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Cholesterol Stimulation of Penetration of Unilamellar Liposomes by Hydrophobic Compounds

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Summary. The incorporation of cholesterol into unilamellar liposomes greatly increased the transmembranous movement of hydrophobic ionophores such as nigericin. In reconstituted liposomes containing rhodopsin as the only protein, the presence of cholesterol lowers by 10-fold or more the amount of nigericin required to eliminate the light-driven proton gradient. These effects are seen both above and below the transition temperature of the phospholipid used for reconstitution.

Cholesterol similarly increases the ability of A-23187, 1799, or NH_4SCN to collapse the proton gradient of bacteriorhodopsin vesicles. Cholesterol also lowers the concentration of nigericin or valinomycin required for a rapid translocation of Rb^+ into protein-free liposomes. It also lowers the concentration of A-23187 required for the release of Ca^{45} trapped in protein-free liposomes. In contrast to these observations and in confirmation of previous findings, we observed that cholesterol decreased the permeability of liposomes for glucose. Thus the effects of cholesterol on the permeability of the membrane vary with the chemical nature of the permeating compounds. We have also confirmed that in multilamellar liposomes cholesterol decreases the permeability of Rb^+ in the presence of valinomycin. It therefore appears that the effect of cholesterol changes with the size and structural features of the model membranes.

In investigations of membrane fluidity physical methods such as differential scanning calorimetry, NMR and ESR spectroscopy and fluorescence polarization have been used [3, 16, 23, 31, 40]. Biological methods, including bacterial growth and enzyme activities [7, 17, 21, 26, 27, 32], have also been used to study the effect of the lipid composition on the fluidity and permeability properties of various membranes. Based on these studies it has been widely accepted that the presence of unsaturated fatty acids increases fluidity and permeability [5, 27], whereas the effect of cholesterol varies with the state of the lipids. Above the transition

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temperature of the phospholipid, cholesterol decreases the fluidity, while below the transition temperature it increases the fluidity [3, 15, 24, 29, 30, 33]. These results were obtained in studies with either unilamellar [3, 33] or multilamellar liposomes [3, 15, 24, 29, 30]. Cholesterol has a similar temperature-dependent effect on the glycerol permeability of multilamellar liposomes, inhibiting above the transition and stimulating below it [6]. Cholesterol decreases the permeability of multilamellar liposomes to small, water-soluble substances above the transition [5, 9–11], and has a similar effect on unilamellar liposomes [33].

When bacteriorhodopsin is incorporated into liposomes a light-driven uptake of protons takes place that is eliminated by proton ionophores [39]. Bacteriorhodopsin vesicles prepared with synthetic phospholipids such as dimyristoyl phosphatidylcholine actively transport protons below and above the transition temperature [37]. In the presence of low concentrations of a mobile proton ionophore such as nigericin, the proton gradient is collapsed above the transition temperature, while below this temperature the ionophore is immobilized and cannot interfere with the pump action.

In the course of studies on the effect of the phospholipid composition on the bacteriorhodopsin proton pump and on the effect of ionophores, it was noted that cholesterol greatly increased the permeability of the membrane to nigericin. In view of the above-mentioned studies this observation was rather unexpected and the phenomenon was further investigated. We shall report in this paper on the effect of cholesterol on the permeability of unilamellar liposomes to a variety of hydrophobic compounds.

Materials and Methods

Dipalmitoyl PC[1], cholesterol, 5α-cholestan3β-ol (dihydrocholesterol), 5α-cholestan3-one, valinomycin, glucuronic acid, HEPES, and gramicidin were obtained from Sigma, egg PC from Grand Island Biological, dimyristroyl PC, dimyristroyl PE, and cholest4-ene3-one from Fluka, A.G, dihexadecyl PC from Calbiochem. Nigericin and A-23187 were donated by Dr. R.J. Hosley of Eli Lilly and 1799 by Dr. P. Heytler of DuPont. Crude soybean phospholipids (asolectin) were obtained from Associated Concentrates and partly purified by extraction with acetone [19].

$^{86}RbCl$ and $^{45}CaCl_2$ were purchased from ICN and ^{14}C-glucose from Schwarz-Mann. Sephadex G-50 was obtained from Pharmacia and Dowex 50W-X8 from J.T. Baker. Brain PE was prepared by the procedure of Papahadjopoulos and Miller [34]. Heart 1-alkenyl,

[1] *Abbreviations:* PC – phosphatidylcholine, PE – phosphatidylethanolamine, HEPES – N-2-hydroxyethylpiperazine N′-2-ethanesulfonic acid, 1799-bis (hexafluoracetonyl)acetone.

2-acyl PC (plasmalogen) and heart diacyl PC were purified as described [13, 41]. Bacteriorhodopsin was purifield from *Halobacterium halobium* S_9 as described by Kanner and Racker [20].

