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EFFECT OF CHOLESTEROL ON THE VALINOMYCIN-MEDIATED UPTAKE OF RUBIDIUM INTO ERYTHROCYTES AND PHOSPHOLIPID VESICLES

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Summary

Human erythrocytes have been treated with lipid vesicles in order to alter the cholesterol content of the cell membrane. Erythrocytes have been produced with cholesterol concentrations between 33 and 66 mol% of total lipid. The rate of valinomycin-mediated uptake of rubidium into the red cells at 37° C was lowered by increasing the cholesterol concentration of the cell membrane. Cholesterol increased the permeability to valinomycin at 20° C of small (less than 50 nm), unilamellar egg phosphatidylcholine vesicles formed by sonication. Cholesterol decreased the permeability to valinomycin at 20° C of large (up to 200 nm) unilamellar egg phosphatidylcholine vesicles formed by freeze-thaw plus brief sonication. It is concluded that cholesterol increases the permeability of small membrane vesicles to hydrophobic penetrating substances while above the transition temperature but has the opposite effect on large membrane vesicles and on the membranes of even larger cells.

Introduction

Cholesterol has nearly always been shown to lower the ability of a wide variety of compounds to penetrate either cellular or artificial phospholipid membranes [1—15]. However, it has been shown that the presence of cholesterol in small (20—50 nm), unilamellar phospholipid vesicles prepared by sonication increased their permeability to hydrophobic ionophores such as valinomycin, nigericin, and A23187 [16]. Since cholesterol has nearly always

lowered the rate at which hydrophilic compounds such as glucose pass through membranes, it seemed that the effect of cholesterol on membrane permeability might be a function of the water solubility of the permeating compound [1–12]. Yet cholesterol also decreased the permeability of large (greater than 100 nm) multilamellar liposomes to hydrophobic ionophores [15,16], which suggested that cholesterol could not increase membrane permeability unless the penetrating compound was hydrophobic and the membrane system consisted of small vesicles. Small vesicles are highly curved and are inherently asymmetric, and either factor might result in anomalous behavior. However, the large multilamellar liposomes are quite heterogeneous in size and possess a large and variable amount of internal lipid that makes the analysis of permeability data difficult [17]. Therefore, it was of interest to study the effect of cholesterol on the permeability of large unilamellar membrane vesicles to ionophores.

One such membrane 'vesicle' is the erythrocyte. The cholesterol content of the erythrocyte can be easily altered [8,18,19]. The erythrocytes are uniform in size, possess a unilamellar membrane, and are extremely large when compared with unilamellar lipid vesicles prepared by sonication. If the effect of cholesterol on the ability of ionophores to penetrate membrane vesicles were a function of vesicle size, then cholesterol would not have the same effect on the ionophore permeability of the erythrocyte as it has on the ionophore permeability of the small sonicated vesicle. As a result, the permeability of the erythrocyte to valinomycin was determined after the cellular cholesterol content had been altered.

The effect of cholesterol on the permeability to valinomycin of relatively large (up to 200 nm), unilamellar phospholipid vesicles was also determined. These vesicles were formed by the freeze-thaw procedure of Kasahara and Hinkle [20], and again if the effect of cholesterol varied with vesicle size it should be apparent from such a study.

Materials and Methods

Cholesterol, dipalmitoyl phosphatidylcholine, egg phosphatidylcholine, bovine serum albumin, valinomycin and ouabain were obtained from Sigma, penicillin from P-L Biochemicals, $^{86}RbCl$ from New England Nuclear, Dowex 50W-X8 (20—50 mesh, H^+ form) from J.T. Baker and Bio-sil HA from BioRad.

Human blood was drawn from healthy, adult volunteers and added at once to 2 vols. of ice-cold sodium citrate (3.02%). The blood was centrifuged at $1000 \times g$ for 10 min and the plasma plus the interfacial leucocytes discarded. The pelleted erythrocytes were resuspended and washed three times in Tris-buffered saline (isotonic, pH 7.2). The washed erythrocyte were resuspended in medium (50 mM Tris-HCl, pH 7.4, 85 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 4 mM sodium phosphate, 10 mM glucose) in a final volume equal to the initial blood volume.

