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ELEMENT CONCENTRATIONS IN THE INTESTINAL MUCOSA OF THE MOUSE AS MEASURED BY X-RAY MICROANALYSIS

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Abstract

Subcellular ion distribution in villus, crypt, Paneth and smooth muscle cells of the mouse small intestine under resting conditions was investigated by X-ray microanalysis of ultrathin cryosections. In addition, the mass distribution was estimated by measuring the optical transmission of the compartments in transmission electron micrographs.

Each cell type is characterized by a special composition in terms of the major monovalent ions Na, K, and Cl. In particular, among crypt epithelial cells, those cells containing small secretion granula (termed crypt A cells) also display cytoplasmic ion concentrations significantly different from crypt epithelial cells lacking secretion granula (crypt B cells). Monovalent ion concentrations in the cytoplasm of Paneth cells, muscle cells, and crypt epithelial cells lacking secretion granula are higher than expected from osmotic considerations. Hence, significant binding of ions to cytoplasmic polyelectrolytes is assumed in these cells.

There are gradients of dry mass and K concentration from the luminal to the basal side of the cell, both in crypt and in villus cells. The terminal web in these cells is rich in Na and Cl. The elemental composition of the large, dark secretion granula in Paneth cells is similar to that of the small dark granula in crypt cells. However, the two morphologically different types of granula within the Paneth cells have a significantly different elemental composition, which might reflect maturation of secretion granula.

KEY WORDS: intestine, ion transport, epithelium, diarrhea, cystic fibrosis, secretion, Paneth cell, X-ray microanalysis, cryoultramicrotomy.

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Introduction

Secretory and absorptive properties of the small intestine have been the focus of a considerable amount of investigation for more than a century, which has led to a generally accepted model of transport in recent years (Field, 1981; Dharmsathaphorn 1987). The active transport of Na by the Na/K-pump is the source of energy for all transport processes. Whereas organic solutes are coabsorbed with Na, water transport is mainly driven by a secondary active transport of Cl (Schultz, 1981). However, other ion transports like those of Ca or K are tightly coupled to Na and Cl movements as well (von Zglinicki, 1986). It is of interest, therefore, to measure not only Na and Cl fluxes through the intestinal epithelium but also the intracellular concentrations of all the ions. Moreover, there is indirect evidence that absorptive and secretory processes are spatially separated. The former take place in the villi and the latter in the crypts (Field, 1981). Clinically, ion transport in the intestine is of interest because in diarrhea abnormal stimulation of chloride and water transport leads to rapid and potentially lethal water loss from the body (Phillips, 1987) whereas in cystic fibrosis transport of chloride and water across the intestinal epithelium is inhibited (Goldstein *et al.*, 1988).

X-ray microanalysis is one of the very few techniques capable of measuring elemental concentrations in individual morphologically well defined cells and their compartments separately. In the case of the intestine, this potential has not yet been fully exploited. Some studies only deal with the absorptive cells of the villi (Gupta *et al.*, 1978; Sjöqvist and Beeuwkes, 1989), whereas in the others only cytoplasmic ion concentrations in villus and crypt cells are compared (Cameron *et al.*, 1979, Spencer *et al.*, 1988). In a study by Carr *et al.* (1987) only peak-to-background values for the elements in enterocytes are given. A more detailed study of element (ion) concentra-

tions in different compartments of the mouse intestinal mucosa was undertaken to serve as a basis of reference for further studies dealing with changes in ion concentrations due to the stimulation or inhibition of transport processes.

Materials and Methods

Mice of both sexes, fed *at libitum* and weighing 20-25 g were killed by cervical dislocation and samples from the proximal part of the jejunum were punched out and simultaneously frozen using a cryobioptic needle (von Zglinicki *et al.*, 1986). Only one sample per animal was taken in order to minimize postmortal ionic changes. Sections of about 0.5 μm thick were cut in an LKB CryoNova at a temperature of 160-180 K. The sections were freeze-dried overnight in a JEOL carbon evaporator at a pressure of about 10^{-3} Torr, carbon-coated and examined at ambient temperature in a Philips EM 400 in transmission mode at 100 kV. Spectra were taken using a Link AN 10000 from the following compartments: basal lamina; muscle cells: cytoplasm, nucleus, and mitochondria; Paneth cells: cytoplasm, rough endoplasmic reticulum (rer), nucleus, mitochondria, dark secretion granula, bright secretion granula; crypt cells containing small secretion granula (crypt A cells): cytoplasm, nucleus, mitochondria, and secretion granula; crypt cells without secretion granula (crypt B cells): brush border, cytoplasm directly beyond the brush border (terminal web), luminal cytoplasm (about halfway between brush border and nucleus), nucleus, basal cytoplasm, and mitochondria; villus cells: brush border, terminal web, luminal cytoplasm, nucleus, basal cytoplasm, and mitochondria. One measurement per compartment was taken per cell, if possible. Specimens from five animals were used.

