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Chitosans for enhanced delivery of therapeutic peptides across intestinal epithelia: in vitro evaluation in Caco-2 cell monolayers

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Abstract

The aim of the study was to evaluate the transport enhancing effects of two chitosan salts, chitosan hydrochloride and chitosan glutamate (1.5% w/v), and the partially quaternized chitosan derivative, *N*-trimethyl chitosan chloride (TMC) (1.5 and 2.5% w/v), in vitro in Caco-2 cell monolayers. The transport of the peptide drugs buserelin, 9-desglycinamide, 8-arginine vasopressin (DGAVP) and insulin was followed for 4 h at pH values between 4.40 and 6.20. All the chitosans (1.5%) were able to increase the transport of the peptide drugs significantly in the following order: chitosan hydrochloride > chitosan glutamate > TMC. Due to its quaternary structure, TMC is better soluble than the chitosan salts and further increases in peptide transport were found at higher concentrations (2.5%) of this polymer. The better solubility of TMC may compensate for its lower efficacy at similar concentrations. The increases in peptide drug transport are in agreement with a lowering of the transepithelial electrical resistance (TEER) measured in the cell monolayers. No deleterious effect to the cell monolayers could be detected with the trypan blue exclusion technique. The enzyme inhibitory effect of chitosan hydrochloride (1.5%) was compared with carbomer (1.5%) [Carbopol® 934P] in transport studies with buserelin in the presence of the endoprotease, α-chymotrypsin. In the presence of α-chymotrypsin the transport of buserelin was decreased markedly (from 4.3 to 1.3% of the total dose applied) with chitosan hydrochloride (1.5%), in contrast with carbomer (1.5%) where the transport remained constant (1.4% of the total dose applied). Also the chitosan derivative TMC was not able to inhibit α-chymotrypsin. It is concluded from this study that chitosans are potent absorption enhancers, and that the charge, charge density and the structural futures of chitosan salts and *N*-trimethyl chitosan chloride are important factors determining their potential use as absorption enhancers for peptide drugs, but that they are unable to prevent degradation from proteolytic

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enzymes. Structural modification of the chitosan molecule may compensate for this shortcoming. © 1997 Elsevier Science B.V.

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1. Introduction

While the oral route is still regarded as the most convenient route of drug administration (Borchard et al., 1996), alternative routes such as the nasal, buccal and rectal routes are becoming increasingly important in peptide drug delivery (Lee, 1991). However, particular difficulties are met in designing effective delivery systems in any of these routes. Apart from physical and chemical instabilities and susceptibility to enzymatic degradation, which negatively influence absorption kinetics, poor absorption at the site of administration is also a major limiting factor in the development of delivery systems for peptide drugs. Peptides and proteins are large molecule substances (MW > 500 Da) and are in general highly hydrophilic in nature. Their size and polarity frequently exclude normal passive diffusion across cell membranes (the transcellular transport pathway), resulting in poor absorption and bioavailability (Lee, 1991).

The absorption of peptide drugs is for the most part limited to the alternative paracellular pathway (Hochman and Artursson, 1994), but the entry of large molecules through the paracellular route is nearly completely restricted by the tight junctions (Gumbiner, 1987). One approach to overcome the restriction of the paracellular transport pathway is the co-administration of absorption enhancing agents (Fix, 1987). Many compounds have been studied which enhance drug transport across the cellular monolayer, but most of them proved to have toxic effects and in general they do not enhance drug transport by interaction with the tight junctions (Muranishi, 1990; Tomita et al., 1996). In recent years significant progress has been made in identifying agents which increase the absorption of drugs through the paracellular transport pathway (Borchard et al., 1996; Lueßen et al., 1997).

In this regard chitosan is of particular interest. Chitosan is a polycationic polymer with numerous applications in the food, agricultural, cosmetic and pharmaceutical industries (Arai et al., 1968; Hirano et al., 1988; Skaugrud, 1989; Li et al., 1992). Chitosan hydrochloride improves the intestinal absorption of buserelin in vivo in rats (Lueßen et al., 1996a) and chitosan glutamate is able to reduce the blood glucose levels after the nasal administration of insulin to rats and sheep (Illum et al., 1994). The enhancement in the absorption of peptide drugs by chitosan is suggested to be a combination of the mucoadhesive properties of chitosan and its ability to regulate tight junction integrity. (Artursson et al., 1994; Illum et al., 1994).

