Chitosans as Absorption Enhancers for Poorly Absorbable Drugs. 1: Influence of Molecular Weight and Degree of Acetylation on Drug Transport Across Human Intestinal Epithelial (Caco-2) Cells

Nicolaas G. M. Schipper,[1,3] Kjell M. Vårum,[2] and Per Artursson[1]

Received May 10, 1996; accepted August 26, 1996

Purpose. Chitosan has recently been demonstrated to effectively enhance the absorption of hydrophilic drugs such as peptides and proteins across nasal and intestinal epithelia (1–3). In this study, the effect of the chemical composition and molecular weight of chitosans on epithelial permeability and toxicity was investigated using monolayers of human intestinal epithelial Caco-2 cells as a model epithelium.
Methods. Eight chitosans varying in degree of acetylation (DA) and molecular weight were studied. The incompletely absorbed hydrophilic marker molecule ^{14}C-mannitol was used as a model drug to assess absorption enhancement. Changes in intracellular dehydrogenase activity and cellular morphology were used to assess toxicity.
Results. Chitosans with a low DA (1 and 15%) were active as absorption enhancers at low and high molecular weights. However, these chitosans displayed a clear dose-dependent toxicity. Chitosans with DAs of 35 and 49% enhanced the transport of ^{14}C-mannitol at high molecular weights only, with low toxicity. One chitosan (DA = 35%; MW = 170kD) was found to have especially advantageous properties such as an early onset of action, very low toxicity, and a flat dose-absorption enhancement response relationship.
Conclusions. The structural features of chitosans determining absorption enhancement are not correlated with those determining toxicity, which makes it possible to select chitosans with maximal effect on absorption and minimal toxicity.

KEY WORDS: mucosal permeability; oral absorption; cell culture; structure-activity.

INTRODUCTION

Passive absorption of drugs across mucosal tissues involves either trans- or paracellular diffusion through one or several epithelial cell layers. The transcellular route is mainly restricted to small hydrophobic compounds capable of passing through the lipophilic cell membrane (4). This leaves the paracellular route for absorption of hydrophilic compounds. The paracellular route, however, comprises only a small part of the epithelium, and passage of molecules is limited by tight junctions that seal epithelial cells at their apical surfaces (5). Consequently, the absorption of orally administered hydrophilic drugs, such as peptides and proteins is severely limited by the low permeability of the intestinal mucosa to such compounds. Many efforts have been made to improve the uptake of poorly absorbed drugs; the coadministration of absorption enhancing compounds is one of the better studied approaches. Numerous classes of compounds that enhance the intestinal uptake of hydrophilic drugs such as antibiotics, peptides, and proteins have been described (6). However, in most cases, drug absorption enhancement is accompanied by mucosal damage induced by the enhancer (7). Nevertheless, a few compounds, including the fatty acid sodium caprate and long chain acylcarnitines, have been shown to improve absorption without obvious harmful effects to the intestinal mucosa (8).

Recently, chitosan has been studied as a potential enhancer of mucosal drug absorption (1–3). Chitosan is a linear polysaccharide made by N-deacetylation of chitin (1:4-linked 2-acetamide-2-deoxy-β-D-glucopyranose (GlcNAc)), resulting in a copolymer of GlcNAc and 2-amino-β-D-glucopyranose (GlcN). Fully water soluble chitosans with degrees of acetylation (DA; i.e., molar content of GlcNAc), from 0 to 60% and known random distribution of GlcNAc and GlcN units have been prepared in order to study the effect of chemical composition of chitosans on their physical and biological properties (9–12). Chitosan is a polycation at acidic pH values, with an intrinsic pKa value independent of DA of approximately 6.5 (13, 14). It has been employed in many studies as a pharmaceutical excipient for the dissolution and sustained release of drugs (15). It is believed to be non-toxic and is approved as a food additive in Japan (15). This is perhaps surprising since other cationic polymers such as poly-L-lysine displayed pronounced toxicity in a variety of studies (16, 17). Chitosan has mucoadhesive properties, which are probably mediated through ionic interactions between positively charged amino groups in chitosan and negatively charged sialic acid residues in mucus or on cell surfaces (18). Studies employing chitosan as an absorption enhancer showed that the absorption of insulin and a decapeptide across nasal and intestinal mucosa, respectively, could be increased (1, 2). The chitosan used in these studies did not cause significant changes in nasal mucosal histology at concentrations that improved nasal insulin absorption. The mechanism of action was proposed to be a combination of mucoadhesion and an effect on tight junctions in the epithelium (3).

