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THE DISTRIBUTION OF ($Na^+ + K^+$)-ATPase ALONG THE VILLUS CRYPT-AXIS IN THE RABBIT SMALL INTESTINE

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The migration of intestinal epithelial cells from the crypts to the tips of villi is associated with progressive cell differentiation. The changes in Na^+-pump levels during migration have been measured in epithelial cells isolated from rabbit small intestine. A significant proportion of ouabain-sensitive ($Na^+ + K^+$)-ATPase in the cell homogenates was latent but could be unmasked by detergent treatment. Highest detergent activation was observed in villus cells. The distribution of pumping sites was also assessed by measuring ouabain binding to intact cells. The kinetics of specific binding was consistent with the interaction of the cardiac glycoside with a single population of binding sites with an apparent K_d of around 10^{-7} M. Both enzyme assay and ouabain-binding measurements suggest that a 2–3-fold increase in the number of Na^+-pumping sites accompanies cell differentiation in rabbit jejunal epithelium. This increase in pumping capacity might be an adaptation of the cells to their absorptive function.

Introduction

Fully developed enterocytes mature from undifferentiated crypt cells during migration along the crypt-villus axis. During differentiation epithelial cells acquire their characteristic morphology [1], enzyme composition [2–4] and transport systems [5,6]. The present report is concerned with the distribution of ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) along the crypt-villus axis. This membrane-bound enzyme, which is thought to be identical with the Na^+ pump, catalyses the exchange of intracellular Na^+ with extracellular K^+ at the expense of ATP [7,8]. The presence of the Na^+-coupled sugar and amino acid transport systems at the brush-border membrane as well as the role of mature cells in Na^+ and water transport must increase traffic of Na^+ across enterocytes; it would then be reasonable to expect that this might increase the need for Na^+ pumping from the enterocytes' cytoplasm.

The presence and properties of ($Na^+ + K^+$)-ATPase in small intestine epithelium have been well documented [9–12]. Contradictory reports have been published, however, concerning its distribution along the villus-crypt axis [13,14]. In the present report we adapt the method described by Weiser [15] to separate small intestinal cells in the rabbit and the methods described by Jørgensen [16] to measure ($Na^+ + K^+$)-ATPase levels after detergent activation, to study the distribution of Na^+ pumps along the crypt-villus axis. Part of these results have been presented at a Physiological Society meeting [17].

Methods

Cell isolation. New Zealand white rabbits, weighing 2.5–3.0 kg and maintained on a standard

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

diet with free access to water, were killed by intravenous injection of pentobarbitone sodium (90 mg/kg body weight). The cells were isolated in sequential populations according to the method of Weiser [15] with some modifications [18]. The jejunum was removed and washed twice with ice-cold phosphate-buffered saline (PBS, Oxoid tablets) containing 1 mM DL-dithiothreitol. The intestine was everted, filled with phosphate-buffered saline and tied at both ends. The gut was incubated for 15 min in a citrate buffer [15]. Succeeding incubations took place in a phosphate-buffered saline-based buffer containing 1.5 mM EDTA, 0.5 mM dithiothreitol and 1% bovine serum albumin (BSA, fraction V from Sigma). Incubations to detach cells sequentially were for 11, 5, 8, 6, 5, 5, 10, 10, 10, 30 and 30 min, respectively, at 37°C with shaking (75 min^{-1}). For enzyme and other assays cells were washed twice in phosphate-buffered saline and homogenised using an Ultra-Turrax homogeniser (20 s, 15000 rpm, 4°C).

Enzyme assays. All assays were done on unfractionated cell homogenates. The activity of γ-glutamyltransferase was assayed following *p*-nitroaniline appearance from L-γ-glutamyl-*p*-nitroanilide using glycylglycine as acceptor [19]. Sucrase was assayed by measuring the liberation of glucose from sucrose [20]. $(Na^+ + K^+)$-ATPase assays were performed according to Jørgensen [16], with minor modifications, at a concentration of protein of 25–50 $\mu g \cdot ml^{-1}$. Detergent preincubated samples were used. The incubation medium (ATPase buffer) had the following composition: 10 mM $MgCl_2$, 120 mM NaCl, 20 mM KCl, 1 mM EDTA, 3 mM ATP (disodium salt), 60 mM imidazole and 60 mM Tris, pH 7.5. Where appropriate 1 mM ouabain was added. After stopping the reaction with ice-cold trichloroacetic acid, liberated inorganic phosphate was assayed by a modification [21] of the Fiske and SubbaRow method. Detergent activation was carried out at room temperature for 30 min at 1–2 $mg \cdot ml^{-1}$ protein concentration. Sodium dodecylsulphate and Triton X-100 were prepared in ATPase buffer while sodium deoxycholate was made up in 60 mM imidazole and 60 mM Tris, pH 7.5. Protein concentrations were determined by a modification [22] of the method of Lowry et al. [23]. DNA was measured by a fluorimetric assay [24].