We have recently shown that a partially quaternized chitosan derivative, *N*-trimethyl chitosan chloride (TMC), was also able to increase the transport of the hydrophilic compounds [^{14}C]mannitol (MW 182.2), a fluorescein isothiocyanate-labelled dextran (FD-4, MW 4400) and the peptide drug buserelin (MW 1299.5) in vitro in Caco-2 cell monolayers (Kotzé et al., 1997a). TMC is a chitosan derivative with highly increased water solubility compared with chitosan and chitosan salts, especially at neutral and basic pH values, and might prove useful in controlled drug release (Kotzé et al., 1997b). The aim of this investigation was to evaluate the effect of different chitosan salts (chitosan glutamate and chitosan hydrochloride) and the chitosan derivative, TMC, on the permeability of intestinal epithelial cells in vitro for their ability to increase the transport of several peptide drugs over the acidic pH range. In addition, TMC was evaluated for enzyme inhibitory effects and the effect of α-chymotrypsin on the absorption enhancing properties of chitosan hydrochloride and carbomer (Carbopol® 934P, a well-known enzyme inhibitor) (Lueßen et al., 1996b), was compared.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

N-Acetyl-L-tyrosine ethylester (ATEE), *N*-acetyl-L-tyrosine (AT), α-chymotrypsin (Type VII from bovine pancreas, TLCK treated, activity 50 U/mg or 54 units/mg protein, lot no. 89F8155), 2-[*N*-morpholino]ethane-sulfonic acid (MES) and trypan blue were obtained from Sigma (Bornem, Belgium). All other chemicals used were at least of reagent grade.

2.1.2. Polymers

Chitosan hydrochloride (SEACURE CL 210) and chitosan glutamate (SEACURE G 210) were kindly provided by Pronova Bioplymer (Drammen, Norway). Both batches have a degree of deacetylation of 83% according to the certificate of analysis and were used as received. TMC (degree of quaternization 12.28%) was synthesized from sieved fractions (< 500 μm) of chitosan base (SEACURE, degree of acetylation ca. 25%) (Pronova Biopolymer) as described previously (Domard et al., 1986). The degree of quaternization of this polymer was calculated from H^1 NMR spectra (600 MHz) obtained with a Bruker DMX-600 spectrometer. Carbomer (Carbopol® 934P; C934P) was a gift from BF Goodrich (Cleveland, OH, USA).

2.1.3. Peptide drugs

The peptide drugs were kindly donated by the following companies: buserelin acetate (MW 1299.5; Hoechst AG, Frankfurt, Germany), 9-desglycinamide, 8-arginine vasopressin (DGAVP; MW 1412; Organon, Oss, The Netherlands) and porcine insulin (sodium salt, MW 5777.6; Diosynth, Oss, The Netherlands).

2.2. Methods

2.2.1. Effect of TMC on α-chymotrypsin activity

The conversion of *N*-acetyl-L-tyrosine ethylester (ATEE) to *N*-acetyl-L-tyrosine (AT) by α-chymotrypsin, in the presence of TMC, was studied based on a previously described method (Lueßen et al., 1996b). TMC was dissolved in concentrations of 1.5–2.5% w/v in a 2-[*N*-morpholino]ethane-sulfonic acid and potassium hydroxide buffer (MES/KOH buffer, pH 6.70) containing 250 mM mannitol. ATEE was dissolved in the different polymer solutions at a concentration of 1 mg/ml. α-Chymotrypsin (1 mg/ml) was dissolved in ice-cold MES/KOH buffer (pH 6.70) and diluted to 1/32 by the double dilution technique. Twenty five μl of the enzyme solution was added to 500 μl of the substrate solutions with the different TMC concentrations (final concentration of α-chymotrypsin = 80 mU/ml incubation medium). The test solutions were incubated at 37°C. Samples of 50 μl were taken at predetermined time intervals up to 4 h into 1 ml stop solution (phosphoric acid. pH 2.0). Both the substrate ATEE and the metabolite AT were detected by HPLC-UV_{232} as described previously (Lueßen et al., 1996b).

