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Cholesterol limits estrogen uptake by liposomes and erythrocyte membranes

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Abstract

Multilamellar vesicles (MLV) were prepared from phospholipids with and without cholesterol in equimolar amounts and [4-^{14}C]estradiol. Unincorporated estrogen was removed by petroleum ether extraction or by aqueous buffer washes. In either case, cholesterol-containing vesicles incorporated one-half the estradiol as vesicles without sterol. Addition of estradiol to preformed vesicles followed by buffer washes showed that vesicles without cholesterol invariably retained more estradiol than those with the sterol. Reduction of the cholesterol content to one-half increased estradiol incorporation. The pattern of estradiol removal from MLV with successive buffer washes indicated that much of the steroid associated with cholesterol-containing vesicles was superficially bound to the membrane but vesicles without cholesterol incorporated the estrogen into the bilayer structure. To test the role of cholesterol in limiting the uptake of an estrogen by cells, right-side out resealed ghosts of ox erythrocytes were prepared. They were partially depleted of cholesterol by exposure to small unilamellar vesicles of dioleoylphosphatidyl choline. A decrease in cholesterol content correlates with an increase in estradiol uptake by red cell ghosts. The experiments described point to a central role of cholesterol in limiting the uptake of steroids. The loss of cholesterol of steroid producing cells caused by tropic hormones may be key to their mode of action in promoting secretion of steroid hormones. Likewise, the long-term genomic responses of steroid target cells may depend upon their cholesterol content and the ease by which the steroid can penetrate the membrane barrier.

Keywords: Cholesterol; Estradiol; Steroid transport; Membrane; Lipid bilayer

1. Introduction

Estradiol (E_2) is a lipid soluble steroid hormone that was thought to cross cell membranes by diffusion. Most of the known receptors for estradiol are intracellular, either cytoplasmic or nuclear [1]. Evidence has accumulated more recently that plasma membrane receptors for estrogens and other steroids may be present as well [2–10]. Evidence for these receptors is based on the kinetics and inhibition of steroid binding to particular cell types or membranes [8–10], although a few membrane proteins associated with steroid binding have been isolated [2–4]. McEwen [4] suggested that steroid binding to membrane proteins is associated with short-term cytophysiological events, such as ion fluxes, but that long-term genomic responses involving intracellular receptors [1,11,12] follow another route determined by membrane lipids.

It is generally assumed that the amount of E_2 that enters cells is determined by the concentration gradient between inside and outside of cells and by the amounts of hormone that may have become tightly bound to intracellular receptors or extracellular transport proteins [13]. Although the membrane has been recognized as a separate compartment in the transfer of estrogens [13], no consideration has been given to the influence of the molecular composition of the membrane on either the amount or the rate of steroid diffusion across the lamella. Bretscher and Munro [14] suggested recently that cholesterol decreases the permeability of lipid bilayers of small molecules, such as

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water or glucose, by decreasing the transient cavitation among unsaturated paraffin side chains of phospholipids.

In this paper, we present evidence that cholesterol embedded in bilayers of single or mixed phospholipids or in native membranes limits the uptake of estradiol. We used multilamellar vesicles (MLV) of varied phospholipid composition and cholesterol content as models of the lipid portion of plasma membranes. We studied both the incorporation of E_2 into vesicles and the amounts that diffuse into and out of vesicles. The results show that phospholipid composition has some influence on the amount of E_2 incorporated or desorbed from MLV, but that cholesterol has a much greater effect. Studies on E_2 uptake by erythrocyte ghosts partially depleted of cholesterol confirm that membranes lacking sterol take up more estrogen than membranes loaded with cholesterol. Since cholesterol is a major component of plasma membranes [15], these findings may have significant physiological and medical implications.

2. Materials and methods

Phospholipids were obtained from Avanti Polar Lipids, Alabaster, AL. [4-^{14}C]Estradiol, specific activity 110.2 Ci/mmol, was purchased from Amersham (Arlington Heights, IL). Cholesterol was recrystallized twice from hot methanol. All other reagents and solvents were of analytical grade and were used without further purification.

MLV were prepared from single phospholipids or from combinations of phospholipids proportioned to reflect the composition of cell membranes from various species. Sheep and bovine erythrocyte membranes were modeled by a 1:1 molar ratio of sphingomyelin (SP) and dioleoylphosphatidylethanolamine (DOPE) [16]. Human erythrocytes were modeled by a 1:1:2 molar ratio of SP, dioleoylphosphatidylcholine (DOPC) and DOPE. For comparison, a lipid mixture typical of plant membranes, 1:1 molar DOPC/DOPE, was also included. Cholesterol was added to appropriate samples in amounts equimolar to phospholipid. Details for the preparation of MLV were described previously [17,18].

For studies of E_2 incorporation into MLV, amounts corresponding to approx. 20000 cpm of undiluted [4-^{14}C]estradiol, dissolved in 20 μl methanol, were added to 500 μg of phospholipids, dissolved in 50 μl of chloroform, with or without 250 μg cholesterol, dissolved in 50 μl of chloroform. The solvents were evaporated under N_2. Vesicles were formed by suspending the dried lipid films in 1.0 ml 0.05 M KH_2PO_4-NaOH buffer, pH 6.8, agitating with a glass bead on a Vortex mixer and then diluting to 1.5 ml with phosphate buffer. The amount of E_2 incorporated into the vesicles was determined in the remainder after extracting the MLV three times, each with 1.5 ml portions of petroleum ether. Estradiol not extractable with petroleum ether was removed by extracting the vesicle suspension three times with 1.5 ml portions of chloroform/methanol (2:1, v/v). Aliquots of all fractions, or of pooled fractions, were subjected to scintillation counting in 5 ml of Ecoscint solution (National Diagnostics, Manville, NJ) in a Beckman LS-7800 scintillation counter (Beckman Instruments, Irvine CA). It was found that the vesicle suspension after petroleum ether extraction could be counted directly without extraction into chloroform/methanol; both methods gave identical results. Controls without lipids were run simultaneously. The validity for the differential solvent extraction of MLV for incorporated or unincorporated steroid has been demonstrated previously [17].

