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# The influence of buffer composition on tissue integrity during permeability experiments "in vitro"

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**Abstract**

A well-balanced incubation saline is necessary for permeability experiments with the rat jejunal tissue in the diffusion chambers. At the same time the investigated substance must be chemically stable and sufficiently soluble in this incubation saline. To investigate whether the absence of some ions in incubation salines influences the tissue viability and integrity or the diffusional characteristics of the epithelial membrane the electrical parameters were monitored and the permeability of fluorescein and acyclovir was evaluated during the experiments in side-by-side diffusion chambers. Our results show that the tissue integrity and viability are seriously impaired when $Ca^{2+}$ and $Mg^{2+}$-free conditions are applied on both sides of the diffusion chambers, but not when only mucosal or only serosal side is $Ca^{2+}$ and $Mg^{2+}$-free. Bicarbonate-free incubation salines can also alter the measured apparent permeability coefficients even though the tissue viability and integrity do not change. This change in the apparent permeability is most likely due to a change in the pH of the mucosal surface and can be prevented if the buffer capacity of the incubation saline is increased.


*Keywords:* Rat jejunum; Diffusion chambers; Modified incubation saline; Permeability; $Ca^{2+}$ and $Mg^{2+}$; Bicarbonate

## 1. Introduction

A well-balanced incubation saline is necessary for viability of the tissue segments during the permeability experiments in the diffusion chambers. It is also important that the saline does not influence the diffusional characteristics of the epithelial membrane and that the investigated substance is chemically stable and sufficiently soluble. There are many different incubation salines used for the experiments with intestinal tissue in Ussing, Sweetana-Grass and other types of side-by-side diffusion chambers, i.e. Ringer buffer/solution (Tai and Decker, 1980; Taylor et al., 2001; Charney et al., 2002) Krebs buffer (Yang et al., 1999; Hayden and Carey, 2000) Krebs-Ringers buffer (Karlsson et al., 1999), Krebs bicarbonate Ringer, HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) Ringer (Charney et al., 2002), TRIS (tris (hydroxymethyl) aminomethane) HCl buffer solution (TBS) (Mineo et al., 2002), Dulbecco's buffer etc. All these salines differ only slightly in the concentrations of $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Cl^-$ or phosphates. In the literature there are also several instances of modified incubation salines. These are most commonly used when the permeability or active transport of electrolytes-ions, which are otherwise also present in the standard incubation salines, is being evaluated (Tai and Decker, 1980; Erba et al., 2001; Taylor et al., 2001).

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Bivalent metallic cations ($Ca^{2+}$ and $Mg^{2+}$) in the incubation salines can interact with some active pharmaceutical compounds like quinolones (Sanchez Navarro et al., 1994; Lomaestro and Bailie, 1995), tetracyclines (Neuvonen, 1976), bisphosphonates (Nicklin et al., 1995; Raiman et al., 2001) and others by chelation or by formation of sparingly soluble salts (ibuprofen, Levis et al., 2003 and indomethacin, Fini et al., 2001). These interactions can influence the drug permeability in vivo and in vitro (Sanchez Navarro et al., 1994). Some difficulties in measuring the apparent permeability of drugs from above mentioned groups could be overcome by using a $Ca^{2+}$ and $Mg^{2+}$-free solution at least on the donor side of diffusion chambers.

A number of studies on natural and on cultured epithelia have shown that the presence of extracellular $Ca^{2+}$ is necessary for normal tight junction function and assembly of tight junction proteins (Lacaz-Vieira, 1997; Ward et al., 2000; Huber et al., 2001). The importance of the extracellular $Ca^{2+}$ for the tight junction integrity in intestinal epithelia has been shown on CaCo-2 monolayers using chelating agents for depleting extracellular $Ca^{2+}$ (Artursson and Magnusson, 1990; Noach et al., 1992, 1993; Collares-Buzato et al., 1994). However, EDTA and EGTA (a chelation agent which binds $Ca^{2+}$ but not $Mg^{2+}$) increase the permeability of CaCo-2 monolayers to hydrophilic molecules and decrease their transepithelial electrical resistance (TEER) more when added to the basolateral side than when added to the apical side (Noach et al., 1992, 1993; Collares-Buzato et al., 1994). In basolateral $Ca^{2+}$-free conditions, the paracellular permeability of CaCo-2 monolayers is increased even when EDTA is not present (Tomita et al., 1994). The addition of EGTA on both sides of rat jejunum mounted in Ussing-type chambers has also been shown to strongly increase passive permeability of mannitol (Pérez et al., 1997) whereas EGTA did not affect the transepithelial resistance when added only on one (any) side of the middle intestine of the eel (Trischitta et al., 2001). Additionally, EDTA increases the tight junction permeability in CaCo-2 monolayers more than in rat jejunum (Tanaka et al., 1995).