2.2.2. Peptide transport across Caco-2 cell monolayers

2.2.2.1. Cell cultures. Caco-2 cells (passages 80–83) were used for the transport experiments with the different peptide drugs. The cells were seeded on tissue culture treated polycarbonate filters (area 4.7 cm^2) in Costar Transwell 6-well plates (Costar Europe, Badhoevedorp, The Netherlands) at a seeding density of 10^4 cells/cm^2. Dulbecco's Modified Eagle's Medium [DMEM, pH 7.4] (Sigma, Bornem, Belgium), supplemented with 1% non-essential amino acids, 10% foetal bovine serum, benzylpenicillin G (160 U/ml) and streptomycin sulphate (100 μg/ml) (all obtained from Sigma), were used as culture medium, and added to both the donor and acceptor compartments. The medium was changed every second day and cell cultures were kept at a temperature of 37°C in an atmosphere of 95% air and 5% CO_2. Filters were used 21–23 days after seeding (Hurni et al., 1993; Noach et al., 1993; Borchard et al., 1996).

2.2.2.2. Measurement of the transepithelial electrical resistance (TEER). A Millicell® ERS meter (Millipore, Bedford, MA), connected to a pair of chopstick electrodes, was used to measure the

effect of the different polymers on the TEER of the filters every hour, while the transport experiments with the different peptide drugs and polymers were in progress (Hurni et al., 1993; Noach et al., 1993; Borchard et al., 1996). Care was taken to assure that the basolateral medium was not contaminated with the apical medium which contain the peptide drug and polymer solution. The TEER values for untreated filters were in the range of 800–1000 Ω cm^2.

2.2.2.3. Viability of Caco-2 cell monolayers. Both the apical and basolateral sides of the cell monolayers were rinsed twice with 0.01 M phosphate-buffered saline (PBS, pH 7.40) after completion of all the transport experiments. The cell monolayers were incubated apically with a solution of 0.1% trypan blue (Sigma) in PBS (Borchard et al., 1996). The basolateral medium was PBS. After 30 min the medium was removed from both sides of the cell monolayers and the cell monolayers were examined by light microscopy for exclusion of the marker. Cells excluding trypan blue were considered to be viable. The trypan blue exclusion technique was also used prior to the start of any transport experiment to validate the viability of the different cell passages used for the transport experiments.

2.2.2.4. Buserelin transport. The effect of chitosan hydrochloride, chitosan glutamate, TMC and carbomer (C934P) on the transport of buserelin in Caco-2 cell monolayers were studied as follows. Solutions of chitosan hydrochloride and chitosan glutamate (1.5% w/v) and TMC (1.5 and 2.5% w/v) were prepared in serum-free DMEM containing 198 μg/ml buserelin. The pH of the polymers solutions was adjusted to 6.20 with 0.1 M HCl. Due to the high viscosity of dispersions of C934P, fast dissolving microparticles were prepared (Akiyama et al., 1996). The microparticles contain a 1:1 mixture of polyglycerol esters of fatty acids and freeze-dried neutralised C934P. Immediately before the start of each experiment the apical medium was removed and the cells were incubated with 2.5 ml of the polymers solutions containing buserelin. In the case of C934P amounts of 75 mg of the microparticles, equivalent to 1.5% w/v C934P, were added to the apical side before applying serum-free DMEM containing the buserelin (pH 6.20). The medium in the acceptor compartment was DMEM buffered at pH 7.40 with 25 mM *n*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid) (HEPES). Samples of 100 μl were taken at several time points for 4 h from the basolateral side. The samples were diluted with 400 μl phosphoric acid (pH 3.00) in HPLC vials and analysed by HPLC-UV_{210} as described previously (Lueßen et al., 1996c). Samples taken from the basolateral side were replaced with an equal volume of DMEM + HEPES. Controls were run in every experiment with solutions containing buserelin without the dissolved polymers or microparticles. All experiments were done in triplicate in an atmosphere of 95% air and 5% CO_2 at 37°C. Results were corrected for dilution and expressed as cumulative transport (% of total dose applied) at time *t*.

Effect of α-chymotrypsin on buserelin transport. The ability of chitosan hydrochloride and C934P to transport buserelin, in the presence of α-chymotrypsin, across Caco-2 cell monolayers was studied as follows. α-Chymotrypsin (20 mU/ml) was dissolved in serum-free DMEM (pH 6.20). Solutions of chitosan hydrochloride (1.5% w/v) and C934P (0.5% w/v) were prepared in the enzyme solution. These solutions were incubated at 37°C for 30 min. Immediately before the start of the experiments the apical medium on the monolayers was removed. In the case of C934P, 50 mg of microparticles (1% C934P) were added on the monolayers. At $t = 0$ buserelin was added to a final concentration of 198 μg/ml in both the preincubated 1.5% chitosan hydrochloride and 0.5% C934P enzyme solutions. These solutions (2.5 ml) were applied to the apical chamber and incubated for 4 h. The final concentration of C934P was 1.5% w/v. Samples of 100 μl were taken at several time points and analysed for buserelin content as described above.