Studies of E_2 uptake by MLV were carried out by preparing vesicles with and without equimolar cholesterol, centrifuging them in 1.5 ml conical bottom tubes for 20 min at 13000 rpm in a microcentrifuge, removing the supernatant (which contained small vesicles that would not sediment at this speed) and resuspending the pelleted vesicles in 1.0 ml 0.05 M phosphate buffer, pH 6.8. At time zero, 20 μl portions of [4-^{14}C]estradiol in methanol solution were added sequentially to samples of MLV prepared with and without cholesterol. Samples were agitated and incubated in a 37°C water bath for time periods varying between 1 and 30 min. To verify the total number of cpm in the samples, 100 μl of the mixed vesicle suspension were removed and counted. At the end of the incubation times, samples were centrifuged, as before, and portions of 100 μl of supernatant were removed and counted. The amount of E_2 bound was taken as the difference between total E_2 recovered and amount remaining in the supernatant after centrifugation. These experiments were repeated at least three times for each lipid combination tested.

Desorption of estrogen from lipid vesicles loaded by exposure to [4-^{14}C]estradiol was studied. Vesicles prepared with and without cholesterol were transferred to microcentrifuge tubes, as described before. 20 μl of methanol with [4-^{14}C]estradiol (ca. 20000 cpm) were added to each tube. Tubes were agitated briefly and incubated at room temperature (22°C) for varying lengths of time from 5 to 30 min prior to centrifuging. A zero time sample was also taken for each group of tubes. At the end of the incubation, samples were centrifuged for 20 to 30 min. Supernatant solutions from each tube were removed. The pelleted vesicles were resuspended in 1.0 ml 0.05 M phosphate buffer, agitated for 20 s and spun down again. This procedure was repeated a total of three times. At the end of the procedure, 100 μl aliquots were removed from each supernatant fraction and counted. The pellets remain-

ing after the last wash also counted in Ecoscint. The E_2 in the first supernatant represented unbound steroid. The amounts in the subsequent three washes were amounts desorbed by diffusion from exogenously loaded vesicles.

Studies of E_2 uptake were done on native membranes with normal and reduced cholesterol content. Whole and cholesterol depleted membranes were produced from right side out, resealed ghosts of ox erythrocytes. Ox erythrocytes were obtained from Carolina Biological Supplies, Burlington, NC. Ghosts were prepared by the method of Hanahan and Ekholm [19] and resealed by the method of Steck [20]. Cholesterol depletion of ghosts followed the protocol of Lange et al. [21]. Small unilamellar vesicles (SUV) were prepared by sonication of MLV prepared from DOPC, 1.0 mg phospholipid/ml. Varying amounts of SUV suspension were incubated with resealed ghosts in 500 μl aliquots (hematocrit = 14%). Samples were run in duplicate and were matched by controls containing the same quantity of ghosts plus a volume of buffer equal to the volume of SUV suspension added to the first group of samples. The paired samples were incubated for 16 h at 37°C with constant shaking. At the end of the incubation time, all samples were centrifuged at 13000 rpm in a microcentrifuge for 10 min. Supernatants were removed and the ghosts were washed once with 500 μl isotonic phosphate buffer. Supernatants from each wash were pooled with initial supernatants and assayed for cholesterol content. Washed samples of ghosts were each resuspended in 500 μl of buffer. 10 μl of methanol with [4-^{14}C]E_2, containing 22500 cpm, were added to half of the control samples and to half of the liposome incubated samples. All samples were incubated at 37°C for 15 min and then centrifuged as before for 10 min. Supernatants were withdrawn from each sample. 200 μl of supernatant solution were removed and counted. Each of the residual ghost preparations were suspended in 200 μl buffer and transferred to scintillation vials for counting. Amounts of E_2 in supernatants and ghosts were determined from the counts in each fraction. Recovery of radioactive label was 100% of the amount added. The cholesterol content of ghosts and supernatants were determined by the methods of Zlatkis et al. [22] and Rose and Oklander [23].

3. Results

Estradiol incorporation into MLV constructed with and without equimolar cholesterol is shown in Figs. 1 and 2. Each figure represents the amount of E_2 remaining in vesicles after three extractions with petroleum ether. In controls without added lipids, 98% of the radioactivity was extractable into the organic phase. No matter which phospholipid or combination of phospholipids were used to construct the vesicles, those without cholesterol always incorporated more steroid than those with cholesterol. Vesicles made from individual phospholipids without cholesterol did not differ from each other in the amount of E_2 incorporated, although they bound one-third to one-half more steroid than vesicles prepared from phospholipid combinations without cholesterol (Fig. 2). MLV made from single species of phospholipid with cholesterol incorporated half or less the amount of E_2 as vesicles made without sterol (Fig. 1). Presence of equimolar cholesterol in MLV halved the amount of E_2 incorporated