Bacteriorhodopsin vesicles were prepared by the sonication procedure [36] in small pyrex test tubes containing the samples as described in the legends of the Figures. The bath-type sonicator described previously [36], which is no longer available, can be replaced by Model G 1225P1, Laboratory Supplies Co., Hicksville, New York. Higher temperatures when specified were used to permit sonication of disaturated phospholipids [37]. Light-dependent proton uptake by the vesicles was assayed as described [39] with the temperature of the assay cell monitored by a thermistor [37].

Ionophore-mediated ^{86}Rb influx into liposomes and ^{45}Ca efflux from liposomes were measured essentially as described [12] with details given in the legends of Figs. 4 and 6.

Glucose uptake into liposomes was measured as described [14] with details given in the legend of Table 2. Multilamellar liposomes were formed by the method of DeGier *et al.* [5].

Results and Discussion

In all experiments described in this paper unilamellar liposomes, prepared by exposing phospholipids to sonication, were used unless stated otherwise. The incorporation of 20 mole percent cholesterol into bacteriorhodopsin liposomes prepared with dimyristoyl PC markedly increased the sensitivity of these liposomes to a variety of hydrophobic ionophores. As can be seen from Fig. 1 *A*, the amount of nigericin required to collapse the proton gradient in the liposomes at 10° decreased by about fourfold in the presence of cholesterol. This finding was not unexpected since, as mentioned earlier, cholesterol is known to increase the "fluidity" of the membrane at temperatures below the transition temperature of the phospholipid. However, the sensitivity of the proton pump to nigericin was similarly increased by cholesterol at 30°, well above the transition temperature of dimyristoyl PC (23°) (Fig. 1 *B*). The activity of the proton pump itself was not altered significantly when cholesterol was incorporated into the liposomes.

The effect of various cholesterol concentrations in the liposomes on the sensitivity of the proton pump to nigericin is shown in Fig. 2. Although the greatest sensitivity was noted at 30 mole percent, concentrations above 25 mole percent were avoided, since it was shown by Newman and Huang [28] that marked changes in the size and shape of liposomes take place under these conditions.

As shown in Table 1 the effect of cholesterol was observed with a variety of phospholipids. Synthetic and natural phospholipids with variations in fatty acid unsaturation and chain-length were tested. The effect of cholesterol was seen with both dipalmitoyl PC (containing two

