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Isolation of epithelial cells, villi and crypts from small intestine of pigeons (*Columba livia*)

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ABSTRACT: The isolation of viable enterocytes, villi and crypts from the small intestine of a feral bird (*Columba livia*) is important for performing physiological experiments in ecologically relevant processes of membrane transport. The effectiveness of mechanical disruption, enzymatic digestion and chelating agents were compared. The objectives were to isolate enterocytes, villi and crypts from the small intestine of young pigeons; to evaluate the viability of the isolated intestinal epithelial cells isolated; and to verify the integrity of enterocytes by biochemical features. Enzymatic and mechanical methods yielded both elongated columnar and spherical cells. With the chelating method villi and crypts were obtained. All methods produced a high yield of intestinal epithelial cells with about 50 % viability. Brush border enzymes (sucrase-isomaltase and alkaline phosphatase) activities were high and, as reported in chickens, they did not differ along the intestinal villus-crypt axis. Although the three methods have good viabilities, the enzymatic technique gives the best yield in cell number, while the chelating method provides the highest populations of morphologically distinctive villi and crypts.

Introduction

The isolation of intestinal epithelial cells, villi and crypts is particularly important for studies of digestive physiology including developmental physiology (Traber, 1999; Uni *et al.*, 1997; King *et al.*, 2000; Uni *et al.*, 2003), membrane transport processes (Audus *et al.*, 1990; Garriga *et al.*, 1997; Artursson and Borchardt, 1997; Wolffram *et al.*, 1998; Angelo *et al.*, 2002), digestive enzyme activity in different maturation stages of the enterocyte (Traber, 1990; Ferraris *et al.*, 1992; Fan and Stoll, 2001; Uni *et al.*, 1998; Semenza *et al.*, 2001) and cellular biotransformation of agrochemicals (Kurihara *et al.*, 1993). In addition, the availability of freshly isolated intestinal epithelial cells and crypts opens the possibility of using relatively undifferentiated enterocytes as starting material for primary cultures and generation of cell lines (Quaroni *et al.*, 1979; Perreault and Beaulieu, 1998; Velge *et al.*, 2002; Weng *et al.*, 2005).

Mammalian intestinal cell isolation for cell culture has been more extensively studied than avian intestinal cell isolation for the same purpose (Quaroni and May, 1980; Booth and O'Shea, 2002; Kaeffer, 2002). Moreover, most avian intestinal cell studies were performed in chickens (Uni *et al.*, 1998; Angelo *et al.*, 2002; Velge *et al.*, 2002) while we are interested in obtaining intes-

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tinal cells from a feral bird (*Columba livia*) for studies on intestinal membrane transport processes with ecological relevance (Caviedes-Vidal, 2003).

The mucosa of the small intestine is arranged into two fundamental structures: villus and crypt. Villi are projections into the lumen covered predominantly with mature, absorptive enterocytes while crypts (of Lieberkhün) are moat-like invaginations of the epithelium around the villi. Stem cells found at the base of the crypts continually divide and provide the source of all the epithelial cells in the crypts and on the villi (Booth and O'Shea, 2002).

Isolation of cells from tissue requires disruption of the cellular junctions by mechanical, enzymatic or chelating methods. There are different techniques for the preparation of isolated intestinal cells by these methods, which provide a large amount of cells and usually allow an adequate separation between villi and crypts (Quaroni and May 1980).

The first objective of this study was the sequential isolation of enterocytes, villi and crypts from the small intestine of young pigeons utilizing shaking, enzymes and chelating agents, and their microscopic examination. The second objective was to compare the viability of the cells obtained with the three classical methods: mechanical (shaking), enzymatic (trypsin in low concentration) and a chelating agent (EDTA). The third objective was to verify the integrity of enterocytes by biochemical features, measuring the specific activities of brush-border digestive enzymes (sucrase-isomaltase and alkaline phosphatase).

Materials and Methods

Materials

Trypsin-EDTA 0.05% was obtained from HyClone (Logan, Utah, USA). Trypan Blue was provided by BioPack (Buenos Aires, Argentina). Glucose and alkaline phosphate detection kits were obtained from Wiener Laboratorios SAIC (Rosario, Argentina). All other reagents were purchased from Anedra S.A. (Buenos Aires, Argentina) and Sigma Chemical Co. (St. Louis, MO, USA).