In a previous study, chitosan increased the transport of a hydrophilic marker molecule across monolayers of a cultured intestinal epithelial cell line (Caco-2) (3). The increase in epithelial permeability was dependent on the pH of the chitosan solutions. The influence of chitosan on transport of the marker molecule was strongest when the pH was well below the pKa of 6.5. This suggests that charge density may be of importance for enhancement of mucosal absorption. Besides pH, the charge density of chitosan is also controlled by varying the DA, since only the GlcN-units are positively charged. In this study, the influence of these parameters on the effects of chitosans on epithelial permeability and toxicity was investigated in monolayers of the human intestinal epithelial cell line Caco-2. Chitosan polymers with DA between 1 and 49% were used. Each was prepared in a low and a high molecular weight form. The results indicate that the structural features determining absorption enhancement are not correlated with those determin-

[1] Department of Pharmaceutics, Uppsala University, Biomedical Center, P.O. Box 580, S-75123 Uppsala, Sweden.
[2] Norwegian Biopolymer Laboratory (NOBIPOL), Dept. of Biotechnology, The Norwegian Institute of Technology (NTH), University of Trondheim, 7034 Trondheim, Norway.
[3] To whom correspondence should be addressed.

0724-8741/96/1100-1686$09.50/0

ing toxicity, which makes it possible to select chitosans showing a maximal effect on absorption with a minimal toxicity.

MATERIALS AND METHODS

Materials

Chitosans were prepared by heterogeneous N-deacetylation, and the acid-soluble fraction prepared as previously described (19). The chitosans were converted to the chloride salts. The average molecular weight was determined from intrinsic viscosity measurements. Chitosan is abbreviated in this work as a C followed by two numbers in brackets. The first number is the degree of acetylation as a percentage in molar basis, the second number the average molecular weight in kD. The chitosans may be divided into 4 pairs with the same degree of acetylation, each pair containing chitosans of low and high molecular weight. ^{14}C-mannitol (271 mCi/g) was obtained from New England Nuclear (Boston, MA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO).

Preparation of Chitosan Solutions

Chitosans were dissolved in a Hanks' balanced salt solution that contained Ca and Mg ions (HBSS). The HBSS solution was buffered with 25 mM 2N-morpholino-ethanesulfonic acid (MES) to pH = 5.5. Clear solutions of 0.5 and 1.0 mg/ml chitosan were obtained by vigorous shaking for 24 to 48 hours at room temperature. Stock solutions were stored at −20°C. Just before the experiments the chitosan solutions were thawed and diluted to the appropriate concentrations. Control solutions contained HBSS pH = 5.5 without addition of chitosan.

Cells

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD). The cells were cultured on polycarbonate filters with a filter diameter of 6.5 mm (Transwell cell culture inserts, Costar, Cambridge, UK) as described by Artursson (20). Cells of passage number 93–105 were used throughout. The cells were used for experiments 21–35 days after seeding.

Transport Studies

All transport experiments were performed in HBSS. Solutions used at the basolateral side of the monolayers were buffered with 25 mM N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) (HEPES) to pH = 7.4. The apical solutions were buffered with 25 mM MES to pH = 5.5. This pH is comparable to the pH of the small intestinal "microclimate" and was necessary to keep all chitosans in solution. Prior to the experiments the cells were washed and equilibrated for 15 min with HBSS pH = 5.5 on the apical side and with HBSS pH = 7.4 on the basolateral side. The experiments were initiated by replacing the apical fluid with a HBSS pH = 5.5 medium containing a ^{14}C-mannitol/chitosan solution. Controls were run in every experiment using ^{14}C-mannitol in HBSS pH = 5.5 without chitosan. Transport studies were performed over 120 min at 37°C and 95% relative humidity in air. Samples were taken at regular intervals from the basolateral side and the radioactivity was measured in a liquid scintillation counter (Tricarb, 1900CA, Packard Instrument, Downers Grove, IL, USA). The apparent permeability coefficient (Papp) was calculated using the following equation: Papp (cm/sec) = dQ/dt/(A.Co), in which dQ/dt (mol/sec) is the rate of appearance of mannitol on the basolateral side, Co (mol/ml) is the initial mannitol concentration on the apical side, and A (cm^2) is the surface area of the monolayer. Since the effects of some chitosans on permeability were time dependent, the Papp values were calculated for each time point separately. In addition, a "mean" Papp was calculated for the first 60 min period of the experiment for easy comparison of the data. In control experiments with pH = 5.5 in the apical and pH = 7.4 in the basolateral HBSS, the permeability of the hydrophilic marker molecule ^{14}C-mannitol was very low with Papp values being between 0.8 and 2.5 × 10^{-7} cm/sec. ^{14}C-mannitol permeabilities at pH = 5.5 or pH = 7.4 in the apical bathing solution were comparable.