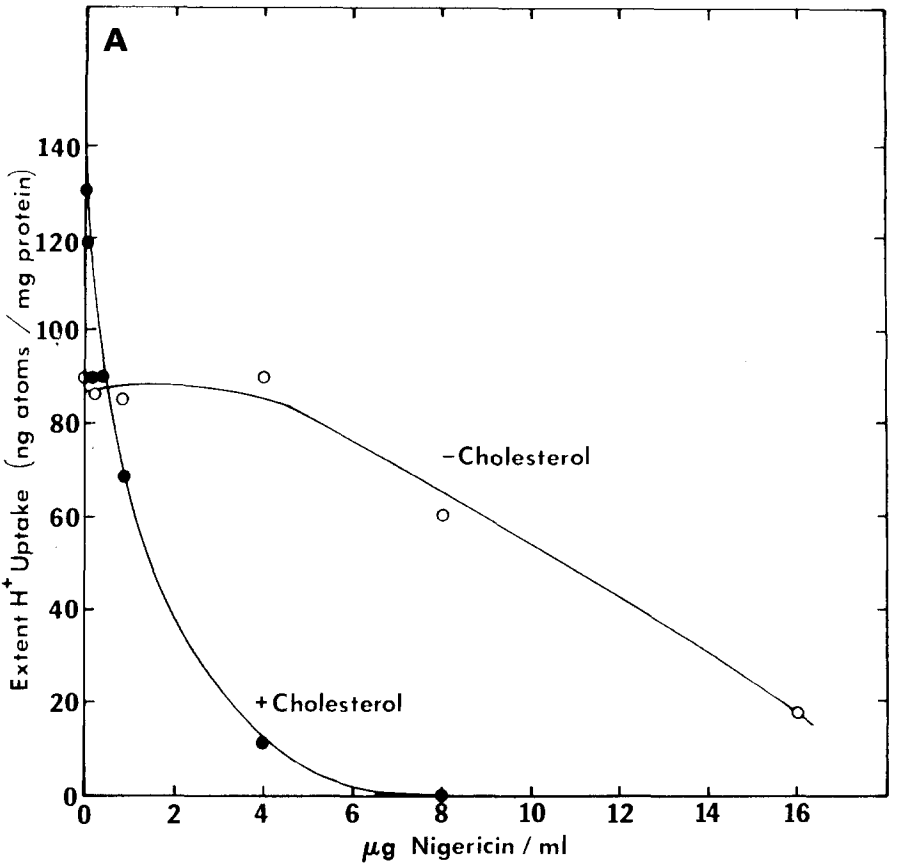
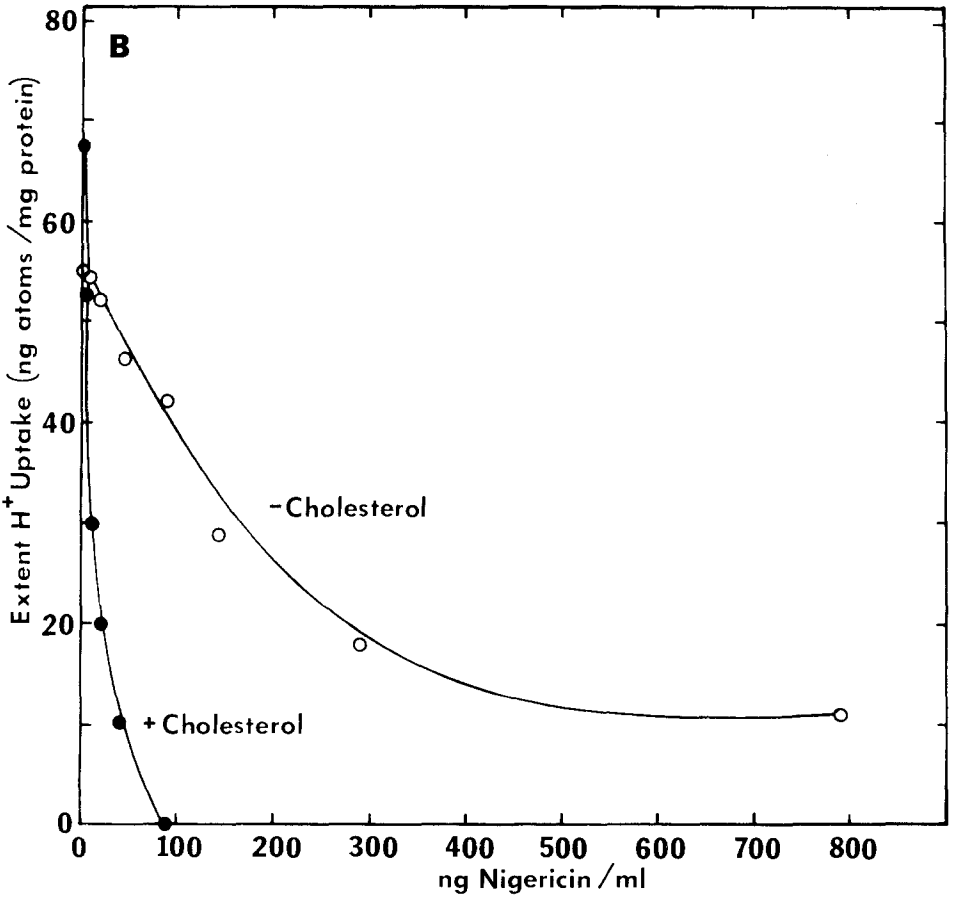


Fig. 1. Effect of temperature and cholesterol on the inhibition of the bacteriorhodopsin proton pump by nigericin. Samples of either 5 μmoles dimyristoyl PC (○—○) or 4 μmoles dimyristoyl PC plus 1 μmole cholesterol (●—●) were suspended in 0.2 ml of 0.15 M KCl and the mixtures were sonicated at 50° until clarified (30 min or more). Bacteriorhodopsin (100 μg) was added to each mixture and the sonication was continued for 15 min at 35°. Samples containing 21 μg of rhodopsin were assayed for light-dependent proton uptake in a final volume of 1 ml of 0.15 M KCl. Increasing concentrations of nigericin were added to the assay mixtures and the extent of proton uptake measured at 10° (*A*) and 30° (*B*)

carboxylate esters) and with dihexadecyl PC (containing no carboxylate esters). The observation with the latter is of interest in the light of evidence that cholesterol interacts with the carbonyl of the fatty acid ester group [44] and suggests another type of interaction. The effect of cholesterol was seen with a pure natural phospholipid (egg PC) and with a crude mixture of soybean phospholipids. Moreover, the effect of cholesterol was not restricted to phosphatidylcholine containing vesicles, but was also observed with pure phosphatidylethanolamine vesicles.



Incorporation of 20 mole percent cholesterol did not increase the effectiveness of gramicidin, which was shown to form a temperature-insensitive channel in bacteriorhodopsin liposomes [37]. Moreover, concentrations of nigericin which completely collapsed the proton gradient of dimyristoyl PC-bacteriorhodopsin liposomes at 30°, had no effect when the temperature was lowered to 10°. It is therefore apparent that the movement of nigericin across the membrane was affected by the presence of cholesterol.