Lipids were extracted from erythrocytes by a procedure derived from that of Nelson [21]. The erythrocytes were added with stirring to 10 vols. of methanol, then 5 vols. of chloroform were added and the mixture centrifuged at low speed to pellet the hemoglobin. The pellets were washed with 3 vols. of chloroform/methanol (1/2), and the combined supernatants were added to 10 vols.

KCl (0.5 M) and 6 vols. chloroform. The resulting two-phase mixtures were then shaken, centrifuged to separate the phases, and the lower, lipid-containing chloroform phases were taken to dryness. The cholesterol content of the lipid extract was determined by the procedure of Brown et al. [22], and the phospholipid content determined by the procedure of Ames and Dubin [23]. When cholesterol was to be purified from the lipid extract before analysis, the lipid extract was applied to a silica gel thin-layer plate and the plate developed with a mixture of hexane/diethyl ether/acetic acid (85 : 15 :2, by vol.). The silica gel was then scraped from different zones of the plate, lipid was extracted from the silica gel by the procedure of Bligh and Dyer [24], and the cholesterol and phospholipid content of the lipid determined. In order to prepare large quantities of pure erythrocyte phospholipid, the erythrocyte lipid was extracted as described above, and the extract was applied to a column of Bio-sil HA (2 g Biosil/200 mg lipid). The cholesterol was eluted with chloroform and the phospholipid eluted with methanol.

Lipid vesicles were prepared by sonication, using a bath-type sonicator from Laboratory Supplies Co., Hicksville, NY (Model G1225 P1). Small (less than 50 nm) unilamellar vesicles were formed by sonication of the lipid mixture as described [16,25]. To form large (up to 200 nM) unilamellar vesicles, the lipid mixture was exposed to sonication, followed by rapid freeze-thaw, and then brief (5—20 s) further sonication [20]. The valinomycin-mediated $^{86}Rb^+$ influx into erythrocytes and vesicles and the $^{86}Rb^+$ efflux from vesicles were measured as described [16,26], with details given in the legends of the figures.

In order to measure the valinomycin partition coefficient for the lipid vesicles by dialysis, phospholipid vesicles were formed as described in the legend of Fig. 2 and then incubated with valinomycin (1 μg/ml) for 15 min at 25° C. The vesicles were then placed in dialysis bags and shaken for 16 h in 10 vols. of phosphate buffer at 25° C. After dialysis, the valinomycin concentration both inside and outside the dialysis bag was determined by measuring the ionophore-stimulated uptake of $^{86}Rb^+$ into phospholipid vesicles. Aliquots of the dialyzed fractions were mixed with egg phosphatidylcholine-cholesterol (3 : 1)-containing vesicles together with $^{86}RbCl$, the mixtures applied to Dowex columns, and the eluted vesicles counted as described in the legend of Fig. 1. The quantity of valinomycin in each dialyzed fraction was determined by comparing the amount of $^{86}Rb^+$ taken up by lipid vesicles in the presence of the dialyzed fractions with the amount taken up by vesicles in the presence of known amounts of valinomycin.

The valinomycin partition coefficient was also measured by means of a centrifugation procedure. Lipid vesicles were formed as described in the legend of Fig. 2, incubated with valinomycin, and then layered onto sucrose density gradients containing layers of 5% (w/v) and 15% (w/v) sucrose. The gradients were centrifuged for 16 h at 150 000 $\times g$ in a Beckman SW 50.1 rotor. Next fractions containing the phospholipid vesicles together with fractions above and below the vesicles were removed from the density gradient tubes and the valinomycin content of the fractions determined as described above.