2.2.2.5. Desglycinamide, 8-arginine vasopressin (DGAVP) transport. The transport of DGAVP across Caco-2 cell monolayers was studied as described for the buserelin transport experiment. Solutions of chitosan glutamate (0.4 and 1.0%

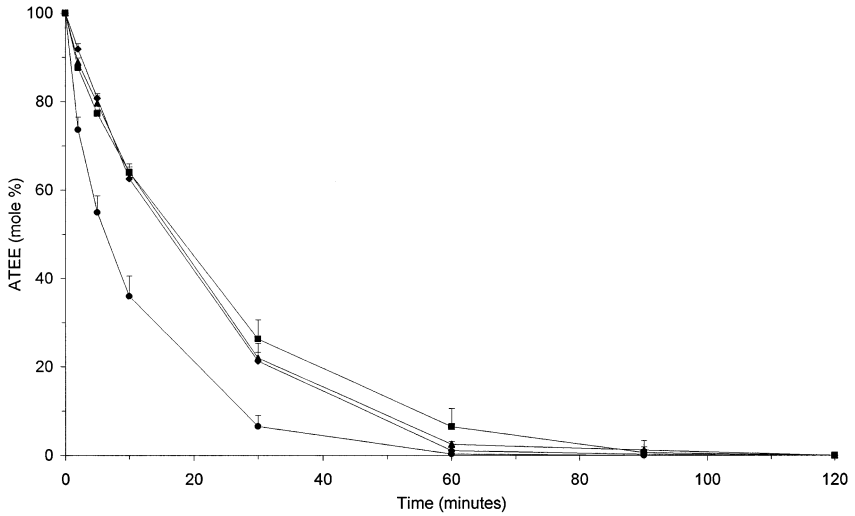

Fig. 1. Effect of TMC on α-chymotrypsin activity at a pH of 6.70. Each point represents the mean ± S.D. of three experiments. Keys: control (●); TMC 1.5% (◆); TMC 2.0% (▲); TMC 2.5% (■).

w/v) and TMC (1.5 and 2.5% w/v) were prepared in serum-free DMEM containing 205 μg/ml DGAVP. The pH of the polymer solutions was adjusted to 5.60 with 0.1 M HCl and incubated on the apical side of the cells for 4 h. Samples of 100 μl were taken at several time points from the acceptor compartment and diluted with 400 μl phosphoric acid (pH 3.00). The samples were analysed for DGAVP content with RP-HPLC as described previously (Lueßen et al., 1996c). Results were corrected for dilution and expressed as cumulative transport (% of total dose applied) at time *t*.

2.2.2.6. Insulin transport. The transport of insulin across Caco-2 cell monolayers was studied as described for the buserelin transport experiments. Solutions of chitosan hydrochloride and chitosan glutamate (1.5% w/v) and TMC (1.5 and 2.5% w/v) were prepared in serum-free DMEM containing 169 μg/ml insulin (sodium salt). The pH of the polymer solutions was adjusted to 4.40 with 0.1 M HCl to assure complete dissolution of the insulin and incubated on the apical side of the cells for 4 h. Samples of 100 μl were taken at several time points from the acceptor compartment and diluted with 400 μl phosphoric acid (pH 3.00). The samples were analysed for insulin content with HPLC-UV_{210} as described previously (Lueßen et al., 1996c). Results were corrected for dilution and expressed as cumulative transport (% of total dose applied) at time *t*.

3. Results

3.1. Effect of TMC on α-chymotrypsin activity

The degradation profile of ATEE by α-chymotrypsin at a pH of 6.70, in the presence and absence of TMC, is given in Fig. 1. Apparently it seems that TMC is able to inhibit the conversion of ATEE to AT by α-chymotrypsin to a low extent. In the control group no ATEE could be detected after 60 min of incubation with α-chymotrypsin. In the presence of TMC (1.5–2.5%) ATEE was completely converted to AT after 90 min of incubation with α-chymotrypsin. This minor enzyme inhibition by TMC is considered to be of no clinical importance compared with other well-known enzyme inhibitors such as carbomer and polycarbophil (Lueßen et al., 1996b).

