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The use of hyperosmolar, intracellular-like solutions for the isolation of epithelial cells from guinea-pig small intestine

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Isolated small intestinal epithelial cells were prepared by using either (a) hyperosmolar, low sodium, high potassium containing (intracellular-like) solutions, or (b) isoosmolar, high sodium, low potassium containing (extracellular-like) solutions. Both (a) and (b) cells show high viability as estimated by Trypan blue exclusion, oxygen consumption, cellular ATP content, lactate-dehydrogenase liberation, intracellular ion concentrations and significant Na^+-dependent alanine and uridine uptakes. Although (a) and (b) cells show in the cold similar ion concentration, after reincubation at 37°C for 30 min (a) cells show intracellular ion concentrations of 31 mM Na, 129 mM K and 88 mM Cl, whilst (b) cells have 71 mM Na, 93 mM K and 102 mM Cl. Cells prepared with (a) concentrate much more alanine and uridine than cells prepared with (b), probably because the latter have a lower Na^+ gradient across the plasma membrane. Cells prepared with intracellular-like solutions would be an ideal system to study Na^+-dependent transport mechanisms and the regulatory systems of intracellular ion concentrations.

Introduction

Isolated small intestinal epithelial cells provide an excellent experimental system to study metabolic and physiological processes at the cellular level. During the last 20 years, methods for the separation of intestinal mucosal cells have been developed involving either mechanical dispersion [1,2], enzymes [3–7] and chelating agents [8,9] or their combination [10,11]. These preparations vary in their yield and viability. Recently, Hegazy et al. [12] described a modification of Weiser's method [11] which allows isolation of epithelial cells with a good yield and high viability. Cells isolated by Hegazy's method actively transported potassium and uridine and incorporated uridine and phenylalanine into macromolecules and their intracellular mitochondria had a well coupled respiration. However, in our hands they were unable to generate a sodium gradient across the plasma membrane of a magnitude similar to that observed in the cells 'in situ' [13,14]. Since the use of intracellular-like solution leads to a superior preservation of organs for transplantation [15], we have decided to modify Hegazy's method by using hyperosmolar isolation media with concentrations high in potassium, low in sodium and low in permeable anions, as suggested for renal cells by Nagineni et al. [16]. In the present paper we evaluate cells obtained with these media and compare them with those obtained with isoosmolar extracellular-like solutions.

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Materials and Methods

[5-^{3}H]Uridine, L-[U-^{14}C]alanine, [^{14}C]inulin were purchased from New England Nuclear (Boston). Collagenase Type I and Dulbecco's medium from Sigma Co. (St. Louis), Oils, di-*n*-butylphthalate and di-*n*-nonylphthalate were from Merck (Darmstadt). All other chemicals of analytical grade were obtained either from Sigma Co. or from Merck.

Solutions. Isolation solutions.

a. Intracellular-like solutions. Solution I contained: 7 mM K_2SO_4, 44 mM K_2HPO_4, 9 mM $NaHCO_3$, 10 mM Hepes and 180 mM glucose (pH 7.4; 340 mosmol/l).

Solution II contained: 0.5 mM dithiothreitol and 0.2 mM ethylendiamine tetracetic acid (EDTA) in addition to solution I.

b. Extracellular-like solutions [11,12]. Solution I contained: 27 mM sodium citrate, 96 mM NaCl, 1.5 mM KCl, 5.6 mM K_2HPO_4 (pH 7.3; 290 mosmol/l).

Solution II contained: 0.5 mM dithiothreitol, 1.5 mM EDTA, 140 mM NaCl, 16 mM Na_2HPO_4 (pH 7.3; 290 mosmol/l).

Dulbecco's modified Eagle's medium complemented with 10 mM K_2SO_4, 10 mM $NaHCO_3$ and 10 mM Hepes (pH 7.4) was used. Its osmolarity was adjusted with mannitol to 320 mosmol/l. The following ionic composition was achieved: 116 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM K_2SO_4, 10 mM Hepes.