Results and Discussion

Washed human erythrocytes were treated with lipid vesicles so as to alter their cholesterol content by the procedure of Cooper et al. [18] (Table I). Vesicles containing dipalmitoyl phosphatidylcholine either with or without cholesterol were formed by sonication of the lipid in medium. The sonicated mixtures were centrifuged to remove any large particulate material into the pellet, and the supernatant vesicles were analyzed for cholesterol and phospholipid and shown to contain nearly all of the initial lipid. The erythrocytes were mixed with either the cholesterol-free or the cholesterol-containing vesicle preparation and also with a control preparation containing only medium. Bovine serum albumin was added to each mixture to guard against hemolysis [8,18] and penicillin added to prevent bacterial growth. The mixtures were shaken at 37°C for several hours, and then centrifuged at low speed to concentrate the cells into the pellet while the vesicles remained in the supernatant. The cells were then washed with isotonic saline and then aliquots were extracted with organic solvent and analyzed for lipid phosphate and cholesterol. As shown in Table I, the erythrocytes added to phosphatidylcholine vesicles lost 21% of their cholesterol after 6 h incubation and 43% after 24 h. Erythrocytes added to phosphatidylcholine-cholesterol vesicles increased their cholesterol content by 78% after 6 h and by 122% after 24 h. The phospholipid content of the cells was unchanged by vesicle treatment.

After the erythrocytes had been separated from the vesicles, aliquots of the

TABLE I

EFFECT OF VESICLE TREATMENT ON THE CHOLESTEROL CONTENT OF ERYTHROCYTES

Either 9 μmol dipalmitoyl phosphatidylcholine or 9 μmol dipalmitoyl phosphatidylcholine plus 23 μmol cholesterol were suspended in 2.25 ml of medium. The mixtures were sonicated under N_2 at 50°C until nearly clear, then centrifuged at 10 000 × *g* for 10 min and the pellets discarded. Washed human erythrocytes (0.35 ml packed cells) were then incubated with and without the lipid vesicle preparations together with bovine serum albumin (2.8 mg/ml), penicillin (45 μg/ml) and medium in a total volume of 5.6 ml. The incubation mixtures were shaken at 37°C for the times indicated and 2.5-ml aliquots were withdrawn, diluted to 12.5 ml with NaCl (0.89%) and centrifuged 10 min at 500 × *g*. The pelleted cells were washed with 10 ml NaCl and extracted with chloroform/methanol as described in Materials and Methods. The cholesterol and phospholipid contents of the cells were determined as described in Materials and Methods.

Vesicle content	Incubation time (h)	μmol cholesterol/ml packed erythrocytes	μmol phospholipid/ml packed erythrocytes	μmol cholesterol/μmol phospholipid
None	6	3.59	3.47	1.03
Dipalmitoyl phosphatidylcholine	6	2.56	3.15	0.81
Dipalmitoyl phosphatidylcholine + cholesterol	6	5.68	3.10	1.83
None	24	3.69	3.60	1.02
Dipalmitoyl phosphatidylcholine	24	1.91	3.30	0.58
Dipalmitoyl phosphatidylcholine + cholesterol	24	8.19	3.63	2.26