Animals and sample collection

Fifteen young feral pigeons (*Columba livia*; Linneo, 1758), five for each treatment, were captured from a nest near the Universidad Nacional de San Luis Campus (San Luis, Argentina) and carried to the laboratory for trials. The birds were anesthetized using ethyl ether, the abdominal cavity was opened and the entire gastrointestinal tract was removed and chilled in ice-cold Hanks' balanced salt solution with mannitol (HBSS-mannitol). The content of the small intestine (SI) was removed, and the SI was cleaned of extraneous tissue, measured for length and weighed. The SI was divided in three parts, proximal (closer to the pylorus) medial and distal. The medial segment was used in the experiments because this part has a high digestive activity (Ciminari *et al.*, 2005). The medial portion of the SI was divided longitudinally into two parts and weighed. One part was destined to the isolation experiment and the other part was immediately frozen to -140ºC and stored for further enzymatic analysis.

Methods of enterocyte isolation

Mechanical shaking:

The intestinal segment from the medial section was washed with ice-cold HBSS-mannitol and cut into small pieces before isolation. The epithelial cells from the crypt-villus axis were isolated by a modification of the method previously described (Velge *et al.*, 2002; Booth and O'Shea, 2002), with mincing and stirring with a magnetic bar at 500 rpm before shaking. In our case, two aliquots of cells were separated at room temperature by vigorous shaking (with vortex) for one minute in HBSS-mannitol. To obtain the first fraction, the tissue underwent one-minute vigorous shaking with vortex, and then the cells were separated from the rest of tissue by filtration through a sieve. The remaining tissue was subjected to another minute of shaking to obtain the second fraction of cells, which were then separated by filtration through a sieve. Dislodged cells in each fraction were harvested.

Enzymatic separation by using low trypsin concentration:

The intestinal segment was washed with chilled HBSS-mannitol before isolation. The epithelial cells from the small intestine were isolated by a modification of the method described by Eade *et al.* (1981) and Booth and O'Shea (2002), (both used 0.25% trypsin). Briefly, the medial portion of the small intestine was cut into five 0.5-cm long segments and incubated in an oven at 37ºC for 30 min in 5 ml of 0.05% Trypsin - EDTA (0.5 g of 1:250/L Porcine Trypsin in HBSS with 1.5 mM EDTA, pH 7.6-8.0) and then the cells were sepa-

rated from the rest of the tissue by filtration through a sieve. The remaining tissue underwent 30 min of incubation in an oven at 37ºC with fresh enzymatic solution to obtain the second fraction of cells, which were then separated from the rest of the tissue by filtration through a sieve. The epithelial cells from both fractions were harvested.

Cell isolation by using chelating agents:

The intestinal segment from the medial section was washed with chilled HBSS-mannitol and cut into small pieces before isolation. The epithelium from the crypt-villus axis was isolated by a modification of the technique reported by Walters (1993) and Booth and O'Shea (2002). Villi were obtained by incubating the segments in 3 mM EDTA with 2 mM dithiothreitol in isotonic solution at 4ºC for one hour, followed by gentle shaking for few seconds. The rest of the tissue was placed in a new dish and fresh chelating solution was added before incubating the tissue at 4ºC for one hour. For isolation of crypts, the remaining tissue underwent an additional 1-hour incubation, which was followed by gentle shaking for few seconds.

Harvesting of cells

The cell fractions were washed three times with HBSS-mannitol by centrifugation at 75 g for 5 min at 4ºC, resuspended in 1 ml of HBSS-mannitol, and dispersed by several passages through a needle prior to cell counting (except for chelating method).

Viability tests

Cell integrity was tested by the method of Trypan blue exclusion (0.4% in physiological saline solution), a method based on the assumption that live cells are impermeable to the dye. Immediately after isolation, cells were counted using a Neubauer chamber.

Enzyme assays

Sample preparation

Intestinal segments (the medial portion without disaggregation and the other used for cell fractioning) or cell fractions were thawed at 4ºC, resuspended in 350 mM mannitol in 1 mM Hepes/KOH buffer (pH 7) (10 ml/g tissue), and homogenized for 30 s using a Fisher Scientific homogenizer (Ciminari *et al.*, 2005). Enzyme activities in these fractions were determined as indicated below.