Quantitative evaluation of spectra was done according to Hall *et al.* (1973) using the QUANTEM software (Gupta and Hall, 1982). A correction for extraneous background was included in the procedure (Roomans, 1988). Frozen-dried cryosections of a gelatine solution containing one of the following salts: Na_2SO_4, $MgSO_4$, KH_2PO_4, KCl, $CaCl_2$ or $ZnCl_2$ in known concentrations in the range from 50 to 400 mmol/kg dry weight were used as standards (Roomans and Sevéus, 1977). Standardization was verified by measuring freeze-dried sections from mouse liver with well known element concentrations (von Zglinicki and Bimmler, 1987).

By measuring the optical transmission of the compartments in the electron micrographs the relative dry mass content was estimated (von Zglinicki *et al.*, 1987). In micrographs of crypt cells, a clear distinction between mitochondria and secretion granules was in general not possible. Therefore, dry mass measurements were done for crypt cells without further differentiation.

Statistical comparison of the data was done by an analysis of variance. Critical differences between means (CDM) were computed at the 5% significance level, i.e., means differing by more than the CDM are taken as significantly different.

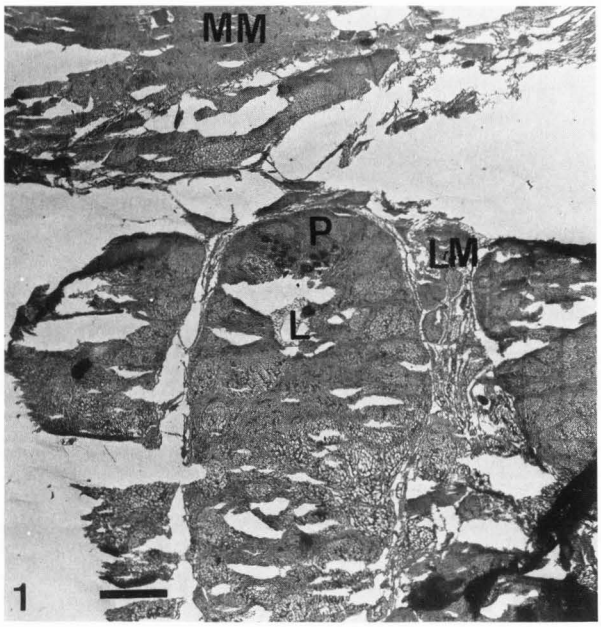
Results

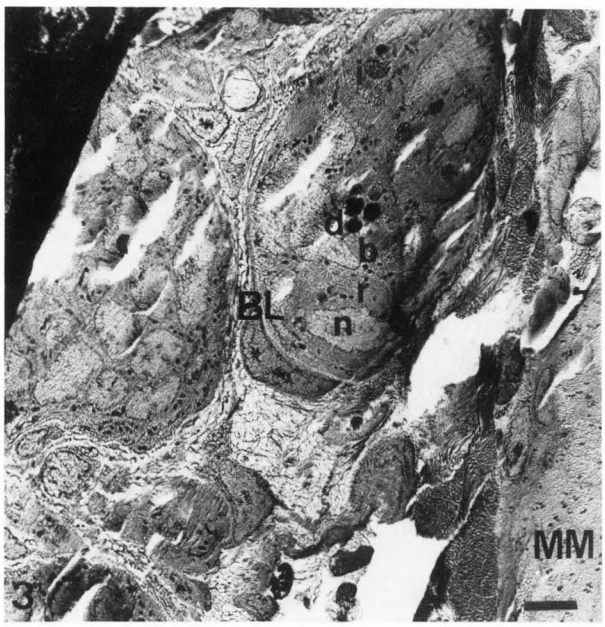
Fig 1 is a low-power micrograph of the deeper parts of the intestinal mucosa showing the underlying muscle layer, lamina propria and the epithelium surrounding the crypt lumen consisting of Paneth and crypt epithelial cells. Due to the high water content of the lamina propria its structure was poorly preserved. X-ray microanalytical measurements in this layer were performed only in the basal lamina.

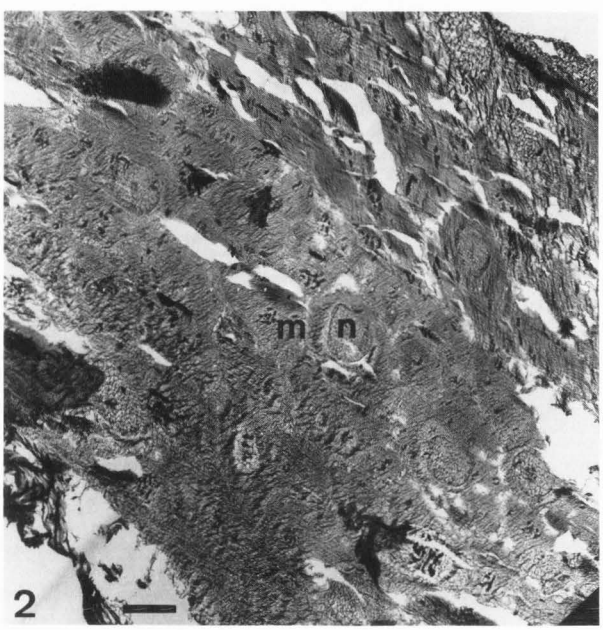
The muscle layer is shown at higher magnification in Fig 2. Nuclei and mitochondria could be discriminated from the cytoplasm.