It has been shown on frog urinary bladder that $Mg^{2+}$ cannot maintain the tight junction seal when no $Ca^{2+}$ is present in the basolateral solution (Lacaz-Vieira, 1997), furthermore excess (20 mM) $Mg^{2+}$ cations in the incubation saline can even enhance the effect of some polycations used as permeability enhancers on monolayers of CaCo-2 cells (Ranaldi et al., 2002).

Bicarbonate-free salines are usually buffered with weak organic acids or bases like HEPES and TRIS (Stewart and Jackson, 1981; Erba et al., 2001; Charney et al., 2002). These buffers have p$K_a$ values closer to the physiologic pH and therefore provide higher buffer capacity than bicarbonate in similar concentrations. Furthermore, they are not sensitive to the equilibrium with $CO_2$ in the gas mixture for saline oxygenation.

The aim of this study was to investigate which incubation salines can be used during permeability experiments in side-by-side diffusion cells without deteriorating the tissue viability. Additionally, we wanted to evaluate the importance of the ions, present in the incubation saline with special emphasis on bivalent cations ($Ca^{2+}$ and $Mg^{2+}$) on the function of the jejunal segment as an absorption barrier. The influence of substituting bicarbonate in Ringer buffer by HEPES on tissue viability and permeability was also evaluated.

For this purpose different electrical parameters (transepithelial potential difference—PD, short circuit current—$I_{sc}$ and transepithelial electrical resistance—TEER) were measured. To get better insight into permeability properties of the rat intestinal tissue mounted in diffusion chambers the apparent permeability coefficients ($P_{app}$) of two substances, acyclovir and fluorescein, which under normal physiologic conditions permeate through the intestinal tissue passively (Kristl and Tukker, 1998) were measured. Recently, it was established that the $P_{app}$ of fluorescein—an acidic drug, is also indicative of alterations in the pH of the mucosal surface—mucus (Kuwayama et al., 2002), which is highly significant for the permeability of ionized drugs (Högerle and Winne, 1983).

## 2. Materials and methods

### 2.1. Materials

Gemfibrozil was obtained from Sigma Aldrich Chemie (Deisenhofen, Germany). Acyclovir was synthesized at the National Institute of Chemistry, Ljubljana, Slovenia (Štimac and Kobe, 1990). Fluorescein sodium was purchased from Fluka (Deisenhofen,

Germany). All chemicals used in this study were of analytical grade.

Incubation salines used for experiments with diffusion chambers were standard Ringer buffer (pH = 7.4) containing (in mM): 140.6 $Na^+$, 5 $K^+$, 1.2 $Ca^{2+}$, 1.2 $Mg^{2+}$, 121.8 $Cl^-$, 25 $HCO_3^-$, 0.4 $H_2PO^{4-}$, 1.6 $HPO_4^{2-}$ and modified Ringer buffers without one or two ions:

- $Ca^{2+}$ and $Mg^{2+}$-free Ringer buffer;
- $Ca^{2+}$-free Ringer buffer;
- $Mg^{2+}$-free Ringer buffer;
- $HCO_3^-/CO_2-$free Ringer buffer;
- $HCO_3^-/CO_2$-free Ringer buffers with 5 mM or 20 mM HEPES instead of bicarbonate;
- phosphate-free Ringer buffer;
- $K^+$-free Ringer buffer.

In modified buffers isoosmolality was achieved by adding sufficient amount of NaCl. Salines containing 25 mM $HCO_3^-$ were bubbled with carbogen (95% $O_2$ and 5% $CO_2$). $HCO_3^-$-free salines were bubbled with $O_2$.

### 2.2. Transport studies across rat jejunum in vitro

The experiments conform to the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Council of Europe No. 123, Strasbourg, 1986).