Intestinal enzyme activities

The activities of two digestive enzymes from the brush border of enterocytes were assayed in homogenates from both isolated fractions of epithelial cells and the portion of the same part of the intestine without disaggregation (see above in animal and sample collection).

To determine the activity of the disaccharidase sucrase-isomaltase (E.C. 3.2.1.48) the colorimetric method developed by Dahlqvist (1984) and modified by Martinez del Río (1990) was used. Aliquots of 40 µl of

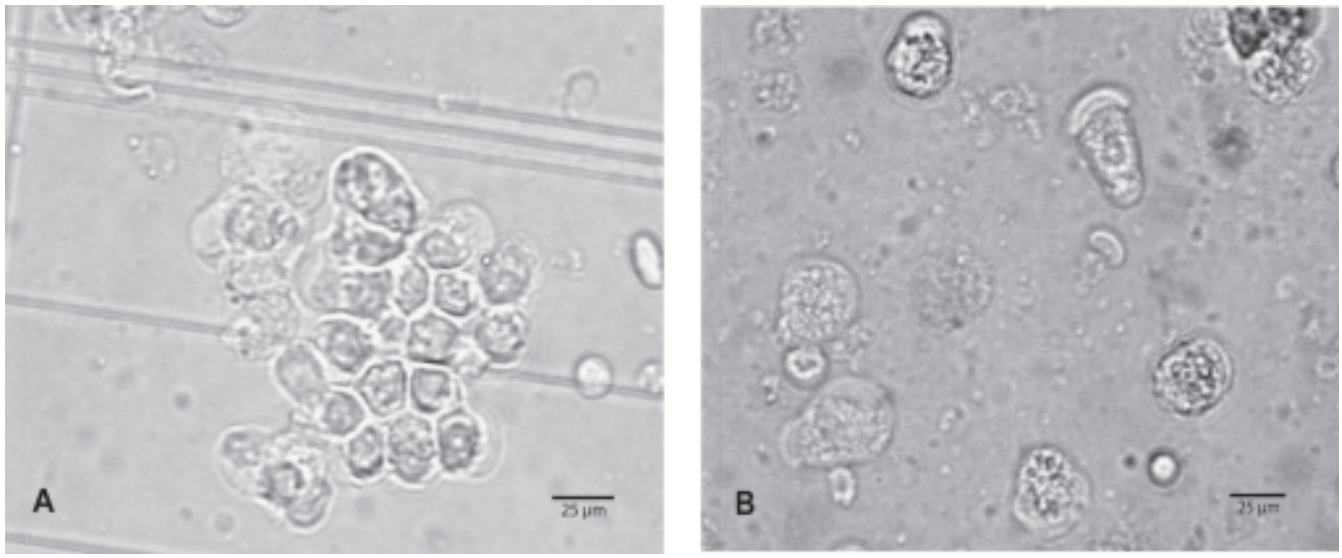

FIGURE 1. Cell isolation by enzymatic method. **A**) Columnar and spherical cells isolated after 30-min incubation with 0.05% trypsin-EDTA, **B**) Spherical cells isolated after 60-min incubation with trypsin 0.05%.

tissue or cell homogenate, appropriately diluted, were incubated with 40 µl of 56 mM sucrose solution in 0.1 M maleate/NaOH pH 6.5. After 10 min incubation at al 40ºC the reaction was stopped by adding 1 ml of Glicemia Enzimática reagent (Wiener Laboratorios SAIC, Rosario-Argentina, with a sensitivity < 10 µg/ml). The enzymatic activity was determined by measuring at room temperature the absorbance at 505 nm with a Spectronic 21D spectrophotometer.

