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The Effect of Monomers and of Micellar and Vesicular Forms of Non-ionic Surfactants (Solulan C24 and Solulan 16) on Caco-2 Cell Monolayers

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Abstract

Measurements of transepithelial electrical resistance (TEER), the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) test and monitoring of poly(ethylene glycol) (PEG) transport have been used to study the effects of the non-ionic surfactants Solulan C24 and Solulan 16, either free in solution or as an integral part of niosome bi-layers, on intestinal epithelial cells from man (Caco-2 cell monolayers).

The effects on epithelial integrity and on the transport of the hydrophilic drug metformin depend on the concentration of the surfactants. At concentrations above 1% the effect on TEER of the surfactant in niosomal form and free in solution were equivalent whereas cell viability was preserved to a higher concentration of Solulans when the Solulans were present in the niosomal form.

It was concluded that the toxic effect of niosomes arises from free surfactant present in the niosome suspension.

A wide variety of surfactants is used to enhance drug absorption across epithelial barriers (Attwood & Florence 1983; Anderberg & Artursson 1993; Anderberg et al 1992, 1993a, b). Although the lower toxicity of non-ionic surfactants makes them more valuable in pharmaceutical formulations than their ionic counterparts, they nevertheless affect the permeability of barrier membranes, such as the intestinal epithelium, in a concentration-dependent manner. There is no clear indication whether or not there is a connection between enhanced permeability and membrane damage or whether changes in permeability are reversible. Recent studies with Caco-2 cell monolayers indicate, however, that several commonly used pharmaceutical surfactants increase permeability by the paracellular pathway, primarily by affecting tight junctions (Anderberg & Artursson 1993; Hochman & Artursson 1994).

Here we are interested in the effect of two non-ionic surfactants presented as monomeric or as micellar solutions and as components of the bi-layers of non-ionic surfactant vesicles (or niosomes). These vesicles consist of one or more bilayers of non-ionic surfactants which can encapsulate both hydrophilic and lipophilic compounds (Florence 1993). Non-ionic surfactant vesicles have been studied for some time as an approach to enhancing the delivery of poorly absorbed drugs, but few studies of the toxicity of such systems have been published.

The purpose of this study was to use the well established Caco-2 cell culture model of intestinal epithelium in man to investigate the effects of the surfactants Solulan C24 and Solulan 16 on the permeability and well-being of intestinal epithelium. Both surfactants were studied in solution and niosomes. The Caco-2 cell line, derived from a colorectal carcinoma in man, is most commonly used in studies of drug absorption and absorption enhancement (Hidalgo et al 1989; Artursson 1990; 1991; Artursson et al 1996).

Solulan C24, a cholesteryl poly(24)oxyethylene ether has been included in vesicle formulations studied in our laboratories for some time (Cable & Florence 1988; Uchegbu & Florence 1995). Their effect was determined by measuring the integrity of the Caco-2 monolayers during and after exposure to increasing concentrations whether in solution or as an integral part of the niosome formulation. The integrity of the monolayers was assessed by measuring trans-epithelial electrical resistance, the transport of ^{14}C-labelled polyethylene glycol through the cell monolayer, and by observing the cells by use of transmission, scanning electron and fluorescence microscopy. An intracellular enzyme activity test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide or MTT test) was used to assess the relative cytotoxicity of these surfactant systems towards enterocytes. Metformin (dimethylbiguanide), an antihyperglycaemic agent which is now used widely for the treatment of non-insulin-dependent diabetes mellitus, was used as a hydrophilic drug marker, the passage of which across the Caco-2 cell monolayers under the influence of the surfactant has been monitored (Noel 1979; Tucker et al 1981).