All solutions were oxygenated with 95% O_2/5% CO_2 for 20 min before their use.

Preparation of cells. Male guinea-pigs weighing 300–350 g were fed with a standard chow and starved for 24 h. Animals were killed by a blow in the head. The small intestine was excised from the ileocecal junction to the duodenal flexure. Food residues and mucus were rinsed from the lumen with solution I. The intestine was filled without pressure with solution I and the two ends were clamped with forceps. The segment was then immersed in 50 ml of solution I for 10 min at 37° C. The luminal content was discarded and the intestine was filled with solution II, clamped and immersed in 50 ml of solution II for 3 min at 37° C. The intestine was then taken out and gently palpated with the fingers for 3 min. The luminal content, which contained isolated cells, was diluted in 100 ml of Dulbecco's medium at 0–4° C and was filtered through a nylon filter with 60 μm pore size. This treatment was repeated once again. The isolated cells were washed by centrifugation at $100 \times g$ for 5 min and the pellet was resuspended in 20 ml of Dulbecco's medium, and was transferred to an incubation chamber at 37° C under oxygenation. Then, 2 mg (800 units) of collagenase type I (Sigma Co.) were added to the suspension which was incubated at 37° C with stirring for 15 min. The suspension was diluted in 80 ml of Dulbecco's medium and filtered through a 30 μm pore size nylon filter and washed twice. The pellet was resuspended in the desired volume of Dulbecco's medium. Cells were stored in the same medium at 0–4° C in plastic tubes without agitation.

Centrifugal filtration. For rapid separation of cells from the incubation medium, the centrifugal filtration method was used. A 400 μl microcentrifuge tube contained 50 μl of the mixture di-*n*-butylphthalate/di-*n*-nonylphthalate (3 : 2, density 1.019). 200–300 μl of medium containing 1–2 mg of cellular protein was placed on top of the oil layer. The density of the phthalate mixture is higher than that of the medium and lower than that of the cells. On centrifugation in a Beckman 52 B microfuge ($13\,000 \times g$) the cells were separated from the incubation medium. Supernatants were removed and the tubes were washed three times with distilled water and then the oil was removed. Pellets were resuspended with 200 μl of water, strongly stirred for 3 min and then 200 μl of 10% trichloroacetic acid was added to precipitate the proteins. The suspensions were centrifuged for 5 min at $13\,000 \times g$. Supernatants were used to determine intracellular contents. Pellets were resuspended with 1 M NaOH and cellular proteins determined by a modified Coomassie blue method [17].

Determination of intracellular water and trapped volume. Measurements of trapped volume were performed by using [^{14}C]inulin. Radioactive labeled solute was added to cell suspensions and the cells were separated from the incubation medium by centrifugal filtration as described above. An aliquot of supernatant was counted to

determine cpm/μl of media. The supernatant was then withdrawn and the tube washed. The pellet was suspended in a known volume of water and proteins were precipitated with trichloroacetic acid. An aliquot of the resuspended pellet was then counted and the total radioactivity trapped by the pellet was calculated. From this value the microliters of media trapped by the pellet were evaluated, and by subtracting that volume from the total pellet water volume the cell water was determined. Total pellet water volume was determined by use of ^3H_2O.

Determination of intracellular potassium, sodium and chloride. Sodium and potassium were determined by flame photometry with a Zeiss PMQ II flame-photometer in aliquots of resuspended pellets prepared as described above. Chloride was measured using a Buchler digital chloridometer. The amounts of sodium, potassium and chloride in the pellets were corrected for the sodium, potassium and chloride contents of the trapped fluid and were divided by the cellular water content to obtain the intracellular ion concentrations.

Oxygen consumption. Oxygen consumption was measured with a Clark electrode. Cells (1–2 mg) were incubated in 3 ml of Dulbecco's medium at 37°C. O_2 consumption was linear with time of incubation for at least 15 min. Experimental zero O_2 concentration in the chamber was obtained by adding sodium dithionite.