Alkaline phosphatase (E.C. 3.1.3.1) activity was determined spectrophotometrically by the rate of sodium phenyl phosphate hydrolysis using an alkaline phosphatase kit (Wiener Laboratorios SAIC, Rosario-Argentina) following the manufacturer's recommendations. Briefly, aliquots of 40 µl of tissue or cell homogenate, appropriately diluted, were incubated with 40 µl of developing reagent (sodium phenyl phosphate in aminomethyl propanol alkaline buffer). Hydrolyzed phenol was detected after a 10-min incubation with 4-aminoantipyrine and ferricyanide at 40ºC. The sensitivity of this method is 10^{-3} units/ml. The samples were read immediately in a Spectronic 21D spectrophotometer at 520 nm.

Protein measurement

The protein concentration of samples was estimated by Lowry's method (1951) using the commercial kit Proti2 Assay (EDTA/Cu Reagent, Wiener Laboratorios SAIC, Rosario-Argentina). Absorbance was read at 540 nm using bovine serum albumin as the standard, with a sensitivity of 20 µg/ml.

Microphotographs of intestinal epithelial cells

Microphotographs of cells fractions, villi and crypts were taken using an Olympus microscope model BX50 and video camera Sony CCD-Iris. For measurements of lengths and widths of isolated enterocytes, the freeware software ImageJ (Rasband, 1997-2006) was employed.

Statistical analysis

Results are given as means ± 1 SEM; n is number of individuals. Standard least-squares methods were used to estimate parameters of linear regression. A Student's *t*-test was used to assess the statistical difference of enzyme activities and protein content between different fractions of isolated cells. One way analysis of variance (ANOVA) was used to compare the viability of cells obtained with enzymatic and mechanical methods. The significance level was set at $P < 0.05$.

Results

Microscopic appearance of isolated epithelial cells

All three methods allowed isolation of morphologically identifiable intestinal epithelial cells. In both fractions, the enzymatic and the mechanical method yielded both elongated columnar and spherical cells. The columnar cells are typically from the villus tip and the spherical ones from mid- and lower villus

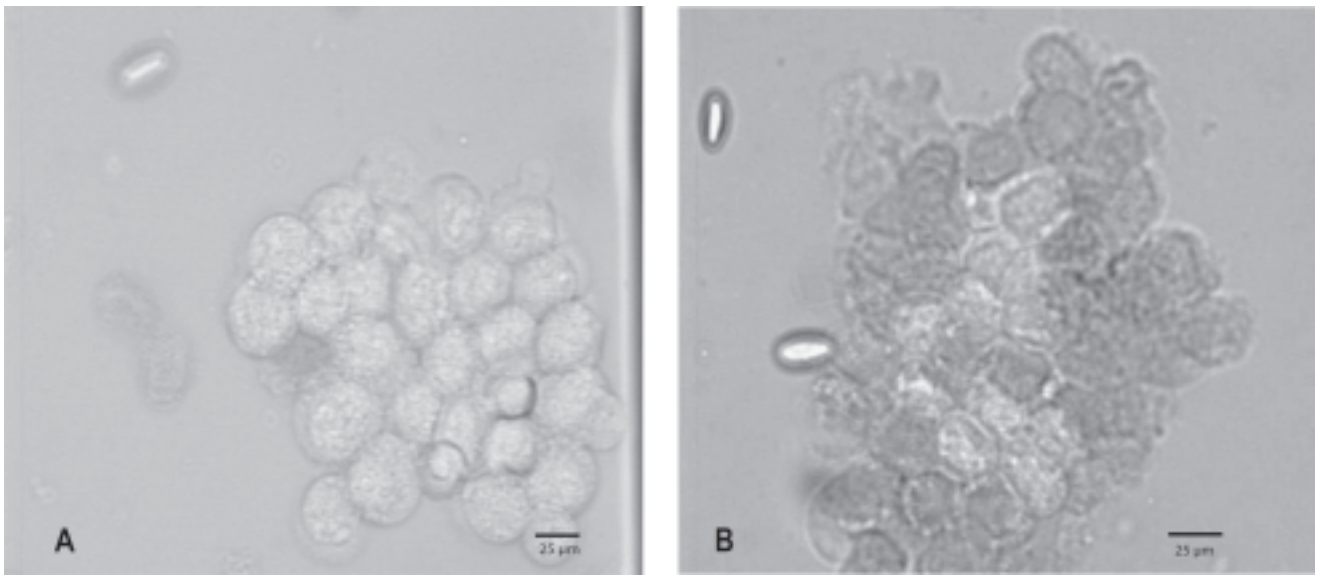

FIGURE 2. Cell isolation by mechanical method. **A**) cluster of cells isolated after 1 min of shaking with vortex, **B**) clusters of cells isolated after 2 min of shaking with vortex.

