Biochemical Pharmacology, Vol. 38, No. 7, pp. 1169–1174, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00
© 1989. Pergamon Press plc

DECREASE IN THE FLUIDITY OF BRUSH-BORDER MEMBRANE VESICLES INDUCED BY GENTAMICIN

A SPIN-LABELING STUDY

TOSHIKI MORIYAMA, HAJIME NAKAHAMA,* YOSHIFUMI FUKUHARA, MASARU HORIO, MASAHIRO YANASE, YOSHIMASA ORITA, TAKENOBU KAMADA, MASARU KANASHIRO† and YOSHIHIRO MIYAKE†

First Department of Medicine, Osaka University Medical School, Osaka 553, Japan; and † Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka 565, Japan

(*Received* 2 *May* 1988; *accepted* 16 *August* 1988)

Abstract—In our previous paper (Horio *et al.*, *Biochim Biophys Acta* **858**: 153–160, 1986), we reported that the addition of gentamicin *in vitro* to rabbit renal brush-border membrane vesicles decreases the apparent V_{max} of Na^+-dependent D-glucose transport without affecting the apparent K_m. In the present study, we investigated the effects of gentamicin on the physical state of spin-labeled rabbit renal brush-border membranes, using electron spin resonance spectrometry. Brush-border membrane vesicles were prepared from outer cortex (mainly contains early proximal tubule) and outer medulla (containing primarily late proximal tubule), and the gentamicin toxicities in both preparations were compared. Significant decreases were observed in the membrane fluidity of 5 mM gentamicin-treated brush-border membranes. The fluidity of outer cortical brush-border membranes was affected at both 25° and 35°, whereas that of outer medullary membranes was affected only at 35°. Two different stearic acid spin labels revealed that gentamicin affected the fluidity only in the superficial region of the membranes. We also demonstrated that the gentamicin-induced decreases in Na^+-dependent D-glucose transport and in the membrane fluidity were recovered by washing gentamicin-treated brush-border membranes. We suggest that gentamicin binds to the superficial region of brush-border membranes and inhibits Na^+-dependent D-glucose transport across brush-border membranes through the decrease in the membrane fluidity.

In our previous study, we demonstrated that gentamicin, added to the isolated renal brush-border membrane vesicles *in vitro*, decreases the V_{max} of Na^+-dependent D-glucose transport in a concentration-dependent manner [1]. Other investigators reported that gentamicin binds to renal brush-border membranes, whose binding sites are thought to be negatively charged phospholipids in the membranes [2, 3]. These observations suggest that gentamicin binding to brush-border membranes affects the functional membrane proteins (such as transporters and enzymes) through a change in the properties of the membranes.

It is reported that the S1 and S2 segments of the proximal tubule are more damaged by gentamicin than the S3 segment, evidenced morphologically [4]. In our vesicle study, the Na^+-dependent D-glucose transport system in early proximal tubule was more vulnerable to gentamicin toxicity than that in late proximal tubule [1]. Therefore, we prepared brush-border membrane vesicles from outer cortex (mainly S1 and S2 segments) and outer medulla (mainly S3 segment).

The spin-label technique is a useful tool for detecting conformational and dynamic changes of biological membranes [5–8]. When a stearic acid spin label with a *N*-oxyloxazolidine ring at the appropriate carbon position is used, electron spin resonance (ESR) spectroscopy depicts the change of freedom of anisotropic motion in the lipid bilayer, so-called "membrane fluidity" [5, 6, 8, 9].

* Correspondence: Hajime Nakahama, M.D., First Department of Medicine, Osaka University Medical School, 1-1-50 Fukushima, Fukushima-ku, Osaka 553, Japan.

‡ HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid.