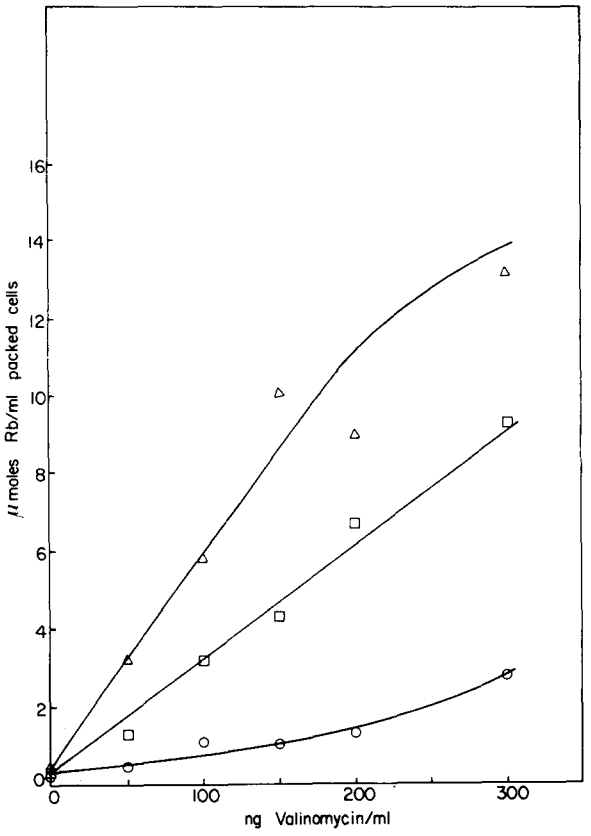

Fig. 1. Valinomycin-mediated $^{86}Rb^+$ uptake into erythrocytes treated with vesicles. Washed erythrocytes were incubated with either dipalmitoyl phosphatidylcholine-containing vesicles (△———△), dipalmitoyl phosphatidylcholine-cholesterol-containing vesicles (○———○), or medium (□———□) for 6 h and washed free of vesicles as described in the legend of Table I. Aliquots of the treated erythrocytes (5 μl packed cells) were incubated with 0.2 ml solutions containing KCl (10 mM), NaCl (150 mM), Tris-HCl (14 mM, pH 7.4). $MgCl_2$ (2.5 mM), ouabain (1.4 mM), $^{86}RbCl$ (2 μCi) and increasing amounts of valinomycin. The mixtures were shaken at 37°C for 15 min and then each incubation mixture was placed on a Dowex 50W-X8 column with a void volume of about 1.2 ml. The erythrocytes were allowed to enter the columns with 0.5 ml 0.25 M added dropwise, and then they were eluted with 3 ml of the sucrose. Samples of the eluted vesicles (1 ml) were counted to determine radioactivity along with a sample of the initial $^{86}Rb^+$ solution.

cells were added to pure water in order to release the hemoglobin, and the hemoglobin concentration determined with the aid of a spectrophotometer. This procedure provided proof that no significant hemolysis had occurred during vesicle treatment. Samples of the erythrocyte lipid extracts were purified by thin-layer chromatography to ensure that the extracts contained no cholesterol ester nor anything else that would interfere with the cholesterol assay.

Erythrocyte aliquots were incubated with $^{86}Rb^+$ and increasing amounts of valinomycin in order to measure the ionophore-mediated ion uptake by the cells. The amount of $^{86}Rb^+$ that entered erythrocytes in the presence of valinomycin was proportional to the length of the incubation period, as would be expected for a transport process. As shown in Fig. 1, the permeability of the

erythrocyte to valinomycin decreased as the cholesterol concentration increased. This effect was more pronounced after the cells had been exposed to vesicles for 24 h than after 6 h exposure. This was consistent with the observation that the longer vesicle treatment had a more dramatic effect on the cellular cholesterol content (Table I). Hardly any $^{86}Rb^+$ penetrated the erythrocytes in the absence of valinomycin, regardless of cholesterol content.

Erythrocytes were also treated with vesicles formed from erythrocyte phospholipid both with and without cholesterol. Again the cells treated with cholesterol-free vesicles lost cholesterol and the cells treated with cholesterol-loaded vesicles gained cholesterol. Again the cellular permeability to valinomycin was lowered by cholesterol. This indicated that the change in cellular permeability to valinomycin shown in Fig. 1 did not result from any alteration in the structure of erythrocyte phospholipid. Such an alteration in phospholipid structure might stem from exchange between erythrocyte phospholipid and the vesicle dipalmitoyl phosphatidylcholine used previously. Some investigators have presented evidence for such exchange [27], but such exchange in irrelevant if the vesicles themselves contain erythrocyte phospholipid.

The data above indicate that cholesterol decreases the permeability of erythrocytes to the hydrophobic ionophore valinomycin, and are in basic accord with results obtained with multilamellar liposomes [15,16] and also with planar bilayers [13,14]. Of course data obtained with erythrocytes must be interpreted with some caution, since vesicle treatment of erythrocytes has been shown to alter membrane protein composition slightly [27]. Effects on sensitivity to ionophores might stem from protein alterations rather than the more massive alterations in cholesterol content.