Rat jejunum was obtained from male Wistar rats (250–320 g). All animals were starved for 18 h before the experiments. After decapitation and laparotomy, the small intestine was immediately excised and placed into the ice-cold 10 mM solution of D-glucose in standard Ringer buffer bubbled with carbogen.

Jejunum 25 cm distally from the pyloric sphincter was used in the experiments. The tissue was rinsed with ice-cold standard Ringer buffer to remove luminal content and cut into 3 cm long segments, excluding visible Peyer's patches. The intestinal segments were opened along the mesenteric border and stretched onto an insert with exposed tissue area of 1 $cm^2$. The insert was then placed between the two EasyMount side-by-side diffusion chambers (Physiologic Instruments, San Diego, CA, USA). Each compartment was filled with 2.5 ml of incubation saline supplemented with 10 mM D-glucose or 10 mM mannitol on serosal and mucosal side of the tissue, respectively. The tissue was kept at 37 °C during the experiments. Incubation salines inside diffusion chambers were oxygenated and circulated by bubbling with carbogen or oxygen. After preincubation for 25 min, the incubation saline on the donor (mucosal) side was replaced with an appropriate buffer solution containing 0.005 mM fluorescein, 1.0 mM acyclovir and 10 mM mannitol. Every 25 min till the end of the experiment (175 min) samples of 250 μl were withdrawn from the acceptor (serosal) compartment and replaced with fresh saline containing 10 mM D-glucose to maintain a constant volume. Effect of compound withdrawal was taken into account for when calculating apparent permeability coefficient ($P_{app}$) values.

### 2.3. Electrical measurements

PD and $I_{sc}$ were measured by a multi channel voltage-current clamp (model VCC MC6, Physiologic Instruments). The diffusion chambers were equipped with two pairs of Ag/AgCl electrodes connected to the chambers via 3 M KCl/3.5% agar bridges, for measuring PD and for passing current, respectively. The experiments were performed under open circuit conditions. The tissue viability was checked by monitoring PD and $I_{sc}$ every 25 min during the experiments and additionally by recording the increase of $I_{sc}$ and PD after the addition of D-glucose (final concentration was 25 mM) to the mucosal compartment at the end of experiments. Because glucose is actively co-transported with $Na^+$ through the apical membrane into the mucosal cells in the viable tissue the absolute value of PD rises in a few minutes (1–5) after the addition of D-glucose to the mucosal side. TEER was determined according to the Ohm's law. $I_{sc}$ and TEER were corrected for fluid resistance prior to mounting the tissue in the diffusion chamber system. The average TEER from 75th to 175th minute of the experiment ($TEER_{75–175}$) was calculated and was used to assess the tissue integrity. Acceptable ranges for viable tissue with good integrity are more than 1.0 mV (absolute value) for $PD_{GLU}$ (PD after the addition of glucose) and between 20 and 40 Ω $cm^2$ for $TEER_{75–175}$.

### 2.4. Analytical procedures

The concentrations of acyclovir and fluorescein in the samples from the transport experiments were

analyzed by a HPLC system (Series 1100, Hewlett Packard, Waldbron, Germany). The column Eurospher C-8 (5 μm, 250 mm × 4 mm; Bia Separations, Ljubljana, Slovenia) was used at 35 °C and a flow of 1 ml/min. The mobile phase for acyclovir and fluorescein was composed of 24% methanol and 76% phosphate buffer (pH = 7.5). For detection of acyclovir UV absorption diode array detector was used at 254 nm. Fluorescein was detected by a fluorescence detector ($\lambda_{EX}$ = 487 nm, $\lambda_{EM}$ = 510 nm) (model RF-535, Shimadzu, Kyoto, Japan).

The mucosal surface pH was measured with a flat membrane pH electrode as described by Legen et al. (2003).

The concentrations of calcium and magnesium were measured by a Perkin-Elmer 2280 atomic absorption spectrophotometer at 422.7 and 285.2 nm, respectively using air-acetylene flame.

### 2.5. Data analysis and statistics

The $P_{app}$ value of the investigated substances was calculated from the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{1}{AC_0}\ (cm/s)$$

where $dQ/dt$ is the steady-state appearance rate on the acceptor side of the tissue, $A$ is the exposed area of the tissue and $C_0$ is the initial concentration of the investigated substance in the donor compartment.