METHODS

Vesicle preparation. Renal outer cortical and outer medullary tissues were obtained from a male albino rabbit weighing about 2 kg, as previously described [1]. Brush-border membrane vesicles were prepared from each tissue in parallel by the Ca^{2+} precipitation method. The final vesicle preparations were resuspended in buffer containing 100 mM mannitol and 10 mM Tris–HEPES‡ (pH 7.4). The relative enrichment of the final vesicle preparations (vesicles/homogenates) was as follows. In the outer cortical preparation, the enrichment factors were 11.2 for alkaline phosphatase and 12.4 for γ-glutamyltranspeptidase, the brush-border membrane markers, while they were 0.29 for $(Na^+$-$K^+)$-ATPase, 0.07 for succinate dehydrogenase and 0.56 for glucose-6-phosphatase, the marker enzymes for basolateral membranes, mitochondria and microsomes respectively. In the outer medullary preparation, the enrichment factors were 10.6 for alkaline phosphatase and 12.1 for γ-glutamyltranspeptidase, while they were 0.43 for $(Na^+$-$K^+)$ATPase, 0.13 for succinate dehydrogenase and 0.79 for glucose-6-

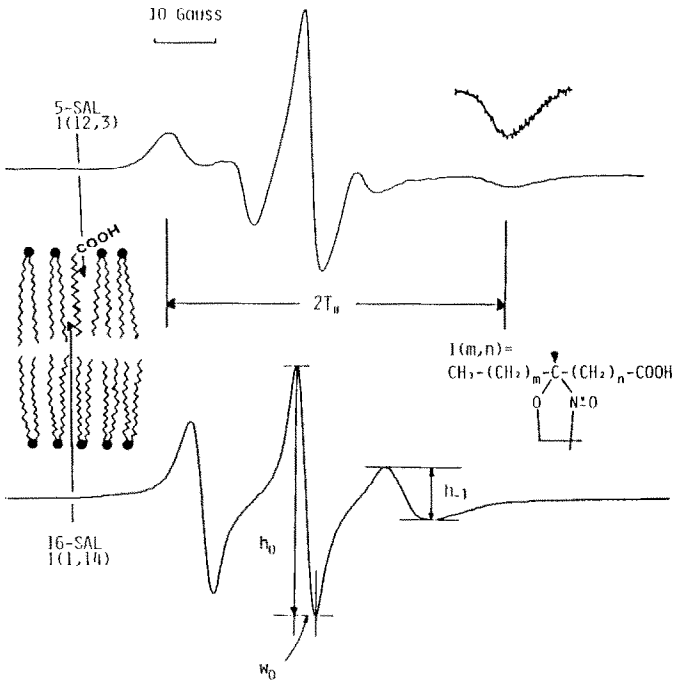

Fig. 1. Chemical formulae for 5- and 16-SAL and the representative electron spin resonance spectra of SAL embedded in outer cortical brush-border membrane vesicles at 25°. Key: SAL, stearic acid spin label; I(m,n), general molecular formula for fatty acid spin labels; ●, phospholipid; h_0, mid-field peak height; h_{-1}, high-field peak height; and W_0, line width of mid-field peak.

phosphatase. The vesicle preparation was then divided into two fractions, and each fraction was incubated with 5 or 10 mM gentamicin, or vehicle, at 37° for 60 min before use.

Preparation of the spin-labeled brush-border membrane vesicles. Two stearic acid spin labels (SAL), 5- and 16-SAL, were purchased from the Syva Co. (Palo Alto, CA). Each has a nitroxide radical ring at the 5th or 16th carbon position, counted from the carboxyl group of the acyl chain (Fig. 1).

One millimolar solutions of the probes in methanol were stored at −60°. Aliquots of the solution were transferred to test tubes and dried under a stream of dry nitrogen. Suspensions of brush-border membrane vesicles (about 10 mg protein/ml) were added to the tube and gently mixed. The ratio of spin label to membrane protein was adjusted to 3 μg spin label/mg protein.

