Mullin, B. R., Pacuszka, T., Lee, G., Kohn, L. D., Brady, R. O., & Fishman, P. H. (1977) *Science (Washington, D.C.) 199*, 77–79.
Rahmann, H., & Hilbig, R. (1979) in *Glycoconjugates: Proceedings of the Fifth International Symposium* (Schauer, R., et al., Eds.) pp 672–673, Georg Thieme Verlag, Stuttgart, Federal Republic of Germany.
Rosenthal, S. N., & Fendler, J. H. (1976) *Adv. Phys. Org. Chem. 13*, 279–424.
Sattler, J., Schwarzmann, G., Staerk, J., Ziegler, W., & Wiegandt, H. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 159–163.
Sillerud, L. O., Prestegard, J. H., Yu, R. K., Schafer, D. E., & Konigsberg, W. H. (1978) *Biochemistry 17*, 2619–2628.
Sillerud, L. O., Schafer, D. E., Yu, R. K., Prestegard, J. H., & Konigsberg, W. H. (1979) *Biophys. J. 25*, 133a.
Sillerud, L. O., Prestegard, J. H., Konigsberg, W. H., Schafer, D. E., & Yu, R. K. (1981) *J. Biol. Chem. 256*, 1094–1098.

Influence of Phospholipid Structure on Sterol Efflux Induced by Albumin–Phospholipid Complexes†

Lester C. Bartholow and Robert P. Geyer*

ABSTRACT: Sterol release from strain L fibroblasts was measured in serum-free medium supplemented with delipidated human serum albumin and various phospholipids. The sterol molecule appears to preferentially interact with the *sn*-2 acyl chain of the phospholipid. The carbonyl oxygen of the phospholipid acyl ester linkage is not required for sterol–phospholipid interactions, while the phosphate and choline groups are required. In the presence of the human serum albumin–phospholipid complex, phospholipids containing *trans*-acyl groups are significantly more effective at removing cellular sterol than the corresponding *cis*-acyl group.

Many studies of sterol–phospholipid interactions involve protein-free model membrane systems. These systems have produced conflicting evidence regarding the nature of apparent hydrogen bonding between the sterol hydroxyl group and either the carbonyl oxygen atom of the phospholipid acyl ester linkage (Huang, 1976; Brockerhoff, 1974) or the phosphate base group (Darke et al., 1972; Verma & Wallach, 1973). In addition, preferential interactions between the sterol molecule and the acyl chains of the phospholipid have been suggested (Huang, 1977; Bloch, 1979; Seelig & Seelig, 1980; Bush et al., 1980) but not confirmed in biological systems.

In the studies reported here, we have investigated sterol–phospholipid interactions in a lipoprotein-like complex of albumin–phospholipid. Previous work from this laboratory (Chau & Geyer, 1978; Bartholow & Geyer, 1981) has established that homogeneous human serum albumin can bind dipalmitoylphosphatidylcholine and that the resulting complex can cause a synergistic release of sterol from human skin and mouse L fibroblasts in tissue culture. Human serum albumin also binds dilinoleoylphosphatidylcholine, but this combination caused significantly less sterol efflux than the saturated phosphatidylcholine–albumin combination. One explanation for the difference in effects between the saturated and unsaturated complexes is that the double bonds of the polyunsaturated phosphatidylcholine introduced sufficient bending (Spritz & Mishkel, 1969) to interrupt hydrophobic interactions between the phospholipid acyl methylene carbon atoms and the sterol molecule. We have attempted to characterize this sterol efflux in terms of sterol–phospholipid interactions and show that (1) the reduction in sterol efflux observed with the polyunsaturated phosphatidylcholine–albumin complex is related to chain length, (2) hydrogen bonding between the carbonyl oxygen atom of the acyl ester linkage and the sterol hydroxyl is not required for sterol efflux, (3) choline and phosphate groups each contribute to the interaction with the sterol molecule, and (4) there appears to be a preferential interaction between the sterol molecule and the *sn*-2 acyl chain.

Experimental Procedures

Compounds. Crystalline human serum albumin was obtained from Miles Laboratories, horse serum from Grand Island Biological Co., and [1-^{14}C]acetate (56 mCi/mmol) from New England Nuclear Corp. The phospholipids were provided by Calbiochem-Behring Corp., with the exception of dielaideoylphosphatidylcholine, which was obtained from Sigma Chemical Co. A standard lipid mixture (mixture B) was obtained from Supelco while silica gel G plates were obtained from Analabs, Inc.