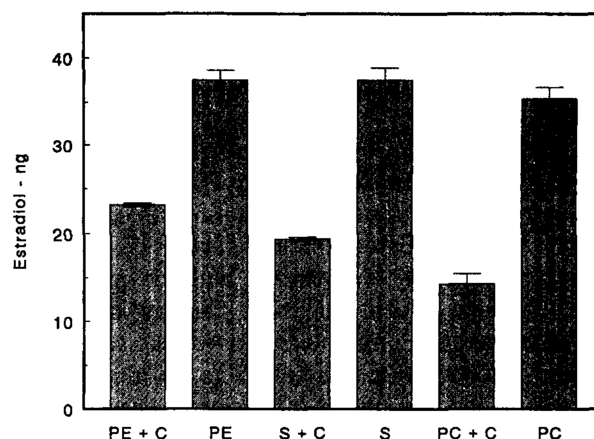

Fig. 1. Estradiol incorporation into MLV prepared from DOPE (PE), DOPC (PC) or sphingomyelin (S), with or without cholesterol (C) equimolar to the phospholipids (PL). MLV were prepared from 500 μg PL and 250 μg C, where indicated, plus 62.5 ng of [4-^{14}C]estradiol in 1.5 ml phosphate buffer. MLV were washed exhaustively with petroleum ether. Radioactive estradiol remaining in the buffer phase was considered as bound to MLV. For details, please see Materials and methods. Each value is the average for three independent trials, performed in triplicate. Bars show standard deviation.

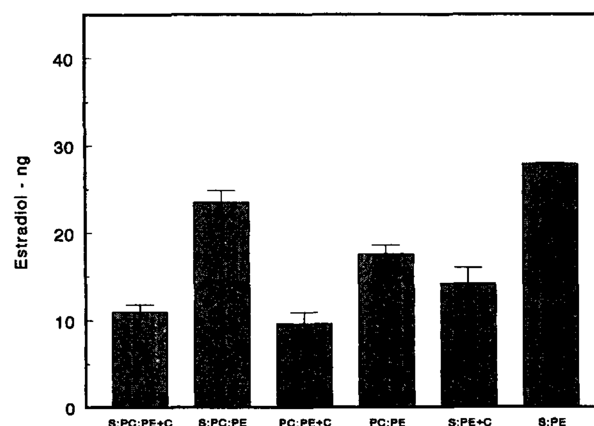

Fig. 2. Estradiol incorporation into MLV prepared from phospholipid mixtures, with and without cholesterol equimolar to the phospholipid. SP/DOPC/DOPE were in a 1:1:2 molar ratio; SP/DOPE and DOPC/DOPE were in a 1:1 molar ratio. Remainder of legend is as in Fig. 1.

into mixed lipid vesicles as well (Fig. 2). Vesicles made with SP in combination with other phospholipids incorporated more E_2 than those composed of DOPC and DOPE.

The uptake of E_2 by MLV made from combinations of phospholipids with and without cholesterol was studied as related to time of incubation. Suspensions of vesicles were incubated for time periods of 1 to 30 min with exogenous [4-^{14}C]estradiol and the phases were then separated by centrifugation. The amounts of MLV and of steroid (62.5 ng) incubated was the same as that in the incorporation studies. The results were expressed as the amount of E_2 bound to vesicles. Data are shown in Figs. 3, 4 and 5. Each of the lipid combinations without cholesterol bound more E_2 than equivalent vesicles with equimolar cholesterol, a result consistent with observations from the incorporation studies. SP/DOPE (1:1 molar ratio) vesicles without cholesterol bound 2.5 times as much steroid as vesicles with cholesterol (Fig. 3). The maximum binding of steroid to vesicles without cholesterol is about the same as the amount incorporated into similar vesicles in the first study. Vesicles with equimolar cholesterol bound less than half the quantity bound by vesicles without sterol. SP/DOPE vesicles bound nearly maximal amounts of E_2 within the first minute of incubation, and the amount bound did not change with time.

Vesicles of SP/DOPC/DOPE (1:1:2 molar ratio) without cholesterol exhibited a similar pattern of steroid binding to SP/DOPE (Fig. 4), but the time dependence of E_2 binding was more pronounced. Incubation for 3 min was required to reach maximal binding. Comparisons of the amounts of E_2 bound by SP/DOPC/DOPE vesicles with the amounts bound by SP/DOPE vesicles are difficult to make because the former did not sediment as uniformly as the latter on centrifugation. There was always some loss of SP/DOPC/DOPE vesicles upon each centrifugation step and, therefore, data for E_2 retention by these vesicles are factitiously low. The amounts of steroid bound by SP/DOPC/DOPE vesicles without sterol are similar to the amounts associated with SP/DOPE vesicles. The amount of steroid bound by SP/DOPC/DOPE vesicles with cholesterol is double that associated with SP/DOPE vesicles with sterol.

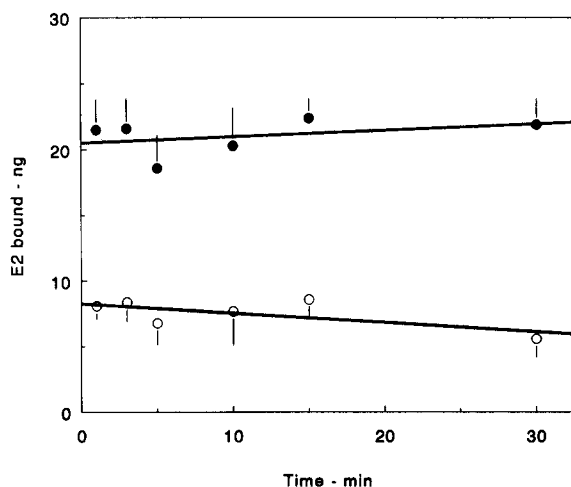

Fig. 3. Estradiol binding to MLV prepared from SP/DOPE (1:1) with and without cholesterol equimolar to the phospholipid, as function of time. MLV were prepared in 1.5 ml buffer, as described under Methods; at the beginning of the trial 62.5 ng of [4-^{14}C]estradiol in 20 μl of methanol were added to each sample of a series. Samples were incubated at 37°C for the indicated time, centrifuged and assayed. Values are the means and standard deviations for five independent trials. Curves were drawn by SlideWrite Plus, version 6, by linear correlation analysis. Closed circles, MLV without cholesterol; open circles, MLV with cholesterol.

