mL]. The samples were treated as described for (A).

dependent on the concentration of filipin as previously noted by these investigators. The amount of cholesterol in the supernatants for this experiment is shown as a function of time in Figure 3B. Although the experimental points are somewhat scattered, it is evident that the major release of cholesterol from the cells corresponds to the time when hemoglobin is released. In addition, the release of cholesterol during the lag period (0–17.5 min) is minimal (1 nmol or ≃5%) even in the presence of 3.5 nmol of filipin. The release of K^+ into the supernatant is shown in Figure 3C. Again, it is evident that the major release of K^+ corresponds to the time of major cell disruption. There also was an initial efflux of K^+ from the cells upon the introduction of filipin. However, it is apparent that the K^+ concentration recedes to the background level before fragmentation occurs. This phenomenon has been confirmed in other similar experiments.

Filipin Binding to Erythrocytes. Figure 4 shows the fraction of filipin bound to erythrocytes as a function of time during the lysing process. The results show that the amount of [^{3}H]filipin associated with the cells is quite constant even though the cells have undergone extensive lysing. It is also evident that the amount of cell-associated filipin is established quite rapidly and is maintained at only ~5% of the total filipin present.

Effect of Filipin Concentration in the Filipin/Cell Ratio on Binding to Erythrocytes. The amount of filipin associated with rat erythrocytes as a function of filipin concentration is shown in Figure 5A. The results indicate that the amount of associated filipin is dependent on the initial filipin concentration. When the ratio of filipin to cells was varied, the amount of associated filipin remained constant as shown in Figure 5B.

Other Aspects of Erythrocyte Lysing. Additional experiments were performed in an effort to elucidate other factors which may affect the lysing of erythrocytes by filipin. It was found that the addition of egg lecithin liposomes had no effect

on the lysing of red cells by filipin. Tests with the filipin–cholesterol complex revealed that the complex does not lyse red blood cells at concentrations that were twice that effective with uncomplexed filipin. In addition, it was also found that the lysing process is inhibited by removal of the supernatant from cells which were ready to lyse, i.e., when unassociated filipin was removed. The existence of a component which causes cell lysis was also eliminated by experiments which exposed intact erythrocytes to both supernatants and cells from samples which were undergoing lysis.

Discussion

The data presented herein show that isotopically labeled filipin interacts with vesicle-bound cholesterol. However, after interaction, very little of the filipin and filipin–cholesterol complex remains with either the lecithin–cholesterol vesicles or the erythrocytes even though an excess of cholesterol is present. In the filtration experiments, the amount of associated filipin was less than 4%. With molecular sieving, the amount of bound filipin was 6% even though excess cholesterol was present. In simple aqueous systems with micromolar amounts of filipin, almost complete binding of filipin to cholesterol with a 1:1 stoichiometry might be expected (Schroeder et al., 1972). This low association of filipin with cholesterol-containing systems is similar to the findings of Van Hoogevest & DeKruijff (1978) with amphotericin B. They found only a small percentage of added amphotericin B (3% above the blank with a standard deviation of ±1.2) associated with egg lecithin–cholesterol vesicles even though cholesterol was in great excess: ~0.0002 μmol of amphotericin B bound per μmol of cholesterol (see their Table I). Similarly, the data of Bittman & Fischkoff (1972) show that the fluorescence polarization of the aqueous filipin–cholesterol complex is low and similar to filipin complexed to lecithin–cholesterol vesicles. This should indicate that filipin did not reside in the vesicle since in a restricted environment the polarization should have increased.

The results of this study indicate that the interaction of filipin with lecithin and lecithin–cholesterol vesicles involves a process whereby only a small amount of the initial filipin is associated with the membranes. The results of Figure 1B suggest that at least two sizes of the filipin–cholesterol complex can exist in aqueous medium, one small and one large.

Some filipin was associated with the cholesterol eluting near the void volume (fractions 15–20, Figure 1B), while most of the cholesterol eluted with filipin in the smaller molecule region. This type of complex might be responsible for the filipin associated with the lecithin–cholesterol vesicles shown in parts A and B of Figure 2. A plausible structure for such a complex might be one in which excess cholesterol is sequestered in the internal portion of the complex and filipin and/or the "primary" filipin–cholesterol complex is on the exterior, providing a hydrophilic surface. This would not invalidate the previous findings that the stoichiometry of binding of filipin to cholesterol in dilute aqueous solutions is 1:1 (Schroeder et al., 1972). Data to be published elsewhere have shown that the ratio of binding of pimaricin to cholesterol varies depending on the experimental conditions.