Intracellular Dehydrogenase Activity

The effect of chitosans on intracellular dehydrogenase activity was determined by the MTT method. MTT is a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in living cells to give a dark blue formazan product (21). Damaged or dead cells show reduced or no dehydrogenase activity. The assay was performed as described earlier (22). Briefly, Caco-2 cells were seeded in 96-well tissue culture plates (Flow Laboratories, Irvine, U.K.) at a density of 50,000 cells/well. After 24 h they were exposed to varying concentrations of chitosans in 50 µl HBSS pH = 5.5 for 60 min at 37°C in air. The cells were washed with 100 µl HBSS pH = 7.4, and incubated for another 60 min at 37°C in air with 50 µl of 1 mg/ml MTT in HBSS pH = 7.4. Formazan crystals were dissolved by addition of 100 µl 1% sodium dodecyl sulphate in 0.02 M HCl/isobutanol 1:1 solution. The developed colour was measured at 590 nm using a multiwell scanning spectrophotometer (Multiscan MCC/340, Labsystems Oy, Helsinki, Finland).

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed to study the effects of the chitosans on cellular morphology. Following exposure to the chitosan solutions for 60 minutes, Caco-2 cell monolayers were washed with PBS and fixed with 1.5% (w/v) glutaraldehyde in PBS and 1% osmium tetroxide in PBS for 1 h. Subsequently, the monolayers were dehydrated with a series of ethyl alcohols at different concentrations and embedded in Epon. Ultrathin sections were contrasted with 1% uranyl acetate in PBS and examined using a Philips 420 electron microscope at 60 keV.

Statistical Evaluation

All data are expressed as the mean ± s.d. Statistical differences were investigated using one-way ANOVA followed by Scheffe's *F*-test for multiple comparisons. Differences between group means were judged significant at $P < 0.05$.

RESULTS

Transport Experiments

Some, but not all, chitosans improved the transport of the hydrophilic model drug ^{14}C-mannitol across Caco-2 cell

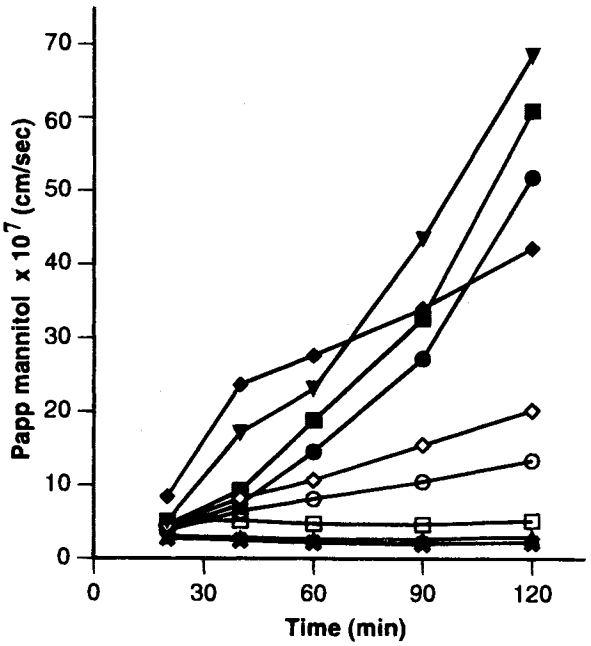

Fig. 1. Time courses for the effects of chitosans (50 μg/ml) on the permeability of mannitol in Caco-2 cell monolayers. Data are given as means of 3–4 experiments. Error bars have been omitted for reasons of clarity. Standard deviations are given in Table I. Control (×); C(1:31) (■); C(1:170) (●); C(15:4.7) (◇); C(15:190) (▼); C(35:12) (▲); C(35:170) (◆); C(49:22) (□); C(49:98) (○).

monolayers. The effects of the chitosans on epithelial permeability were concentration dependent and became more pronounced with longer exposure times (Fig. 1, Table I).

At a concentration of 10 μg/ml, ^{14}C-mannitol permeability was not changed by any chitosan but C(15:190). The latter increased the transport of the marker approximately two fold. At a concentration of 50 μg/ml, absorption enhancement was dependent on both the DA and the molecular weight of the chitosan polymer (Fig. 1; Table I). Thus, chitosans with a low DA (1 and 15%) showed a clear effect on mannitol transport independently of the molecular weight of the chitosans in the range investigated (4.7–190 kD). Exposure for 60 min to 50 μg/ml C(1:31), C(1:170), C(15:4.7), and C(15:190) increased the Papp values of ^{14}C-mannitol approximately 3 to 6-fold, compared to control (Table I). C(35:12) and C(49:22) did not influence mannitol transport significantly at concentrations of 50 μg/ml. However, the corresponding chitosans of higher molecular weights significantly increased the permeability of ^{14}C-mannitol. C(35:170) and C(49:98) increased the average Papp after 60 min about 8-fold and 2.5-fold, respectively (Fig. 1; Table I). At a concentration of 250 μg/ml, the 1% and 15% acetylated chitosans increased the average Papp after 60 min to more than 10 times the control value. A similar increase was found for C(35:170). However, while the chitosans with low acetylation (1 and 15%) showed strong concentration dependence in their effect on epithelial permeability, the permeability of mannitol in solution containing the more acetylated chitosan C(35:170) appeared to reach a plateau at concentrations of 50 and 250 μg/ml (Table I). At 250 μg/ml, C(49:98) improved the average Papp after 60 min approximately 5-fold as compared to control (Table I). The low molecular weight compounds C(35:12) and C(49:22) were without effect even at the higher concentration of 250 μg/ml.