Figs 3 and 4 display details of the crypt region. The base of the crypts is formed mainly by Paneth cells (Fig 3) characterized by their pyramidal shape, abundant rough endoplasmic reticulum and the existence of large secretion granula. Even if some of the differences as seen between secretion granula in Fig 3 are due to differences in ice crystal formation, it is evident that there are at least two groups of secretion granula with clearly different dry mass fractions sometimes within one cell.

Slightly more towards the lumen (Fig 4), the crypt lumen is surrounded mainly by undifferentiated epithelial cells, sometimes seen in mitosis. Mucus-secreting goblet cells with secretion granula smaller than those of Paneth cells were also found. However, goblet cells were relatively scarce and were not examined by X-ray microanalysis. Some of the undifferentiated cells, but not all, contain small granula undiscernible from mitochondria in frozen-dried cryosections. A clear distinction could only be made by X-ray microanalysis which showed an elemental composition of these granules very similar to that of Paneth cell secretion granules (Table 3). Therefore, measurements of dry mass fractions by microdensitometry of micrographs of crypt cells were pooled together. On the other hand, significant differences between element concentrations in the cytoplasm of crypt cells were found, depending on whether the cells contained secretion granula or not. The cells were termed crypt A cells (with secretion granula) and crypt B cells (without secretion granula), respectively.







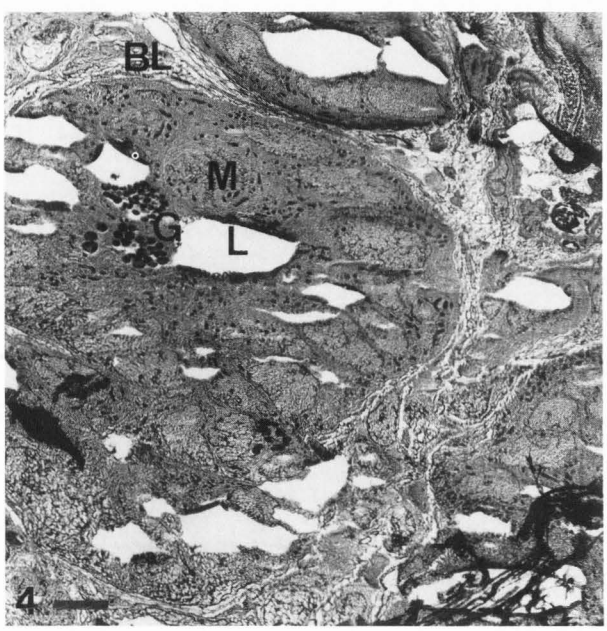


Fig 1: Frozen-dried cryosection of the intestinal mucosa. MM = smooth muscle layer, LM = lamina propria, L = crypt lumen, P = Paneth cell. The bar corresponds to 10 μm.

Fig 2: Smooth muscle layer at higher magnification. n = nucleus, m = mitochondrion. Plasma membranes are only occasionally visible in spite of the small size of ice crystal remnants. The bar corresponds to 3 μm.

Fig 3: Cross-section through the crypt base showing Paneth cells with different secretion granula. MM = smooth muscle layer, BL = basal lamina, n = nucleus, r = rer, d = dark, b = bright secretion granule, and i = secretion granule with large ice crystal remnants. Bar = 3 μm.

Fig 4: Cross-section through the crypt slightly above the level shown in Fig 3. Two goblet cells (G) and an epithelial cell in mitosis (M) are seen. Crypt A and Crypt B cells (see text) are present but cannot be distinguished by their morphological appearance. L = crypt lumen, BL = basal lamina. Bar = 3 μm.

Table 1

Element concentrations (in mmol/kg dry weight, mean ± sem) and dry mass fractions (DM) relative to that of the cytoplasm of crypt cells (mean ± sem) in the cytoplasm of different cells and in the basal lamina of the intestinal mucosa. Numbers of measurements n_{El} and n_{DM} and the critical differences between means (CDM) are given.

Compartment	Na	Mg	P	Cl	K	Ca	n_{El}	DM	n_{DM}
basal lamina	297±25	15±3	399±82	409±37	500±47	7±3	19		
muscle cell	192±24	33±5	344±26	342±14	787±65	7±4	15	0.74±0.09	14
Paneth cell	123±19	50±6	1264±92	133±13	1030±68	14±6	22	1.28±0.09	20
crypt A cell	68± 6	28±2	740±44	100± 9	611±42	13±2	22	1	
crypt B cell	114± 7	44±2	976±25	106± 6	873±20	9±3	37	1	
villus cell	84± 8	30±2	793±17	46± 4	645±17	6±2	15	1.14±0.11	14
CDM	37	10	154	39	123	n.s.		0.26	

Table 2

Element concentrations (in mmol/kg dry weight, mean ± sem) in compartments of intestinal mucosa cells and dry mass fractions (DM) relative to the cytoplasm or the luminal cytoplasm of the respective cells (mean ± sem). Numbers of measurements n_{El} and n_{DM} and the critical differences between means (CDM) are given.

