Biochimica et Biophysica Acta, 330 (1973) 8–26

BBA 76489

ROLE OF CHOLESTEROL IN MEMBRANES
EFFECTS ON PHOSPHOLIPID–PROTEIN INTERACTIONS, MEMBRANE PERMEABILITY AND ENZYMATIC ACTIVITY

DEMETRIOS PAPAHADJOPOULOS[a], MARK COWDEN[a] and HAROLD KIMELBERG[b]

[a] *Department of Experimental Pathology and* [b] *Department of Neurosurgery, Roswell Park Memorial Institute, Buffalo, N.Y. 14203 (U.S.A.)*

(Received June 25th, 1973)

SUMMARY

The effect of cholesterol on the interaction of proteins with phospholipid membranes was studied using three independent techniques: effects on vesicle permeability, monolayer expansion, and phospholipid-dependent $(Na^+ + K^+)$-ATPase activity. The proteins studied were: cytochrome *c*, albumin, hemoglobin, lysozyme, myelin basic protein, myelin proteolipid apoprotein, and the polypeptide gramicidin A. The results were as follows:

1. All the proteins in this study produced a large increase in the permeability of phospholipid vesicles to Na^+. When cholesterol was mixed with phospholipid in equimolar proportions, most proteins produced only a comparatively small increase in permeability. This inhibitory effect of cholesterol on the permeability of phospholipid-protein membranes, was 38-fold for cytochrome *c* and 10-fold for hemoglobin. The only protein that was not affected by cholesterol was the myelin proteolipid apoprotein. Experiments with cytochrome *c* indicated that the above effects were unlikely to be due to inhibition of its binding to phosphatidylserine vesicles.

2. The presence of cholesterol in phosphatidylserine monolayers inhibits the area expansion produced by the addition of cytochrome *c* to the bulk phase. The inhibition of monolayer expansion by cholesterol was shown to be considerably larger than that obtained by equivalent dilution of the surface charges of phosphatidylserine by phosphatidylcholine.

3. The presence of cholesterol inhibits the ability of phospholipids to activate a delipidated preparation of $(Na^+ + K^+)$-ATPase. The degree of inhibition produced by nearly equimolar mixtures of cholesterol with brain phosphatidylserine and dioleylphosphatidylglycerol was approximately 60% while in mixtures with dipalmitoylphosphatidylglycerol, it was more than 90%.

4. The biological significance of these data are discussed in relation to the possible effects of increased cholesterol levels in cell membranes during the process of aging and the development of atherosclerosis.

Abbreviations: DPPG, dipalmitoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; DOPG, dioleylphosphatidylglycerol; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

INTRODUCTION

The role of cholesterol as a component of biological membranes has been studied extensively with phospholipid model membranes. Such studies, with well-defined membrane systems (monolayers, bilayers and vesicles) have been important in relating the physicochemical properties of phospholipid–cholesterol mixtures to the possible physiological role of cholesterol in cell membranes. Recent reviews[1,2] have discussed in detail the effects of cholesterol on the structure of phospholipid membranes, such as the "condensation" of the area per molecule in monolayers, the inhibition of motion of the acyl chains within the outer segment of the phospholipid bilayer, the increase of the width of the bilayer and the increased perpendicular orientation of the acyl chains.

Permeability studies with phospholipid vesicles and bilayers support and extend the above studies. It has been observed that incorporation of cholesterol decreases the permeability of phospholipid vesicles to Cl^-(ref. 3), to glucose[4], to other glycols and sugars[5,6] and to monovalent cations[7]. Furthermore it has been shown with bilayer membranes (black lipid films) that the presence of cholesterol decreases the water permeability[8] and increases the electrical capacitance and resistance[7,9,10].

Studies with biological membranes have indicated that partial removal of cholesterol from erythrocyte membranes increases the osmotic fragility and glycerol permeability[11]. On the other hand, guinea pig erythrocytes with increased cholesterol/phospholipid ratio show a decreased permeability to non-electrolytes, as well as active and passive Na^+ transport[12]. Similarly, increased cholesterol in the membranes of *Acholeplasma laidlawii* produces a decrease in the permeability rate of glycerol[13]. These studies are in accord with the effects of cholesterol on phospholipid membranes. However, other studies produced conflicting evidence indicating that partial removal of cholesterol has no appreciable effect on the hydraulic conductivity[14] or non-electrolyte permeability[15] of erythrocytes.

The studies with model membranes discussed earlier, strongly suggest that cholesterol could regulate the permeability of biological membranes by affecting the internal viscosity and molecular motion of the lipids within the membrane. Evidence on the possible role of cholesterol on "facilitated" transport was reported in relation to the inhibitory effect of cholesterol on the action of macrotetralide actins on bilayers[16]. A similar effect of cholesterol was shown with another "ionophore", valinomycin, on the rate of Rb^+ efflux through vesicles[17].

In this report we describe the influence of cholesterol on the interaction of several proteins with phospholipid membranes. The results indicate that the presence of cholesterol generally inhibits the ability of proteins to increase the permeability of phospholipid vesicles and inhibits the ability of these proteins to "expand" or "penetrate" phospholipid monolayers. It also inhibits the activity of a reconstituted preparation of $(Na^+ + K^+)$-ATPase. The results are discussed in relation to their physiological implications. A hypothesis is advanced relating the effects of cholesterol on membrane function to the process of atherogenesis.

MATERIALS

All phospholipids were prepared in this laboratory and were pure by the

criterion of thin layer chromatography on silica gel H and a solvent of chloroform–methanol–7M ammonia (230/90/15; v/v/v). Phosphatidylserine from beef brain, and phosphatidylcholine from egg yolk were prepared as described[18]. Dipalmitoylphosphatidylglycerol (DPPG) was synthesized from dipalmitoylphosphatidylcholine (DPPC), by minor modifications[19] of the method of Dawson[20]. DPPC was synthesized according to Robles and Van den Berg[21]. Dioleylphosphatidylglycerol (DOPG) was synthesized similarly to DPPG. Cholesterol was obtained from Sigma and recrystallized twice from methanol. All lipids were stored in chloroform under nitrogen in sealed ampoules at −50 C, at a concentration of approx. 10 μmoles/ml. Each ampoule was newly opened for each experiment.

Cytochrome *c* (horse heart, Type VI) was obtained from Sigma; albumin (human serum, crystallized) was obtained from Miles-Pentex; hemoglobin (human, twice crystallized) from Mann Research; gramicidin A (activity 100%) from Nutritional Biochemicals; myelin basic protein (A1) was a gift from Dr E. H. Eylar and was prepared from bovine brain[22]; myelin proteolipid apoprotein (N2) was a gift from Dr M. Moscarello, and was prepared from human brain myelin[23]. It was used as a solution in water at a concentration of 7 mg/ml. ATPase was prepared from frozen rabbit kidney outer medullas (obtained from Pell-Freez Biologicals, Inc.) as before[24].

