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MOLECULAR BASIS OF THE NEPHROTOXICITY OF AMINOGLYCOSIDE ANTIBIOTICS ; A FOURIER TRANSFORM INFRARED SPECTROSCOPIC INVESTIGATION

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ABSTRACT

The aminoglycoside antibiotics are the oldest known class of broad-spectrum antibiotics with activity against gram-positive and gram-negative organisms. Unfortunately their clinical use is associated with oto- and nephrotoxic side effects. The higher susceptibility of kidney and inner ear cells seems to be related to the presence of high concentrations of phosphatidylinositols (PI) in their cytoplasmic membranes due to an active metabolism of phosphoinositides. An early and significant step in aminoglycoside-induced nephrotoxicity is the loss of activity of lysosomal phospholipases A and sphingomyelinase. The interaction of the aminoglycoside gentamicin with negatively charged and zwitterionic phospholipids was studied by FTIR spectroscopy. Gentamicin binds to PI bilayers and the associated modifications of the lipid bands are consistent with a tightening of the lipid network resulting from head group neutralization by gentamicin. The changes observed at the level of carbonyl groups, phosphate groups and acyl chain packing are unique to the interaction with PI. They result in an alteration in the "quality" of the interface, the change in the critical charge density and membrane fluidity leading to a modification of the interface that is not conducive to incorporation of phospholipase A.

The lipopeptide daptomycin has been reported to reduce *in vivo* the nephrotoxicity of aminoglycoside antibiotics (Wood, C.A., Finkbeiner, H.C., Kohlhepp, S.J., Kohnen, P.W. and Gilbert, D.N. [1989] Influence of daptomycin on Staphylococcal abscesses and experimental tobramycin nephrotoxicity.

Antimicrob. Agents Chemother., **33**, 1280-1285; Beauchamp, D., Pellerin, M., Gourde, P., Pettigrew, M. and Bergeron, M.G. [1990] Effect of daptomycin and vancomycin on tobramycin nephrotoxicity in rats. *Antimicrob. Agents Chemother.*, **34**, 139-147). A recent dialysis study confirmed the existence of an electrostatic interaction between daptomycin and tobramycin (Couture, M., Simard, M., Gourde, P., Lessard, C., Gurnani, K., Lin, L., Carrier, D., Bergeron, M.G. and Beauchamp, D. [1994] Daptomycin may attenuate experimental tobramycin nephrotoxicity by electrostatic complexation to tobramycin. *Antimicrob. Agents Chemother.*, **38**, 742-749). The interaction of gentamicin with daptomycin and PI dispersions was investigated by FTIR spectroscopy. We found no evidence of a direct interaction involving the neutralization of the aspartate residues of daptomycin by gentamicin and the amide I band of daptomycin did not reveal significant conformational changes of its peptidic moiety. On the other hand, daptomycin readily inserts within bilayers of PI, dimyristoylphosphatidylglycerol or dipalmitoylphosphatidylcholine, as judged from its influence on the fluidity of these bilayers. The incorporation of daptomycin into PI bilayers has only a slight effect on the lipopeptide amide I band. The affinity of the aminoglycoside for PI is slightly increased in the presence of daptomycin, in agreement with the results of the dialysis study mentioned above. Gentamicin induces a slight narrowing of the amide I band of daptomycin bound to PI bilayers. The fluidity of the lipid bilayers corresponds to that seen in the absence of both drugs. It is proposed that in the presence of the lipopeptide antibiotic, the critical charge density and membrane

fluidity required for optimal enzyme activity is restored, explaining its nephroprotective capabilities.

The mechanism of nephroprotection by poly-L-aspartic acid is different from that of daptomycin. Dialysis studies have indicated an optimal binding between gentamicin and polyaspartic acid at acidic pH. We found nc evidence of a direct interaction involving the neutralization of the carboxylates of polyaspartic acid by gentamicin and the amide I band of polyaspartic acid did not reveal significant conformational changes. On the other hand, polyaspartic acid had no effect on the spectral features of PI bilayers. A reduction in the changes induced by gentamicin in the lipid head group and interfacial region suggests that the affinity of the aminoglycoside antibiotic decreases in the presence of polyaspartic acid.

Résumé

Les antibiotiques aminoglycosidiques forment la plus ancienne classe d'antibiotiques à large spectre d'activité et efficaces contre les organismes Gram positif et Gram négatif. Malheureusement, leur utilisation clinique s'accompagne souvent d'effets oto- et néphrotoxiques. La plus grande susceptibilité des cellules du rein et de l'oreille interne semble reliée à la présence de hautes concentrations de phosphatidylinositols (PI) dans leurs membranes cytoplasmiques en raison d'un métabolisme actif des phosphoinositides. Une étape précoce et significative de la néphrotoxicité induite par les aminoglycosides est la perte d'activité des sphingomyélinases et phospholipases A des liposomes. L'interaction de l'aminoglycoside gentamicine avec les phopholipides chargés négativement ou zwitterioniques a été étudiée par spectroscopie infrarouge à transformée de Fourier (FTIR). La gentamicine se lie aux bicouches de PI et les modifications des bandes du lipide suggèrent un resserrement du réseau lipidique consécutif à la neutralisation des têtes polaires par la gentamicine. Les changements observés au niveau des groupements carbonyles et phosphate et de l'empilement des chaînes acyles sont propres à l'interaction avec PI. Il semble y avoir altération de la "qualité" de l'interface membranaire du point de vue de la densité de charges critique et de la fluidité des chaînes acyles, ce qui rend la membrane peu propice à l'incorporation des phospholipases A. Le lipopeptide daptomycine réduit *in vivo* la néphrotoxicité des antibiotiques aminoglycosidiques (Wood, C.A., Finkbeiner, H.C., Kohlhepp, S.J., Kohnen, P.W. et Gilbert, D.N. [1989] Influence of daptomycin

on staphylococcal abscesses and experimental tobramycin nephrotoxicity. *Antimicrob. Agents Chemother.*, **33**, 1280-1285; Beauchamp, D., Pellerin, M., Gourde, P., Pettigrew, W. et Bergeron, M.G. [1990] Effect of daptomycin and vancomycin on tobramycin nephrotoxicity in rats. *Antimicrob. Agents Chemother.*, **34**, 139-147). Une récente étude de dialyse a confirmé l'existence d'une interaction électrostatique entre la daptomycine et la tobramycine (Couture, M., Simard, M., Gourde, P., Lessard, C., Gurnani, K., Lin, L., Carrier, D., Bergeron, M.G. et Beauchamp, D. [1994] *Antimicrob. Agents Chemother.*, **38**, 742-749). L'interaction de la gentamicine avec la daptomycine et des dispersions de PI a été étudiée par spectroscopie FTIR. Nous n'avons trouvé aucune preuve d'interaction directe impliquant la neutralisation des groupements carboxylate de la daptomycine par la gentamicine et la bande amide I de la daptomycine n'a révélé aucun changement conformationnel significatif de sa portion peptidique. Par contre, la daptomycine s'insère facilement au sein des bicouches de PI, de dimyristoylphosphatidylglycérol et de dipalmitoylphosphatidyl-choline, si l'on en juge par son influence sur la fluidité de ces bicouches. L'incorporation de la daptomycine dans les bicouches de PI n'affecte que légèrement la bande amide I du lipopeptide. L'affinité de l'aminoglycoside pour PI augmente sensiblement en présence de daptomycine, en accord avec les résultats de dialyse mentionnés plus haut. La gentamicine cause un léger rétrécissement de la bande amide I de la daptomycine liée aux bicouches de PI. La fluidité des chaînes acyles des bicouches lipidiques est semblable à celle observée en l'absence des deux

drogues. Ceci suggère qu'en présence du lipopeptide, la densité de charge critique et la fluidité des chaînes acyles requises pour une activité enzymatique optimale sont restaurées, ce qui explique son effet néphroprotecteur.

Le mécanisme de néphroprotection de l'acide polyaspartique est différent de celui de la daptomycine. Des études de dialyse ont indiqué une liaison optimale entre la gentamicine et l'acide polyaspartique à pH acide. Les résultats de la présente étude ne fournissent aucune preuve de neutralisation des fonctions carboxylate de l'acide polyaspartique par la gentamicine et la bande amide I du polypeptide n'a révélé aucun changement conformationnel significatif. Par ailleurs, l'acide polyaspartique n'a causé aucune modification des caractéristiques spectrales des bicouches de PI. Une réduction des changements induits par la gentamicine sur la tête polaire du lipide et sur sa région interfaciale suggère une diminution de l'affinité de l'antibiotique pour PI en présence d'acide polyaspartique.

DEDICATION

To my husband, my daughter and my family

who through their support, love and encouragement

made this work possible.

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To my family, a special word for their loving support.

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ABBREVIATIONS

DMPC	Dimyristoylphosphatidylcholine
DMPG	Dimyristoylphosphatidylglycerol
DOPC	Dioleoylphosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
DTGS	Deuterated triglycine sulphate
FTIR	Fourier transform infrared
MCT	Mercury cadmium telluride
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PLA1	Phospholipase A1
PT	Proximal tubule
T_m	Gel-to-fluid phase transition temperature

Chapter 1

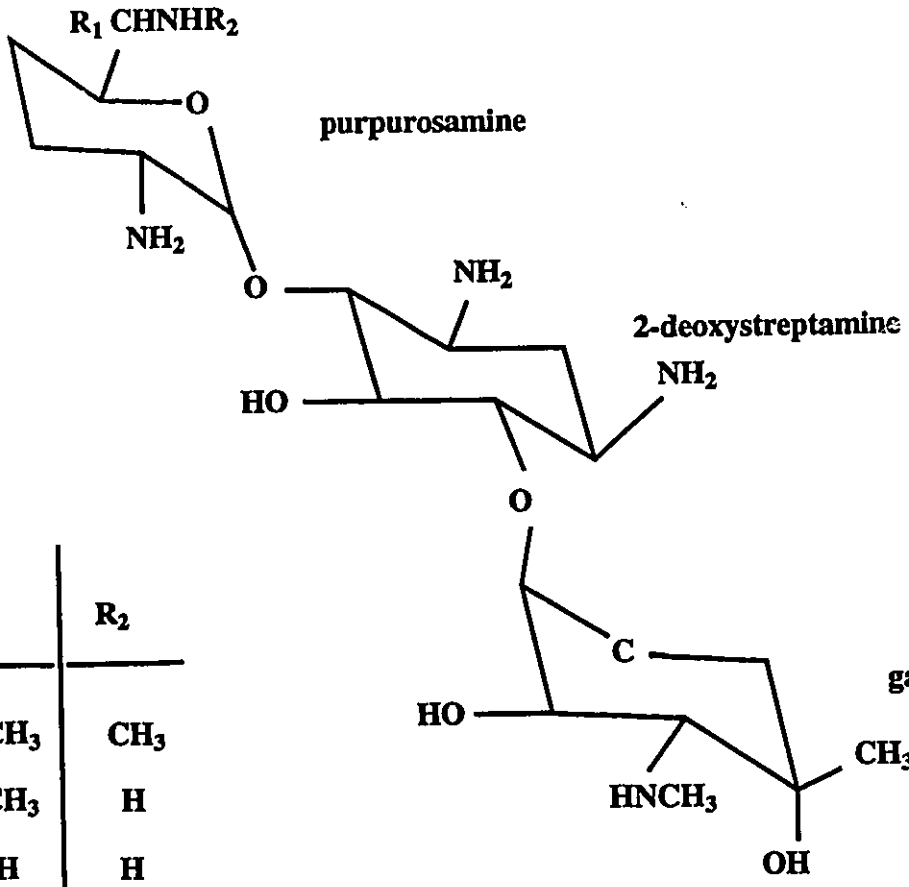
INTRODUCTION

1.1 *Aminoglycoside Antibiotics*

The aminoglycoside or aminocyclitol antibiotics are the oldest known class of broad-spectrum antibiotics with activity against gram-positive and gram-negative organisms as well as Mycobacteria. However, they are not active against fungi or anaerobic bacteria. The aminoglycosides that are currently used in clinical practice include gentamicin, tobramycin, neomycin, kanamycin, netilmicin, sisomycin and amikacin (Spelman, D.W., McDonald, M. and Spicer, W.J., 1989). Except for streptomycin and dihydrostreptomycin, which are derivatives of streptamine, they are all glycosides of 2-deoxystreptamine and are products of two genera of bacteria, *Streptomyces* and *Micromonospora*. Amikacin and netilmicin are semisynthetic derivatives of kanamycin and sisomycin.

Gentamicins were discovered by Weinstein and co-workers in 1963 (Weinstein, M.J., Leudemann, G.M., Oden, E.M. and Wagman, G.H., 1963). These antibiotics were unique because they were the first aminoglycosides to be isolated from a source other than streptomyces, that is *Micromonospora*. Gentamicin is an antibiotic complex consisting of three closely related components, gentamicin C1, C2 and C1a, and also gentamicin A which differs from the other members of the complex but is structurally similar to kanamycin C (Fig. 1.1.1).

Fig. 1.1.1
Chemical Structure of Gentamicin which consists of three closely related components, C1, C2 and C1a. Structures contain 2-deoxystreptamine linked to two saccharide units, garosamine and purpurosamine.



gentamicin	R_1	R_2
C_1	CH_3	CH_3
C_2	CH_3	H
C_3	H	H

1.1.1 Mechanism of Action

Studies on the mechanism of action of aminoglycoside antibiotics have revealed a remarkably pleiotropic set of effects of which four appear to be essential: ribosomal blockade, misreading in translation, membrane damage and irreversible uptake of the antibiotic (Davis, B.D., 1987). The primary target of aminoglycosides is the bacterial ribosome. It has been shown that the 30S ribosomal subunit is the site of aminoglycoside attachment. Once attached to the ribosome, they demonstrate a number of important effects including the blockage of protein synthesis at the stage of initiation (Luzzatto, L., Apirion, D. and Schlessinger, D., 1968). Other investigators have reported that aminoglycosides block protein synthesis by inhibiting polypeptide chain elongation (Modolell, J. and Davis, B.D., 1969). Modolell *et al.* proposed that the aminoglycoside binds to the 30S ribosomal subunit and distorts the A site, leading to an impairment of binding of both aminoacyl-tRNA and peptidyl-tRNA. Another consequence of antibiotic interaction with the ribosome is a greater frequency of misreading of the genetic code due to incorrect codon-anticodon interaction (Davies, J., Gorini, L. and Davis, B.D., 1965). The bacterial cell is, therefore, unable to synthesize proteins required for its vital processes after exposure to lethal concentrations of the antibiotic. Further studies confirmed that aminoglycoside antibiotics block the initiation complex and not chain elongating ribosomes, although they do act on the latter, decreasing both the rate and accuracy of translation. The incorporation of misread

proteins into the membrane is probably responsible for membrane damage and increased uptake of the drug.

The structural requirements for the antimicrobial activity of aminoglycosides have been determined by chemical modification or degradation of the antibiotics. These studies have revealed that specific numbers and locations of amino groups on sugars attached to the 2-deoxystreptamine ring were required for the aminoglycoside to bind to the bacterial ribosomes. Uptake of aminoglycosides is an energy dependent process that requires oxygen. Facultative organisms, which can survive under both aerobic and anaerobic conditions are, therefore, resistant to aminoglycosides under anaerobic conditions (Verklin, R.M., Jr. and Mandell, G.L., 1977).

1.1.2 Mechanism of Resistance

Bacteria can acquire resistance to aminoglycoside therapy by an alteration of the target site (ribosomal resistance), by an alteration in the permeability to aminoglycoside or by enzymatic modification of aminoglycosides by phosphotransferases, acetyltransferases and adenyltransferases.

1.1.3 Spectrum of Activity

They are active against most facultative gram-negative organisms, including *E.coli, Proteus spp., Klebsiella spp., Enterobacter spp.* and *Neisseria spp* (Spelman *et al.*, 1989). Streptococci are resistant to aminoglycosides, but

combination with penicillin shows *in vitro* synergism against this organism. This is because the cell wall of streptococci acts as a barrier to uptake of aminoglycosides. Agents like penicillin that impair cell wall synthesis result in enhanced uptake of aminoglycoside and the bactericidal activity observed is related to this interaction. Anaerobic gram-negative bacteria are resistant to aminoglycosides. These antibiotics are also not active in abscess cavities because of the low pH found therein.

1.1.4 Pharmacology of Aminoglycosides

Aminoglycosides are water soluble, stable over a wide pH range and relatively heat resistant. They are usually administered intramuscularly or intravenously. Tissue levels are low compared to serum levels except the high concentrations found in renal cortical tissue. Their levels in the cerebrospinal fluid, pleural fluid, saliva, prostatic tissue and vitreous humour are low, therefore, local instillation is required to reach therapeutic concentrations to treat infections. Aminoglycosides are not metabolized and they are removed from the body by glomerular filtration, although some tubular reabsorption does occur. The major toxic reactions of aminoglycoside therapy include nephrotoxicity, ototoxicity and other uncommon effects such as neuromuscular blockade and allergic reactions. The higher susceptibility of kidney and inner ear cells seems to be related to the presence of high concentrations of phosphatidylinositols in their cytoplasmic membranes, due to an active metabolism of phosphoinositides (Schacht, J., 1986).

The high concentrations of the drug in the glomerular filtrate promotes their nephrotoxicity. The drug produces nonoliguric acute renal failure that is associated with hypoosmolarity and progressive decline in glomerular filtration rate.

1.1.5 Renal Tubular Transport and Intrarenal Aminoglycoside Distribution

There is evidence to indicate that net reabsorption of gentamicin occurs in the kidney along the proximal tubule. Pastoriza-Munoz *et al.* used the microinjection technique to demonstrate that ^{3}[H]-gentamicin was absorbed along the proximal convoluted tubule and loop of Henle of superficial nephrons (Pastoriza-Munoz, E., Bowman, R.L. and Kaloyanides, G.J., 1979). The mode of entry into proximal tubular cells is most likely by adsorptive pinocytosis from the tubular lumen. Adsorptive pinocytosis is a process by which substances bind to the pericellular membrane and are thereafter included into intracytoplasmic vacuoles that arise from the membrane and that eventually fuse with the lysosomes. This process is responsible for the efficient uptake of several exogenous compounds into the lysosomes. Although renal accumulation of gentamicin reflects the transport of gentamicin across both apical and basolateral membranes of proximal tubular epithelium, experimental evidence indicates that apical membrane transport is the dominant route of uptake. Cortical accumulation of gentamicin in isolated perfused rat kidney was reduced significantly when glomerular filtration was suppressed but renal blood flow was maintained (Collier, V., Lietman, P.S. and Mitch, W.E., 1979). It was also shown that renal cortical

accumulation of gentamicin was depressed in rat kidneys subjected to an ischemic insult designed to suppress glomerular filtration, whereas in the same kidney, the uptake of p-aminohippurate, which is a basolateral membrane transport function, remains unchanged from control levels (Chiu, P.J.S. and Long, J.F., 1979). More direct evidence comes from autoradiography of microdissected nephrons which showed an association of the drug with the brush border and then with apical vacuoles, prior to its intralysosomal deposition (Silverblatt, F.J. and Kuehn, C., 1979; Morin, J.P., Viotte, G., Vandewalle, A., Van Hoof, H., Tulkens, P.M. and Fillastre, J.P., 1980).

1.1.6 **The Four Stages of Aminoglycoside Nephrotoxicity**

Stage I Functional Impairment

A rapid increase in urinary β2-microglobulin occurs 24-48 hours after exposure to aminoglycoside. At this stage no other histologic changes can be detected in proximal tubule (PT) cells, but increased β2-microglobulin excretion indicates initial failure of the PT cell to reabsorb these small proteins (Schentag, J.J., Sutfin, T.A., Plaut, M.E. and Jusko, W.J., 1978).

In normal animals, glucose is filtered at the glomerulus but almost all of it is reabsorbed by the proximal tubule. Another indication of the deterioration of PT transport processes is the

appearance of glucose in the urine (glucosuria), although normal levels of glucose are seen in the serum (Ginsburg, D.S., Quintanilla, A.P. and Levin, M., 1976).

Stage II Structural Damage

Microscopic evidence of PT cellular damage appears after three to five days. A decrease in the number and height of microvillae of the brush border membranes, accumulation of cytosegresomes and dilation of cisternae of rough endoplasmic reticulum in the proximal tubular cells can be observed. Nephrons still exposed to these drugs will develop additional structural damage: brush border fragments, extruded myeloid bodies, membrane vesicles and cytoplasmic debris are seen within the tubular lumen. The damage to cell membranes and intracellular organelles is probably the explanation for the sequential appearance of urinary enzymes, followed by renal tubular casts consisting of cellular proteins and aminoglycosides around days 6-10 (Schreiner, G.E., 1957).

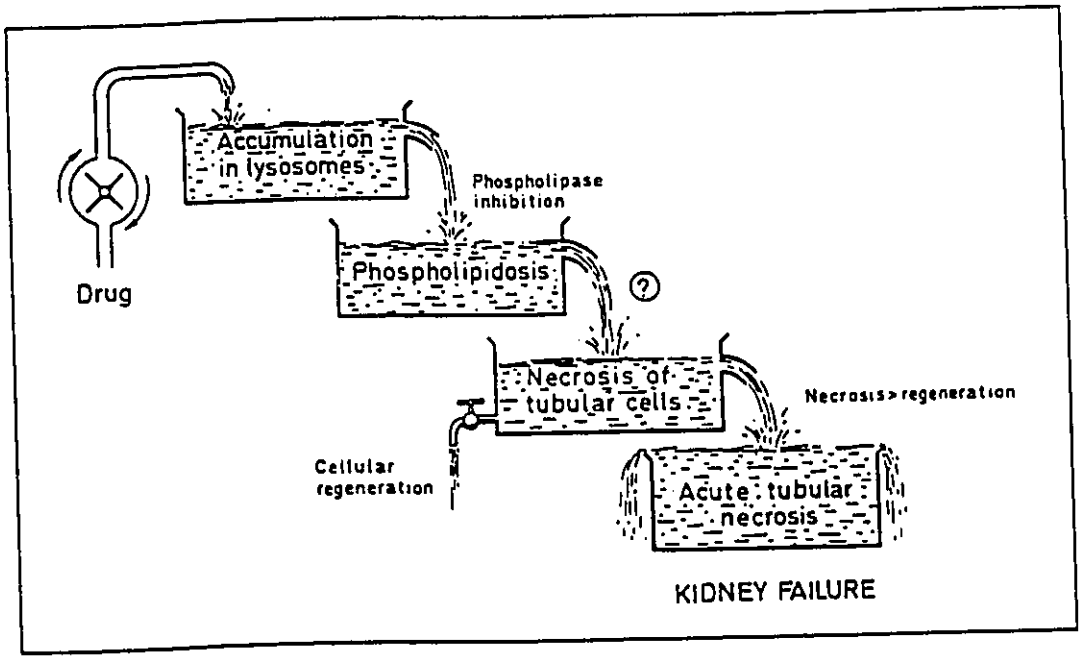
The most important structural change is seen within the lysosomes of proximal tubular cells. There is a marked increase in the total volume of lysosomes which accumulate electron dense, lamellar structures called myeloid bodies containing concentrically arranged, densely packed membranes. The aminoglycosides, which are known to accumulate within the lysosomes, inhibit the activities of lysosomal sphingomyelinase and phospholipase A1 leading to the

accumulation of undegraded phospholipids, a condition described as phospholipidosis (Laurent, G., Carlier, M.B., Rollman, B., Van Hoof, F. and Tulkens, P.M., 1982; Tulkens, G.A., Van Hoof, F. and Tulkens, P.M., 1979). The sequence of events from endocytosis, intralysosomal accumulation of these antibiotics within renal proximal tubular cells, inhibition of the activities of phospholipase A1 and sphingomyelinase causes the formation of myeloid bodies. The growth of these tightly apposed lipidic bilayers results in swelling of the lysosomes and ultimately in their bursting and the release of large amounts of the aminoglycoside, of lysosomal enzymes and of phospholipids into the cytosol. Therefore, the aminoglycoside first induces phospholipidosis and a general alteration of lysosomal enzyme function (Tulkens *et al.*, 1979; Laurent *et al.*, 1982). Secondly, the postulated rupture of the lysosomes is likely to induce rapid cell death because of the release of lysosomal enzymes and aminoglycoside molecules which are then free to attack mitochondria and endoplasmic reticulum. Necrosis of the proximal tubular cells follows, leading to impairment of renal function (Fig. 1.1.2).

Alterations in renal plasma membrane structure and function

The initial interaction of the aminoglycoside with renal proximal tubule cells occurs to the brush border membrane. To test for the presence of aminoglycoside antibiotic binding sites, the binding of ^{3}[H]-gentamicin to brush border membrane vesicles was studied. Proteolytic enzymes and protein modifying agents did not decrease ^{3}[H]-gentamicin binding to brush border membrane vesicles.

Fig. 1.1.2
The aminoglycoside nephrotoxicity cascade. Each alteration induces the next one only after a certain threshold has been reached. Thus, necrosis develop only after the phospholipidosis has reached a critical level, below which the cell is able to cope with such an alteration. Similarly acute tubular necrosis develops only after focal necrosis are no longer compensated by regeneration (from Tulkens, P.M., 1986).


Drug
Accumulation in lysosomes
Phospholipase inhibition
Phospholipidosis
?
Necrosis of tubular cells
Necrosis > regeneration
Cellular regeneration
Acute tubular necrosis
KIDNEY FAILURE

Phospholipases A and C, however, decreased ^{3}[H]-gentamicin binding, confirming the phospholipidic nature of the binding site (Sastrasinh, M., Knauss, T.C., Weinburg, J.M. and Humes, H.D., 1982). The binding between ^{3}[H]-gentamicin and phospholipids appears to be an electrostatic interaction between the cationic, polybasic antibiotic and the anionic, acidic phospholipid. When the acidic phospholipid content of the brush border membrane vesicles was increased by increasing the amount of phosphatidylinositol, there was an increase in ^{3}[H]-gentamicin binding. In fact, Schacht was the first to propose that polyphosphoinositides, a metabolically active member of acidic phospholipids, were membrane receptors for the drug in both inner ear and kidney, the main sites of aminoglycoside toxicity (Schacht, J. 1976). The binding of aminoglycosides to PI has also been confirmed by gel filtration, conformational analysis, and increase in turbidity of liposomes containing various phospholipids extracted from rat renal cortex. The maximum increase in turbidity was observed for phosphatidylinositol-4, 5 diphosphate and phosphatidylinositol (Brasseur, R., Laurent, G., Ruysschaert, J.M. and Tulkens, P.M., 1984; Aramaki, Y. and Tsuchiya, S., 1989). The binding of the aminoglycoside to its receptor on the membrane surface results in certain structural and functional consequences. Spin-labeling studies have revealed a decrease in the fluidity of brush border membranes in gentamicin treated animals (Moriyama, T., Nakahama, H., Fukuhara, Y., Horio, M., Yanase, M., Orita, Y., Kamada, T., Kanashiro, M. and Miyake, Y., 1989). This leads to a decrease in the sodium dependent D-glucose transport across brush border membrane. Na^{+}-K^{+}-

ATPase, a basolateral membrane enzyme, couples the hydrolysis of ATP with the expulsion of sodium and uptake of potassium against their respective electrochemical gradients. This enzyme maintains an intracellular sodium gradient that is responsible for almost all solute reabsorption. Glucose and phosphate are among the many solutes that are dependent upon sodium for luminal uptake. The ATP levels and the activity of the enzyme Na^+-K^+-ATPase play an important role in solute reabsorption and it was suggested that gentamicin affects both by altering the fluidity of the phospholipid annulus surrounding the enzyme complex. PI and its mono- and diphosphates were able to prevent the inhibition of ATPase by the aminoglycoside suggesting that the aminoglycoside may specifically interact with the phosphatidylinositols (Lipsky, J.J. and Lietman, P.S., 1980; Aramaki, Y., Takahashi, M., Inaba, A., Ishii, Y. and Tsuchiya, S., 1986).

The activity of another membrane bound enzyme, adenylate cyclase, which is critical in the generation of the intracellular second messenger cyclic AMP, has also been shown to depend on its phospholipid environment. Aminoglycosides were found to inhibit the activity of adenylate cyclase (Williams, P.D., Holohan, P.D. and Ross, C.R., 1981).

Gentamicin has been shown to inhibit renal mitochondrial oxidative phosphorylation *in vivo* before any functional and morphological evidence of severe renal injury (Simmons, C.F., Jr., Bogusky, R.T. and Humes, H.D., 1980). This inhibitory effect could be specific for renal mitochondria since liver mitochondria were unaffected by gentamicin therapy. Gentamicin appears to compete with

magnesium at the inner mitochondrial membrane surface and induces an increase in mitochondrial membrane monovalent cation permeability (confirmed by swelling of mitochondria during gentamicin therapy).

Gentamicin also showed an inhibition of glutamate dehydrogenase in the direction of glutamate synthesis, while it did not affect glutamate deamination (Bryla, J., Lietz, T., Jarzyna, R., Michalik, M. and Pietkiewicz, J., 1992). The inhibitory effects of aminoglycoside antibiotics on the activity of ornithine decarboxylase, the rate limiting enzyme involved in polyamine synthesis, have been documented (Kaloyanides, G.J. and Ramsammy, L.S., 1993). This inhibition was reversed by the addition of pyridoxal-5-phosphate. The depletion of the co-factor may be responsible for the inhibition of the enzyme. Since polyamines participate in the regulation, proliferation and repair, disruption in polyamine metabolism might play a role in the pathogenesis of aminoglycoside nephrotoxicity.

As mentioned earlier, the damage to cell membranes and intracellular organelles is probably the explanation for the sequential appearance of urinary enzymes (enzymuria) during nephrotoxicity. Excretion of alanylaminopeptidase, a brush border membrane enzyme, and other lysosomal enzymes such as N-acetyl-β-glucosaminidase, β-galactosidase and α-fucosidase are indicative of loss of brush border membranes of the PT cells secondary to alteration in their structure and function induced by direct binding of aminoglycoside to membrane and rupture of lysosomes.

Aminoglycosides were found to induce renal K^+ and Mg^{++} wasting with hypokalemia and hypomagnesemia due to alterations in membrane transport and permeability of these ions. Aminoglycoside renal tissue concentration begins to decline as the nephron enters stage III, probably because the drug laden cells begin to slough off into the urine, carrying large amounts of tissue bound drug with them. The nephrons with PT lining cells in stage II cannot maintain their function to reabsorb water, electrolytes and proteins. Continued glomerular filtration would be life threatening. The protective mechanism linking individual nephron glomerular filtration rate with proper renal tubular cell function must be activated to avoid large fluid and electrolyte losses. This activation marks the onset of stage III.

Stage III Complete Shutdown of Glomerular Filtration

Reduction in glomerular filtration rate is manifested by the retention of the nitrogenous end products of metabolism and is monitored by a rise in serum creatinine and blood urea nitrogen together with a fall in creatinine clearance. The decline in glomerular filtration rate due to aminoglycoside therapy has been well demonstrated in humans (Wilfert, J.N., Burke, J.P., Bloomer, H.A. and Smith, C.B., 1971). Although it is viewed as an adverse development, glomerular filtration rate must cease when the nephron loses its proximal tubular lining cells. The return of nephron filtration before PT cells regenerate would severely disrupt electrolyte balance. The progression of

stage III cessation of GFR can be monitored only by serum creatinine. There are no casts or β2-microglobulin in the urine of Stage III patients. This is because non-filtering nephrons can no longer be expected to excrete β2-microglobulin, casts, enzymes or electrolytes.

Stage IV Regeneration and resistance to further damage

When the nephron enters stage III, it also apparently enters stage IV since nephron renal tubular cell regeneration begins as soon as exposure to the aminoglycoside ends at the cessation of glomerular filtration. Stage IV is difficult to study because there are no available markers other than microscopy. An increase in the incorporation of ^{3}[H]-thymidine in proximal tubules is an indication of a regenerative response to necrosis. Results of histoautoradiography further reveal that this increase corresponds to the labelling of a large number of nuclei. Studies have also demonstrated a decrease in the number of peroxisomes of proximal tubular cells. Peroxisomes are characteristic organelles in fully differentiated proximal tubular cells, but they become sparse in regenerating cells (Böti, Z.S., Ivanyi, B., Kobor, J. and Ormos, J., 1979). Regenerating proximal tubule cells are temporarily less sensitive to further damage by aminoglycosides or other renal tubular toxins. Although these cells do not reabsorb normally during resolving tubular necrosis, they do accumulate aminoglycosides without further injury. This indicates that

proximal tubular cells transiently tolerate previously lethal aminoglycosides antibiotic levels (Table 1.1.1).

1.1.7 **Risk Factors for Aminoglycoside Nephrotoxicity** Aminoglycosides are used to treat patients with serious gram-negative infections. Since the nephrotoxic and ototoxic adverse effects limit their clinical use, recognition of risk factors associated with greater incidence of renal damage is important for the safer use of these antibiotics (Table 1.1.2). The nephrotoxicity of aminoglycosides is determined by two major variables: the intrinsic potential of the drug to damage subcellular structures and the amount of drug accumulation in the renal cortex (Kaloyanides, G.J. and Pastoriza-Munoz, E., 1980).

Any factor that increases the renal uptake of aminoglycosides is a risk factor for nephrotoxicity. The duration of exposure of the proximal tubular cells to aminoglycosides is a critical factor since it determines the extent of uptake. Persistent exposure undoubtedly results in increased renal drug levels. Dosage regimen is another important determinant of the extent of cortical aminoglycoside concentrations. Nephrotoxicity caused by gentamicin is more severe when the total daily dose is divided or given by continuous infusion than when it is given as a single bolus (Powell, S.H., Thompson, W.L., Luthe, M.A., Stern, R.C., Grossniklaus, D.A., Bloxham, D.D., Groden, D.L., Jacobs, M.R., DiScenna, A.O., Cash, H.A. and Klinger, J.D., 1983; Verpooten, G.A., Giuliano, R.A., Verbist, L., Eestermans, G. and De Broe, M.E., 1989).

Table 1.1.1
Salient Features of Aminoglycoside Nephrotoxicity (adapted from Tulkens, P.M., 1989).

SALIENT FEATURES OF AMINOGLYCOSIDE NEPHROTOXICITY

Fate of the Drug
- Glomerular filtration
- Binding to anionic Phospholipids of brush border membrane
- Adsorptive pinocytosis
- Intralysosomal sequestration

Early Alterations
- Inhibition of activities of lysosomal phospholipases and sphingomyelinase
- Phospholipidosis and formation of myeloid bodies
- Glucosuria
- β-microglobulinuria

Established Alterations
- Shedding of brush border enzymes (e. g. alanylaminopeptidase)
- Excretion of lysosomal enzymes (e. g. N-acetyl- β-glucosaminidase)
- Decreased glomerular filtration
- Increase in blood urea nitrogen and creatinine
- Necrosis, Acute Renal Failure

Regenerative Lesions
- Tubular cell proliferation
- Tubular dilatation

Table 1.1.2
Risk Factors for Aminoglycoside Nephrotoxicity (adapted from Appel, G.B., 1990).