Analysis of ESR spectra. ESR spectra were measured with a Varian EPR spectrometer, model E-109, equipped with a Varian temperature controller, model E-257. A sample (80 μl) was put in a flat quartz cell (JEOL, JES-Lc-11), of which the temperature was monitored by a copper-constantan thermocouple attached to the surface of the cell. The experimental conditions were as follows: time constant, 0.5 sec; scan rate, 200 G per 8 min; modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 20 mW; microwave frequency, 9.08 gHz. The representative spectra of SAL spin labels embedded in the brush-border membrane vesicles are shown in Fig. 1. 5-SAL gave the upper spectrum where we could clearly define the outermost peak positions and the fluidity could be estimated by the order parameter $S(T_{11})$ expressed as Equation 1. Treatment with gentamicin did not alter the spectrum except for the increase of the distance between the outermost peaks as shown in Fig. 2. The observed values of the outermost hyperfine splitting ($2T_{11}$ in Gauss) were used to calculate the order parameter $S(T_{11})$ in 5-SAL according to Equation 1 [10]. Here, T_{xx} and T_{zz} are the hyperfine elements of the static interaction tensor (T_{11}) parallel to the static Hamiltonian (H) principal nuclear hyperfine axes x and z respectively. The x axis is parallel to the N—O bond direction, and the z axis is parallel to the nitrogen 2p orbital. The elements of T used in this study were determined previously by incorporating nitroxide derivatives into host crystals as substitutional impurities; (T_{xx}, T_{zz}) = (6.1, 32.4) Gauss [10]. However, 16-SAL embedded in the deepest region of the membrane gave the lower spectrum in Fig. 1, where the outermost peaks collapsed into inner ones corresponding to its pronounced fluidity. Therefore, we could no longer calculate the order parameter $S(T_{11})$, which compelled us to estimate the membrane fluidity by the rotation correlation time (τ_0) formulated as Equation 2.

$$S(T_{11}) = 1/2\left[\frac{3(T_{11} - T_{xx})}{(T_{zz} - T_{xx})} - 1\right] \qquad (1)$$

$T_{xx} = 6.1$ Gauss; $T_{zz} = 32.4$ Gauss; and T_{11}, outer hyperfine splitting.

$$\tau_0 = K \cdot W_0\left[(h_0/h_{-1})^{1/2} - 1\right] \qquad (2)$$

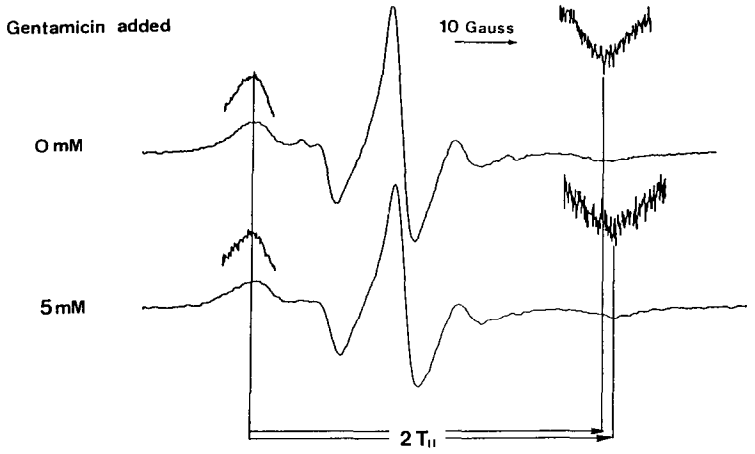

Fig. 2. ESR spectra of brush-border membrane labeled with 5-stearic acid at 25° in the presence of 0 mM (upper) and 5 mM (lower) gentamicin.

$K = 6.5 \times 10^{-10}$ sec, W_0, line width of mid-field peak; h_0, mid-field peak height; and h_{-1}, high-field peak height. When the value of the order parameter $S(T_{11})$ or rotation correlation time (τ_0) is big, the freedom of anisotropic motion of spin label in the membranes, i.e. the membrane fluidity, is small [9].