Both the extent and the kinetics of absorption enhancement were dependent on the structural properties of the chitosans. C(35:170) had the most rapid onset of action resulting in the strongest effect on mannitol permeability after the first 20 min compared with chitosans with lower and higher degrees of acetylation (Table II). Due to the rapid onset of absorption enhancement, the Papp values obtained for C(35:170) remained the highest during the first 60 min of the experiment. However, the effect of the 1 and 15% acetylated chitosans became stronger at longer exposure times (Fig. 1). The effects of C(49:98) on permeability also increased with time but remained lower than those of the other chitosans.

Effects of Chitosans on Intracellular Enzyme Activity

A clear dose-dependent effect on intracellular dehydrogenase activity was observed for C(1:31), C(1:170), C(15:4.7),

Table I. Concentration-dependent Effects of Chitosans on the Mean Permeability of ^{14}C-Mannitol Across Caco-2 Cell Monolayers[a]

Chitosan[b]	Papp × 10^7 (cm/sec) 10 μg/ml	Ratio[c]	Papp × 10^7 (cm/sec) 50 μg/ml	Ratio[c]	Papp × 10^7 (cm/sec) 250 μg/ml	Ratio[c]
control	1.3 ± 0.2	1	2.4 ± 0.2	1	1.8 ± 0.2	1
C(1:31)	1.5 ± 0.1	1.2	10.8 ± 2.2[d]	4.5	18.0 ± 4.6[d]	10.0
C(1:170)	1.5 ± 0.2	1.2	8.6 ± 0.6[d]	3.6	19.6 ± 6.6[d]	10.9
C(15:4.7)	1.3 ± 0.1	1.0	7.7 ± 2.2[d]	3.2	27.8 ± 5.7[d]	15.4
C(15:190)	2.5 ± 0.3[d]	1.9	15.1 ± 6.3[d]	6.3	20.3 ± 1.3[d]	11.2
C(35:12)	1.3 ± 0.2	1.0	2.7 ± 0.2	1.1	3.4 ± 1.0	1.9
C(35:170)	1.3 ± 0.2	1.0	19.9 ± 2.2[d]	8.3	16.2 ± 1.1[d]	9.0
C(49:22)	1.4 ± 0.2	1.1	1.6 ± 0.3	0.7	1.8 ± 0.6	1.0
C(49:98)	1.4 ± 0.1	1.1	6.0 ± 2.0[d]	2.5	8.4 ± 2.5[d]	4.7

[a] The mean Papp values for ^{14}C-mannitol are calculated over a 60 min period. The Papp values are given as the mean ± s.d. of 3–4 experiments.
[b] Chitosans are abbreviated as C, the degree of acetylation (%) is given by the first number in brackets, the second number stands for the molecular weight in kD.
[c] The ratio Papp chitosan/Papp control is given.
[d] Significantly different from control ($P < 0.05$).

Table II. Effect of Chitosans at a Concentration of 50 μg/ml on the Permeability of Mannitol Across Caco-2 Cell Monolayers After 20 Min Exposure

Chitosan	Papp × 10^7 (cm/sec)	Ratio[a]
control	2.7 ± 0.3	1
C(1:31)	4.4 ± 0.5[b]	1.6
C(1:170)	3.9 ± 0.4[b]	1.4
C(15:4.7)	4.3 ± 0.1[b]	1.6
C(15:190)	5.0 ± 0.4[b]	1.9
C(35:12)	2.9 ± 0.2	1.1
C(35:170)	8.4 ± 1.8[b,c]	3.1
C(49:22)	1.7 ± 0.3	0.6
C(49:98)	3.7 ± 0.9[b]	1.4

[a] The mean Papp values for ^{14}C-mannitol are calculated over a 20 min period. The Papp values are given as the mean ± s.d. of 3–4 experiments. The ratio Papp chitosan/Papp control is given.
[b] Significantly different from control ($P < 0.05$).
[c] Significantly different from all other treatments ($P < 0.05$).

and C(15:190), with the high molecular weight C(15:190) showing the lowest effect (Fig. 2). C(35:170) and C(49:98) decreased enzyme activity only at concentrations higher than 250 μg/ml, i.e., above the concentrations necessary to improve mannitol transport. None of the other chitosans reduced the enzyme activity significantly in the concentration range studied (1–500 μg/ml).