Erythrocytes also cannot be completely depleted of cholesterol without suffering hemolysis, and so the data in Fig. 1 demonstrate only the effect of increasing the cholesterol concentration from about 33 mol% of total lipid to about 66 mol%. Previous work has shown that an increase in the cholesterol concentration of small unilamellar vesicles from 0 mol% to 30 mol% greatly increases the permeability to ionophores, but hardly any data was obtained using vesicles containing more than 30 mol% cholesterol [16]. Data shown in Fig. 2 indicate that cholesterol increased the permeability of small unilamellar vesicles to valinomycin only when the cholesterol concentration was kept below 33 mol%. When the cholesterol concentration of these vesicles was raised from 33 mol% to 66 mol% the ionophore permeability actually decreased slightly.

Although one must be careful not to draw too many conclusions concerning erythrocyte behavior from experiments with phospholipid vesicles, one might well ask whether or not cholesterol would increase the valinomycin permeability of erythrocytes containing less than 30 mol% cholesterol if such erythrocytes could be formed. In order to help answer this question relatively large unilamellar vesicles were formed by the freeze-thaw procedure of Kasahara and Hinkle [20]. If cholesterol really did make highly curved membranes more permeable to ionophores and largely flat membranes less permeable, then an increase in vesicle size would alter the effect.

Data in Table II indicate that the freeze-thaw procedure greatly increased the internal volume of the vesicles. Vesicles were produced with the internal $^{86}Rb^+$

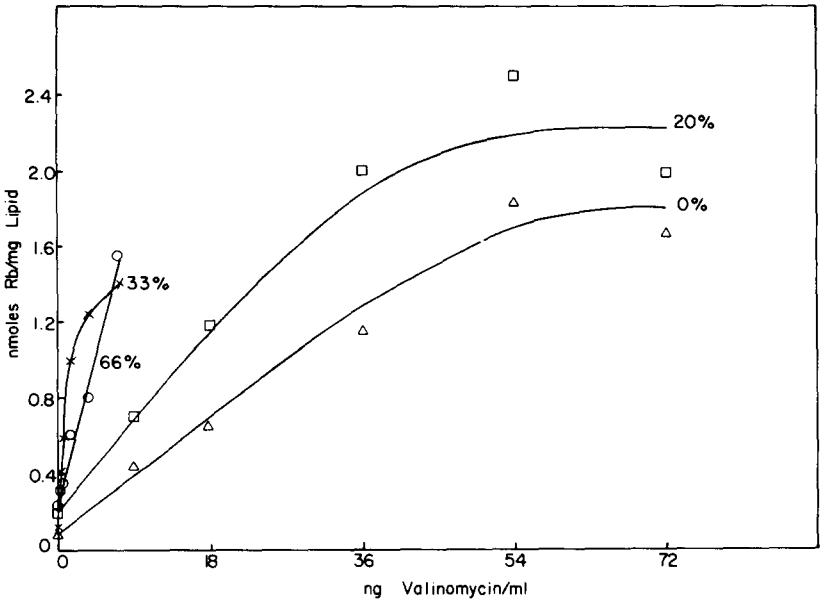

Fig. 2. Effect of cholesterol on the valinomycin-mediated uptake of $^{86}Rb^{+}$ into small unilamellar vesicles. Mixtures of egg phosphatidylcholine and cholesterol containing 0 (△———△), 18 (□———□), 33 (X———X), and 66 (○———○) mol% cholesterol and consisting of 9 mg total lipid were suspended in 1.8 ml of phosphate buffer (5 mM potassium phosphate, 45 mM sodium phosphate, pH 7.2) and the mixtures were sonicated to clarify them (20—40 min). Aliquots of the vesicles (0.25 ml) were incubated with 25 μl phosphate buffer containing $^{86}RbCl$ (4 μCi) and the indicated amounts of valinomycin at 20°C. After 6 min, the incubation mixtures were placed on Dowex columns and eluted as described in Fig. 1.