Water was twice distilled, the second time in all-glass apparatus. Water used for the monolayer experiments was distilled two more times in all-glass apparatus once over $KMnO_4$ (pre-final). Salts and chemicals were all analytical reagent grade. *N*-tris (hydroxymethyl)-methyl-2-amino-ethane sulfonic acid (TES) and histidine were from Sigma. $^{22}Na^+$ was obtained from New England Nuclear and silicic acid from Mallinckrodt (A.R. 100 mesh). Crystalline ATP (disodium salt) and ouabain were obtained from Sigma. $(NH_4)_2SO_4$ and Tris were special enzyme grade from Mann Research.

METHODS

Phospholipid concentration was assayed by measuring inorganic phosphorus after $HClO_4$ digestion, with ammonium molybdate (1%, w/v) and Elon (1 in 10 dilution of 30 g $NaHSO_3$ + 10 g *p*-dimethylaminophenol sulfate per l). Protein concentration was measured by the biuret reaction[25] or by the method of Lowry[26]. ATPase activity was assayed under conditions identical to those described previously[24].

Phospholipid vesicles were prepared as previously described[7,27]. The method involves sonication for one hour in a bath type sonicator, in a closed glass tube placed under a nitrogen atmosphere at 24 C. Vesicles of DPPG were sonicated at 42 C. All vesicles were prepared in an aqueous solution of NaCl (either 100 mM or 10 mM as specified in each figure), buffered by histidine (2 mM) *plus* TES (2 mM) at pH 7.4 (or 6.5 in the case of N2) and containing EDTA (0.1 mM). Permeability properties (efflux of $^{22}Na^+$) were studied as before[7] by a procedure based on the original method of Bangham *et al.*[28]. Proteins were added to the pre-formed vesicles after passage through Sephadex, as before[29].

Expansion of monolayers

Expansion of phospholipid monolayers was performed in a 62-ml capacity

teflon trough, with a total surface area of 60 cm^2 (20 cm × 3 cm). The bulk phase (10 mM NaCl, 0.1 mM EDTA, 2 mM histidine, 2 mM TES, pH 7.4) was stirred with a small teflon bar-magnet. Constant temperature (25 °C) was maintained by running the experiments inside a water-cooled Lucite box and by running water through a glass coil in the teflon trough, both being regulated by a constant-temperature circulation bath. Phosphatidylserine monolayers were formed by applying a 5–10 μl-solution in hexane to the surface of the water, which had been cleaned by sweeping with a teflon barrier. Surface pressure measurements were performed with a platinum foil (0.5 cm wide, sanded with 400 grit, silicon carbide paper) suspended from a Cahn RG electro-balance connected to a recorder which was calibrated in dynes/cm. Constant film pressure (25 ± 1 dynes/cm) was maintained by manually positioning the teflon barrier. Area measurements were calculated from ruler readings with a ruler attached to the side of the trough. Expansion experiments were initiated by injecting 5 μl (125 μg) of cytochrome *c* solution below the surface film. The final concentration of cytochrome *c* was 2.0 μg/ml or 160 pmoles/ml.

Injection of cytochrome *c* under phosphatidylserine films results in a substantial increase in the film pressure[30,31] at constant area. Such an increase in pressure ($\Delta\pi$) has been taken as a measure of "penetration" of the injected substance into the monolayer[32]. Measurements of $\Delta\pi$, however, are difficult to interpret with mixed

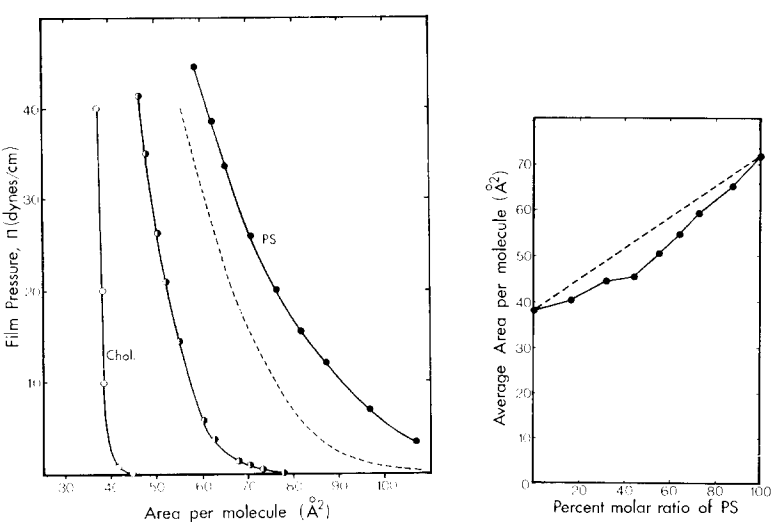

Fig. 1. Surface pressure–area curves for monomolecular films of phosphatidylserine (PS), cholesterol and an equimolar mixture. Each film was formed by spreading a dilute solution of each lipid in hexane, on a clean air–water (10 mM NaCl, pH 7.4) interface. The films were compressed by a teflon barrier. ○, cholesterol. ●, phosphatidylserine (PS), ◑, mixture consisting of 55% phosphatidylserine, 45% cholesterol (mole %). The broken line represents the calculated area of phosphatidylserine in the mixed film indicating considerable "condensation". The area per phosphatidylserine molecule in the mixed film was obtained by subtracting the relative area of cholesterol, which was assumed to be the same as in films of pure cholesterol.

Fig. 2. Average area per molecule for monolayer of phosphatidylserine (PS), cholesterol and mixtures. The points shown were obtained at 25 dynes/cm and 25 °C. – – –, the average area per molecule expected from ideal mixing of the two components.

films of different constitution, due to differences in the relationship of π to area per molecule for different substances, as shown in Fig. 1. In the experiments presented here the pressure of the films was kept constant, and the area was expanded, to compensate for the effect of the interacting protein. This method of isopiezic expansion[33] gives a more unambiguous picture of the effect of the same protein on different films. A constant film pressure of 25 dynes/cm was chosen as representing an area per phospholipid molecule (72 $Å^2$) which is similar to that calculated for phospholipid bilayers by X-ray diffraction[34,35]. Fig. 1 shows the area per molecule at different surface pressures for films of pure phosphatidylserine, cholesterol and a mixture of each at equimolar amounts. In accordance with previous results obtained with phosphatidylcholine/cholesterol mixtures[36–38], the area of the mixed film is less than the total area of each component calculated from pure films, indicating a "condensation" of approx. 20%. Fig. 2 shows the deviation from additivity for several phosphatidylserine/cholesterol mixtures at the experimental conditions of 25 dynes/cm and 25 °C.