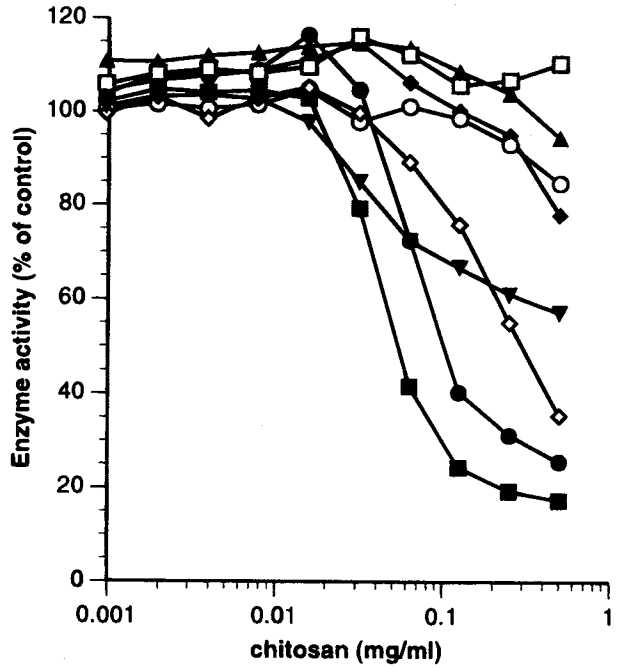

Fig. 2. Effects on intracellular dehydrogenase activity after 60 min exposure to chitosans. The data are given as the mean of 3 experiments, and are represented as a percentage of control data. Error bars have been omitted for reasons of clarity. Relative standard deviations were between 10 and 15%. C(1:31) (■); C(1:170) (●); C(15:4.7) (◇); C(15:190) (▼); C(35:12) (▲); C(35:170) (◆); C(49:22) (□); C(49:98) (○).

Effects of Chitosans on Caco-2 Cell Monolayer Morphology

Using TEM, the typical morphology of untreated Caco-2 cells comprised well differentiated epithelial cells with microvilli, tight junctions, and a well organized terminal web, excluding cell organelles (Fig. 3a). Untreated Caco-2 cell monolayers incubated at an apical pH of 5.5 for 60 minutes were indistinguishable from those incubated at pH = 7.4 (data not shown) (25). Exposure of monolayers to 50 μg/ml chitosans C(1:31) and C(1:170) for 60 min resulted in discontinuities in and a reduced number of microvilli (Fig. 3b). The terminal web of the epithelial cells showed a disorganized pattern and contained small vesicle-like structures. The tight junctions, however, looked normal. No gross effects on the cell membrane, such as membrane discontinuities, were observed for these two chitosans. Treatment with 50 μg/ml C(35:170) and 250 μg/ml C(49:98) resulted in less pronounced changes in microvilli and terminal web (Fig. 3c). The number of microvilli was higher than observed for C(1:31) and C(1:170), and the terminal web and tight junctions appeared to be normal. Chitosans showing no effect on mannitol transport did not change cell morphology compared to controls (data not shown). Exposure of Caco-2 cells to 15% acetylated chitosans was not studied by TEM, due to lack of material.

DISCUSSION

The potential adverse effects of many absorption enhancing agents on epithelial integrity, morphology and function are of major concern (7). Ideally, the action of an absorption enhancer should be immediate and should coincide with the presence of the drug at the absorption site, which is often difficult to achieve (23). For a thorough evaluation of absorption enhancing compounds it is therefore important not only to study the extent of improved drug absorption, but also to assess the absorption enhancement-time profile and the toxicity of the enhancer (24).

In the present study it was found that the structural properties of chitosan, i.e., molecular weight and degree of acetylation, dictated absorption enhancing properties and toxicity to a large extent. Chitosans with a low molecular weight (22,000) and a high DA (≥35%) lacked absorption enhancement activity, whereas chitosans with a low DA and/or a high molecular weight increased epithelial permeability. The relation between molecular weight, DA, and effect on epithelial permeability is summarized in Figure 4. We conclude that structural properties of chitosans are very important for the absorption enhancement of hydrophilic drugs across mucosal tissues.

The onset of action and saturability of absorption enhancement were also dependent on the structural properties of the chitosan. For example, C(35:170) increased the permeability of the epithelial cell monolayer more rapidly than the other chitosans. Moreover, the effect of C(35:170) levelled off at the higher dose. That is, epithelial permeability did not change as strongly with concentration as it did with other chitosans. Absorption enhancement with this chitosan in vivo is therefore expected to be less sensitive to the actual chitosan concentration reaching the absorption site. This should give C(35:170) more controllable and reliable absorption enhancing characteristics. In general, chitosans had a shallow dose-response relationship as compared to other absorption enhancing agents studied in