Materials and Methods

Materials

Dulbecco's modified Eagle medium, gentamicin (10 mg mL^{-1}), non-essential amino acids, foetal calf serum, trypsin/EDTA and Hank's balanced salt solution were obtained from Gibco Life Technologies, Paisley, Scotland. Solulan C24 (cholesterol poly(24)oxyethylene ether) and

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Solulan 16 (poly(16)oxyethylene ether of lanolin alcohols) were obtained from Anstead UK. $C_{16}G_2$ was a gift from L'Oreal, Paris. Sorbitan monooleate (Span 80), cholesterol, trypan blue and diphenyltetrazolium bromide were obtained from Sigma, UK, and used as received. [^{14}C]Poly(ethylene glycol) (PEG; MW-4000; 60 mCi mmol^{-1}) was obtained from Amersham International, UK. Hionic-fluor liquid scintillation fluid was obtained from Packard Instrument Company. Transwell polycarbonate cell culture inserts were obtained from Costar, Cambridge. [^{14}C]Metformin hydrochloride (66 mCi mmol^{-1}) was a gift from Lipha Pharmaceuticals, UK.

Preparation of niosomes

In the first group of experiments Solulan C24 and Solulan 16 were used as components of niosomes; sorbitan monostearate (Span 80) was the primary vesicle-forming surfactant. Typically the weights used were: Span 80, 0·028 g; cholesterol, 0·026 g; Solulan C24 or Solulan 16, 0·020 g. The surfactant-cholesterol mixture was dissolved in chloroform in a round-bottomed flask and the solvent was removed at room temperature under reduced pressure in a rotary evaporator. The dried surfactant film was hydrated with Hanks balanced salt solution (2 mL) at 55–60°C with gentle agitation giving a concentration of 1% of Solulan C24 or Solulan 16.

In the second group of experiments the three components were used for the preparation of niosomes: cholesterol, hexadecyl diglyceryl ether ($C_{16}G_2$) and Solulan C24. The dried lipid film prepared as above was hydrated with Hank's balanced salt solution (10 mL). Five different formulations were prepared in the molar ratios Solulan C24-cholesterol-$C_{16}G_2$: 40:30:30, 30:35:35, 20:40:40, 10:45:45 and 5:47·5:47·5, providing a range of concentrations of Solulan C24 from 0·43% to 3·46%. The size of niosomes was determined by laser-light scattering (Mastersizer X, Malvern Instruments, UK).

Determination of critical micellar concentration (CMC)

A Cahn DCA-312 System was used to determine the surface tension of solutions of Solulan C24 and Solulan 16 in distilled water at 23 ± 1°C. The CMC values, obtained from plots of surface tension against log (surfactant concentration) were 0·009% and 0·01% w/v for Solulans C24 and 16, respectively.

Osmolality measurement

The osmolality of the different samples of niosomes and free Solulan C24 was determined with an electronic osmometer (Knauer), calibrated with a 400 milliosmols kg^{-1} solution of NaCl.

Cells

Caco-2 cells were maintained in Dulbecco's modified Eagle medium supplemented with 1% non-essential amino acids, 10% foetal calf serum and 50 μg mL^{-1} gentamicin. Caco-2 cells of passage number 70–75 were used. If the cell viability was satisfactory (assessed by the trypan blue exclusion method; Freshney 1991) the cells were cultured on a permeable cell culture insert (Transwell, diameter 12 mm, pore diameter 0·4 μm). A cell suspension (0·5 mL; 1 × 10^6 cells mL^{-1}) was added to the apical sides of the cell culture inserts and the cells were allowed to grow and differentiate for up to 30 days; re-feeding was performed on alternate days.

Transepithelial electrical resistance (TEER)

The integrity of the mono-layers was determined by measurement of the potential difference between their apical and the basolateral sides, as described previously (Artursson 1990). TEER was measured each hour during the experiments and was expressed as a percentage of TEER at t = 0.