RISK FACTORS FOR AMINOGLYCOSIDE NEPHROTOXICITY

Drug Related
- Long duration and short dosing interval
- Prior aminoglycoside treatment
- Choice of aminoglycoside in high risk patient
- Coadministration of other potentially nephrotoxic drugs, loop diuretics and volume depleting agents

Patient Related
- Advanced age
- Female gender
- Liver disease
- Volume depletion, Hypotension, Shock

Advanced age has also been suggested as a risk factor for aminoglycoside nephrotoxicity. This is because aging is accompanied by a decreased capacity in renal function and a marked decline of the regenerative response to drug induced cell injury. Other patient-related risk factors include pre-existing renal disease, female gender, liver disease, hypotension, shock and volume depletion. Treatment-related risk factors such as prior courses of aminoglycoside therapy, prolonged duration of therapy, greater total dose, coadministration of other potentially nephrotoxic drugs, loop diuretics and volume depleting agents have been associated with higher incidence of renal damage (Appel, G.B., 1990).

1.1.8 Relative Nephrotoxicity of Various Aminoglycosides

Streptomycin is known to have minimum nephrotoxic potential. Neomycin exhibits the highest nephrotoxic potential and is no longer used in clinical medicine. Among the other 2-deoxystreptamine-containing aminoglycosides endowed with broad spectrum and currently in clinical use, amikacin and tobramycin exhibit the lowest nephrotoxic potential in experimental animals, whereas gentamicin and sisomycin show the highest. However, differences in the nephrotoxic potential among aminoglycosides remain limited and none of them can be given to humans or animals without eliciting some toxic reaction. Moreover, patient susceptibility and variability may defeat the advantage of being able to choose one aminoglycoside over others at the level of the individual.

1.1.9 Clinical Use of Aminoglycosides

Aminoglycosides have found extensive use in clinical medicine. They have been a major benefit in the therapy of serious infections, particularly those that have been hospital acquired and due to multi-resistant bacteria. They are used for upper respiratory tract infections, infections after surgery of the neck, pleuropulmonary infections, urinary tract infections, gram-negative sepsis, intraabdominal infections, cardiovascular infections, infections of the nervous system, skin and soft tissue infections, brain abscess, burns, gastrointestinal, gynaecologic, bone and joint infections. It is important that these drugs be used only to treat infections in which they have had a clearly demonstrated beneficial effect and after careful consideration of their toxicities.

1.2 Phospholipids and Phospholipases

Acidic phospholipids have been identified as the renal brush border membrane binding sites for the aminoglycoside antibiotics. This binding is due to a charge interaction between the acidic, anionic phospholipids and the polybasic, cationic aminoglycoside antibiotic. *In vitro* studies have revealed that the binding of the aminoglycoside to its receptor on the membrane surface results in the decrease of the activity of lysosomal phospholipases towards neutral phospholipids. Alterations in phospholipid metabolism affect the integrity of plasma membrane and intracellular membranes causing cell injury and ultimately leading to cell death. It would therefore be interesting to review the normal functioning of

phospholipids and phospholipases in intact cells and how disturbances in their normal functioning lead to cell injury and death.

Lipids form the basic framework of all biological membranes. The most abundant lipids in animal cell membranes are phospholipids. Phospholipids are formed by the esterification of two fatty acids, such as palmitic acid, myristic acid or oleic acid, to the hydroxyl groups of carbon atoms 1 and 2 of glycerol. The third hydroxyl group is esterified to phosphoric acid forming a phosphoglyceride. The phosphoglyceride is then esterified via the phosphoric acid to the polar head group molecule to form the phospholipid. Since they possess a polar head group in addition to their non polar hydrocarbon moieties, they are called amphipathic, amphiphilic or polar lipids. The parent compound of the phospholipids is phosphatidic acid which contains no polar alcohol head group. It occurs in very small amounts in cells but it is an important intermediate in the biosynthesis of phosphoglycerides. The most abundantly occurring phospholipids in plant and animal cells are phosphatidylethanolamine and phosphatidylcholine, which contain as head groups the amino alcohols ethanolamine and choline, respectively. In phosphatidylinositol, the head group is a six-carbon sugar alcohol inositol. In phosphatidylserine, the hydroxyl group of the amino acid L-serine is esterified to phosphoric acid. In phosphatidylglycerol, the head group is a molecule of glycerol. The properties of phospholipids are largely dependent on the size and electric charge of their polar head groups. With the exceptions of phosphatidylcholine and

phosphatidylethanolamine which are zwitterionic, the other phospholipids described above are anionic at neutral pH.

Phospholipid Asymmetry in Membranes

Sphingomyelin, phosphatidylglycerol and phosphatidylcholine are located mostly in the outer leaflet of the plasma membrane. On the other hand phosphatidylserine, phosphatidylethanolamine and phosphatidic acid are found on the inner half of the bilayer. Phosphatidylinositol-4, 5-diphosphate and phosphatidylinositol-4-monophosphate are found exclusively in the inner leaflet whereas some phosphatidylinositol is also found in the outer leaflet.

Phospholipases

Phospholipases are a class of ubiquitous enzymes that are classified into two broad categories: i) acyl hydrolases which include phospholipases A1, A2, B and lysophospholipases, and ii) phosphodiesterases which include phospholipases C and D. Phospholipase A1 specifically removes the fatty acid from the first position and phospholipase A2 from the second position. Removal of one fatty acid molecule yields a lysophosphoglyceride. Lysophosphoglycerides are intermediates in phospholipid metabolism and are found only in trace amounts in tissues; at high concentrations they are toxic and injurious to membranes. Phospholipase B is a mixture of phospholipases A1 and A2, it can bring about successive removal of the two fatty acids of the phosphoglyceride. Phospholipase C hydrolyzes the bond between glycerol and phosphoric acid, while phospholipase D removes the polar head group to leave phosphatidic acid.

In addition to their role in hydrolysis of dietary phospholipid, phospholipases also have an important role in regulatory functions. Phospholipase A2 is associated with the arachidonic cascade that provides substrates for further metabolism to bioactive molecules such as eicosanoids (prostaglandins, thromboxanes, leucotrienes, lipoxins) and platelet activating factor. Likewise phospholipase C is involved in the phosphatidylinositol cycle. Phospholipases also play an important role in maintaining the acyl composition of membrane phospholipids in the deacylation-reacylation cycle. The activity of membrane associated enzymes (Na^+-K^+-ATPase, Ca^{++}-Mg^{++}-ATPase) is dependent on its phospholipid environment and is regulated by phospholipases.

Since aminoglycoside antibiotics interact with anionic phospholipids and inhibit phospholipase activity, an understanding of the mechanism by which these drugs inhibit phospholipase activity is vital to explain how phospholipid catabolism is restored by nephroprotectants. Two models have been proposed for the drug-lipid(substrate) interaction. Mingeot-Leclercq *et al.* have shown that the hydrolysis of phosphatidylcholine by lysosomal extracts at pH 5.4 is critically dependent on the negative charges carried by the bilayer (Mingeot-Leclercq, M.P., Laurent, G. and Tulkens, P.M., 1988). This hydrolysis that is carried on by phospholipases A1 and A2 increases markedly when the PI content is raised from 10 to 30% of the total phospholipids. Addition of gentamicin decreases the activity of these enzymes, and the effect is inversely proportional to the amount of PI present in the bilayer. They proposed a "charge neutralization model" according to which

gentamicin inhibits the activity of lysosomal phospholipases towards phosphatidylcholine (PC) by binding to anionic phospholipids and decreasing the available negative charges on the surface of the bilayer.

Hostetler *et al.* proposed a "substrate depletion model" according to which gentamicin and tobramycin bind to phosphatidylinositol (PI), a non substrate, apparently interfering with access of phospholipase A1 (PLA1) to dioleoylphosphatidylcholine (DOPC) at the surface of the substrate vesicle (Hostetler, K.Y and Jellison, E.J., 1990). The inhibition could be overcome by increasing the concentration of the DOPC/PI vesicles. They attributed this inhibition to substrate "depletion". When the number of substrate molecules is increased, the aminoglycosides are no longer able to adequately cover or alter the surface, and hydrolysis of DOPC resumes at rates comparable to control.

The experimental conditions of these two models differed in several respects such as the enzyme source (liver versus kidney) and nature of the enzyme (native lysosomal extract versus purified, delipidated phospholipase A1), and the composition of the lipid vesicles (those containing PC, cholesterol, PI and sphingomyelin versus those containing PC and PI only (Piret, J., Kishore, B.K. and Tulkens, P.M., 1992). Experiments using a single enzyme source (rat liver lysosomes) have shown that the environment of PC in lipid vesicles influences the activity of PLA1, its regulation by the presence of negatively charged phospholipids and the nature of its inhibition by gentamicin. Regulation (stimulation) of activity of PLA1 by negatively charged phospholipid (Mingeot-Leclercq *et al.* 1988) is only

observed when cholesterol and/or sphingomyelin are also present in vesicles, in addition to PC and PI. It is possible that the presence of cholesterol and sphingomyelin in vesicles used by Mingeot-Leclercq *et al.* modifies the chemical composition and physical organization of the bilayer. Both "charge neutralization" and "substrate depletion" models are true within the framework of their respective experimental designs. However, since the composition of the lipid vesicles as well as the nature of the enzyme preparation (whole lysosomal extract) in the "charge neutralization" model is closer to *in vivo* conditions, this model may be more relevant to the *in vivo* situation.

1.3 Protection against Aminoglycoside-Induced Nephrotoxicity

Several approaches have been envisaged to reduce the occurrence of aminoglycoside-induced nephrotoxicity. Among them correction of the risk factors described earlier, close monitoring of the aminoglycoside serum levels and administration of the aminoglycoside in only one (or two) injections per day have been found to be effective in reducing the incidence of toxicity. Since the drug shows a marked post-antibiotic effect, that is no bacterial regrowth is seen for several hours after withdrawal of the antibiotic, once daily administration of the aminoglycoside was expected to provide a sufficient efficacy and it has been found to reduce the occurrence of toxicity. Efforts are also being made towards selection or design of less toxic antibiotics. This requires a better understanding of the molecular aspects of the aminoglycoside binding to brush border membranes and

of their inhibitory effects inside proximal tubular cells. An alternative strategy is the pre or coadministration of an inhibitor. Hence oral supplementation of calcium has been found to reduce gentamicin nephrotoxicity, the cation acting as a competitive inhibitor of the drug on renal membrane binding sites (Humes, H.D., Sastrasinh, M. and Weinberg, J.M., 1984; Bennett, W.M., Elliott, W.C., Houghton, D.C., Gilbert, D.N., DeFehr, J. and McCarron, D.A., 1982). The applicability of calcium supplementation for clinical use is questionable since high amounts are required to get protection. Nitrendipine, a calcium channel inhibitor, was found to be effective in protecting rats from gentamicin toxicity (Lee, S.M. and Michael, U.F., 1985). Latamoxef, an oxacephem antibiotic, has been shown to protect rat from nephrotoxicity induced by the aminoglycoside tobramycin. Latamoxef reduces tobramycin intrarenal level by inhibiting its binding to brush border membranes (Kojima, R., Ito, M. and Suzuki, Y., 1989). It has been shown to react chemically with tobramycin *in vitro* (Kojima, R., Ito, M. and Suzuki, Y., 1988). Pyridoxal-5'-phosphate (vitamin B6) also protects against gentamicin induced nephrotoxicity by mechanisms similar to latamoxef (Kacew, S., 1989). On the other hand, a protection of renal function can also be afforded without any reduction of aminoglycoside cortical level. Hence coadministration of either polyaspartic acid or daptomycin with gentamicin prevented functional renal failure, in spite of up to ten-fold increase of the level of accumulated aminoglycoside.

1.3.1 Protection against Aminoglycoside-Induced Nephrotoxicity by Poly-L-Aspartic Acid

Study in this field was pioneered by P.D. Williams and G.H. Hottendorf who found that polyamino acids including polylysine, polyasparagine and polyaspartic acid inhibited the binding of ^{3}[H]-gentamicin to brush border membranes from rat renal cortex (Williams, P.D. and Hottendorf, G.H., 1985). *In vivo* studies, however, revealed that although polyaspargine and polyaspartic acid provided nephroprotection, polylysine did not significantly alter the nephrotoxicity of gentamicin. Coadministration of polyaspartic acid with gentamicin protected against biochemical, functional and morphological changes in renal cortex caused by gentamicin therapy. Polyaspartic acid prevented the depression of creatinine clearance and attenuated the severity of proximal tubular cell necrosis despite the fact that renal cortical concentration of gentamicin in rats injected with gentamicin plus polyaspartic acid was higher than in rats injected with gentamicin alone (Ramsammy, L.S., Josepovitz, C., Lane, B.P. and Kaloyanides, G.J., 1989). Such high aminoglycoside levels were also reported by other investigators (Gilbert, D.N., Wood, C.A., Kohlhepp, S.J., Kohnen, P.W., Houghton, D.C., Finkbeiner, H.C., Lindsley, J., and Bennett, W.M., 1989). In addition, normal serum creatinine values, no increase in the excretion of the lysosomal enzyme N-acetyl-β-D-glucosaminidase and absence of cell necrosis and tubular regeneration was observed in rats given gentamicin and polyaspartic acid (Gilbert *et al.*, 1989). Beauchamp and co-workers reported that polyaspartic acid protected against the

decrease in sphingomyelinase activity and increase in lipid phosphorus observed during gentamicin therapy. The four-fold increase in ^{3}[H]-thymidine incorporation into cortical DNA observed during gentamicin treatment was also significantly reduced in the presence of polyaspartic acid (Beauchamp, D., Laurent, G., Maldague, P., Abid, S., Kishore, B.K. and Tulkens, P.M., 1990a).

Morphological studies revealed that rats given polyaspartic acid either alone or with gentamicin showed the presence of intracytoplasmic vacuoles. Optically clear vacuoles were detected in rats given polyaspartic acid alone, while in rats given gentamicin and polyaspartic acid the vacuoles contained an electron-dense material (Gilbert *et al.*, 1989; Beauchamp *et al.*, 1990a). Gentamicin alone induced enlargement of lysosomes into which appeared prominent electron-dense myeloid bodies made of tightly apposed lamellae. In the presence of polyaspartic acid, the electron-dense bodies were smaller and exhibited a non lamellar structure. Animals receiving polyaspartic acid alone showed no alteration in their lysosomes (Beauchamp *et al.*, 1990a).

In vitro experiments to study the interaction between gentamicin and polyaspartic acid at pH 5.4, the intralysosomal pH, and pH 7.5, the pH prevailing at the brush border membrane surface, revealed optimal binding at pH 5.4 (Kishore, B.K., Kallay, Z., Lambricht, P., Laurent, G. and Tulkens, P.M., 1990a). Polyaspartic acid also displaced bound gentamicin from negatively charged liposomes and restored the activity of lysosomal phospholipase A1 towards phosphatidylcholine included in negatively charged liposomes. Studies at pH 7.0

using purified brush border membrane vesicles revealed that polyaspartic acid binds to gentamicin and displaces it from brush border membrane vesicles. Phospholipidosis, one of the early signs of aminoglycoside nephrotoxicity, has been explained by the binding of the aminoglycoside to negatively charged phospholipids in membranes. This binding leads to an inhibition of lysosomal phospholipases towards phosphatidylcholine, resulting in an accumulation of undegraded phospholipids in lysosomes. Since both polyaspartic acid and gentamicin could be recovered from the lysosomal fractions of cortex of rats administered with these two compounds, Kishore *et al.* proposed that the intralysosomal complexation of polyaspartic acid and gentamicin prevented phospholipidosis and other cortical alterations, conferring protection against gentamicin induced nephrotoxicity.

The ability of poly-L-aspartic acid to protect against aminoglycoside-induced nephrotoxicity was compared to that of two other anionic polypeptides, poly-L-glutamic acid and poly-D-glutamic acid. All three polyanions bound to gentamicin to the same extent with optimal binding at pH 5.4. They also displaced bound gentamicin from negatively charged liposomes (Kishore, B.K., Lambricht, P., Laurent, G., Maldague, P., Wagner, R. and Tulkens, P.M., 1990b). *In vivo* studies, however, revealed that only poly-L-aspartic acid and poly-D-glutamic acid conferred protection against aminoglycoside-induced nephrotoxicity as judged by biochemical parameters (namely increase in lipid phosphorus and decrease of lysosomal sphingomyelinase activity), and morphological studies of lysosomes

(presence of electron-dense lamellar myeloid bodies). On the basis of these biochemical and morphological changes, poly-L-glutamic acid failed to confer nephroprotection. The three polypeptides showed a difference in their capacity to resist degradation by lysosomal proteases. Poly-L-glutamic acid was 15-fold more susceptible to hydrolysis at pH 5.4 compared to the other two polyanions. On the other side, poly-D-glutamic acid also caused a lysosomal storage disorder consisting of the accumulation of osmiophilic, non-lamellar material within the lysosomes. The stability of the polyanionic peptide is important in determining its nephroprotective effect and not all polyanions resistant to lysosomal proteases could function as nephroprotectants since they could cause other side effects (Kishore, B.K., Ibrahim, S., Lambricht, P., Laurent, G., Maldague, P., Tulkens, P.M., 1992).

The *in vitro* interaction of polyaspartic acid and different aminoglycosides (gentamicin, tobramycin, neomycin and amikacin) was also demonstrated by Ouchterlony's double immunodiffusion technique, equilibrium dialysis and changes in the optical density of the solution after addition of polyaspartic acid (Kohlhepp, S.J., McGregor, D.N., Cohen, S.J., Kohlhepp, M.E. and Gilbert, D.N., 1992). Optimal interaction occurred at acidic pH, in agreement with earlier reports. The interaction was inhibited by serum proteins and was unaffected by physiological concentrations of sodium, potassium, calcium or magnesium. In contrast to earlier reports where polyaspartic acid did not affect the antimicrobial activity of

gentamicin, this group reported that the aminoglycoside loses its antimicrobial activity after its interaction with polyaspartic acid.

Studies to explore the duration of nephroprotection conferred by polyaspartic acid revealed a stepwise reduction in nephroprotection as the dosage interval was prolonged (Swan, S.K., Gilbert, D.N., Kohlhepp, S.J., Leggett, J.E., Kohnen, P.W. and Bennett, W.M., 1992). The nephroprotection provided by polyaspartic acid was specific for aminoglycoside-induced renal injury, no protection being observed from the nephrotoxic effects of mercuric chloride or cis-platinum (Swan, S.K., Kohlhepp, S.J., Kohnen, P.W., Gilbert, D.N. and Bennett, W.M., 1991).

1.3.2 Protection against Aminoglycoside-Induced Nephrotoxicity by Daptomycin

Daptomycin has been proposed as an alternative to vancomycin (Eliopoulos, G.M., Willey, S., Reiszner, E., Spitzer, P.G., Caputo, G. and Moellering Jr., R.C., 1986; Benson, C.A., Beaudette, F. and Trenholm, G., 1987), a glycopeptidic antibiotic used in combination with aminoglycosides to enhance their bactericidal activity but which also aggravates their nephrotoxicity (Wood, C.A., Kohlhepp, S.J., Kohnen, P.W., Houghton, D.C. and Gilbert, D.N., 1986; Marre, R., Schulz, E., Hedtke, D. and Sack, K., 1985). Daptomycin, formerly known as LY146032, is a lipopeptidic antibiotic active against gram-positive bacteria in which it inhibits lipotechoic acid synthesis (Canepari, P., Boaretti, M., Mar Lleó, M.D and Salta, G., 1990). Daptomycin has been found to prevent the development of

aminoglycoside-induced functional renal failure (Wood, C.A., Finkbeiner, H.C., Kohlhepp, S.J., Kohnen, P.W. and Gilbert, D.N., 1989). Similar to polyaspartic acid, it inhibited lysosomal phospholipidosis and increased cellular regeneration in the presence of similar or even higher aminoglycoside levels (Beauchamp, D., Pellerin, M., Gourde, P., Pettigrew, M. and Bergeron, M.G., 1990b). Daptomycin did not affect renal function (blood urea nitrogen or serum creatinine), sphingomyelinase activity and cellular regeneration, but large clear vacuoles were observed in proximal tubular cells of all daptomycin treated animals. A recent dialysis study indicated an interaction of daptomycin with tobramycin (Couture, M., Simard, M., Gourde, P., Lessard, C., Gurnani, K., Lin, L., Carrier, D., Bergeron, M.G. and Beauchamp, D., 1994). The effects were more pronounced at pH 5.4, the intralysosomal pH. The dependence on ionic strength confirmed the electrostatic character of this interaction. These results were consistent with the intralysosomal complexation between daptomycin and the aminoglycoside, preventing an inhibitory effect of aminoglycoside towards phospholipid metabolism. It was proposed that the protective effect of daptomycin was due to neutralization of toxicity of the aminoglycoside antibiotic rather than to their redistribution. The clinical use of daptomycin needs to be assessed.

Aminoglycosides are, and will probably remain, an essential member of our therapeutic armamentarium in severe gram negative infections. The reduction of aminoglycoside toxicity is a concern among clinicians and a lot of research has focused on understanding the toxicity mechanism, design of less toxic

aminoglycosides, together with identification of drugs with increased clinical efficacy. These lines of investigation have included molecular manipulation of the aminoglycoside nucleus, pharmacokinetic study of these drugs in the serum and various body tissues, comparative toxicity and efficacy studies in experimental animals and human subjects, mechanism of drug-induced toxicity (particularly interactions with phospholipids and intracellular alterations) and protection against drug induced toxicity by other compounds. In the present study, the interaction of the aminoglycoside antibiotic gentamicin with negatively charged and zwitterionic phospholipids was studied by Fourier transform infrared spectroscopy. FTIR spectroscopy is an excellent technique to probe molecular interactions because the vibrational modes that are examined are often affected by changes in molecular segment conformation, charge and hydrogen bonding. The changes observed in the infrared spectra of the lipid bilayers in the interfacial and head group region as well as the fluidity of the membrane in the presence of the aminoglycoside antibiotic gives an insight into the mechanism of toxicity of these drugs. Although the clinical use of nephroprotectants such as daptomycin, polyaspartic acid, calcium and several others needs to be assessed, an understanding of the mechanism by which they confer nephroprotection could contribute to a better control of the toxicity of these drugs and aid in the design of less toxic drugs. We examined i)the influence of daptomycin/polyaspartic acid on the spectral features of PI bilayers and looked for spectral evidences of ii)a direct interaction between

daptomycin/polyaspartic acid and gentamicin in solution and of iii) a possible influence of daptomycin/polyaspartic acid on gentamicin binding to lipidic membranes.

Chapter 2

THEORY OF INFRARED SPECTROSCOPY

An infrared spectrum is obtained when a sample absorbs radiation in the region of the electromagnetic spectrum known as infrared. Energy is transferred from the incident radiation to the molecule causing the electron to be promoted to a higher energy level. The difference in energy between the two energy levels is proportional to the frequency of light absorbed and is expressed as $E=h\nu$ where h is the universal Planck's constant ($6.624X10^{-27}$ erg.s) and ν is the frequency of light in Hz. The frequency of light is related to the wavelength, λ, of light by the expression $\nu=c/\lambda$, where c is the velocity of light in vacuum, $3x10^{10}$ cm/s. Wavenumber is also used in the description of spectra. The relationship is $\upsilon=1/\lambda$ where υ is the wavenumber in cm^{-1} (reciprocal centimeters or Kaysers). It is common for spectroscopists to use the word frequency instead of wavenumber, although the quantity that is actually being expressed is the wavenumber in cm^{-1}.

2.1 Molecular Vibrations

There are two kinds of vibrations for molecules: stretching vibrations, in which the distance between the two atoms increases or decreases but the atoms remain in the same bond axis, and bending or deformation, in which the position of the atoms changes relative to the original bond axis. In-plane bending vibrations include scissoring and rocking modes and out-of-plane bending

vibrations include wagging and twisting modes (Fig. 2.1.1). The stretching and bending vibrations occur at certain quantized frequencies. When infrared light of that same frequency is incident on the molecule, energy can be absorbed and the amplitude of vibration is increased. When the molecule goes back from the excited state to the original ground state, the absorbed energy is released as heat. Bending vibrations require less energy and occur at lower wavenumbers than stretching vibrations. The stretching frequency (υ in cm^{-1}) of a bond is related to the masses of atoms (M_a and M_b in grams), the velocity of light c, and the force constant of the bond (k in dynes/cm) by the expression,

$$\upsilon = \frac{1}{2\Pi c}\sqrt{\frac{k}{M_a M_b / (M_a + M_b)}}$$

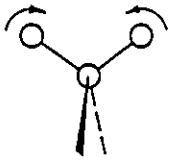
There are 3N -6 normal modes of vibration for a non-linear molecule, where N is the number of atoms. This can be explained as follows: each atom has three degrees of motional freedom, that is, motions along the x, y and z directions. Thus N atoms have 3N independent motions. When the atoms are linked in a molecule, the motions are no longer independent. Three motions are translational, where all the atoms move simultaneously in the x, y, or z direction. Another three are rotations, where all atoms rotate in phase about the x, y or z axis. This leaves 3N-6 motions, in which internuclear distances and bond angles change but the centre of gravity of the molecule does not move. A linear molecule has two independent rotational degrees of freedom about two mutually perpendicular axis,

Fig. 2.1.1
Vibrations of a group of atoms (+ and - signify vibrations perpendicular to the plane of the paper (from Dyer, J.R., 1965).

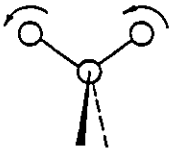
Symmetric

Asymmetric

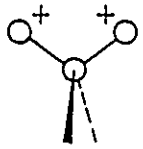
STRETCHING VIBRATIONS



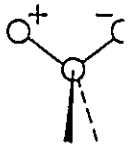
Scissoring



Rocking



Wagging



Twisting

IN-PLANE BENDING VIBRATIONS

OUT-OF-PLANE BENDING VIBRATIONS

perpendicular to the molecular axis. Rotation of a linear molecule about the molecular axis is not considered a degree of freedom of motion since no displacement of nuclei are involved. Thus there are 3N-5 modes of vibration for a linear molecule.

In order for a particular vibration to result in the absorption of infrared energy, that vibration should cause a change in the dipole moment of the molecule. Molecules that contain symmetry elements display simplified spectra (Dyer, J.R., 1965).

2.2 Dispersive and Fourier Transform Infrared Spectrometers

An infrared spectrometer is comprised of a source of infrared radiation, a detector and a monochromator.

2.2.1 Source of Infrared Radiation

The source of infrared radiation is usually some solid material heated to incandescence by an electric current. It is usually a coil of wire of high resistance such as nichrome, a rod of partially conductive material such as silicon carbide (Globar) or rare earth oxides (Nernst glower).

2.2.2 Infrared Detector

The infrared detector is a device that measures the infrared energy of the source after it has passed through the sample. Commonly used detectors are the

DTGS and MCT detectors. The DTGS detector is a thermal, pyroelectric detector. It consists of a thin pyroelectric crystal, deuterated triglycine sulphate (DTGS), which develops electrical polarity with changes in temperature. Electrodes on the crystal faces collect the charges so that the device acts as a capacitor across which voltage appears, the intensity of which is sensitive to the temperature of the device. The pyroelectric detector operates at room temperature and possesses essentially a flat wavelength response ranging from near infrared through the far infrared.

Another commonly used detector with a very rapid response and high sensitivity is the mercury cadmium telluride detector (MCT) which is cooled with liquid nitrogen. It is a photoconductive cell which shows an increase in electrical conductivity when illuminated with infrared light. This detector utilizes photon energy to promote bound electrons in the detector material to free states which results in increased electrical conduction. There is a long wavelength limit to the response, however, because photons with wavelength longer than a certain limit will not have sufficient energy to excite the electrons.

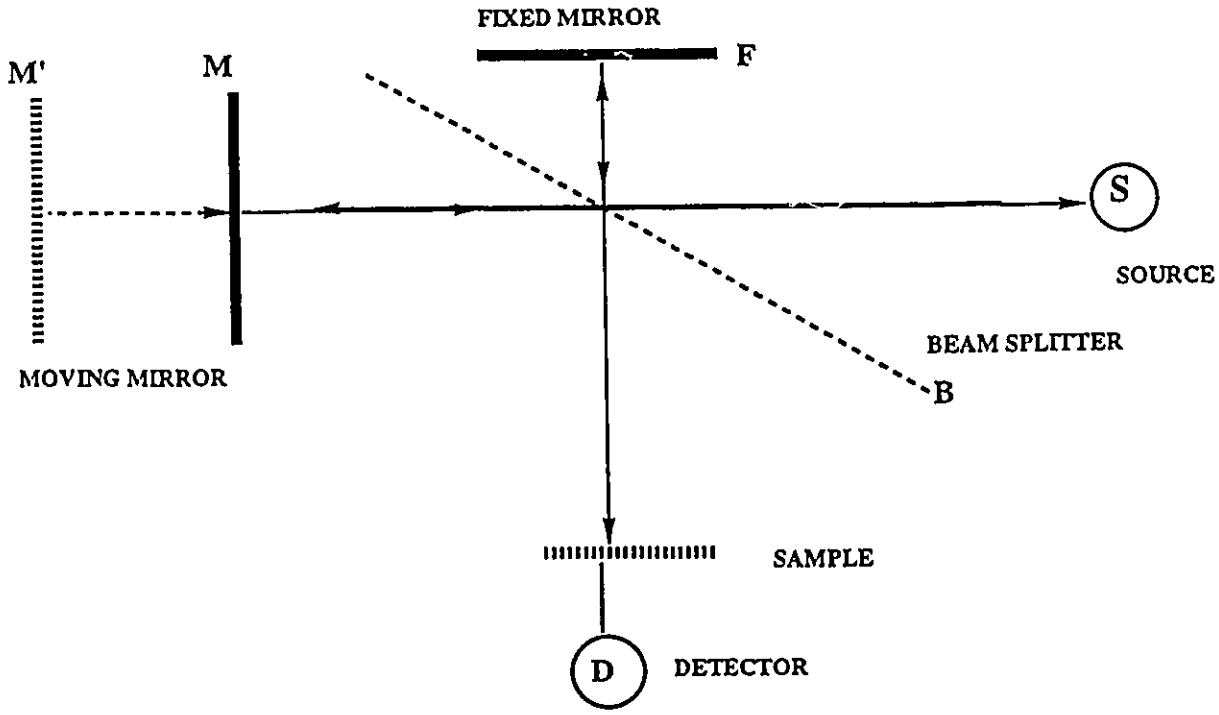
2.2.3 **The Monochromator**

Between the infrared source and the detector, there is a device to analyze the radiation so that an intensity can be evaluated for each wavelength resolution element. There are two basic types, the monochromators used in dispersive instruments and the interferometers used in Fourier transform instruments. The

monochromator disperses the continuous radiation into its spectrum of monochromatic components. Prisms were used earlier but today gratings are found in all dispersive spectrometers. The component frequencies are passed sequentially and continuously by a mechanical scanning device to the detector. In this way, the detector can sense which frequencies have been absorbed or partially absorbed by the sample and which frequencies have been unaffected. The radiation enters the monochromator through a slit and, after dispersion, leaves through another slit. The width of the entrance slit determines how much energy enters the monochromator, and the width of the exit slit determines the width of the narrow band of frequencies simultaneously reaching the detector.

Fourier transform infrared spectrometry relies on the principles of interferometry and Fourier transformation for its speed and sensitivity. Michelson first described the interferometer that now bears his name. In this instrument a polychromatic beam is directed on to a beam splitter, an example of a beam splitter that is commonly used is a thin layer of germanium on a IR transmitting support. The beam splitter transmits half the radiation and reflects the other half. The transmitted and reflected beams from the beam splitter strike two plain mirrors, one fixed and one moveable at right angles to each other. The two beams are recombined at the same beamsplitter, so that on an average half the beam returns to the source and the other half passes to the detector, after passing through the absorbing sample (Fig. 2.2.1). The effect of the moving mirror is to introduce a path difference, and hence a phase difference between the two beams

Fig. 2.2.1
Schematic representation of a Michelson interferometer (adapted from Susi, H. and Byler, D.M., 1986).


FIXED MIRROR
F
M'
M
S
SOURCE
BEAM SPLITTER
B
MOVING MIRROR
SAMPLE
D
DETECTOR

on recombination at the beam splitter. When the optical path difference or optical retardation is zero or an integral multiple of λ, the two beams will recombine at the beam splitter in phase. Due to constructive interference, the signal at the detector will be of maximum intensity and this region is called the "centerburst". When the optical path difference has any other value, the two beams will be partially out of phase, resulting in destructive interference and decreased detector signal. When the fixed mirror is in position and the moveable mirror moves at constant velocity v through some distance r, the signal observed at the detector is a cosine wave whose frequency ν is given by the expression, $\nu = v/\lambda = v\upsilon$ (where υ is the wavenumber of the incident radiation). The amplitude or intensity of this signal as a function of optical retardation is called interferogram. A dispersive infrared spectrometer records the spectrum in the frequency domain, whereas interferometers record the spectrum in the time or Fourier domain. The interferogram has to be transformed to the frequency domain by means of Fourier transformation. The sample is usually placed between the interferometer and the detector and the spectrum generated is a single beam spectrum where the vertical coordinate is the intensity of radiation at the source after it has passed through the sample, measured as a function of wavenumber or wavelength. A single scan of the moveable mirror produces a complete single beam spectrum. Usually a number of scans are taken and signal averaged by a computer, which reduces the noise since the signal-to-noise ratio is proportional to the square root of the number of scans.

There are numerous advantages to measuring a spectrum interferometrically: 1. FTIR spectrometers are more sensitive than dispersive instruments because the throughput of the incident light is not slit limited, therefore, for a given source of light, more energy will reach the detector. The signal-to-noise (S/N) ratio of the resulting spectrum is high since the infrared beam is not attenuated by monochromation. 2. The data collection time is reduced for FTIR spectrometers compared to dispersive spectrometers since the length of time required to produce a spectrum in dispersive instruments depends on the time required to select each frequency to be directed to the sample while FTIR spectrometers simultaneously encode all spectral frequencies to give a complete spectrum in a matter of seconds. 3. Since the signal-to-noise ratios are good and high wavenumber precision is possible, FTIR spectra can be manipulated easily and efficiently by a computer. This facilitates mathematical procedures such as Fourier self-deconvolution, calculation of derivatives, curve fitting and spectral subtraction (Colthup, N.B., Daly, L.H. and Wiberley, S.E., 1990).

2.3 Resolution Enhancement by Deconvolution and Derivative Techniques

Many of the absorption bands in the infrared spectrum are broad and complex because they result from two or more overlapping bands. The width of these bands are such that they cannot be separated by instrumental resolution. Examples of such bands include the amide I band of proteins and the ester carbonyl stretching vibrations of lipids. Several approaches have been developed

to resolve these composite bands into their component bands. Although this data processing is referred to as resolution enhancement, it does not increase instrumental resolution, but it does increase the degree to which individual component bands can be visualized. Fourier self-deconvolution was introduced to infrared spectroscopy in 1981 (Kauppinen, J.K., Moffatt, D.J., Mantsch, H.H. and Cameron, D.C., 1981) and is the most effective procedure for narrowing infrared absorption bands. The line shape used in infrared studies is Lorentzian, this is because experimental and theoretical studies have shown that infrared lines are close to Lorentzian. The Fourier transform of a Lorentzian line located at frequency υ, is an exponentially decaying cosine wave. The periodicity of the cosine wave is related to the frequency of the band maxima, while the way in which the cosine wave decays is related to the line shape. A broader line decays more rapidly in the Fourier domain while a narrow line decays slowly (Cameron, D.G., Moffatt, D.J., 1984). Mathematically, the absorbance of a Lorentzian band at wavenumber υ is given by,

$$A(\upsilon) = A^{\circ}\gamma^2 / [\gamma^2 + (\upsilon-\upsilon^{\circ})^2]$$

where A° is the maximum absorbance of the band, υ° is the wavenumber for A° and γ is the half-width of the band at half maximum intensity.