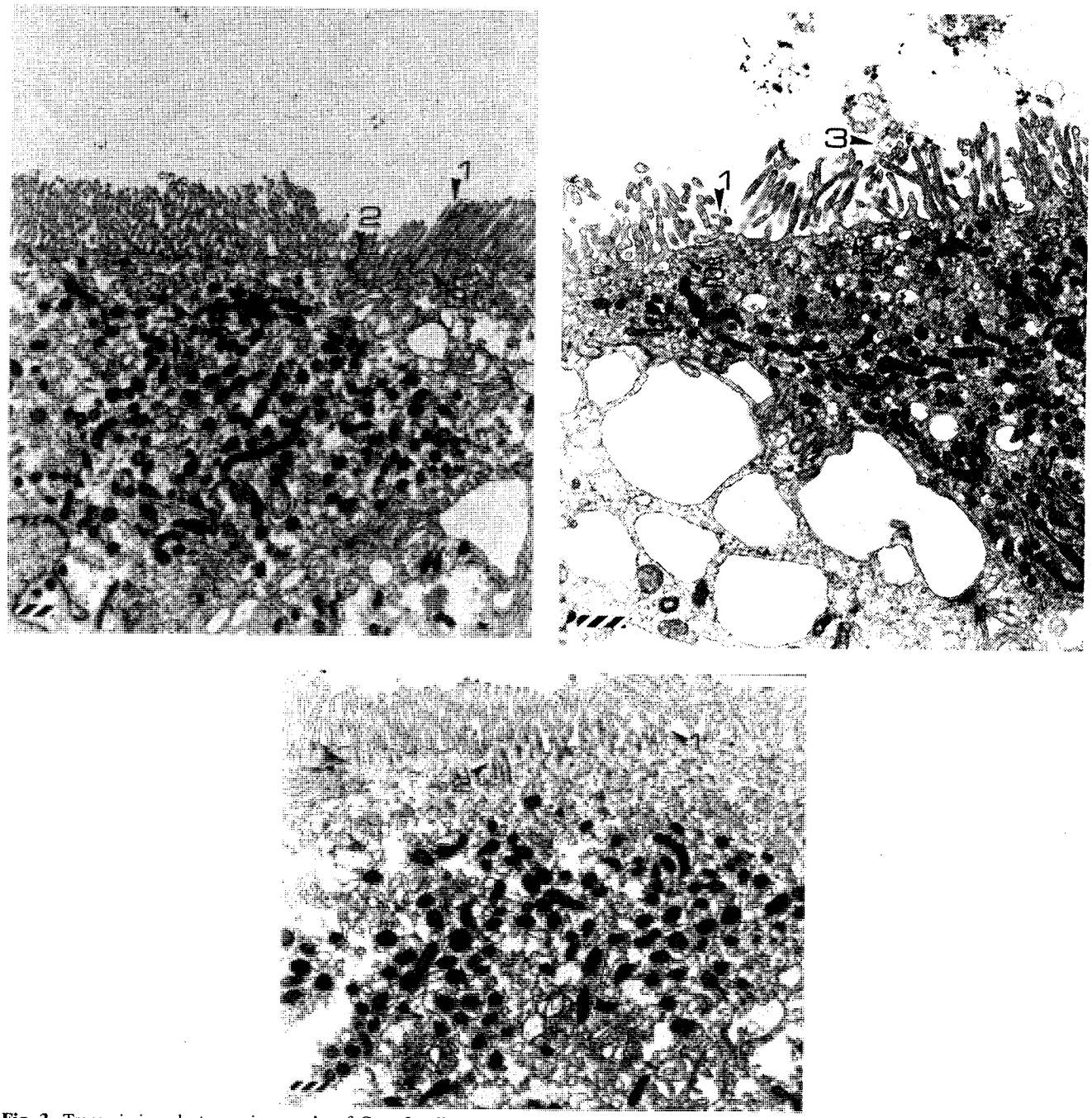

Fig. 3. Transmission electron micrographs of Caco-2 cells. (a) Untreated cells with microvilli (1), tight junctions (2), and a well organized terminal web (3). (b) Caco-2 cells treated for 60 min with 50 μg/ml C(1:31). Note the discontinuities in the microvilli (1) and the presence of small vesicle-like structures in the terminal web (2) and outside the cells (3). (c) Caco-2 cells treated for 60 min with 50 μg/ml C(35:170). Some of the microvilli show discontinuities (1) but less frequently than for C(1:31). Note that the terminal web excludes vesicles and is well preserved (2). The scale bars indicate 1 μm.

the Caco-2 cell model (22, 25). In an earlier preliminary study the commercially available Seacure chitosan (DA about 10–15%) also enhanced the mannitol permeability across Caco-2 cell monolayers to the same extent at concentrations of 2.5 and 5 mg/ml (3). Various anionic surface active substances such as sodium dodecyl sulphate and sodium taurodihydrofusidate, on the other hand, resulted in more than 100-fold increases in absorption rates following small increases in the enhancer concentration (22, 25).