Absorption studies

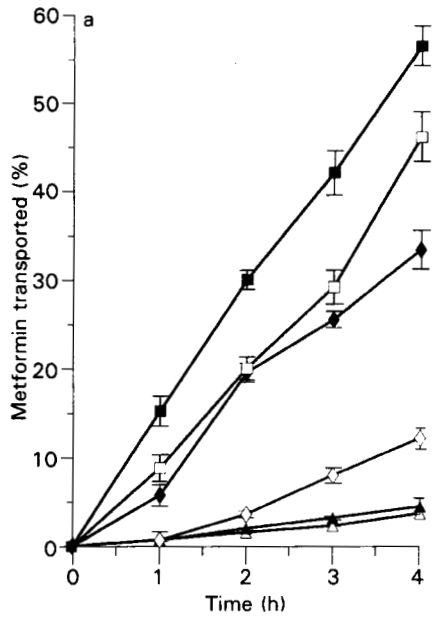
The transepithelial transport of a macromolecular marker, poly(ethylene glycol) (MW 4000) was measured to assess mono-layer integrity. The antihyperglycaemic agent metformin was used as a model hydrophilic drug. [^{14}C]PEG was added to the apical side of the mono-layers and the transport of the radiolabelled marker was followed for up to 3 h at 37°C. Solutions were prepared from a solution of the radiolabelled isotope [^{14}C]metformin and the corresponding unlabelled compound in Dulbecco's modified Eagle medium to give a final concentration of 100 mM. Absorption was followed for 4 h. The radiolabelled drug solutions (control) and mixtures of metformin and surfactants, Solulan C24 and Solulan 16, were added to the apical chamber. Samples (450 μL) were taken from each basolateral chamber (and replaced with fresh medium) and measured in a liquid scintillation counter (Beckman). Inserts without cells were used also to determine the transport of PEG during the same time period across the filter alone. The results were expressed as percentage of the dose transported from the apical to basolateral chambers.

Intracellular enzyme activity (cell viability)

Intracellular dehydrogenase activity was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-2H-tetrazolium bromide) test (Mosmann 1983). MTT is reduced by mitochondrial succinate dehydrogenase in living, but not dead, cells to give a dark-blue product, formazan. The amount of formazan produced is proportional to the number of viable cells present. Immediately after incubation MTT (5 mg mL in PBS; 100 μL) was added and the plates were incubated for another 5 h (37°C, 10% CO_2). The solutions were removed and dimethylsulphoxide (100 μL) was added to dissolve the formazan. The optical density at 550 nm was measured on a microtitre plate-reader.

Results and Discussion

Caco-2 cells are derived from a colonic adenocarcinoma in man, but when grown on permeable supports they have morphological as well as functional similarities to intestinal enterocytes. Assessment of the integrity of the Caco-2 cell monolayers was performed by measuring PEG transport and TEER (Hidalgo et al 1989; Artursson 1990). Our PEG transport study indicated that very little PEG (less than 0·065% h^{-1}) crossed the cell monolayer during the period of incubation. Under the experimental conditions the trans-epithelial electrical resistance was high (> 300 Ω cm^{-2}). Resistances ranging from 150 Ω cm^{-2} to up to 600 Ω cm^{-2} have been reported (Artursson et al 1996). Therefore, the results showed that the monolayers were intact and the tight junctions fully developed.



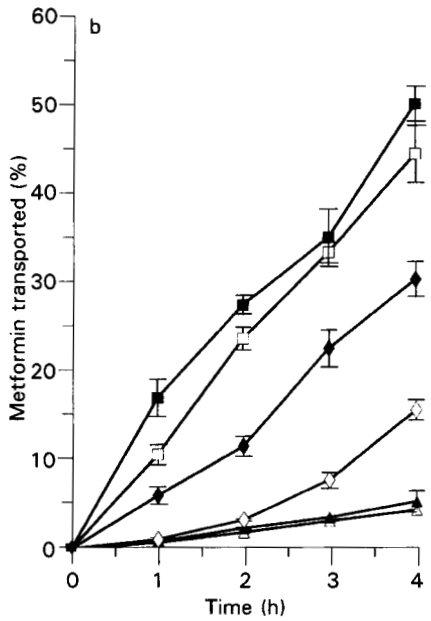
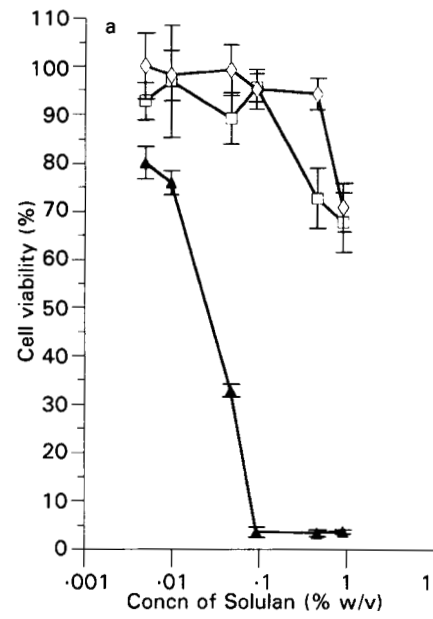