Because expansion is not seen with films of either phosphatidylcholine or cholesterol alone after injection of cytochrome *c* into the subphase, the percent expansion of phosphatidylserine/phosphatidylcholine and phosphatidylserine/cholesterol films were expressed as percent area expansion per phosphatidylserine area only. In phosphatidylserine–cholesterol films when phosphatidylserine is condensed by cholesterol, the uncondensed area was used in the expansion calculation (mole % phosphatidylserine × 72 $Å^2$ × total number of molecules in film). The total number of molecules present in the film was obtained from the initial area of each film and the average area per molecule given in the graph of Fig. 2.

RESULTS AND DISCUSSION

(*a*) *Effect of cholesterol on protein-induced permeability changes*

The influence of cholesterol on the ability of proteins to increase the permeability of phospholipid vesicles was studied with several purified proteins and polypeptides, a partial list of which is given in Table I. The test system involved measurements of the rate of efflux of $^{22}Na^+$ out of phosphatidylserine vesicles and vesicles containing equimolar amounts of phosphatidylserine and cholesterol. The proteins were added outside the vesicles at different concentrations, and the rate of $^{22}Na^+$ efflux was monitored by dialysis. As shown in the first two columns of Table I, all the proteins used in this study tend to increase the permeability of the vesicles to $^{22}Na^+$. However, the self-diffusion rates obtained with the same amount of protein were much smaller when the vesicles contained cholesterol. The effect of cholesterol in the absence of protein was much smaller (Lines 1 and 2, Columns 1 and 2). The ratio of $^{22}Na^+$ self-diffusion rates obtained with each protein (phosphatidylserine vesicles over phosphatidylserine-cholesterol vesicles) is given in Column 3. From this last column, we can see that in the presence of cytochrome *c* the permeability of phosphatidylserine vesicles to Na^+ was 115 times larger than that of phosphatidylserine-cholesterol vesicles. As shown by the appropriate control in the absence of protein (Lines 1 and 2, Column 3) the difference in permeability between phosphatidylserine vesicles alone and with cholesterol is only 2-fold (at 24 °C) or 3-fold (at 36 °C).

Similarly large differences in Na^+ diffusion rates were obtained when the effect of other proteins was studied with the two types of vesicles, indicating a pro-

TABLE I

EFFECT OF VARIOUS PROTEINS ON Na^+ PERMEABILITY OF PHOSPHATIDYLSERINE VESICLES WITH OR WITHOUT CHOLESTEROL

Each reaction mixture was 1 ml and contained approx. 1 μmole phosphatidylserine (with or without 1 μmole cholesterol) and the amount of protein indicated in Column 1. Self-diffusion rates: 1st h after addition of proteins. The figures given in Lines 1 and 2 (lipid vesicles alone) are averages of several runs, taken from Papahadjopoulos *et al.*[7]. The results shown in the subsequent lines are averages of two different experiments, with a deviation usually within 10% of the average value.

Protein	*$^{22}Na^+$ self-diffusion rate (% per h)*		*Column 1/Column 2*
	Phosphatidylserine	*Phosphatidylserine/cholesterol*	
None*,†	0.02	0.01	2
None**, ***	0.06	0.02	3
Cytochrome *c**** (10 mg/ml)	46.9	0.41	115
Basic myelin protein A1* (0.65 mg/ml)	40.6	0.92	44
Serum albumin HSA*** (50 mg/ml)	76.8	1.25	61
Hemoglobin** (0.1 mg/ml)	21.5	1.12	19
Gramicidin A** ($2 \cdot 10^{-7}$ M)	13.1	1.32	10
Myelin proteolipid protein N2† (0.36 mg/ml)	15.0	4.9	3

* 100 mM NaCl, pH 7.4, 24 °C.
** 100 mM NaCl, pH 7.4, 36 °C.
*** 10 mM NaCl, pH 7.4, 36 °C.
† 10 mM NaCl, pH 6.5, 26 °C.

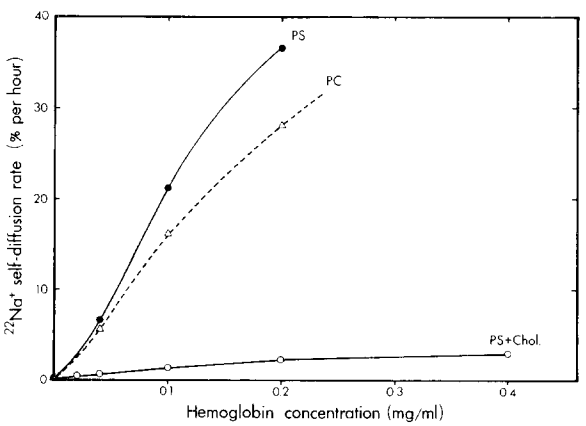

Fig. 3. Effect of hemoglobin on $^{22}Na^+$ self-diffusion rate through phospholipid vesicles with and without cholesterol. Hemoglobin was added at the indicated concentration outside the vesicles, in 100 mM NaCl, pH 7.4, at 36 °C. The amount of phospholipid in each mixture was approximately 1 μmole and the total volume 1 ml. ●, phosphatidylserine vesicles; △, phosphatidylcholine vesicles; ○, phosphatidylserine/cholesterol (1/1 molar ratio) vesicles. Points were taken from single experiments, with appropriate duplicates. Separate experiments deviate within 20% of the given values.

nounced inhibitory effect induced by the presence of cholesterol. The ratios of the Na^+ self-diffusion rates (Column 3; Table I) indicate a 44-fold inhibition by cholesterol for the effect of the basic myelin protein (A1), 61-fold inhibition for human serum albumin, and 19-fold inhibition for hemoglobin.

Fig. 3 gives more details on the effect of different concentrations of hemoglobin on the Na^+ diffusion rates through phosphatidylserine vesicles with and without cholesterol. It is clear that the inhibitory effect produced by cholesterol is sustained over a wide range of concentrations. Similar results were obtained with lysozyme but not shown here. As shown earlier[39], albumin was much more effective in increasing the permeability of phosphatidylserine vesicles at low protein concentrations, when the buffer contained small amounts of Ca^{2+}(0.5 mM). A similar influence of Ca^{2+} was found more recently for the effect of the basic myelin protein (A1) (unpublished). In both of the above cases, incorporation of cholesterol into the phospholipid vesicles inhibits the effect of either albumin or A1 in the presence of Ca^{2+}, as was seen above in the absence of Ca^{2+}.

