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IN-VIVO INHIBITION BY POLYCATIONS OF SMALL INTESTINAL ABSORPTION OF METHYL α-D-GLUCOSIDE AND LEUCINE IN THE RAT*

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Abstract—Polycations are able to inhibit active transport processes in rat small intestine *in vitro*. Whether this effect can also be confirmed *in vivo* is the concern of this study. Therefore, the effect of various polycations, e.g., protamine and polylysine, on the absorption of methyl α-D-glucoside and leucine was investigated *in vivo* by single-pass perfusion of rat jejunum. The inhibition of absorption of methyl α-D-glucoside and leucine by the polycations was strongly dose dependent. At a substrate concentration of 1 mmol/l a 50% inhibition was achieved with a protamine concentration of 3.2 mg/ml. The inhibition increased as the chain length of the polycation increased. In the presence of protamine the concentration-dependent leucine absorption was reduced at leucine concentrations below 60 mmol/l, but was increased at 100 mmol/l. Absorption of mannitol and 2-deoxy-D-glucose was significantly enhanced by the polycations. These results demonstrate that polycations inhibit active transport and increase passive diffusion processes in the rat small intestine *in vivo*. In addition, pretreatment of rats with a polycation added to the drinking water impaired the small intestinal absorption of methyl α-D-glucoside as subsequently measured by the tissue accumulation technique *in vitro*. Since polycations are hardly absorbed in the intestine, but do attach to negatively charged groups at the mucosal surface, polycations may be useful to study the influence of these negative groups on the absorption of nutrients and drugs.

Inhibitors of the small intestinal absorption of nutrients have theoretical aspects. Some of these substances are of practical and therapeutical value. The theoretical interest in transport inhibitors is reflected by their use in experiments with the aim to elucidate the mechanisms of absorption processes, e.g., sugar derivatives were useful to determine the structure–activity relationship of the small-intestinal glucose carrier [1–3]. The practical and therapeutical value of absorption inhibitors derives from their ability to impair or to retard the absorption of special food components in certain diseases [4], e.g., sucrose absorption in diabetes by α-glucosidase inhibitors [5, 6], or cholesterol absorption in hypercholesterolemia by sitosterol [7]. In this context it should be referred to experimental work about the reduction of carbohydrate absorption by α-amylase inhibitors [8], and the more general retardation of nutrient absorption by carbohydrate gelling agents [9, 10].

Recently, we have shown that polycations such as polylysine, protamine and polyethyleneimine inhibit the active transport of sugars and amino acids in rat small intestine *in vitro* [11]. These experiments were performed for short periods of up to 35 min. The *in vitro* preparations of the small intestine which were used to study the inhibition of the absorptive processes were supplied with metabolic energy and oxygen via the incubation solution. *In vitro* intestinal absorption is often characterized by the rate of uptake of the substrate into the mucosal tissue, e.g., in the tissue-accumulation technique [12], or it is reflected by the rate at which a substrate is transported into a serosal compartment, e.g., in everted-sac preparations [13]. Thus, the conditions under which absorption and its inhibition are measured *in vitro* certainly differ from those *in vivo*. For instance, the interference of polycationic compounds with cation-related membrane transport systems is supposed to depend on the accessibility of superficial binding sites for cations, but the ionic environment of the mucosal surface or of the unstirred water layer *in vitro* may be different *in vivo* due to the different ways of energy and oxygen supply, the *in vivo* effect of the polycations may differ from that *in vitro*. Therefore, the present study investigates *in vivo* at low perfusion rates and under more physiological conditions to what extent polycations impair the absorption of sugars and amino acids. A preliminary report on the results was given at the 8th meeting of the European Intestinal Transport Group in Seggau, Austria, 1987 [14].

MATERIALS AND METHODS

All experiments were carried out using female Wistar rats (Zentralinstitut für Versuchstiere, Hannover, F.R.G.) weighing between 160 and 200 *g*. Non-fasted animals were used throughout the study. The methods employed are only briefly described here since they are well established and were recently presented in detail [10, 11].