All data are presented as means ± S.D. To investigate the effect of incubation saline composition on the values of $P_{app}$ of fluorescein and acyclovir and on $TEER_{75–175}$, ANOVA was used. Least significant difference (LSD) was applied for pairwise comparisons.

## 3. Results and discussion

The values for $P_{app}$ of fluorescein and acyclovir and electrical parameters ($TEER_{75–175}$ and $PD_{GLU}$) obtained under different experimental conditions are given in Table 1. Fig. 1 shows the time-course of PD and TEER of tissue segments under control conditions.

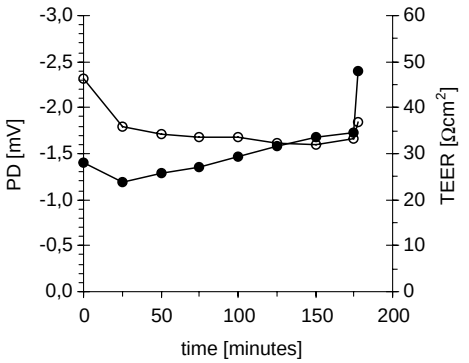


Fig. 1. Tracks of PD (●) and TEER (○) vs. time for jejunal segments incubated in normal Ringer buffer (control) during the experiment. Each point is an average of measurements on 27 rat jejunal segments.

Table 1
The influence of saline composition on the values of $P_{app}$ of fluorescein and acyclovir, on average TEER from 75 min till the end of experiment and on the transepithelial potential after the addition of glucose in the mucosal compartment

| | $n$ | $P_{app}$ (FLU) ($\times 10^{-6}$ cm/s) | $P_{app}$ (ACV) ($\times 10^{-6}$ cm/s) | $TEER_{75–175}$ ($\Omega$ cm$^2$) | $PD_{GLU}$ (mV) |
|---|---|---|---|---|---|
| Ringer buffer on both s[a] | 27 | 5.6 ± 1.5 | 10.0 ± 1.6 | 32.9 ± 14.5 | −2.4 ± 0.8 |
| $Ca^{2+}$ and $Mg^{2+}$-free on both s | 6 | 10.4 ± 2.7 | 18.5 ± 3.1 | 9.9 ± 8.6 | −0.1 ± 0.3 |
| $Ca^{2+}$ and $Mg^{2+}$-free on S s | 6 | 5.8 ± 1.0 | 11.1 ± 1.2 | 33.8 ± 6.3 | −1.7 ± 1.3 |
| $Ca^{2+}$ and $Mg^{2+}$-free on M s | 3 | 4.4 ± 0.3 | 7.5 ± 0.9 | 36.5 ± 1.3 | −2.3 ± 1.0 |
| $Ca^{2+}$-free on both sides | 3 | 5.0 ± 1.1 | 10.9 ± 2.8 | 24.5 ± 13.8 | −1.4 ± 1.1 |
| $Mg^{2+}$-free on both sides | 3 | 4.1 ± 1.8 | 8.4 ± 2.7 | 36.1 ± 15.0 | −2.8 ± 0.3 |
| $HCO_3^-$-free | 6 | 15.1 ± 5.9 | 11.4 ± 1.3 | 24.0 ± 10.5 | −0.6 ± 0.3 |
| HEPES (5 mM) | 3 | 13.9 ± 2.6 | 9.6 ± 1.3 | 22.9 ± 8.4 | −2.7 ± 0.9 |
| HEPES (20 mM) | 3 | 4.8 ± 1.2 | 9.9 ± 1.6 | 28.1 ± 7.7 | −2.0 ± 0.3 |
| Phosphate-free | 3 | 6.3 ± 4.4 | 10.5 ± 2.6 | 27.7 ± 8.7 | −3.0 ± 0.8 |
| $K^+$-free | 6 | 11.7 ± 3.8 | 9.0 ± 2.6 | 39.0 ± 19.3 | −2.0 ± 0.6 |

Data are presented as means ± S.D.; $n$ is the number of determinations on different rat jejunal segments. $P_{app}$ (FLU) is the permeability coefficient of fluorescein; $P_{app}$ (ACV) is the permeability coefficient of acyclovir.
[a] Control experiment.