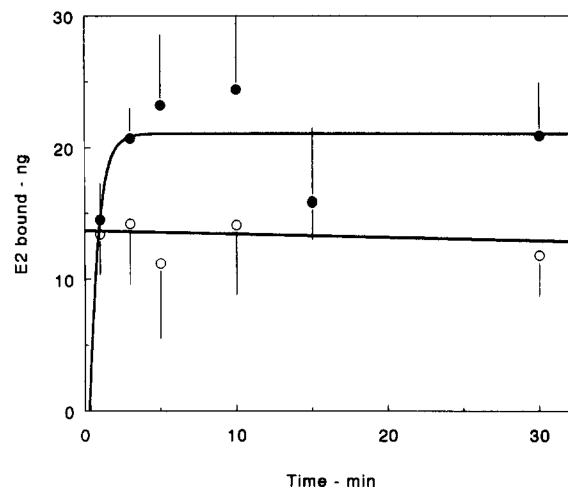

Fig. 4. Estradiol binding to MLV prepared from SP/DOPC/DOPE (1:1:2), with and without cholesterol equimolar to the phospholipid, as function of time. Procedure as in Fig. 3. Closed circles, MLV without cholesterol; open circles, MLV with cholesterol.

Vesicles made from DOPC/DOPE (1:1 molar ratio) which had incorporated the least amount of E_2 in the incorporation study took up the largest amounts in the binding study (Fig. 5). Vesicles without cholesterol bound twice as much E_2 as vesicles with sterol; maximal binding occurred within 5 min of adding steroid to the vesicle suspension. In vesicles without cholesterol, the amount of E_2 bound to DOPC/DOPE was greater than the amount bound to SP/DOPE and approximately equal to the amounts bound by SP/DOPC/DOPE vesicles. In vesicles with cholesterol, DOPC/DOPE bound one-third more steroid than SP/DOPE, but about the same amount as SP/DOPC/DOPE vesicles.

The desorption of E_2 from vesicles incubated with exogenous [4-^{14}C]estradiol is shown in Figs. 6–8. The bars in these figures indicate the initial binding of E_2 to MLV, the quantity of E_2 removed in each of three successive washes with buffer and the amount remaining in the final pellets. As control, [4-^{14}C]estradiol was

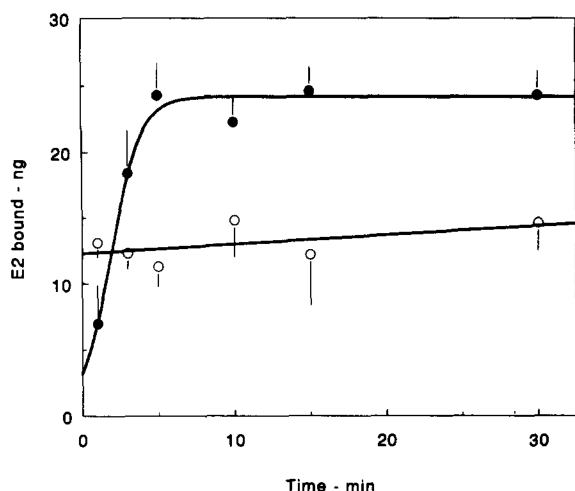

Fig. 5. Estradiol binding to MLV prepared from DOPC/DOPE (1:1), with and without cholesterol equimolar to the phospholipid, as function of time. Procedure as in Fig. 3. Closed circles, MLV without cholesterol; open circles, MLV with cholesterol.

added to buffer alone, with no MLV, and the results are shown in Fig. 6. Of 62.5 ng added at time zero, only 2 ng remained with the lipid phase after the initial centrifugation and separation of phases. This small amount may represent steroid adhering to the plastic walls of the tubes and most of it was removed in the first wash so that less than 0.05 ng remained. Vesicles made from SP with equimolar amounts of cholesterol, retained 4 ng of E_2 after phase separation (Fig. 6). Most of the small amount remaining, 2.6 ng, was removed in the first wash, 0.3 ng in the second, and 0.2 ng in the third. The final pellet retained 0.5 ng E_2.

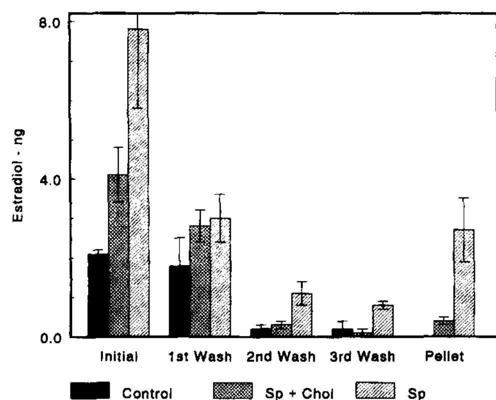

Fig. 6. Removal of estradiol from MLV prepared from Sp, with and without equimolar cholesterol, after their incubation with the estrogen. MLV were incubated with [4-^{14}C]estradiol for 15 min at 37°C, separated from the supernatant and washed successively with 1.5 ml portions of buffer. As control, [4-^{14}C]estradiol alone, without MLV, was incubated. Radioactivity was determined in all fractions, including the remaining pellets. Details are described under Materials and methods. The initial values show the total estrogen associated with the MLV and were obtained by difference between the amount added and the amount in the first supernatant. All values are means and standard deviations for three experiments.