FIG. 1. Transport of [^{14}C]metformin across Caco-2 cell mono-layers as a function of the concentration of Solulan C24 (a) and Solulan 16 (b). △ Control, ▲ 0·005, ◇ 0·01, ◆ 0·05, □ 0·1, ■ 0·5%.



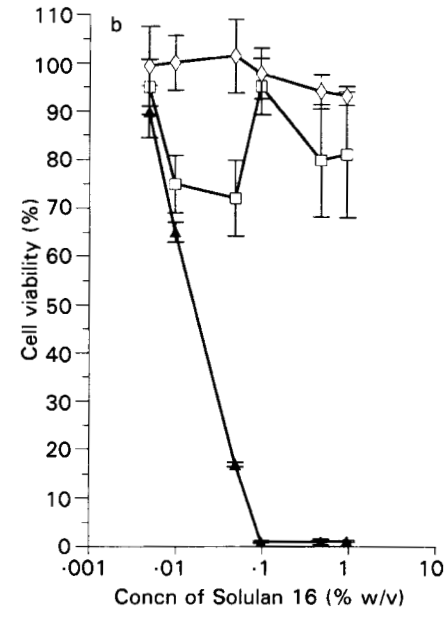


FIG. 2. Cytotoxicity of Solulan C24 (a) and Solulan 16 (b). ▲ Solulan, ◇ Solulan in 300-nm niosomes, □ Solulan in 500–1000-nm niosomes (incubated for 3 h).

The absorption-enhancing effect of Solulan C24 and Solulan 16 was evaluated by use of [^{14}C]metformin. Transport of hydrophilic molecules across epithelial mono-layers is mainly limited to the paracellular route. The osmolality of the incubation medium (Solulan C24 or Solulan 16 at concentrations 0·005, 0·01, 0·05, 0·1 or 0·5%) and solutions of metformin (control) were determined before each experiment to establish that hypo- or hyper-osmolality was not the reason for any change in the transport of metformin. The osmolality of all solutions was approximately 300 mosmol kg^{-1}, i.e. they were isoosmotic.

Exposure to different concentrations of Solulan C24 resulted, as expected, in transport of metformin that was dependent on Solulan concentration (Fig. 1a). Large increases in the transport of metformin were related to the effect of Solulan C24 on the epithelial cell monolayers, especially for the concentrations 0·05, 0·1 and 0·5%. Fig. 2a shows the results obtained from use of MTT assay to measure viability or toxicity. A significant reduction in cell viability was observed as the concentration of Solulan C24 was increased. At concentrations of Solulan C24 of 0·1 and 0·5% no viable cells survived in the test; at a concentration of 0·05% only 30% of