Compartment	Na	Mg	P	Cl	K	Ca	n_{El}	DM	n_{DM}
Muscle cell									
cytoplasm	192±24	33±5	344±26	342±14	787± 65	7±4	15	1	
nucleus	251±50	37±7	946±98	380±37	981±122	0±5	14	0.95±0.09	8
mitochondria	166±35	20±3	652±28	331±30	627± 50	11±7	13		
CDM	n.s.	16	177	n.s.	240	n.s.		n.s.	
Paneth cell									
cytoplasm	123±19	50±6	1264±92	133±13	1030± 68	14±6	22	1	
rer	48±17	40±4	1149±43	131±19	810± 29	9±3	12		
nucleus	104±16	43±4	992±43	160±13	1013± 35	3±6	14	0.68±0.04	18
mitochondria	59± 9	28±4	692±32	163±12	614± 42	2±3	15		
CDM	53	14	193	33	170	n.s.		0.08	
Crypt A cell									
cytoplasm	68± 6	28±2	740±44	100± 9	611± 42	13±2	22	1	
nucleus	80±10	29±5	594±65	82± 7	645± 94	5±2	7		
mitochondria	61± 8	25±3	635±19	122± 6	571± 14	3±4	5		
CDM	n.s.	n.s.	164	n.s.	n.s.	7			
Crypt B cell									
brush border	202±16	16±2	341±10	275±22	708± 37	11±2	14		
terminal web	137±10	36±4	537±44	144± 9	893± 37	-1±2	9		
luminal cytopl	103±14	38±2	919±46	69± 7	813± 31	7±4	9	1	
nucleus	92±12	35±4	842±47	123±11	851± 52	2±3	23	0.76±0.04	21
basal cytopl	90±15	40±4	1066±43	93±16	1010± 51	3±2	7	0.77±0.09	8
unident cytopl	126±14	48±5	971±57	126±15	853± 60	12±3	21		
mitochondria	79±8	32±3	664±81	179±14	612± 58	4±4	12		
CDM	46	14	153	45	181	8		0.14	
Villus cell									
brush border	137±13	16±3	330±20	154±20	615±31	10±2	12	1.13±0.10	20
terminal web	116± 8	25±3	471±37	73±14	607±15	6±2	9	0.80±0.06	20
luminal cytopl	95± 9	27±2	793±20	44± 5	609±15	6±2	9	1	
nucleus	74±15	31±4	832±40	41± 4	687±49	6±3	6	0.95±0.07	20
basal cytopl	68± 7	35±3	792±33	48± 5	700±32	5±2	6	1.11±0.05	20
mitochondria	101±13	26±3	670±18	96±12	488±29	4±1	5		
CDM	31	9	85	47	88	n.s.		0.18	

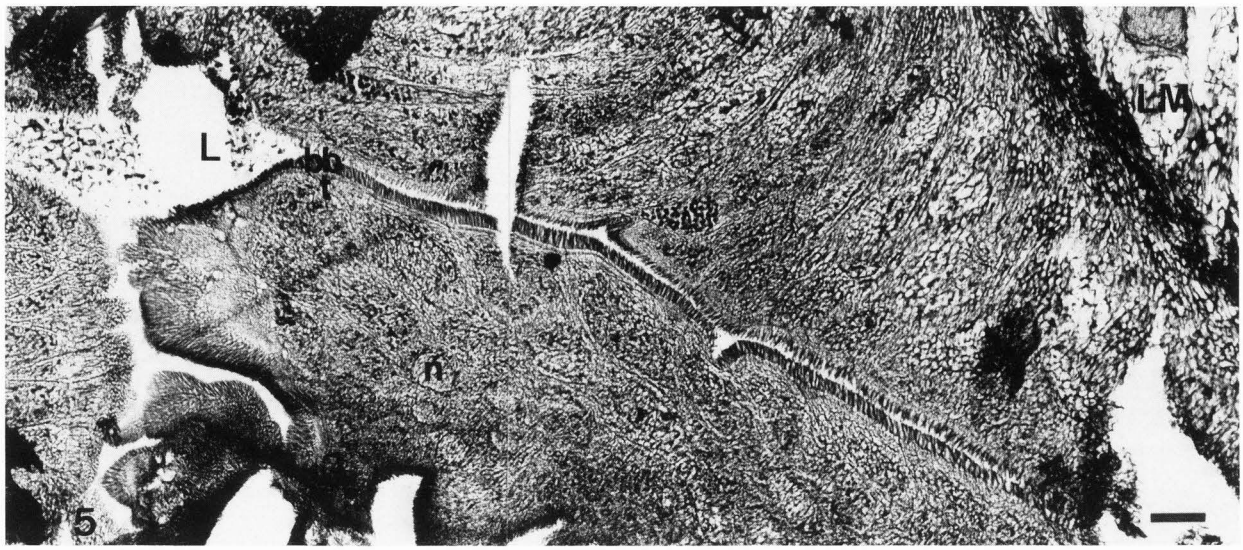


Fig 5: Frozen-dried cryosection of the villus tip region of the intestine. L = lumen, LM = lamina propria, n = nucleus, bb = brush border, t = terminal web. Bar = 3 μm.