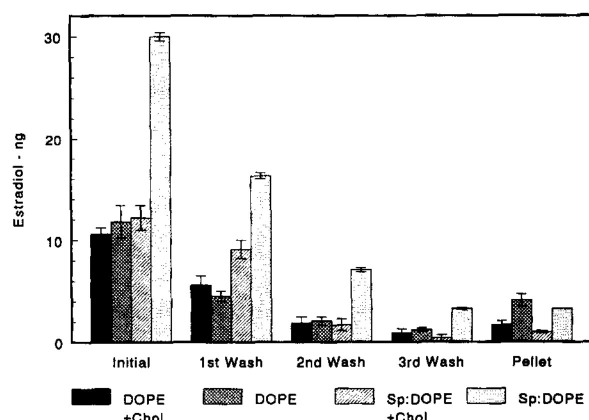

Fig. 7. Removal of estradiol from MLV prepared from DOPE and SP/DOPE (1:1), with and without equimolar cholesterol, after their incubation with the estrogen. Conditions are described in Fig. 6.

Vesicles made from SP without cholesterol retained 7.8 ng of the initial 62.5 ng E_2 to which they were exposed. After three washes, 3.7 ng remained in the pellet. MLV without cholesterol retained 7.4-times more estrogen than vesicles without sterol.

A similar experiment with DOPE vesicles is shown in Fig. 7. The pattern of results was similar to that obtained with SP, but the amount of steroid initially bound to vesicles was higher. Results for phospholipid combinations of SP/DOPE, SP/DOPC/DOPE and DOPC/DOPE with and without equimolar cholesterol are summarized in Figs. 7 and 8. The amounts of E_2 initially bound to MLV of phospholipids and cholesterol were always less than amounts bound to vesicles without cholesterol; the most striking difference occurred in the SP/DOPE vesicles (Fig. 7). DOPE vesicles and vesicles with more than one species of phospholipid bound 2- to 5-times as much steroid as SP vesicles (Figs. 7 and 8). The difference between the initial amount of steroid bound and the amount re-

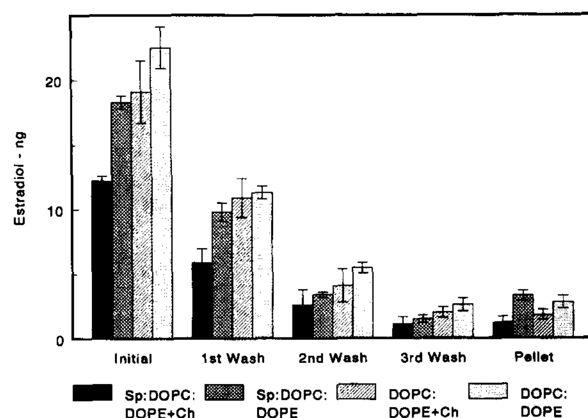

Fig. 8. Removal of estradiol from MLV prepared from SP/DOPC/DOPE (1:1:2) and DOPC/DOPE (1:1), with and without equimolar cholesterol, after their incubation with the estrogen. Conditions are described in Fig. 6.

Table 1
Release of estradiol from MLV exposed to exogenous estrogen

Lipid composition of MLV	Estradiol bound initially (%)	Estradiol removed (%)		
		1st wash	2nd wash	3rd wash
Sp/DOPE + Chol	18.1 ± 0.2	76.1 ± 1.8	51.9 ± 3.7	23.1 ± 7.7
Sp/DOPE	48.0 ± 0.6	54.3 ± 1.5	51.8 ± 1.5	50.0 ± 1.5
Sp/DOPC/DOPE + Chol	19.5 ± 0.6	66.4 ± 2.0	51.2 ± 2.4	45.0 ± 5.0
Sp/DOPC/DOPE	29.3 ± 0.8	53.6 ± 3.8	40.0 ± 2.4	29.4 ± 5.9
DOPC/DOPE + Chol	30.6 ± 3.8	57.0 ± 5.2	37.8 ± 9.8	35.1 ± 7.0
DOPC/DOPE	36.0 ± 2.6	50.2 ± 2.2	49.1 ± 3.6	45.6 ± 8.8

maining with the final pellet is the amount released from the vesicles. MLV with cholesterol always released less E_2 than those lacking the sterol. The incorporation and desorption studies show that cholesterol limited the amount of E_2 that associated with lipid bilayers, but the location of the steroid in the bilayers was not apparent.

An analysis of the results allowed us to describe the topography of E_2 with respect to structure of membrane lipids. Estradiol may have been intercalated between the acyl side chains of phospholipids or it may have been loosely bound to the surface of the bilayers or both events may occur to some extent. If all of the steroid were intercalated into the inner bilayer structure, repeated washing of vesicles with buffer should produce a consistent partition pattern of steroid into the aqueous phase, i.e., the percentage of E_2 removed from MLV on each successive wash should be the same. If the steroid were loosely bound to the surface, the first wash should remove a high percentage and subsequent washes successively smaller percentages. The assumption is that the surface of bilayers consists of a more heterogeneous population of binding sites than the hydrocarbon inner core of the bilayer. If there were two or more sub-populations of E_2 associated with vesicles, most of the superficially bound steroid should be removed in the first wash, along with a small amount of incorporated steroid that partitioned into the aqueous phase. In this case, the initial wash would remove a larger percent of E_2 associated with MLV than subsequent washes. The percentage of E_2 removed in subsequent washes should be identical.

To distinguish among these possibilities, the percent of steroid removed in each buffer wash as a percent of total estradiol in that fraction was calculated from the data presented graphically in Figs. 6, 7 and 8 and is shown in Tables 1 and 2. The percent of E_2 initially incorporated or bound as function of total added was determined. The difference between the initial amount of E_2 added (62.5 ng) and the sum of the amounts in the three washes plus the amount in the final pellet was numerically equal to the amount in the first buffer supernatant. The percent of E_2 removed in the 1st wash was calculated by dividing the amount of E_2 in the supernatant by the total amount of E_2 in that fraction, calculated by difference of the total amount in the previous fraction and the amount removed in its supernatant.