The adverse effects of chitosans, as determined by intracellular enzyme activity, varied between clear dose-dependent toxicity to no effect at all in the concentration ranges studied. The effects of chitosans on intracellular enzyme activity were comparable to or less than those observed with other commonly used absorption enhancers, such as sodium taurohydrofusidate and bile salts (22). Chitosans with a low DA (<35%) had the largest effect on enzyme activity, and at DA>35%, enzyme activity was decreased only at dosages well above the concentra-

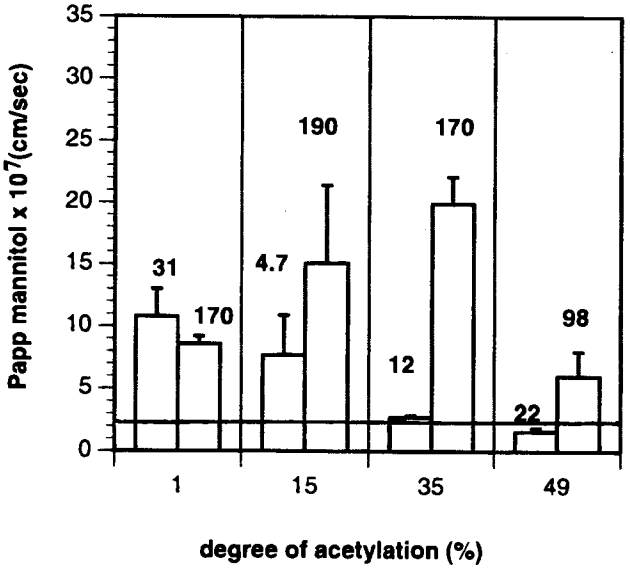

Fig. 4. The mean Papp of mannitol across Caco-2 cell monolayers during 60 min exposure to 50 μg/ml chitosan. The numbers associated with the bars in the graph show the molecular weight of the studied chitosans in kD. The Papp of mannitol across untreated monolayers was 2.4 ± 0.2 ($\times 10^{-7}$) cm/sec, and is indicated in the figure by the horizontal line. Data are given as the mean of 3–4 experiments. Error bars represent standard deviations.

tion necessary for absorption enhancement. These findings were confirmed by TEM studies of the morphological effects of chitosans on Caco-2 cell monolayers. Gross effects on the cell membrane, such as the discontinuities observed after exposure to sodium dodecyl sulphate (25), were not found for any chitosan studied herein. Nevertheless, clear changes in the number of microvilli and the organisation of the terminal web were observed after exposure to chitosans with a low DA (1%). In contrast, the morphological changes following exposure to chitosans with higher DAs were small. The decreased adverse effects of chitosans with increasing DA could possibly be explained by an increased lysozyme degradation of these particular chitosans, as observed by in-vitro studies with purified lysozyme by Nordtveit *et al.* (12).

Thus the toxicity of chitosans appears to be related to the DA and, consequently, the positive charge density of the polymer. This is in agreement with the well-known cell lytic and toxic properties of cationic polymers with high charge density, such as poly-L-lysine, poly-L-arginine, and protamine (16, 26). Recently, it was shown that poly-L-lysines with a reduced charge density caused less cell toxicity (17), similar to that observed with the chitosans of low charge density in the present study.

The positive charge of the chitosan polymer also makes possible a charge interaction between the chitosan and the negatively charged surface of the epithelial cell membrane. Such an interaction may be important for drug absorption enhancement. Other cationic polypeptides such as poly-L-lysine, poly-L-arginine, and protamine also alter the permeability of a number of endothelial and epithelial tissues (27–30). The changes in permeability as found for these compounds were more dependent on the amount of positive charge in the polycations than the type of cationic polypeptide used, and were related to alterations in membrane properties (30) as well as tight junctional integrity (28, 29). The influence of chitosans on membrane or tight junctional permeability, and the dependence of these effects on the molecular weight and DA are under investigation. Preliminary studies with chitosans have indicated that both membrane and tight junctional barrier properties may be altered. As was the case with absorption enhancement, these effects were dependent on the structural properties of the chitosans. In agreement with the results of the present study, the transepithelial electrical resistances of the Caco-2 cell monolayers were rapidly reduced after exposure to 50 μg/ml C(35:170) while the effect of C(1:31) was slower (manuscript in preparation).

In conclusion, the ability of chitosan to improve transport of hydrophilic compounds across mucosal epithelia is dependent on the polymer structure and charge density. A low DA and/or a high molecular weight appears to be necessary to increase epithelial permeability. Adverse effects of chitosan, such as decreased cellular metabolism, and changes in cell morphology are also dependent on the polymer structure and charge. A low DA (<35%) results in clear adverse effects. Molecular weight does not seem to influence the severity of side effects as strongly. Thus, it is possible to select chitosans with both good absorption enhancing characteristics and low cytotoxicity. C(35:170), a chitosan with an intermediate DA and a high molecular weight, was found to fulfill these criteria.

ACKNOWLEDGMENTS

We are grateful to Mr. Tapio Nikkilä for preparing the electron micrographs, and Ms. Susanne Olsson for skilful technical assistance. This work was supported by the Swedish Medical Research Council (9487), the Swedish Fund for Medical Research without Animal Experiments, Centrala försöksdjursnämnden (93-11), and Astra AB.