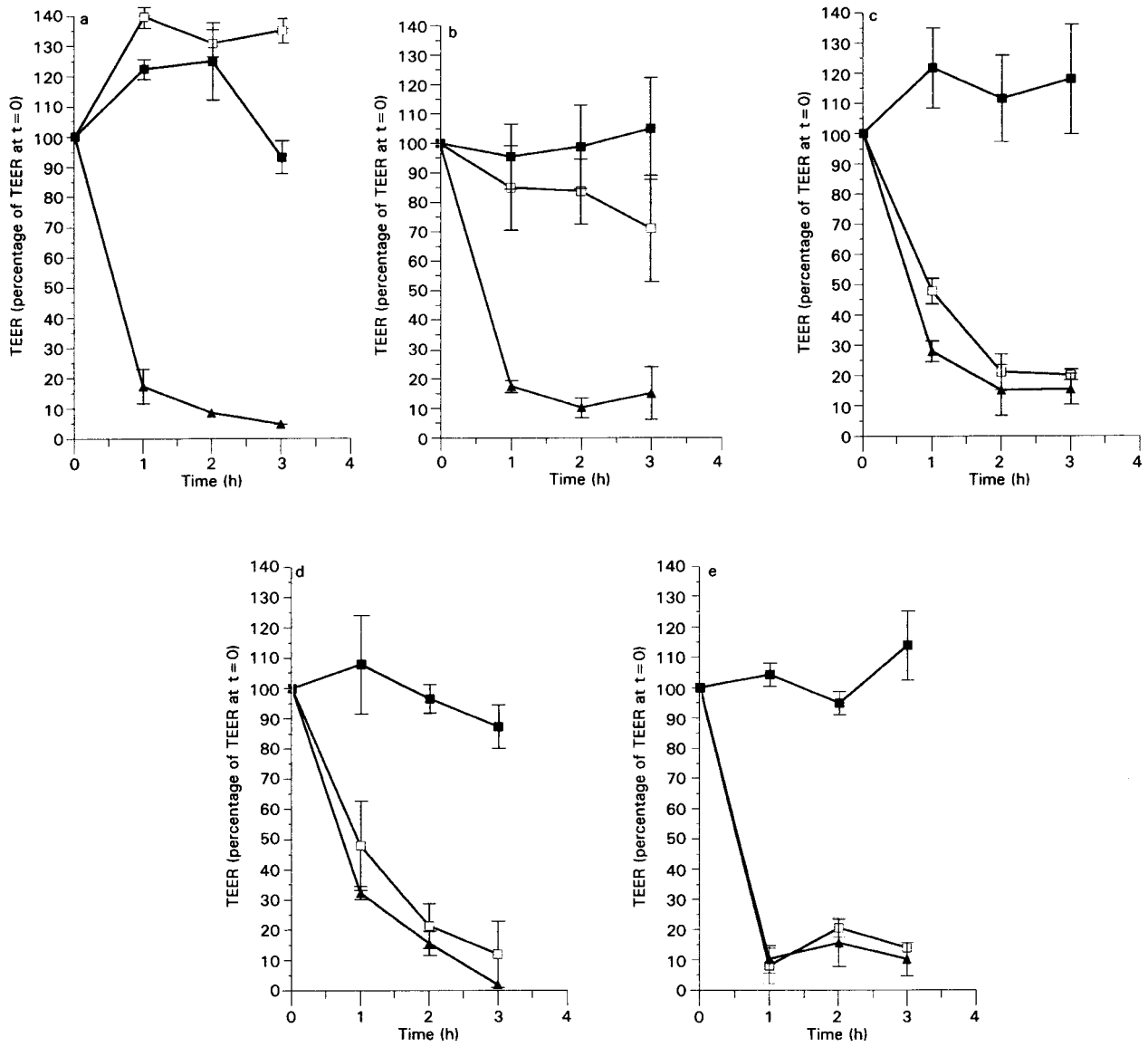


FIG. 3. Effect of Solulan C24 on the transepithelial electrical resistance of Caco-2 cell monolayers as a function of time and surfactant concentration. a. 0·43, b. 0·8, c. 1·7, d. 2·6 and e. 3·46%, w/v. ▲ Solulan C24, □ Solulan C24 in niosomes, ■ control cells.

the cells were viable. Similar effects on cell-layer permeability, metformin transport and cell viability were found with Solulan 16 (Figs 1b and 2b).

Cholesteryl poly(24)oxyethylene ether (Solulan C24) can increase the absorption of octreotide (a somatostatin analogue) across Caco-2 monolayers after oral administration to rats and man (Drewe et al 1993). Administration of octreotide in the presence of Solulan C24 resulted in an approximately 23-fold increase in bioavailability in rats and an 8-fold increase in man, but no detailed mechanistic and cytotoxicity study has been reported to date. It is known that surfactants exert their effects on drug absorption by modification of mucosal permeability or interaction with the drug (Florence 1981; Attwood & Florence 1983). In general, surfactants increase the permeability of the intestinal membrane in a concentration-dependent manner, but not all non-ionic surfactants enhance the absorption of the drug when the surfactants are present at concentrations above their critical micelle concentration (CMC). When a small amount of surfactant is present (below the CMC) the molecules of detergent are incorporated into the membrane 'fluidizing' the structure. Higher concentrations result in the occurrence of mixed protein-lipid-surfactant micelles in equilibrium with surfactant micelles and free surfactant molecules (Gulik-Kraywicki 1975; Helenius & Simons 1975). This results in membrane solubilization. According to surface-tension measurements the CMCs for Solulans C24 and 16 were 0·009 and 0·01%, respectively. Toxic effects were most pronounced above the CMCs (Figs 1b, 2b and 3).