Single-pass perfusion. Rats were anesthetized intraperitoneally with 70 mg sodium pentobarbital per kg body weight. The abdominal cavity was

* This work is dedicated to Prof. Dr Fritz Wagner on the occasion of his 60th birthday.

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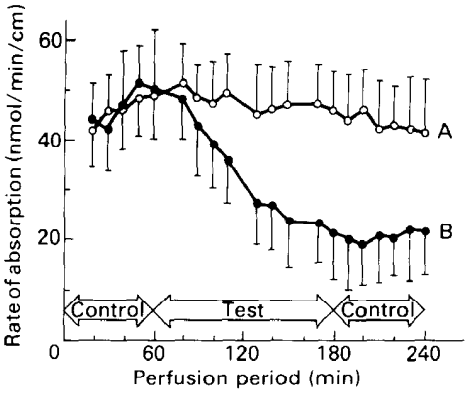

Fig. 1. Time course of the inhibition of jejunal methyl α-D-glucoside absorption *in vivo* by protamine. Intestinal absorption was determined by single-pass perfusion of rat jejunum *in vivo*; α-Me-Glc concentration: 10 mmol/l; protamine concentration: 3.2 mg/ml. (A) without, and (B) with protamine added during the test period. The second control period (from 180–240 min) was added to look for the reversibility of the protamine effect. Values are mean ± SD (N = 6).

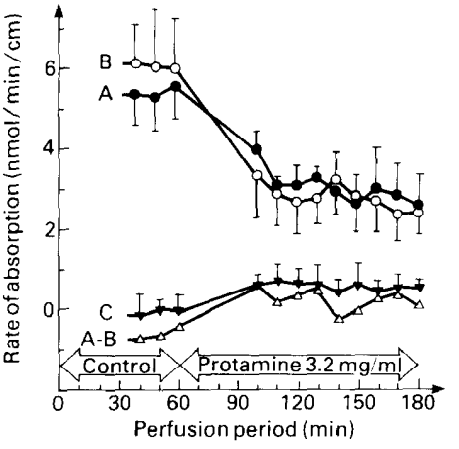

Fig. 2. Time course of the effect of protamine on the jejunal absorption of methyl α-D-glucoside and mannitol *in vivo*. Single-pass perfusion of rat jejunum using tritiated PEG 4000 (A and C) or mannitol (B) as impermeable volume marker; (A) [^{14}C]α-Me-Glc (1 mmol/l) + [^{3}H]PEG 4000; (B) [^{14}C]α-Me-Glc (1 mmol/l) + [^{3}H]mannitol (1 mmol/l); and (C) [^{14}C]mannitol (1 mmol/l) + [^{3}H]PEG 4000. A−B represents the difference between the curves A and B. Values are mean ± SD (N = 5).

opened and a jejunal segment was cannulated proximally and distally to provide a perfusion segment of approx. 20 cm in length. The proximal end of the jejunal segments was about 10 cm distal to the duodenojejunal flexure. The perfusion medium was Krebs–Henseleit phosphate buffer (KH buffer) with a pH of 7.3–7.4. The transport substrates, e.g. methyl α-D-glucoside (α-Me-Glc) and leucine, were added at the expense of sodium chloride if their concentrations exceeded 1 mmol/l. Since the transport substrates were ^{14}C-labeled, [^{3}H]PEG 4000, or [^{3}H]mannitol was used as impermeable volume marker. When a polycation was added as free base, as it occurred occasionally in the case of protamine, the pH of the buffer had to be readjusted slightly by the addition of 0.1 mM HCl. However, the hydrochlorides (lysine), hydrobromides (Lys_{20} and Lys_{400}) or sulfates (protamine) of the polycations were used when available. Lys_5 was obtained as acetate.