Fig 5 is an example of the villus epithelium. In the absorptive cells of the villi, microvilli were well developed and the terminal web was clearly seen. In these untreated animals, the mean dry mass of nuclei was not very different from that of the cytoplasm. No lateral spaces between absorptive cells are seen. Goblet cells are again scarce.

By X-ray microanalysis, concentrations of Na, Mg, P, S, Cl, K, Ca, and Zn were measured. Values for S measured at ambient temperature in the electron microscope are known to be significantly biased (Cantino *et al.*, 1986, von Zglinicki and Uhrik, 1988) and are therefore not dealt with here. Concentrations of Zn were found to be significantly above zero only within secretion granula.

Mean cytoplasmic element and dry mass content are compared with those of the basal lamina in Table 1. As expected, Na and Cl were highest in the basal lamina, whereas Mg and K are lowest. The muscle cell cytoplasm was characterized by the highest Na and Cl concentrations of all cells investigated and by low concentrations of P. The dry mass of muscle cells was also low. Paneth cells displayed the highest concentrations of K and P in their cytoplasm and a significantly higher dry mass content than the epithelial cells. Crypt and villus epithelial cells were not significantly different in terms of dry mass but displayed interesting similarities and differences in their element contents. Concentrations of Cl were low in villus cells but comparatively high in both types of crypt cells. In contrast, concentrations of the cations Na, Mg and K were low both in crypt A cells and in the absorptive cells of the villi, but high in crypt cells without secretion granula. These differences were significant at the 5% level with the exception of Na in the villus cell cytoplasm. All cells contained rather high amounts of Ca.

Table 2 summarizes the intracellular distribution of elements and water. The following results are evident from these data: mitochondria of the cells in the epithelium always contained less P and K than the cytoplasm in that particular cell type. Quite unexpectedly, the concentrations of Cl in mitochondria were rather high and even significantly higher than in the cytoplasm in crypt B cells. Element concentrations in nuclei are not different from those in the cytoplasm with the sole exception of K in muscle cells. The water content of nuclei was in general higher than that of the cytoplasm, the difference being significant in Paneth and crypt cells.

The brush border in crypt B and villus cells was found to be rich in Na and Cl. Low concentrations of Mg and P were measured there. The terminal web contains intermediate concentrations of Na, Cl, and P. In absorptive cells, the water content of the terminal web was significantly higher than that of the cytoplasm.

There were gradients of the dry mass and K concentration between luminal and basal cytoplasm both in crypt B and villus cells. However, whereas K concentrations were lower at the luminal cytoplasm in both cell types, this contained less water than the basal pole in crypt B cells but more water in absorptive cells.

Elemental concentrations in secretion granula are summarized in Table 3. The element content of the large, dark appearing secretion granula in Paneth cells was

Table 3

Element concentrations (in mmol/kg dry weight, mean ± sem) in secretion granula of Paneth and crypt A cells. The number of measurements n and the critical difference between means CDM are given.

Secr granula	Na	Mg	P	Cl	K	Ca	Zn	n
Paneth dark	48± 4	13±2	78±33	170±22	258±26	106±10	3±1	23
Paneth bright	35±10	9±4	88±21	275±38	481±36	70± 6	-2±3	7
crypt A	59± 9	7±1	73±12	135±13	205±14	88± 8	2±1	25
CDM	n.s.	5	n.s.	51	58	24	4	

very similar to that of the small granula in crypt A cells. However, dark and bright appearing secretion granula within Paneth cells were of clearly different element composition.

Discussion

It has been shown that the preparation and measuring techniques used here are capable of giving unbiased estimations of intracellular element and water distributions (for review, see von Zglinicki, 1988a). The sole exception is S which suffers from radiation damage at ambient temperature (Cantino *et al.*, 1986, von Zglinicki and Uhrik, 1988).

In crypt A and absorptive cells, the sum of the concentrations of Na, K, and Cl is less than 800 mmol/kg dry weight and is therefore in the range expected from osmotic considerations. This value is also in accord with a total of about 200 mmol/kg wet weight in the cytoplasm of absorptive cells as measured by Gupta *et al.* (1978). However, ion concentrations in crypt B cells and especially in the cytoplasm of Paneth and smooth muscle cells are considerably higher, mainly due to the high K concentration in Paneth and crypt B cells and to high Na and Cl concentrations in muscle cells (see Table 1). Although the water content of muscle cells is higher than that of epithelial cells, this difference is too small to account fully for the increase in ion concentrations. The water content of Paneth cells is even lower than that of the epithelial cells. Ion concentrations in muscle cells as measured here are in good accord with those measured in other smooth muscle cells (Somlyo *et al.*, 1979).

It might be concluded that significant amounts of intracellular ions have reduced osmotic activity, i.e., are electrostatically bound to polyelectrolyte charges in the cytoplasm. Binding of K to muscle proteins (von Zglinicki, 1988b) and to ribonucleoproteins , and especially to ribosomes, in rat liver (von Zglinicki and Bimmler, 1987) has been demonstrated. The latter might be the case also in Paneth cells and to a lesser extent in crypt B cells due to the existence of a well developed rer which cannot always be discriminated from the surrounding cytoplasm in frozen-dried cryosections. High concentrations of P as found in the cytoplasm of these two cell types are typical for the existence of a large number of ribosomes. Cl concentrations exceeding by far those expected for a Donnan distribution have been demonstrated in smooth muscle cells and attributed to the existence in the plasma membrane of a Cl pump (Somlyo *et al.*, 1979).