Preparation of Compounds. Both the human serum albumin and the horse serum were delipidated by the method of Rothblatt et al. (1976), as previously reported (Bartholow & Geyer, 1981). The aqueous proteins were then sterilized by filtration through a 0.22-μm Millipore filter before addition to the cells.

The phospholipids were dissolved in heptane and evaporated to dryness under N_2. NaCl (0.9%) was added to give a concentration of 1 mg/mL. The solution was then sonicated at 60% of full power under N_2 for two 1-min bursts with a Biosonik sonicator having a microtip of 8 mm. The resulting dispersions, prepared in 20-mL sterile glass scintillation vials, were slightly opaque. The sonicated lipids were used immediately after preparation at a final concentration in the incubation medium of 50 μg/mL. Thin-layer chromatography of 50 μg of each phospholipid gave a single spot with iodine visualization.

Cell System. Stock cultures of mouse fibroblasts (L strain) were grown in suspension at 37 °C in 50 mL of modified

† From the Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts 02115. *Received June 2, 1981.* This work was supported by the Harvard Nutrition Fund of the Department of Nutrition.

0006-2960/82/0421-1271$01.25/0

Table I: Sterol Efflux from Strain L Fibroblasts in Serum-Free Medium Supplemented with Delipidated Human Serum Albumin and Various Phospholipids

phospholipid	sterol efflux[a]		
	PL[b] (%)	HSA + PL (%)	*P* value[c]
1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	10.4 ± 2.2	58.8 ± 3.6	<0.001
1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine	14.6 ± 1.9	47.1 ± 1.7	<0.001
1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine	30.3 ± 7.3	39.8 ± 1.7	ND
1,2-dioleoyl-*sn*-glycero-3-phosphocholine	8.6 ± 0.8	44.1 ± 4.7	<0.001
1,2-dielaideoyl-*sn*-glycero-3-phosphocholine	13.3 ± 1.4	57.3 ± 2.2	<0.001
cardiolipin, bacterial	19.7 ± 2.8	31.6 ± 3.4	NS
cardiolipin, bovine	10.6 ± 1.0	50.1 ± 1.9	<0.001

[a] All values in this table are the mean ± SD; $n = 3$. Serum-free medium supplemented with human serum albumin alone gave a percent efflux of 18.9 ± 4.7 ($n = 39$), while sterol efflux to unsupplemented serum-free medium gave a percent release of 8.8 ± 3.9 ($n = 18$) (mean ± SD). [b] Abbreviations: NS, not significant; ND, not determined; HSA, human serum albumin; PL, phospholipid. [c] Two-way analysis of variance was performed to determine if a synergistic interaction between albumin and phospholipid had occurred. The *P* value is the probability that such interaction could occur by chance.

Waymouth medium supplemented with 2.5% delipidated horse serum, as previously reported (Chau & Geyer, 1978). Cell growth was exponential with a doubling time of 24 h; cell density was between 1×10^5 and 1×10^6 cells/mL. Cell number and size were monitored with an electronic counter and size distribution plotter (Coulter Electronics).

Strain L fibroblasts were labeled with [1-^{14}C]acetate (56 mCi/mmol) for 24 h. Label (20 μCi) was added to each 50-mL inoculum at an initial cell density of 400 000 cells/mL. After the labeling period was completed, the cell number was determined; the cells were centrifuged at 600*g* for 10 min, washed twice with serum-free medium, and then resuspended in 10 mL of serum-free medium supplemented with albumin, phospholipid, or albumin–phospholipid mixtures. The final albumin and phospholipid concentrations were 5 mg/mL and 50 μg/mL, respectively, or a 1:1 molar ratio. The cell number at the start of the depletion period was 400 000 cells/mL. At the end of a 12-h depletion period, triplicate 10-mL cell cultures were centrifuged as before, and the lipids of the medium and the cell pellet were extracted by the method of Slayback et al. (1977). The sterols present were iolated by thin-layer chromatography on silica gel G plates and were developed in a hexane/diethyl ether/glacial acetic acid mixture (65:30:1). A standard lipid mixture (mixture B, Supelco, Inc.) was chromatographed with the experimental samples. Radioactive assays were performed with a Beckman Model LS-250 scintillation counter with 5 mL of Betafluor as the scintillator.

We subjected the data to two-way analysis of variance utilizing the Minitab statistical computing system (Pennsylvania State University). The *P* value reported in the table is the probability that an interactive, or synergistic, effect between albumin and the phospholipid could have occurred by chance. Synergism, as used here, refers to the sterol efflux in excess of the additive effect of albumin and phospholipid.