TABLE II

INTERNAL VOLUMES OF UNILAMELLAR VESICLES

Mixtures containing egg phosphatidylcholine and increasing amounts of cholesterol and consisting of 2.25 mg total lipid were suspended in 0.5 ml of phosphate buffer (5 mM potassium phosphate, 45 mM sodium phosphate, pH 7.2) containing $^{86}RbCl$ (7.5 μCi), and the mixtures were sonicated to clarify them (20—40 min). Aliquots of vesicles (0.2 ml) were applied to Dowex columns and eluted as described in Fig. 1. The remaining vesicles were frozen with liquid N_2, thawed and sonicated for 20 s. Again 0.2 ml aliquots of the vesicles were applied to Dowex columns and eluted. The internal vesicle volume was calculated from the equation: $V_i = (cpm_i)\ V_t/(cpm_t)\ m$, with V_i, internal vesicle volume/mg lipid; cpm_i, amount of ^{86}Rb that is eluted from the Dowex column; V_t, total volume of ^{86}Rb-vesicle preparation before application to Dowex; cpm_t, total amount of ^{86}Rb in the preparation before application to Dowex, and m, mg of lipid in the vesicle preparation.

Percent cholesterol in vesicles	Internal volume of vesicles			
	Before freeze-thaw		After freeze-thaw	
	cpm in vesicles ($\times 10^{-3}$)	Vesicle vol. (μl/mg lipid)	cpm in vesicles ($\times 10^{-3}$)	Vesicle vol. (μl/mg lipid)
0	14.7	0.85	67.2	3.88
20	13.3	0.77	79.0	4.56
33	18.0	1.04	123	7.12
40	19.6	1.13	92.3	5.32
50	16.2	0.93	88.2	5.09
66	25.0	1.30	75.4	3.95

concentration equal to the external concentration, simply by adding $^{86}Rb^+$ to the lipid before sonication. After their formation the vesicles were applied to a Dowex column and the external $^{86}Rb^+$ was absorbed by the Dowex. The internal $^{86}Rb^+$ passed through the column and could be easily measured. An increase in the amount of internal $^{86}Rb^+$ indicated an increase in internal vesicle volume and a corresponding increase in vesicle size. When vesicles formed by simple sonication were subjected to freeze-thaw followed by very brief sonication, the internal volume increased by 5—7 fold. Cholesterol increased the size of the small vesicles slightly, and had a more complex but not substantial effect on the size of the large vesicles.

Both small and large vesicles, with and without cholesterol, were examined for leakage. The vesicles were formed with internal $^{86}Rb^+$, diluted several fold with sucrose (0.25 M), and incubated for up to 20 min before being applied to Dowex columns. The same amount of $^{86}Rb^+$ was found inside the vesicles after the 20 min incubation as was present before the incubation. It was concluded that no leakage occurred with any preparation of vesicles. Cholesterol markedly decreased the permeability of large, unilamellar vesicles to valinomycin (Fig. 3) even at low concentrations.

The valinomycin partition coefficient has been determined by several investigators for such phospholipid preparations as planar bilayers [28—31] and multilamellar liposomes [32]. If the valinomycin partition coefficient for unilamellar vesicles was similar to the partition coefficient for multilamellar liposomes (0.022 cm), then under the conditions of the experiments reported herein, over 99% of the valinomycin added to each preparation would be incorporated into the vesicles and hardly any would remain free in solution. The valinomycin partition coefficient for unilamellar sonicated vesicles has been determined by two procedures. Initially the vesicles were mixed with valinomycin and dialyzed in order to allow the free aqueous valinomycin to reach an equilibrium concentration on either side of the dialysis membrane. This concentration was determined by measuring the ability of the ionophore to stimulate $^{86}Rb^+$ uptake into lipid vesicles as described in Materials and Methods. The partition coefficient was determined from the equation [32]

$$K_p = \frac{\text{mol valinomycin in lipid vesicles/cm}^2 \text{ lipid surface}}{\text{mol valinomycin in solution/ml solution}}$$

The area of the lipid surface for unilamellar vesicles can be calculated as about 3072 $cm^2/\mu mol$ lipid from the known dimensions of the lipid molecule(51 $Å^2$/molecule) [33]. The partition coefficient was further measured by means of a centrifugation assay. Unilamellar lipid vesicles were mixed with valinomycin and separated from aqueous phase by exposure to a force of 150 000 $\times g$ for 16 h. This procedure was shown by Carroll and Racker to completely separate phospholipid vesicles from the aqueous phase [34]. The final valinomycin concentration in both the aqueous phase and the vesicles was determined as described in Materials and Methods. The valinomycin partition coefficient as determined by either the dialysis procedure or by centrifugation was essentially the same: 0.03—0.08 cm. No significant difference in the partition coefficient was produced by the addition of cholesterol to the phospholipid vesicles, nor was the partition coefficient in small sonicated vesicles different from the value