In all perfusion experiments the perfusion rate was 0.7 ml/min. In general, a pre-equilibration period of 30 min (in order to reach a steady state for water and solute absorption) was followed by a 30-min control period (perfusing the KH buffer without the polycation) during which three 10-min collections of the outflowing perfusate were obtained for analysis of radioactivity. This control period was followed by a second equilibration period of at least 50 min (see Results) and subsequently by the test period employing in both these periods the polycation to be tested. Again, three 10-min collections were made during the test period. Absorption was calculated from the three samples of the control and test period to provide a single value for each animal under control and test conditions, respectively.

Determination of enzyme activities. To check whether mucosal cells became leaky or damaged during perfusion, the activities of lactate dehydrogenase, LDH, and maltase were measured in the outflowing perfusates of the control and test periods; during each perfusion period three separate 20-min collections were made. The enzyme activities were also determined in mucosal homogenates to obtain a kind of initial enzyme activity of the jejunal mucosa. LDH activity was determined according to the manual of a commercially available test kit (Merckotest No. 3339, Merck, Darmstadt, F.R.G.) and maltase activity was measured by the method of Dahlqvist [15].

Expressed as total units per cm of length, the mucosal enzyme activities of the jejunum were 11.2 U/cm for LDH and 1.5 U/cm for maltase (mean of mucosal samples from three rats). Under control conditions a total of 3% of the LDH and of 4.5% of the maltase activity was obtained in the perfusate after 1 hr. The enzyme activities in each of the 20-min collections were rather similar indicating a continuous release over time. In the presence of the polycations (3.2 mg/ml), the enzyme activities increased. After a 1-hr test period a total of 7.3% of the LDH activity (Lys_{20}) and of 5.6% (Lys_{20}) or of 7.0% (protamine) of the maltase activity were found in the perfusate; again, enzyme activities were quite continuously released over time. Since under control conditions the appearance of the enzyme activities in the perfusate did not cause a significant reduction of the absorption rates (not even after 4 hr, see Fig. 1, Results), the increased enzyme activities in the presence of the polycations do not indicate an extent of a cellular damage which could have been responsible for the polycation-mediated inhibition of absorption.

Tissue accumulation method (everted jejunal rings). These *in vitro* experiments were performed by incubating rings of everted jejunum from controls and pretreated animals. Pretreatment was as follows: 60 hr prior to the absorption experiments twelve rats were divided into two groups, a control and a test

Table 1. Changes in the passive absorption of mannitol and 2-deoxy-D-glucose (dGlc) by protamine

Substrate (1 mmol/l)		Rate of absorption (nmol/min/cm) Control (KH buffer)	Test (+ Protamine)
Mannitol	(N = 5)	0.0 ± 0.3	0.6 ± 0.3*
dGlc	(N = 10)	0.7 ± 0.3	1.4 ± 0.4*

Absorption rates were measured *in vivo* by single-pass perfusion of rat jejunum; protamine concentration: 3.2 mg/ml. Control rates were measured during a first control period (without adding protamine to the perfusate), and the effect of protamine was established in a subsequent second test period. For further details see Materials and Methods. Values are mean ± SD; * $P < 0.05$.

group of six animals each. During this 60-hr period both groups were fed the same pelleted diet (C 1013, Altromin, Lage, F.R.G.) containing 22% casein, 57% starch, 10% sucrose, 3% soy bean oil (refined), 6% minerals and trace elements, and 2% vitamin mixture *ad lib.* and had free access to tap water. For the test group the polycation poly[(*N,N*-dimethyl)-3,5-dimethylene-piperidinium chloride] (PDDP) was dissolved in the drinking water at a concentration of 0.5 mg/ml. PDDP was chosen for this experiment because its polymer structure is formed only by C—C bonds which are unlikely to be cleaved by gastrointestinal enzymes. Water intake was on average 56 ml per 60 hr per animal which amounted to a total dose of 28 mg PDDP per rat. Both the food and the water intake were monitored during the pretreatment period. Body weights and intestinal parameters, such as intestinal wet weight, mucosal protein and DNA content (measured as previously reported [16]), and maltase activity [15], were measured at the end of the pretreatment period. The intestinal parameters were determined in a 20-cm segment of the midjejunum, a segment distally adjacent to the one used for the transport experiments (see below).