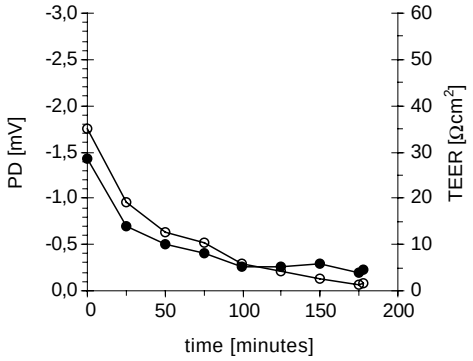


Fig. 2. Tracks of PD (●) and TEER (○) vs. time for jejunal segments incubated in $Ca^{2+}$ and $Mg^{2+}$-free Ringer buffer on both sides of diffusion chambers. Each point is an average of measurements on three rat jejunal segments.

The results clearly show, that in the case of a Ringer buffer without $Ca^{2+}$ and $Mg^{2+}$ on both sides of the isolated jejunal segment, the tissue was not viable during the experiment ($t = 175$ min). In 50 min after the beginning of the experiment TEER and PD decreased below 20 Ω and below −0.5 mV, respectively. After 175 min of incubation in $Ca^{2+}$ and $Mg^{2+}$-free saline the transepithelial potential was low and did not respond to the addition of glucose (Fig. 2). It is reported that the TEER of CaCo-2 monolayers, can recover after the treatment with EDTA if sufficient $Ca^{2+}$ is added (Artursson and Magnusson, 1990), while on the contrary in our experiments the viability and the integrity of the rat intestinal tissue segments did not improve when the $Ca^{2+}$ and $Mg^{2+}$-free saline in the diffusion chambers was replaced by standard Ringer buffer. This shows that when the lack of bivalent cations was sufficient to cause a disruption of the paracellular barrier the rat intestinal epithelium was irreversibly damaged. Consequently the $P_{app}$ values of fluorescein and acyclovir were significantly higher and the $TEER_{75–175}$ was significantly lower compared to the control conditions ($P < 0.001$ for all three parameters). This can indicate that the ability of intestinal tissue to regenerate in "in vitro" conditions is not as good as that of cultured cancer cells.

The tissue was viable throughout the experiment when both $Ca^{2+}$ and $Mg^{2+}$, were absent from the incubation saline only on mucosal or only on serosal side of the tissue. TEER values as well as the $P_{app}$ values of fluorescein and acyclovir were in the same range as in the case of control experiment (Table 1). It was shown (Tomita et al., 1994) that CaCo-2 monolayers are not influenced by $Ca^{2+}$-free conditions on apical side if EDTA is not used. On the other hand, the basolateral $Ca^{2+}$-free conditions cause a significant increase in CaCo-2 monolayer permeability even when no EDTA is added (Tomita et al., 1994). It was shown on different cultured epithelia that a morphological change in uvomorulin is the major if not the only cause of the disruption of paracellular barrier induced by $Ca^{2+}$ chelation (Collares-Buzato et al., 1994). The cellular location of this $Ca^{2+}$-dependant molecule, which is distributed over the lateral membrane, could thus explain the sensitivity of the junctional complex to basal $Ca^{2+}$-free conditions and the lack of sensitivity to apical $Ca^{2+}$-free conditions. On the other hand, the basolateral side of the unstripped isolated segment of rat jejunum is protected by underlying muscular and connective tissue, which could make it less susceptible to the lack of bivalent cations than the basolateral side of monolayers of cultured cells which is directly exposed to the incubation saline.