Data obtained for MLV prepared from mixed phospholipids, with and without equimolar cholesterol, and with [4-^{14}C]E_2 are shown in Table 1. For all three types of vesicles examined (SP/DOPE, 1 : 1; SP/DOPC/DOPE, 1:1:2; and DOPC/DOPE, 1:1; the values expressed as molar ratios), the percent of E_2 initially incorporated was at least twice as great if cholesterol was lacking. SP/DOPC/DOPE vesicles incorporated more E_2 than the other types. The data for E_2 removal by successive washes of vesicles lacking sterol were consistent. For any given type of sterol-free MLV, the percent E_2 removal in each wash was the same within the experimental limits. Vesicles with cholesterol showed a different pattern. The percent E_2 removed in the first wash from vesicles of SP/DOPE with equimolar cholesterol was 1.5-times higher than the percent removed in the second wash. The percent

Table 2
Estradiol extractable from MLV prepared with the estrogen

Lipid composition of MLV	Estradiol Associated with MLV (%)	E_2 removed (%)		
		1st wash	2nd wash	3rd wash
Sp/DOPE + Chol	11.8 ± 0.0	75.7 ± 1.3	50.0 ± 1.8	22.2 ± 11.1
Sp/DOPE + Chol *	17.8 ± 6.1	68.5 ± 20.7	57.1 ± 25.7	46.7 ± 13.3
Sp/DOPE	29.4 ± 1.0	57.1 ± 2.2	54.4 ± 3.8	47.2 ± 2.8
Sp/DOPC/DOPE + Chol	23.2 ± 2.1	63.4 ± 8.3	54.7 ± 3.8	50.0 ± 4.2
Sp/DOPC/DOPE + Chol *	28.5 ± 1.8	60.7 ± 3.9	61.4 ± 10.0	63.0 ± 0.0
Sp.DOPC/DOPE	41.8 ± 0.1	46.4 ± 0.2	45.0 ± 1.4	42.9 ± 1.3
DOPC/DOPE + Chol	15.5 ± 2.2	66.0 ± 3.1	63.6 ± 36.4	25.0 ± 8.3
DOPC/DOPE + Chol *	38.7 ± 5.8	43.3 ± 4.1	47.6 ± 3.1	45.6 ± 13.2
DOPC/DOPE	35.0 ± 0.8	49.8 ± 9.1	49.1 ± 0.9	46.4 ± 3.6

* These samples were prepared with 1/2 molar equivalent of cholesterol, rather than equimolar amounts.

removed in the second wash was twice as high as that removed in the third wash. Vesicles of other lipid combinations gave similar patterns. Data for all vesicles prepared without cholesterol were consistent with a model of estradiol where the steroid is intercalated between lipid bilayers, whereas data for vesicles prepared with cholesterol were those expected for a model of estradiol bound to the surface of the phospholipid bilayer.

The differing patterns of E_2 desorption from vesicles containing or lacking sterol indicated that cholesterol competes with E_2 for a position within the bilayer, preventing the internal membrane binding of estradiol and facilitating the surface binding of the estrogen. With this hypothesis, reduction of the molar ratio of cholesterol to phospholipid should result in less surface binding and increased incorporation of E_2. Data for vesicles prepared with a 0.5 : 1 cholesterol to phospholipid ratio are shown in Table 2. The percentage of E_2 initially associated with vesicles was increased compared to the percentage associated with vesicles made with a 1 : 1 molar ratio of cholesterol to phospholipid. DOPC/DOPE vesicles with 1/2 the molar amount of cholesterol bound as much E_2 as vesicles without any sterol. The percent E_2 removed from vesicles by buffer washes was approximately the same for each successive wash, suggesting that E_2 was intercalated into the lipid structure rather than surface bound.

To further explore the possibility that cholesterol limits incorporation of E_2 into lipid bilayers and increases surface binding, we prepared the same vesicles used in the previous study with and without equimolar cholesterol, but without incorporating [4-^{14}C]estradiol into the lipid mixtures. These vesicles, once prepared, were incubated with exogenous [4-^{14}C]estradiol and then centrifuged. Supernatants were discarded and percentages of E_2 initially bound and removed by successive buffer washes were determined. Data are shown in Table 2. Except in the case of SP/DOPC/DOPE, the percent of estradiol bound was higher than in the experiments were the steroid was added to the lipid mixture prior to preparing the vesicles. Here again, the percent of E_2 initially bound by vesicles without cholesterol was higher than the percent bound by vesicles with cholesterol. Determination of the pattern of extraction into successive buffer washes showed that vesicles with cholesterol lost a greater percentage of E_2 in the initial wash than in subsequent washes, whereas vesicles without cholesterol tended to lose the same percentage of E_2 in each wash. MLV prepared from SP/DOPC/DOPE and exposed to estrogen may present an anomaly because these vesicles are difficult to sediment. Formed vesicles exposed to E_2 follow the same pattern of E_2 release into buffer as vesicles prepared in presence of the estrogen. These data confirm that vesicles with cholesterol are associated with two or more populations of E_2, one loosely bound to heterogeneous binding sites on the surface, the other incorporated into the hydrocarbon core of the bilayers. Vesicles without cholesterol incorporate E_2 into the inner hydrocarbon core.