Lactate dehydrogenase determination. Lactate dehydrogenase was measured according to the procedure of Bergmeyer and Bernt [18]. The enzyme that leaked out of the cells was determined in the supernatant of centrifuged samples. Total lactate dehydrogenase activity was measured after cells lysis by using 0.1% of Triton X-100.

Colorant exclusion. It was tested by mixing equal volumes of cell suspension and 0.5% Trypan blue solution in 150 mM NaCl at room temperature. At least 400 cells per field were counted under the microscope.

Alanine and uridine uptake. Alanine and uridine uptake were measured in the presence and absence of sodium in the incubation medium. Choline was used instead of sodium. The cells after isolation were washed three times with their respective uptake medium and resuspended in the same medium at the desired concentration. Cells were preincubated for 15 min at 37°C with stirring and oxygenation. In the alanine uptake experiments, cells were preincubated for 15 min with 2.5 mM (aminooxy)acetate, an inhibitor of alanine metabolism [19]. Uptakes were initiated by adding 1 ml of preincubated cells (4–5 mg) to 3 ml of incubation medium containing the appropriate solute and isotopic tracer. Incubation was performed at 37°C and under oxygenation. Samples of 300 μl were taken out at desired intervals and each sample was immediately centrifuged. The intracellular radioactivity was determined as described above.

ATP determination. The ATP concent was determined with hexokinase and glucose-6-phosphate dehydrogenase [20] in $HClO_4$ cellular extracts.

Electron microscopy. Isolated cells in suspension were fixed with 2% glutaraldehyde (v/v) in 100 mM cacodylate buffer (pH 7.4) for 1 h at 4°C. Cells were precipitated by centrifugation at 100 × *g* for 5 min. The pellet was dehydrated and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate. They were observed in a Zeiss EM 10 microscope.

Results

Cell morphology and viability

There was no difference in the morphology and viability of cells prepared either with intracellular-like solution with extracellular-like solution. Fig. 1 shows a electron micrograph of isolated small intestinal epithelial cells. By counting, under light and electron microscopy, was determined that approx. 80% of the cells keep their polarity (brush border, basolateral plasma membrane, basal nucleus), while 20% of the cells lose it (microvilli distribute around the cell and the nucleus is laterally located). Microvilla show normal structure with the microfilaments extending into the cytoplasma. Mitochondria and other intracellular organelles are well preserved. Therefore, with our procedure isolated cells appear as structurally intact showing some of the morphological characteristics of intestinal epithelial cells 'in situ'. Cell viability was judged by the following criteria. (i) At the end of the isolation procedure, at most 1% of the total cell population took up Trypan blue

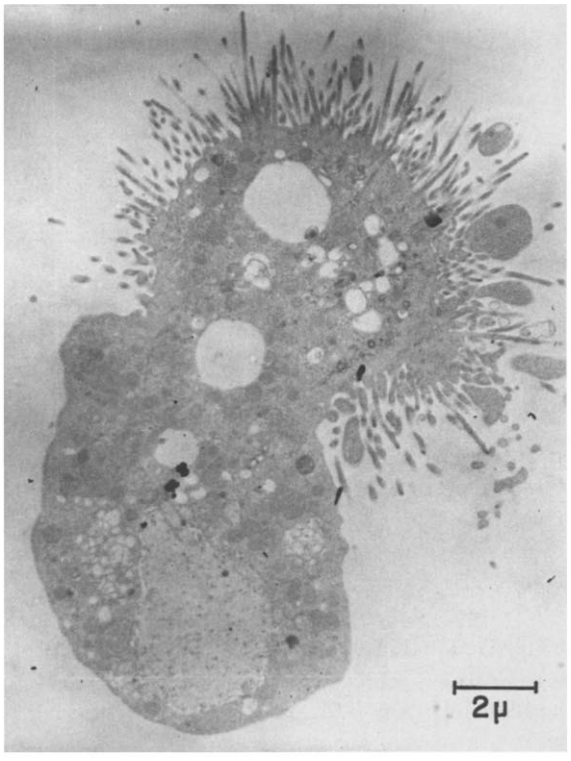

Fig. 1. Electron micrograph of isolated intestinal cells. This polarized cell constitutes approximately 80% of the preparation.