After the pretreatment period the animals were killed by stunning and decapitation, and the proximal end of the jejunum was identified approx. 10 cm beyond the duodenojejunal flexure. A 30-cm segment was quickly excised, everted over a plastic rod, chilled in oxygenated (100% O_2) KH buffer, and cut into rings (0.5–1.0 cm in length). The incubations were carried out in oxygenated KH buffer at 37°. [^{3}H]mannitol together with unlabeled mannitol (1 mmol/l) was used to correct for the extracellular space. Uptake of the transport substrate into the tissue (tissue accumulation) was expressed as a distribution ratio: S_T/S_M, where S_T equals the substrate concentration in the intracellular fluid volume and S_M the substrate concentration in the incubation medium [11].

Statistical treatment of results. The unpaired Student's *t*-test was used to determine whether significant differences existed between groups.

Materials. ^{14}C-labeled transport substrates, and ^{3}H-labeled mannitol and PEG 4000 were purchased from NEN Chemicals GmbH (Dreieich, F.R.G.), poly[(*N,N*-dimethyl)-3,5-dimethylenepiperidinium chloride] (PDDP) from EGA-Chemie (Steinheim, F.R.G.), pentalysine (Lys_5) from Serva GmbH (Heidelberg, F.R.G.), and the other polycations from Sigma Chemie GmbH (München, F.R.G.). Other materials were obtained from standard commercial sources.

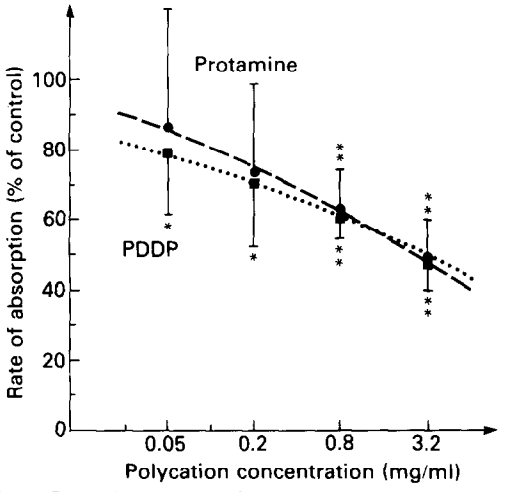

Fig. 3. Dose dependency of the polycation-mediated inhibition of rat jejunal absorption of methyl α-D-glucoside *in vivo*. Single-pass perfusion of rat jejunum; α-Me-Glc concentration: 1 mmol/ml. Note that the abscissa is on a log scale. PDDP: poly[(*N,N*-dimethyl)-3,5-dimethylene-piperidinium chloride]. Values are mean ± SD (N = 6); * $P < 0.05$, ** $P < 0.005$.

RESULTS

Time dependency and reversibility of inhibition

When absorption rates are measured under different *in-vivo* perfusion conditions, their comparison requires that absorption had reached a steady state. Therefore, the time course of the effect of protamine on the absorption of α-Me-Glc was investigated. The experiment was also performed to check the reversibility of possible effects by re-establishing control conditions. The results, as depicted in Fig. 1, show that a new, lower steady-state absorption was achieved approx. 1 hr after addition of the polycation

Table 2. Effect of chain length of lysines on the inhibition of jejunal absorption of methyl α-D-glucoside

Test cation (3.2 mg/ml)	Rate of absorption (nmol/min/cm) Control (KH buffer)	Test (+ test cation)	% of control
Lys	5.1 ± 0.6	4.8 ± 0.9	94
Lys_5	5.6 ± 0.7	5.0 ± 0.6	89
Lys_{20}	5.0 ± 0.9	2.4 ± 0.5*	48
Lys_{400}	6.3 ± 0.7	2.6 ± 0.5*	41

Absorption rates were measured *in vivo* by single-pass perfusion of rat jejunum; α-Me-Glc concentration: 1 mmol/l. Control rates were obtained from a first control period (in the absence of the test cations), and the effect of the lysines was determined in a subsequent second test period. For further details see Materials and Methods. Values are mean ± SD (N = 5); * $P < 0.05$.