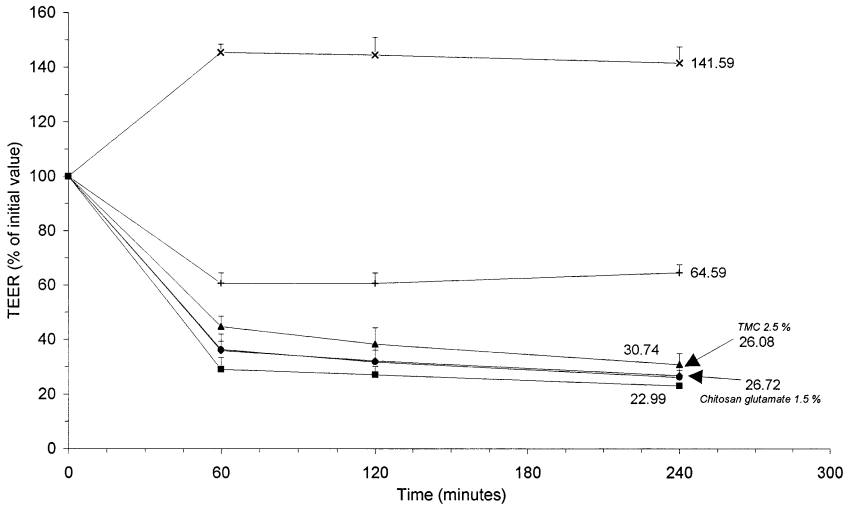

Fig. 2. Effect of chitosan salts, TMC and C934P on the TEER of Caco-2 cell monolayers at a pH of 6.20. Each point represents the mean ± S.D. of three experiments. Keys: control (×); C934P 1.5% (+); TMC 1.5% (▲); TMC 2.5% (◆); chitosan glutamate 1.5% (●); chitosan hydrochloride 1.5% (■).

3.2. Effect of the different polymers on the TEER

Fig. 2 shows the decrease in the TEER of the Caco-2 cell monolayers measured in the filters during the transport study with buserelin. Incubation on the apical side of the monolayers with the different polymers lead to a pronounced reduction in TEER values compared with the control group. The reduction in TEER, 1 h after the start of the experiment, with 1.5% concentrations of the polymers, was in the following order: chitosan hydrochloride ($71 \pm 4\%$ reduction) > chitosan glutamate ($64 \pm 6\%$ reduction) > TMC ($55 \pm 4\%$ reduction) > C934P ($39 \pm 4\%$ reduction). Prolonged incubation, up to 4 h, only results in a slight decrease in TEER values, compared to the initial reduction measured after 1 h. In a concentration of 2.5%, TMC was able to cause a further decrease in the TEER, compared with the reduction measured with the 1.5% concentration. Both chitosan salts and C934P could not be prepared in concentrations above 1.5% due to its high viscosity and low solubility and was therefore only tested in 1.5% concentrations. Similar results were obtained in the transport studies with DGAVP and insulin, where the decrease in TEER values and the order of reduction were comparable with the results described for buserelin.

3.3. Peptide transport

The transport of buserelin across the Caco-2 cell monolayers at pH 6.20 is depicted in Fig. 3. All the polymers were able to improve the transport of buserelin significantly. In the control group nearly no buserelin was transported up to 4 h of incubation (0.04% of the total dose applied). In agreement with the reduction measured in the TEER with 1.5% concentrations of the chitosan salts and TMC, the increase in the transport of buserelin up to 4 h was in the following order: chitosan hydrochloride ($4.3 \pm 0.3\%$ of the total dose applied) > chitosan glutamate ($3.0 \pm 0.9\%$) > TMC ($1.4 \pm 0.2\%$). However, at a 2.5% concentration of TMC the transport of buserelin was improved further ($2.7 \pm 0.3\%$ of the total dose applied), compared with the transport found at 1.5% concentrations. This increase in the transport of buserelin was comparable with the transport found with 1.5% chitosan glutamate.