ndicating a $99 \pm 0.8\%$ viability. Viability decreased to $91 \pm 0.9\%$ after 60 min of incubation at 37 °C with agitation and oxygenation. (ii) Fig. 2 shows the lactate dehydrogenase released to the medium as a percentage of the intracellular lactate dehydrogenase activity. At most 5% of the intracellular lactate dehydrogenase activity was lost during a 30 min incubation at 37 °C with oxygenation which was our usual incubation time. Lactate dehydrogenase loss increased with further incubation. Lack of oxygenation resulted in a higher lactate dehydrogenase loss during the same incubation periods. These results add support to the idea that little cell damage is produced during 30 min incubation at 37 °C with agitation and oxygenation.

Oxygen consumption is shown in Table I. The basal oxygen consumption, 12.8 ± 1.53 nmol O_2/mg cell protein per min, was not stimulated by addition of 0.5 mM ADP or 5 mM succinate, corroborating the integrity of the plasma membrane. Respiration was approx. 90% inhibited by 10 μg/ml oligomycin an inhibitor of mitochondrial ATP-synthetase. Subsequent addition of carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler, increased the oxygen consumption by more than 120% of the

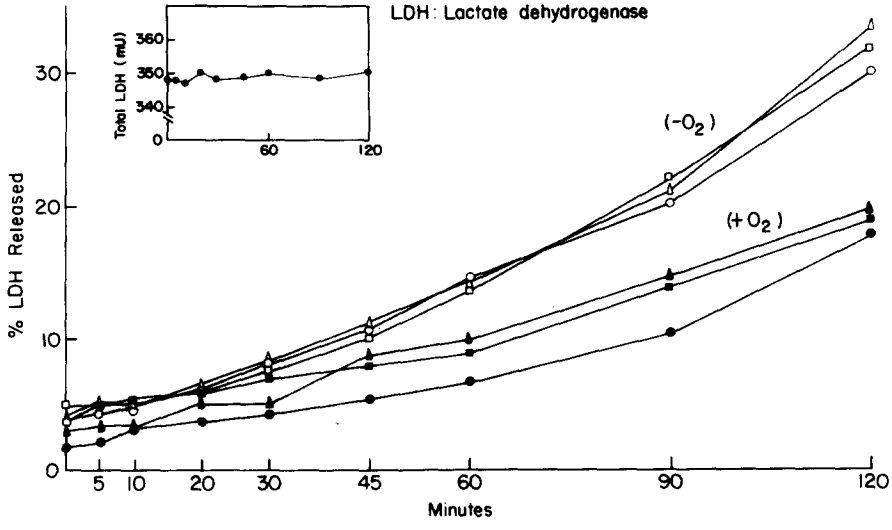

Fig. 2. Time-course of lactate dehydrogenase liberation to medium from cells incubated at 37 °C with or without oxygenation, as percentage of total lactate dehydrogenase activity measured after cell lysis by 0.1% Triton X-100. The latter does not change during incubation time. Values from three different experiments are presented.

TABLE I

OXYGEN CONSUMPTION BY ISOLATED INTESTINAL CELLS

Oxygen consumption was measured using a Clarck electrode at 37 °C. Values are means ± S.E. of three experiments.