In the light of the possibility of intracellular monovalent ion binding it is not clear at present whether the K and water gradients in crypt B and absorptive cells could have any significance for the transport of electrolytes through the epithelium. The fact that the water content in absorptive cells is lower at the basal pole where the highest K concentration was found, suggests some K binding also in these cells. On the other hand, the distribution of water parallels that of K in crypt B cells. Intracellular ion gradients during osmotic water flow have been demonstrated in toad urinary bladder epithelium (Rick and DiBona, 1987). Gupta *et al.* (1978) measured hypertonicity in the terminal web and in a narrow zone of peripheral cytoplasm surrounding the lateral intracellular spaces between absorptive cells and concluded that this peripheral zone including the terminal web might be preferentially involved in electrolyte and water absorption in the intestine. Our results of high Na and Cl concentrations and high water content in the terminal web (see Table 2) are in line with this observation.

However, there is evidently no strong absorption of electrolytes through the villus epithelium of the unstimulated animals. Fluid-filled lateral interspaces as typical for the electrolyte absorbing intestinal epithelium (Trier and Madara, 1981; Gupta *et al.*, 1978) were never observed. Moreover, concentrations of Na, K, and Cl are low in villus cells (see Table 1) in contrast to what would be expected for actively absorbing cells (Schultz, 1981).

Similar ion concentrations and a higher degree of hydration in nuclei as compared to the cytoplasm are as expected. However,

there are differences in mean nuclear dry mass between the cell types examined. The degree of chromatin condensation as measured by the relative dry mass content of nuclei is low in muscle and crypt cells, intermediate in Paneth cells and high in absorptive cells.

Mitochondrial element concentrations as measured here should be considered with caution. Because relatively large sections were used, ice crystal size was not always small compared to the size of mitochondria (compare Fig 5) which may make it difficult to obtain reliable results at very high resolution (von Zglinicki, 1988a). Ionic redistribution between mitochondria and surrounding cytoplasm cannot be excluded. Moreover, in order to obtain large areas, the section thickness chosen was in the order of the mitochondrial diameter. There might be some cytoplasmic contribution to intentionally mitochondrial spectra, therefore. However, these effects could only mask, not create, concentration differences. *In vivo* elemental gradients between mitochondria and cytoplasm must be at least as high as measured here. This is true also for the high Cl concentrations found in mitochondria of all cell types examined.Cl values above electrochemical equilibrium in mitochondria have been found by a number of investigators (see, for example, Somlyo *et al.*, 1979; von Zglinicki *et al.*, 1986) and sequestration of Cl within or a secondary active transport of Cl into mitochondria have been assumed (von Zglinicki and Bimmler, 1987).

High Ca concentrations similar to those measured in the secretory granula in crypt A and Paneth cells were found in serous and mucous granula of a number of exocrine glands (Müller and Roomans, 1985). However, secretory granula in the intestinal crypts are special with respect to their low Mg and high K concentrations. K and Cl are found in abundance in Paneth cell secretion granula with low dry mass content, but these elements are to a significant extent replaced by Ca and Zn in the more densely packed granula. Because Ca and Zn have a higher efficiency in shielding repulsive electrostatic forces than monovalent ions, this replacement is as expected in the process of the final concentration of secretory products and can be interpreted as a sign of maturation of secretion granula.

The similarity of element concentrations between secretion granula in crypt A cells and mature granula in Paneth cells does not necessarily mean that the secretory products of both cells are the same. Crypt A cells might be seen as undifferentiated precursors of Paneth cells. However, cytoplasmic ion concentrations of Paneth and crypt A cells are much more different than those of Paneth and crypt B cells.

Therefore, clear precursor-successor relationships cannot be deduced from these data. Moreover, the data presented here do not indicate which of the cells measured actually are engaged in electrolyte secretion. In order to establish this, a study of the intestine under secretory conditions is necessary. This will be dealt with in a forthcoming publication.

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Discussion with Reviewers

B.L. Gupta: Your method of measuring dry mass by optical densitometry of the electron micrographs would require that the conditions of exposure and photographic processing are uniform and that there is no differential shrinkage or beam-distortion of the sections. Furthermore the areas used for elemental measurements are not the same as used for densitometry. How much uncertainty is introduced by this dual and indirect approach in the estimation of ionic concentrations, especially when comparing subcellular compartments and different cell types?

If the Link system has been used for converting X-ray data into mmol/kg dry weight by the Hall method then it must have included a measure of local continuum (= dry mass). Why can one not use this continuum value for estimating dry mass directly for every site analysed, particularly when your dry mass (DM) estimates are only relative and not absolute? Furthermore, why can one not use the mass thickness standard in the Quantem/FLS file to measure absolute dry mass?