The cosine Fourier transform of the band $A(\upsilon)$ described in the equation above is given by,

$$I(v) = F[A(\upsilon)] = 0.5A^{\circ}\gamma \cos(2\Pi\upsilon^{\circ}v) \exp(-2\Pi\gamma v)$$

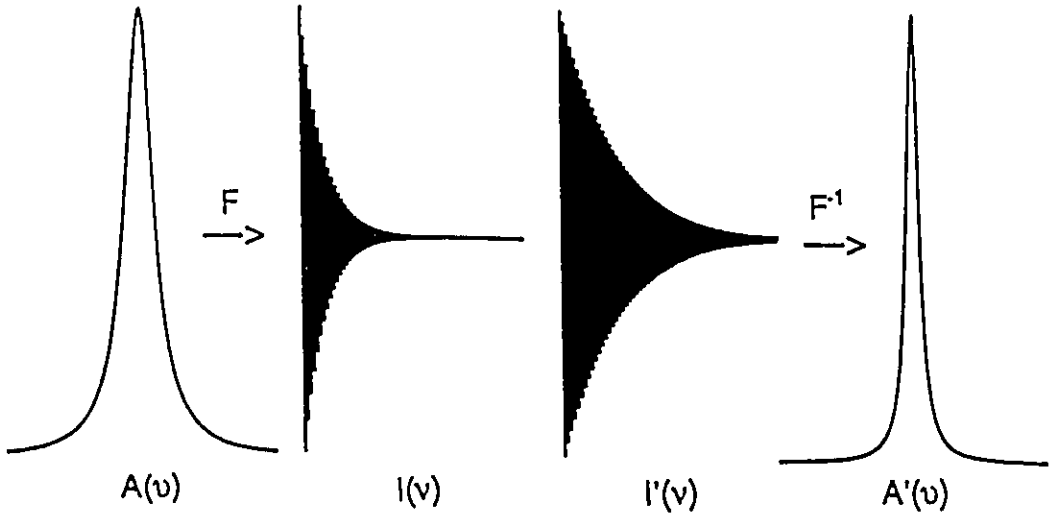
The latter equation can be split into three parts: the coefficient $0.5A^{\circ}\gamma$, a cosine term $\cos(2\Pi\upsilon^{\circ}\nu)$ and an exponential decay term, $\exp(-2\Pi\gamma\nu)$. The coefficient $0.5A^{\circ}\gamma$ is directly proportional to the area of the band. The cosine term has a frequency which is dependent on υ°, the centre wavenumber of the band. The exponential decay term $\exp(-2\Pi\gamma\nu)$, is determined by the γ value, that is, the wider the absorbance band, the more rapidly its Fourier transform decays. Therefore, if the rate of decay of this exponential term is decreased, the width of the absorbance bands will be reduced. This is achieved by multiplying $I(\nu)$ with an exponential that increases with time, $(2\Pi\gamma'\nu)$, to yield a new function $I'(\nu)$.

$$I(\nu) = I(\nu)\ \exp\ (2\Pi\gamma'\nu)$$

$$= 0.5A^{\circ}\gamma\ \cos(2\Pi\nu^{\circ}\nu)\exp\ [-2\ \Pi(\gamma - \gamma')\nu]$$

On performing the reverse Fourier transform on $I'(\nu)$, i.e. $F^{-1}\{I'(\nu)\} = A'(\upsilon)$, we obtain a new absorbance band which is narrower than $A(\upsilon)$ and the width of the band at half-maximum peak intensity will be reduced to $2(\gamma - \gamma')cm^{-1}$ (Fig. 2.3.1). The ratio of $\gamma/\gamma' = r$ is defined as the resolution enhancement factor. In practice, this procedure also enhances the noise and the new function $I'(\nu)$ is often smoothed by multiplication with an apodization function (Griffiths, P. and Pariente, G.L., 1986; Kauppinen *et al.*, 1981; Susi and Byler, 1986; Surewicz and Mantsch 1988).

Fig. 2.3.1
Band narrowing by Fourier self-deconvolution. The cosine Fourier transform of the original infrared band A(υ) with a half-width at half maximum intensity γ yields the function I(ν). Multiplication in the Fourier domain of I(ν) with an exponential that increases with time gives I'(ν) which, after reverse Fourier transformation leads to the new infrared band A'(υ) whose half-width at half maximum intensity is now (γ - γ') cm^{-1} (from Surewicz, W.K. and Mantsch, H.H., 1988).


F
F-1
A(υ)
I(ν)
I'(ν)
A'(υ)

An alternate approach to enhance the resolution of overlapping absorption bands in infrared spectra is by derivatization which analyzes the change of slope of the band directly from the spectrum and gives us accurately the value of peak frequencies. This technique can be applied in wavenumber and Fourier domain. Both derivation and deconvolution are similar in the respect that they produce their result by applying a weighing function to the Fourier transform of the spectrum. Typically the power of the derivative is kept at the default value of 3 with a smoothing breakpoint in the range 0-1. By varying the breakpoint the derivative can be subjectively optimized when noting the noise level in the resulting Fourier transform band (Moffatt, D.J., Kauppinen, J.K., Cameron, D.G., Mantsch, H.H. and Jones, R.N., 1986; Cameron, D.G. and Moffatt, D.J., 1984).

Caution needs to be exercised for Fourier self-deconvolution to select the correct parameters that would give maximum band narrowing while keeping the increase in noise and/or appearance of side lobes at a minimum. Features in the spectra such as absorption bands due to water vapour and random noise are amplified during deconvolution and derivation and they may show up as artifacts that are difficult to distinguish from real component bands. Therefore, complete elimination of water vapour bands by purging water vapour from the spectrometer and sample cell and a high signal-to-noise ratio are important while acquiring spectra.

2.4 Infrared Spectroscopic Study of Lipids

Phospholipids are amphipathic molecules which spontaneously self-associate in water to form the bilayer structures which are the basis of all biological membranes. Such membranes are not static, rigid structures, but highly fluid and dynamic with most of the component lipids in a liquid crystalline state. Synthetic membranes of simple composition can be shown to exist in either this liquid crystalline state or in a more rigid, gel-like state, depending upon the temperature. The infrared spectra of membrane lipids can be classified into spectral regions which originate from molecular vibrations of the hydrophobic, hydrocarbon tail and those that originate from the hydrophilic head group. Since water shows absorption bands in the spectral regions of interest, lipid dispersions are prepared in ^2H_2O instead of H_2O.

There are many groups in the lipid molecule that are infrared active (Table 2.4.1). Stretching, bending, wagging and scissoring vibrations are all observed for the methylene groups of acyl chains. The CH_2 symmetric and asymmetric stretching modes are observed at 2850 and 2920 cm^{-1}, respectively. The frequencies of these modes are sensitive to conformation and respond to temperature induced changes of trans/gauche ratio in acyl chains. The symmetric and asymmetric vibrational modes due to terminal methyl groups are found at 2870 and 2950 cm^{-1} respectively. The olefinic =C-H stretching bands due to unsaturation in acyl chains is found around 3010 cm^{-1}. Infrared bands due to CH_2 bending or scissoring are found around 1470 cm^{-1}; the number of these bands

Table 2.4.1
Characteristic frequencies for important absorptions in lipids.

VIBRATION	FREQUENCY (cm^{-1})
=C-H stretch	3010
CH_3 asymmetric stretch	2956
CH_2 asymmetric stretch	2920
CH_3 symmetric stretch	2870
CH_2 symmetric stretch	2850
C=O stretch	1700-1745
CH_2 bending or scissoring	1450-1470
CH_3 asymmetric bend	1460
CH_3 symmetric bend	1378
CH_2 wagging band progression	1200-1400
PO_2^- asymmetric stretch	1220
PO_2^- symmetric stretch	1085

and their frequencies give us information about acyl chain packing and conformation. A series of bands in the region 1190-1380 cm^{-1} are due to CH_2 wagging band progression. They are difficult to observe in the spectra of phospholipids because of overlapping with asymmetric PO_2^- stretching band around 1220 cm^{-1}. The interfacial region of lipids gives rise to strong absorption bands from ester C=O groups in the 1700-1780 cm^{-1} region. For diacyl lipids we observe at least two component bands in the deconvolved spectra. The high frequency component represents "free" carbonyl groups and the low frequency component represents hydrogen bonded carbonyl groups. The polar head groups of the lipid molecules gives rise to absorption bands due to phosphate groups (in phospholipids) and other groups such as choline, sulphocholine, ethanolamine or serine group vibrations. The bands due to asymmetric and symmetric phosphate stretching modes occur at 1220 cm^{-1} and 1085 cm^{-1}, respectively.

These lipid molecules exist in different polymorphic forms, the particular polymorphic form that predominates depends not only on the structure of the lipid molecule and on its degree of hydration but also upon variables such as temperature, pressure and pH. Infrared spectroscopy has been used to study the thermal phase behaviour of lipids. The main endothermic transition of phospholipid-water systems results in an abrupt change in the frequency of the CH_2 stretching vibrations of acyl chains. The frequency of the symmetric stretching vibration of methylene groups of acyl chains can be used to probe membrane fluidity. The presence of slight impurities decreases the cooperativity

and amplitude of transition. The spectral changes observed in the acyl chain bands at T_m (transition temperature) are indicative of a transition from a conformationally and motionally ordered gel phase to a disordered liquid-crystalline phase containing a high proportion of gauche conformers (Casal, H.L. and Mantsch, H.H., 1984).

2.5 Infrared Spectroscopic Study of Proteins

Proteins are long polymers formed from twenty commonly occurring amino acids. Folding of the protein polypeptide chain results in the formation of two major classes of regular secondary structures, α-helices and β-sheets. α-Helical structures are characterized by a corkscrew-like arrangement of the polypeptide chain stabilized with hydrogen bonds between residues favourably aligned along the helix axis. On the other hand, β-sheets are extended, pleated structures, which are cross linked by interchain hydrogen bonds. The peptide linkage in proteins gives rise to absorption in the infrared spectra of proteins known as Amide absorption. The most useful band for the spectroscopic analysis of the secondary structure of proteins in aqueous media is the Amide I band between 1600-1700 cm^{-1}. This band arises from the stretching of the C=O band in the amide linkage (80%) coupled with C-N stretching and in plane N-H bending modes. The amide II band located at 1565-1535 cm^{-1} represents N-H in-plane deformation and C-N stretching modes. Sensitivity of the Amide II band to polypeptide backbone conformation is not well established. The Amide III band,although sensitive to

secondary structure gives rise to a weak IR band. The exact frequency of the Amide I band depends on the nature of H-bonding involving the CO and NH groups and on the geometry of the molecule. This in turn depends on the secondary structure adopted by the protein. The conformation sensitive Amide I band is a composite band consisting of overlapping component bands originating from different structures such as α-helices, β-strands, turns and non-ordered polypeptide fragments. Resolution enhancement techniques allow us to identify the component bands and determine the proportion of various conformers (Surewicz and Mantsch, 1988). Table 2.5.1 summarizes the typical amide I frequency ranges found for the major conformers, as determined from the spectra of proteins for which the secondary structure has been deduced from X-ray diffraction data.

Table 2.5.1
Amide I frequencies for the protein secondary structural elements.

Secondary Structure	Frequency (cm^{-1})
3_{10} helix	1658-68
α helix	1648-55
Unordered	1640-45
β-sheet	1630-40
	1680-90
turns	1670-80

Chapter 3

MATERIALS AND METHODS

Materials

The sodium salt of L-α-phosphatidylinositol from soya bean was obtained from Sigma Chemical Co. (St. Louis, MO). Gentamicin was kindly donated by Schering Canada Inc. (Pointe-Claire, Québec, Canada) whereas daptomycin was a generous gift of Eli Lilly Canada Inc. (Scarborough, Ontario, Canada). The sodium salt of poly-L-aspartic acid (MW 14,400) was purchased from Sigma Chemical Co. (St. Louis, MO) and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidyl-glycerol (sodium salt) was from Avanti Polar Lipids, Inc. 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid, were obtained from Sigma Chemical Co. (St. Louis, MO). Deuterated water was from MSD isotopes (Montréal, Québec, Canada).

Methods

The lipidic dispersions (10%) for infrared measurements were prepared by mixing the desired amount of lipid and solvent (H_2O or ^2H_2O). In order to ensure proper organization of the bilayers, the lipidic dispersions were heated for several minutes to some 10° C above the T_m of the corresponding lipid, vortexed while warm, and allowed to cool below the T_m before commencing the measurements. (Casal and Mantsch, 1984). At least three such freeze-thaw cycles were done to prepare lipidic dispersions. Experiments were performed at pH 5.4, the pH found

in lysosomes where aminoglycosides accumulate in kidney cells (De Duve, C., Okhuma, S., Poole, B. and Tulkens, P.M., 1978) and at 7.5, the pH prevailing at the surface of brush border membranes. NaO^2H and ^2HCl were used to adjust the p^2H of the samples prepared in ^2H_2O to pH 5.0 and 7.1, as read on the pH meter (p^2H 5.4 and 7.5 respectively according to Glasoe, P.K. and Long, F.A., 1960). Similarly the pH of samples prepared in H_2O was adjusted with NaOH and HCl. Additional freeze-thaw cycles were also performed after additions of the required amount of 5% gentamicin solution, 10% daptomycin solution or 15% poly-L-aspartic acid solution in H_2O or ^2H_2O, after appropriate pH adjustment.

Infrared spectra were recorded at room temperature on a Digilab FTS-40 Fourier-transform spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride (MCT) detector. Samples were placed between two calcium fluoride windows separated by a 6 μm spacer. A total of 256 interferograms were co-added for each spectrum, with spectral resolution of 2 cm^{-1}. To eliminate spectral contributions of atmospheric water vapour, the instrument was purged continuously with dry air. Experiments directed towards studying the thermotropic phase behaviour of lipids and the effect of various antibiotics on their phase behaviour involved collecting the infrared spectra of the sample at various temperatures and monitoring changes in spectral parameters as a function of temperature. A thermostated cell mount and variable temperature cell bath was used. The temperature range and increments had to be preselected according to

the sample under study. Some of the experiments at variable temperature were realized on a Digilab FTS-60 Fourier-transform spectrometer.

Data treatment was performed as described in the previous chapter for resolution enhancement by deconvolution and derivative techniques. Data processing was achieved using RAMOP, a software package written by Doug Moffatt (National Research Council of Canada). Band narrowing by Fourier self-deconvolution for carbonyl bands of various lipids was performed using a bandwidth of 13 and resolution enhancement factor of 1.75 or 1.5. The frequencies for the C-H stretching vibrations of methylene and olefinic groups in acyl chains of various lipids were obtained from the spectra after Fourier derivation with a power of 3 and a breakpoint in the range 0-1. The spectra were plotted using GPLOT, a general purpose plotting package written by Doug Moffatt.

Chapter 4

INTERACTION OF AMINOGLYCOSIDE ANTIBIOTICS WITH LIPIDS

4.1 Introduction

The mechanism of aminoglycoside-induced toxicity is related to their ability to alter membrane structure and function. The initial interaction of the aminoglycosides with renal proximal tubular cells occurs at the brush border membrane. In an attempt to identify the aminoglycoside binding site, the kinetics of [^{3}H]-gentamicin binding to isolated rat renal brush border membranes was assessed. These studies revealed that [^{3}H]-gentamicin binds to rat renal brush border membranes in a saturable fashion. Proteolytic enzymes and protein modifying agents did not decrease [^{3}H]-gentamicin binding to brush border membranes. Phospholipases A and C however, decreased [^{3}H]-gentamicin binding, confirming the phospholipidic nature of the aminoglycoside binding site (Sastrasinh *et al.*, 1982). The study of the interaction between the lipid components of the membrane and these drug molecules has been a subject of great interest. *In vitro*, gentamicin was shown by gel permeation to bind to phospholipid bilayers (liposomes) under conditions which mimic lysosomal environment (acidic pH and presence of phosphatidylinositol). Reversibility of binding upon an increase in pH and ionic strength suggested that it was mainly mediated by electrostatic forces. The effect of pH was interesting since it could contribute to the selective action of the drug towards lysosomes (Laurent *et al.*,

1982). The interaction of aminoglycoside antibiotics with negatively charged lipid layers was also demonstrated by conformational analysis (Brasseur *et al.*, 1984). Variation of the phosphatidylinositol content from 0-27% of the total phospholipids caused an increase in aminoglycoside binding. Interaction of aminoglycosides with phospholipids was also estimated by an increase in the turbidity of liposomes consisting of various phospholipids. An increase in turbidity occurred with liposomes consisting of acidic phospholipids, the highest increase was observed for phosphatidylinositol-4, 5-diphosphate and phosphatidylinositol (PI); no increase in turbidity was found in liposomes containing the neutral phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

The mode of interaction between aminoglycosides and negatively charged phospholipids plays a critical role in the inhibition of lysosomal phospholipases by these antibiotics and, therefore, in their nephrotoxicity. Infrared spectroscopy is an excellent technique to detect interactions localized at individual functional groups. We have used this technique to study the interaction of the aminoglycoside antibiotic gentamicin with phosphatidylinositol (Fig. 4.1.1) phosphatidylglycerol (Fig. 4.1.2) and phosphatidylcholine bilayers (Fig. 4.1.3).

The simplest model membrane systems to study are liposomes composed of single lipid species and much of the early spectroscopic information concerning lipid structure in membranes was obtained from such systems. There are two features in the lipid spectrum that are particularly useful: its carbonyl stretching

Fig. 4.1.1
Chemical structure of phosphatidylinositol.

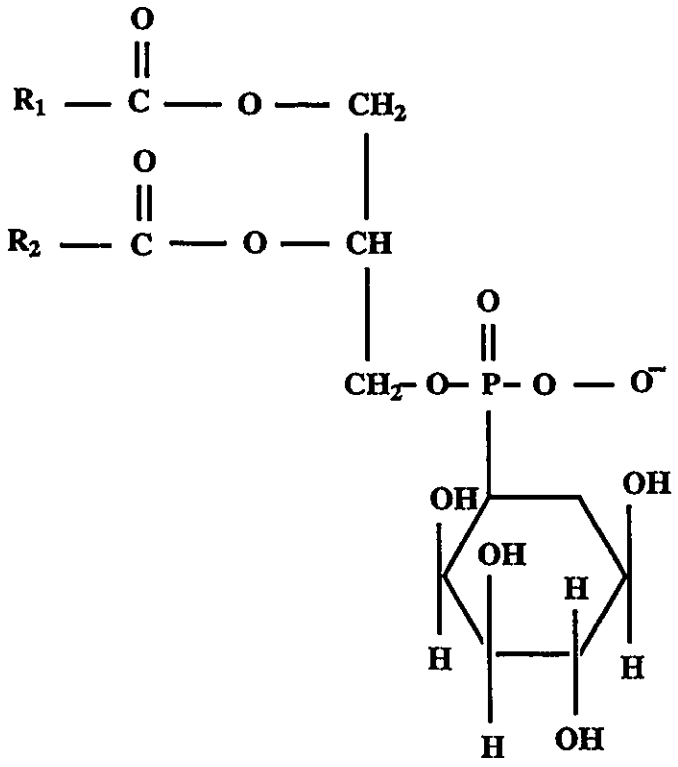


Fig. 4.1.2
Chemical structure of phosphatidylglycerol.

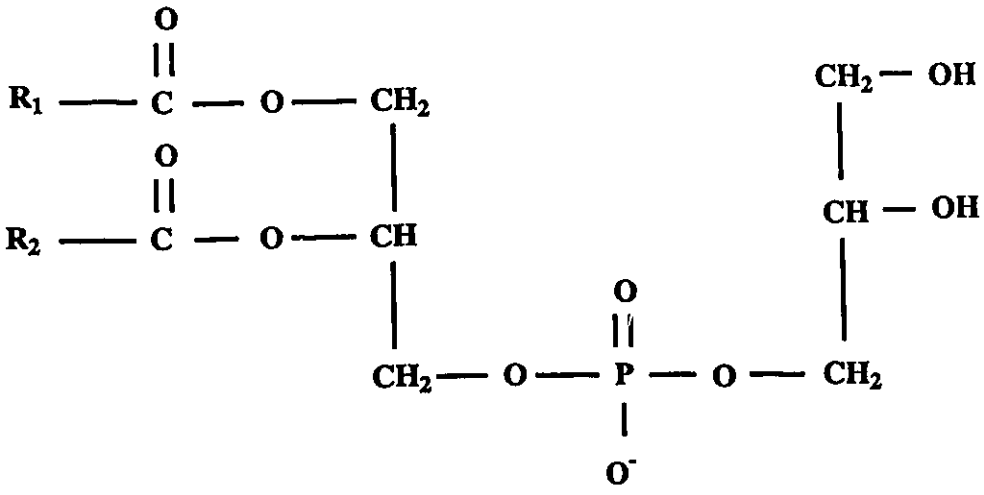
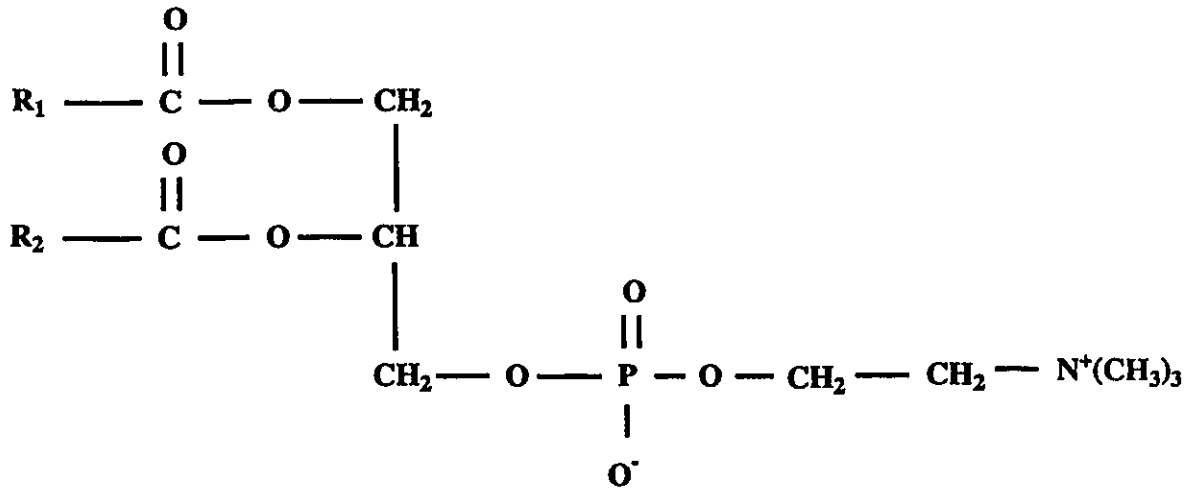


Fig. 4.1.3
Chemical structure of phosphatidylcholine.



band and its phosphate antisymmetric stretching band, both occurring in spectral regions free of gentamicin contributions.

4.2 The Lipid C=O Stretching Region (1700-1800 cm^{-1})

The carbonyl stretching region of 1, 2-diacyl phospholipids consists of a broad band which on Fourier self-deconvolution is found to contain two overlapping components. Up to recently, the high frequency component band was attributed to the stretching vibration of the *sn*-1 C=O group while the low frequency component band was assigned to the *sn*-2 C=O group. It was originally suggested that the appearance of two ester C=O stretching vibrations in phospholipids resulted from the structural inequivalence of the two acyl chains at the lipid interface region (Mushayakarara, E. and Levin, I.W., 1982). However, recent experiments with ^{13}C=O substituted lipids have shown this not to be so (Mantsch, H.H., Moffatt, D.J. and Casal, H.L., 1988; Hübner, W., Mantsch, H.H. and Casal, H.L., 1990). Substitution of the *sn*-2 C=O with ^{13}C=O would be expected to shift the low frequency component by about 44 cm^{-1} to around 1680 cm^{-1}. This should result in two well resolved bands. However, this procedure actually produces four C=O stretching bands, both the *sn*-1 C=O and *sn*-2 ^{13}C=O groups giving rise to two absorptions (Hübner *et al.*, 1990). It is believed that in each case the high frequency absorption represents "free" C=O groups, with the lower absorption arising from hydrogen bonded C=O groups.

The carbonyl stretching band of the ester functions of phosphatidylinositol in aqueous suspension is presented in Fig. 4.2.1A and B (solid line). The original spectrum consists of an asymmetric band. Two components are revealed after resolution enhancement using Fourier self-deconvolution (4.2.1A and B, dashed line). The high frequency component, at 1745 cm^{-1}, pertains to carbonyl groups that are not hydrogen bonded and the peak at 1725 cm^{-1} corresponds to hydrogen bonded carbonyl groups (Mantsch *et al.*, 1988; Hübner *et al.*, 1990). The addition of gentamicin to PI suspension in a molar ratio of 2 PI:1 gentamicin does not result in any significant frequency shift of these two components, but an increase of the intensity ratio of non bonded to bonded carbonyl bands is observed (Fig. 4.2.2A and B, dashed line). Gentamicin thus induces an increase in the proportion of carbonyl groups that are not hydrogen bonded. Replacing the solvent ^2H_2O by H_2O resulted in a 1 cm^{-1} increase in the frequency of hydrogen bonded carbonyl band while the free carbonyl component remained unaffected (not shown). This frequency shift indicates that the carbonyl hydrogen bonding involves solvent molecules. The decrease in the population of hydrogen bonded carbonyl groups shows that gentamicin reduces the penetration of water to the bilayer interface region. It is consistent with a tightening of the lipidic network following neutralization of PI molecules by gentamicin. The same behaviour is observed at pH 5.4 and 7.5.

The carbonyl stretching band of the ester functions of phosphatidylglycerol (PG) in aqueous suspension is presented in Fig. 4.2.3A and B(solid line). The

Fig. 4.2.1
Carbonyl stretching region of the infrared spectra of dispersions of phosphatidylinositol in ^2H_2O at p^{2}H 5.4(A) and 7.5(B). Original spectra (solid line) of dispersions of the lipid alone are superimposed with spectra obtained after a resolution enhancement using Fourier self-deconvolution with a bandwidth of 13 and a resolution enhancement factor of 1.5.

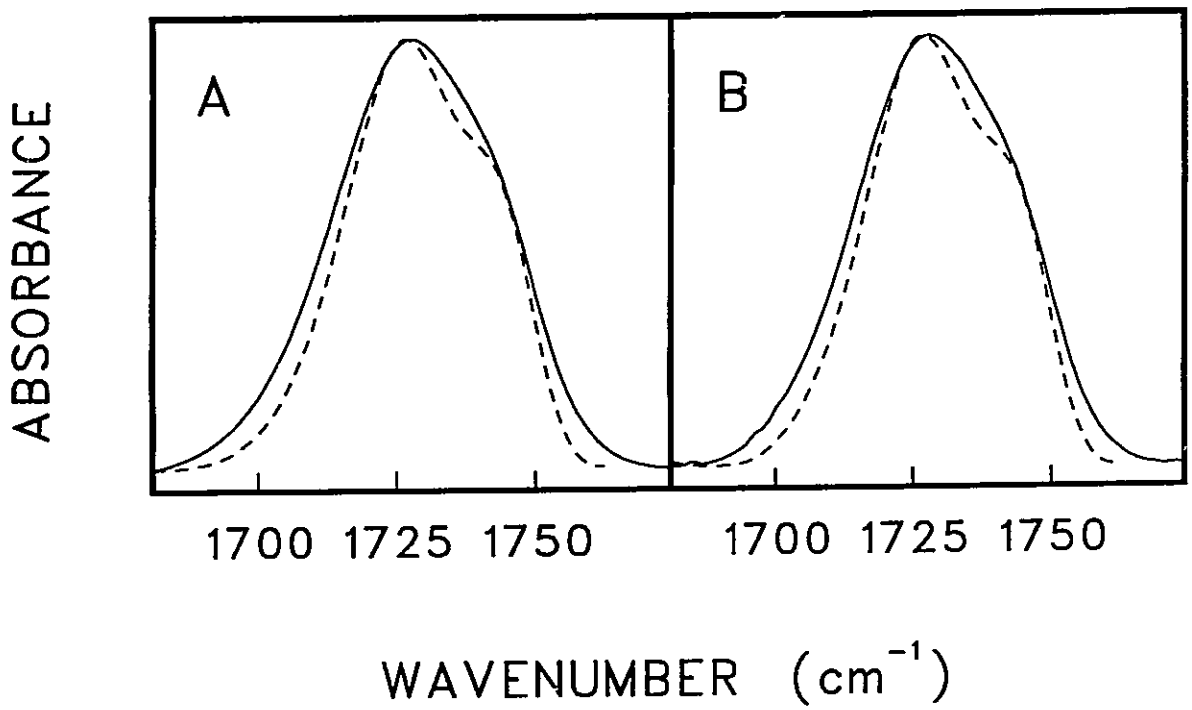

A
B
ABSORBANCE
1700 1725 1750
1700 1725 1750
WAVENUMBER (cm^{-1})

Fig. 4.2.2
Carbonyl stretching region of the infrared spectra of dispersions of phosphatidylinositol alone (solid line) or with gentamicin (small dashes) at PI:gentamicin molar ratio of 2:1, in ^2H_2O at p^{2}H 5.4(A) and 7.5(B). The spectra were obtained after band narrowing by Fourier self-deconvolution using a bandwidth of 13 and a resolution enhancement factor of 1.5.

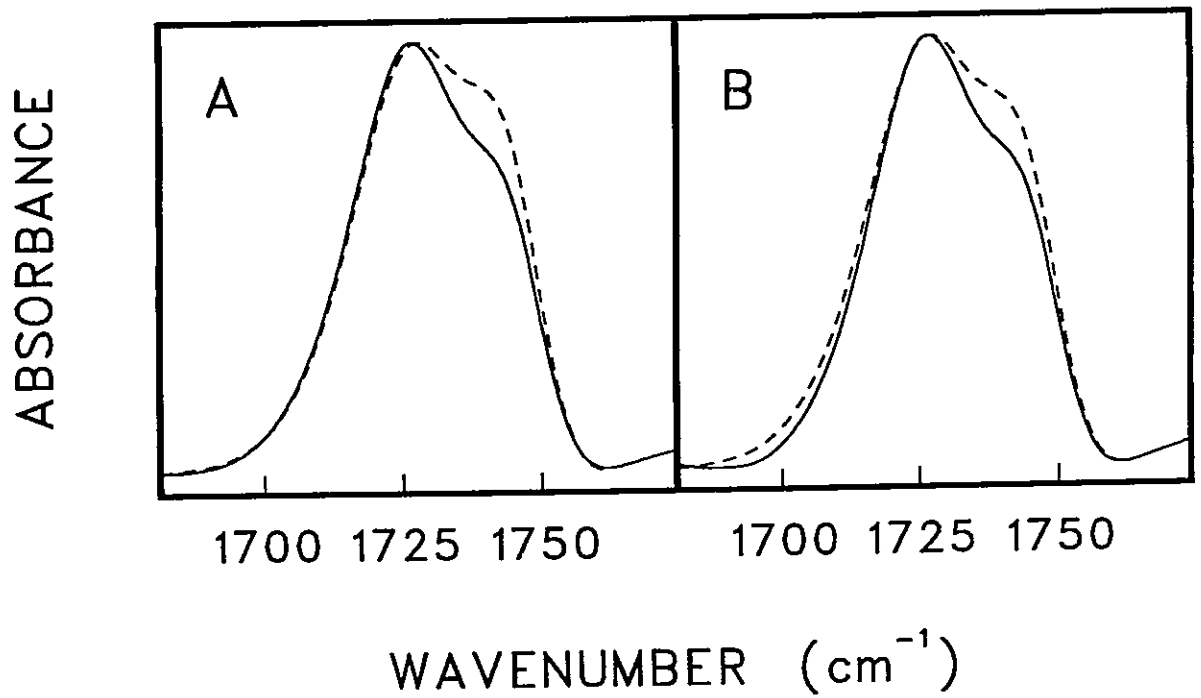
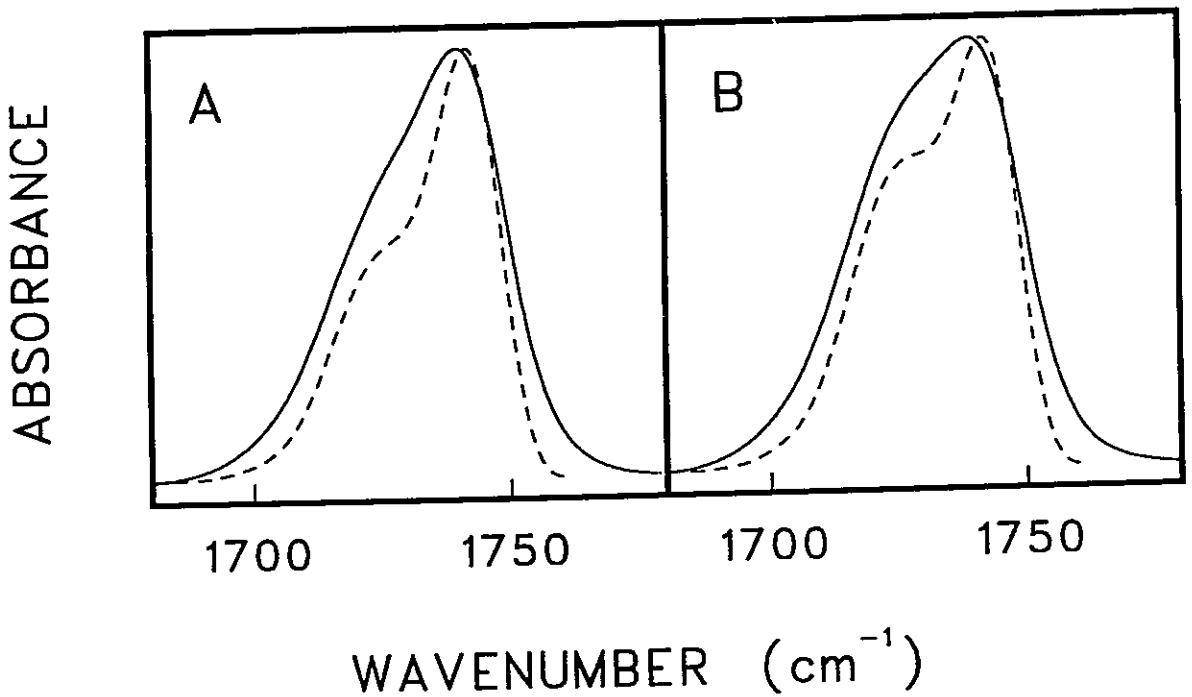

ABSORBANCE
A
B
1700 1725 1750
1700 1725 1750
WAVENUMBER (cm^{-1})

Fig. 4.2.3
Carbonyl stretching region of the infrared spectra of dispersions of dimyristoylphosphatidylglycerol in ^2H_2O at p^{2}H 5.4(A) and 7.5(B). Original spectra (solid line) of dispersions of the lipid alone are superimposed with spectra obtained after a resolution enhancement using Fourier self-deconvolution with a bandwidth of 13 and a resolution enhancement factor of 1.75.