To compare the effect of free Solulan C24 and Solulan 16 in solution with the effect of niosomes containing the Solulans, vesicles of span 80 and cholesterol containing a range of concentrations of Solulan C24 and Solulan 16 were studied. In our study we used two niosome preparations, one with a mean size of 300 nm, another with a broader distribution and mean

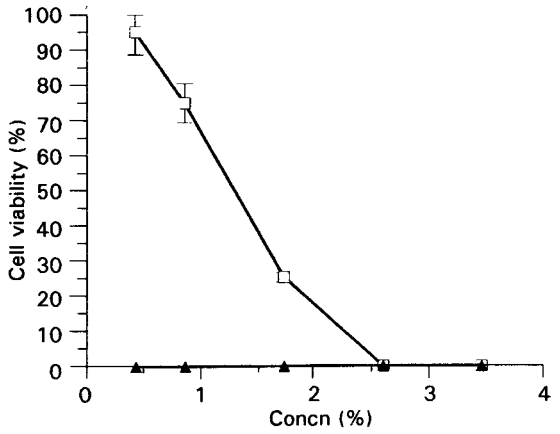


FIG. 4. Cytotoxicity of Solulan C24. ◆ Solulan C24 in solution (samples 1–5), □ Solulan C24 in niosomes (samples 1–5) (incubated for 3 h).

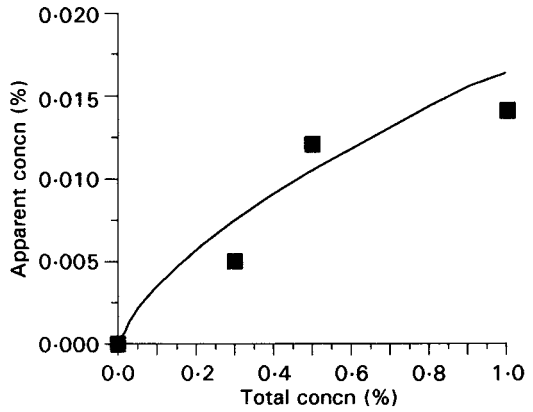


FIG. 6. The apparent free concentration of Solulan C24 as function of the total concentration of Solulan in niosomes. The data have been derived from Fig. 2a assuming that the main niosomal components are inactive in the system. The line can be considered to be representative of the thermodynamic activity of the Solulan in the vesicular system.

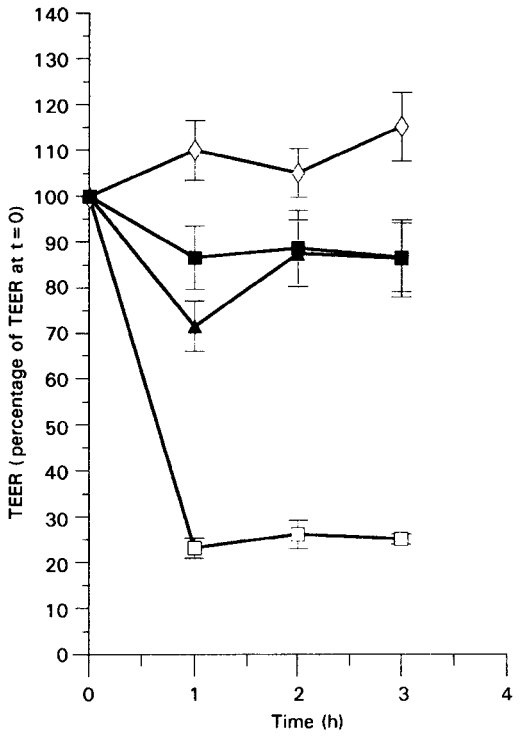


FIG. 5. Effect of pellet, supernatant and niosomes with no Solulan C24 on trans-epithelial electrical resistance. ▲ Pellet (sample 1), □ supernatant (sample 1), ◇ niosomes with no Solulan C24, ■ control cells.