Table 3
Ratio of estradiol release from MLV prepared with cholesterol and MLV prepared without cholesterol

Phospholipid composition	Exposure to E_2 *	Ratio
Sp/DOPE (1:1)	Internal	2.5
Sp/DOPE (1:1)	External	2.6
Sp/DOPC/DOPE (1:1:2)	Internal	1.8
Sp/DOPC/DOPE (1:1:2)	External	1.4
DOPC/DOPE (1:1)	Internal	2.1
DOPC/DOPE (1:1)	External	1.1

* MLV were prepared in the presence of estradiol (internal) or the MLV were prepared and then exposed to estradiol (external).

Data for desorption of E_2 from either endogenously or exogenously loaded MLV illustrate one other point with regard to the amounts of estrogen that cross the bilayers. Vesicles without cholesterol incorporate more steroid than those with equimolar amounts of cholesterol. After three washes with buffer, vesicles still retain steroid in roughly the same proportions as the initially loaded vesicles. But if the difference between the amount of E_2 bound initially and the amount of E_2 in final pellets is calculated, vesicles without sterol lose between 1.1 and 2.6-times more E_2 than vesicles with sterol (Table 3). Since all of the vesicle preparations were washed for the same lengths of time under the same conditions, the data imply that the quantity of estrogen desorption from bilayers lacking cholesterol is higher than the quantity of estrogen desorption from similar bilayers but including cholesterol.

If the steroid estrogen enters cells by diffusing through the lipid bilayer portion of plasma membranes, a depletion of cholesterol from plasma membranes should increase the amount of steroid entering cells. We tested this hypothesis by preparing right side out, resealed ghosts of ox erythrocytes and partially depleting one-half of them of cholesterol by incubating with DOPC vesicles. 500 μl of ghosts (hematocrit = 14%) were incubated for 16 h with 100, 300 or 500 μl of small unilamellar vesicles of DOPC, at 37°C, washed with buffer, centrifuged and separated. The ghosts were made up to 500 μl with buffer. Untreated ghosts contained 68 to 75 μg cholesterol per experimental sample; ghosts incubated with various amounts of DOPC vesicles had between 54 and 68 μg cholesterol. [4-^{14}C]Estradiol, 62.5 ng and 20500 cpm, were added to all samples of ghosts and these were incubated at 37°C for 15 min, centrifuged and separated. The radioactivity of untreated and cholesterol-depleted ghosts

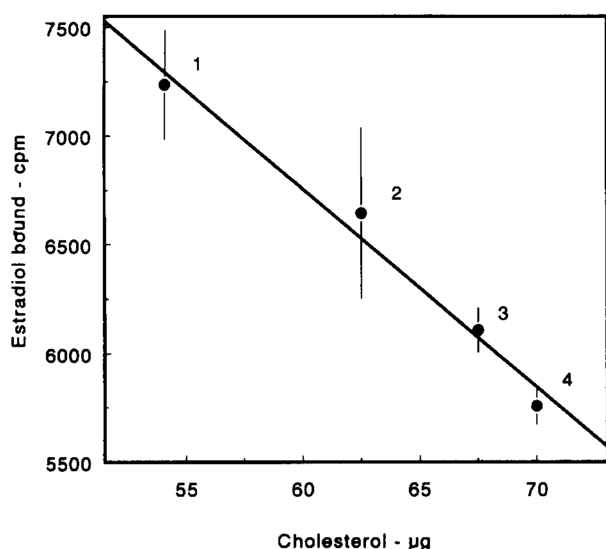

Fig. 9. Binding of [4-^{14}C]estradiol to right-side-out resealed membranes of cholesterol-depleted ox erythrocytes. 500 μl of a membrane suspension in phosphate buffer at pH 6.8, with a membrane volume of 14%, were incubated with varying amounts of liposomes of DOPC, containing 1 mg phospholipid per ml. After 16 h at 37°C, the membranes were separated and washed once with buffer. They were then made up to 500 μl with buffer and incubated for 15 min at 37°C with 62.5 ng of [4-^{14}C]estradiol. Membranes were separated from supernatant and their cholesterol and estradiol contents were measured. Further details are given under Methods. Points 1, 2 and 3 show data, respectively, for incubations with 500, 300 and 100 μl of liposome suspension. Point 4 is for controls incubated with buffer alone. Points are averages for three determinations and show standard deviations as vertical bars.

were determined. Results are shown in Fig. 9. Untreated ghosts took up about one-third of the labeled steroid. Cholesterol-depleted ghosts incorporated more of the steroid, proportional to the quantity of cholesterol removed; i.e., the less cholesterol in the membranes, the greater the amount of E_2 incorporated.

4. Discussion

Estrogens are unionized and lipophilic at physiological pH. Existing in the non-polar state, they are generally thought to cross the lipid matrix of the cell membrane by diffusion [24]. Although membrane receptors for steroids have been identified, it is not at all clear whether such bound steroids can enter the cell. Steroid-receptor complexes have been observed in the cytoplasm and in the nucleus, bound to DNA [1] where they presumably act as enhancers to regulate the activity of selected genes [25]. Estrogenic effects on gene expression are thus dependent on two factors: the ability of the hormone to enter the cell and the likelihood that it will bind to its internal receptor.

Studies of adsorption of E_2 to mixed lipid vesicles showed that steroid was rapidly picked up by vesicles prepared with and without cholesterol, but the presence of sterol limited the total amount of E_2 bound. The phospholipid composition of the vesicles had little effect upon the binding of E_2. Studies of desorption of steroid from MLV into which E_2 had been incorporated or to which E_2 had been added exogenously yielded two interesting observations. First, vesicles made with cholesterol bound and retained less E_2 than vesicles made without sterol; and second, they exhibited a much greater propensity for surface binding of E_2 than vesicles without sterol. The latter observation can well account for the saturable estrogen binding to low density subcellular fractions of hepatocyte plasma membranes, described by Pietras and Szego [26]. The authors attributed this binding to a cell surface receptor but the receptor was never identified.