Since the aim of this study was to establish whether an incubation saline-free of bivalent cations, which could interact with some tested compounds inside the donor solutions could be used for the experiments with rat jejunum mounted in the diffusion chambers we determined the amount of these cations that appears in the "$Ca^{2+}$ and $Mg^{2+}$-free" donor solution during the experiment. The appearance of $Ca^{2+}$ and $Mg^{2+}$ could be either due to the diffusion from the acceptor side (where standard Ringer buffer with 1.2 mM of each bivalent cation was present) or from the tissue segment itself. The donor side can be serosal or mucosal side depending on whether m-to-s (mucosal to serosal.) or s-to-m (serosal to mucosal) drug permeability is being measured. Therefore, the concentrations of $Ca^{2+}$ and $Mg^{2+}$ at the end of a 175 min experiment were measured in both mucosal and serosal "$Ca^{2+}$ and $Mg^{2+}$-free" saline. The $Ca^{2+}$ concentrations determined were only $34 \pm 8$ μM (mean ± S.D.; $n = 3$) and $27 \pm 9$ μM on mucosal and on serosal "$Ca^{2+}$-free" side, respectively. Similarly the $Mg^{2+}$ concentrations rose to $37.7 \pm 1.7$ μM and to $24.5 \pm 1.1$ μM on mucosal and on serosal "$Mg^{2+}$-free" side of side-by-side diffusion chambers, respectively. $Ca^{2+}$ and $Mg^{2+}$ at concentrations this low probably would not cause any important interactions with the tested compounds,

which are usually applied in much higher concentrations (i.e. 1 mM) in the donor solutions.

The absence of only $Ca^{2+}$ or only $Mg^{2+}$ from incubation salines in both compartments of the diffusion chambers did not affect the tissue integrity since TEER and $P_{app}$ values for fluorescein and acyclovir were not affected, compared to the control experiment (Table 1). The observation that the absence of $Ca^{2+}$ does not cause any changes in tissue integrity is rather surprising since $Ca^{2+}$-free conditions on both sides (apical and basolateral) cause deterioration in the integrity of cultured epithelia—an opening of tight junctions (Tomita et al., 1994). When a similar test on rat jejunum was performed (Pérez et al., 1997), 5 mM EGTA was used to provide $Ca^{2+}$-free conditions and the mannitol flux was dramatically increased (the control of tissue viability was not reported). In our experiments the $Ca^{2+}$ reserves of the tissue segments themselves were not depleted by any chelating agent and might have been sufficient to maintain the tissue viability and integrity while the incubation saline contained $Mg^{2+}$ in normal concentration. It was shown (Lacaz-Vieira, 1997) that $Mg^{2+}$ itself probably cannot replace $Ca^{2+}$ in its protein binding sites, however our results indicate that $Mg^{2+}$ must have some role in maintaining the tissue integrity and viability in the absence of $Ca^{2+}$. The results, which show that the absence of only $Mg^{2+}$ from incubation saline on both sides of the isolated jejunal segment does not alter the function of the epithelial barrier are in accordance with previously reported observations, when similar permeability experiments with rat small intestine were performed (Emi et al., 1998).

When bicarbonate-free buffer with bicarbonate substituted by an equimolar amount of NaCl was tested, the value for TEER was in acceptable range and $P_{app}$ of acyclovir was similar to that obtained in control conditions, indicating that the function of the tight junctions between epithelial cells was not affected (Fig. 3, Table 1). However, the transepithelial potential was much lower than in control conditions, and the $P_{app}$ of fluorescein was significantly ($P < 0.001$) higher than in control experiments. The measured transepithelial potentials were probably low due to the inwardly directed proton gradient across the intestinal tissue. The pH of this bicarbonate-free saline at time zero was 7.50 on both sides of the tissue. In 175 min (the duration of the experiment), the saline pH has

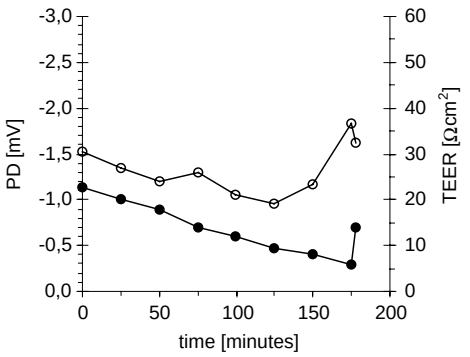


Fig. 3. Tracks of PD (●) and TEER (○) vs. time for jejunal segments incubated in $HCO_3^-$−free Ringer buffer on both sides of diffusion chambers. Each point is an average of measurements on three rat jejunal segments.