Authors: For the estimation of element concentrations according to the Hall method the X-ray continuum after correction for extraneous background was used as a measure of local dry mass in the very same compartment. An estimation of the relative water content of the compartments was given as additional information. Densitometry rather than X-ray microanalysis was used for this because it has been shown that the variance of densitometrical measurements is considerably smaller (Linders *et al.*, 1982; von Zglinicki *et al.* 1987) due to count-rate depending variations and problems in the proper subtraction of the external background. Differential shrinkage and beam distortion might contribute to the variance (and would do so both for the densitometrical and X-ray microanalytical estimations) but does not introduce a systemic bias under proper freezing and freeze-drying conditions (von Zglinicki *et al.*, 1987; von Zglinicki 1988a).

A.J. Spencer: There is clearly a large difference in the degree of ice crystal damage in villus tip and crypt regions. How does this affect relative dry mass measurements between these two areas?

Authors: The ice crystal size was always small compared to the area measured and should therefore not affect dry mass measurements by densitometry.

A.T. Marshall: Perhaps you could give an estimate of the electron dose in your analysis? Have you considered the possibility that differential mass loss might occur at ambient temperature thereby influencing your dry mass measurements? Or

were densitometry measurements made on low magnification micrographs taken prior to X-ray microanalysis?

R. Rick: One of the major conclusions from your paper is that there is significant binding of intracellular ions in several cell types investigated, resting on the observation of exceedingly high concentration values of monovalent ions when quantified according to the Hall method. While I cannot rule out intracellular binding of monovalent cations I think that other potential explanations for your result should be discussed. For example, what is your evidence that there is no significant mass loss in your sections? Would not a loss in the continuum signal without a parallel loss in the element characteristic radiation also lead to higher than expected concentrations?

B.L. Gupta: If a loss of sulphur occurs under the beam then there must be loss of organic mass (continuum) and this is not likely to be uniform in different compartments. How can then one accept your measured differences in ionic concentrations as valid? This is apart from the other factors you have carefully discussed in your review.

Authors: The electron dose was not measured, but the conditions of analysis (count rate about 700 cps, 100 s live time) correspond to those commonly used in biological thin section analysis. Dry mass measurements were done on low-power micrographs taken before analysis. It has been shown (von Zglinicki *et al.*, 1987) that there is no differential mass loss between mitochondria and an about 25% protein solution during analysis at room temperature. Moreover, results of ion concentration measurements in different compartments of liver cells at ambient and liquid nitrogen temperature were identical (von Zglinicki and Uhrik, 1988). Total mass and sulfur losses are not very closely related as demonstrated by the fact that sulfur but not mass loss can be blocked at low temperature (Cantino *et al.*, 1986). In the same paper, rather uniform mass loss was shown to occur in different kinds of biological specimens, especially if measured at room temperature. It is likely, therefore, that there is also no different mass loss between the different cell types or compartments and standards. The correctness of the quantitation was tested by using liver and, occasionally, red blood cells. In those cases, the expected concentrations were found.

B.L. Gupta: In your estimates of intracellular osmolarity you have used Na+K+Cl. This excludes electroneutrality and the contributions from anionic organic solutes. Strictly one should (as done in classical physiology) estimate intracellular osmolarity by [Na+K]x2. This should give you a better estimate of putatively "bound" ions. Any comments?

Authors: You are correct. However, Na+K+Cl is evidently the weakest criterium one can use. Since we do not know the actual water content of the cells, we felt it to be more appropriate to use this criterium in order to be on the safe side with our conclusions.

A. Warley: The concentrations of K are quite high particularly in the Paneth cell. Do you think that is related to the secretory function of these cells?

Authors: We do not see a direct relation of high K content to secretory function (crypt A cells are rather low in K but also possess secretion granula). However, there is a good correlation between K and P not only in the cytoplasm but also in the nuclei of the different cell types. This is in accordance with results of Cameron *et al.* (1989) and von Zglinicki *et al.* (1989). As we discussed (von Zglinicki and Bimmler, 1987) there might be a correlation of K with the functional status of the cell via the ribosome content in the cytoplasm.

B.L. Gupta: There are numerous studies (e.g., Cremaschi *et al.* (1982) Biochim Biophys Acta **688**: 271-274; Gordon JI (1989) J Cell Biol **108**: 1187-1198; Berridge MJ (1984) A general Survey of the mechanism and control of intestinal fluid. In: Basic Science in Gastroenterology (ed. Palade *et al.*) Glaxo, Ware, pp 225-249) which have established that the transport (absorptive) competence of the enterocytes increases progressively from the crypt/-villus junction to the tip of the villus. Only the enterocytes in the upper (luminal) one-third of the villus have full ion, water and solute absorption capacity. Our unpublished data indicates that the Na, K and Cl composition of enterocytes along the length of the villus is substantially different. Have you tried to distinguish the position of the enterocytes along the villus?

Authors: It is difficult to establish the exact location of a cell in the villus because villi are tilted more or less randomly with respect to the plane of sectioning. The existence of a well-developed brush border and terminal web was taken as the ultimate criterium. We think that the data were collected mainly from enterocytes in the upper half of the villi.

A. Warley: The authors conclude from their measurements of elemental concentrations on a dry weight basis that there are gradients between the cytoplasm and mitochondria. If the differences in water content between mitochondria and cytoplasm are taken into account would this gradient still exist?