(Hartmann *et al.*, 1982). Cells occurred singly and in clusters (Figs. 1 and 2). The first fraction of the chelating method allowed obtaining mainly villus units or portions of villi (Fig. 3A) while the second fraction produced crypts or portions of crypts (Fig. 3B). In all cases, the suspensions of isolated intestinal epithelial cells were minimally contaminated by mesenchymal and plasma elements.

Intestinal epithelial cell isolation

The dimensions of isolated pigeon jejunal enterocytes were: for columnar cells (Fig. 1; n = 10), 49.19 ± 1.1 μm length and 24.86 ± 0.81 μm of diameter, and for spherical cells (Fig. 1; n = 50) 33.41 ± 0.67 μm of diameter.

Cell counts and viabilities for mechanical and en-

TABLE 1.

Yields (in number of cells, villus or crypts per ml) and viabilities of intestinal epithelial cell isolates from pigeons, separated by mechanical, enzymatic and chelating methods. Values are means ± SEM (n=5). * $P < 0.032$.

	Mechanical method	**Enzymatic method**	**Chemical method**
Total cells or villus x 10^6 fraction I	6.4 ± 2.1	3.6 ± 1.8*	0.013 ± 0.87
Total cells or crypts x 10^6 fraction II	4.2 ± 1.4	20.7 ± 4.5*	0.038 ± 1.46
Viability (%) fraction I	48 ± 8	50 ± 13	~50
Viability (%) fraction II	46 ± 10	77 ± 8	~50

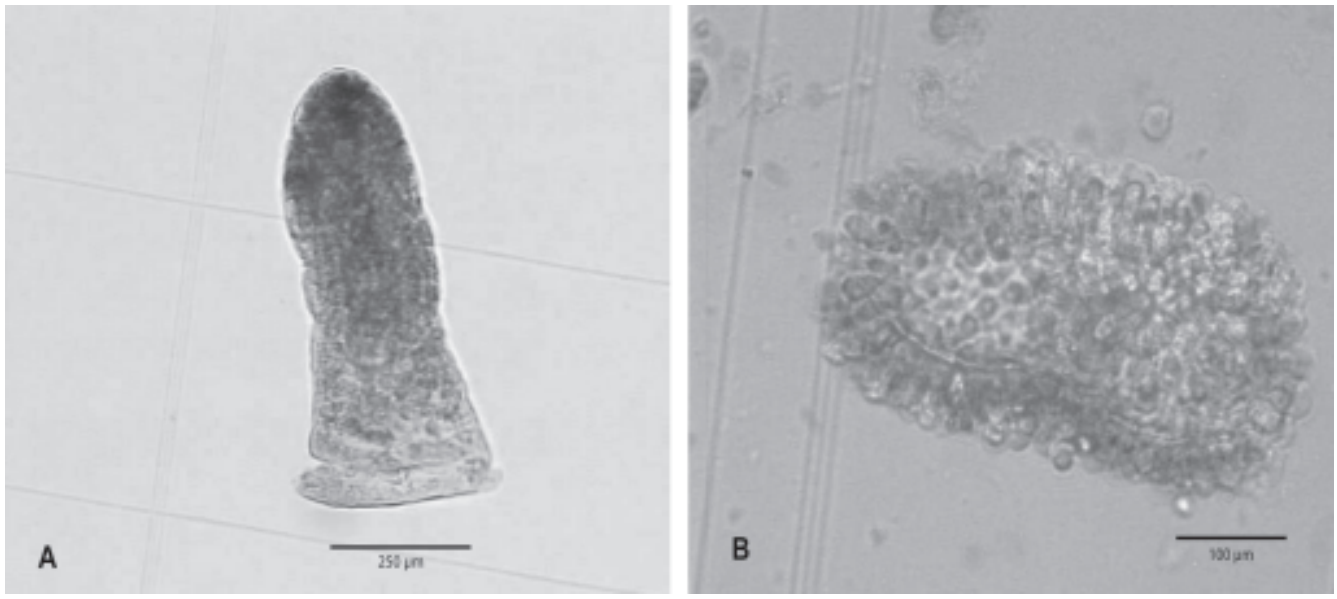

FIGURE 3. Cell isolation by chemical method. **A**) Units of villus isolated after 1-hour incubation in an EDTA solution, **B**) Units of crypts isolated after two hours in EDTA solution.