Results and Discussion

Effects of Double Bonds. Compared to dipalmitoylphosphatidylcholine, introduction of one or two polyunsaturated acyl chains into the phospholipid progressively reduced sterol efflux in the presence of human serum albumin (Table I). Since both the saturated and unsaturated phospholipids bind to albumin (Kitagawa et al., 1976; Zborowski et al., 1977; Bartholow & Geyer, 1981), binding in itself is not sufficient to cause maximal sterol efflux. Dilinoleoylphosphatidylcholine alone caused a reduction in cell number from 400 000 to 320 000 cells/mL during the depletion period; the sterol release measured in the presence of this phospholipid, therefore, may reflect some toxicity. In the presence of human serum albumin this phospholipid had no adverse effects on cell number and size; however, the possibility of more subtle residual toxicity cannot be ruled out. In any case, the synergistic potential between this phospholipid and albumin, therefore, could not be statistically assessed by two-way analysis of variance.

When used in the absence of human serum albumin, none of the phospholipids other than dilinoleoylphosphatidylcholine (Table I), 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (Table III), and 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (Table III) caused any cell toxicity. Although there were small significant differences in sterol release induced by the various phosphatidylcholines individually, the magnitude of the response in the presence of the human serum albumin–phospholipid complexes greatly exceeded these base line differences. The physiological basis for these base line differences is not known.

The progressive reduction in sterol efflux observed as the number of double bonds was increased suggested that the bending of the acyl chains caused by these double bonds could interrupt sterol–phospholipid interactions. To test this hypothesis, we measured sterol release in the presence of human serum albumin and phospholipids of the same carbon number but differing in cis or trans configuration (Table I). The trans phospholipid–albumin complex was more effective in removing cellular sterol than was the cis phospholipid–albumin complex. Since cis double bonds cause bending of acyl chains, which reduces their effective length (Spritz & Mishkel, 1969), these results suggest that chain length is an important variable in the observed sterol efflux. The sterol efflux observed with the bacterial and bovine cardiolipins further emphasizes the importance of the saturation of the acyl chains (Table I). In the presence of human serum albumin and bovine cardiolipin, which contain primarily saturated acyl chains, there was a significantly greater efflux of sterol than with the unsaturated bacterial cardiolipin.

Binding of the Sterol Hydroxyl Group to the Phospholipid. The data in Table II show that removal of the choline group from dipalmitoylphosphatidylcholine significantly reduces, but does not abolish, the sterol-removing capability of the resulting phosphatidic acid. Removal of the phosphate group, however, eliminates any synergistic component and reduces the sterol efflux to that observed with albumin alone. Apparently both the choline and phosphate groups are required to achieve the maximal sterol efflux of 60% observed in this test system. Since the sterol release induced by the dihexadecylphosphatidylcholine–albumin combination was equal to that induced by the ester-linked phospholipid complex, hydrogen bonding between the carbonyl oxygen of the acyl ester linkage and the sterol hydroxyl group is not required for sterol–

Table II: Sterol Efflux from Strain L Fibroblasts in Serum-Free Medium Supplemented with Delipidated Human Serum Albumin and Various Phospholipids

phospholipid	sterol efflux PL	sterol efflux HSA + PL	*P* value[a]
1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	10.4 ± 2.2	58.8 ± 3.6	<0.001
1,2-dipalmitoyl-*sn*-glycerol 3-phosphate	6.3 ± 0.9	29.8 ± 4.0	<0.05
1,2-dipalmitoyl-*sn*-glycerol	15.7 ± 1.5	21.7 ± 4.0	NS
1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine	9.1 ± 0.6	57.6 ± 2.7	<0.001
1-*O*-octadec-1,9-enyl-2-*O*-hexadecyl-*sn*-glycero-3-phosphocholine	18.4 ± 4.0	57.7 ± 0.8	<0.001

[a] The *P* value is the probability that an interaction, or synergism, between albumin and phospholipid occurred by chance.

Table III: Sterol Efflux from Strain L Fibroblasts in Serum-Free Medium Supplemented with Human Serum Albumin and the Indicated Phospholipid

phospholipid	sterol efflux (%)
1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine	19.3 ± 2.6
1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (acetal)	34.0 ± 0.4
2-palmitoyl-1-lyso-*sn*-glycero-3-phosphocholine	40.4 ± 4.0
1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	58.8 ± 3.6

[a] Prelabeled L strain fibroblasts were placed in serum-free medium supplemented with HSA and phospholipid.

