overcoming bacterial permeability barriers in clinical practice.

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Résumé. In vivo aussi bien qu'in vitro, la triéthylène tétramine (TRIEN), agent qui forme des chélats avec les cations bivalents, renforce significativement l'activité antibactérienne de l'antibiotique carbénicilline envers la résistante *Pseudomonas aeruginosa.*

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Phlorizin Binding to Bilayer Vesicles of Phospholipids and Phospholipid-Cholesterol

Phlorizin inhibits sugar transport in kidney, intestine, and erythrocytes[1,2]. In erythrocytes, it also inhibits anion exchange and increases anion conductance[3,4]. However, the mechanism of its interaction with cell membranes is unclear. In renal cell membranes, phlorizin was found to bind not only to a specific, high-affinity protein receptor[5], but also to low-affinity sites of unknown nature[5,6], possibly including lipid. In this context, it seemed useful to study the binding of phlorizin to sonicated bilayer vesicles of phospholipids and phospholipid-cholesterol.

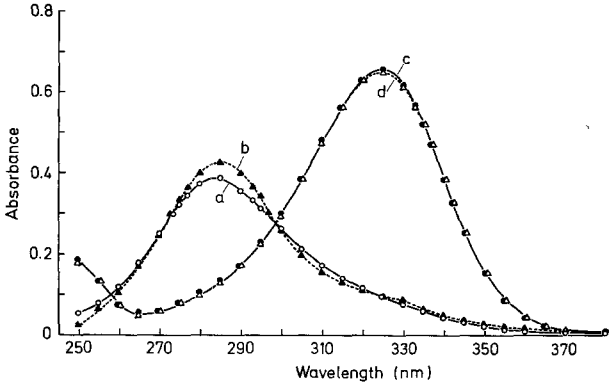

Fig. 1. Phlorizin absorbance spectra at pH 4.5 and 9.5 and the effect of PC vesicles. Phlorizin, 25 μM; phospholipid, 2 mM. At pH 4.5: a) keto-phlorizin; a_M, 1.62×10^4 M^{-1} cm^{-1} (285 nm); b) phlorizin and PC. At pH 9.5: c) enol-phlorizin; a_M, 2.73×10^4 M^1 cm^{-1} (325 nm); d) phlorizin and PC. To amplify changes in spectra, lipid concentrations were increased to 4 times that used in the binding studies.

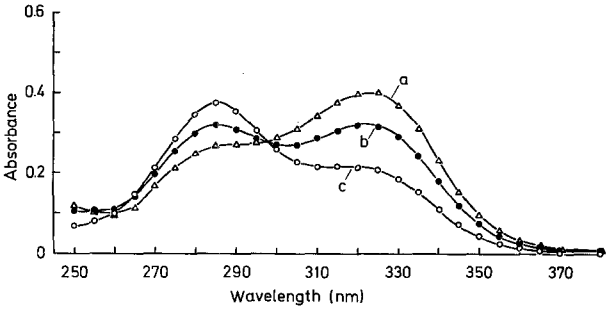

Fig. 2. Effect of PC and PS vesicles on the phlorizin absorbance spectrum at pH 7.2. Phlorizin, 25 μM; phospholipid, 2 mM. a) phlorizin; b) phlorizin and PS; c) phlorizin and PC.

Methods. Chromatographically pure egg yolk phosphatidylcholine (PC) and ox-brain phosphatidylserine (PS) were obtained from Lipid Products (South Nutfield, U.K.). Cholesterol (99%) (Merck) was recrystallized from methanol. Phlorizin (Roth) was purified to remove all traces of phloretin[4].

PC and PS dispersions in 50 mM KCl and 30 mM Glycylglycine buffer at pH 7.2 were sonicated at 5 °C under N_2 to minimum optical density[7,8]. Phlorizin binding to lipid vesicles was measured spectrophotometrically in double-chamber mixing cuvettes (Hellma) at 25 °C. The 2 compartments of the reference cuvette were filled with equal volumes (1 ml) of lipid vesicles and buffer. The sample cuvette contained vesicles in one chamber and phlorizin (20–75 μM) in the other. The absorbances of keto-phlorizin at 285 nm and of enol-phlorizin at 325 nm were measured before mixing and within 2 min afterwards. On the assumption that the enolic form of phlorizin is not bound to the lipid vesicles, the concentrations of free and bound keto-phlorizin were calculated from these absorbances[9].

Results and discussion. The absorption spectra of the keto and enol tautomers[10] of phlorizin are shown in Figure 1 (Curves a and c). Also shown is the effect of PC vesicles on the spectra. The spectrum of phlorizin plus lipid at pH 9.5 is identical to that of the enolic species alone. The spectrum of phlorizin plus lipid at pH 4.5 differs slightly from that of the ketonic species alone. These observations suggest that an association of the ketonic species with the phospholipid occurs, accompanied by a change in the molar absorbancy index (a_M). The effect of PS on a_M was qualitatively similar, but smaller. In the calculation of free and bound phlorizin concentrations, corrections were made for this small change in a_M.