Spectral examination of the two complexes showed that their ultraviolet absorption spectra are the same. This would seem to indicate that, at least in aqueous medium, the simple, or what might be called the primary complex, and the larger complexes are spectroscopically detectable as proposed by Schroeder et al. (1972). The data do not support the existence of primary filipin–cholesterol complexes which are spectroscopically undetectable as proposed by DeKruijff & Demel (1974), but our data are consistent with formation of two types of complexes. One would be the simple or primary one with a 1:1 cholesterol stoichiometry; this could be the water-soluble one that does not reside in the vesicles or membranes. The other could be the larger one proposed above and could be the large aggregate proposed by DeKruijff et al. (1974a,b) and DeKruijff & Demel (1974); it also could be involved in the filipin-induced lipid rearrangements proposed by Kinsky and co-workers (1966, 1967b).

The data show that micromolar concentrations of filipin cause the lysis of rat erythrocytes even though the amount of erythrocyte-bound cholesterol is much greater than the total amount of filipin present. The lysis occurs under conditions where 5% or less of the filipin associates with the erythrocyte membrane. Furthermore, the amount of filipin bound to the cells was constant during the lysing process even though the cell/filipin ratio was varied (as done in Figure 5B in which the cholesterol/filipin ratio exceeded 10).

It is apparent from Figures 3A–C that filipin interaction with erythrocytes ends in a gross fragmentation of the cells. In addition, Figure 3B would seem to eliminate a mechanism whereby cholesterol was continually extracted from the membrane, eventually causing gross fragmentation. However, Figure 3C suggests that fragmentation is not the only cause of permeability changes in red cell membranes. It appears that, upon initial interaction with filipin, there is a transient efflux of K^+ into the supernatant. Subsequently, the erythrocytes recovered most of the lost K^+ before fragmentation of the cells occurred. This observation is consistent with the mechanism proposed by Tillack & Kinsky (1973), especially if filipin either removes or somehow changes the normal role of a small quantity of cholesterol which is critical for membrane integrity, i.e., for the formation of large aggregates. The initial loss of K^+ could then be a result of this filipin–cholesterol interaction.

The results of the experiment shown in Figure 2 indicate that dispersed lecithin is able to compete for some cholesterol which was already complexed with filipin. This is indicative of an equilibrium competition between phospholipid and filipin for cholesterol which is not as one-sided toward filipin as previously thought. This result is not unexpected since we have shown previously that detergents such as deoxycholate and lauryl sulfate can prevent the interaction of polyene antibiotics with sterols (Patterson et al., 1979).

Acknowledgments

We thank J. M. Patterson for the preparation of some of the filipin used in this study and M. R. Olinger for his helpful discussions.

References

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Batzri, S., & Korn, E. D. (1973) *Biochim. Biophys. Acta 298*, 1015–1019.

Bittman, R., & Fischkoff, S. A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3795–3799.

Bittman, R., Chen, W. C., & Anderson, O. R. (1974a) *Biochemistry 13*, 1364–1373.

Bittman, R., Chen, W. C., & Blau, L. (1974b) *Biochemistry 13*, 1374–1379.

Chen, P. S., Jr., Terepka, A. R., & Lane, K. (1964) *Anal. Biochem. 8*, 34–42.

DeKruijff, B., & Demel, R. A. (1974) *Biochim. Biophys. Acta 339*, 57–70.

DeKruijff, B., Gerritsen, W. J., Oerlemans, A., Demel, R. A., & Van Deenen, L. L. M. (1974a) *Biochim. Biophys. Acta 339*, 30–43.