Condition	Oxygen consumption (nmol O_2/mg cellular protein per min)
Basal	12.8 ± 1.53
(+) 0.5 mM ATP	13.2 ± 1.73
(+) 5 mM succinate	12.5 ± 1.40
(+) 10 μg/ml oligomycin	1.4 ± 0.16 **
(+) 0.5 μM FCCP	28.2 ± 3.05 **
(+) 1.0 μM rotenone	1.0 ± 0.09 **

** $P < 0.01$.

basal value. Rotenone, which blocks the respiratory chain electron transport between NADH and ubiquinone, inhibited respiration almost completely, and subsequent addition of 10 mM succinate had no effect on residual respiration (data not shown). These results indicate that the mitochondrial respiration is intact, in agreement with the normal ATP content of the isolated cells which was determined to be 12.8 ± 1.1 nmol ATP/mg cellular protein (intracellular concentration: 2.9 mM).

Cell ion and water content

Intracellular Na^+ and K^+ concentration are among the most sensitive criteria of viability. We compared Na^+, K^+, Cl^--and water content of intestinal isolated cells prepared either with intracellular-like solution or with extracellular-like solution, first in cells which were stored as a concentrate suspension for 30 min at 0–4 °C; and then in cells that were rewarmed to 37 °C for 30 min after a period of 30 min at 0–4 °C. Results are shown in Table II. Notice that after incubation in the cold there is no difference in water and ion contents of cells prepared either with intracellular-like solution or extracellular-like solution.

Upon transfer to 37 °C for 30 min, cells prepared either with intracellular-like solution or with extracellular-like solution reduce their Na^+, Cl^- and water content and increase their K^+ content. However, cells prepared with intracellular-like solution have higher Na^+ and K^+ gradients across the plasma membrane than cells prepared with extracellular-like solution. In both cases Na^+ and K^+ gradients across the plasma membrane were abolished by 1 mM ouabain (data not shown).

TABLE II

INTRACELLULAR CONTENT AND CONCENTRATION OF Na^+, K^+, Cl^- AND WATER OF ISOLATED SMALL INTESTINAL CELLS

Cells were collected in ice-cold Dulbecco's modified Eagle's medium at the end of preparative procedure and washed twice by centrifugation 100 × g. After this step, the cells were incubated for 30 min at 4 °C. Then, they were reincubated at 37 °C for an additional period of 30 min. Values are means ± S.E. of four experiments. ICLS, cells prepared with intracellular-like solutions; ECLS, cells prepared with extracellular-like solutions.

	Cellular content (nmol/mg cell protein)		concentration [a]	
	ICLS	ECLS	ICLS	ECLS
At 4 °C				
Na^+	714 ± 26.3	735 ± 40.6	113	113
K^+	232 ± 16.8	221 ± 10.8	37	34
Cl^-	697 ± 12.2	710 ± 41.9	110	109
H_2O [b]	6.34 ± 0.16	6.25 ± 0.34	–	–
At 37 °C				
Na^+	135 ± 10.6	376 ± 15.3	31	71
K^+	568 ± 17.5	493 ± 29.9	129	93
Cl^-	389 ± 14.5	541 ± 20.7	88	102
H_2O [b]	4.4 ± 0.11	5.3 ± 0.21	–	–

[a] Cellular concentration is expressed in mmol/l cell water.
[b] Cell water is expressed in μl/mg cellular protein.

Differences between cells prepared with intracellular-like solution or extracellular-like solution are also observed when studying Na^+-dependent transport processes. Fig. 3 shows alanine (A) and uridine (B) uptake by isolated intestinal cells as a function of time. The Na^+-independent uptake was very similar in cells isolated with intracellular-like solution or with extracellular-like solution. However, the Na^+-dependent uptake was significantly higher in the former than in the latter group. Thus the intracellular to medium concentration ratio for alanine was 12 in cells prepared with intracellular-like solution and 4 in those prepared with extracellular-like solution, after 30 min incubation. For uridine, intracellular to medium concentration ratio was 10 for the cells