The effect of cholesterol was not restricted to nigericin. The ionophore A-23187 exchanges Ca^{2+} for protons [35]. Cholesterol greatly increased the effectiveness of A-23187 when tested with bacteriorhodopsin liposomes (Fig. 3*A*). Similarly the uncoupler of oxidative phosphorylation 1799 (Fig. 3*B*), and NH_4SCN (Fig. 3*C*) were strikingly more effective

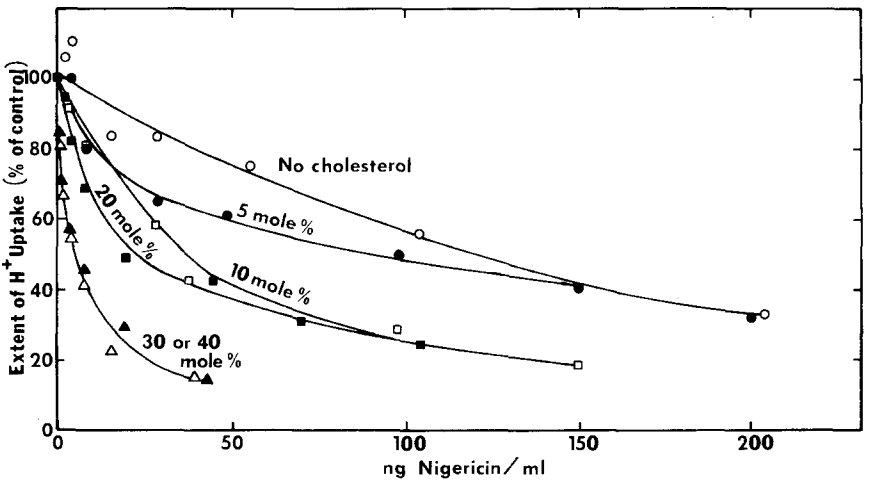


Fig. 2. Effect of different cholesterol concentrations on the inhibition of the bacteriorhodopsin proton pump by nigericin. Bacteriorhodopsin vesicles were prepared with egg PC and different amounts of cholesterol, and the extent of proton uptake was measured at 40° as indicated in the legend of Fig. 1. Increasing amounts of nigericin were added to the assay mixtures containing vesicles with 0 (○—○), 5 (●—●), 10 (□—□), 20 (■—■), 30 (△—△), and 40 (▲—▲) mole % cholesterol

Table 1. Effect of phospholipid composition and cholesterol on the nigericin inhibition of the bacteriorhodopsin proton pump[a]

Phospholipid	Assay temperature C°	ng Nigericin required to inhibit proton uptake by 50%	
		Without cholesterol	With cholesterol (20 mole %)
Dimyristoyl PC	30	456	33
Dipalmitoyl PC	40	216	22
Egg PC	40	121	24
Heart PC	40	128	16
Heart plasmalogen PC	40	48	4
Crude soybean phospholipid	25	1	0.1
Dihexadecyl PC	40	60	10
Dimyristoyl PE	40	44	10

[a] Bacteriorhodopsin vesicles were prepared with the phospholipids listed in the Table. Light-dependent proton uptake was determined in the presence of increasing amounts of nigericin as described in the legend of Fig. 1. When dipalmitoyl PC was used as the phospholipid, bacteriorhodopsin vesicles were prepared by sonication at 41°.

in collapsing the proton gradient when cholesterol was present in the reconstituted bacteriorhodopsin liposomes.

Although it seemed unlikely in view of the above experiments that the effect of cholesterol was predicated on the presence of bacteriorhodopsin, the permeability of protein-free liposomes to hydrophobic iono-