In the presence of C934P, $1.4 \pm 0.2\%$ of the total buserelin dose applied was transported across the cell monolayers. This is comparable with the transport found with TMC at a similar concentration (1.5%). In the presence of α-chymotrypsin C934P was able to transport the same

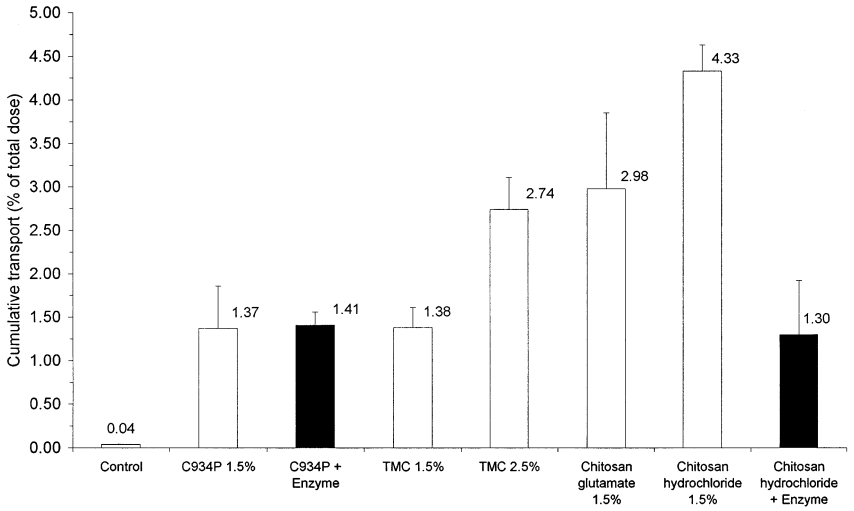

Fig. 3. The effect of chitosan hydrochloride, chitosan glutamate, TMC and C934P on the cumulative transport of buserelin at a pH of 6.20 (white bars). Black bars represents the effect of α-chymotrypsin on the transport enhancing properties of chitosan hydrochloride and C934P (1.5%). Each point represents the mean $\pm$ S.D. of three experiments.

amount of buserelin ($1.4 \pm 0.2\%$ of the total dose applied), compared to the group where no enzyme was present. In contrast with C934P, the transport of buserelin was decreased markedly after 4 h in the presence of α-chymotrypsin at a similar concentration (1.5%) of chitosan hydrochloride, where the transport was decreased from 4.3 ± 0.3 to $1.3 \pm 0.6\%$ of the total dose of buserelin applied.

Fig. 4 shows the transport of insulin at pH 4.40 across the Caco-2 cell monolayers. No transport in the control group could be detected up to 4 h. Incubation for 4 h with both chitosan salts (1.5%) and TMC (1.5%) resulted in insulin transport in the following order: chitosan hydrochloride ($1.2 \pm 0.4\%$ of the total dose applied) > chitosan glutamate ($0.6 \pm 0.2\%$) > TMC ($0.3 \pm 0.1\%$). At a concentration of 2.5% w/v, TMC was again able to increase the transport of insulin to $0.8 \pm 0.1\%$ of the total dose applied, which was comparable with the transport found with chitosan glutamate. The increase in the permeation of insulin was, as with the buserelin transport study, in agreement with the reduction in TEER values measured (data not shown) while the insulin experiments were in progress.

Fig. 5 shows that both chitosan glutamate and TMC were able to improve the permeation of DGAVP at a pH of 5.60 across the cell monolayers. In the control group a very low permeability ($0.19 \pm 0.29\%$ of the total DGAVP dose applied) was found. In contrast with the TEER results (measured directly in the filters used for the DGAVP transport experiments, data not shown) and the transport results described for buserelin and insulin, no differences were found in DGAVP transport between these two polymers. Chitosan glutamate (0.4 and 1.0%) improves the transport of DGAVP to 1.13 ± 0.08 and $1.19 \pm 0.14\%$ of the total dose applied, respectively. Similar results were obtained with TMC. TMC (1.5 and 2.5%) increases the transport to 0.96 ± 0.28 and $1.09 \pm 0.08\%$ of the total DGAVP dose applied, respectively.

Staining with trypan blue, after completion of the transport experiments with all the peptide drugs at any polymer concentration, does not show any intracellular uptake of this dye. These results, together with the TEER values measured during all the transport studies, indicate that the cell membranes were still intact and functionally well.