A
B
ABSORBANCE
1700
1750
1700
1750
WAVENUMBER (cm^{-1})

original spectrum consists of an asymmetric band. Two components are revealed after resolution enhancement using Fourier self-deconvolution (Fig. 4.2.3A and B, dashed line). The high frequency component, at 1743 cm^{-1}, pertains to carbonyl groups that are not hydrogen bonded and the peak at 1721 cm^{-1} corresponds to hydrogen bonded carbonyl groups. The addition of gentamicin to PG suspension in a molar ratio of 2 PG:1 gentamicin does not result in any significant frequency shift or change in the intensity ratio of non bonded to bonded carbonyl groups in the gel state (Fig. 4.2.4A and B, dashed line). The same behaviour is observed at pH 5.4 and 7.5. At temperatures above the lipid gel-to-fluid transition, we do observe an increase in the proportion of free carbonyl groups (not shown).

The carbonyl stretching band of the ester functions of phosphatidylcholine (PC) in aqueous suspension is presented in Fig. 4.2.5A and B (solid line). The original spectrum consists of an asymmetric band and reveals two components after resolution enhancement using Fourier self-deconvolution (Fig. 4.2.5A and B, dashed line). The high frequency component, at 1742 cm^{-1}, pertains to "free" carbonyl groups and the peak at 1726 cm^{-1}, corresponds to hydrogen bonded carbonyl groups. The addition of gentamicin to PC suspension in a molar ratio of 2 PC:1 gentamicin does not result in any significant frequency shift or change in the intensity ratio of non bonded to bonded carbonyl groups. However, we do observe a slight broadening of the band at pH 7.5 (Fig. 4.2.6A and B, dashed line). The same results were obtained at temperatures above the lipid gel-to-fluid transition (not shown).

Fig. 4.2.4
Carbonyl stretching region of the infrared spectra of dispersions of dimyristoylphosphatidylglycerol alone (solid line) or with gentamicin (small dashes) at PG:gentamicin molar ratio of 2:1, in ^2H_2O at p^{2}H 5.4(A) and 7.5(B). The spectra were obtained after band narrowing by Fourier self-deconvolution using a bandwidth of 13 and a resolution enhancement factor of 1.75.

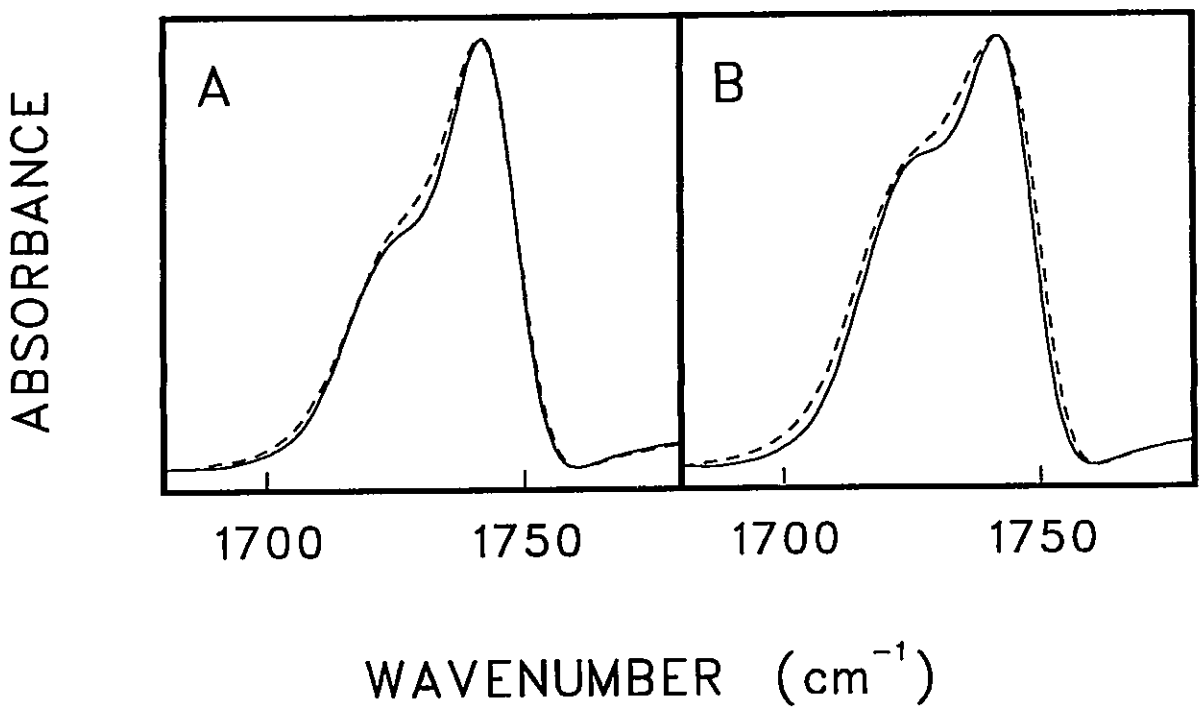

A
B
ABSORBANCE
1700
1750
1700
1750
WAVENUMBER (cm^{-1})

Fig. 4.2.5
Carbonyl stretching region of the infrared spectra of dispersions of dimyristoylphosphatidylcholine in ^2H_2O at p^{2}H 5.4(A) and 7.5(B). Original spectra (solid line) of dispersions of the lipid alone are superimposed with spectra obtained after a resolution enhancement using Fourier self-deconvolution bandwidth of 13 and a resolution enhancement factor of 1.75.

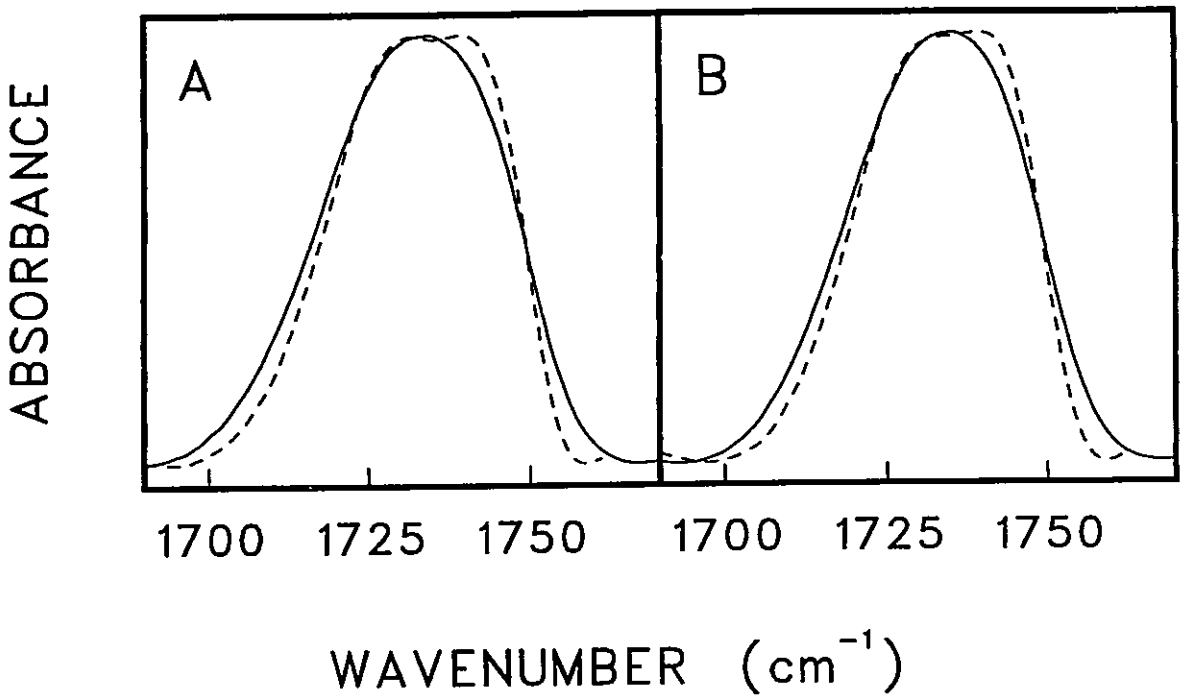
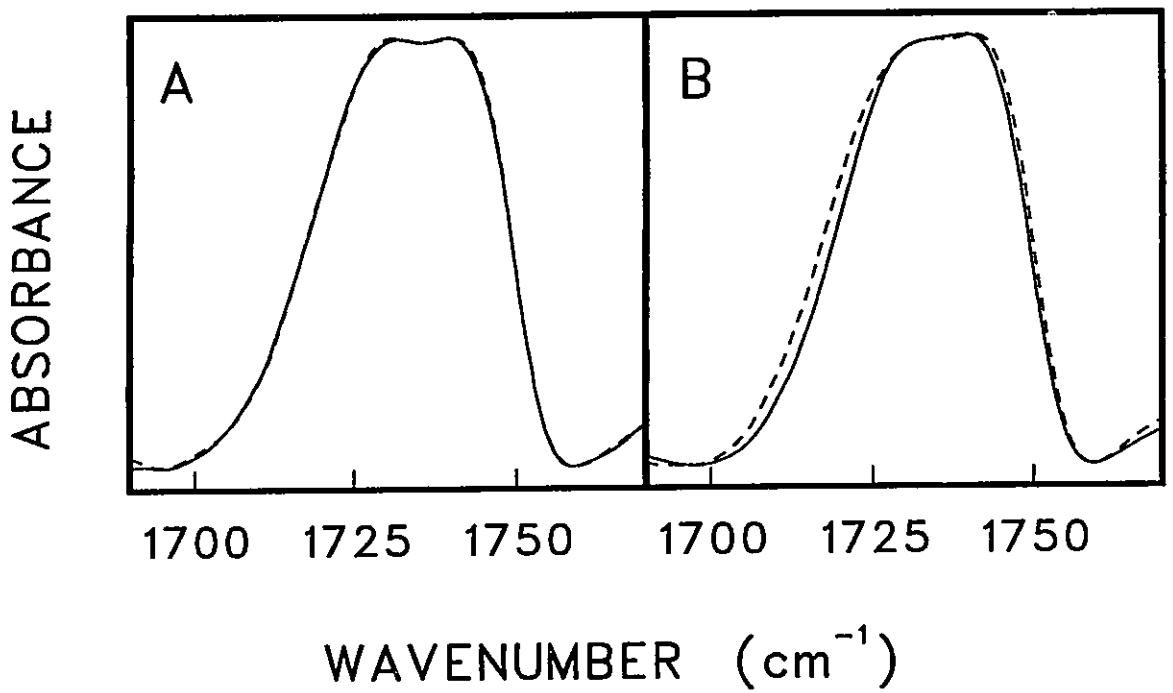

A
B
ABSORBANCE
1700
1725
1750
1700
1725
1750
WAVENUMBER (cm^{-1})

Fig. 4.2.6
Carbonyl stretching region of the infrared spectra of dimyristoylphosphatidylcholine alone (solid line) or with gentamicin (small dashes) at DMPC:gentamicin molar ratio of 2:1, in $^{2}H_{2}O$ at p^{2}H 5.4(A) and 7.5(B). The spectra were obtained after band narrowing by Fourier self-deconvolution using a bandwidth of 13 and a resolution enhancement factor of 1.75.

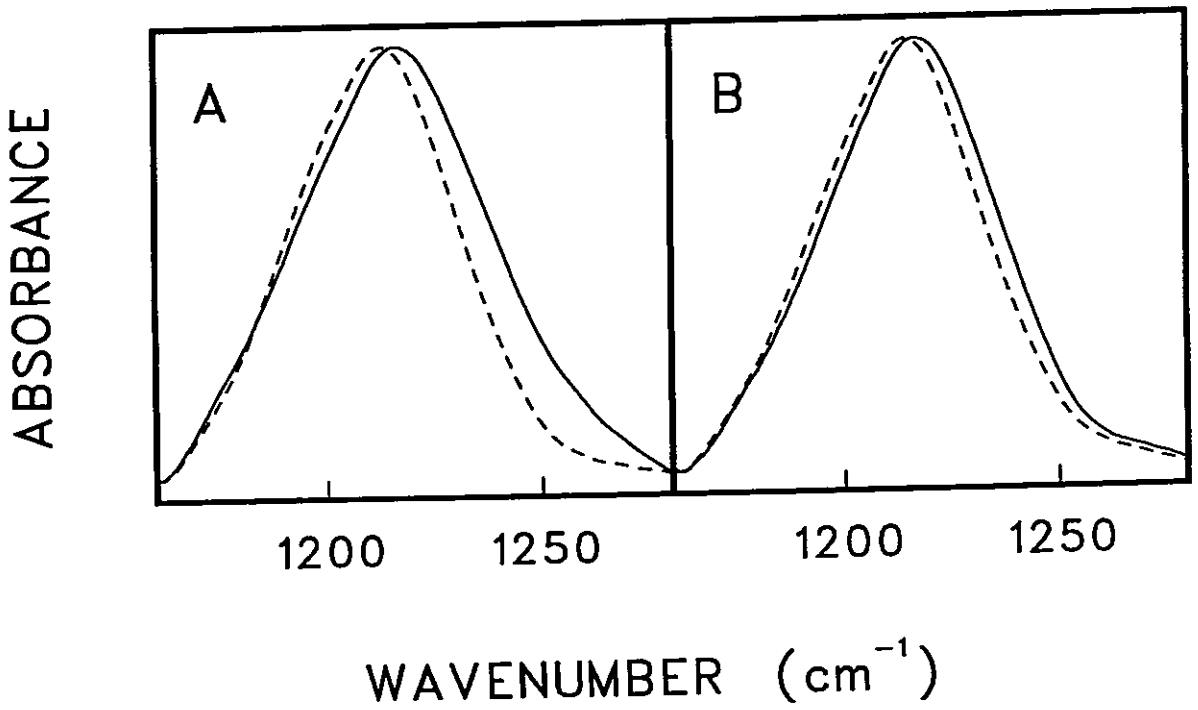

A
B
ABSORBANCE
1700
1725
1750
1700
1725
1750
WAVENUMBER (cm^{-1})

4.3 The Lipid PO_2^- Stretching Region (1000-1300 cm^{-1})

Infrared bands characteristic of the phosphate group in phospholipids occur in the region 1000-1300 cm^{-1}. The complex profile of the infrared bands in this region is due to the superposition of the progressional bands from the CH_2 twisting and wagging modes onto the symmetrical and antisymmetrical PO_2^- stretching region (Snyder, R.G. and Schachtschneider, J.H., 1963). There are two strong bands around 1250 cm^{-1} and 1085 cm^{-1} due to double bond PO_2^- stretching (PO_2^- antisymmetric and symmetric stretch, respectively), while the single bond P-O stretching modes are found at lower frequencies.

The phosphate region must be studied in the absence of ^2H_2O to avoid interferences from its bending mode in the 1200 cm^{-1} region. The frequency of the phosphate antisymmetric stretching vibration usually varies between 1220 and 1270 cm^{-1}, depending on the extent of hydrogen bonding (Casal and Mantsch, 1984). A lipidic dispersion of PI yielded a broad band with a maximum at 1216 cm^{-1} (Fig. 4.3.1A and B, solid line), for both pH 5.4 and 7.5. Since the asymmetric double bond stretching frequency of a non-hydrogen bonded PO^{2-} group is approximately 1260 cm^{-1}, it is clear that in this sample the phosphate group is involved in very strong hydrogen bonds. The addition of gentamicin resulted in a decrease of approx. 1-2 cm^{-1} of the frequency of the maximum of that band at pH 7.5 and 2-4 cm^{-1} at pH 5.4. (Fig. 4.3.1A and B, dashed line). Actually, this frequency shift seems to result mostly from a marked decrease of the intensity on the high frequency side of the broad band, corresponding to lipid molecules with

Fig. 4.3.1
Phosphate antisymmetric stretching region of the infrared spectra of aqueous dispersions of phosphatidylinositol alone (solid line) or with gentamicin (small dashes) at a PI:gentamicin molar ratio of 2:1, at pH 5.4(A) and 7.5(B).


A
B
ABSORBANCE
1200
1250
1200
1250
WAVENUMBER (cm^{-1})

weaker hydrogen bonds. The frequency shift was smaller for a molar ratio of 8 PI:1 gentamicin instead of 2:1(spectra not shown). The strengthening of phosphate hydrogen bonds is also consistent with a decrease of PI intermolecular distance consecutive to charge neutralization by gentamicin.

A lipidic dispersion of PG yielded a broad band with a maximum at 1208 cm^{-1} (Fig. 4.3.2A and B, solid line), for both pH 5.4 and 7.5. The addition of gentamicin did not bring about a significant frequency shift of the maximum of the band.

The phosphate antisymmetric stretching vibration of phosphatidylcholine in aqueous suspension is presented in Fig. 4.3.3A and B, (solid line), for both pH 5.4 and 7.5. In the presence of the polycationic aminoglycoside drug gentamicin we observed a narrowing of the band as well as a downward shift of the frequency maximum (Fig. 4.3.3A and B, dashed line). The effect was greater at pH 5.4 than 7.5.

4.4 Effect of Gentamicin on the Fluidity of Membranes

The fluidity of the lipid bilayer plays an important role in cellular functions. Maintenance of the fluidity of the lipid matrix is important for a number of reasons. Firstly, fluidity will determine the degree of structural stability of the bilayer. Too great a degree of conformational disorder will destabilize the bilayer which will either lose its selective permeability characteristics, or worse, collapse. Secondly, fluidity regulates the activity of many intrinsic proteins. If the bilayer is too rigid,

Fig. 4.3.2
Phosphate antisymmetric stretching region of the infrared spectra of aqueous dispersions of dimyristoylphosphatidylglycerol alone (solid line) or with gentamicin (small dashes) at a PG:gentamicin molar ratio of 2:1, at pH 5.4(A) and 7.5(B).

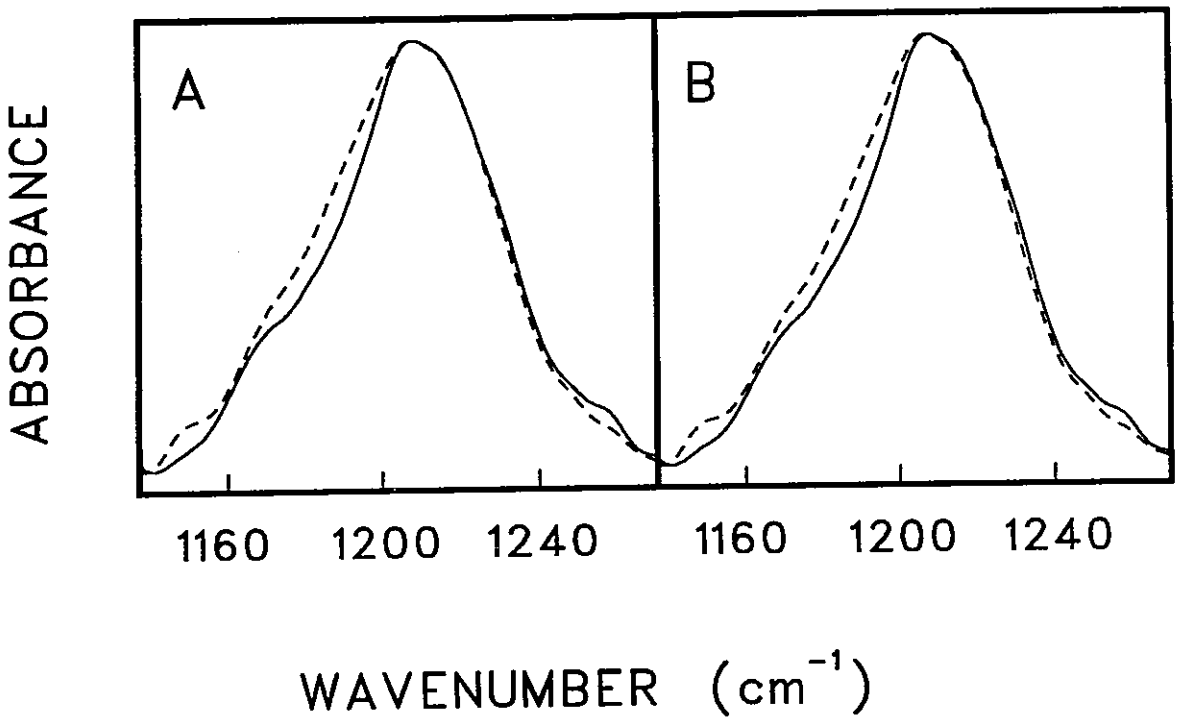
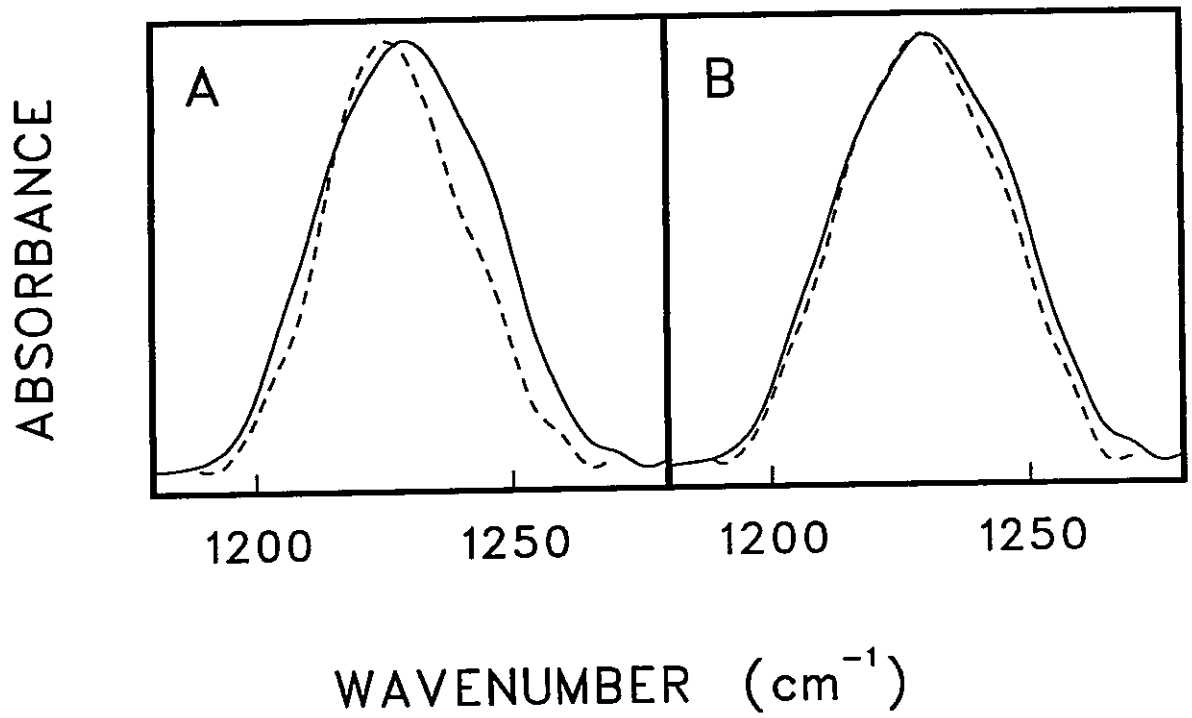

A
B
ABSORBANCE
1160
1200
1240
1160
1200
1240
WAVENUMBER (cm^{-1})

Fig. 4.3.3
Phosphate antisymmetric stretching region of the infrared spectra of aqueous dispersions of dimyristoylphosphatidylcholine alone (solid line) or with gentamicin (small dashes) at PC:gentamicin ratio molar ratio of 2:1, at pH 5.4(A) and 7.5(B).


A
B
ABSORBANCE
1200
1250
1200
1250
WAVENUMBER (cm^{-1})

conformational transitions necessary for catalytic activity may be restricted or prevented, resulting in inactivation of enzymes.

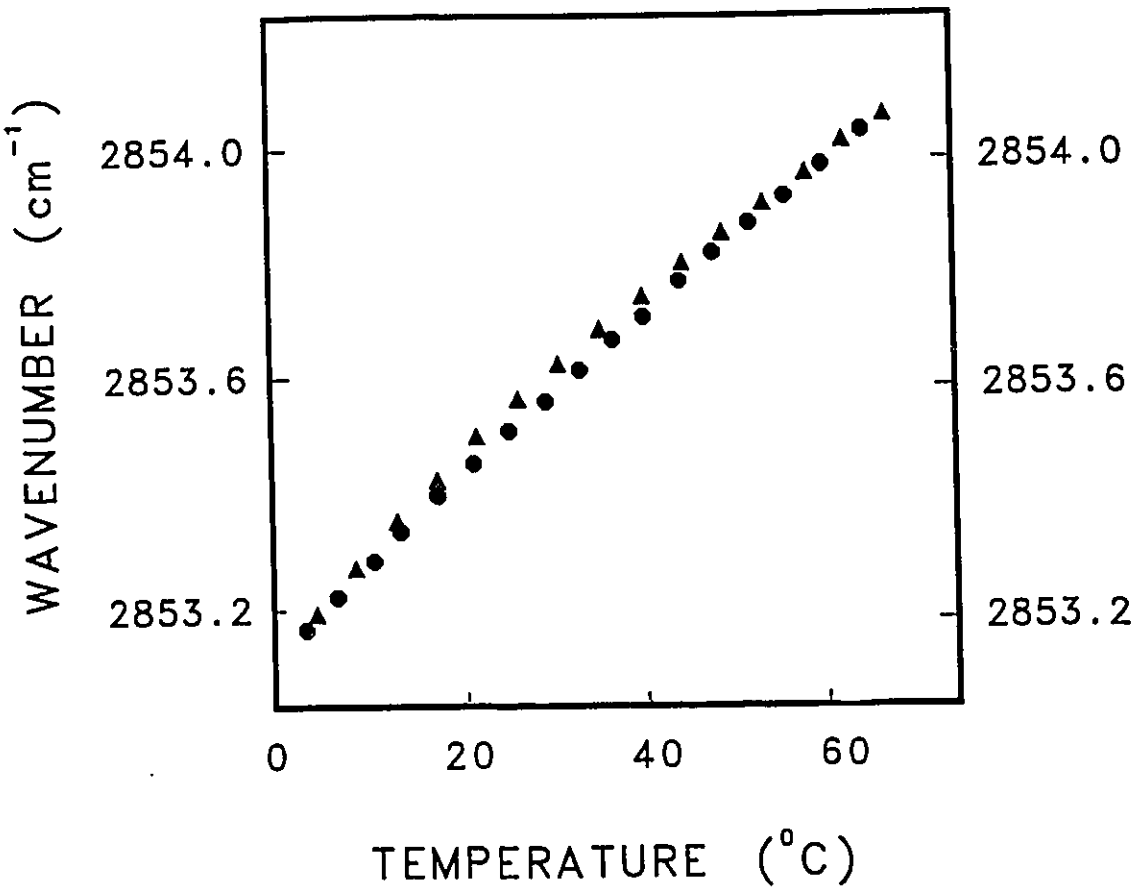
Membrane lipids are polymorphic, that is, they can exist in a variety of different kinds of organized structures, especially when hydrated. The particular polymorphic form that predominates depends not only on the structure of the lipid molecule itself and on its degree of hydration but upon such variables as temperature, pressure, ionic strength and pH (Gennis, R.B., 1989). FTIR spectroscopy has been extensively used to study the transition of one lipidic phase to another. The lamellar gel-to-liquid crystalline or chain melting phase transition has been the most intensively studied lipid phase transition. This cooperative transition involves the conversion of a relatively ordered gel-state bilayer, in which the hydrocarbon chains exist predominantly in their rigid, extended, all trans conformation, to a relatively disordered liquid crystalline bilayer, in which the hydrocarbon chains contain a number of gauche conformers and exhibit greatly increased rates of intra- and intermolecular motions. The gel-to-liquid crystalline phase transition is accompanied by a pronounced lateral expansion and a concomitant decrease in the thickness of the bilayer, as well as a small increase in the total volume occupied by the lipid molecules. Studies have also indicated that the number of water molecules bound to the surface of the lipid bilayer increase upon chain melting (Lewis, R.N.A.H. and McElhaney, R.N., 1991).

A number of drugs are known to produce their characteristic effects by interacting with the lipid bilayer of biological membranes. Some of these

molecules produce their effects by changing the thermotropic properties, i.e. the degree of fluidity of the membrane. Such molecules can modify the transition profile by a change in transition temperature and cooperativity of transition. We, therefore, studied the effect of the aminoglycoside antibiotic gentamicin on the thermotropic properties of negatively charged and neutral liposomes at pH 5.4.

Since soya bean PI contains a heterogeneous mixture of various acyl chains, the most common ones being palmitate (16:0) and linoleate (18:2), we do not observe cooperativity in the gel-to-fluid transition. The CH_2 stretching vibrations give rise to bands at frequencies near 2850 and 2920 cm^{-1}, attributed to symmetric and antisymmetric stretching modes respectively. These vibrations are sensitive to conformational changes in the acyl chains of lipid bilayers and can be used to probe membrane fluidity. It can be seen in Fig. 4.4.1 (triangles) that the frequency of the symmetric C-H stretching vibration of methylene groups of PI liposomes increases gradually upon raising the temperature from 5 to 60 °C. The effect of gentamicin is subtle and difficult to detect in the temperature profile of the methylene symmetric stretching vibration (Fig. 4.4.1., circles). We can observe that gentamicin causes a decrease in the fluidity of PI liposomes. Similar results have been reported by Ramsammy, L.S. and Kaloyanides, G.J., (1987) using differential scanning calorimetry. This observation is consistent with the neutralization of the negative charges on PI due to an electrostatic interaction with the cationic amino groups of the aminoglycoside which leads to increased lipid packing. The ordering effect of gentamicin is also seen in the inner region of the

Fig. 4.4.1
Temperature dependence of the frequency of the symmetric C-H stretching vibration of the methylene groups of PI in aqueous dispersions, in the absence (triangles) and in the presence (circles) of gentamicin at a PI:gentamicin molar ratio of 2:1. The coordinates on the right side of the figure apply to circles. The frequencies were obtained from the spectra after Fourier derivation with a power of 3 and a breakpoint of 0.3.


WAVENUMBER (cm^{-1})
2854.0
2853.6
2853.2
2854.0
2853.6
2853.2
0
20
40
60
TEMPERATURE (°C)

bilayer. The frequency of the C-H stretching mode of alkenyl groups of PI liposomes increases gradually upon raising the temperature from 5 to 60 °C (Fig. 4.4.2., triangles). We also observed a decrease in membrane fluidity in the presence of gentamicin (Fig. 4.4.2., circles). This indicates that the interaction of gentamicin with the phospholipid head group affects the deeper regions of the bilayer.

The gel-to-fluid transition for bilayers of dimyristoylphosphatidylglycerol is shown in Fig. 4.4.3, (triangles). We observe a sudden increase in the frequency (1.5 cm^{-1}) of symmetric stretching vibration of methylene groups at the transition temperature. Increase in the CH_2 stretching frequency of this magnitude are characteristic of the increase in the conformational disorder which occurs upon melting of the polymethylene chains and have previously been observed at the gel-to-liquid crystalline phase transition of a number of different phospholipids. The addition of gentamicin does not produce a significant change in the transition temperature (Fig. 4.4.3, circles).

An increase in the ratio of bonded to non bonded carbonyl groups is observed above the transition temperature for both DMPG with and without gentamicin (data not shown). This is consistent with an increase in the penetration of water to the bilayer interface above the transition temperature.

The gel-to-fluid transition for bilayers of dimyristoylphosphatidylcholine is shown in Fig. 4.4.4 (triangles). The main transition occurs at 23.7 °C which involves the introduction of conformational disorder (that is gauche conformers) in

Fig. 4.4.2
Temperature dependence of the frequency of the symmetric C-H stretching vibration of the olefinic groups of PI in aqueous dispersions, in the absence (triangles) and in the presence (circles) of gentamicin at a PI:gentamicin molar ratio of 2:1. The coordinates on the right side of the figure apply to circles. The frequencies were obtained from the spectra after Fourier derivation with a power of 3 and a breakpoint of 0.15.

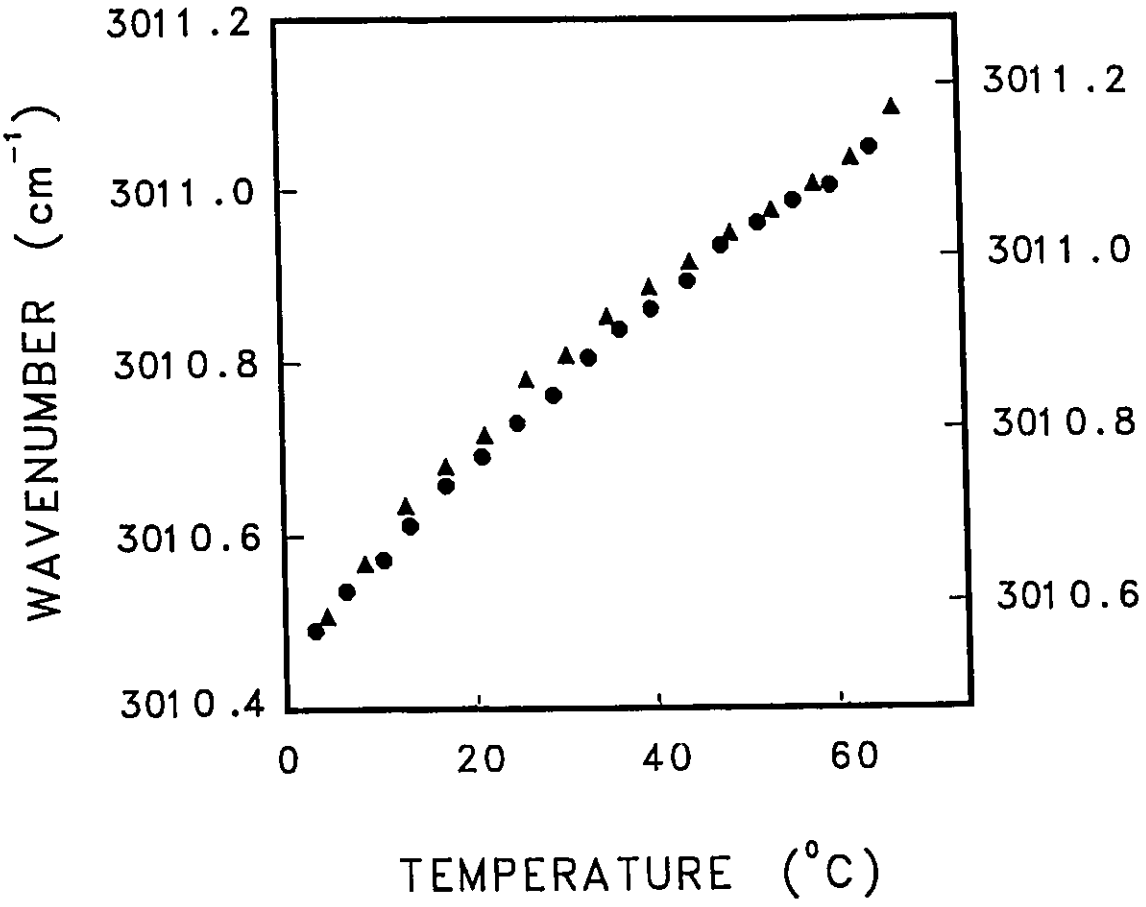

3011.2
3011.0
3010.8
3010.6
3010.4
WAVENUMBER (cm^{-1})
0
20
40
60
TEMPERATURE (°C)

Fig. 4.4.3
Temperature dependence of the frequency of the symmetric C-H stretching vibration of the methylene groups of DMPG in aqueous dispersions in the absence (triangles) and in the presence (circles) of gentamicin at a DMPG:gentamicin molar ratio of 2:1. The coordinates on the right side of the figure apply to circles. The frequencies were obtained from the spectra after Fourier derivation with a power of 3 and a breakpoint of 0.3.