Authors: Yes, generally it would. If we assume in particular Cl to be freely

soluble, the gradient would even be considerably reinforced due to the lower water content of mitochondria as compared to the cytoplasm. Only Na and K gradients in muscle, Paneth and crypt B cells would decrease.

B.L. Gupta: Have you excluded the possibility of selective and focal Cl absorption during freeze-drying as a cause for high Cl in mitochondria?
Authors: Dow *et al.* (1981) demonstrated Cl absorption in high density tissue compartments when the sections were left overnight within the scanning electron microscope vacuum and the anticontaminator was allowed to warm up to room temperature. No contamination was found after freeze-drying for 30 min with the anticontaminator chilled. These results are comparable to those obtained by freeze-drying and storing rat liver cells for different time intervals in a scanning transmission electron microscope (von Zglinicki and Zierold, 1989). There is evidently a certain risk of contamination (and also of ultrastructural changes) which is significantly increased when the anticontaminator is allowed to warm up and degas. However, no significant changes were found even on an ultrastructural level for up to 12 h under vacuum, as long as the anticontaminator was chilled all the time. As an example, Cl concentrations in rat liver mitochondria were found to be the same whether the sections were freeze-dried according to the protocol as used in the present study or whether they were freeze-dried within the electron microscope for 30 min with a chilled anticontaminator (von Zglinicki and Uhrik, 1988). Therefore, it is concluded that selective contamination with Cl can be avoided for at least 12 h under vacuum, if the sections are not exposed to outgassing anticontamination traps.

B.L. Gupta: The organic composition of the basal lamina is cell-type specific and therefore its ion sequestration (K, Ca) and exclusion (Na, Cl) abilities would be expected to differ locally. Where did you measure your basal lamina?
Authors: Basal lamina measurements were done in the crypt regions.

R. Rick: You report surprisingly high concentrations of P and K in the basal lamina. What is the source of the high P and K in such an entirely extracellular compartment? Is the lateral resolution power sufficient to exclude cellular stray contributions?
B.L. Gupta: Your measurements of high K, Mg, and Ca, and low Na and Cl in the basal lamina is in accord with the first reports by Gupta *et al.* (see Gupta and Hall (1978) Fed Proc **38**: 144 for references) on insect tissues where no connective tissue is present outside the basal lamina. Given the poor preservation of lamina propria in your samples, can you be sure that your basal lamina sites do not include "invisible" contributions from connective tissue cells, e.g., fibroblasts?
Authors: The lateral resolution in a 0.5 μm thick frozen-dried cryosection should be about equal to the section thickness and should be sufficient to exclude contributions from the cells of the epithelium (see, e.g., Fig 3). However, due to the rather poor preservation of the lamina propria (Fig 1), precipitation of unidentified constituents onto the basal lamina might occur.

A.J. Spencer: Was any distinction made between longitudinal and circular smooth muscle for analysis and if so, were there any significant differences between the two populations of cells?
Authors: No distinction between the two muscle layers was made.

R. Rick: Isn't the clearly different element composition of light and dark granules largely a reflection of a differential water content?
Authors: One should expect that even the monovalent ions are bound in such densely packed compartments. However, as long as this has not been demonstrated, it cannot be ruled out that the decreased Cl and K concentrations in dark secretion granula are due to the lower water content of these granula. However, Ca is certainly bound, and there is an increase in Ca concentration. It might be more appropriate to see the decreased water content as the result of changes in ion composition.

A.J. Spencer: Staley and Trier (1965) demonstrated that Paneth cell granules in mice are structurally heterogeneous, often comprising a central core surrounded by a halo, which contain neutral and acidic mucopolysaccharides, respectively. Close examination of the Paneth cell granules in Fig 1 shows that some of these granules have a relatively electron lucent core. Would it be possible to probe or scan across the core of such granules to determine whether these regions were elementally similar to the bright Paneth granules?
Authors: A scan across Paneth cell granula is technically possible in a scanning transmission electron microscope (STEM). We would expect that a closer examination of secretory granules would reveal the presence of all kinds of intermediate element concentrations and possibly even heterogeneity within one granule.

A.J. Spencer: Could the dark granules in crypt A cells be lysosomes, which are also present in Paneth cells?
Authors: Two facts argue against this assumption. First, it is known that some

of the crypt epithelial cells contain secretory granules (Trier and Madara 1981). Second, there is the great similarity of element concentrations between granules in crypt A cells and secretory granules in Paneth cells. However, a study of the distribution of lysosomal enzymes was not carried out.

A.T. Marshall: Would not the high Na and Cl concentration of the brush border be partially attributable to salt migration from the lumen during freeze-drying?
Authors: There certainly is a precipitation of luminal salts unto the brush border membrane during freeze-drying. This would affect measurements of Na and Cl in the brush border, because membranes or even lumen could not always be excluded from the measurement area. However, a migration of salts into the cytoplasm, whether brush border or terminal web, does not take place. Steep gradients between cytoplasm and extracellular space are normally found in widely differing cell types. Therefore, intermediate ion concentrations as found in the terminal web in fact reflect a special property of this structure, not a migration artefact.

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