zymatic methods and the yield of villi (or at least portions of villi) and crypts for the chelating method are shown in Table 1. The viability of the chelating method is an approximation, because we analyzed units or major parts of villi or crypts. There are no significant differences between methods in viability and number of cells obtained in both fractions, except for the enzymatic method ($P < 0.032$).

Protein content

The protein concentration (mg per gram of tissue) in the cells isolated by the three different methods was not statistical different between each fraction with any method: 48.9 ± 2.3 vs 50.2 ± 6.5 ($P > 0.64$) for the mechanical method, 56.6 ± 7.8 vs. 61.9 ± 4.9 ($P > 0.54$) for the enzymatic method and 48.69 ± 4.2 vs 50.75 ± 4.1 ($P > 0.84$) for the chelating method. The ANOVA comparison for all methods gives no difference in protein content ($P > 0.32$).

Levels of brush border enzyme activity

Brush border enzyme activities (sucrase-isomaltase and alkaline phosphatase) of intestinal cells were determined, in both cell fractions, after isolating cells by enzymatic, mechanical and chemical methods. The activities of sucrase-isomaltase and alkaline phosphatase were high in both fractions of cells and they represent around 75% of the activities of the whole tissue used for comparison (whole intestinal segment - see animal and sample collection in materials and methods) (Tables 2 and 3). However, these differences were not statistically significant (all $P > 0.10$).

TABLE 2.

Specific activity of sucrose-isomaltase ($\mu mol \cdot min^{-1} \cdot g^{-1}$ protein) in whole tissue and cells fractions. Values are means ± SEM (n=5).

	Mechanical method	**Enzymatic method**	**Chemical method**
Whole intestinal segment	27.34 ± 6.14	40.79 ± 10.01	41.56 ± 8.51
Cell fraction 1	18.36 ± 3.62	31.64 ± 8.12	28.75 ± 7.99
Cell fraction 2	19.71 ± 5.21	30.88 ± 5.36	40.08 ± 8.46

TABLE 3.

Specific activity of alkaline Phosphatase (UI phenol$\cdot g^{-1}$ protein) in whole tissue and cells fractions. Values are means ± SEM (n=5).

	Mechanical method	**Enzymatic method**	**Chemical method**
Whole intestinal segment	8.18 ± 1.93	7.37 ± 1.05	10.5 ± 4.11
Cell fraction 1	6.91 ± 0.88	6.57 ± 0.68	4.36 ± 2.22
Cell fraction 2	7.78 ± 0.52	4.51 ± 0.96	3.77 ± 1.37

Discussion

The small intestinal epithelium constitutes a system in constant renewal. Enterocytes comprise up to 90% of epithelial cells in the crypt and over 95% of villus cells (Cheng and Leblond, 1974). The typical enterocyte from the villus-tip is recognizable under light microscopy by its oblong shape and conspicuous brush border (Fig. 1B). The spherical cells are typical for mid- and lower villus and crypts (Fig. 1A; Hartmann *et al.*, 1982). In the mechanical and enzymatic methods, spherical cells constituted the main cell type isolated in both cell fractions (Figs. 1 and 2).

An interesting point of the present results concerns the dimensions of isolated pigeon jejunal enterocytes where columnar cells, typical for villus-tip, were 49.19 ± 1.1 μm in length and 24.86 ± 0.81 μm in diameter. These dimensions are larger than those of enterocytes isolated from the jejunum of rat and fish, but agree with dimensions of duodenal enterocytes obtained from young chickens and reptiles (Table 4).

The differences in size have significance in aspects like absorption and digestion, because a large surface area may enhance nutrient digestion and uptake. On the other hand, a smaller number of enterocytes per unit of intestinal surface area might be associated with fewer cell junctions and, therefore, with fewer potential paths for paracellular absorption.