between 0·5 and 1 μm. Fig. 2a shows that the viability of Caco-2 cells treated with Solulan C24 in niosomes was approximately 100% for all Solulan C24 concentrations irrespective of niosome size. A slight reduction in viability of up to 30% was noticed with 0·5 and 1% of Solulan C24 in niosomes. Similar effects were observed with Solulan 16 (Fig. 2b). The smaller niosomes appeared to exert less effect on cell viability than the larger vesicles. Free Solulan C24 or 16 at a level of 0·1% was very toxic to the cells, whereas the same concentrations of Solulan C24 or 16 in niosomes had no effect on the cell viability. However, some toxicity was observed for higher levels of Solulan C24 and 16 in niosomal form.

In the second part of our study we used several formulations of Solulan C24, cholesterol and $C_{16}G_2$ to compare the toxicity of Solulan C24 in niosomes and in a solution. Five samples of each form were prepared with increasing concentrations of Solulan C24 (0·43%, 0·8%, 1·7%, 2·6% and 3·46%). Osmolality measurement showed that all samples were isoosmotic. The size of the niosomes was approximately 6 μm. The TEER changes shown in Fig. 3 mainly reflect changes in paracellular, but also in transcellular, permeability. If the cell membrane is made permeable, e.g. by an absorption enhancer, the passive ion permeability across the cell membrane will increase, with a concomitant reduction in TEER (Anderberg et al 1992). Free Solulan C24 caused a significant reduction of TEER, to approximately 5% of the initial value (Figs 3a, b), whereas Solulan C24 at the same concentrations in niosomes did not have any significant toxic effects (assessed by TEER and MTT tests). For Solulan C24 concentrations of 1·7, 2·6 and 3·46% a reduction of TEER and cytotoxicity (MTT) was observed with the surfactant free in solution or as part of niosomes (Figs 3c, d, e and 4). In all experiments the TEER of control samples (with no Solulan C24 added) did not changes with incubation time. In fact, above a Solulan content of 1%, their presence in the vesicles or in the free form made little difference to their effect. Cytotoxicity measurements (MTT) (Fig. 4) have shown that free Solulan C24 was toxic at all concentrations used whereas a declining number of viable cells was observed with increasing concentration of Solulan C24 in niosomes.

To study the cause of the toxic effect, we prepared niosomes with the highest concentration of Solulan C24 (3·46% of Solulan C24, sample 1) and niosomes without Solulan C24 ($C_{16}G_2$-cholesterol-DCP in mol% 49:49:2). Sample 1 was centrifuged twice-1 h at 50 000 rev min^{-1} (supernatant was withdrawn) and then for 20 min at 20 000 rev min^{-1} (at 4°C) to separate the niosomes. The niosomes was resuspended in Hank's balanced salt solution buffer. The osmolalities of the three samples (niosomes without Solulan C24, pellet, supernatant) were approximately 300 mOsm kg^{-1}. Each sample (0·5 mL) was added to the cell culture inserts and the TEER was followed for 3 h (Fig. 5). A reduction of TEER was

observed with the supernatant whereas the pellet and the niosomes with no Solulan C24 had no significant toxic effect. It can be concluded that the toxic effect of niosomes is principally a result of the amount of free surfactant present in the niosomes suspension.

Solulan C24, as an example, is incorporated to the bilayer structure of the vesicles. In the vesicular preparations it is not free to act: its thermodynamic activity is reduced. Some concentration-dependence of effect of Solulan in vesicles is apparent from Fig. 2a. In Fig. 6 these data are plotted as the apparent free concentration of surfactant against the total concentration in the niosome suspension. This assumes that the sorbitan monooleate (Span 80) as the niosome-forming surfactant is biologically inactive in the test system.

Acknowledgements

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