Two possible alternative explanations of the above results are that the effect of cholesterol is due to an inhibition of protein binding (resulting from a decrease in surface charge density), or due to an inhibition of protein "penetration" into the bilayer (resulting from an increase in membrane viscosity). The following two experiments are relevant to this question. The amount of cytochrome *c* bound to phosphatidylserine and phosphatidylserine/cholesterol (1/1) vesicles was estimated as 115 and 80 (nmoles cytochrome *c* per μmole phosphatidylserine), respectively. The procedure for measuring bound cytochrome *c* was as described before[29]. It involves spectrophotometric determinations of the amount of cytochrome *c* and phosphate present in the pellets obtained by high speed centrifugation of sonicated phosphatidylserine and phosphatidylserine/cholesterol vesicles in the presence of cytochrome *c* (original concentrations: 12 mg/ml cytochrome *c* and 0.77 μmoles phosphatidylserine/ml in 10 mM NaCl, pH 7.4). From the results obtained, it is clear that cholesterol does have an inhibitory effect (30%) on the amount of cytochrome *c* bound per phosphatidylserine. However, this effect is relatively small compared to the permeability effects and is unlikely to account for the 115-fold inhibition in cytochrome *c*-induced permeability.

The second experiment relating to the question of charge dilution was a comparison of the effect of hemoglobin on phosphatidylserine and phosphatidylcholine vesicles (shown in Fig. 3). It is clear that hemoglobin produces a large increase in Na^+ permeability with the neutral phosphatidylcholine vesicles, which is almost as great as with the negatively charged phosphatidylserine vesicles. This result indicates that the hemoglobin effect is not strictly related to the surface charge density of the vesicles, as is the case for cytochrome *c* (ref. 29). Following this argument, it can be concluded that the 19-fold inhibition of the hemoglobin effect on permeability by cholesterol could not be due to the dilution of the surface charge density of the phosphatidylserine vesicles.

The last two lines in Table I describe the influence of cholesterol on the interaction of gramicidin A and myelin proteolipid apoprotein (N2) with phospholipid vesicles. In contrast to the results with the other proteins, the presence of cholesterol has much less effect. The ratio of the increase in Na^+ self-diffusion rates (Line 3) is 10 for gramicidin and only 3 for myelin proteolipid apoprotein (N2). These results

indicate that the presence of cholesterol has only a minimal effect on the ability of N2 to increase the permeability of phospholipid membranes to Na^+ and only a small effect on that of gramicidin A. The results with gramicidin A are in agreement with the work of Krasne *et al.*[41] which showed that gramicidin A was effective in increasing the conductance of bilayer membranes equally well below or above the temperature at which the fatty acyl chains freeze (T_c).

The ability of the myelin proteolipid apoprotein to interact and increase the permeability of phospholipid membranes equally well in the presence or absence of cholesterol is of considerable biological interest, because of the high amount of cholesterol present in myelin membranes. The lack of cholesterol effect in this case could perhaps be attributed to the high "hydrophobicity" of this protein. Thus, proteolipid protein is extracted into organic solvents with lipids, it can remain soluble in organic solvents after removal of most lipids and can become water soluble only after prolonged dialysis procedures[23,42]. Its chemical composition indicates the presence of a high percentage of non-polar amino acids, as well as covalently linked fatty acids[23,42]. It is thus reasonable to speculate that the predominantly non-polar nature of this protein favors its penetration into the interior of the lipid bilayer even when the viscosity of the hydrocarbon chain is increased due to the incorporation of cholesterol. The subject of the penetration of protein into phospholipid membranes will be discussed in more detail later in relation to the monolayer studies.

(b) Effect of acyl-chain fluidity and phase transitions

If the cholesterol effect on lipid-protein interactions discussed above is due to the stabilization of the membrane, induced by immobilization of the acyl chains interacting with cholesterol, it should be possible to obtain similar results by simply freezing the acyl chains, *i.e.* lowering the experimental temperature below the T_c for the lipids involved. The experiments presented in this section were designed to test this possibility.

In an earlier publication we have presented studies with vesicles composed of DPPG showing a large increase in Na^+ efflux between 30 °C and 38 °C which coincided with a decrease in the polarization of fluorescence of perylene embedded in the same vesicles[19]. It was concluded from this evidence that these vesicles undergo a phase transition at this temperature range (mid-point 38 °C) from a solid to liquid-crystalline state. More recent measurements in this laboratory with differential scanning calorimetry have verified this conclusion by indicating a temperature of 37.8 °C for the rising part of the endothermic peak with sonicated preparations of DPPG vesicles. We have therefore utilized this system to study the effect of cytochrome *c* on the Na^+ efflux at different temperatures, below and at the phase transition region.

Fig. 4A shows the effect of low concentrations of cytochrome *c* on the Na^+ efflux through DPPG vesicles at 24, 30 and 36 °C. The lower (broken) line indicates the self-diffusion rates without cytochrome *c* and the upper (solid) line in the presence of 1 mg/ml cytochrome *c*. The numbers in parenthesis over each set of points represents the fold increase in permeability induced by cytochrome *c*. It can be seen that at temperatures of 24 and 30 °C there was only 3-fold increase, while at 36 °C the increase was 17-fold. This indicates that cytochrome *c* is much more effective in increasing the Na^+ diffusion rates at temperatures near the T_c. In a similar experiment with higher concentration of cytochrome *c* (5 mg/ml, Fig. 4B) the increase in perme-

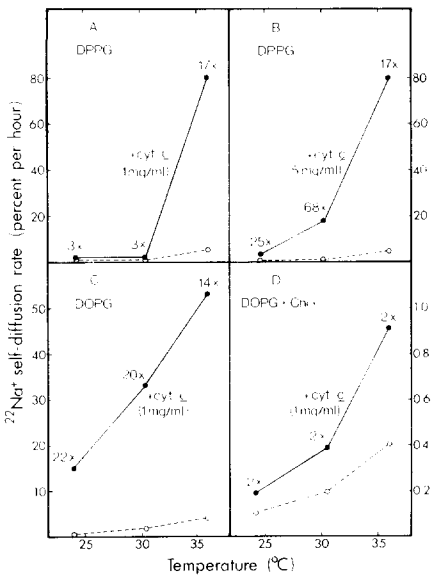

Fig. 4. Effect of cytochrome *c* on $^{22}Na^+$ self-diffusion rate through phospholipid vesicles at different temperatures. Cytochrome *c* was added at the indicated concentrations outside the vesicles, in 10 mM NaCl, pH 7.4, at room temperature. The amount of phospholipid in each mixture was approx. 1 μmole, and the total volume 1.0 ml; the mixture was then immediately dialysed at the indicated temperature, against 10 ml of 10 mM NaCl buffer, which was changed at 1-h intervals. The points shown in Fig. 4 were obtained during the second hour and represent single experiments with appropriate duplicates. ●, vesicles with cytochrome *c* added; ○, vesicles alone. (A) Vesicles of dipalmitoylphosphatidylglycerol (DPPG) (1 mg cytochrome *c* per ml); (B) vesicles of dipalmitoylphosphatidylglycerol (DPPG) (5 mg cytochrome *c* per ml); (C) vesicles of dioleylphosphatidylglycerol (DOPG) (1 mg cytochrome *c* per ml); (D) equimolar mixture of dioleylphosphatidylglycerol (DOPG) and cholesterol (1 mg cytochrome *c* per ml).