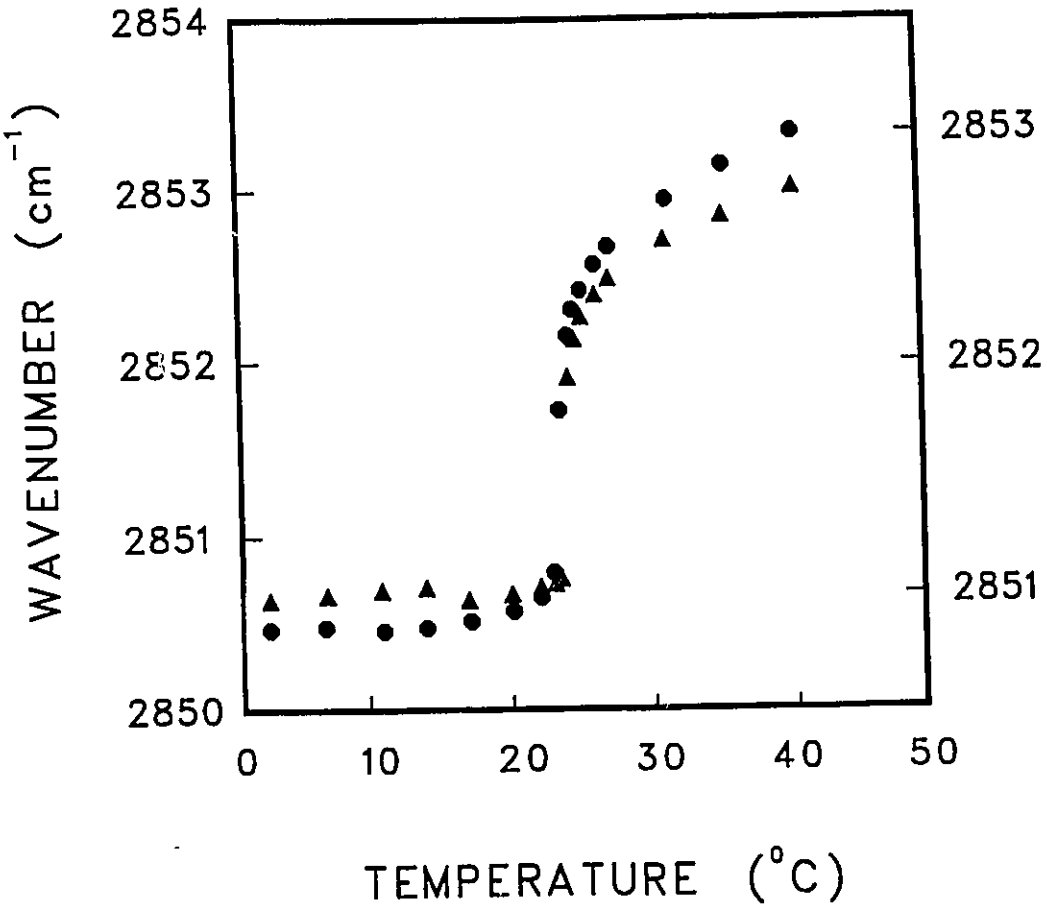
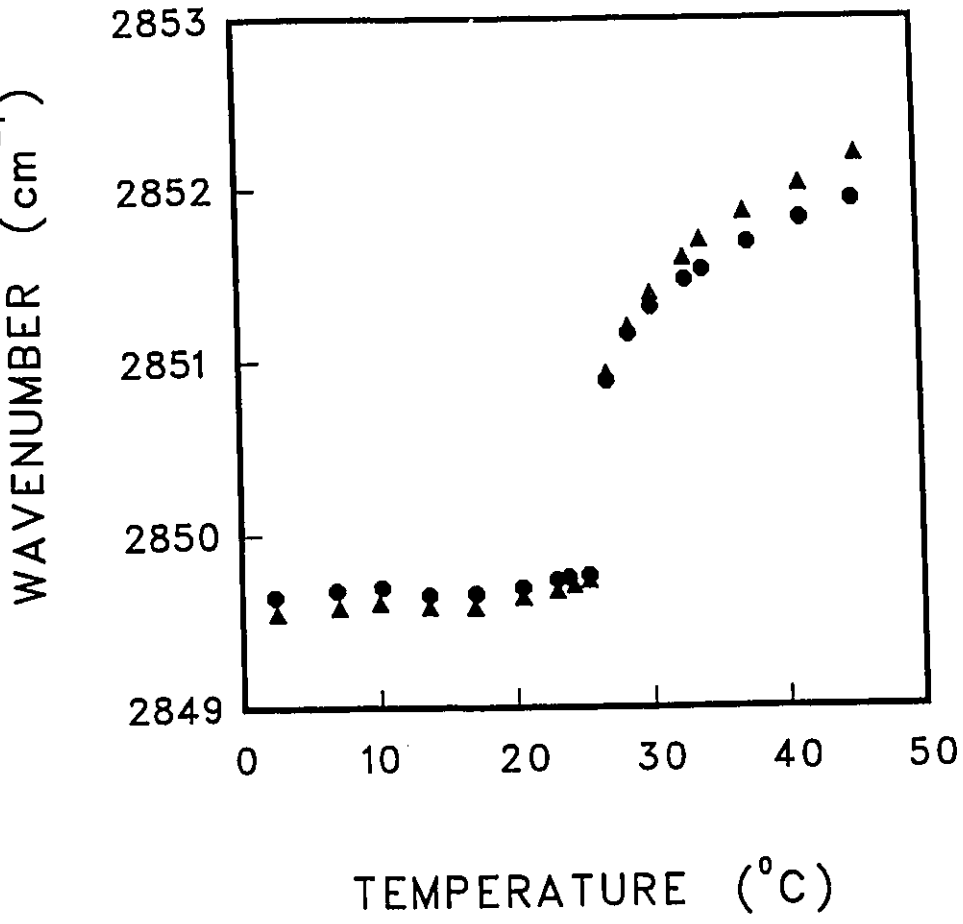

2854
2853
2852
2851
2850
WAVENUMBER (cm^{-1})
2853
2852
2851
0
10
20
30
40
50
TEMPERATURE (°C)

Fig. 4.4.4
Temperature dependence of the frequency of the symmetric C-H stretching vibration of the methylene groups of DMPC in aqueous dispersions, in the absence (triangles) and in the presence (circles) of gentamicin at a DMPC:gentamicin ratio of 2:1. The frequencies were obtained from the spectra after Fourier derivation with a power of 3 and a breakpoint of 0.3.


2853
2852
2851
2850
2849
WAVENUMBER (cm^{-1})
0
10
20
30
40
50
TEMPERATURE (°C)

acyl chains. This acyl chain melting phase transition involves large structural rearrangements which lead to considerable changes in the infrared spectrum. A sharp increase in the frequency of the symmetric stretching vibration of methylene groups occurs at the transition temperature. The addition of gentamicin does not produce an increase or decrease in the transition temperature although we do observe a decrease in the span of transition (Fig. 4.4.4, circles).

Similar to DMPG, we observed an increase in the ratio of bonded to non bonded carbonyl groups above the transition temperature, indicative of an increase in the penetration of water to the bilayer interface(data not shown).

4.5 Discussion

The kidney is the major site of toxicity for the aminoglycoside antibiotics. The initial site of drug interaction with the kidney occurs at the plasma membrane of renal proximal tubular cell. Interactions between aminoglycosides and negatively charged phospholipids occurring *in vivo* have been proposed to be the initial event leading to inhibition of activity of lysosomal phospholipases. This is considered to be an early and significant step in the development of aminoglycoside-induced nephrotoxicity (Brasseur *et al.*, 1984).

Our purpose was to study the interactions between aminoglycosides and different phospholipids to determine the molecular basis of the nephrotoxicity of these drugs. Most natural membranes contain an array of negatively charged phospholipids, and aminoglycosides have been demonstrated to bind to several

of them, in particular phosphatidylserine, phosphatidylglycerol, phosphatidic acid and phosphoinositides. However, the kidney is much more suceptible than most other organs to the toxic damage by aminoglycosides. This is because the content of acidic phospholipids (especially PI) is substantially higher in kidney than in other tissues (Schacht, J., 1976). In addition, the concentration of gentamicin exposed to proximal tubular cells is appreciably higher than the serum levels and exceeds the K_d of the membrane-antibiotic interaction (Sastrasinh *et al.*, 1982).

Phosphatidylinositol is the major binding site for gentamicin in the renal brush border membranes. Earlier studies have shown that the inhibitory potency of gentamicin on the activity of lysosomal phospholipases, measured towards phosphatidylcholine included in negatively charged liposomes, is markedly influenced by the nature of the acidic phospholipid used [PI, PS or PA], (Mingeot-Leclercq, M.P., Piret, J., Brasseur, R. and Tulkens, P.M., 1990a). It was also demonstrated that the energy of interaction between gentamicin and PI was the highest compared to energies of interaction with other acidic lipids (Mingeot-Leclercq, M.P., Piret, J., Tulkens, P.M. and Brasseur, R., 1990b). Our findings indicate that the neutralization of the negative charge on the phosphate groups of PI by the cationic amino groups of gentamicin leads to a tightening of the lipidic network which is manifested by a decrease in the penetration of the solvent to the bilayer interface region. The strengthening of the phosphate hydrogen bonds is also consistent with a decrease of PI intermolecular distance consecutive to charge neutralization by gentamicin. A tightening of the lipidic network would alter the

thermotropic behaviour of the membrane lipids and we did observe a decrease in the fluidity of the membrane in the presence of the aminoglycoside.

The narrowing of the antisymmetric phosphate stretching band of DMPC in the presence of the polycationic drug gentamicin could be attributed to a change in the phosphocholine head group conformation which is known to be sensitive to electric charges on the membrane surface. The P-N dipole in phosphatidylcholine is approximately parallel to the membrane surface in pure phospholipid membrane. Molecules with a positive charge will position their charge in the vicinity of the negative end of the $^{-}$P-N^{+} dipole. This will cause the positive end of the phosphocholine dipole to be pushed out into the aqueous medium (Scherer, P.G. and Seelig, J., 1989). Negative charges at the membrane surface have an opposite effect, forcing the N^{+} end towards the membrane interior.

The phosphoglycerol head group of DMPG is less sensitive to membrane surface charge than the phosphocholine head group. However, it does undergo a unique conformational change under the influence of modifications of surface charge which are probably due to head group dipoles (that is, OH or CO) seeking to align themselves with the direction of the electrical field at the surface (Marassi, P.M. and Macdonald, P.M., 1991). The response of other phospholipid polar groups (including phosphatidylinositol) to surface charge remains unknown. We are not aware of the extent to which these effects would contribute to the changes seen in the antisymmetric phosphate band of PI in the presence of gentamicin. However, our interpretation of the strengthening of phosphate hydrogen bonds is

consistent with changes seen in other regions of the spectra consecutive to charge neutralization by the aminoglycoside. The changes seen in the infrared spectra of DMPG in the presence of gentamicin have been attributed to a slight and insignificant interaction. The slight change seen in the carbonyl stretching band of DMPG in the presence of gentamicin is considered insignificant since this variation is within the changes observed between different DMPG spectra at the same pH. A running integral of the band in the presence and absence of gentamicin revealed that the ratio of intensity acquired for bonded to non bonded carbonyl groups is essentially the same. A similar explanation is valid for the 0.4 °C difference in the transition temperature of DMPG in the presence of gentamicin. The effect is termed "insignificant" because this variation was within the change observed between the transition temperature of different DMPG samples at the same pH. For the zwitterionic phospholipid DMPC, we observed no change in the interfacial region or on the fluidity and conformation of acyl chains indicative of an interaction with the aminoglycoside. A running integral of the band in the presence and absence of gentamicin revealed that the ratio of intensity acquired for bonded to non bonded carbonyl groups is essentially the same. Interpretation of changes observed in the infrared spectra of PI in the presence of gentamicin were made simple due to the high reproducibility of different PI spectra at either pH studied.

Although equilibrium dialysis experiments have failed to demonstrate significant differences in the binding parameters of the drug towards different negatively charged phospholipids (Mingeot-Leclercq *et al.*, 1990a), we have shown

that at the molecular level there are significant differences in the drug-phospholipid interaction and the changes observed at the level of carbonyl groups, phosphate groups and acyl chain packing are unique to the interaction with phosphatidyl-inositol.

Chapter 5

EFFECT OF DAPTOMYCIN ON PHOSPHATIDYLINOSITOL BILAYERS

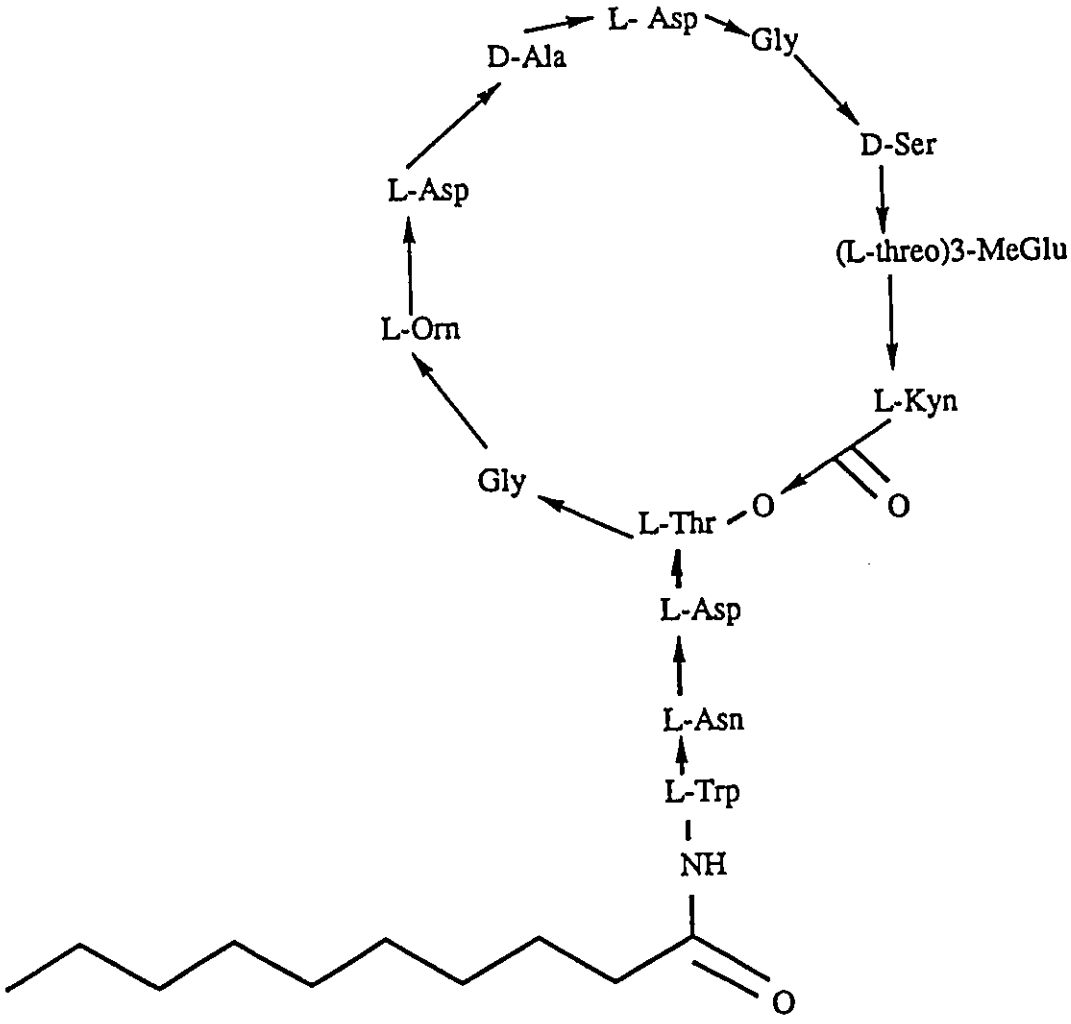
5.1 Introduction

Daptomycin is a lipopeptidie antibiotic produced by chemical modification of a compound isolated from *Streptomyces roseosporus* (Smith, K., Cobbs, G., Dill, R., Lyon, D., Graves, A. and Avent, K., 1990). Its antibacterial activity against gram-positive organisms is due to its inhibition of the synthesis of lipoteichoic acid, an early step in cell wall biosynthesis (Allen, N.E., Hobbs, J.N., Jr. and Alborn, W.E., 1987). Daptomycin has been shown to protect kidney cells from gentamicin induced nephrotoxicity (Beauchamp *et al.*, 1990b). Several possibilities can be considered in order to explain the protective effect of daptomycin on aminoglycoside-induced nephrotoxicity. An interaction between daptomycin and PI could lead to an impairment of the binding of aminoglycosides to brush border membranes. The effect of daptomycin on phosphatidylinositol bilayers was investigated using FTIR spectroscopy.

5.2 Effect of Daptomycin on the Fluidity of Phospholipid Bilayers

The 3-methyl glutamate and three aspartate residues found in this cyclic lipopeptide give it a net negative charge in the pH range studied (Fig. 5.2.1). Although one would expect electrostatic repulsion between the peptidic head group of daptomycin and PI bilayers, the decanoyl chain of the lipopeptide will also

Fig. 5.2.1
Structure of the lipopeptide antibiotic LY146032 (daptomycin).
Kyn represents kynurenine, Orn, ornithine and MeGlu, methyl glutamate


L- Asp
Gly
D-Ala
D-Ser
L-Asp
(L-threo)3-MeGlu
L-Orn
L-Kyn
Gly
O
O
L-Thr
L-Asp
L-Asn
L-Trp
NH
O

give rise to an entropy driven effect. This would mean that the lipidic character of daptomycin could dominate the thermodynamic balance of forces and lead to the insertion of the fatty acyl moiety into the membrane.

Several drugs have been shown to interact with membranes and to affect their thermotropic properties. The effect of daptomycin on the fluidity of phospholipid membranes was first investigated. As discussed in the previous chapter, the frequency of the C-H stretching vibration of acyl chains in lipids can be used to probe membrane fluidity (Casal and Mantsch, 1984). Soya bean PI contains a mixture of various acyl chains, the most common being palmitate [16:0] and linoleate [18:2] (Justin, A.M., Demandre, C. and Mazliak, P., 1989). This heterogeneity abolishes cooperativity in the gel-to-fluid transition. It can be seen in Fig. 5.2.2 (triangles) that the frequency of the symmetric stretching vibration of the methylene groups of PI liposomes increases gradually upon raising the temperature from 5 to 60 °C. The frequency of that mode changes dramatically at the gel-to-fluid transition for bilayers of dimyristoylphosphatidylglycerol (DMPG) (Fig. 5.2.3, triangles) and dipalmitoylphosphatidylcholine (DPPC) (Fig. 5.2.4, triangles).

If a hydrophobic effect is the driving force of the interaction of daptomycin with PI, then the lipopeptide is likely to undergo the same type of interaction with any type of phospholipidic bilayer. We therefore examined the effect of daptomycin on the thermotropic properties of DMPG and DPPC. Indeed, daptomycin induces a 5 °C decrease in the transition temperature of DMPG

Fig. 5.2.2
Temperature dependence of the frequency of the symmetric C-H stretching vibration of the methylene groups of PI in aqueous dispersions, in the absence (triangles) and in the presence (circles) of daptomycin at a PI:daptomycin ratio of 2:1. The coordinates on the right side of the figure apply to circles. The frequencies were obtained from the spectra after Fourier derivation with a power of 3 and a breakpoint of 0.15.

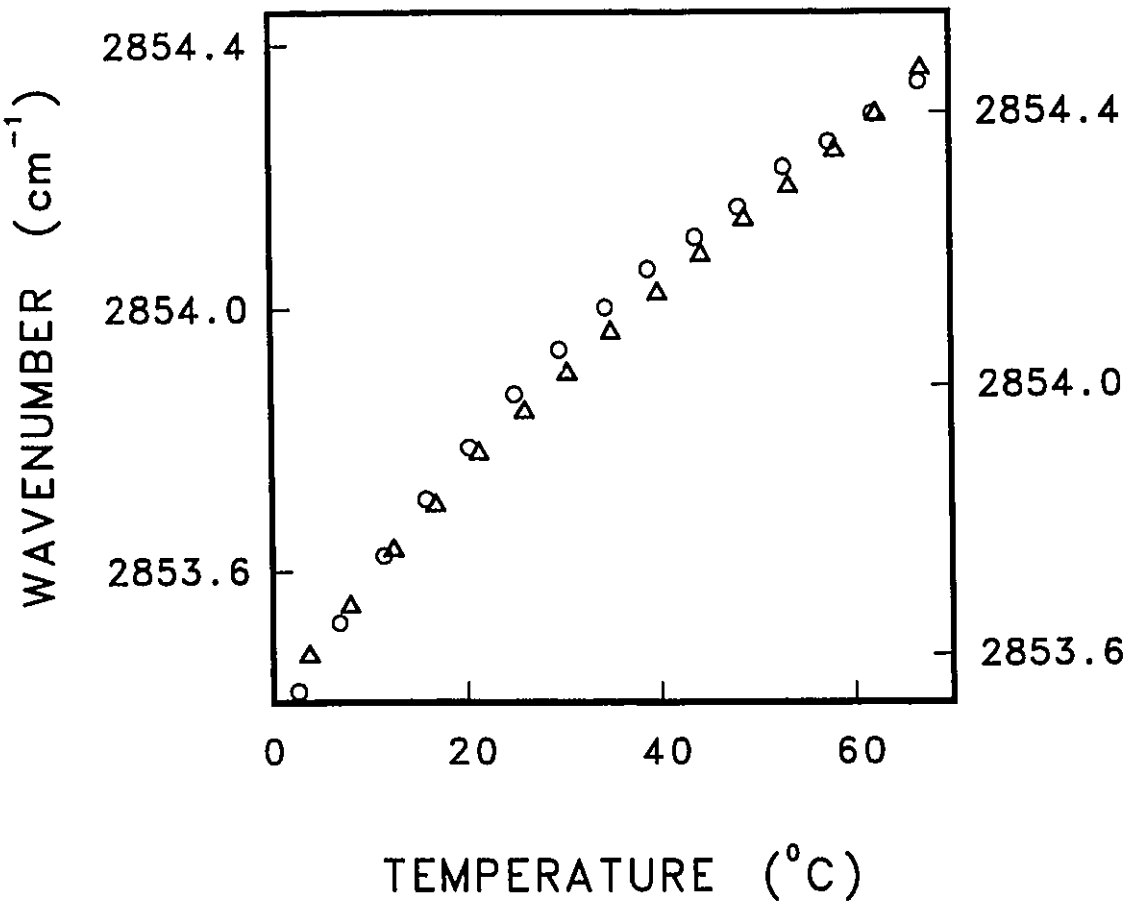

2854.4
2854.0
2853.6
WAVENUMBER (cm^{-1})
2854.4
2854.0
2853.6
0
20
40
60
TEMPERATURE (°C)

Fig. 5.2.3
Temperature dependence of the frequency of the symmetric C-H stretching vibration of the methylene groups of DMPG in aqueous dispersions, in the absence (triangles) and in the presence (circles) of daptomycin at a DMPG:daptomycin ratio of 2:1. The frequencies were obtained from the spectra after Fourier derivation with a power of 3 and a breakpoint of 0.3.

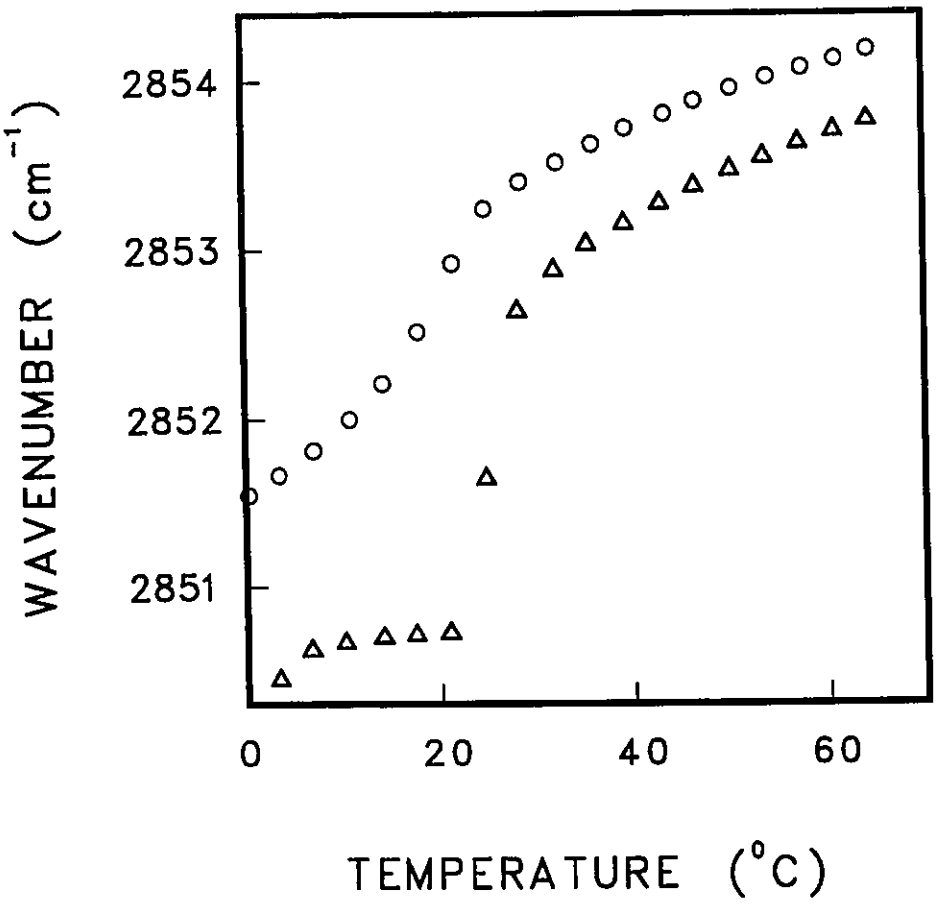
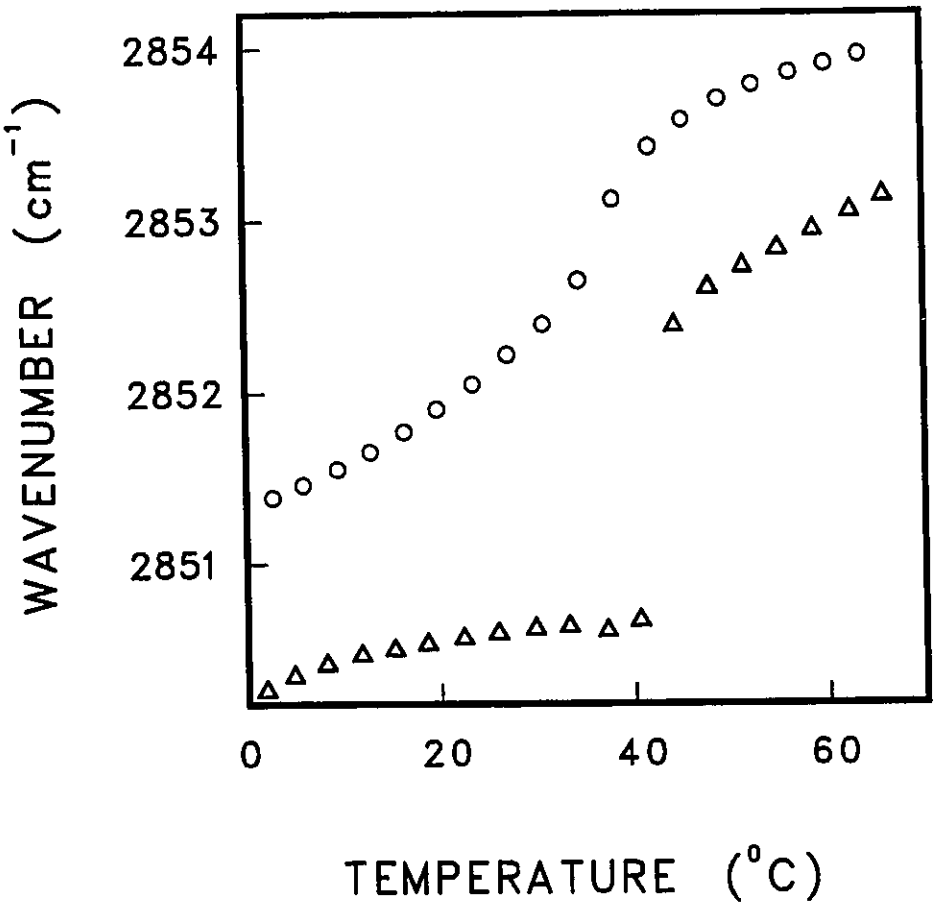
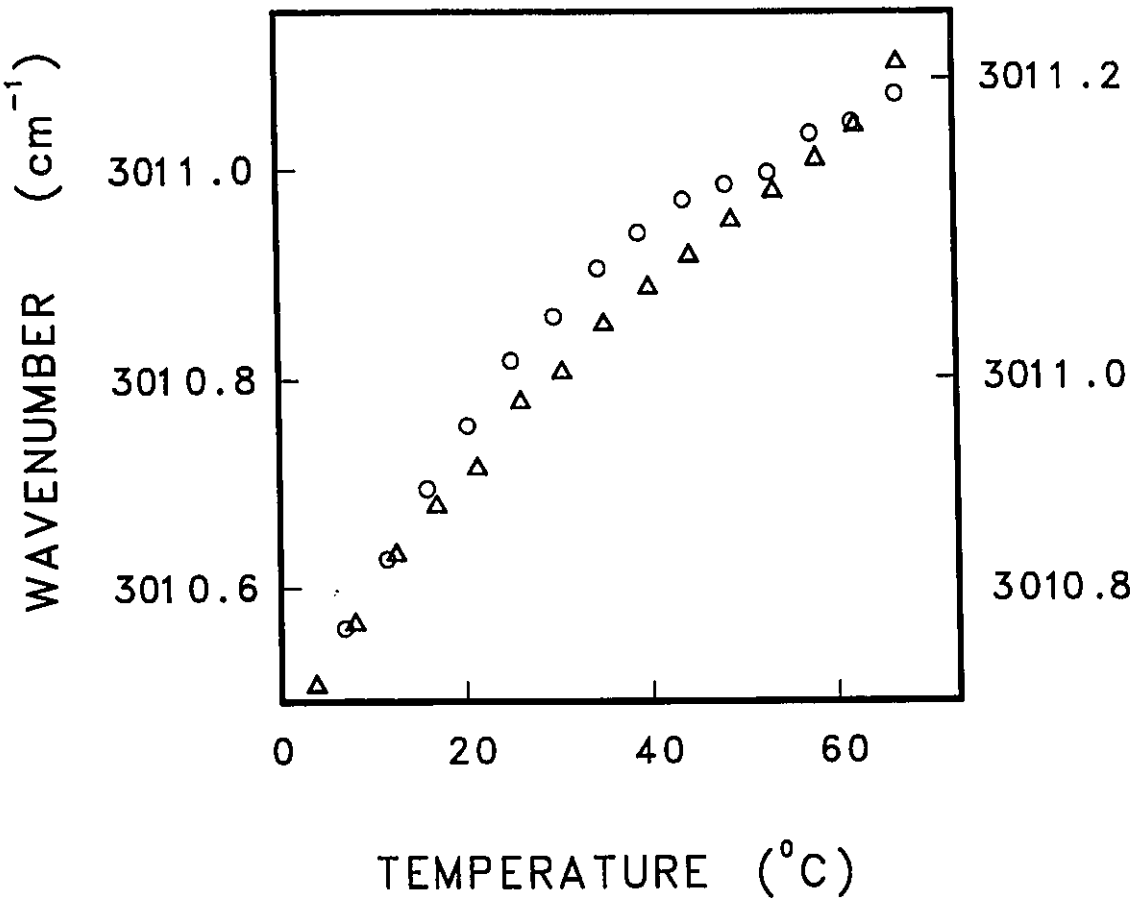

2854
2853
2852
2851
WAVENUMBER (cm^{-1})
0
20
40
60
TEMPERATURE (°C)

Fig. 5.2.4
Temperature dependence of the frequency of the symmetric C-H stretching vibration of the methylene groups of DPPC in aqueous dispersions, in the absence (triangles) and in the presence (circles) of daptomycin at a DPPC:daptomycin ratio of 2:1. The frequencies were obtained from the spectra after Fourier derivation with a power of 3 and breakpoint of 0.3.


2854
2853
2852
2851
WAVENUMBER (cm^{-1})
0
20
40
60
TEMPERATURE (°C)

bilayers (Fig. 5.2.3 circles) and a 7 °C decrease of that of DPPC bilayers (Fig. 5.2.4, circles), as well as a decrease of the cooperativity and amplitude of that transition. The decanoyl chain of daptomycin is shorter than myristoyl and palmitoyl chains, so its insertion into the bilayer hydrophobic core impedes a perfect packing of the acyl chains on the entire length and consequently destabilizes the gel phase. This results in an increase in the fluidity of the membrane as manifested by a decrease in the transition temperature. The disordering effect of daptomycin insertion is more subtle and difficult to detect in the temperature profile of the methylene symmetric stretching vibration (Fig. 5.2.2). Interestingly, the effect of daptomycin is more pronounced when monitored using the frequency of the C-H stretching mode arising from the vibrations of alkenyl groups of linoleyl acyl chains in PI (Fig. 5.2.5, circles). The double bonds of linoleyl chains in PI are found at positions 9 and 12, which are most likely located below the terminal methyl group of daptomycin. The inner region of the bilayer core experiences the greatest disordering effect because the space between PI acyl chains is larger at that level when daptomycin is present in the upper part. Moreover, the acyl chain portion adjacent to the decanoyl chain of daptomycin is being squeezed and their order is actually increased. The methylene stretching frequency reflects an average of the properties of upper and deeper regions whereas the C-H stretching frequency of alkenyl groups probes only the inner region, where the disturbing influence of daptomycin is maximal.

Fig. 5.2.5
Temperature dependence of the frequency of the symmetric C-H stretching vibration of the olefinic groups of PI in the absence (triangles) and in the presence (circles) of daptomycin at a PI:daptomycin ratio of 2:1. The coordinates on the right side of the figure apply to circles. The frequencies were obtained from the spectra after Fourier derivation with a power of 3 and breakpoint of 0.15.


WAVENUMBER (cm^{-1})
3011.0
3010.8
3010.6
3011.2
3011.0
3010.8
0
20
40
60
TEMPERATURE (°C)

5.3 *Effect of Daptomycin on the Head Group and Interfacial Region of PI Bilayers*

The insertion of daptomycin into PI bilayers may also affect the lipid head group and interface. The ester carbonyl stretching vibration of phosphatidylinositol in aqueous suspension is a broad asymmetric band. Resolution enhancement using Fourier self-deconvolution (Fig. 5.3.1, solid line) reveals two components at 1745 cm^{-1} and 1725 cm^{-1}, attributed to carbonyl groups that are "free" and hydrogen bonded respectively. The frequency of the two components of PI carbonyl stretching band, obtained after derivatization of the original spectra, are not affected by daptomycin. A small increase of the proportion of non hydrogen bonded carbonyl groups is observed at both pH 5.4 and 7.5 (Fig. 5.3.1, dashed line), suggesting that the interaction of daptomycin results in a higher cohesion of the bilayer at the interface level.