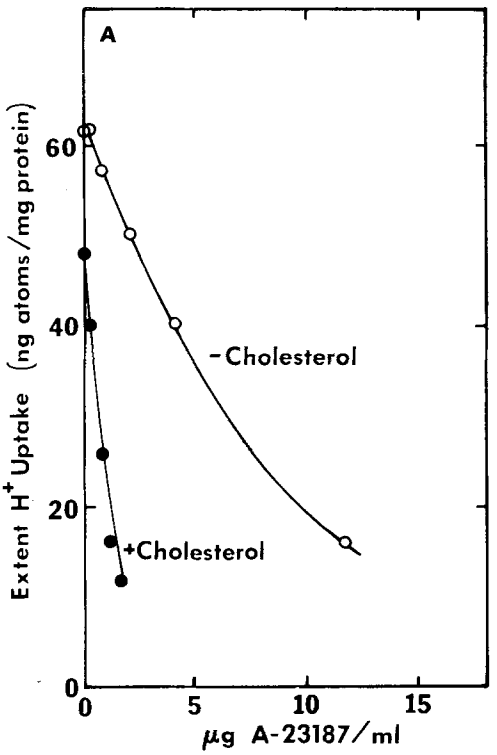
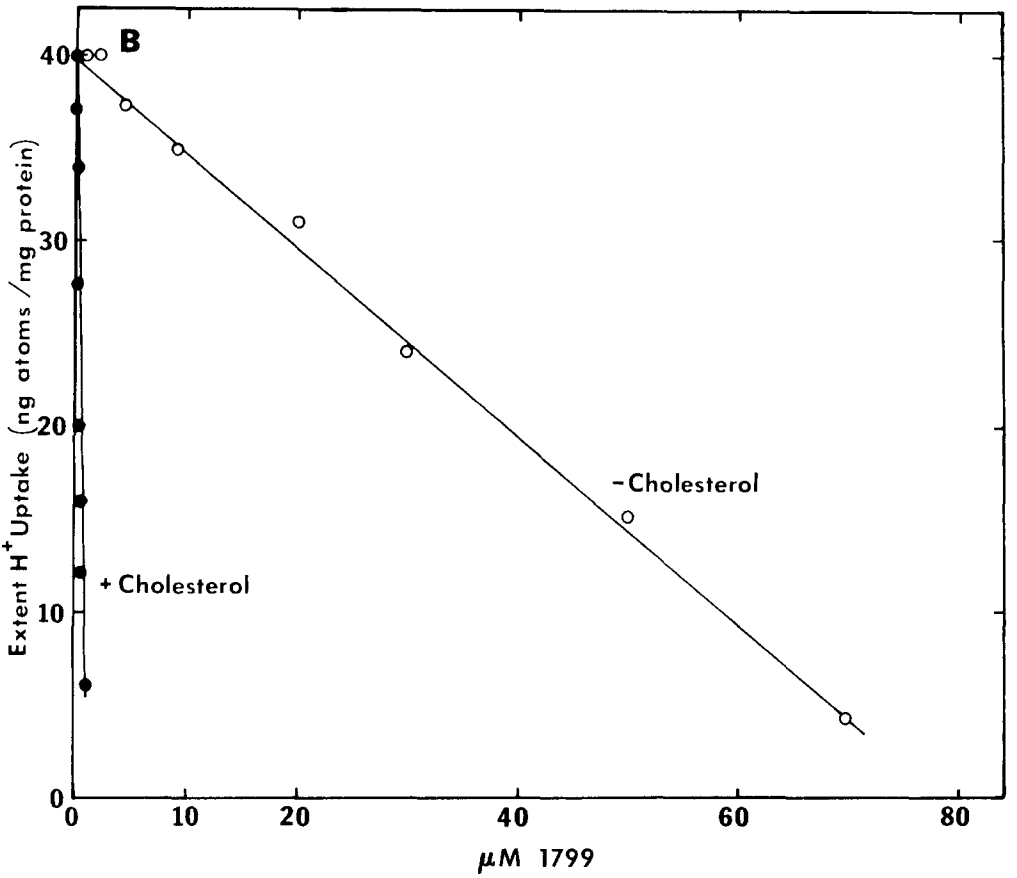


Fig. 3. Effect of cholesterol on the inhibition of the bacteriorhodopsin proton pump by proton conductors. Bacteriorhodopsin vesicles were prepared with dimyristoyl phosphatidylcholine and the extent of light-dependent proton uptake was measured at 30° as described in the legend of Fig. 1 with (●—●) and without (○—○) 20 mole % cholesterol. A-23187+2 mM $CaCl_2$ (*A*), 1799 (*B*), and NH_4SCN (*C*) were added in the amounts indicated

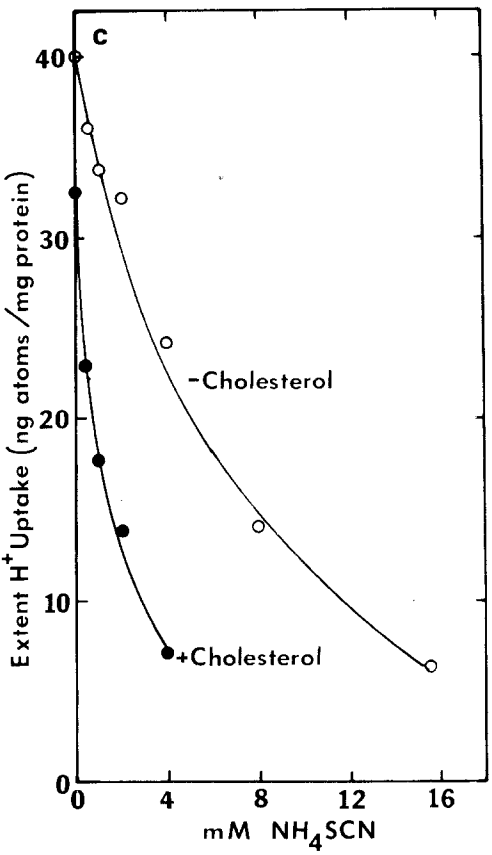
phores was examined. The permeability was measured by determining the amount of nigericin or valinomycin required to catalyze the uptake of $^{86}Rb^+$ into egg PC liposomes. Cholesterol significantly decreased the amount of nigericin (Fig. 4) or of valinomycin (Fig. 5) required for the transport of Rb^+ into these vesicles. Cholesterol also markedly lowered the amount of A-23187 required to release Ca^{2+} from $^{45}Ca^{2+}$-loaded liposomes (Fig. 6).