ability was high at all temperatures, 25-fold at 24 °C, 68-fold at 30 °C and 17-fold (80% per h self-diffusion rate) at 36 °C. This might be indicating that at high concentrations, cytochrome *c* can induce structural rearrangements in the phospholipid bilayer, effectively interfering with the cooperative melting of the acyl chains. An effect of cytochrome *c* on the phase transition of mycoplasma phospholipids was reported recently[43] and has been verified more recently in this laboratory with DPPG–cytochrome *c* mixtures. In this latter case, the presence of cytochrome *c* tends to broaden the endothermic peak of DPPG, lowers the T_c, and reduces the overall heat of transition per phospholipid molecule.

In order to verify the above conclusions, we have performed similar experiments with DOPG vesicles which are above their T_c at all three experimental temperatures. As shown in Fig. 4C, low concentrations of cytochrome *c* (1 mg/ml) give a large increase in the Na^+ diffusion rates through DOPG vesicles; 22-fold at 24 °C, 20-fold at 30 °C and 14-fold at 36 °C. When the same amount of cytochrome *c* was added to DOPG vesicles containing equimolar amounts of cholesterol (Fig. 4D), the increase in permeability was minimal (2-fold) and the same at all temperatures. The same results, with only a 2-fold increase in permeability at all temperatures was obtained with DPPG and cholesterol (1/1) vesicles, not shown in the figure. It thus appears

from the results shown in Fig. 4 that freezing of the acyl chains of a phospholipid bilayer (similarly to the incorporation of cholesterol) can inhibit the ability of a protein such as cytochrome *c* to produce a large increase in Na^+ permeability. However, the effect of freezing can be overcome by increasing the concentration of cytochrome *c*. This is in contrast to cholesterol-containing bilayers, which exhibit low permeability to Na^+ even in the presence of high concentrations of this protein.

(c) Effect of cholesterol on the activity of $(Na^+ + K^+)$*-ATPase*

The experiments described above indicate that cholesterol can have a large inhibitory influence on the ability of some proteins to increase the passive Na^+ permeability of phospholipid membranes. A more biologically relevant question is whether the presence of cholesterol can similarly influence the specific function of membrane enzymes, especially those involved in transport phenomena. To examine this possibility we have investigated the influence of cholesterol on the ability of several phospholipid vesicles to reactivate a de-lipidized preparation of $(Na^+ + K^+)$-ATPase. Results described earlier have indicated that this enzyme can be re-activated specifically by the addition of vesicles composed of phosphatidylserine or phosphatidylglycerol[24]. Furthermore, it was shown[24] that the activity is sensitive to changes in lipid viscosity as indicated by a sharp change of the slope of the Arrhenius plot of the enzyme activity at 32 °C for dipalmitoylphosphatidylglycerol ("viscotropic effect"). The temperature is close to the experimentally determined T_c for this lipid, 38 °C.

We have re-examined the influence of membrane viscosity on the activity of the $(Na^+ + K^+)$-ATPase by studying the effect of cholesterol on the reactivation obtained with three purified phospholipids. Bovine brain phosphatidylserine has a T_c at approximately 15 °C (ref. 44). Dioleylphosphatidylglycerol probably has a T_c lower than −22 °C (from a comparison of the results of Phillips *et al.*[45], for dioleylphosphatidylcholine and the results of Papahadjopoulos *et al.*[19], with DPPG and DPPC) and dipalmitoylphosphatidylglycerol has a T_c of 38 °C (ref. 19). The results are presented in Table II. The second and third columns give the specific activity of the preparation as Mg^{2+}-stimulated and $(Na^+ + K^+)$-stimulated ATPase at 37 °C.

As observed earlier[24], the addition of phosphatidylserine vesicles induces a more than 10-fold increase of the $(Na^+ + K^+)$-stimulated part of the activity, with a negligible effect on the Mg^{2+}-dependent part. Similar activation is given by DOPG, and considerably less but still significant (5-fold) activation is given by DPPG. When the same experiments were performed with vesicles containing nearly equimolar amounts of cholesterol to phospholipid, the levels of $(Na^+ + K^+)$-ATPase were significantly lower than those obtained with pure phospholipids. Column 4 in Table II gives the percent inhibition of the $(Na^+ + K^+)$-ATPase activity induced by the presence of cholesterol. It can be seen that there is approx. 60% inhibition of the activation by phosphatidylserine and DOPG and more than 90% inhibition of the activation by DPPG. Similar degrees of inhibition were obtained, irrespective to whether the $(Na^+ + K^+)$-ATPase activity was obtained by subtracting the ouabain controls from the totals (as shown in Table II), or by subtracting the K^+-free controls from the totals.

In order to test the possibility that the inhibitory effect of cholesterol was due to the dilution of the phosphatidylserine or phosphatidylglycerol molecules (effectively decreasing the surface charge density) we studied the activation of the $(Na^+ + K^+)$-

TABLE II

EFFECTS OF CHOLESTEROL ON PHOSPHOLIPID-DEPENDENT ATPase ACTIVITY

The phospholipid/cholesterol ratios indicated are mole ratios. The amounts of phospholipid added were: phosphatidylserine, 0.64 μmoles; DOPG, 0.105 μmole; DPPG, 0.59 μmoles. ATPase activities were assayed as described under Methods + ouabain. 0.07 mg protein was present in 1.5 ml total volume. All measurements were at 37 °C except for DOPG which was at 38 °C. The % inhibition for ($Na^+ + K^+$)-ATPase in the presence of cholesterol refers to the control activity for the same phospholipid. Figures in parenthesis represent the number of separate experiments for each lipid mixture. Values in table are averages of this number of results, or simply averages of the appropriate duplicates within each experiment.