The phosphate antisymmetric stretching band of phosphatidylinositol in aqueous dispersion is presented in Fig. 5.3.2, solid line. The frequency of this mode is 1216 cm^{-1}, indicating that the hydrogen bonds undergone by hydrated PI are exceptionally strong. The phosphate antisymmetric stretching band is slightly shifted to higher frequencies (Fig. 5.3.2, dashed line), by approx. 1 cm^{-1}. This could be due to steric constraints put on the head group by the bulky peptidic moiety of daptomycin.

Fig. 5.3.1
Carbonyl stretching region of the infrared spectra of dispersions of phosphatidylinositol alone in ^2H_2O (solid line) or with daptomycin (small dashes) at PI:daptomycin molar ratio of 4:1. The spectra were obtained after band narrowing by Fourier self-deconvolution using a bandwidth of 13 and resolution enhancement factor of 1.5.

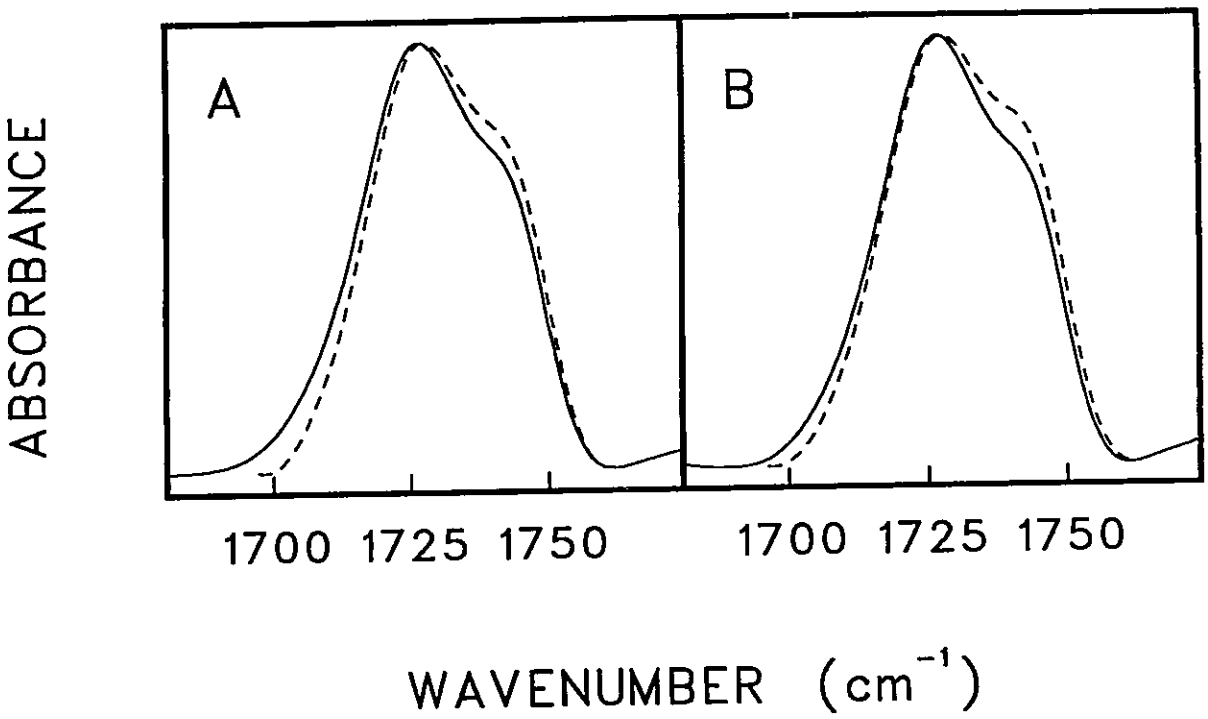
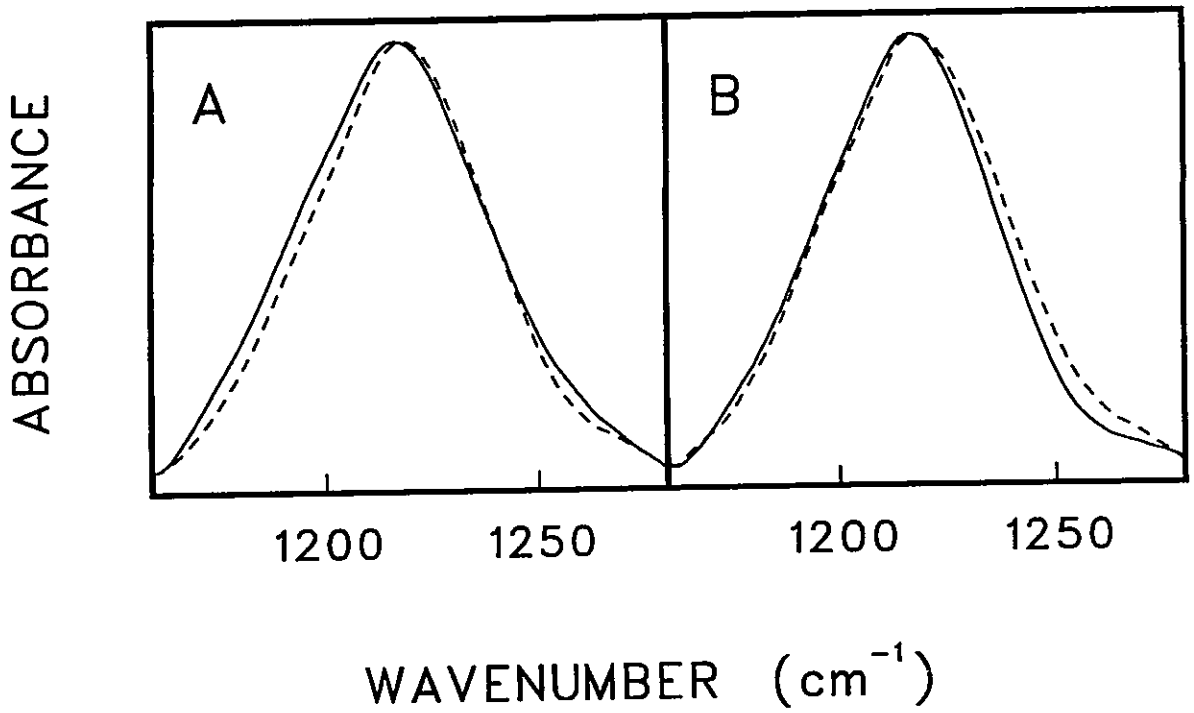

A
B
ABSORBANCE
1700 1725 1750
1700 1725 1750
WAVENUMBER (cm^{-1})

Fig. 5.3.2
Phosphate antisymmetric stretching region of the infrared spectra of aqueous dispersions of phosphatidylinositol alone (solid line) or with daptomycin (small dashes) at a PI:daptomycin molar ratio of 4:1, at pH 5.4 (A) and 7.5 (B)


ABSORBANCE
A
B
1200
1250
1200
1250
WAVENUMBER (cm^{-1})

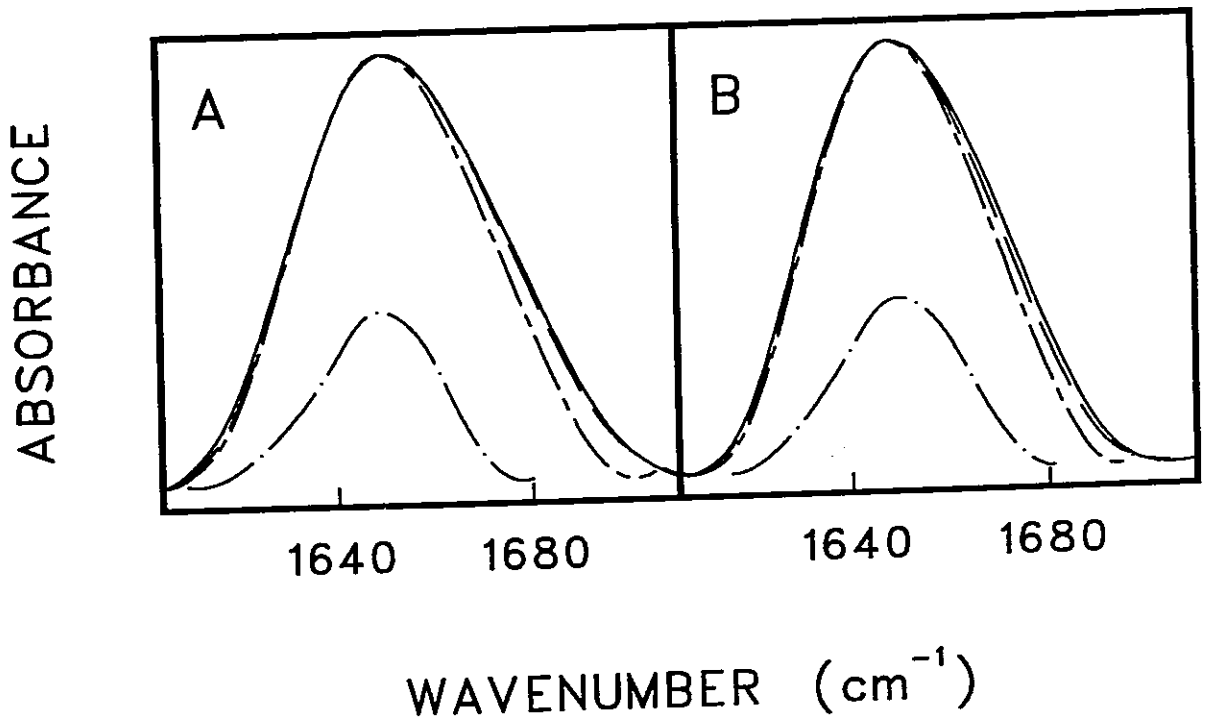
5.4 The Amide I Region

The insertion of the fatty acyl moiety of daptomycin into the hydrophobic core of lipid bilayers could affect the properties of this lipopeptide antibiotic. We examined the amide I band of daptomycin in the presence of PI to check for any conformational constraints in the peptidic head group, once it has bound to the membrane. The width of the band found in the amide I region decreases in the presence of PI (Fig. 5.4.1, long and short dashes). However, the spectral contribution of C=C stretching mode of the linoleyl chains of phosphatidylinositol should be considered (Fig. 5.4.1, dot-long dash). After the contributions of the lipid have been subtracted from the spectrum of PI/daptomycin, the corrected amide I band reveals no significant change in the overall shape and frequency due to PI (Fig. 5.4.1, long dashes).

5.5 Discussion

The spectroscopic data presented here revealed some very interesting features about the interactions of daptomycin with phosphatidylinositol. Daptomycin incorporates into lipidic membranes, owing to the hydrophobic character conferred by its decanoyl chain, notwithstanding the repulsion between its peptidic moiety and the negatively charged head groups of DMPG, DPPC or PI. The insertion of the short decanoyl chain of daptomycin molecules in the hydrophobic core of the bilayer results in an increased order of the portion of the acyl chains that is adjacent to the decanoyl chains, whereas the terminal portion

Fig. 5.4.1
Amide I region of the infrared spectra of daptomycin alone (solid line) or with PI (short-long dashes) in ^2H_2O at p^{2}H 5.4 (A) or 7.5 (B), at a PI:daptomycin molar ratio 4:1. The spectrum of a dispersion of PI is also presented (dot-long dash) to show the contribution of the C=C stretching of its acyl chains. This spectral contribution can be subtracted from the spectrum of PI/daptomycin to obtain the corrected amide I band of daptomycin (long dashes).


A
B
ABSORBANCE
1640
1680
1640
1680
WAVENUMBER (cm^{-1})

of the long lipidic acyl chains (mostly palmitate and linoleate) becomes more fluid. On the other hand, the conformation of the peptidic moiety of daptomycin experiences no significant change, as seen from the amide I band (Fig. 5.4.1., long dashes). The steric constraints put on the PI head group by the bulky peptidic moiety of daptomycin cause the antisymmetric phosphate stretching band to be shifted to higher frequencies. It remains to be seen whether the insertion of daptomycin into the hydrophobic core of membranes impedes the binding of aminoglycosides to negatively charged PI bilayers.

Chapter 6

DIRECT INTERACTION OF DAPTOMYCIN AND GENTAMICIN

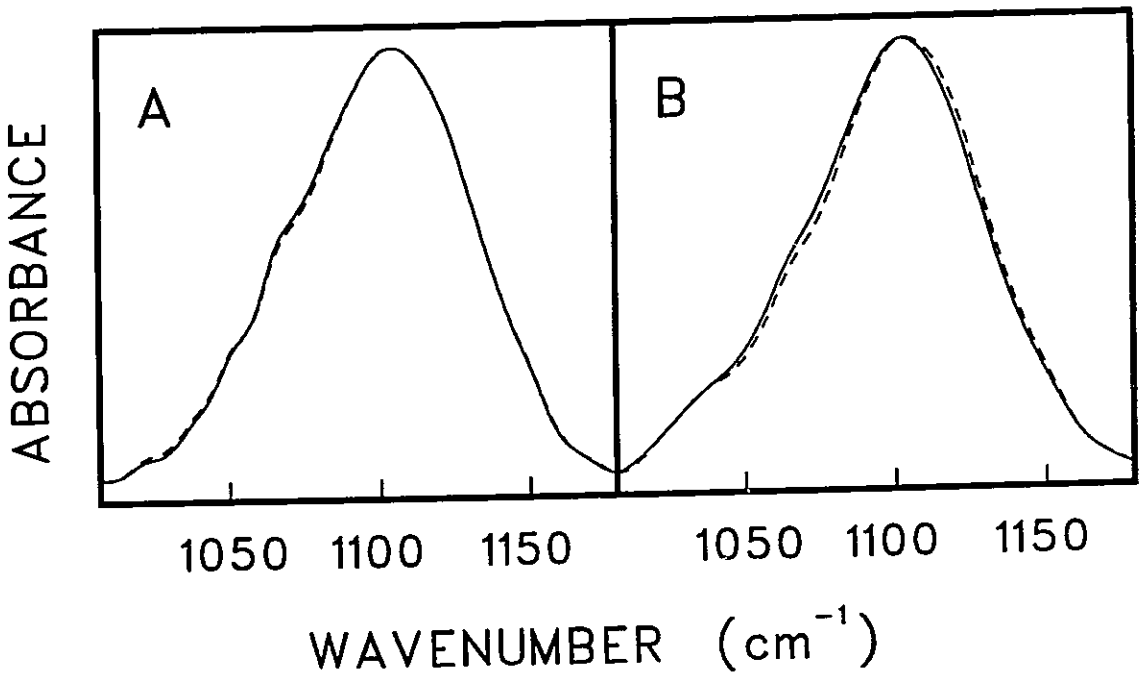
6.1 *Introduction*

Daptomycin has been found to be a potentially useful alternative to vancomycin, a glycopeptidic antibiotic which was combined with aminoglycosides to enhance bactericidal activity (Eliopoulos *et al.*, 1986; Benson *et al*, 1987). Vancomycin amplified the risk of aminoglycoside nephrotoxicity in animal studies and clinical trials (Wood *et al.*, 1986). The bactericidal activity of daptomycin and vancomycin are similar, in addition daptomycin has been shown to protect kidney cells against aminoglycoside-induced nephrotoxicity (Beauchamp *et al.*, 1990b). As mentioned previously, several possibilities could be considered to explain the protective effect of this lipopeptide drug. The aim of this chapter is to examine the possibility of a direct interaction between daptomycin and gentamicin in solution, that could lead to an impairment of the aminoglycoside binding to brush border membranes.

6.2 *The 1105 cm^{-1} Band of Gentamicin*

The infrared spectrum of gentamicin below 2000 cm^{-1} comprises a single well defined band, at 1105 cm^{-1} approx. (Fig. 6.2.1, solid line). The C-N stretching from the primary and secondary amine groups, C-O stretching and C-C stretching vibrations contribute to the intensity of the 1105 cm^{-1} band. This composite band

Fig. 6.2.1
Infrared spectrum of gentamicin in the C-O, C-C and C-N stretching region at pH 5.4(A) and 7.5(B). Solid lines represent aqueous solutions of gentamicin and dashed lines, aqueous solutions of gentamicin in the presence of daptomycin at a gentamicin:daptomycin molar ratio of 2:1. Spectral contributions of daptomycin have been subtracted.

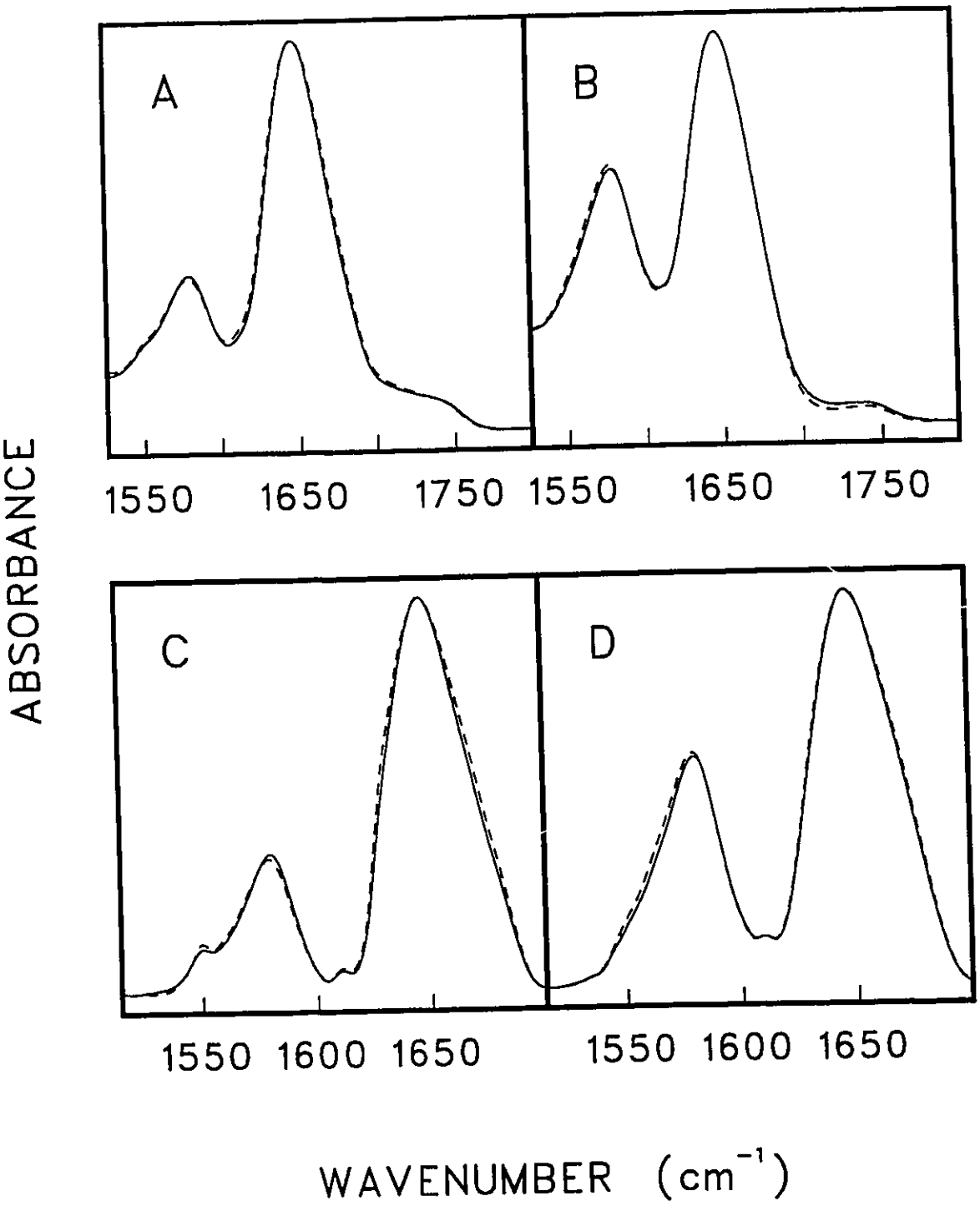

A
B
ABSORBANCE
1050
1100
1150
1050
1100
1150
WAVENUMBER (cm^{-1})

remains the same at pH 7.5 and 5.4. The contributions of C-N, C-O and C-C stretching vibrations of daptomycin in that region do not give rise to a well defined band. The addition of daptomycin to gentamicin did not result in any significant alteration of the frequency or overall shape of gentamicin 1105 cm^{-1} band at either pH (Fig. 6.2.1, dashed line).

6.3 Carbonyl Stretching of Daptomycin

The infrared spectrum of the lipopeptide antibiotic daptomycin contains many features that are sensitive to changes in conformation or ionization of the molecules. The 3-methyl glutamate and the three aspartate side chains confer a net negative charge on the molecule at neutral pH. The carbonyl stretching vibrations of these groups give a band of moderate intensity at 1580 cm^{-1} at both pH 5.4 and 7.5, consistent with the prevalence of the ionized state in that pH interval (Fig. 6.3.1A and B, solid line). At lower pH, neutralization of carboxylates causes the carboxylate band to lose intensity whereas the protonated acid band grows up at 1710 cm^{-1}. Another carbonyl stretching band is also found at approx. 1744 cm^{-1} at all pHs. This peak is due to the C=O stretching vibration of the ester function of the peptidic ring and of the aromatic ketone group of the L-kynurenine residue. This feature is of little diagnostic value. On the other hand, the amide I band seen at 1649 cm^{-1} is known to be sensitive to peptide backbone conformation. In proteins and polypeptides, its shape and frequency are used to characterize secondary structure. The cyclic structure of daptomycin peptidic

Fig. 6.3.1
Carbonyl stretching region of the infrared spectra of daptomycin alone (solid line) or with gentamicin (dashed line) in solution, at a gentamicin:daptomycin molar ratio of 2:1 in aqueous solutions, at pH 5.4 (left panels) and 7.5 (right panels). The original spectra (A and B) were deconvolved using a bandwidth of 13 and a resolution enhancement factor of 1.75 (C and D).


A
B
C
D
1550
1650
1750
1550
1650
1750
1550
1600
1650
1550
1600
1650
ABSORBANCE
WAVENUMBER (cm^{-1})

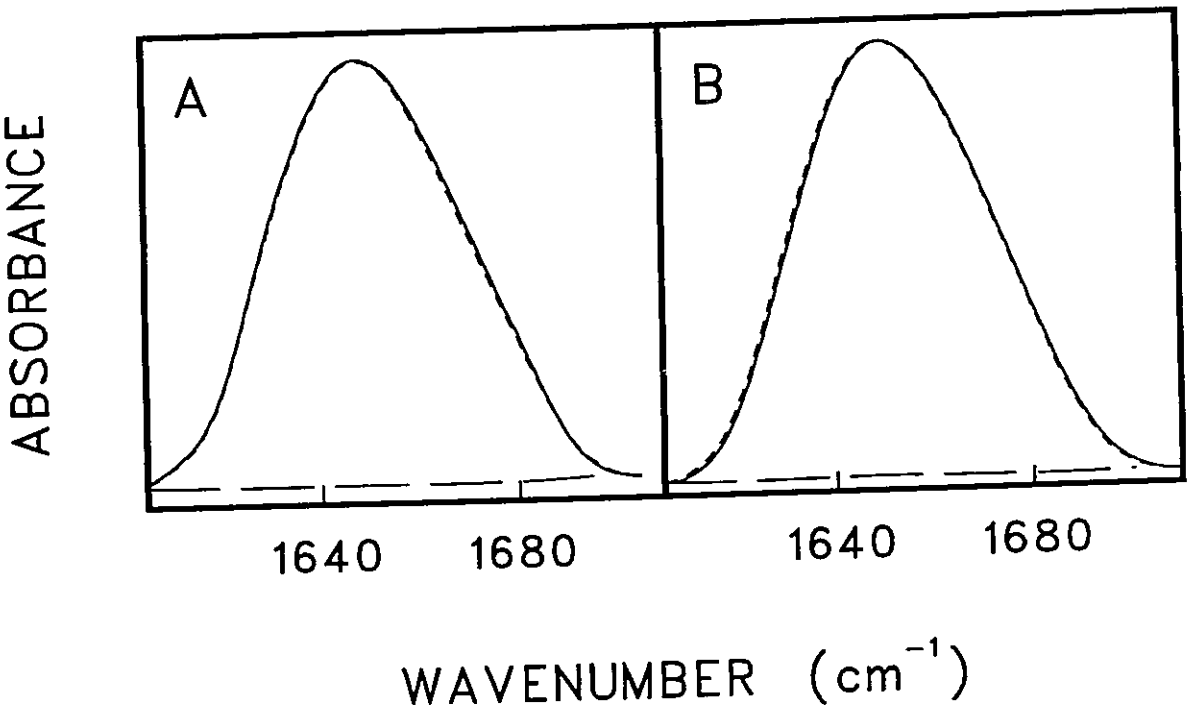
portion severely restrains the backbone flexibility and impedes the formation of sufficiently long α-helices or β-sheets for example. Nevertheless, interactions at the side chains and changes in environment can still be reflected in spectral variations of the amide I band because they can affect the backbone geometry. The shape and frequency of the amide I band are not affected upon going from pH 5.4 to 7.5 and addition of gentamicin also fails to induce any significant changes (Fig. 6.3.2, dashed line).

Although the neutralization of a fraction of the carboxylates produced upon lowering the pH from 7.5 to 5.4 was reflected in a marked decrease of the intensity of the band at 1579 cm^{-1}, no consistent change of that band was noted when gentamicin was added (Fig. 6.3.1, dashed line). If there is an electrostatic interaction between daptomycin and gentamicin, it can only be a loose one, extending through the hydration spheres of these molecules and causing no constraints on their conformation.

6.4 Discussion

A recent dialysis study indicated a direct interaction of daptomycin with gentamicin (Couture *et al.*, 1994). The dependence on ionic strength and pH confirmed the electrostatic character of this interaction. Interestingly, the results showed an increased binding of gentamicin and tobramycin after incorporation of daptomycin into liposomes containing phosphatidylinositol. In the present study we found no spectral evidence of a direct interaction of daptomycin with gentamicin

Fig. 6.3.2
Amide I region of the infrared spectra of daptomycin alone (solid line) or with gentamicin (short dashes) in ^2H_2O at p^{2}H 5.4(A) or 7.5(B). Long dashes represent the spectral contributions of gentamicin.


A
B
ABSORBANCE
1640
1680
1640
1680
WAVENUMBER (cm^{-1})

in solution. The absence of significant spectral changes and the fact that both compounds remain in solution after mixing preclude tight interactions and direct neutralization of daptomycin's carboxylates. Nevertheless, the formation of a loose complex cannot be excluded, it would justify the dialysis results and still be compatible with our study.

Chapter 7

EFFECT OF DAPTOMYCIN ON GENTAMICIN-PHOSPHATIDYLINOSITOL INTERACTION

7.1 Introduction

As seen in the previous chapters, the addition of the aminoglycoside gentamicin to phosphatidylinositol bilayers results in a tightening of the lipidic network following neutralization of PI molecules by the drug. This is manifested by a decrease in the population of hydrogen bonded carbonyl groups which indicates that gentamicin reduces the penetration of water to the bilayer interface. The lipopeptide drug, daptomycin, incorporates into lipidic membranes owing to the hydrophobic character conferred by its decanoyl chain. The next logical step was to examine the effect of daptomycin on gentamicin-phosphatidylinositol interaction in order to propose a mechanism for the nephroprotective action of the lipopeptide antibiotic.

7.2 Study of Supernatants

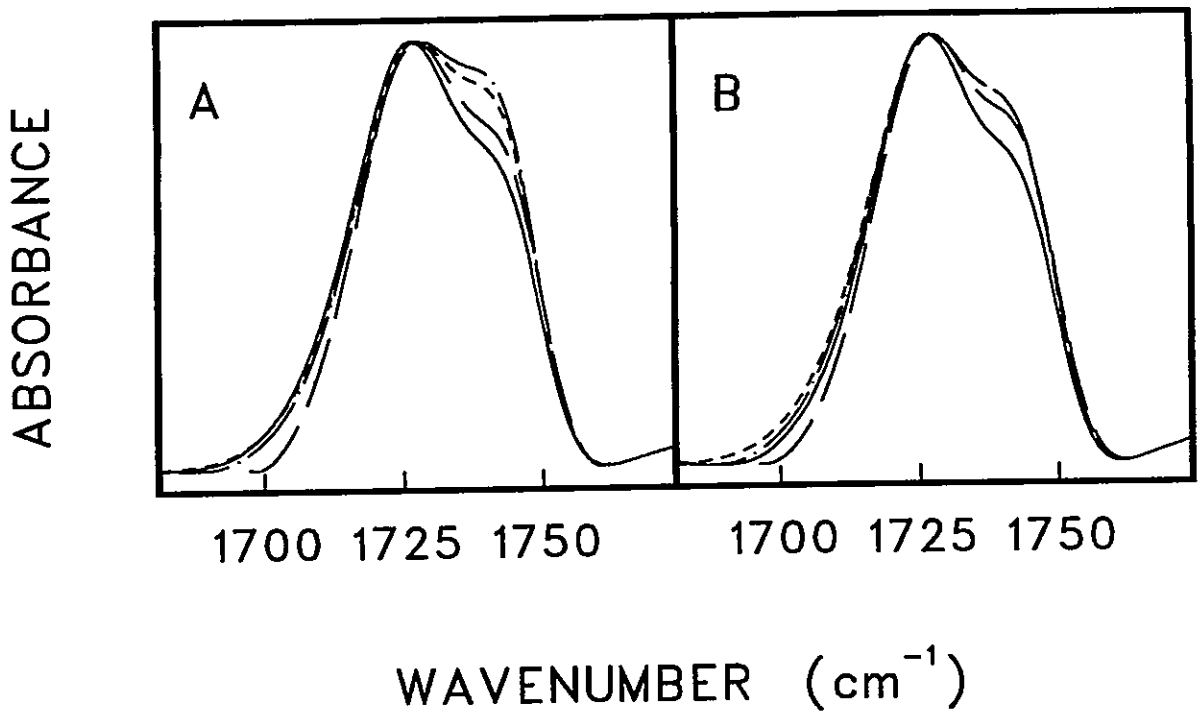
Gentamicin acts as a polycation with PI bilayers. The addition of the aminoglycoside to that negatively charged lipid results in a fast and massive aggregation. The formation of these big, flaky aggregates is due to the binding of gentamicin to bilayers from different liposomes. We also observe precipitation when daptomycin is added to PI liposomes, but the aggregates are not bulky. A

clear supernatant, free of PI, is obtained. A simple test to check if the presence of daptomycin within PI bilayers would impede or affect in any way gentamicin binding or if premixing of daptomycin and gentamicin would prevent the fixation of the aminoglycoside on the lipidic surface was to check for the presence of antibiotics in the supernatant. The C-O and C-N stretching band (1105 cm^{-1}) was used as a rough estimate of gentamicin concentration and the amide I band for daptomycin monitoring. Surprisingly, we consistently found less gentamicin in the supernatant in samples containing daptomycin (not shown). All mixtures presented spectral evidence of large amounts of both antibiotics in the precipitate. Therefore, daptomycin does not impede the binding of gentamicin to phosphatidylinositol.

7.3 The Lipid C=O Stretching Region

The deconvolved carbonyl stretching band of the ester functions of phosphatidylinositol in aqueous suspension is presented in Fig. 7.3.1A and B (solid line). Gentamicin induces an increase in the proportion of carbonyl groups that are not hydrogen bonded (Fig. 7.3.1A and B, dashed line). The insertion of daptomycin into PI bilayers results in a small increase of the proportion of non hydrogen bonded carbonyl groups at pH 5.4 and 7.5 (Fig. 7.3.1A and B, long dashes). The lipid carbonyl stretching region of the infrared spectrum of PI-gentamicin-daptomycin at a molar ratio of 4:2:1 is presented in Fig. 7.3.1 (dot-long dash). We observed that at pH 5.4 there is a more pronounced decrease of hydrogen bonded PI carbonyl groups than in the presence of gentamicin only. The

Fig. 7.3.1
Carbonyl stretching region of the infrared spectra of dispersions of phosphatidylinositol alone in ^2H_2O (solid line), or with gentamicin (small dashes) at a PI:gentamicin molar ratio of 2:1, daptomycin (long dashes) at a PI:daptomycin molar ratio 4:1, or both gentamicin and daptomycin (dot-long dash) at a PI:gentamicin:daptomycin molar ratio 4:2:1, at p^{2}H 5.4(A) and 7.5(B). All spectra have been obtained after a resolution enhancement using Fourier self-deconvolution with a bandwidth of 13 and a resolution enhancement factor of 1.5.


ABSORBANCE
A
B
1700 1725 1750
1700 1725 1750
WAVENUMBER (cm^{-1})

result is essentially the same if daptomycin is added after or before gentamicin, or if they are mixed prior to addition to PI dispersion. As for PI-daptomycin and PI-gentamicin mixtures, there is no significant shift of the frequency of the two carbonyl stretching components. It thus appears that hydrogen bonding of PI carbonyl groups still involves solvent molecules, but the proportion of bonded groups is smaller because gentamicin and daptomycin work in conjunction to tighten up the lipidic network, squeezing out more water molecules. The basic factor, of course, is charge neutralization of PI head groups.

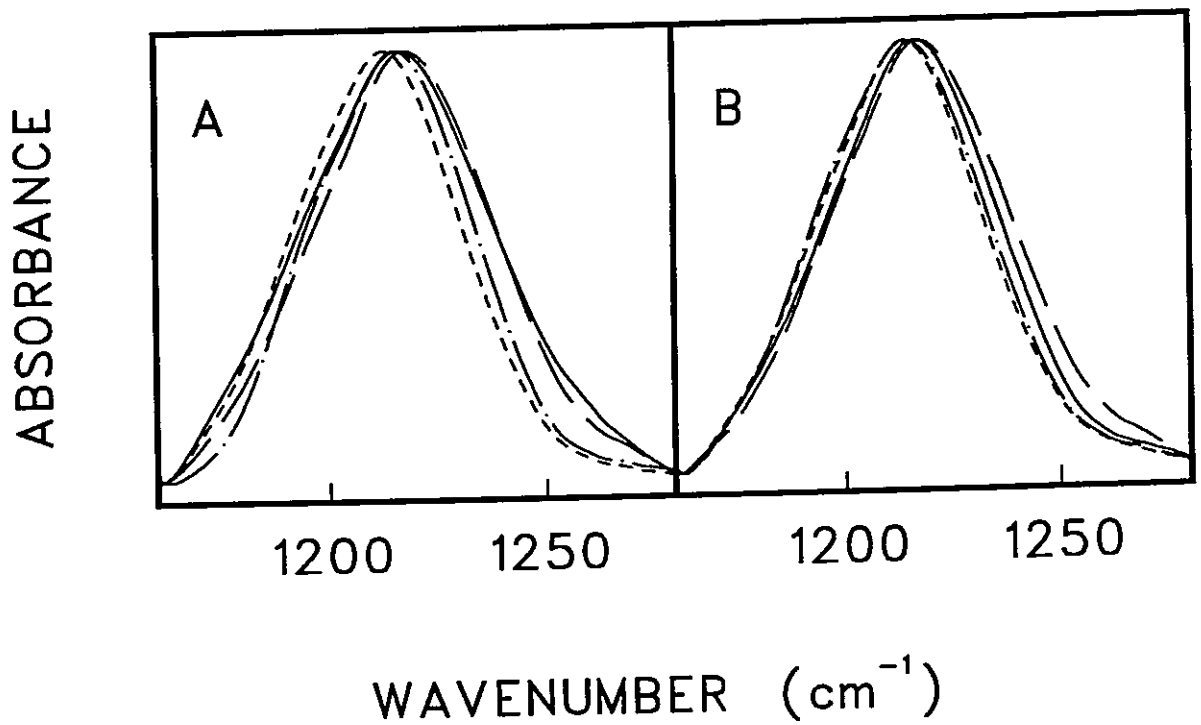
7.4 The Lipid Antisymmetric PO_2^- Stretching Region

As described earlier, daptomycin causes a high frequency shift of the antisymmetric stretching bands of PI phosphate group (Fig. 7.4.1A and B, long dashes), whereas gentamicin induces a decrease of its frequency and bandwidth at both pH 5.4 and 7.5 (Fig. 7.4.1A and B, small dashes). In the presence of both gentamicin and daptomycin, there is a low frequency shift resembling that obtained with gentamicin alone but of a smaller magnitude (Fig. 7.4.1A and B, dot-long dash). The spectral changes analyzed up to this point are consistent with a synergy of effects of daptomycin and gentamicin.