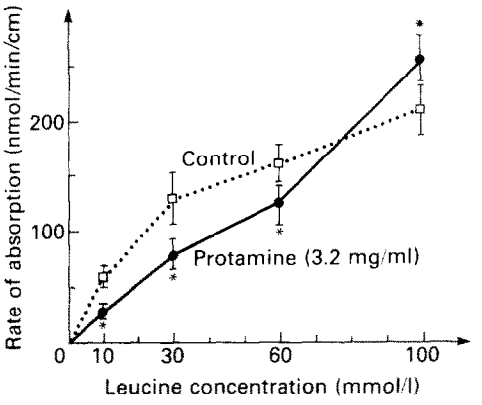

Fig. 4. Effect of protamine on the concentration-dependent leucine absorption in the rat jejunum *in vivo*. Values are mean ± SD (N = 6); * $P < 0.05$.

to the perfusate. The steady state, however, was not completely stable. The absorption tended to decrease further when the test period was extended for another hour. Using a protamine concentration of 3.2 mg/ml and a sugar concentration of 10 mmol/l the absorption rate decreased by about 45%. A significant reversibility was not observed in the second 1-hr control period. Almost identical results were obtained when PDDP (3.2 mg/ml) and α-Me-Glc (10 mmol/l) were perfused (data not shown).

Effect on passively absorbed compounds

In a second set of experiments besides PEG 4000 (Fig. 2, curve A) also mannitol (Fig. 2, curve B) was used as volume marker for the measurement of α-Me-Glc absorption. Assuming that the absorption of the sugar was independent of the kind of volume marker applied, the difference between the two curves in Fig. 2, A − B, suggested an increase of mannitol absorption in the presence of protamine. In fact, protamine in the perfusate increased the rate of mannitol absorption from zero to about 0.6 nmol/min/cm (Fig. 2, curve C) when mannitol absorption was measured with respect to PEG 4000 as volume marker. In the presence of the polycation, again, the rate of α-Me-Glc absorption dropped by about 50%. Thus, employing equal concentrations of either α-Me-Glc or mannitol as transport substrate (1 mmol/l) in the perfusate, in the presence of the polycation the absorption of α-Me-Glc is still four to five times higher than that of mannitol. In addition to mannitol also dGlc was employed in this kind of experiment. Table 1 shows that protamine increases the absorption rates of both these passively transported substrates.

Dose-dependent inhibition

Increasing concentrations of the polycations, as shown for protamine and PDDP in Fig. 3, resulted in an increased inhibition of the steady-state absorption rate of α-Me-Glc as measured after 50 min of pre-equilibration in the 30-min test period. The same kind of dose dependency was found for the inhibition of leucine absorption by these polycations (data not shown).

Influence of the degree of polymerization

The effect of the chain length of the polycation on the absorption rate of α-Me-Glc was investigated with oligo- and polymers of lysine (Table 2). For each lysine derivative tested five animals were employed. As before, the jejunum was perfused first with the inhibitor-free control medium in order to determine the unaffected steady-state absorption rate of α-Me-Glc in each group, and then with the perfusion medium to which the lysine derivative was added. The free amino acid (Lys) and the pentapeptide (Lys_5) caused only a minor reduction of the α-Me-Glc absorption. Significant inhibition of absorption, however, was observed when the degree of polymerization of the lysine derivative reached 20 or more.

Inhibition of the concentration-dependent leucine absorption

For further elucidation of the inhibitory effect of the polycations, the concentration-dependent leucine absorption was determined in the presence and absence of protamine (Fig. 4). The addition of the polycation to the perfusate produced a significant decrease of the absorption rate at leucine concentrations below 60 mmol/l, but an increase at 100 mmol/l. Furthermore, the saturation character of the leucine absorption, as demonstrated under control conditions, was changed into an almost non-saturable process within the range investigated, i.e. a more linear relationship between substrate concentration and absorption rate was found.