Phospholipid added	*ATPase activity* (*μmoles P_i/mg protein/h*)			*% inhibition of* ($Na^+ + K^+$)-*ATPase*
	Total	Mg^{2+}	$Na^+ + K^+$	
None	21.5 (2)	18.2	3.3	-
Phosphatidylserine	56.7 (4)	19.2	37.5	Control
Phosphatidylserine/cholesterol (1/1.1)	39.7 (2)	25.4	14.3	62
DOPG	45.8	10.6	35.2	Control
DOPG/cholesterol (1/1.5)	37.2	22.3	14.9	58
DPPG	43.8 (2)	26.0	17.8	Control
DPPG/cholesterol (1/1)	26.8 (2)	25.4	1.4	92
Phosphatidylserine/phosphatidylcholine (1/1)	60.6	16.8	43.8	-

ATPase by vesicles comprised of an equimolar mixture of phosphatidylserine and phosphatidylcholine. As shown in Table II, the activation obtained with this mixture was slightly higher than that given by pure phosphatidylserine, indicating that surface charge density is not an important factor within the limits of this experiment. It is thus reasonable to conclude that the inhibitory effect of cholesterol is related to the inhibition of molecular motion within the phospholipid bilayer, shown by NMR experiments[46].

It should be noted that the ATPase activity in the presence of unsaturated phospholipids (phosphatidylserine and DOPG) is inhibited only partly by cholesterol while the activity in the presence of DPPG is almost completely inhibited. This result is in good agreement with previous work on the ability of cholesterol to condense the area per molecule of different phospholipid monolayers at the air water interface[37,38,47]. These studies have indicated that the cholesterol "condensing effect" is related to the fluidity of the phospholipids and it is maximal when the phospholipids are close to their transition point (T_c). Thus, cholesterol shows much less of a condensing effect with polyunsaturated phospholipids compared to phospholipids containing mixtures of both saturated and unsaturated fatty acyl chains. In the case of DPPG membranes, cholesterol would tend to expand the area per molecule at temperatures below the T_c and condense above the T_c. At the experimental temperature (37 °C) the DPPG vesicles are very close to their transition point, while DOPG and phosphatidylserine are, respectively, 50 and 22 degrees higher than their T_c. It appears then that the inhibition of the ($Na^+ + K^+$)-ATPase activity in this system could be ascribed to a "viscotropic" effect, similar to that seen earlier[24] with DPPG at temperatures below the T_c.

(d) Effect of cholesterol on protein-induced expansion of monolayers

In a previous publication we reported an apparent correlation between the ability of proteins to increase the permeability of phospholipid vesicles and their ability to increase the film pressure of phospholipid monolayers[31]. The increase in film pressure is usually interpreted as "penetration" of the protein into the monolayer[30,32,33,48]. The effect can also be measured by an expansion of the monolayer area if the experiments are performed under constant film pressure *i.e.* isopiezically[33]. In the series of experiments reported in this paper, we have studied the ability of cytochrome *c* to expand phosphatidylserine monolayers in the presence of different amounts of cholesterol. Parallel experiments were also run with monolayers of phosphatidylserine mixed with different amounts of phosphatidylcholine in order to examine the effect of charge dilution on the penetration of cytochrome *c*.

Fig. 5 shows the degree of expansion of different monolayers at different time intervals following the injection of cytochrome *c* under each monolayer, kept at a constant surface pressure of 25 dynes/cm. The expansion is expressed as a percentage increase of the original area of the phosphatidylserine component in each monolayer. After a delay of approximately 2 min possibly due to mixing, a linear increase in percentage of expansion was observed until approx. 30 min. A plateau region was obtained in most cases after 60 min following injection. The degree of expansion obtained at equilibrium (60 min) was very different for each monolayer. Thus pure phosphati-

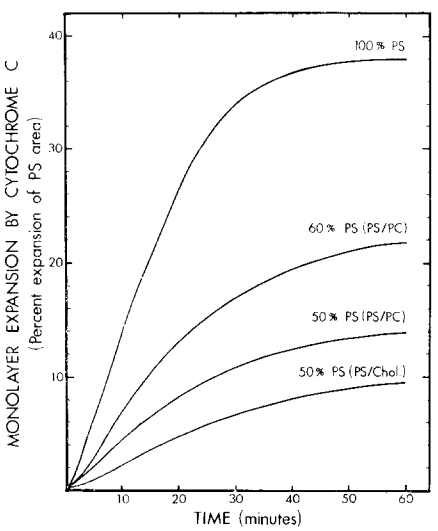

Fig. 5. Expansion of the area of monolayers by cytochrome *c* with time. Monolayers composed of either pure phosphatidylserine (PS) or mixtures with phosphatidylcholine (PC) or cholesterol, were spread on a clean surface of water containing NaCl (10 mM) at pH 7.4, and compressed to a film pressure of 25 dynes/cm. Cytochrome *c* was added under the monolayer, and the barrier containing the film was moved so as to keep the surface pressure constant at 25 dynes/cm ± 1 dyne/cm. The increase of the area of the film was expressed as a percent expansion of the area of the phosphatidylserine component in each mixed film. All experiments at 25 °C. The percentages of phosphatidylserine in the films are in moles. The curves shown are average values obtained from 2 to 4 different experiments, with a deviation within 10% of the average value.

dylserine was expanded by 38%, while when phosphatidylserine was mixed with cholesterol at equimolar proportions the expansion of the phosphatidylserine area was only 9.5%. In equimolar mixtures with phosphatidylcholine, the area of phosphatidylserine was expanded by 14%, while the expansion of the area of phosphatidylserine in a mixed monolayer consisting of 60% phosphatidylserine and 40% phosphatidylcholine was expanded by 22%. As indicated by calculations presented in Methods, the area of phosphatidylserine in a monolayer consisting of an equimolar mixture of phosphatidylserine and cholesterol is approx. 60%. This calculation takes into account the area per molecule of phosphatidylserine (72Å^2) and cholesterol (38Å^2) at 25 dynes/cm and the degree of "condensation" produced by cholesterol. Consequently, for a proper comparison of the effect of cytochrome *c* on the phosphatidylserine–cholesterol monolayer, the percentage expansion (9.5%) should be related to its effect on the 60/40 phosphatidylserine/phosphatidylcholine monolayer (22%). From the large difference in percent expansion between these two monolayers, it can be concluded that the inhibition of phosphatidylserine expansion observed in the presence of cholesterol is not simply due to the dilution of surface charge.