Measurement of D-*glucose uptake.* The procedure for uptake measurement was as previously described [1]. Briefly, a 50-μl aliquot of vesicles (3–4 mg protein/ml) was incubated with 100 μl of "incubation medium" containing D-[^{3}H]glucose and other constituents as required. After 1 sec, the reaction was terminated by the addition of a 10-fold dilution of ice-cold stop solution using a fast sampling apparatus. After addition of the stop solution, the vesicles were applied to a Millipore filter (HAWP 0.45 μm) under light suction. The filter was then washed with another 4.5 ml of the stop solution, dissolved in scintillation fluid, and counted along with samples of the incubation medium and appropriate standards. The Na^+-dependent component of D-glucose flux was calculated from the total flux by subtracting the Na^+-independent flux measured with choline replacing sodium. The stop solution contained 150 mM NaCl, 1 mM phlorizin and sufficient mannitol to compensate for intravesicular osmolarity, in 10 mM Tris–HEPES (pH 7.4). All experimental points were carried out, at least in triplicate, at 25°.

Chemicals. D-[^{3}H]Glucose was from the New England Corp. (Boston, MA). Choline chloride (3× crystallized) was from the Sigma Chemical Co. (St. Louis, MO). Gentamicin sulfate was from Essex Japan (Osaka, Japan). The other chemicals were of the highest purity available from commercial sources. Tukey's multiple comparison analysis was used in the statistical evaluation of data, and P values of less than 0.05 were taken to indicate a statistically significant difference.

RESULTS

Effects of gentamicin on the fluidity of brush-border membrane vesicles. Table 1 shows the results using 5-SAL as a spin label. The order parameter $S(T_{11})$ in 5 mM gentamicin-treated outer cortical brush-border membrane vesicles was significantly higher than that in the control at both 25° and 35°. In the gentamicin-treated outer medullary brush-border membrane vesicles, $S(T_{11})$ values were higher than control values at 35°, whereas there was no difference in $S(T_{11})$ at 25°. These results indicate that gentamicin decreased the anisotropic motion of 5-SAL in outer cortical brush-border membranes more than in the outer medullary preparation. Table 2 shows the effects of 5 mM gentamicin on the rotation correlation time (τ_0) of 16-SAL-labeled brush-border membrane vesicles. As shown in Table 2, 5 mM gentamicin had no effect on the rotation correlation time in outer cortical or outer medullary brush-border membrane vesicles at either temperature tested. 16-SAL is thought to reflect the condition of a more hydrophobic region (i.e. deeper sites) of the brush-border membrane than 5-SAL [5]. These results indicate that gentamicin mainly affects the physical state of the superficial region of outer cortical brush-border membranes.

Effect of washing on Na^+*-dependent* D-*glucose uptake in 10 mM gentamicin-treated brush-border membrane vesicles.* In our previous paper, we demonstrated that the V_{max} values of Na^+-dependent D-glucose transport in outer cortical and outer medullary brush-border membrane vesicles are decreased by gentamicin treatment *in vitro* [1]. Here, recovery from the gentamicin-induced decrease in Na^+-dependent D-glucose transport was tested by washing gentamicin-treated vesicles. Either outer cortical or outer medullary brush-border membrane vesicles were divided into two fractions. One was treated with 10 mM gentamicin and the other with vehicle at 37° for 60 min before use. Each fraction was divided into two subfractions. One was used for transport measurement as it was (pre-wash). The other was ultracentrifuged twice (43,000 *g*, 20 min), replacing the supernatant fraction with gentamicin-free buffer (washed), and was used for transport measurement.

Table 1. Order parameters $S(T_{11})$ of outer cortical and outer medullary brush-border membranes labeled with the stearic acid spin label I (12, 3)

Membrane preparation	Order parameter $S(T_{11})$ 25°	35°
Outer cortex		
Untreated	0.797 ± 0.004	0.694 ± 0.004
Incubated with 5 mM gentamicin	0.825 ± 0.004*	0.723 ± 0.004†
Outer medulla		
Untreated	0.783 ± 0.004‡	0.668 ± 0.005‡
Incubated with 5 mM gentamicin	0.794 ± 0.004‡	0.694 ± 0.004*‡

* $P < 0.01$ vs untreated.
† $P < 0.001$ vs untreated.
‡ $P < 0.05$ vs respective outer cortical preparation.