The addition of liposomes to phlorizin at pH 7.2, close to the pK_a of phlorizin, lowers the absorbance at 325 nm and increases that at 285 nm (Figure 2). Together with

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Number and affinity of phlorizin binding sites of lipid bilayer vesicles

Preparations	K (l/mole) $\times 10^4$		n (μmoles/mmole PL)	
	PC	PS	PC	PS
Pure Phospholipids (5)	2.1 ± 0.2	1.0 ± 0.2	98 ± 8	112 ± 14
Phospholipids plus Cholesterol (1:1) (5)	1.5 ± 0.2	1.6 ± 0.3	86 ± 11	67 ± 13

The association constant (K) and the number of binding sites (n) were calculated from the least squares regression lines of the Scatchard plot. Values are means $\pm$ S.E. In parentheses number of measurements. PL represents total phospholipid. Lipid phosphorus was determined according to BARTLETT[15].

the data in Figure 1, the inverse variation of enolic and ketonic peaks strongly suggests that the uncharged, ketonic form of phlorizin binds rapidly to the phospholipid bilayers.

Figure 3 shows the relationship between bound phlorizin and the concentration of the free ketonic species. A Scatchard plot of the data is shown in the inset. As shown in the Table, the association constants of pure PC and PS bilayers differ significantly ($p < 0.005$), while the corresponding number of binding sites do not ($p > 0.4$). Since at pH 7.2 the vesicles of PC are isoelectric and those of PS negatively charged[11], the small difference in affinities suggests that the charge of the phospholipid head groups plays a minor role in the binding process.

Control experiments indicated that phlorizin penetration of the vesicles during the binding measurements is negligible. Thus, if it is assumed that all the phospholipid is present in the form of single bilayer vesicles of 250 Å diameter[8,12], about 70% of the total phospholipid would be on the outer vesicle surface[7,13], and hence be available for binding. Accordingly, PC and PS bilayer vesicles bind maximally about 150 μmoles/mmole phospholipid or 1 phlorizin per 7 phospholipid molecules.

Cholesterol (1:1 molar ratio) lowers the phlorizin affinity of PC bilayers and raises that of PS to resultant values which are not significantly different from each other ($p > 0.7$). For the range of phlorizin concentrations studied, Scatchard analysis again indicated a homogeneous population of binding sites. The presence of cholesterol, does not alter the binding capacity per phospholipid molecule of PC bilayers significantly ($p > 0.4$); however, it tends to reduce that of PS bilayers by about 40% ($p < 0.05$). If one assumes a size of about 250–300 Å for the cholesterol containing vesicles[14], one can calculate that cholesterol-PS vesicles bind maximally only 96 μmoles ketophlorizin/mmole PS or 1 phlorizin per 10 PS molecules.

Zusammenfassung. Spektrophotometrische Messungen an Phospholipid-Doppelschichten zeigten, dass die Keto-Form von Phlorizin bevorzugt gebunden wird. Phosphatidylcholin und Phosphatidylserin besitzen beide eine Bindungskapazität von etwa 1 Molekül Phlorizin pro 7 Moleküle Phospholipid. Ihre Affinitäten für Phlorizin sind 2.1×10^4 bzw. 1.0×10^4 l/mole. Die Anwesenheit von Cholesterin verändert die Bindungsparameter.

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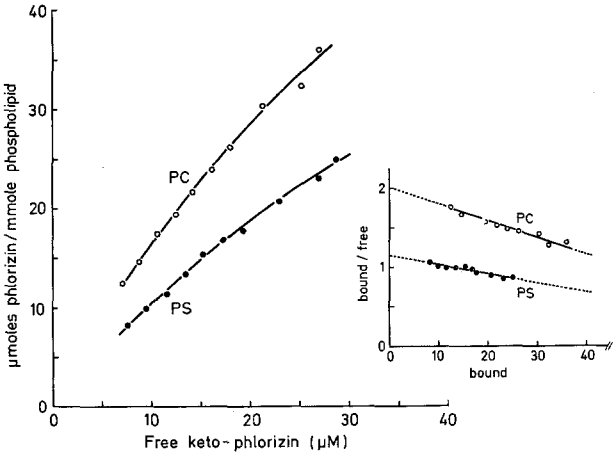

Fig. 3. Concentration dependence of phlorizin binding. Graph depicts data of a representative experiment. A Scatchard plot of the data is shown in the inset with ordinate: bound phlorizin (μmole/mmole)/free keto-phlorizin (μM); abscissa: μmoles phlorizin bound/mmole total phospholipid. Phlorizin, 20–75 μM; phospholipid, 0.5 mM.

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