REFERENCES

1. L. Illum, N. F. Farraj, and S. S. Davis. *Pharm. Res.* **11**:1186–1189 (1994).
2. C. O. Rentel, C. M. Lehr, J. A. Bouwstra, H. L. Luessen, and H. E. Junginger. *Proceed. Intern. symp. control. Rel. Bioact. Mater.* **20**:446–447 (1993).
3. P. Artursson, T. Lindmark, S. S. Davis, and L. Illum. *Pharm. Res.* **11**:1358–1361 (1994).
4. M. J. Jackson. In L. R. Johnson (ed.), *Physiology of the Gastrointestinal Tract. Second edition*, Raven Press, New York, 1987, pp. 1597–1621.
5. J. L. Madara and J. S. Trier. In L. R. Johnson (ed.), *Physiology of the Gastrointestinal Tract. Second edition*, Raven Press, New York, 1987, pp. 1251–1266.
6. P. L. Smith, D. A. Wall, C. H. Gochoco, and G. Wilson. *Adv. Drug Del. Rev.* **8**:253–290 (1992).
7. E. S. Swenson and W. J. Curatolo. *Adv. Drug Del. Rev.* **8**:39–92 (1992).
8. J. H. Hochman and P. Artursson. *J. Control. Rel.* **29**:253–267 (1994).
9. K. M. Vårum, M. W. Anthonsen, H. Grasdalen, and O. Smidsrød. *Carbohydr. Res.* **211**:17–23 (1991).
10. K. M. Vårum, M. W. Anthonsen, H. Grasdalen, and O. Smidsrød. *Carbohydr. Res.* **217**:19–27 (1991).
11. M. W. Anthonsen, K. M. Vårum, and O. Smidsrød. *Carbohydr. Polymers* **22**:193–201 (1993).
12. R. J. Nordtveit, K. M. Vårum, and O. Smidsrød. *Carbohydr. Polymers* **23**:253–260 (1994).
13. A. Domard. *Int. J. Biol. Macromol.* **9**:98–104 (1987).
14. M. W. Anthonsen and O. Smidsrød. *Carbohydr. Polymers* **26**:303–305 (1995).

15. C. J. Brine, P. A. Sandford, and J. P. Zikakis. *Advances in Chitin and Chitosan*, Elsevier Applied Science, London, 1992.
16. S. W. Chang, J. Y. Westscott, J. E. Henson, and N. V. Voelkel. *J. Appl. Physiol.* **62**:1932–1943 (1987).
17. H. M. Ekrami and W. C. Shen. *J. Drug Target.* **2**:469–475 (1995).
18. C. M. Lehr, J. A. Bouwstra, E. H. Schacht, and H. E. Junginger. *Int. J. Pharm.* **78**:43–48 (1992).
19. K. M. Vårum, M. W. Anthonsen, M. H. Ottøy, H. Grasdalen, and O. Smidsrød. In: C. J. Brine, P. A. Sandford, and J. P. Zikakis (eds.), *Advances in Chitin and chitosan*, Elsevier Applied Science, London, 1992, pp. 127–136.
20. P. Artursson. *J. Pharm. Sci.* **79**:476–482 (1990).
21. K. Lappalainen, I. Jääskeläinen, K. Syrjänen, A. Urtti, and S. Syrjänen. *Pharm. Res.* **11**:1127–1131 (1994).
22. E. K. Anderberg, C. Nyström, and P. Artursson. *J. Pharm. Sci.* **81**:879–887 (1992).
23. A. G. DeBoer and D. D. Breimer. In A. G. DeBoer (ed.), *Drug Absorption Enhancement: Concepts, Possibilities, Limitations and Trends*, Harwood Academic Publishers, Chur, 1994, pp. 155–175.
24. E. S. Swenson, W. B. Milisen, and W. Curatolo. *Pharm. Res.* **11**:1132–1142 (1994).
25. E. K. Anderberg and P. Artursson. *J. Pharm. Sci.* **82**:392–398 (1993).
26. A. Santana, S. Hyslop, E. Antunes, M. Mariano, Y. S. Bakhle, and G. DeNucci. *Agents Actions* **39**:104–110 (1993).
27. I. Westergren and B. B. Johansson. *Acta Physiol. Scand.* **149**:99–104 (1993).
28. G. T. A. McEwan, M. A. Jepson, B. H. Hirst, and N. L. Simmons. *Biochim. Biophys. Acta* **1148**:51–60 (1993).
29. C. J. Bentzel, M. Fromm, C. E. Palant, and U. Hegel. *J. Membr. Biol.* **95**:9–20 (1987).
30. C. J. Tzan, J. R. Berg, and S. A. Lewis. *Am. J. Physiol.* **265 (Cell Physiol. 34)**:C1637–1647 (1993).