Table 2. Rotational correlation times of renal outer cortical and outer medullary brush-border membranes labeled with the stearic acid spin label I (1, 14)

Membrane preparation	Rotational correlational time $(\tau_0) \times 10^{10}$ sec 25°	35°
Outer cortex		
Untreated	19.08 ± 0.27	16.91 ± 0.45
Incubated with 5 mM gentamicin	18.84 ± 0.27	16.81 ± 0.32
Outer medulla		
Untreated	18.21 ± 0.63	15.96 ± 0.21
Incubated with 5 mM gentamicin	18.15 ± 0.51	16.05 ± 0.25

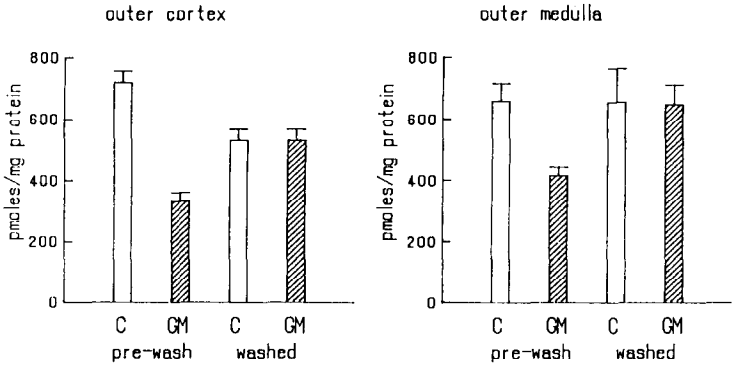

Fig. 3. Effect of washing procedure on Na^+-dependent D-glucose flux into outer cortical (left panel) and outer medullary (right panel) brush-border membrane vesicles. Vesicles (C: control; GM: 10 mM gentamicin-treated) were centrifuged twice (43,000 *g*, 20 min) with buffer without gentamicin. The final pellet was suspended in the same buffer described in Methods. The incubation buffer contained 0.5 mM D-glucose. Na^+-dependent D-glucose flux was measured for 1 sec.

In outer cortical brush-border membrane vesicles, Na^+-dependent D-glucose transport was decreased to 46% of the control values by gentamicin treatment, whereas it was recovered to 100% of the control values after washing. In outer medullary brush-border membrane vesicles, the results were much the same as those in outer cortical preparations (Fig. 3).

Effect of washing on the physical state of gentamicin-treated brush-border membrane vesicles. Gentamicin was shown to decrease the fluidity of brush-border membrane vesicles. The recovery from gentamicin-induced decrease in membrane fluidity was tested by washing. In this experiment, we used outer cortical brush-border membrane vesicles,

whose membrane fluidity was more susceptible to gentamicin toxicity than that of the outer medullary preparation. The experimental procedure was the same as above. ESR measurement was done at 25°.

As shown in Table 3, the order parameter $S(T_{11})$ was significantly higher in 10 mM gentamicin-treated vesicles than in control vesicles. After washing, there was no significant difference in the order parameter between 10 mM gentamicin-treated and control vesicles.

DISCUSSION

In our previous paper, we demonstrated that gentamicin inhibits Na^+-dependent D-glucose transport in rabbit renal brush-border membrane vesicles in a concentration-dependent (1.0 to 9.9 mM) manner. We reported K_i values of Na^+-dependent D-glucose transport inhibition of 4 and 7 mM for the outer cortical and outer medullary brush-border membrane vesicles respectively. The Na^+-dependent D-glucose transport system in outer cortical brush-border membrane vesicles was, therefore, more susceptible to gentamicin than that in outer medullary membranes.