It is apparent from these findings that the mobility of a variety of hydrophobic ionophores is enhanced by cholesterol irrespective of the nature of the cation that is carried across the membrane and irrespective of the presence of a charge (e.g., valinomycin) or the absence of a charge (e.g., nigericin) in the permeating ionophore-ion complex.

A survey of the literature revealed that these findings are in apparent contradiction with virtually all observations on the effect of cholesterol on liposome permeability. Van Deenen and his collaborators [6] and



others [18, 25] have demonstrated that cholesterol affects the permeability of liposomes to water-soluble compounds in the same way as it affects the "fluidity" of the liposomes. Some of the observations were made with unsonicated, multilamellar vesicles [6, 9–11, 18, 25], others with sonicated vesicles [33]. Cholesterol has been shown to make red blood cells and microorganisms less permeable to small water soluble compounds [2, 7, 8, 22, 27]. Moreover, Szabo *et al.* [42] have reported that cholesterol decreased the ability of macrotetralide antibiotics to lower the resistance of phospholipid bilayer membranes, although much higher concentrations of cholesterol were used in these studies compared to the present ones. Furthermore, DeGier *et al.* [4] have shown that cholesterol made multilamellar liposomes of egg PC less permeable to K^+ in the presence of valinomycin, while Bakker *et al.* [1] observed no effect of cholesterol on the permeability of such vesicles to 1799. In view



of these reports we used our experimental procedure to test the permeability of multilamellar liposomes to K^+ in the presence of valinomycin both with and without cholesterol. As can be seen from Fig. 7, the presence of cholesterol in multilamellar liposomes decreased the sensitivity to valinomycin confirming the results obtained by DeGier [4]. It appears therefore that the effect of cholesterol on the mobility of hydrophobic compounds is a function of the size and structural features of the liposomes.

We have also confirmed the reports [9–11] that cholesterol renders liposomes less permeable to glucose (Table 2) when assayed above the transition temperature of the phospholipid. Thus there is a clear difference in the effect of cholesterol on the mobility of hydrophilic and hydrophobic compounds.