Water absorption

It should be noted here that in the *in-vivo* experiments described above water absorption during the test periods was not significantly different from that in the control periods, i.e. the concentration of PEG 4000 in the perfusate did not change due to the presence of the polycations. For instance, the mean value (N = 6) of the net water absorption was 4 ± 17 μl/min/cm under control conditions (α-Me-

Glc concentration in the perfusate: 10 mmol/l) and -3 ± 21 µl/min/cm in the presence of protamine (3.2 mg/ml).

Effect of pretreatment

In addition to these *in-vivo* perfusion studies rats were orally pretreated with a polycation added to the drinking water. This experiment was designed to show whether or not there is a persistent inhibition of intestinal absorption after *in-vivo* polycation pretreatment. Subsequent *in-vitro* absorption experiments were performed without adding the polycation to the incubation medium. In fact, a 60-hr pretreatment with PDDP, a polycation which is not likely to be cleaved in the gastrointestinal tract, resulted in the inhibition of the uptake of α-Me-Glc by everted jejunal rings *in vitro* (Table 3). Inhibition was significant only at low sugar concentrations (2 mmol/l) and amounted to about 30%. Water and food intake were not different between the two groups during this 60-hr pretreatment period. Furthermore, body weights and intestinal parameters such as intestinal wet weight, mucosal protein and DNA content, and maltase activity were not significantly altered by the administration of PDDP (Table 3). Using groups of four rats each, lower PDDP concentrations and shorter periods of pretreatment did not reveal a significant inhibition of the sugar uptake *in vitro* (unpublished results).

DISCUSSION

The results of the present study clearly demonstrate that the various polycations tested are able to inhibit the *in-vivo* absorption of α-Me-Glc and leucine. A comparison with previous *in-vitro* experiments [11] employing a greater variety of transport substrates and polycations shows a good agreement between the *in-vitro* and *in-vivo* results. This suggests that, most probably, the mechanism of inhibition of the polycations on the intestinal transport of the tested substrates is not different between the two experimental conditions. Furthermore, it suggests that the present *in-vivo* findings using only α-Me-Glc and leucine as actively transported substrates can be extrapolated to the other actively transported sugars and amino acids tested *in vitro* such as glucose, galactose and phenylalanine. Along with the inhibition of the active transport of the substrates tested, an increase in the ability of the small intestine to transport solutes by passive diffusion was observed in the jejunum. Mannitol absorption is increased from virtually zero to about 0.6 nmol/min/cm and dGlc absorption is doubled in the presence of protamine which suggests that the absorption of α-Me-Glc and leucine in the presence of polycations is mainly due to passive diffusion. In fact, this is also demonstrated by the *in-vivo* effect of protamine on the kinetics of leucine absorption and presumably on those of glucose absorption. The presence of the polycation in the perfusate changed the saturable process under control conditions into a nonsaturable one. It is admitted that the standard polycation concentration of 3.2 mg/ml used in the present study is high. However, this does not mean that the inhibitory effect is only seen at that concentration. Significant inhibition of intestinal absorption can be observed even at concentrations of 0.05 mg/ml as in case of PDDP (Fig. 3).

The mechanisms by which polycations interfere with the active transport of sugars and amino acids in the small intestine are not established yet. Certain is the binding of the polycations to the acidic groups of the negatively charged microvillous surface and to the glycocalyx [17]. Still, there are several possibilities by which the polycations may interfere with active, Na^+-dependent transport mechanisms.

Firstly, polycations may compete for anionic sites near or directly at the brush-border membrane which are necessary for the cation-dependent active transport processes. The hypothesis of a competition between Na^+ and other cations for these sites is supported by the fact that replacement of residual Na^+-ions at the binding sites by Mg^{2+}-ions in a Na^+-free perfusion medium is more effective in reducing intestinal glucose uptake than the use of mannitol [18]. If the polyanionic nature of the small intestinal surface is essential for the Na^+-dependent active transport of sugars and amino acids, the reaction of polycations with polyanions [19] can certainly alter the cationic environment adjacent to the brush border. This may not only apply to the cation-dependent solute transport, but also to the intestinal absorption of cations [20].