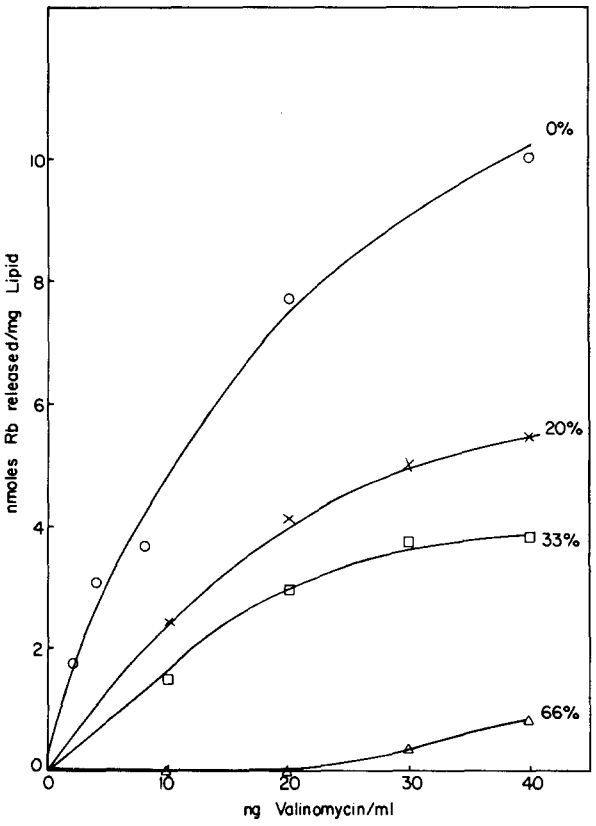

Fig. 3. Effect of cholesterol on the valinomycin-mediated efflux of ^{86}Rb from large unilamellar vesicles. Mixtures of egg phosphatidylcholine and cholesterol containing 0 (○———○), 20 (X———X), 33 (□———□), and 66 (△———△) mol% cholesterol and consisting of 2.5 mg total lipid were suspended in 0.45 ml phosphate buffer (5 mM potassium phosphate, 45 mM sodium phosphate, pH 7.2) containing $^{86}RbCl$ (7.5 μCi) and the mixtures were sonicated to clarify them (20—40 min). The vesicles were frozen in liquid N_2, thawed, sonicated for 5 s and then aliquots of the vesicles (50 μl) were incubated with 0.2 ml phosphate buffer and increasing amounts of valinomycin at 20°C. After 6 min, the incubation mixtures were placed on Dowex columns and eluted as described in Fig. 1.

obtained with large unilamellar vesicles formed by the freeze-thaw procedure. Under the experimental conditions described in Figs. 2 and 3, over 99% of the valinomycin would, therefore, be absorbed by the vesicles and not be free in solution. If cholesterol did alter this value slightly it could not alter the concentration of vesicular ionophore enough to account for the pronounced effects on permeability observed in Figs. 2 and 3. Cholesterol changes vesicle permeability and not merely the valinomycin partition coefficient.

It appears that above the phospholipid transition temperature cholesterol can increase the permeability of membranes only when the penetrating species is hydrophobic, when the cholesterol concentration is below 33 mol% and when the membrane is highly curved [16]. Presumably any amount of cholesterol would lower the permeability of the relatively large erythrocyte to valinomycin. These results indicate that data obtained from the study of relatively large freeze-thaw vesicles might be more relevant to the study of rather flat

membranes (as found in the erythrocyte), while data obtained from the study of relatively small vesicles formed by sonication might be relevant to the study of highly curved membranes (as found in mitochondrial cristae).