Van Deenen and his collaborators have shown that certain cholesterol

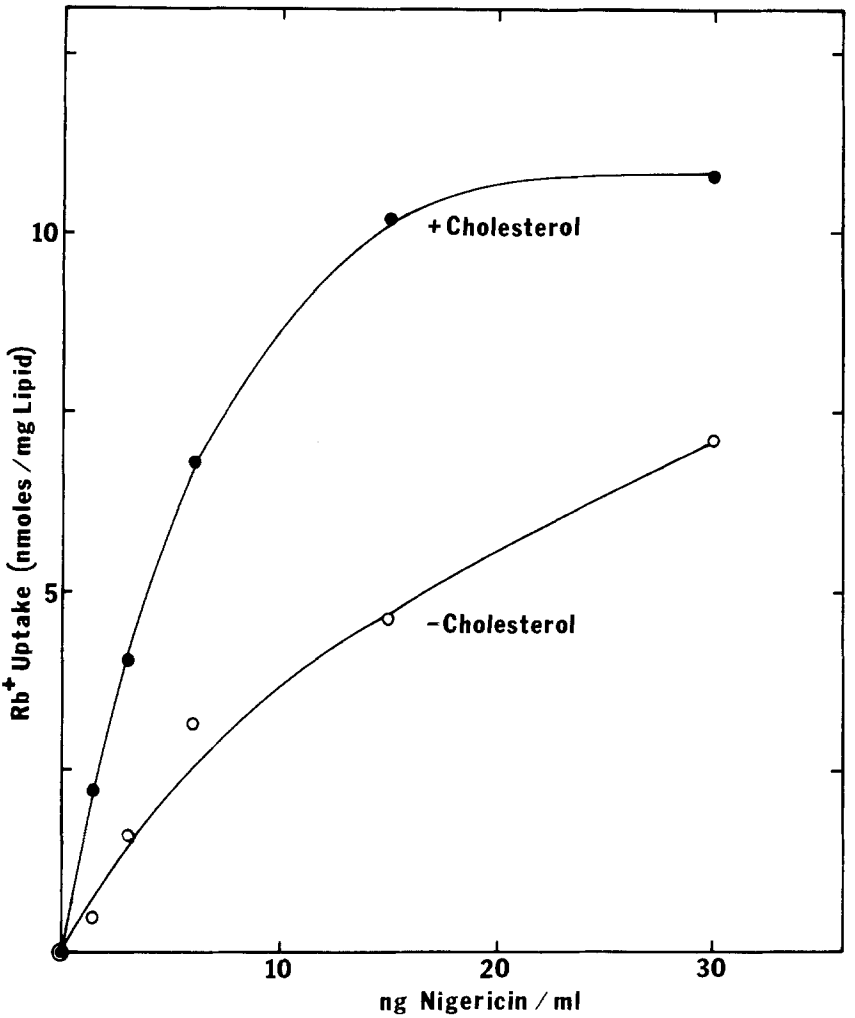


Fig. 4. Effect of cholesterol on the nigericin-mediated uptake of ^{86}Rb into liposomes. Either 18 μmoles egg PC (○—○) or 14.4 μmoles egg PC plus 3.6 μmoles cholesterol (●—●) were suspended in 0.9 ml of 50 mM potassium phosphate (pH 7.4), and the mixtures were sonicated to clarify them. Aliquots of the vesicles (125 μl) were incubated with 150 μl, 50 mM potassium phosphate solutions containing $^{86}RbCl$ (5 μCi) and the indicated amounts of nigericin at 20°. After 6 min, 100 μl samples of the incubation mixtures were placed on Dowex 50 columns, each with a void volume of about 1.2 ml. The vesicles were allowed to enter the columns with 0.5 ml 0.25 M sucrose added dropwise, and then they were eluted with 3 ml of the sucrose. Samples of the eluted vesicles (1 ml) were dried and counted

analogs do not have the same effect on permeability of liposomes to hydrophilic compounds (e.g., glycerol) as does cholesterol [2, 8, 9]. The sensitivity of bacteriorhodopsin vesicles to nigericin is increased by cholesterol analogs as well as by cholesterol (Table 3).

The only report we are aware of which appears in line with the

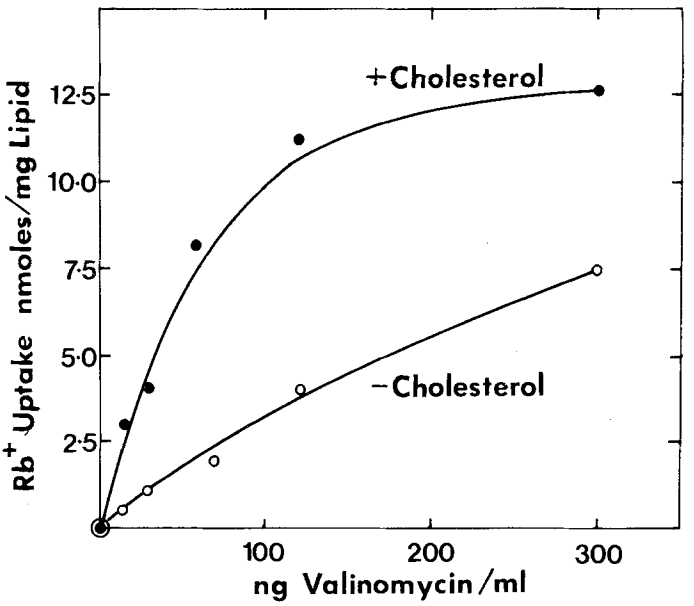


Fig. 5. Effect of cholesterol on the valinomycin-mediated uptake of ^{86}Rb into liposomes. Liposomes were prepared with (●—●) or without (○—○) cholesterol and assayed for valinomycin-mediated ^{86}Rb uptake as described in the legend of Fig. 4

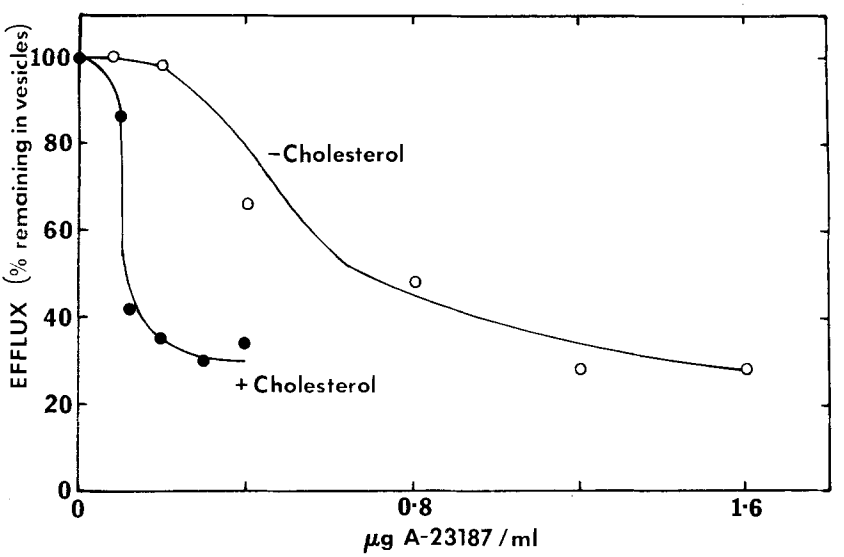


Fig. 6. Effect of cholesterol on the A-23187-mediated efflux of ^{45}Ca from liposomes. Either 4 μmoles egg PC (○—○) or 3.2 μmoles egg PC plus 0.8 μmoles cholesterol (●—●) were suspended in 0.2 ml of buffer containing 0.1 M potassium glucuronate (pH 7.4), 2 mM $CaCl_2$, 5 mM potassium HEPES, and 85 μCi $^{45}CaCl_2$. The mixtures were sonicated until clarified and 25 μl aliquots were incubated with solutions containing 0.25 ml of isotope-free buffer and the indicated amounts of A-23187 at 20°. After 15 min, 100 μl samples were applied to Dowex columns, eluted with 3.5 ml sucrose, and the eluants were counted as described in the legend of Fig. 4