7.5 The Amide I Region

The insertion of the fatty acyl moiety of daptomycin into the hydrophobic core of PI bilayers resulted in no significant change in the overall shape and

Fig. 7.4.1
Phosphate antisymmetric stretching region of the infrared spectra of aqueous dispersions of phosphatidylinositol alone (solid line) or with gentamicin (small dashes) at a PI:gentamicin molar ratio of 2:1, daptomycin (long dashes) at a PI:daptomycin molar ratio of 4:1 or both gentamicin and daptomycin (dot-long dash) at a PI:gentamicin:daptomycin molar ratio of 4:2:1, at pH 5.4(A) and 7.5(B).

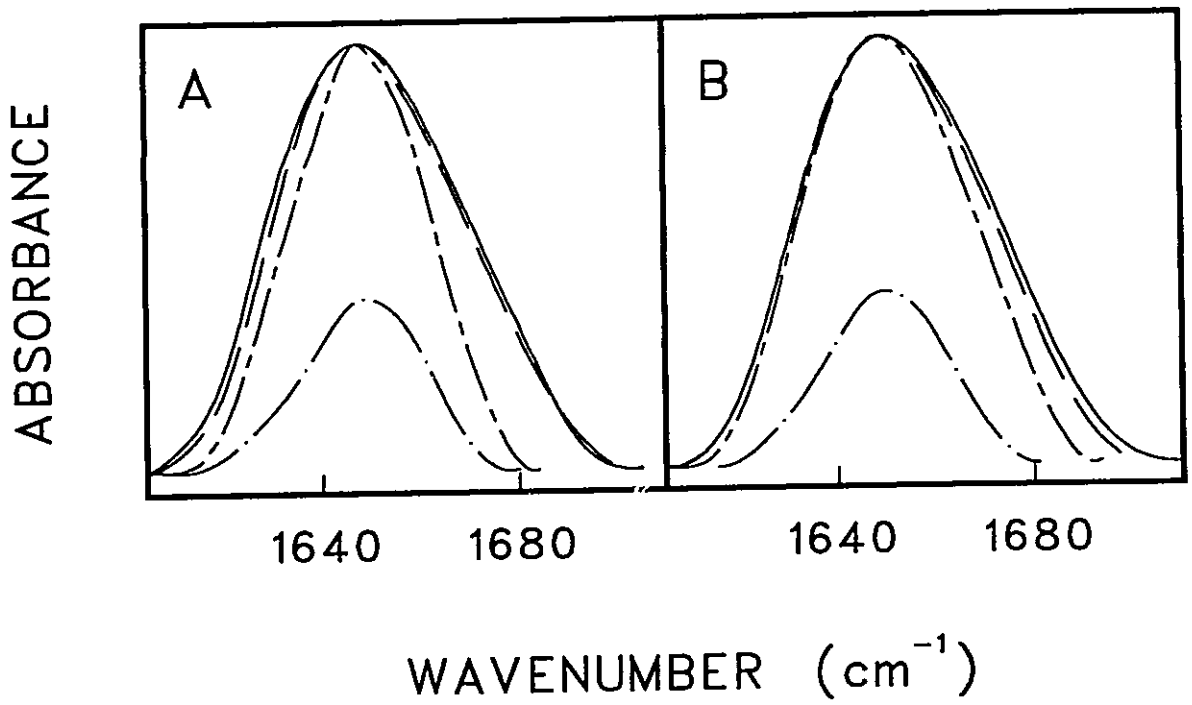

ABSORBANCE
A
B
1200
1250
1200
1250
WAVENUMBER (cm^{-1})

frequency of the amide I band (Fig. 5.4.1A and B, long dashes). The addition of gentamicin also failed to induce any significant change (Fig. 6.3.1). The steric constraints between the gentamicin molecules adsorbed onto the lipidic surface and the peptidic rings stemming out of the bilayer could possibly result in conformational changes of the peptidic portion. The amide I region of the infrared spectrum of daptomycin is presented in Fig. 7.5.1A and B, solid line. In the ternary complex, the width of band observed in the amide I is reduced (Fig. 7.5.1, short-long dashes). It is important to consider the spectral contribution of the C=C stretching mode of the linoleyl chains of phosphatidylinositol because the narrowing effect could be due to the contribution of the lipid band (Fig. 7.5.1, dot-long dash). After the contributions of the lipid have been subtracted from the spectrum of PI/daptomycin/gentamicin, we observe a slight narrowing of the corrected amide I band in the presence of PI (Fig. 7.5.1, long dashes).

7.6 Bilayer Fluidity

As seen in the previous chapters, the insertion of the short decanoyl chain of daptomycin into the hydrophobic core of PI bilayers causes an increase in the fluidity of the lipidic acyl chains (mostly palmitate and linoleate). The aminoglycoside antibiotic causes a decrease in the fluidity of the membrane following head group neutralization by the cationic drug. In the presence of both antibiotics, the decrease in membrane fluidity previously observed in the presence of gentamicin is abolished. In fact, the membrane fluidity corresponds to that seen

Fig. 7.5.1
Amide I region of the infrared spectra of daptomycin alone (solid line) or with both gentamicin and PI (short-long dashes) in ^2H_2O at p^{2}H 5.4(A) or 7.5(B) at a PI:gentamicin:daptomycin molar ratio of 4:2:1. The spectrum of a dispersion of PI is also presented (dot-long dash) to show the contribution of the C=C stretching of its acyl chains. This spectral contribution can be subtracted from the spectrum of PI/gentamicin/daptomycin to obtain the corrected amide I band (short-long dashes).


A
B
ABSORBANCE
1640
1680
1640
1680
WAVENUMBER (cm^{-1})

in the absence of both drugs. This is evident from the temperature profile of the frequency of the methylene symmetric stretching mode which reflects an average of the properties of upper and deeper regions of bilayer (Fig. 7.6.1). The inner region of the bilayer core shows a fluidity similar to that obtained with gentamicin, that is, a slight decrease in the fluidity of acyl chains but of a smaller magnitude compared to that seen with gentamicin alone (Fig. 7.6.2).

7.7 Discussion

The spectral changes analyzed up to this point indicate that the presence of the bulky peptidic group of daptomycin at the bilayer surface does not prevent subsequent binding of gentamicin and reversely, daptomycin interacts with the bilayer in spite of prior treatment with gentamicin. The slower aggregation observed when pre-mixed gentamicin and daptomycin are added to PI dispersions suggests that achieving appropriate spatial organization of the three components is an arduous task. The amide I band of the infrared spectrum of daptomycin is unchanged in the presence of gentamicin nor does it show any change when the lipopeptide inserts in a lipidic environment. In the ternary complex, we observed a slight narrowing of the amide I band which may be due to steric constraints between the gentamicin molecules adsorbed onto the lipidic surface and the peptide rings stemming out of the bilayer. With the data available, it is impossible to determine how the peptide rings and gentamicin molecules are organized at the bilayer surface. The restoration of the membrane fluidity in the presence of

Fig. 7.6.1
Temperature dependence of the frequency of the symmetric C-H stretching vibration of the methylene groups of PI in aqueous dispersions, in the absence (triangles) and in the presence (circles) of a mixture of gentamicin and daptomycin at a PI:gentamicin:daptomycin molar ratio of 4:2:1. The coordinates on the right side of the figure apply to circles. The frequencies were obtained from the spectra after Fourier derivation with a power of 3 and a breakpoint of 0.3.

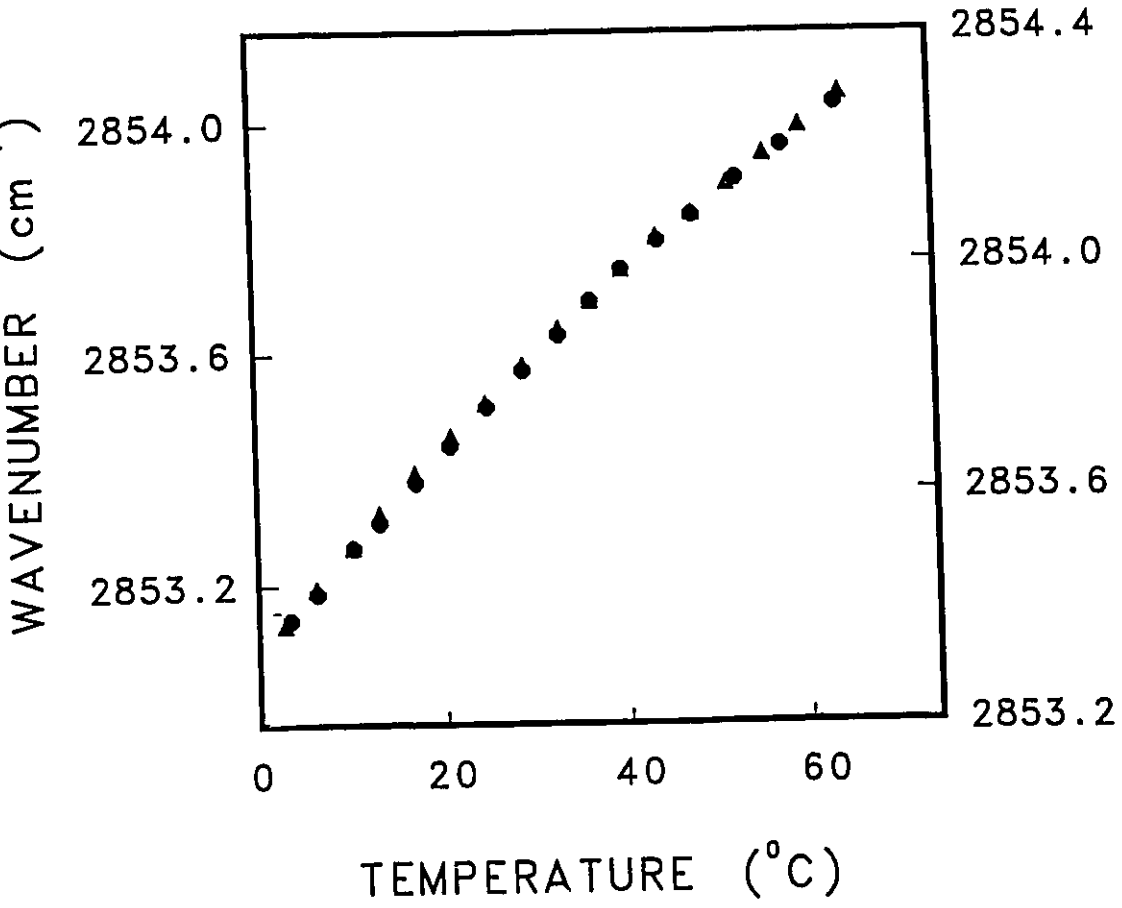
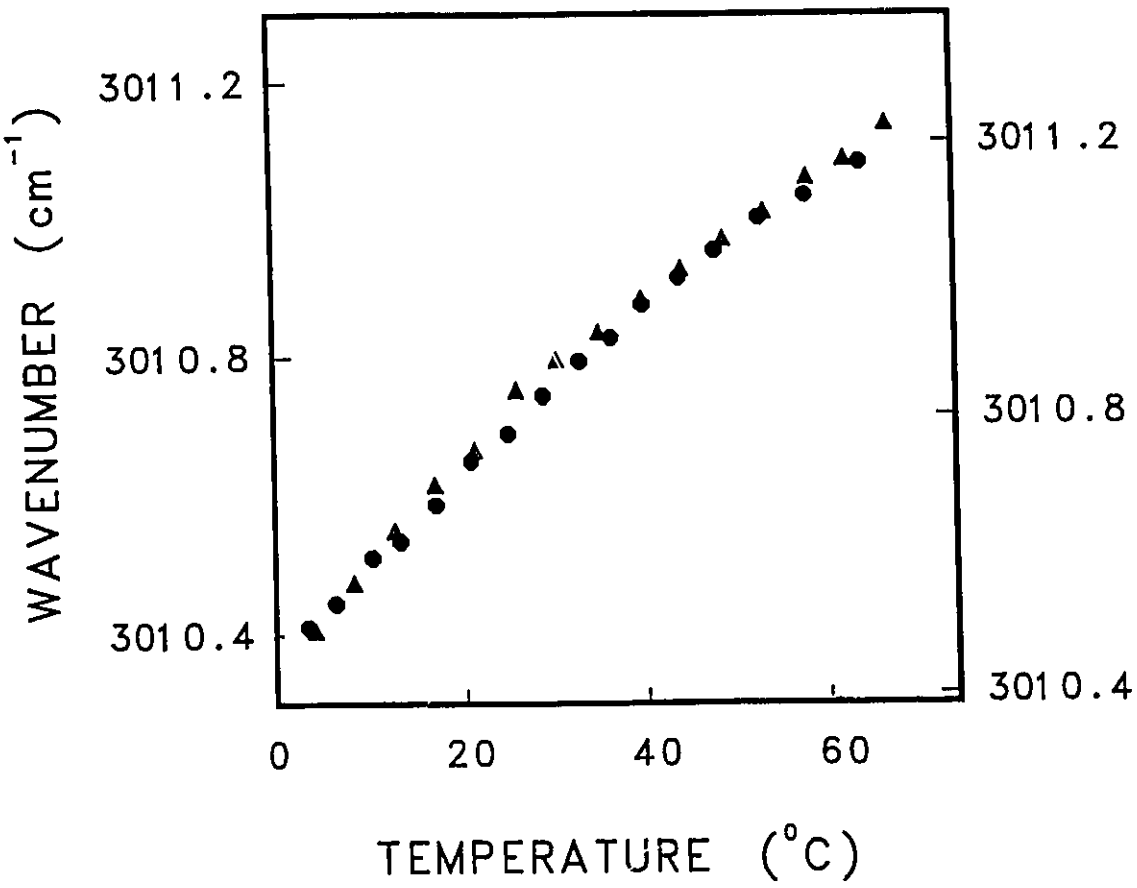

WAVENUMBER (cm^{-1})
2854.0
2853.6
2853.2
2854.4
2854.0
2853.6
2853.2
0
20
40
60
TEMPERATURE (°C)

Fig. 7.6.2
Temperature dependence of the frequency of the symmetric C-H stretching vibration of the olefinic groups of PI in aqueous dispersions, in the absence (triangles) and in the presence (circles) of a mixture of gentamicin and daptomycin at a PI:gentamicin:daptomycin molar ratio of 4:2:1. The coordinates on the right side of the figure apply to circles. The frequencies were obtained from the spectra after Fourier derivation with a power of 3 and a breakpoint of 0.3.


3011.2
3010.8
3010.4
WAVENUMBER (cm^{-1})
0
20
40
60
TEMPERATURE (°C)
3011.2
3010.8
3010.4

daptomycin will help us propose a model for the restoration of phospholipase activity in the presence of the lipopeptide antibiotic.

Chapter 8

MECHANISM OF PROTECTION AFFORDED BY POLYASPARTIC ACID AGAINST GENTAMICIN INDUCED NEPHROTOXICITY

8.1 Introduction

Coadministration of poly-L-aspartic acid has been shown to protect rats against most histopathological, biochemical and functional signs of nephrotoxicity of aminoglycoside antibiotics (Williams, P.D. and Hottendorf, G.H., 1985; Ramsammy *et al.*, 1989; Beauchamp *et al.*, 1990a), despite an up to ten-fold increase of the level of accumulated aminoglycoside (Gilbert *et al.*, 1989). Equilibrium dialysis studies demonstrated an optimum binding of poly-L-aspartic acid to gentamicin at acidic pH [5.4] (Kishore *et al.*, 1990a). The polyanion was also found to displace gentamicin from negatively charged liposomes and to restore the activity of gentamicin inhibited lysosomal phospholipase A1. Experiments performed at pH 7.0 revealed that polyaspartic acid binds and displaces gentamicin from purified brush border membrane vesicles, causing an apparent decrease in the affinity of gentamicin for these membranes (Kishore *et al.*, 1990a). In addition, coadministration of polyaspartic acid did not alter the intrarenal distribution of gentamicin and undegraded polyaspartic acid was recovered in the lysosomal fraction (Kállay, Z. and Tulkens, P.M., 1989). Based on the assumption that polyaspartic acid gains access to lysosomes of proximal tubular cells, as many low molecular weight proteins and polypeptides do, Kishore

et al. (1990a) proposed that protection against gentamicin induced nephrotoxicity is obtained by the binding of the aminoglycoside to the polyanion in lysosomes, preventing the development of phospholipidosis and therefore interfering with the cascade of events leading from drug accumulation to nephrotoxicity.

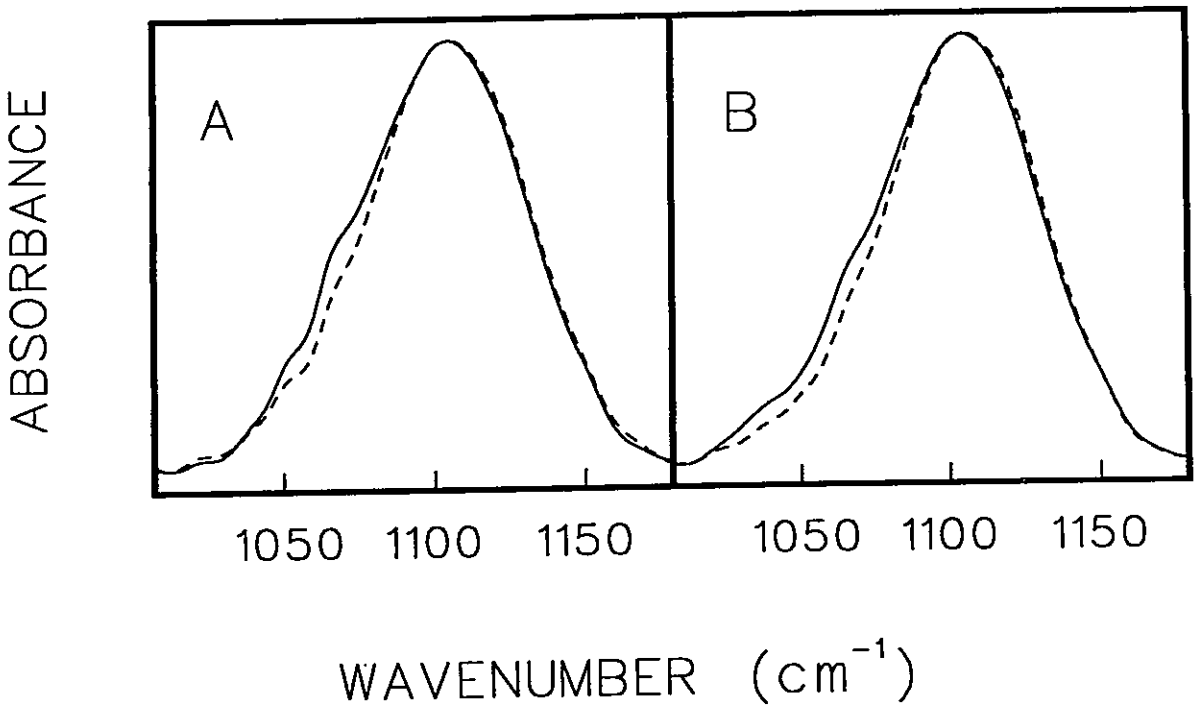
The aim of our study was to gain an insight into the mechanism by which poly-L-aspartic acid confers nephroprotection. This would improve our understanding of the mechanism of aminoglycoside-induced nephrotoxicity and aid in the design of less toxic antibiotics.

We looked for spectral evidence of (i) a direct interaction between polyaspartic acid and the aminoglycoside antibiotic gentamicin, (ii) the effect of polyaspartic acid on the spectral features of PI bilayers, and (iii) a possible influence of polyaspartic acid on gentamicin binding to lipidic membranes.

8.2 Direct Interaction of Polyaspartic acid and Gentamicin

The infrared spectrum of gentamicin below 2000 cm^{-1} comprises a single well defined band at 1105 cm^{-1} approx. (Fig. 8.2.1). The C-N stretching vibration from primary and secondary amine groups, C-O stretching and C-C stretching contribute to the intensity of this band which remains unchanged when the pH is increased from 5.4 to 7.5. Studies were performed under conditions of pH 5.4, mimicking those prevailing in lysosomes, and at pH 7.5, which is the pH at the surface of the brush border membranes. The contributions of C-N, C-O and C-C stretching vibration of polyaspartic acid does not give rise to a well defined band

Fig. 8.2.1
Infrared spectrum of gentamicin in the C-O, C-C and C-N stretching region at pH 5.4(A) and 7.5(B). Solid lines represent aqueous solutions of gentamicin and dashed lines, aqueous solutions of gentamicin in the presence of polyaspartic acid at a gentamicin:aspartic acid residue molar ratio of 1:10. Spectral contributions of polyaspartic acid have been subtracted.


ABSORBANCE
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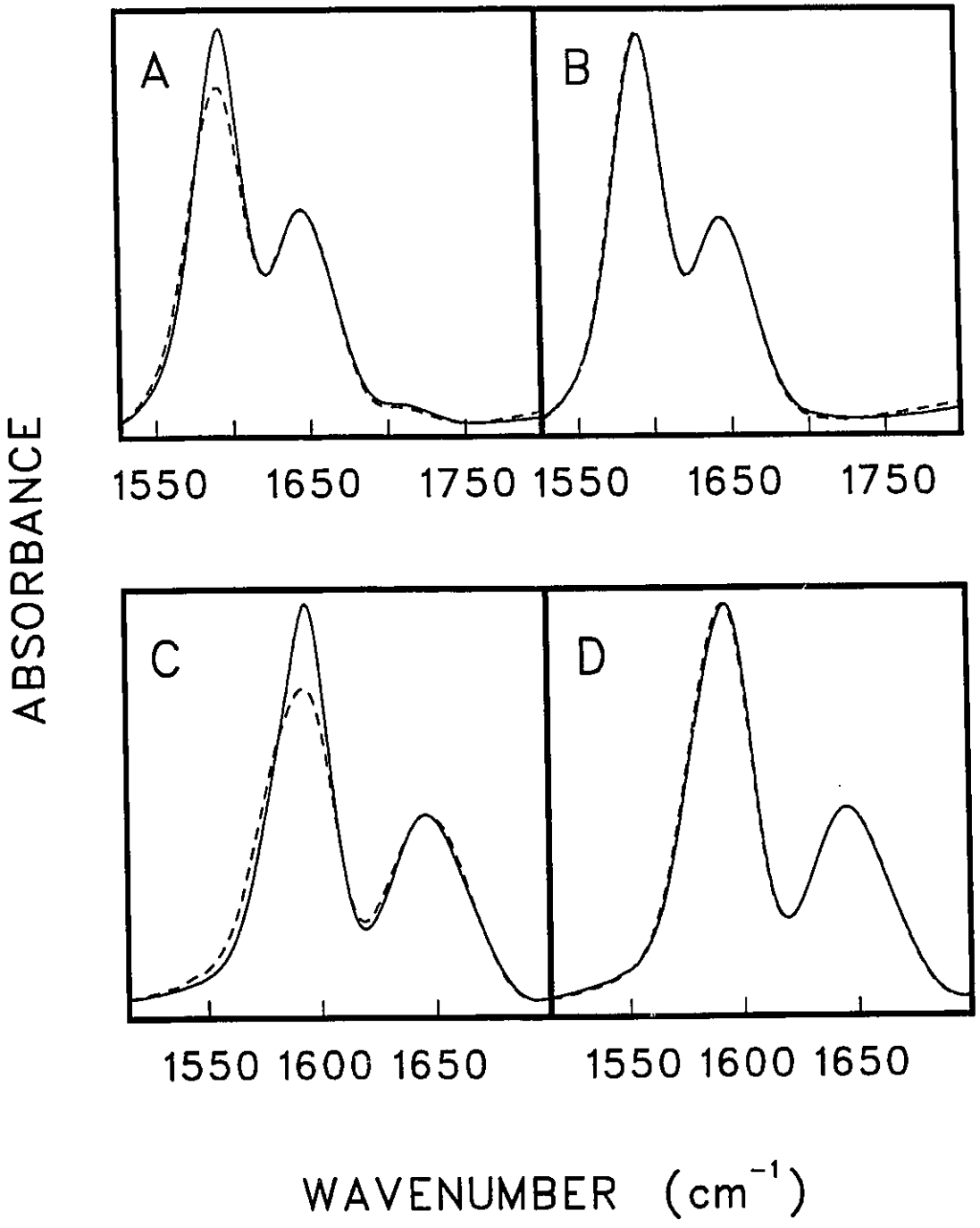
WAVENUMBER (cm^{-1})

in this region. With the addition of polyaspartic acid to gentamicin we did not observe a significant alteration of the frequency or overall shape of the gentamicin 1105 cm^{-1} band at either pH (Fig. 8.2.1A and B).

The infrared spectra of polyaspartic acid contains many features that can be sensitive to ionization and changes in conformation of the molecule. At neutral pH, the carboxylate function of the side chains confer a net negative charge on the molecule. The carbonyl stretching vibrations of the ionized carboxylates gives rise to a band of moderate intensity at 1590 cm^{-1} at both pH 5.4 and 7.5 (Fig. 8.2.2), consistent with the prevalence of the ionized state in that pH interval. When the pH is lowered, the carboxylate band loses intensity and a band due to protonated carboxylates grows up at 1710 cm^{-1}. The amide I band which represents primarily the C=O stretching vibrations of the amide groups coupled to the in-plane NH bending and C-N stretching modes is observed at 1645 cm^{-1}. The sensitivity of the amide I band to polypeptide backbone conformation is well established. The exact frequency of the amide I band depends on the nature of hydrogen bonding involving the C=O and NH groups, which in turn, is determined by the secondary structure adopted by the protein. Changes in the conformation, interactions at the side chains and alterations in the environment are reflected in spectral variations of the amide I band. Although the overall shape of the amide I band remains the same, there is a slight increase in the frequency (2-3 cm^{-1}) of this band upon going from pH 5.4 to 7.5. Addition of gentamicin to polyaspartic acid in a gentamicin:aspartic acid residue molar ratio of 1:10 did not induce any significant

Fig. 8.2.2
Carbonyl stretching region of the infrared spectra of polyaspartic acid alone (solid line) or with gentamicin (dashed line) in solution, at a gentamicin:aspartic acid residue molar ratio of 1:10 in aqueous solutions, at pH 5.4 (left panels) and 7.5 (right panels). The original spectra(A and B) were deconvolved using a bandwidth of 13 and a resolution enhancement factor of 1.75(C and D).


A
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ABSORBANCE
WAVENUMBER (cm^{-1})

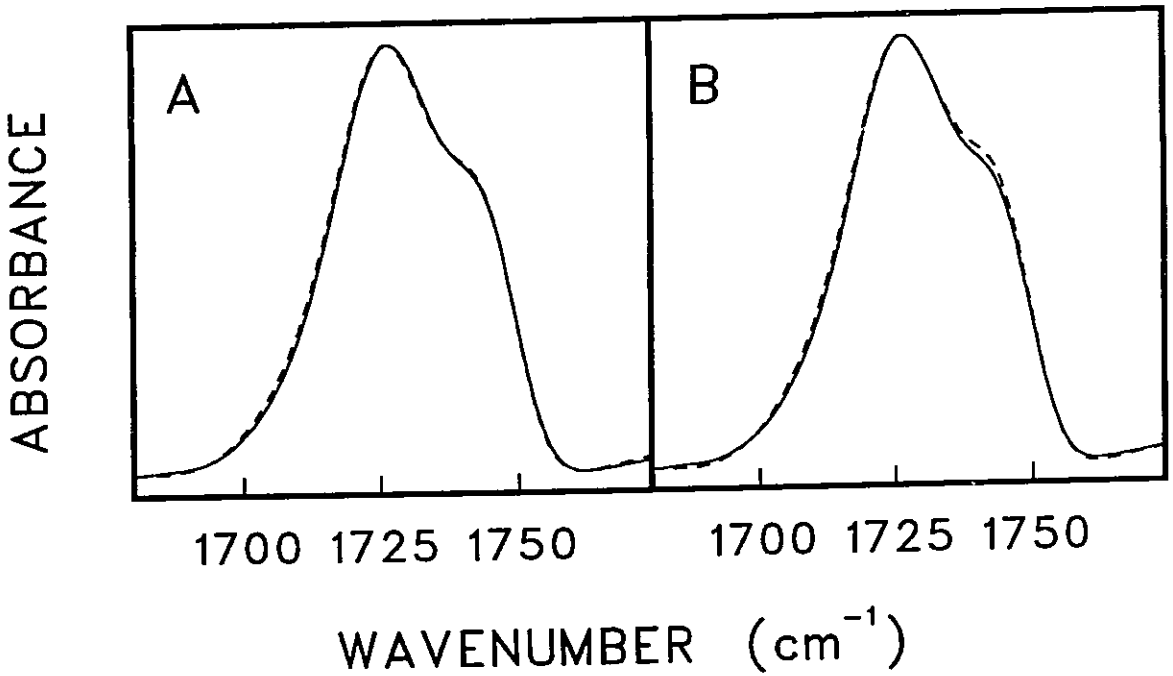
change in the frequency or shape of the amide I band (Fig. 8.2.2, dashed line) When the pH is lowered from 7.5 to 5.4, neutralization of a fraction of the carboxylate groups was reflected by a marked decrease of the intensity of the band at 1590 cm^{-1}; no consistent change of that band was noted when gentamicin was added to polyaspartic acid.

8.3 Effect of Polyaspartic acid on Phosphatidylinositol Bilayers

Since polyaspartic acid bears a net negative charge at both pH 7.5 and 5.4, we do not expect any kind of interaction with PI bilayers, in fact there would be electrostatic repulsion.

The antisymmetric stretching mode of the phosphate group esterified to the inositol ring and the ester carbonyl stretching vibrations in the interfacial region are good diagnostic tools to study the effect of the polyaminoacid on the lipid bilayers. The deconvolved carbonyl stretching band of the ester functions of phosphatidylinositol in aqueous suspension is presented in Fig. 8.3.1A and B (solid line). The high frequency component, at 1745 cm^{-1}, pertains to "free" carbonyl groups and the peak at 1725 cm^{-1} corresponds to hydrogen bonded carbonyl groups (Mantsch *et al.*, 1988; Hübner *et al.*, 1990). The addition of polyaspartic acid to PI suspension in a molar ratio of 1PI:5 aspartic acid residues does not result in any significant frequency shift or change in the intensity ratio of non-bonded to bonded carbonyl groups at either pH studied (Fig. 8.3.1, dashed line).

Fig. 8.3.1
Carbonyl stretching region of the infrared spectra of dispersions of phosphatidylinositol alone in ^2H_2O (solid line), or with polyaspartic acid (dashed line) at a PI:aspartic acid residue molar ratio of 1:5 at p^{2}H 5.4(A) and 7.5(B). The spectra were obtained after band narrowing by Fourier self-deconvolution using a bandwidth of 13 and a resolution enhancement factor of 1.75.


A
B
ABSORBANCE
1700 1725 1750
1700 1725 1750
WAVENUMBER (cm^{-1})

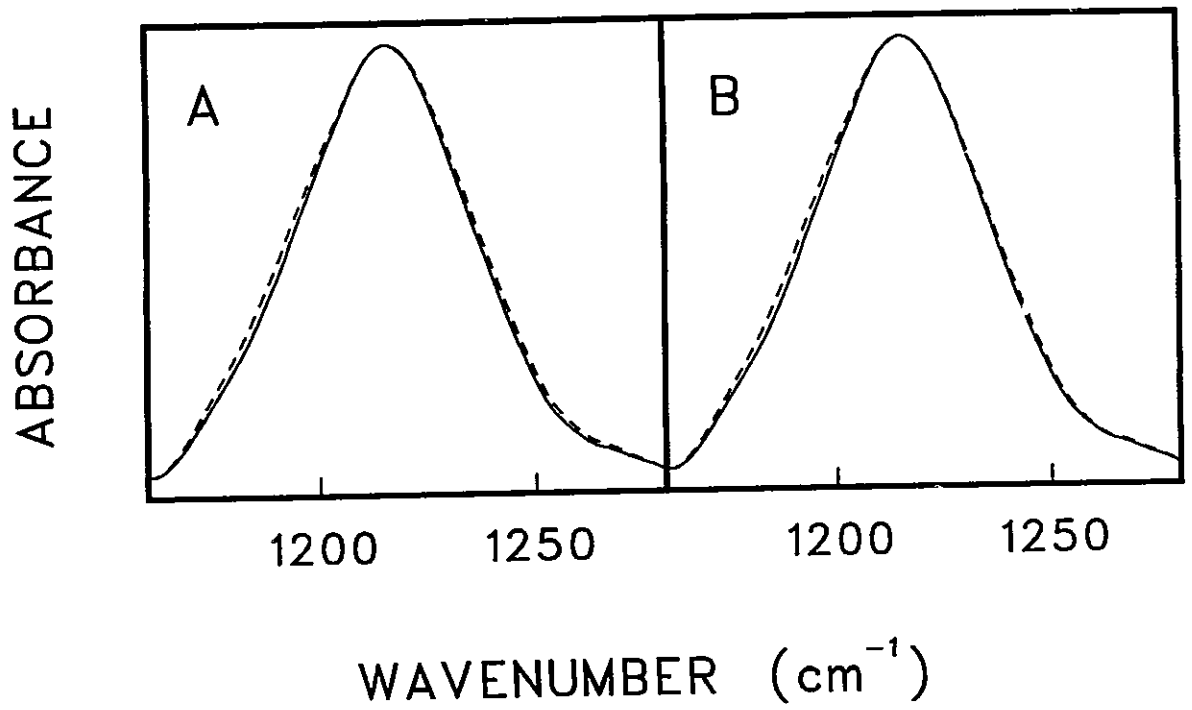
Infrared spectra to study the phosphate region are recorded in the absence of ^2H_2O to avoid interference from its bending mode in the 1220 cm^{-1} region. The frequency of the phosphate antisymmetric stretching vibration usually varies between 1220 and 1270 cm^{-1}, depending on the extent of hydrogen bonding (Casal and Mantsch, 1984). The frequency of this mode for a lipidic dispersion of PI is 1216 cm^{-1} (Fig. 8.3.2, solid line), indicative of strongly hydrogen bonded phosphate groups. The overall shape and frequency of this vibration remains the same in the presence of polyaspartic acid (Fig. 8.3.2, dashed line).