changed to $6.67 \pm 0.01$ and to $7.81 \pm 0.14$ on mucosal and on serosal side, respectively. This pH gradient also explains the increased $P_{app}$ of fluorescein. Although fluorescein is used as a marker molecule for paracellular permeability its $P_{app}$ is influenced by the pH gradient across the apical membrane and is markedly increased when an inwardly directed proton gradient is present (Kuwayama et al., 2002). By replacing bicarbonate with NaCl the buffer capacity (as calculated by the Van Slyke equation) is decreased from 0.00331 (standard Ringer buffer) to 0.00103. It seems that the changes in saline pH during the experiment described above could be simply the consequence of the insufficient buffer capacity of this bicarbonate-free saline. However, when bicarbonate in Ringer buffer was replaced by 5 mM HEPES and not by NaCl, the calculated buffer capacity was 0.00391 but the $P_{app}$ of fluorescein was still significantly higher ($P < 0.001$) then under the control conditions (Table 1). Furthermore, when a buffer with normal bicarbonate concentration but without phosphates (phosphate-free Ringer buffer, Table 1) was tested both $P_{app}$ values and all electrical parameters were similar to those obtained under control conditions although the buffer capacity of this phosphate-free saline was only 0.00228. These results indicate that there must be another mechanism, most probably connected with the presence of bicarbonate ions in the saline, which influences the permeability of fluorescein. A possible explanation is given by Legen and Kristl (2003) who suggest that bicarbonate-free conditions activate NHE3 $Na^+/H^+$ exchangers on the apical enterocyte membrane. Increased activity of

these exchangers then causes a drop in the pH value at the mucosal surface. Consequently, low pH of the mucosal surface (mucus) is most probably the reason for increased $P_{app}$ of the acidic marker fluorescein. When bicarbonate in Ringer buffer was replaced with NaCl the buffer capacity of the incubation saline was insufficient to resist the change in pH and the acidification was not limited to the mucosal surface microclimate as it was when bicarbonate was replaced with 5 mM HEPES.

To insure a constant pH of bicarbonate-free incubation saline and to minimize the acidification of the mucosal surface during the experiment, the incubation saline's buffer capacity must be much higher. The $P_{app}$ value of fluorescein in a bicarbonate-free incubation saline was similar to the value obtained under control conditions only when bicarbonate was replaced with 20 mM HEPES and the buffer capacity was 0.0126. When salines were buffered with 20 mM HEPES instead of bicarbonate/$CO_2$ the viability, TEER and the values of $P_{app}$ for both, fluorescein and acyclovir were similar as under control conditions.

The effect of HEPES, used instead of bicarbonate in the incubation saline, on the mucosal surface pH was further confirmed by pH measurements of mucosal surface after incubation of tissue segments in standard Ringer buffer or in 20 mM HEPES buffered incubation saline. The surface pH of rat jejunal mucosae was significantly (*t*-test; $P < 0.01$) lower even when a 20 mM HEPES buffered incubation saline at pH = 7.5 was used ($6.88 \pm 0.17$) compared to standard Ringer buffer at pH = 7.5 ($7.14 \pm 0.10$).

During the experiments with the incubation saline without potassium the tissue was viable, the values of $P_{app}$ for acyclovir and TEER were similar to those obtained under control conditions. This shows that the paracellular barrier (tight junctions) was functioning normally. The $P_{app}$ of fluorescein, however was significantly higher ($P < 0.001$) then under the control conditions. This higher permeability of fluorescein cannot be explained by the data obtained by now. Further experiments are necessary.

## 4. Conclusions

Different ions present in Ringer buffer play a very important role in viability and permeability properties of the isolated rat intestine "in vitro". The withdrawal of an ion from this buffer or its substitution with another ionized substance could cause unexpected changes in permeability of some substances (i.e. fluorescein).

On the other hand, it seems that passively permeable substances that cannot be transported by any other mechanism (i.e. acyclovir) are not so susceptible to the ionic composition of the incubation saline unless tissue is not viable during the experiment or tight junctions are opened.

Regarding the tissue viability and the opening of tight junctions, one must pay special attention to bivalent cations, $Ca^{2+}$ and $Mg^{2+}$ in the composition of incubation salines. However, unilateral $Ca^{2+}$ and $Mg^{2+}$-free conditions could be used when necessary for m-to-s and s-to-m experiments with compounds, which would otherwise interact with these cations. Although the passive permeability of nonionized compounds ($P_{app}$ of acyclovir) through paracellular route is not significantly affected when bicarbonate is removed or substituted by other buffers like HEPES, such changes of salines could significantly influence the $P_{app}$s of weak acids or bases.

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