Secondly, polycations may interact with negatively charged proteins or groups of proteins at the surface of the absorbing epithelium to form clusters. This can alter the functions of the membrane proteins and, thus, the properties of the membrane, e.g., the fluidity of the membraneous lipid phase [21]. Since the carriers of active sugar and amino acid transport are functional proteins [22], it is likely that cluster formation and changes in the membrane fluidity, or both, are able to interfere with the transport properties of such carriers.

Thirdly, intracellular events of active transport processes may not be as important in the polycation-mediated inhibition as interactions at the luminal surface. This is indicated by the fact that the membrane transport of polycations is assumed to be low [23] and that the *in-vitro* binding of phlorizin to the sugar carrier is inhibited by polycations [11].

Assuming a reaction between polycations and negatively charged groups of the cell surface or the glycocalyx, species differences in the character and in the amount of these negative groups [24] should lead to a different susceptibility of various species to the inhibitory action of the polycations. A destabilization of the microvillous membrane by polycations in the gallbladder of the rabbit [25] and the disruption of the luminal cell membranes in the kidney proximal tubule [26] hints towards a different susceptibility of the organs with regard to such influences. Morphological studies on the effect of polylysine (1 mg/ml) at the brush border of rat small intestine did not reveal any alteration of the microvillous membrane [17]. That rat small intestinal epithelium may be less susceptible towards deleterious effects of polycations is also suggested by present perfusion experiments in which intracellular (LDH) and superficial enzyme (maltase) activities were determined in the perfusate to indicate the extent of

Table 3. Effect of oral pretreatment of rats with a polypiperidinium chloride (PDDP) in the drinking water on the body weight, several intestinal parameters and the jejunal *in-vitro* uptake of methyl α-D-glucoside (α-Me-Glc)

Parameter	Control (untreated)	Test (pretreated)
Body weight (g)	186 ± 11	182 ± 13
Intestinal weight (mg/cm)	62 ± 6	60 ± 3
Mucosal protein (mg/cm)	4.5 ± 0.5	4.8 ± 0.3
Mucosal DNA (μg/cm)	188 ± 18	180 ± 23
Maltase (mU/mg protein)	289 ± 29	271 ± 33
α-Me-Glc uptake (S_T/S_M) at:		
2 mmol/l	1.22 ± 0.10	0.85 ± 0.09*
32 mmol/l	0.138 ± 0.028	0.126 ± 0.038

Prior to the *in-vitro* absorption measurement test rats received PDDP with the drinking water (0.5 mg/ml) for 60 hr. Tissue uptake of α-Me-Glc (at two concentrations) was determined by means of the tissue accumulation technique using everted jejunal rings (for further details see Materials and Methods). Values are mean ± SD (N = 6); * $P < 0.05$.

mucosal damage. Definitely, the presence of the polycations increased the enzyme activities in the perfusates. However, with respect to the enzyme activities released into the perfusate under control conditions, which were not correlated with any changes in the absorption (Fig. 1, A), the increases in enzyme activities measured in the presence of the polycations do not match with the strong inhibition of absorption. The different amount of negatively charged structures on the tissue surface may be one reason for these differences of the polycation effect in different organs. Also due to the large amounts of mucus associated with the glycocalyx of the intestinal surface probably higher polycation concentrations are needed to produce an effect in the intestine as compared to the gallbladder or the kidney proximal tubule.

In conclusion, the polycation effect in the small intestine obtained under *in vivo* conditions is in agreement with that established *in vitro*. One may assume that polycations which are essentially not absorbed from the small intestine are able to change the ionic milieu close to the absorbing surface or even at the transport sites so that active, cation-dependent transport processes are impaired. Since polycations can be synthesized with a great variety of properties [27], and since *in-vivo* pretreatment with polycations produces a similar effect as co-administration, polycations may be useful as experimental tools for studies of cation-dependent intestinal transport processes and of the absorption of cations themselves.

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