The amide I band of polyaspartic acid is sensitive to changes in the conformation, interaction at the side chains and alterations in the environment which are reflected in spectral variations of this band. A narrowing of the amide I band is observed in the presence of PI (Fig. 8.3.3, dashed line). This is due to the contribution of the C=C stretching mode of the linoleyl chains of phosphatidylinositol (Fig. 8.3.3, dot-long dash). After the contributions of the lipid have been subtracted, we observe no significant, reproducible change in the overall shape and frequency of the corrected amide I band in the presence of PI (Fig. 8.3.3, long dashes).

8.4 Effect of Polyaspartic acid on gentamicin-PI interaction

The addition of gentamicin to PI dispersions results in an increase of the intensity ratio of non-bonded to bonded carbonyl bands (Fig. 8.4.1, dashed line). The lipid carbonyl stretching of the infrared spectrum of PI-gentamicin-aspartic acid

Fig. 8.3.2
Phosphate antisymmetric stretching region of the infrared spectra of aqueous dispersions of phosphatidylinositol alone (solid line) or with polyaspartic acid (dashed line) at a PI:aspartic acid residue molar ratio of 1:5, at pH 5.4(A) and 7.5(B).


A
B
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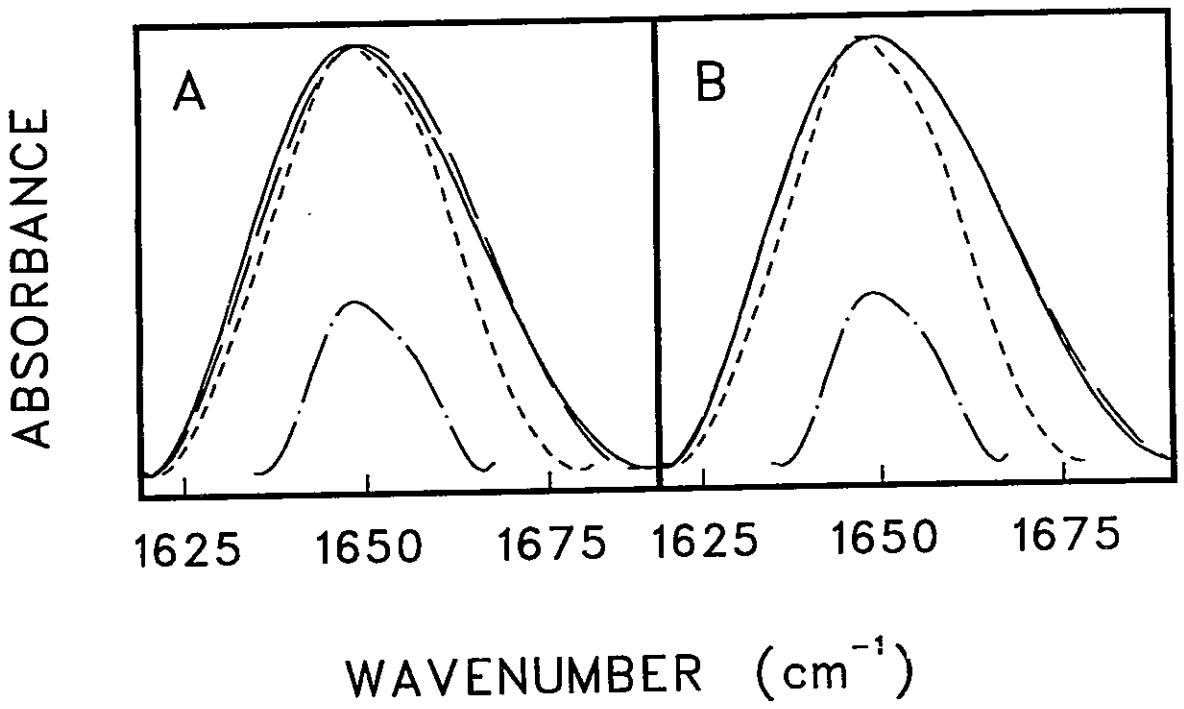
1200
1250
1200
1250
WAVENUMBER (cm^{-1})

Fig. 8.3.3
Amide I region of the infrared spectra of polyaspartic acid alone (solid line) or with PI (short dashes) in ^2H_2O at p^{2}H 5.4(A) or 7.5(B) at a PI:aspartic acid residue molar ratio of 1:5. The spectrum of a dispersion of PI is also presented (dot-long dash) to show the contribution of the C=C stretching of its acyl chains. This spectral contribution can be subtracted from the spectrum of PI/polyaspartic acid to obtain the corrected amide I band of polyaspartic acid (long dashes).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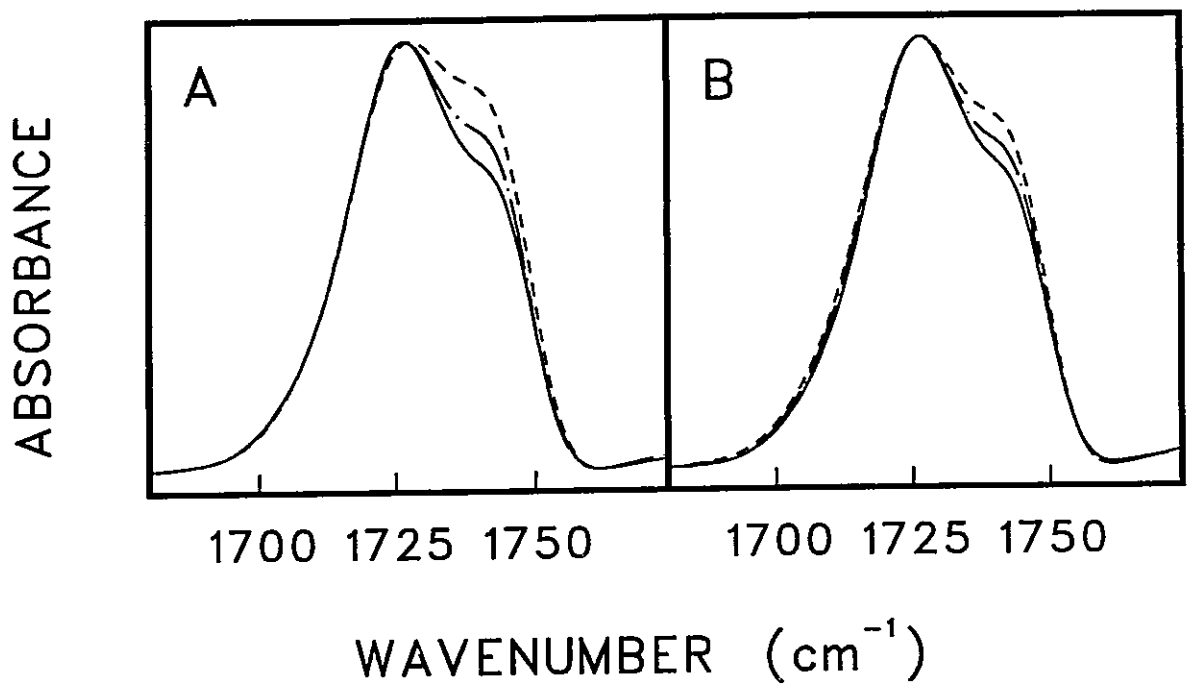
1650
1675
1625
1650
1675
WAVENUMBER (cm^{-1})

Fig. 8.4.1
Carbonyl stretching region of the infrared spectra of dispersions of phosphatidylinositol alone in ^2H_2O (solid line), or with gentamicin (small dashes) at a PI:gentamicin molar ratio of 2:1, or both gentamicin and polyaspartic acid (dot-long dash) at a PI:gentamicin:aspartic acid residue molar ratio of 2:1:10 at p^{2}H 5.4(A) and 7.5(B). The spectra were obtained after band narrowing by Fourier self-deconvolution using a bandwidth of 13 and a resolution enhancement factor of 1.75.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1700 1725 1750
WAVENUMBER (cm^{-1})

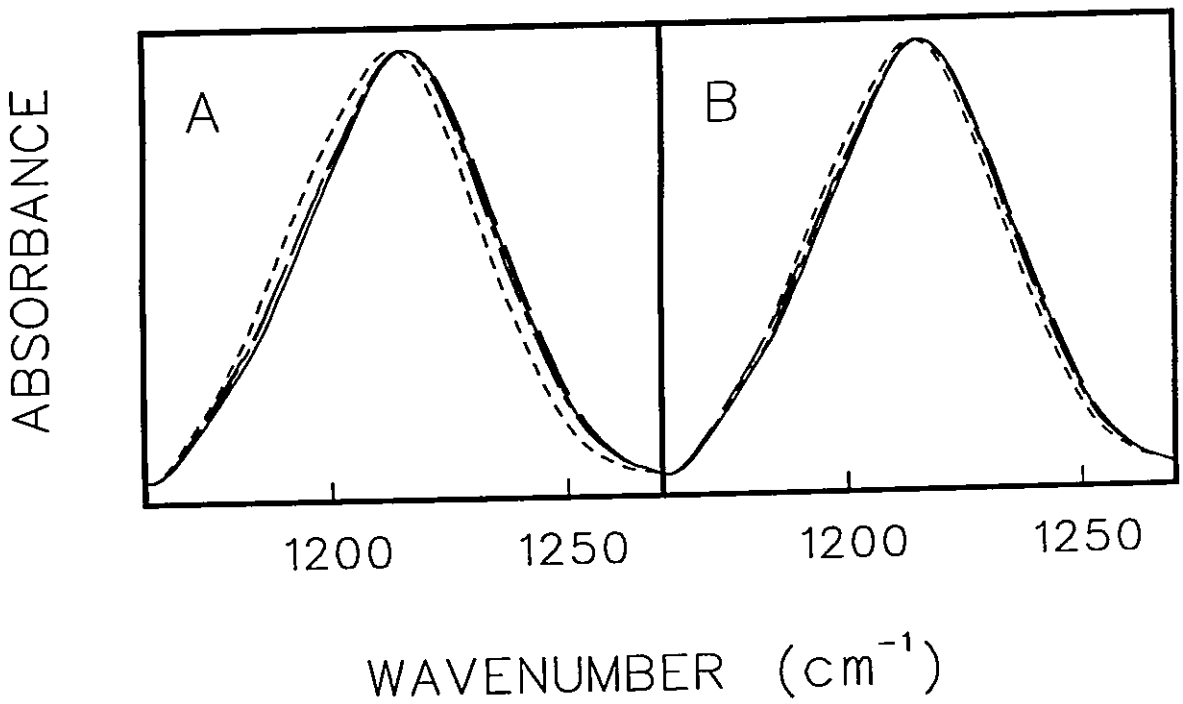
residue at a molar ratio of 2:1:10 is presented in Fig. 8.4.1 (dot-long dash). At both pH 5.4 and 7.5, there is a decrease of the intensity ratio of non bonded to bonded carbonyl bands relative to the effect seen with the aminoglycoside. The dampening effect of gentamicin is more pronounced in samples where polyaspartic acid is added first and is lighter in samples where gentamicin is added first. The effect is intermediate when polyaspartic acid and gentamicin are mixed prior to addition to PI dispersions.

As mentioned in the previous chapters, gentamicin induces a 2 cm^{-1} downward shift in the frequency maximum of the antisymmetric phosphate stretching mode, consistent with the strengthening of phosphate hydrogen bonds consecutive to charge neutralization of head groups (Fig. 8.4.2, dashed line). The addition of polyaspartic acid to PI dispersions does not result in a significant change in the overall shape and frequency of this vibration (Fig. 8.4.2, long dashes). In the presence of both gentamicin and polyaspartic acid, there is a shallow downward frequency shift. Although very small, the effect was reproducible and was found to be more pronounced in samples where polyaspartic acid had been added first and smaller in samples where gentamicin had been added first.

8.5 Discussion

Co-administration of poly-L-aspartic acid has been shown to protect rats against most histopathological, biochemical and functional signs of nephrotoxicity

Fig. 8.4.2
Phosphate antisymmetric stretching region of the infrared spectra of aqueous dispersions of phosphatidylinositol alone (solid line) or with gentamicin (small dashes) at a PI:gentamicin molar ratio of 2:1, polyaspartic acid (long dashes) at a PI:aspartic acid residue molar ratio of 1:5 or both gentamicin and polyaspartic acid (long-short dashes) at a PI:gentamicin:aspartic acid molar ratio of 2:1:10, at pH 5.4(A) and 7.5(B).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of aminoglycoside antibiotics. *In vitro* experiments revealed an optimal interaction between gentamicin and polyaspartic acid at pH 5.4, the intralysosomal pH. Kishore *et al.*(1990a) proposed that the intralysosomal complexation of polyaspartic acid and gentamicin prevented phospholipidosis and other cortical alterations, conferring protection against gentamicin induced nephrotoxicity.

Dialysis studies have indicated a direct interaction of polyaspartic acid with gentamicin (Kishore *et al.*, 1990a). The dependence on pH and ionic strength confirmed the electrostatic character of this interaction. We have no spectral evidence of a direct interaction of polyaspartic acid with gentamicin in solution. The absence of significant spectral changes and the fact that both the compounds remain in solution after mixing preclude tight interactions and direct neutralization of polyaspartic acid's carboxylates. Nevertheless, the formation of a loose complex cannot be excluded, it would justify the dialysis studies and still be compatible with our spectroscopic study.

The addition of polyaspartic acid to PI dispersions did not affect the spectral features of the lipid bilayers. The precipitation observed when polyaspartic acid is added to PI dispersions is due to a change in the ionic strength by the addition of the polyanion to the lipid.

Although study of supernatants did not provide us with spectral evidence of decreased binding of gentamicin to PI bilayers in the presence of polyaspartic acid, the changes induced at the head group and interfacial region by the aminoglycoside drug are reduced in the presence of polyaspartic acid. The effect

was more pronounced when polyaspartic acid was added first and smaller when gentamicin was added first. The result was intermediate when polyaspartic acid and gentamicin were mixed prior to addition PI dispersions. We conclude that the mechanism of nephroprotection by polyaspartic acid is different from that of daptomycin. Assuming that polyaspartic acid gains access to lysosomes of proximal tubular cells, as many small low molecular weight proteins and polypeptides do, the intralysosomal sequestration of gentamicin by polyaspartic acid is probably responsible for a reduction in the changes induced at the head group and interfacial region of PI bilayers by gentamicin .

Chapter 9

GENERAL DISCUSSION

Aminoglycosides have found extensive use in clinical medicine in the last three decades since the discovery of streptomycin. In many situations, the availability of an aminoglycoside antibiotic has meant the difference between survival and death. The use of these clinically invaluable drugs is associated with dose related ototoxic and nephrotoxic side effects. In recent years, research has focussed on understanding the toxicity mechanism, design of less toxic aminoglycosides, together with identification of drugs with increased clinical efficacy.

An early and significant step in aminoglycoside-induced nephrotoxicity is a loss of activity of lysosomal phospholipases A1, A2 and sphingomyelinase, resulting in lysosomal phospholipidosis, with a non specific accumulation of polar phospholipids in myeloid bodies (Carlier, M.B., Laurent, G., Claes, P.J., Vanderhaeghe, H.J. and Tulkens, P.M., 1983; Laurent *et al.*, 1982). The lysosome is an organelle responsible for the degradation of extracellular as well as intracellular materials. These digestive activities play a role in several cellular metabolic pathways, in the normal turnover of biological molecules, in host defence against pathogens and in the removal of necrotic material from the blood stream and from tissues. Physiologically, pericellular and intracellular membranes

segregate in lysosomes by autophagia and are degraded therein by the combined action of lysosomal enzymes including phospholipases.

It seems relevant at this point to review the mechanism by which lipolytic enzymes such as phospholipases act on their substrates in order to propose a mechanism for the inhibition and reactivation of these enzymes by drugs and other molecules. The most intensively studied class of enzymes is phospholipase A2, which catalyzes hydrolysis of the *sn*-2 ester bond in 1, 2-diacyl-*sn*-3-phosphoglycerides to form fatty acid and lysophospholipid products. Extracellular phospholipases A2 have been isolated primarily from cobra and rattlesnake toxins and from bovine and porcine pancreas. Intracellular phospholipases A are not well characterized because of their poor stability, low specific activity and scarcity in tissues. Phospholipases A2 are small water soluble proteins with molecular weights around 14,000. All extracellular phospholipases have an absolute requirement for calcium, and His-48 in the catalytic site plays an important role in hydrolysis. X-ray crystallography has revealed a rigid, three dimensional structure stabilized by several disulphide bridges.

Interfacial catalysis by phospholipase A2 is a necessary consequence of the amphipathic character of phospholipids. The hydrophobic effect provides the driving force for amphipathic molecules in water to form organized structures such as bilayers, monolayers, micelles and emulsions. The rate of hydrolysis for solitary monomeric phospholipid molecules is very low and up to 10^4-fold activation is observed at interfaces depending on the structural organization and dynamics of

the substrate. The catalytic cycle of phospholipase A2 occurs in two distinct steps. The enzyme in the aqueous phase, E, binds to the substrate interface to give E*. The enzyme in the interface then binds a molecule of phospholipid at the catalytic site to give the Michaelis complex, E*S, which goes on to generate the products of hydrolysis and free enzyme in the interface, E* + P. Following this step there could be two different possibilities with significantly different kinetic consequences. After each or a few catalytic turnover cycles in the interface E* could leave the interface via the aqueous phase to another interface, that is, the intervesicle exchange of the enzyme is rapid and the enzyme in effect "hops" from one interface to another via the aqueous phase. The other extreme case would be that E* "scoots" from one substrate molecule to the other without leaving the interface during several thousand catalytic turnover cycles. These two extreme modes of interfacial catalysis have been termed as "hopping" and "scooting" respectively by Jain and colleagues (Jain, M.K., Rogers, J., Jahagirdar, D.V., Marecek, J.F. and Ramirez, F., 1986a; Jain, M.K., DeHaas, G.H., Marecek, J.F. and Ramirez, F., 1986b). Under optimal conditions for catalysis, scooting predominates and hopping tends to slow down the catalytic turnover due to the slow nature of the desorption and resorption of the enzyme intrinsic to the E to E* step (Jain, M.K., Rogers, J. and DeHaas, G.H., 1988). The catalytic site of phospholipase A2 is presumed to be functionally and topologically distinct from the "interfacial recognition region" along which the enzyme binds to the interface. Therefore interfacial binding is a distinct step from catalytic turnover. The fact that binding

of the substrate to the catalytic site of PLA2 is not required for its binding to its interface has been demonstrated by the fact that alkylated PLA2 in which the His-48 at the catalytic site has been modified and is catalytically inactive binds to the bilayer interface with high affinity (Jain, M.K., Ranadive, G., Yu, B.Z. and Verheij, H.M., 1991).

Ionic interactions play an important role in the binding of phospholipase A2 to the bilayer interface. This is indicated by the fact that binding affinity of PLA2 to anionic bilayers is about ten orders of magnitude larger than its binding affinity to zwitterionic bilayers and the enzyme bound to the anionic interface exchanges with excess vesicles only in the presence of salt (Jain, M.K., Maliwal, B.P., DeHaas, G.H. and Slotboom, A.J., 1986c; Bayburt, T., Yu, B.Z., Lin, H.K., Browning, J., Jain, M.K. and Gelb, M.H., 1993). In addition, hydrophobic interactions promote the binding of the enzyme to the interface. These interactions occur along a face of the enzyme, with a hydrophobic collar and a ring of cationic residues, through which the catalytic site is accessible to the substrate molecule in the bilayer (Ramirez, F. and Jain, M.K., 1991).

Inhibition of phospholipase activity by an inhibitor could occur by either of the following modes:

(i) The drug could exist predominantly in the bilayer where it interacts with the enzyme bound to the interface (E*) and competes with the substrate for binding to the catalytic site.

(ii) The drug does not bind directly to the enzyme but changes the physical organization of the bilayer in a way that promotes desorption of the bound enzyme or impedes its binding.

(iii) The drug binds directly to either the active site or the interfacial binding site of PLA2 and prevents the enzyme from binding to the substrate.

The first and third possibilities can be disregarded because studies have not been able to demonstrate a direct binding between aminoglycosides and phospholipase A under conditions which were identical to the conditions used to measure aminoglycoside-induced inhibition (Hostetler and Jellison, 1990). The second possibility seems the most likely explanation for the inhibition of phospholipase A by gentamicin. This enzyme is known to be sensitive to the distribution and density of anionic charges on the substrate interface required for binding of the enzyme, that is, the E to E* step. A perturbation of the charge distribution in the substrate interface can shift the proportion of the bound enzyme by several orders of magnitude (Burack, W.R., Yuan, Q., Biltonen, R.L., 1993). In addition cationic groups on phospholipase A2 in the N-terminus region (Arg-6, Lys-10 and His-17) have been implicated in its interfacial activation (Jain *et al.*, 1986c). Intervesicle exchange of the bound enzyme is promoted in the presence of anions in the aqueous phase which were found to bind to phospholipase A2 and thus compete with the anionic interface for binding to the enzyme. Anionic additives to the bilayer however activated phospholipase A2 (Jain *et al.*, 1986c). The neutralization of the negative charges on the phospho groups of PI by the

aminoglycoside antibiotic would result in a perturbation of the distribution and density of anionic charges required for interfacial activation. This explains the inhibition of the enzyme by the polycationic drug gentamicin.

Action of PLA2 on vesicles of zwitterionic phospholipids is characterized by an initial burst of product release, followed by a latency period before the fast steady-state rate of hydrolysis is attained. The accelerated hydrolysis that follows the latency phase of the reaction progress curve was due to product assisted binding of the enzyme to the substrate bilayer (Apitz-Castro, R., Jain, M.K. and DeHaas, G.H., 1982). Addition of lysophosphatidylcholine to preformed vesicles of diacyl phospholipids reduced the latency phase and enhanced the binding of phospholipase A2 to the vesicles. In contrast, the binary codispersions prepared from diacyl phospholipids premixed with lysophosphatidylcholine did not exhibit enhanced susceptibility to the phospholipase. This effect was attributed to organizational defects created by asymmetrical incorporation of lysophospholipid molecules into the outer monolayer of vesicles: the action of phospholipase was not observed when the additive was equilibrated in both the monolayers of the vesicles (Jain, M.K. and DeHaas, G.H., 1983). In addition, the lag time as a function of temperature showed a pronounced minimum at the gel-to-liquid crystalline phase transition temperature of the substrate and was altered by the addition of a variety of hydrophobic or amphipathic molecules, including the products of hydrolysis of zwitterionic phospholipids (Apitz-Castro *et al.*, 1982). The hydrolysis of phosphatidylcholine liposomes by lysosomal phospholipase A was

also reported to be maximal at the phase transition temperature of the lipid (Vaudenbranden, M., De Gand, G., Brasseur, R., Defrise-Quertain, F. and Ruysschaert, J.M., 1984).

A negative charge in the interface is a necessary but not a sufficient condition for interfacial catalysis. This is because fatty acids alone in DMPC vesicles do not promote hydrolysis until lysophospholipids are also present. This is probably because PLA2 anchored to the anion in the interface cannot bind to the substrate unless the substrate can be destabilized enough to bind to the catalytic site of PLA2. Destabilization of the substrate molecule in the bilayer is probably promoted by organizational defects or instabilities. The E to E* step is the anchoring step in which the anionic binding site binds to the anionic group in the interface. The E* + S to E*S step requires destabilization of the substrate molecule in the interface. The dual sensitivity of PLA2 to membranes physical structure (as demonstrated by the temperature effect on lag time) and composition suggests that lateral phase separation plays an important role in the regulation of PLA2 activity. The neutralization of PI molecules by gentamicin, the tightening of the lipidic network which reduces the penetration of water into the bilayer interface region (as indicated by a decrease in the population of hydrogen bonded carbonyl groups) and the decrease in fluidity of acyl chains result in a modification of the "quality of the interface" that is not conducive to the incorporation of the enzyme.

In order to unravel the toxicity mechanism, we studied the interactions between the aminoglycoside antibiotic gentamicin and different phospholipids at

the molecular level. Natural membranes contain an array of negatively charged phospholipids and aminoglycosides have been demonstrated to bind to several of them, in particular phosphatidylserine, phosphatidylglycerol, phosphatidic acid and phosphoinositides. The kidney is much more susceptible to the toxic damage by aminoglycosides than other organ systems. This is related to the high concentration of phosphatidylinositol in its membranes due to an active metabolism of phosphoinositides (Schacht, J., 1986). In addition, these antibiotics are not metabolized and are eliminated from the body by the kidney only. The resulting high concentrations in the glomerular filtrate promotes their nephrotoxicity. Phosphatidylinositol is the major binding site for gentamicin in the renal brush border membranes. Our results indicate that the neutralization of the negative charges of PI head groups by the cationic amino groups of gentamicin leads to a tightening of the lipidic network which is manifested by a decrease in the penetration of the solvent to the bilayer interface region. The strengthening of the phosphate hydrogen bonds is also consistent with a decrease of PI intermolecular distance consecutive to charge neutralization by gentamicin. A tightening of the lipidic network would alter the thermotropic behaviour of the membrane lipids and a decrease in the fluidity of the membrane is observed in the presence of gentamicin. The infrared spectra of DMPG and DMPC do not show significant changes in the lipid interface region or thermotropic properties in the presence of gentamicin. The narrowing of the antisymmetric phosphate stretching band of DMPC in the presence of gentamicin is due to a change in the phosphocholine

head group conformation which is known to be sensitive to electric charges on the membrane surface (Scherer and Seelig, 1989). The phosphoglycerol head group of DMPG is less sensitive to membrane surface charge than the phosphocholine head group. However, it does undergo a unique conformational change under the influence of modifications of surface charge which are probably due to head group dipoles (that is, OH or CO) seeking to align themselves with the direction of the electrical field at the surface (Marassi, P.M. and Macdonald, P.M., 1991). Gentamicin induces no significant change in the ratio of bonded to non bonded carbonyl groups of DMPG in the gel state. However an increase in the proportion of free carbonyl groups is observed at temperatures above the gel-to-fluid transition. This confirms the existence of an electrostatic interaction between the negatively charged DMPG head group and the cationic drug. Despite this decrease in water penetration, there was no significant change in the fluidity of DMPG bilayers. Equilibrium dialysis experiments demonstrated a binding of the drug to different negatively charged phospholipids (Mingeot-Leclercq *et al.*, 1990a), but the interaction with PI was the one leading to the greatest loss of phospholipases A activity. It is thus tempting to relate this greater inhibitory effect seen with PI with a specificity of the decrease in fluidity. We have shown that at the molecular level there are significant differences in the drug phospholipid interaction and the changes observed at the level of carbonyl groups, phosphate groups and acyl chain packing are unique to the interaction with phosphatidylinositol. Different aminoglycosides in clinical use vary in their

nephrotoxic potential. Future work involving a study of the interaction between PI and various aminoglycosides with different nephrotoxic potentials would help us to correlate the extent of toxicity with changes observed at the molecular level.

We examined several possibilities to unravel the mechanism of nephroprotection by the lipopeptide antibiotic daptomycin. A recent dialysis study indicated a direct interaction of daptomycin with gentamicin (Couture *et al.*, 1994) The dependence on ionic strength and pH confirmed the electrostatic character of this interaction. The results also showed an increased binding of gentamicin and tobramycin after incorporation of daptomycin into liposomes containing phosphatidylinositol. In the present study we found no spectral evidence of a direct interaction of daptomycin with gentamicin in solution. The absence of significant spectral changes and the fact that both compounds remain in solution after mixing preclude tight interactions and direct neutralization of daptomycin's carboxylates. Nevertheless, the formation of a loose complex cannot be excluded, it would justify the dialysis results and still be compatible with our study.

We looked into a possible interaction between daptomycin and PI, leading to an impairment of the binding of aminoglycosides to brush border membranes. Our spectroscopic data revealed that daptomycin incorporates into lipidic membranes, owing to the hydrophobic character conferred by its decanoyl chain. The interaction between the lipopeptide antibiotic and phospholipid membranes occurs despite the repulsion between its peptidic moiety and the negatively charged head groups of DMPG or PI. Insertion of the short decanoyl chain of

daptomycin molecules into the hydrophobic core of the bilayer results in an increased order of the portion of the acyl chains that is adjacent to the decanoyl chains, whereas the terminal portion of the long lipidic acyl chains (mostly palmitate and linoleate) becomes more fluid. The conformation of the peptidic moiety of daptomycin, however, experiences no significant change as seen from the amide I band. The steric constraints put on the PI head group by the bulky peptidic moiety of daptomycin causes the antisymmetric phosphate stretching band to be shifted to higher frequencies.

The next logical step was to examine the effect of daptomycin on gentamicin-phosphatidylinositol interaction. The spectral changes indicate that the presence of the bulky peptidic group of daptomycin at the bilayer surface does not prevent subsequent binding of gentamicin and reversely, daptomycin interacts with the bilayer in spite of prior treatment with gentamicin. The slower aggregation observed when pre-mixed gentamicin and daptomycin are added to PI dispersions suggests that achieving proper spatial organization of the three components is an arduous task. The amide I band of the infrared spectrum of daptomycin is unchanged in the presence of gentamicin nor does it show any change when the lipopeptide inserts in a lipidic environment. In the ternary complex, a slight narrowing of the amide I band is observed which may be due to steric constraints between gentamicin molecules adsorbed onto the lipidic surface and the peptide rings stemming out of the bilayer. The restoration of the fluidity of the membrane in the presence of daptomycin is probably an important effect in its role as a

nephroprotectant. There have been no reports in the literature on the effects of daptomycin on phospholipase A2 activity. Studies have indicated that daptomycin is safe and no deleterious side effects that are associated with enhanced phospholipase A2 activity have been identified during drug administration. Presumably there is a range of critical charge density and membrane fluidity optimal for PLA2 activity. This is not altered significantly by the insertion of the decanoyl chain of daptomycin into the hydrophobic core of phospholipid bilayers.

When both antibiotics are present in the lipid bilayer, we observe that the decrease in membrane fluidity previously observed in the presence of gentamicin is abolished. In fact, the fluidity of the membrane corresponds to that seen in the absence of the aminoglycoside drug. This is evident from the temperature dependence of the frequency of the methylene symmetric stretching mode of lipid acyl chains in samples with and without the mixture of antibiotics. The inner region of the bilayer core shows a fluidity resembling that obtained with gentamicin but the decrease of fluidity is of a smaller magnitude. As discussed earlier, a perturbation in the distribution and density of anionic charges required for interfacial activation and a decrease in the fluidity of membranes induced by the neutralization of PI head groups by gentamicin is probably responsible for the inhibition of phospholipase A2 activity. It is proposed that when both the aminoglycoside and lipopeptide drugs are present in the bilayer, the membrane is sufficiently destabilized for optimal phospholipase activity. In addition, the negative charges conferred by the 3-methyl glutamate and three aspartate residues in this

cyclic lipopeptide drug restores the critical charge density required for anchoring of the enzyme to the interface.

With the data available, it is impossible to determine how the peptide rings and gentamicin molecules are organized at the bilayer surface. Fluorescence studies have indicated a calcium dependent interaction between daptomycin and phospholipid membranes (Lakey and Ptak, 1988). The lipopeptide initially bound to the phospholipid membrane by its lipid tail is drawn further into the membrane by calcium bound to its negative residues. In addition, binding of daptomycin to bacterial cell walls and membranes requires calcium. FTIR spectroscopy can be used to study organization of the peptide rings at the bilayer surface in the presence of calcium and how the divalent cation affects its role as a nephroprotectant.

The mechanism of nephroprotection by poly-L-aspartic acid was also studied by Fourier transform infrared spectroscopy. Dialysis studies have demonstrated a direct interaction of polyaspartic acid with gentamicin (Kishore *et al.*, 1990a). The dependence on pH and ionic strength confirmed the electrostatic character of this interaction. Like with daptomycin, we have no spectral evidence of a direct interaction of polyaspartic acid with gentamicin in solution. The absence of significant spectral changes and the fact that both compounds remain in solution after mixing preclude tight interactions and direct neutralization of polyaspartic acid's carboxylates. Nevertheless, the formation of a loose complex cannot be

excluded, it would justify the dialysis studies and still be compatible with our spectroscopic study.

Although study of supernatants did not provide us with spectral evidence of a decreased binding of the aminoglycoside to PI bilayers in the presence of polyaspartic acid, the changes induced at the head group and interfacial region by the aminoglycoside are reduced in the presence of polyaspartic acid. The effect was more pronounced when polyaspartic acid was added first and smaller when gentamicin was added first. The result was intermediate when polyaspartic acid and gentamicin were mixed prior to addition to PI dispersions. The mechanism of nephroprotection by polyaspartic acid is different from that of daptomycin. The intralysosomal complexation of polyaspartic acid with gentamicin is probably the reason for decreased binding of the aminoglycoside to membranes. This would explain the reduction in the changes induced by gentamicin at the head group and bilayer interface in the presence of polyaspartic acid.

Conclusion

The mechanism underlying the inhibition of phospholipase A activity by gentamicin involves a modification of the "quality of the interface" which is not conducive to the incorporation of the enzyme. The neutralization of negative charges of PI head groups by cationic amino groups of gentamicin leads to a tightening of the lipidic network which is manifested by a decrease in the penetration of the solvent to the bilayer interface region. The decrease in the fluidity of PI bilayers induced by gentamicin is indicative of an increase in the order of the acyl chains consecutive to head group neutralization. Such an alteration in the distribution and density of negative charges and a decrease in the fluidity of membranes induced by gentamicin may be responsible for the inhibition of phospholipase activity by gentamicin. Although equilibrium dialysis experiments have failed to demonstrate significant differences in the binding parameters of the drug towards different negatively charged phospholipids (Mingeot-Leclercq *et al.*, 1990a), we have shown that at the molecular level there are significant differences in the drug-phospholipid interaction and the changes observed at the level of carbonyl groups, phosphate groups and acyl chain packing are unique to the interaction with phosphatidylinositol.

The mechanism of the inhibition of gentamicin nephrotoxicity by daptomycin involves an incorporation of the lipopeptide antibiotic into the hydrophobic core of the bilayer. Daptomycin does not impede the binding of gentamicin to PI bilayers

and gentamicin induces a slight change in the amide I band of daptomycin bound to the bilayers. The restoration of membrane fluidity in the presence of daptomycin is probably an important effect in its role as a nephroprotectant. In addition, the negative charges conferred by the 3-methyl glutamate and three aspartate residues in this cyclic lipopeptide drug restores the critical charge density required for anchoring of the enzyme to the interface.

The mechanism of protection against gentamicin induced nephrotoxicity by polyaspartic acid is different from that of daptomycin. The addition of polyaspartic acid to PI dispersions did not affect the spectral features of the lipid bilayers. However, the changes induced at the head group and interfacial region of the lipid by the aminoglycoside drug are reduced in the presence of polyaspartic acid. Based on the assumption that polyaspartic acid gains access to the lysosomes of proximal tubular cells as many small polypeptides do, protection against gentamicin induced nephrotoxicity may be obtained by the binding of the aminoglycoside to the polyanion in lysosomes, preventing phospholipidosis and interfering with the cascade of events leading to drug induced toxicity.

Different aminoglycosides in clinical use vary in their nephrotoxic potential. Future work involving a study of the interaction between PI and various aminoglycosides with different nephrotoxic potentials would help to correlate the extent of toxicity with changes observed at the molecular level.

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