The above studies were extended to a series of mixed monolayers with different percentages of cholesterol and phosphatidylcholine in phosphatidylserine. The degree of expansion obtained after injection of cytochrome *c*, at equilibrium, with different monolayers is shown in Fig. 6. The two curves give the percent expansion relative to the area of the phosphatidylserine component in each monolayer as a function of the composition of the monolayer in percent moles of phosphatidylserine. It can be seen that the phosphatidylserine/phosphatidylcholine curve gives higher expansion at all points compared to the phosphatidylserine/cholesterol curve. It is interesting that both

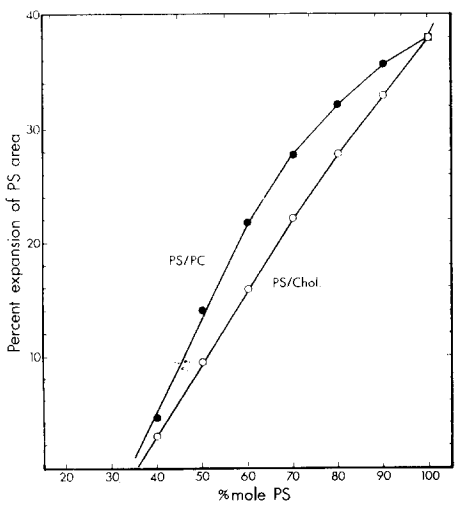

Fig. 6. Cytochrome *c*-induced expansion of monolayers of phosphatidylserine (PS) mixed with different amounts of phosphatidylcholine (PC) or cholesterol. Experimental details as in Fig. 5 and Methods. The values of expansion were obtained at equilibrium, 60 min after injection of cytochrome *c*. Expansion of the area is expressed as a percentage of the area of PS in each film. ●, mixtures of phosphatidylserine with phosphatidylcholine; ○, mixtures of phosphatidylserine with cholesterol; ⊥, pure phosphatidylserine. Points represent averages of two experiments.

curves extrapolate to zero expansion with monolayers of approx. 35% phosphatidylserine. It should be noted here that the values of expansion shown in Fig. 6 are calculated on the basis of the phosphatidylserine component present within each mixed monolayer and not on the basis of the total area. Consequently if the area of each molecule of phosphatidylserine was expanded to the same degree in each mixture, the percent expansion should be constant in the different mixtures.

The fact that both curves shown in Fig. 6 extrapolate to zero expansion below approx. 35% mole phosphatidylserine indicates that cytochrome *c*-induced expansion could be due to multiple-point attachment of the protein at neighboring phosphatidylserine molecules. In any membrane composed of two different molecules where one is negatively charged (such as phosphatidylserine) and the other neutral (such as phosphatidylcholine), the lowest energy configuration would tend to minimize contacts between the charged molecules. In a hexagonally packed array of such molecules of similar size, if the negatively charged component (phosphatidylserine) is $\leq 33.3\%$ of the total, each molecule will be completely surrounded, on the average, by molecules of the other component (Fig. 7A). At concentrations of phosphatidylserine more than 33% (or one phosphatidylserine for two phosphatidylcholine as shown in Fig. 7A) there will be an increasing number of neighboring phosphatidylserine molecules. The lack of any expansion below the 35% phosphatidylserine mixtures might therefore indicate that the cytochrome *c*-induced expansion depends on the concentration of

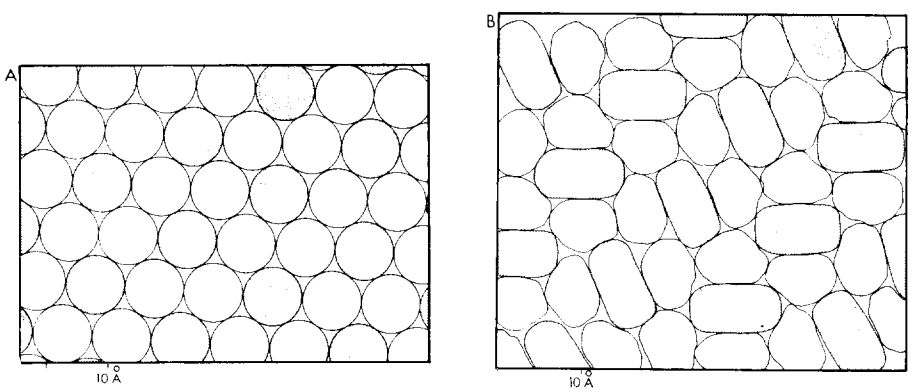

Fig. 7. (A) A schematic representation of hexagonally packed molecules of equal size. The dark circles represent the negatively charged component phosphatidylserine, the open circles represent the neutral component phosphatidylcholine. The molecular ratio in this arrangement (one phosphatidylserine for every two phosphatidylcholine or 33.3% phosphatidylserine) is the maximal ratio which allows for no contacts between phosphatidylserine molecules. The actual shape of each molecule is not necessarily circular but it can be considered as such for simplicity and because of the relatively high degree of molecular motion at the liquid crystalline phase. The area per molecule of either component, as determined by the area of monomolecular film at 25 dynes/cm is 72 Å^2. (B) A schematic representation of a possible packing arrangement of molecules of phosphatidylserine and cholesterol. The shape of the cholesterol molecule was taken from VandenHeuvel[19] and was determined by projection of space-filling models (approximately 38 Å^2). The area of phosphatidylserine is only an approximate representation of the area that the two chains might occupy when "condensed" by cholesterol (approximately 58 Å^2 calculated from the data of Fig. 2). The pattern of molecular packing shown here represents a molecular ratio of one phosphatidylserine for every 2 cholesterols (33.3% phosphatidylserine) and allows for no contacts between phosphatidylserine molecules.

clusters of more than one molecule of phosphatidylserine. If this is indeed the case, it also appears that cytochrome *c* does not tend to concentrate or segregate the phosphatidylserine molecules in clusters under the conditions of these experiments. The same argument holds for the packing within mixed phosphatidylserine cholesterol monolayers although in this case the approximate shape of the molcules has to be taken into account. As shown in Fig. 7B, the 1 to 2 molecular ratio (33.3% phosphatidylserine in cholesterol) is again the maximal ratio with no neighboring phosphatidylserine molecules.

The effect of cholesterol on phosphatidylserine monolayer expansion was also studied with two other proteins, both obtained from myelin. Results with the basic myelin protein (A1) at concentrations of 1.6 μg/ml in a 0.1 M NaCl bulk phase at pH 7.4 indicated an expansion of 65% at a constant film pressure of 25 dynes/cm. The expansion of the phosphatidylserine area of a film of phosphatidylserine–cholesterol (1/1) under similar conditions and concentrations of A1 was only 42%. On the other hand, injection of a similar concentration of the myelin proteolipid apoprotein (N2) under the same conditions, resulted in an expansion per phosphatidylserine area of approx. 60% after 60 min, with both phosphatidylserine and phosphatidylserine–cholesterol (1/1) monolayers. These results are in good agreement with the effects of these proteins on the permeability of vesicles with and without cholesterol. Thus there appears to exist a correlation for the inhibition of protein-induced expansion of monolayers, and of protein-induced increase in Na^+ permeability of vesicles, as previously suggested[31].