Table IV: Summary of Effects of Structural Modification of 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine on Sterol Efflux

group removed from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	sterol efflux[a] (%)
none	58.8
sn-1 and *sn*-2 acyl chain carbonyl oxygens[b]	57.7
sn-1 acyl chain[c]	40.4
sn-3 choline[b]	29.8
sn-3 choline phosphate[b]	21.7
sn-2 acyl chain[c]	19.3

[a] Measured in the presence of human serum albumin and phospholipid. [b] See data in Table III. [c] See data in Table II.

phospholipid interaction in this test system.

Preferential Interaction of the Sterol Molecule and the sn-2 Acyl Chain. The greater sterol release in the presence of the ether analogue with an 18:2 acyl chain in the *sn*-1 position and a 16:0 in the *sn*-2 position (Table II) compared to the 1-palmitoyl-2-linoleoylphosphatidylcholine (Table I) suggested a preferential interaction between the sterol molecule and the *sn*-2 position acyl chain. To investigate this possible interaction, we tested three phospholipids containing a single acyl chain for their ability to remove cellular sterol in the presence of human serum albumin (Table III). Both the 2-lyso and acetal phospholipids were toxic to the cells if human serum albumin was not present; hence, an interaction term could not be calculated. However, in the presence of human serum albumin, cell size and number were normal. The ability of the *sn*-2 acyl chain phospholipid, but not the *sn*-1, to induce significant sterol efflux in the presence of human serum albumin again argues for a preferential interaction with the *sn*-2 acyl chain. The intermediate sterol efflux observed with the acetal phospholipid (Table III), whose acyl chain can be considered equidistant between the *sn*-1 and the *sn*-2 position acyl chains, is consistent with a preferential interaction between the sterol molecule and the *sn*-2 position acyl chain. The possible role of phospholipases A_1 and A_2 on sterol flux by generation of their respective lysophosphatidylcholines remains to be determined.

Table IV summarizes these effects of phospholipid structure on sterol efflux in the presence of human serum albumin. Removal of the *sn*-3 choline group or the *sn*-1 acyl chain reduces sterol efflux by about 30–40% of control values, while removal of the *sn*-2 acyl chain or the *sn*-3 choline phosphate groups reduces sterol efflux to that induced by human serum albumin alone. Further studies are necessary to elucidate the mechanism of action of these observed changes in sterol movement. We have shown, however, that direct hydrogen bonding is not a significant source of phospholipid–sterol interactions and that there appears to be a preferential interaction between the *sn*-2 phospholipid acyl chain and the sterol molecule. Further, the reduction in sterol release with the introduction of double bonds and the greater release induced by *trans*- vs. *cis*-acyl chains both argue that chain length is an important variable in the sterol efflux observed in these investigations.

Acknowledgments

We thank Dr. Albert Owen for his helpful discussions. We appreciate the help of Jeanne MacLaren with the preparation of the manuscript.

References

Bartholow, L. C., & Geyer, R. P. (1981) *Biochim. Biophys. Acta 665*, 40–47.
Bloch, K. E. (1979) *CRC Crit. Rev. Biochem. 7*, 1–5.
Brockerhoff, H. (1974) *Lipids 9*, 645–650.
Bush, S. F., Levin, H., & Levin, I. W. (1980) *Chem. Phys. Lipids 27*, 101–111.
Chau, I., & Geyer, R. P. (1978) *Biochim. Biophys. Acta 542*, 214–221.
Darke, A., Finer, E. G., Flook, A. G., & Phillips, M. C. (1972) *J. Mol. Biol. 63*, 265–279.
Huang, C. H. (1976) *Nature (London) 259*, 242–244.
Huang, C. H. (1977) *Chem. Phys. Lipids 19*, 150–158.
Kitagawa, T., Inoue, K., & Nojima, S. (1976) *J. Biochem. (Tokyo) 79*, 1147–1155.
Rothblatt, G. H., Arbogast, L. Y., Ouellette, L., & Howard, B. V. (1976) *In Vitro 12*, 554–557.
Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.
Slayback, J. R. B., Cheung, L. W. Y., & Geyer, R. P. (1977) *Anal. Biochem. 83*, 372–384.
Spritz, N., & Mishkel, M. A. (1969) *J. Clin. Invest. 48*, 78–86.
Verma, S. P., & Wallach, D. F. M. (1973) *Biochim. Biophys. Acta 330*, 122–131.
Zborowski, J., Roerdink, F., & Scherphof, G. (1977) *Biochim. Biophys. Acta 529*, 115–122.