DeKruijff, B., Gerritsen, W. J., Oerlemans, A., Van Dijck, P. W. M., Demel, R. A., & Van Deenen, L. L. M. (1974b) *Biochim. Biophys. Acta 339*, 44–56.
Feingold, D. S. (1965) *Biochem. Biophys. Res. Commun. 19*, 261–267.
Heider, J. G., & Boyett, R. L. (1978) *J. Lipid Res. 19*, 514–518.
Holland, J. F., Teets, R. E., & Timnick, A. (1973) *Anal. Chem. 45*, 145–153.
Huang, C. (1969) *Biochemistry 8*, 344–352.
Kinsky, S. C. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 1049–1056.
Kinsky, S. C. (1963a) *Arch. Biochem. Biophys. 102*, 180–188.
Kinsky, S. C. (1963b) *Antimicrob. Agents Chemother. (1962)*, 387–394.
Kinsky, S. C., Avruch, J., Permutt, M., Rogers, H. B., & Schonder, A. A. (1962) *Biochem. Biophys. Res. Commun. 9*, 503–507.
Kinsky, S. C., Luse, S. A., & Van Deenen, L. L. M. (1966) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 25*, 1503–1510.
Kinsky, S. C., Demel, R. A., & Van Deenen, L. L. M. (1967a) *Biochim. Biophys. Acta 135*, 835–843.
Kinsky, S. C., Luse, S. A., Zopf, D., Van Deenen, L. L. M., & Haxby, J. (1967b) *Biochim. Biophys. Acta 135*, 844–861.
Lampen, J. O., Arnow, P. M., Borowska, Z., & Laskin, A. I. (1962) *J. Bacteriol. 84*, 1152–1160.
Norman, A. W., Demel, R. A., DeKruijff, B., & Van Deenen, L. L. M. (1972a) *J. Biol. Chem. 247*, 1918–1929.
Norman, A. W., Demel, R. A., DeKruijff, B., Geurts Van Kessel, W. S. M., & Van Deenen, L. L. M. (1972b) *Biochim. Biophys. Acta 290*, 1–14.
Norman, A. W., Spielvogel, A. M., & Wong, R. G. (1976) *Adv. Lipid Res. 14*, 127–170.
Patterson, J. M., Olinger, M. R., Holland, J. F., & Bieber, L. L. (1979) *J. Antibiot.* (in press).
Rose, H. G., & Oklander, M. (1965) *J. Lipid Res. 6*, 428–434.
Schroeder, F., & Bieber, L. L. (1975) *Insect Biochem. 5*, 201–221.
Schroeder, F., Holland, J. F., & Bieber, L. L. (1972) *Biochemistry 11*, 3105–3111.
Schroeder, F., Holland, J. F., & Bieber, L. L. (1973) *Biochemistry 12*, 4785–4789.
Steck, T. L. (1974) *Methods Membr. Biol. 2*, 245–281.
Tillack, T. W., & Kinsky, S. C. (1973) *Biochim. Biophys. Acta 323*, 43–54.
Van Hoogevest, P., & DeKruijff, B. (1978) *Biochim. Biophys. Acta 511*, 397–407.
Verkleij, A. J., DeKruijff, B., Gerritsen, W. F., Demel, R. A., Van Deenen, L. L. M., & Ververgaert, P. H. J. (1973) *Biochim. Biophys. Acta 291*, 577–581.
Weber, M. M., & Kinsky, S. C. (1965) *J. Bacteriol. 89*, 306–312.

25-Hydroxyvitamin D_3 26,23-Lactone: A New in Vivo Metabolite of Vitamin D†

Joseph K. Wichmann, Hector F. DeLuca,* Heinrich K. Schnoes, Ronald L. Horst,‡ Richard M. Shepard, and Neal A. Jorgensen

ABSTRACT: A major vitamin D metabolite was isolated in pure form from the blood plasma of chicks given either maintenance levels or large doses of vitamin D_3. The isolation involved methanol–chloroform extraction and five column chromatographic procedures. The metabolite purification and elution position on these columns were followed by a competitive protein binding assay. The metabolite was identified, using high- and low-resolution mass spectrometry, 270-MHz proton nuclear magnetic resonance spectrometry, ultraviolet absorption spectrophotometry, Fourier transform infrared spectrophotometry, and specific chemical reactions, as 3β,-25-dihydroxy-9,10-seco-5,7,10(19)-cholestatrieno-26,23-lactone. The trivial names 25-hydroxyvitamin D_3 26,23-lactone or calcidiol 26,23-lactone are suggested for this compound.

The metabolism of vitamin D_3 to physiologically active forms has been well established (DeLuca & Schnoes, 1976; Kodicek, 1974; DeLuca, 1974). Vitamin D_3 is first hydroxylated in liver to 25-hydroxyvitamin D_3 (25-OH-D_3).[1] 25-OH-D_3 is then hydroxylated in kidney to form either 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the most active form of the vitamin, or 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$]. Other known metabolites include 25,26-dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$] and 1,24,25-trihydroxyvitamin D_3 [1,24,25-$(OH)_3D_3$]. These compounds, however, do not represent all vitamin D_3 metabolites. Other metabolites have been detected, several of which appear to have their circulating levels influenced by dietary factors such as calcium and phosphorus (Ribovich & DeLuca, 1978a–c).

During the course of developing an assay procedure for determining plasma levels of 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$, a previously unidentified compound was detected in chick plasma by using the competitive protein binding radioassay technique (Horst et al., 1979; Shepard et al., 1979). We wish to report here the isolation of this vitamin D_3 metabolite from plasma of chicks receiving maintenance and

† From the Department of Biochemistry and the Department of Dairy Science, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received March 28, 1979.* Supported by National Institutes of Health Program-Project Grant AM-14881 and Grant GM-00236 and the Harry Steenbock Research Fund.
‡ Present address: National Animal Disease Center, ARS, U.S.D.A., Ames, Iowa 50010.

[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,-24,25-trihydroxyvitamin D_3; MLP, plasma from chicks on maintenance levels of vitamin D_3; HDP, plasma from chicks given high doses of vitamin D_3; LC, high-pressure liquid chromatography; FT-IR, Fourier transform infrared spectroscopy; Me_3Si, trimethylsilyl; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide.

0006-2960/79/0418-4775$01.00/0