All three experimental systems discussed so far have indicated that cholesterol can inhibit the interaction of several proteins with phospholipids, with a concomitant decrease in passive and active (by extrapolation from the ATPase results) transport of monovalent cations. The following section will consider some possible biological implications of these results.

(*e*) *Biological implications: aging and atherosclerosis*

Although cholesterol is a major constituent of many biological membranes, its physiological role is still not well understood. Some evidence indicates that partial removal of cholesterol from erythrocyte membranes increases the osmotic fragility and glycerol permeability[11]. Conversely, guinea pig erythrocytes with increased cholesterol-to-phospholipid ratio show decreased permeability to non-electrolytes, and Na^+ transport[12]. Similarly, increased cholesterol in the membranes of *Acholeplasma laidlawii* produces a decrease in the permeability rate of glycerol[13].

The studies discussed earlier involving phospholipid vesicles and other model membranes, strongly indicate that cholesterol could regulate the permeability of biological membranes by affecting the internal viscosity and molecular motion of the lipids within the membrane. The degree of cohesion between the lipid molecules will in turn determine the motional freedom and localization of membrane enzymes, "carriers" and "gating" systems involved in transport and other membrane functions. The role of cholesterol can thus be considered as a stabilizing or a "dampening" mechanism, inhibiting structural changes in the membrane due to thermal, mechanical and other stresses. It is probably not coincidental that the most stable and metabolically inactive membrane (myelin) has the highest (one to one) cholesterol to phospholipid ratio[50,51]. Other plasma membranes vary from a ratio of approx. 0.9 for

various species of erythrocytes[52,53] to ratios of 0.6 to 0.7 for the more metabolically active plasma membranes of liver[54,55] and non-myelinated nerve[56]. Intracellular membranes have much lower ratios[55] compared to plasma membranes.

We suggest that while cholesterol is needed in order to provide a generally stable membrane framework, it is excluded from highly functional areas of membranes such as those containing $(Na^+ + K^+)$-ATPase. These areas are likely to require greater structural fluctuations and molecular motion than more functionally inert areas. Following the same argument, it can also be suggested that an increased cholesterol to phospholipid ratio, along with decreased unsaturation (which would tend to enhance the cholesterol condensing effect) could be detrimental to the function of the plasma membrane and indirectly to the cellular metabolism. Increased cholesterol content and also decreased unsaturation have been observed to correlate with aging and the process of atherosclerosis[57–59]. Moreover, a rise in the free cholesterol content is one of the earlier events involving changes of aortic lipid composition in the pre-atherotic lesions, the fatty streaks[60–62].

The above evidence, combined with the studies on model membranes discussed earlier, and the well known "atherogenic" effect of cholesterol-feeding in animals[63,64] suggests the possibility that increased cholesterol content in the membranes of aortic cells could be involved as an initiator of the process of atherogenesis. A hypothesis to this effect has been advanced recently[65], proposing that increased incorporation of cholesterol into the plasma membranes of arterial intima cells (induced by high levels of circulating plasma β-lipoproteins and/or endothelial injury), could have a critical inhibitory effect on several important membrane enzymes, with consequent alterations of the metabolic state of the cells. Inhibition of the pump ATPase will result in loss of intracellular K^+, and consequently to inhibition of protein synthesis and other intracellular enzyme systems, followed by lysis due to osmotic imbalance and eventual cell death. Impairment of the adenyl cyclase and the resulting reduction of cyclic AMP levels, will produce inhibition of several intracellular enzyme systems and secretory mechanisms[66] and would also induce cell proliferation[67]. Many of the early and subsequent events in the development of atherosclerosis could thus be accounted for, by the initial inhibitory effects of increased membrane cholesterol. Some preliminary evidence on the effect of exogenous cholesterol on the behavior of cells in culture (Mayhew and Papahadjopoulos, unpublished), is in good agreement with some of the above hypothesis. It was found that when mouse fibroblasts were incubated with phospholipid vesicles containing high amounts of cholesterol, there was an accumulation of microscopically visible bodies in their cytoplasm, concomitant with a 60–70% inhibition of rate of K^+ uptake from the medium. This indicates a possible relationship between exogenous cholesterol and the $(Na^+ + K^+)$-ATPase activity of mammalian cells.

Concluding remarks

The studies reported here have indicated that the presence of cholesterol can inhibit the interaction of many proteins with phospholipid membranes, although some fairly hydrophobic proteins such as the myelin proteolipid apoprotein are less affected. This inhibitory effect of cholesterol appears to be similar to the effect of freezing the acyl chains of the phospholipid bilayers below their T_c. Both these effects were manifested as: a marked decrease in the ability of proteins to enhance the perme-

ability of phospholipid vesicles to Na^+, and a substantial decrease in the ability of proteins to "expand" phospholipid monolayers. Furthermore, the presence of cholesterol produced a partial or total inhibition of the activity of phospholipid-activated $(Na^+ + K^+)$-ATPase, depending on the degree of unsaturation of the phospholipid component. Complete inhibition of re-activation was obtained when cholesterol was mixed with saturated phospholipids.

The results are in good agreement with the available information concerning the physicochemical properties of cholesterol–phospholipid mixtures. It would appear that the inhibitory effects of cholesterol on phospholipid–protein interactions are the result of the increased stability of the bilayers containing cholesterol. It is possible then that the presence of cholesterol, which condenses the area per phospholipid molecule and drastically decreases the molecular motion of the acyl chains, makes the "penetration" or "deformation" of the bilayer by many proteins energetically unfavorable.

The fluidity of the fatty acyl chains of phospholipids has been shown to be a requirement for several transport systems[68-71] and also of the $(Na^+ + K^+)$-ATPase[24]. The fluidity of the acyl chains probably provides the required motional freedom, allowing proteins within membranes to undergo conformational changes and rotational and/or translational movements associated with their activity. Cholesterol could thus have a regulatory role in these processes by controlling the fluidity of the acyl chains.

The physiological role of cholesterol could thus be that of a "dampening" agent, or stabilizing force, needed for the overall integrity of the cell plasma membrane. However, an increased level of cholesterol in membranes due to a pathological situation, could interfere with some vital functions of the membrane, with detrimental effects to cell metabolism and viability.

ACKNOWLEDGEMENTS

We wish to thank Mrs R. Lazo and Mr T. Isac for expert technical assistance. Supported in part by grants from the American Heart Association (69730), National Institutes of Health (5R01 GM-18921) and N.A.S.A. NGR-33015016. The work on monolayers constitutes part of the requirements for the Ph. D. program of M. Cowden at the Department of Biochemistry, Roswell Park Division, State University of New York at Buffalo, and supported by Public Health Service Training Grant CAO-5016-16 and a New York State Department of Health fellowship line 79502.

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