In these studies, we examined the incorporation and desorption of estradiol from MLV constructed from phospholipid mixtures typical of various cell membranes, with and without cholesterol equimolar to phospholipid. We also looked at the binding of estradiol to resealed erythrocyte ghosts partially depleted of cholesterol. Other studies in our laboratory using [4-^{14}C]cholesterol as marker indicated that over 90% of cholesterol mixed with phospholipid in the construction of MLV is incorporated into the vesicles (data not shown). The concentrations of phospholipid and of cholesterol in media utilized to make MLV were approx. $6.7 \cdot 10^{-4}$ M. The concentration of estradiol was $2.3 \cdot 10^{-7}$ M, or 3000-times less than the molar concentration of cholesterol. Mixed phospholipid vesicles without cholesterol incorporated between 27 and 45% of added estradiol, whereas vesicles with cholesterol incorporated 15 to 22%.

The observation that cholesterol did not completely block incorporation of estradiol into vesicles is consistent with a model of cholesterol in which the sterol reduces cavitation among acyl side chains in a lipid bilayer [14]. Cavities would be expected to favor entry of estradiol. An alternative interpretation of the data suggests the existence of a competition between cholesterol and estradiol for the same position in the bilayer. Neither of these possibilities explains why, in the presence of a large excess of cholesterol, estradiol can accumulate at or near the surface of the bilayer without entering the cell. This phenomenon was especially noticeable in bilayers containing sphingomyelin (Tables 2 and 3). Perhaps the 3-hydroxy group of cholesterol interacts with the amide group linking the fatty acyl side chain of sphingomyelin and creates a favorable binding site for the estrogen. This could lead to a stacking effect of estradiol.

It has been stated that the principal reason for having cholesterol in the plasma membrane is to make the bilayer less permeable to small water-soluble molecules, such as glucose [14]. Cholesterol promotes

the ordering of phospholipid side chains in a bilayer and in its absence fatty acid side chains gain in flexibility. Estrogens are not considered components of biological membranes, yet during their passage across the membrane layer they must become transiently associated with the phospholipid components. The vacancy of cavitational sites created by the removal of cholesterol may present increased opportunities for the association of estrogens and the fatty acid side chains of the bilayer.

The desorption studies showed that vesicles with no or reduced cholesterol gave up quantitatively more estrogen than vesicles with an equimolar ratio of cholesterol to phospholipid. On examining the uptake of E_2 by erythrocyte ghosts with depleted cholesterol content, we found that native membranes mirror the behavior of lipid vesicles: the less cholesterol in the membrane, the more E_2 was bound. Cholesterol limits the amount of estradiol that can enter or transverse the lipid portion of a biological membrane.

The amount of estradiol that circulates in human blood is in the order of 21–68 pg/ml plasma in normal females [27], equivalent to $2.5 \cdot 10^{-10}$ M at the highest. The number of E_2 molecules that a cell will encounter is not large. Any impediment in the membrane to hinder steroid crossing will decrease the opportunity for cytosolic binding of steroid molecules to their receptors.

The notion that the loss of cellular cholesterol accompanies the action of tropic hormones upon their target tissue is well recognized. Sayers et al. [28] noted in 1944 that synthesis and secretion of corticosteroids in the adrenal cortex in response to ACTH was accompanied by a pronounced drop in the cholesterol content of the adrenocortical tissue. Since the adrenal cortex contains 5% cholesterol, a substantial amount of the sterol is lost. Sayers et al. [28] suggested that cholesterol might be used in the synthesis of corticoids. It is generally thought at present that although cholesterol is the precursor of corticoids it is likely that this cholesterol arises mainly from de novo synthesis starting with acetate and only to a small extent from storage or plasma cholesterol [29]. Studies by Hochberg et al. [30] showed that cholesterol sulfate in the adrenal gland can be converted to steroids without loss of the sulfate group. Whether cholesterol or its sulfate are the steroid precursors in adrenal tissue, the quantity of cholesterol converted to corticosteroid is insufficient to account for the amount of cholesterol lost from tissue stimulated by ACTH.

The depletion of cholesterol from ovarian tissue as a response to LH stimulation of estrogen synthesis and secretion has been described [25,31–33]. Both acetate and cholesterol function as estrogen precursors in ovarian tissue [34,35], but the available evidence shows that steroid synthesis occurs largely from cholesterol synthesized de novo [36,37]. Steroid synthesis cannot account for the cholesterol depletion observed in response to LH stimulation. The rapid loss of cholesterol from adrenal and ovarian cells in response to regulatory hormones may represent a mechanism for allowing the steroid to leave the cells. We suggest that one of the effects of peptide hormones is to deplete membrane cholesterol and thereby promote the secretion of steroid hormones. A similar mechanism can be envisioned for changes in lipid composition of target cell membranes when a steroid hormone enters, facilitated by loss of membrane cholesterol.

Steroid hormones mediate a wide variety of developmental and reproductive outcomes by activating genes in selected target tissues or by some as yet unknown mechanism. In related events, peptide hormones trigger steroid synthesis and/or release from their target cells. The cell regulatory, developmental and reproductive effects elicited by steroid hormones require that they enter target tissue cells, while biosynthetic operations are followed by steroid release from cells of their origin. Estrogens are suspected cocarcinogens in breast carcinoma [38]. Growth of neoplastic tissues may be prompted when a cell with genetically altered growth control is further stimulated by the inappropriate uptake of steroid. We propose that the cholesterol content of membranes is the common regulator in all of these seemingly disparate phenomena. The experiments described in this paper demonstrate that steroid passage across biological membranes is a controlled process and that its modulation occurs via alterations of the cholesterol content of the lipid bilayer.

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