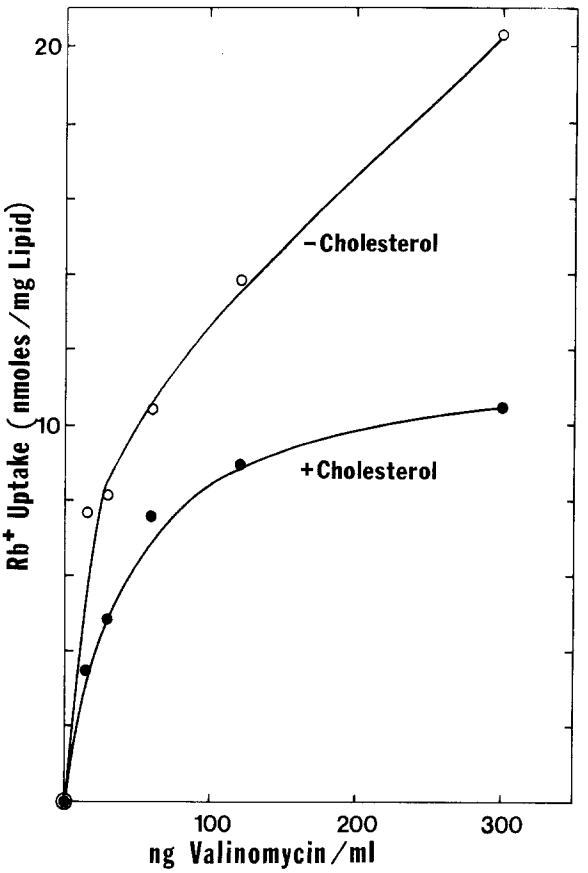


Fig. 7. Effect of cholesterol in the valinomycin-mediated uptake of ^{86}Rb into multilamellar liposomes. Either 18 μmoles egg PC (o—o) or 14.4 μmoles egg PC plus 3.6 μmoles cholesterol (●—●) were suspended by stirring at 37° in 0.9 ml of 50 mM potassium phosphate (pH 7.4). The multilamellar liposomes were assayed for valinomycin-mediated $^{86}Rb^+$ uptake as described in the legend of Fig. 4

observations reported here is a recent paper by Tsong [43] who reported that cholesterol stimulated the penetration of sonicated liposomes by 8-anilino-1-naphthalene sulfonate both above and below the transition temperature of the phospholipid.

It is apparent from the results reported here and from other laboratories that cholesterol can profoundly alter the permeability properties of phospholipid bilayers. From a quantitative point of view the effect of cholesterol on the mobility of hydrophobic ionophores reported here is the most striking one. It will be of considerable interest to study the effect of cholesterol on reconstituted ion pumps and on ionophores isolated from ion pumps [38].

Table 2. Effect of cholesterol on the uptake of glucose into liposomes[a]

Incubation time (min)	Glucose uptake (pmoles/mg lipid)	
	Without cholesterol	With cholesterol
0	3.8	4.8
15	42.2	19.8
30	61	32

[a] Either 3 μmoles egg PC or 2.4 μmoles egg PC plus 0.6 μmole cholesterol were suspended in 0.2 ml of buffer containing 10 mM Tris-HCl (pH 7.4), 0.15 M KCl, and 1 mM $MgSO_4$, and sonicated until clarified (usually 15–20 min). Then 1 μl of ^{14}C-glucose (20 mM, 50 μCi/μmole) was added to the vesicles (100 μl), and the mixture was incubated at 20° for the times indicated. The entire sample mixture was placed on a Sephadex G50 column (26 × 1 cm) and eluted at 0° with the buffer used above. The vesicles were eluted from the column in the void volume and they were collected and counted.

Table 3. Effect of cholesterol analogs on the nigericin inhibition of the bacteriorhodopsin pump[a]

Analog	ng Nigericin required to inhibit proton uptake by 50%
None	121
Cholest4-ene3-one	0.6
5α-cholestan3-one	10
5α-cholestan3β-ol	13

[a] Various cholesterol analogs (30 mole %) were used in the preparation of bacteriorhodopsin vesicles along with egg PC, and the ability of the vesicles to transport protons in the presence of increasing amounts of nigericin was determined as described in the legend of Fig. 1. Both the sonication and the assay were performed at 40° and by a Postdoctoral Fellowship (5F22/AM01095-02) from the National Institute of Arthritis, Metabolic and Digestive Diseases.

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