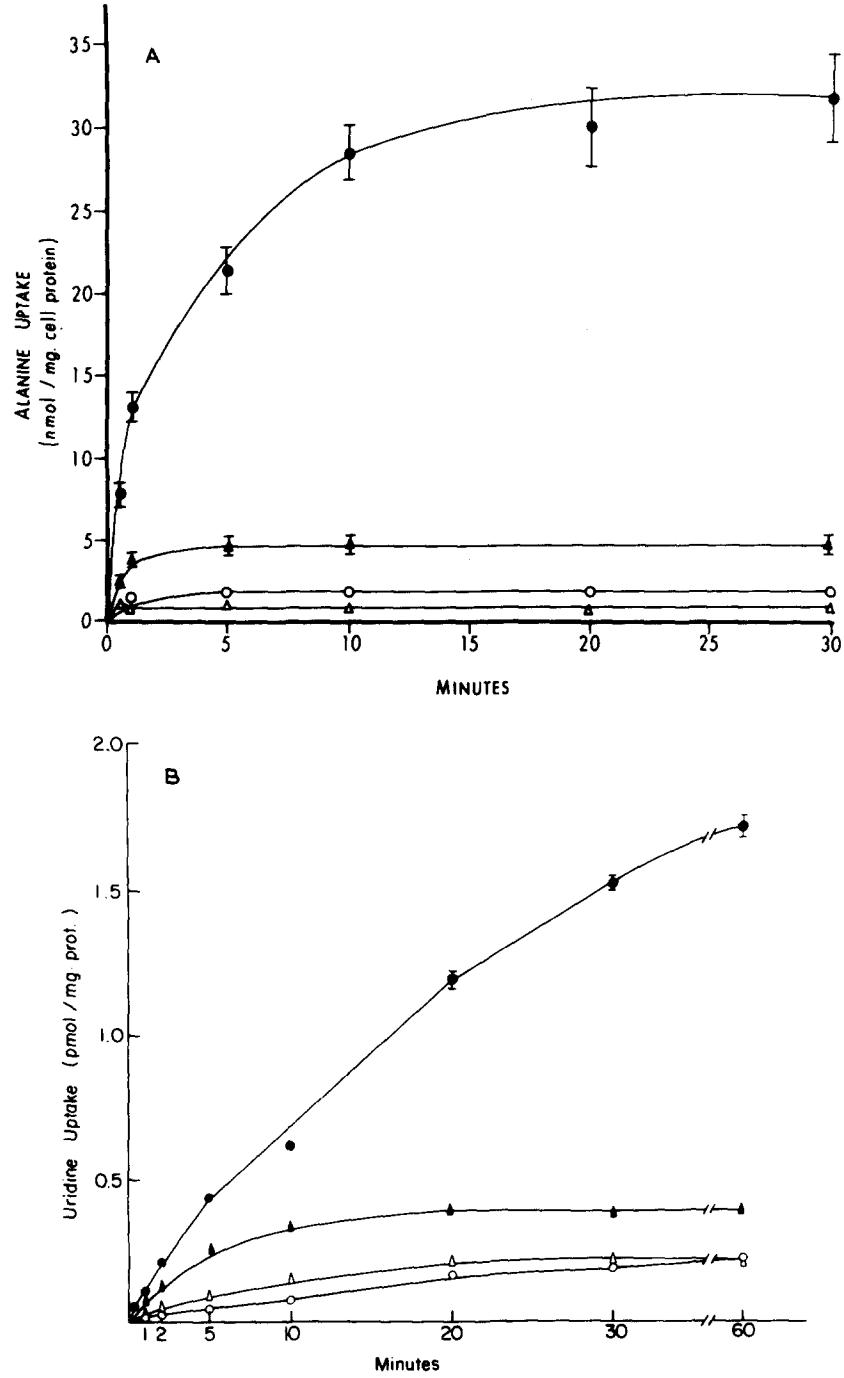

Fig. 3. Alanine (A) and uridine (B) uptake by isolated intestinal cells, prepared with intracellular-like (○) or extracellular-like (△) solutions. Na^+-independent uptake was measured in a medium where choline replaced Na. Na^+-dependent uptake is the difference between the uptake in a medium with Na^+ (●, ▲) minus the uptake in a medium without Na^+ (○, △). Alanine concentration: 1 mM. Uridine concentration: 0.03 mM. Values presented are means ± S.E. of three experiments.

isolated with intracellular-like solution and 3 for those prepared with extracellular-like solution.