In the present study, we present a series of experiments which investigate the effects of gentamicin on the physical state of renal brush-border membranes *in vitro*. It has already been reported that gentamicin changes membrane fluidity, observed by incorporating a fluorescent membrane probe, 1-anilino-8-naphthalene sulfonate, into the membrane [11]. To obtain more detailed information on the membrane fluidity, we used a spin label experiment where two stearic acid spin labels different in the location of a *N*-oxyloxazolidine ring were incorporated into the membrane. One spin label (5-SAL) informs us of the fluidity of the superficial region and the other (16-SAL) that of the deeper region. As shown in Table 1, the membrane fluidity of outer cortical brush-border membrane vesicles apparently was affected by gentamicin treatment more than was that of outer medullary preparations at any given temperature. The order of the gentamicin-induced changes in the membrane fluidity of outer cortical and outer medullary preparations was in good agreement with our previous results on Na^+-dependent D-glucose transport described above. In addition, the order parameters $S(T_{11})$ of outer cortical brush-border membranes in the presence or absence of gentamicin were significantly different from those of outer medullary brush-border membranes in the presence or absence of gentamicin at either 25° or 37°. This suggests that the outer cortical brush-border membranes are more rigid than in the outer medullary ones.

In general, the transporter is an intrinsic protein. Therefore, the lipid environment around the transporter may affect the property of the transporter. In fact, Fernandez *et al.* [12] reported, using a series of *n*-aliphatic alcohols, that in intestinal brush-border membranes a 3% increase in the membrane fluidity is associated with an 80% inhibition of D-glucose uptake. In renal brush-border membranes, Carriere and Grimmelec [13] reported that the addition of benzyl alcohol progressively increases the membrane fluidity and markedly inhibits the Na^+-dependent D-glucose transport. In these papers, they demonstrated that membrane fluidizing agents could decrease D-glucose transport. More recently, Mitjavila *et al.* [14] reported in intestinal brush-border membranes that the decrease in membrane fluidity caused by *n*-phenylcarbamate treatment, as well as the increase in membrane fluidity caused by *n*-aliphatic alcohols, inhibits Na^+-dependent D-glucose transport. To explain these contradictory results, they suggested that an optimum ordering state may be necessary to maintain normal membrane activity. In our study, we demonstrated that the gentamicin-induced decrease in the membrane fluidity was accompanied by a decrease in Na^+-dependent D-glucose transport in renal brush-border membrane vesicles. Taken together, our data suggest that the gentamicin-induced decrease in the membrane fluidity of spin-labeled brush-border membrane vesicles is correlated with the inhibition of Na^+-dependent D-glucose transport.

In the experiments using 16-SAL as a spin probe, we did not observe any difference in the values of rotation correlation time (τ_0) between control and 5 mM gentamicin-treated vesicles (Table 2). The spectra of 16-SAL embedded in the brush-border membrane vesicles reflected the anisotropic mobility of deeper sites, in lipid bilayer, as shown in Fig. 1. These results indicate that gentamicin affects the region relatively close to the surface of brush-border membranes. This supposition agrees with the configuration of gentamicin-phosphatidylinositol mixed bilayer predicted by computer analysis [3, 15].

Sastrasinh *et al.* [2] demonstrated that gentamicin binds to isolated brush-border membranes and that gentamicin binding sites are primarily composed of

Table 3. Order parameters of renal outer cortical brush-border membranes labeled with the stearic acid spin label I (12, 3): Effect of washing on control and gentamicin-treated vesicles

	Order parameter $S(T_{11})$, 25°	
Membrane preparation	Pre-wash	Washed
Control	0.792 ± 0.007	0.792 ± 0.007
Incubated with 10 mM gentamicin	0.820 ± 0.007*	0.794 ± 0.004

* $P < 0.05$ vs other values.

acidic phospholipids. Since acidic phospholipids have a negative charge, gentamicin is supposed to bind to phospholipids by charge–charge interaction. The influence of the pH on the binding of aminoglycoside to negatively charged liposomes also suggests the charge interaction between gentamicin and membranes [15]. The results of the experiments in which gentamicin-treated brush-border membranes were washed indicate the correlation between gentamicin binding to the membranes and its inhibitory potencies (Table 3, Fig. 3). Thus, we postulate that gentamicin binds to the superficial region of brush-border membranes, presumably to acidic phospholipids, and inhibits the Na^+-dependent D-glucose transport of brush-border membrane through a decrease in membrane fluidity.