Suspensions of isolated intestinal epithelial cells, minimally contaminated by non-epithelial elements are relatively easy to obtain. However, there is controversy about the viabilities of cells after different mechanical, enzymatic and chelating methods (Eade *et al.*, 1981; Ferraris *et al.*, 1992; Uni *et al.*, 1998). In this study, high viabilities of cells (see Table 1) were found with all methods. Moreover a better result in cell number and viability was shown for the enzymatic technique with a low concentration of trypsin in the second fraction. The chemical method with chelating agents provided enriched populations of morphologically distinctive villi and crypt units, which are free from contamination by non-epithelial elements, such as fibroblasts. The advantage of the chelating method was that crypts were easily obtained and the isolated crypts appeared to maintain their structure and proliferative potential. This is remarkable since crypts are widely used in digestive research (Pothier and Hugon, 1980; Ferraris *et al.*, 1992; Uni *et al.*, 1998; Fan *et al.*, 2001; Angelo *et al.*, 2002; Weng *et al.*, 2005).

Four approaches are available to study the differentiation-dependent expression of enterocyte function *in vivo*: serial sectioning technique, quantitative cytochemical analysis, quantitative immunohistochemical analysis, and sequential cell isolation in combination with biochemical analysis (Smith, 1985). In the present case, the serial sectioning, cytochemical analysis and

TABLE 4.

Dimensions of enterocytes in different taxa.

Animal	Section	Length (μm)	Width (μm)	Reference
Birds				
Feral pigeons (juvenals)	Jejunum	49.19 ± 1.1	24.86 ± 0.81	This study
Broiler chicken (juvenals)	Jejunum	46		Geyra *et al.*, 2001
Mammals				
Swiss-Webster mice (adults)	Jejunum	16.1 ± 1.5	12.0 ± 1.1	Ferraris *et al.*, 1992
Rat	Jejunum	18	11	MacKenzie, 1985
Fish				
Gilthead seabream	Anterior intestine	5.50 ± 0.22	4.59 ± 0.20	Dopido *et al.*, 2004
Reptiles				
Burmese python (juvenals)	Medial portion	40	4.2	Lignot *et al.*, 2005

immunohistochemical analysis techniques are difficult to quantify and limited by availability of specific antibodies. Sequential cell isolation in combination with biochemical assays enables kinetic analyses of digestive enzyme activity in enterocytes under various conditions. This approach has been used previously in rabbits and rodents (Raul *et al*., 1977; Rowling and Sepulveda, 1984).

In mammals, cells in the enterocyte lineage divide several times as they migrate up the crypts. As they migrate towards the villus, they differentiate further, expressing all the transport proteins and enzymes characteristic of absorptive cells (Weiser, 1973; Traber, 1999). Hence, decreasing activities of alkaline phosphatase and sucrase-isomaltase along villus-crypt axis have been described in mammals (Pothier and Hugon, 1980; Ferraris *et al.*, 1992; Fan *et al.*, 2001). This contrasts with the lack of clear definition of the zones of enterocyte differentiation in chicken (Uni *et al.*, 1998) which indicates that in this case further proliferation of enterocytes takes place after they leave the crypt. Consequently, the activity of sucrase-isomaltase and alkaline phosphatase shows high levels of activity along the crypt-villus tip axis, but alkaline phosphatase decreases in crypts (Uni *et al.*, 1998). In pigeons high activities of sucrase-isomaltase and alkaline phosphatase were here found with all methods for both fractions, although as already stated, the chemical method yields mostly crypts (see Fig. 3). These results agree with those reported by Uni *et al.* (1998) in that, in avian intestine, enterocyte differentiation is not precisely localized. In contrast with Eade *et al.* (1981) who recommend careful selection of a method for cell isolation because some methods (e.g. mechanical and enzymatic) could affect the integrity of some proteins of the cellular surface, no differences between brush border sucrase-isomaltase and alkaline phosphatase activities in the whole tissue and the isolated enterocytes were found in the present work.

The isolation of viable enterocytes, villi and crypts from the small intestine of a feral bird raises the possibility of using relatively undifferentiated enterocytes as the starting material for cell culture. It also allows physiological experiments in membrane transport processes with ecological relevance.

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