Discussion

In the present paper we have evaluated the structural and functional integrity of the isolated cells prepared with hyperosmolar, high K, low Na, containing solutions in which anions were used that only slowly permeate the cell membrane. Probably, most of the cells originated from the tip of the intestinal villi and not form the crypts. As judged from electron micrographs, as that shown in Fig. 1, isolated cells keep some of the morphological characteristics of the intestinal epithelial cells 'in situ'. Thus most of the cells (80%) keep their polarity showing a brush border, basolateral plasma membrane, basal nucleous and well preserved mitochondria while the rest lose their polarity. At the moment, we do not know if the loss of polarity is accompanied by some functional damage. Plasma membrane impermeability to trypan blue is preserved (99% exclusion) and the inability of succinate (5 mM) and ADP (0.5 mM) to stimulate the basal oxygen consumption indicates that the plasma membrane integrity is maintained [21]. High viability continues during incubation time, as indicated by the small lactate dehydrogenase released to the medium (Fig. 2).

Mitochondrial function state was evaluated. Basal oxygen consumption was similar to that reported for other viable preparations. Inhibition by oligomycin (90%) indicates a good energetic coupling. The stimulation by carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (120% over the basal consumption) suggests the existence of an excess of oxidable substrates in the basal condition. A respiratory control ratio of 2.2 was obtained; it is higher than the respiratory control ratio reported for hepatocytes, 1.5 [21]. Most of the oxidable substrates feed the electron transport chain at site 1, as indicated by the almost total inhibition of the oxygen consumption by rotenone. The excellent mitochondrial functional state is confirmed by the ATP content of the cells, 2.9 mM.

Although the morphological and functional characteristics mentioned above are very similar for cells prepared with intracellular-like solution or extracellular-like solution, significant differences are observed when intracellular ion concentrations under different incubation conditions are studied after reincubation at 37 °C. Cells prepared with intracellular-like solution have higher Na^+ and K^+ gradients across the plasma membrane than those prepared with extracellular-like solution (Table II), and their Na^+ and K^+ concentrations are very similar to those reported for intestinal mucosal sheets [14]. These differences have functional consequences, as deduced from the observation that cells prepared with intracellular-like solution concentrate more alanine and uridine than those prepared with extracellular-like solution, in a phenomenon where the affected process is only the Na^+-dependent uptake which depends directly on the Na^+ gradient across the plasma membrane [22]. The maintenance of higher Na^+ gradients is probably due to a better preservation of the plasma membrane integrity, as a consequence of the use of high K containing solutions to isolate the cells. Thus in 1965, Stern and Reilly [23] reported that the presence of K^+ in the isolation and suspending media was essential for the maintenance of the cellular function as measured by respiration and glycolysis, and Nagineni et al. [16] have demonstrated that the use of intracellular-like solutions in the isolation procedure yields a functionally more viable renal cell preparation.

Finally, we have added to the preparative procedure described by Hegazy et al. [12] a treatment with collagenase type I or IV, which transforms preparations with 80–90% viability into preparations with 99% viability. This treatment has been tried in isolated renal and hepatic cells with very good results [24]. At the moment, we do not know the mechanism by which the treatment is effective but we think that collagenase, and probably other proteases participate in the destruction of the non-viable cells when incubated in the indicated conditions.

In short, by using intracellular-like solution we have obtained an intestinal cellular preparation with high viability and yield which preserves much of the morphological and functional characteristics of the intestinal cells 'in situ'.

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