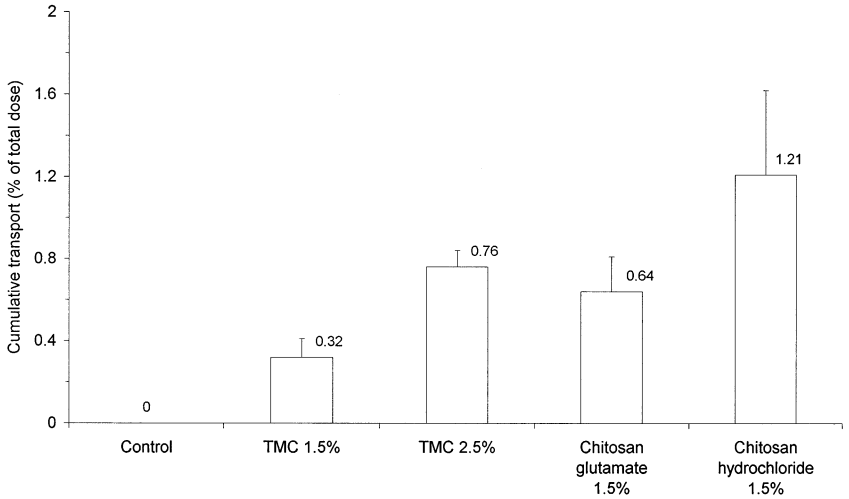

Fig. 4. The effect of chitosan hydrochloride and chitosan glutamate (1.5% w/v) and TMC (1.5 and 2.5% w/v) on the cumulative transport of insulin at a pH of 4.40. Each point represents the mean ± S.D. of three experiments.

4. Discussion

Our results clearly demonstrate that chitosan hydrochloride and chitosan glutamate are able to decrease the TEER of Caco-2 monolayers. Measurement of TEER are believed to be a good indication of the tightness of the junctions between cells. Incubation of the monolayers with these polymers resulted in a marked accumulation of the peptide drugs studied, in the acceptor compartments. The increase in the transport of these compounds is also in good agreement with a decrease in the TEER which was measured directly in the filters at several time points during the transport studies. Solutions above 1.5% w/v of chitosan hydrochloride and chitosan glutamate could not be prepared in acidic medium due to their low solubility and high viscosity.

Chitosan is a weak base and a certain amount of acid is required to transform the glucosamine units into the positively charged, water-soluble form. However, potential reactive groups make chitosan a versatile polymer with unique properties for utilisation in drug release technology. TMC is a partially quaternized derivative of chitosan with superior solubility compared with other chitosan salts, especially at neutral and basic pH values (Kotzé et al., 1997b). Due to its quaternary structure, TMC easily dissolves in much higher concentrations in both acidic and alkaline mediums.

TMC was also able to improve the transport of all the peptide drugs (buserelin, DGAVP, insulin) significantly, compared with the control groups. Apparently it seems that chitosan hydrochloride is more effective than chitosan glutamate followed by TMC at similar weight concentrations. However TMC is superior in solubility and similar results could be obtained at higher concentrations of this polymer. Since the degree of deacetylation of each salt is the same (certificate of analysis), this difference in effect has been explained in terms of the equivalent weights of each repeating unit in the polymer backbone of the respective polymers (theoretically 197 for chitosan hydrochloride, 308 for chitosan glutamate and 239 for TMC monomer units), thus determining the amount of free chitosan base and therefore the density of the amino groups available for protonation at similar weight concentrations. About 50% of chitosan glutamate by weight is the glutamate salt whereas for chitosan hydrochloride the salt part by weight constitute only a small fraction (5–10%). Additionally, the attached methyl groups on the C-2 position of TMC probably cause steric effects and also partially hide the