Physical measurements have also shown that large lipid vesicles differ from smaller vesicles in terms of phase transitions and other properties [17]. Smaller vesicles have been shown to incorporate proteins more readily than do larger ones [35], and it has been suggested that this might be relevant to the study of protein incorporation into biological membranes and membrane biogenesis [35]. Certainly all investigators who work with small, unilamellar lipid vesicles must be aware that some of the properties of these systems may change as the vesicle size changes.

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References

1 DeGier, J., Mandersloot, J.G. and Van Deenen, L.L.M. (1969) Biochim. Biophys. Acta 173, 143–145
2 Demel, R.A., Bruckdorfer, K.R. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 321–330
3 Demel, R.A., Geurts van Kessel, W.S.M. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 266, 26–40
4 Demel, R.A., Kinsky, S.C., Kinsky, C.B. and Van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 150, 655–665
5 Inoue, K. (1974) Biochim. Biophys. Acta 339, 390–402
6 LeLievre, J. and Rich, G.T. (1973) Biochim. Biophys. Acta 298, 15–16
7 Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T. (1973) Biochim. Biophys. Acta 311, 330–348
8 Bruckdorfer, K.R., Demel, R.A., DeGier, J. and Van Deenen, L.L.M. (1969) Biochim. Biophys. Acta 183–334–345
9 DeKruyff, B., DeGreef, W.J., Van Eyk, R.V.W., Demel, R.A. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta. 298, 479–499
10 DeKruyff, B., Demel, R.A. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 331–347
11 Kroes, J. and Ostwald, R. (1971) Biochim. Biophys. Acta 249, 647–650
12 McElhaney, R.N., DeGier, J. and Van der Neut-Kok, E.C.M. (1973) Biochim. Biophys. Acta 298, 500–512
13 Szabo, G., Eisenman, G. and Ciani, S. (1969) J. Membrane Biol. 1, 346–382
14 Benz, R. and Cross, D. (1978) Biochim. Biophys. Acta 506, 265–280
15 DeGier, J., Haest, C.W.M., Mandersloot, J.G. and Van Deenen, L.L.M. (1970) Biochim. Biophys. Acta 211, 373-375
16 LaBelle, E.F. and Racker, E. (1977) J. Membrane Biol. 31, 301–315
17 Van Dijck, P.W.M., DeKruijff, B., Aarts, P.A.M.M., Verkleij, A.J. and DeGier, J. (1978) Biochim. Biophys. Acta 506, 183–191
18 Cooper, R.A., Arner, E.C., Wiley, J.S. and Shattil, S.J. (1975) J. Clin. Invest. 55, 115–126
19 Hope, M.J., Bruckdorfer, K.R., Hart, C.A. and Lucy, J.A. (1977) Biochem. J. 166, 255–263
20 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
21 Nelson, G.J. (1967) J. Lipid Res. 8, 374–379
22 Brown, H.H., Zlatkis, A., Zak, B. and Boyle, A.J. (1954) Anal. Chem. 26, 397–399
23 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769–775
24 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917
25 Racker, E. (1973) Biochem. Biophys. Res. Commun. 55, 224–230
26 Gasko, O.D., Knowles, A.F., Shertzer, H.G., Suolinna, E.M. and Racker, E. (1976) Anal. Biochem. 72, 57–65

27 Bouma, S.R., Drislane, F.W. and Huestis, W.H. (1977) J. Biol. Chem. 252, 6759—6763
28 Stark, G. and Benz, R. (1971) J. Membrane Biol. 5, 133—153
29 Stark, G., Ketterer, B., Benz, R. and Lauger, P. (1971) Biophys. J. 11, 981—94
30 Lauger, P. (1972) Science 178, 24—30
31 Gambale, F., Gliozzi, A. and Robello, M. (1973) Biochim. Biophys. Acta 330, 325—334
32 Blok, M.C., DeGier, J. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 367, 210—224
33 Wolff, D., Caessa-Fischer, M., Vargas, F. and Diaz, G. (1971) J. Membrane Biol. 6, 304—314
34 Carroll, R.C. and Racker, E. (1977) J. Biol. Chem. 252, 6981—6990
35 Eytan, G.D. and Broza, R. (1978) FEBS Lett. 85, 175—178