The different susceptibilities to gentamicin toxicity of outer cortical and outer medullary preparations may reflect the different lipid and protein compositions of these membranes. As has been shown by Sastrasinh *et al.* [2], gentamicin binds mainly to acidic phospholipids but not to proteins in brush border membrane vesicles. We assume, therefore, that any effect of gentamicin-protein binding on the different susceptibilities should be negligible. Phospholipid analysis, therefore, would be required for further understanding.

REFERENCES

1. Horio M, Fukuhara Y, Orita Y, Nakanishi T, Nakahama H, Moriyama T and Kamada T, Gentamicin inhibits Na^+-dependent D-glucose transport in rabbit kidney brush-border membrane vesicles. *Biochim Biophys Acta* **858**: 153–160, 1986.
2. Sastrasinh M, Knauss TC, Weinberg JM and Humes HD, Identification of the aminoglycoside binding site in rat renal brush border membranes. *J Pharmacol Exp Ther* **222**: 350–358, 1982.
3. Brasseur R, Laurent G, Ruysschaert J and Tulkens P, Interactions of aminoglycosde antibiotics with negatively charged lipid layers. *Biochem Pharmacol* **33**: 629–637, 1984.
4. Houghton DC, Plamp CE III, DeFehr JM, Bennett WM, Porter G and Gilbert D, Gentamicin and tobramycin nephrotoxicity—A morphologic and functional comparison in the rat. *Am J Path* **93**: 137–152, 1978.
5. Hubbell WL and McConnell HM, Molecular motion in spin-labeled phospholipids and membranes. *J Am Chem Soc* **93**: 314–326, 1971.
6. Ito T and Ohnishi S, Ca^{2+}-induced lateral phase separations in phosphatidic acid-phosphatidylcholine membranes. *Biochim Biophys Acta* **352**: 29–37, 1974.
7. Gordon LM and Sauerheber RD, Studies on spin-labelled egg lecithin dispersions. *Biochim Biophys Acta* **466**: 34–43, 1977.
8. Henry SA and Keith AD, Membrane properties of saturated fatty acid mutants of yeast revealed by spin labels. *Chem Phys Lipids* **7**: 245–265, 1971.
9. Eletr S and Keith AD, Spin-label studies of dynamics of lipid alkyl chains in biological membranes: Role of unsaturated sites. *Proc Natl Acad Sci USA* **69**: 1353–1357, 1972.
10. Seelig J, Spin label studies of oriented smectic liquid crystals (A model system for bilayer membranes). *J Am Chem Soc* **92**: 3881–3887, 1970.
11. Au S, Weiner N and Schacht J, Membrane perturbation by aminoglycosides as a simple screen of their toxicity. *Antimicrob Agents Chemother* **30**: 395–397, 1986.
12. Fernandez Y, Boigegrain RA, Cambon-Gros C and Mitjavila S, Sensitivity of Na^+-coupled D-glucose uptake, Mg^{2+}-ATPase and sucrose to perturbations of the fluidity of brush-border membrane vesicles induced by *n*-aliphatic alcohols. *Biochim Biophys Acta* **770**: 171–177, 1984.
13. Carriere B and Grimellec C, Effects of benzyl alcohol on enzyme activities and D-glucose transport in kidney brush-border membranes. *Biochim Biophys Acta* **857**: 131–138, 1986.
14. Mitjavila S, Fernandez Y, Boigegrain RA and Deltour P, Modulation of Na^+-coupled D-glucose uptake of enterocyte brush-border by the perturbation in ordering state of lipids. In: *Ion Gradient-Coupled Transport* (Eds. Alvarado F and Van Os CH), pp. 153–156. Elsevier, Amsterdam, 1986.
15. Tulkens PM, Ruysschaert JM, Brasseur R, Carlier MB, Laurent G, Claes JP and Vanderhaeghe HJ, Computer models and structure–activity data for the prediction of aminoglycoside-induced nephrotoxicity. In: *Renal Heterogeneity and Target Cell Toxicity* (Eds. Bach PH and Lock EA), pp. 303–313. John Wiley, New York, 1985.