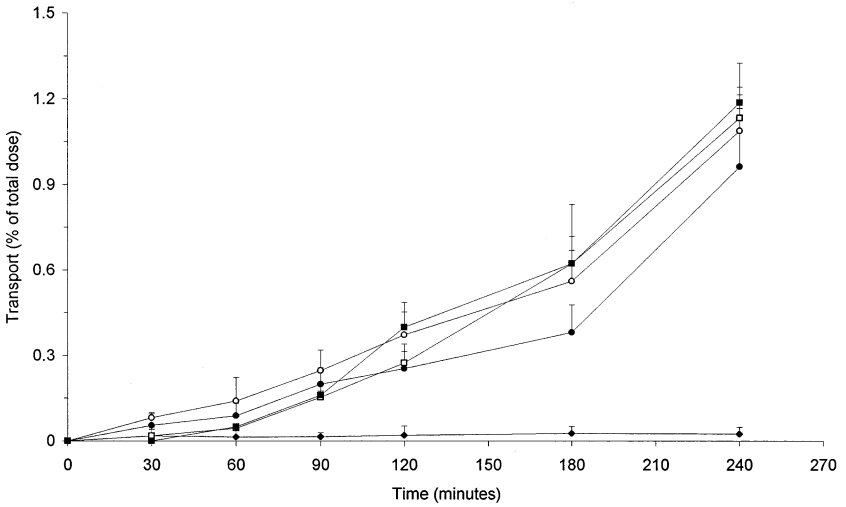

Fig. 5. The effect of chitosan glutamate (0.4 and 1.0% w/v) and TMC (1.5 and 2.5% w/v) on the cumulative transport of DGAVP at a pH of 5.60. Each point represents the mean ± S.D. of three experiments. Keys: control (◆); chitosan glutamate 1.0% (■); chitosan glutamate 0.4% (□); TMC 2.5% (○); TMC 1.5% (●).

positive charge on the quaternary amino groups, thereby altering the time needed to interact with the negatively charged cell membranes and tight junctions (Kotzé et al., 1997b). In this regard, calculation of chitosan in terms of equivalent base, to enable clearer comparison between these polymers, could not be made.

Tight junctions play a crucial part in maintaining the selective barrier function of cell membranes and in sealing cells together to form a continuous cell layer, through which even small molecules cannot penetrate. Furthermore, the paracellular route occupies only a very small surface area compared with the transcellular route (Madara, 1989). However, tight junctions are permeable for water, electrolytes and other charged or uncharged molecules up to a certain size (Madara, 1989; Wilson and Washington, 1989). Tight junctions are known to respond to changes in calcium concentration, c-AMP, osmolarity, pH and the status of the cytoskeleton (Cereijido et al., 1993).

It has been proposed that chitosan salts open the tight junctions in a reversible way (Artursson et al., 1994; Kotzé et al., 1997b). Chitosan acts primarily by an interaction between the positively charged amino groups on the C-2 position with negatively charged sites on the cell membranes and tight junctions to allow for opening of the tight junctions. It has been shown that chitosan glutamate was able to induce changes in the F-actin distribution (Artursson et al., 1994). It is also known that pharmacological agents which interact with cytoskeletal F-actin simultaneously increase the paracellular permeability (Meza et al., 1982). This is in agreement with the hypothesis that F-actin is directly or indirectly associated with the proteins in the tight junctions (Madara, 1987). TMC most likely has a similar action on the junctional complex.

Carbomer was also able to improve the transport of the peptide drug buserelin. Carbomer is a poly(acrylate) derivative and a polyanionic polymer. The effect of the fatty acids, used in preparing carbomer containing microspheres, have not yet been evaluated and they may also contribute to the absorption enhancing effect described for carbomer. However, it has been reported that carbomer has a high affinity for binding of Ca^{2+} ions, especially above a pH of 4 (Lueßen et al., 1996b). The improvement in the transport of buserelin at pH 6.20 could be explained by complexation of Ca^{2+}-ions by the carboxylic groups of the polymer, thereby altering the integrity of

the tight junctions which is sensitive to changes in calcium concentration (Cereijido et al., 1993). In a similar way carbomer was able to inhibit the activity of α-chymotrypsin. This enzyme is an endoprotease and a representative of the group of serine protease's, containing Ca^{2+} as an essential co-factor in its structure (Lueßen et al., 1996b). In contrast with carbomer, chitosan hydrochloride and the novel derivative TMC were not able to inhibit α-chymotrypsin activity. This is in agreement with a previous study in which chitosan glutamate was not able to prevent the degradation of *N*-α-benzoyl-L-arginine ethylester by trypsin (Lueßen et al., 1997). Although both classes of polymers were able to improve the transport of buserelin, the chitosan salts and TMC proved to be more effective absorption enhancers. These results clearly suggest that they act by different mechanisms to improve the paracellular transport of peptide drugs. While carbomer mainly acts as a protease inhibitor and calcium complexing agent, the chitosans improve peptide transport mainly by increasing the paracellular permeability. The versatility of chitosans as absorption enhancing agents is clear from the results presented here with different peptide drugs at different pH values across the acidic pH range.

Staining with trypan blue after completion of all the TEER experiments did not result in any visible intracellular uptake of this marker. The absence of intracellular trypan blue, after prolonged incubation with the different polymers, implies that the Caco-2 cell monolayers remained undamaged and functionally intact. As the dye was excluded from the cells we concluded that the viability of the monolayers was not affected by any of the polymers. Present studies are directed to clarify the role of the degree of quaternization (charge density) of TMC on the permeability of intestinal epithelial cells at neutral and basic pH values.

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