Ouabain-binding measurements. Cells used were isolated as described above but washed and resuspended in a K^+-free medium of the following composition: 1.3 mM $CaCl_2$/0.5 mM $MgCl_2$/4.2 mM $NaHCO_3$/143 mM NaCl/0.1% bovine serum albumin/5.5 mM D-glucose/10 mM Hepes, pH 7.2. In all binding experiments cells were preincubated for 15 min at 37°C; cell concentrations in the final suspension were 4–5 mg cell protein per ml. Binding was initiated by mixing equal volume aliquots of preincubated cells with prewarmed medium containing labelled ouabain. Cells were separated from the medium by centrifugation through oil as described before [25]: at timed intervals 100 μl samples were transferred to ice-cold plastic centrifuge tubes containing an upper layer of 900 μl of buffer and a 300 μl bottom layer of a mixture of di-*n*-butylphthalate and dinonylphthalate (3 : 2, by vol.) and immediately centrifuged for 30 s at 12000 × *g*. Under the conditions used there was no partition of tritiated ouabain into the oil phase. Samples were taken for counting from the aqueous phase, the remaining aqueous layer as well as most of the oil being discarded. The tips of the centrifuge tubes were placed in scintillation vials and counted. Oil carried into the counting vials together with the pellets produced quenching. The external standard facility in the scintillation counter was used to correct for this. The amount of ouabain associated with the cells was calculated taking into account the specific activity in the aqueous phase.

Materials. [G-^{3}H]Ouabain (32 Ci/mmol) was obtained from Amersham International PLC, Bucks, England. Unlabelled ouabain and bovine serum albumin (Fraction V) came from Sigma, Poole, Dorset, England. All other reagents were of A.R. grade.

Results

Cell isolation

The activity of γ-glutamyltransferase and sucrase in the different cell fractions isolated from rabbit intestine is shown in Fig. 1. A marked decrease in activity was seen from the early to the late samples. The pattern was similar to that reported before for rat jejunum [18] and reveals that

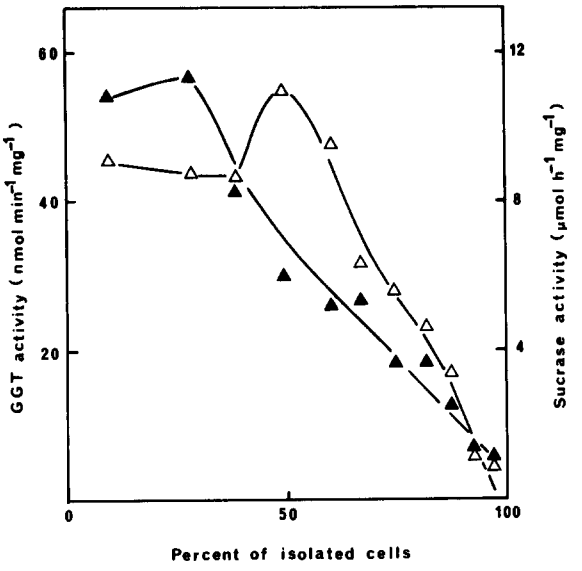

Fig. 1. Activity of γ-glutamyltransferase (GGT) and sucrase in epithelial cells from rabbit jejunum. Cells were isolated in eleven sequential populations from villus to crypt as described in the text. Left-hand axis GGT activity (▲———▲), right-hand axis sucrase activity (△———△), expressed on a per mg total cell protein basis. The percent of isolated cells is based on the cumulative protein content of the sequentially isolated cell fractions.

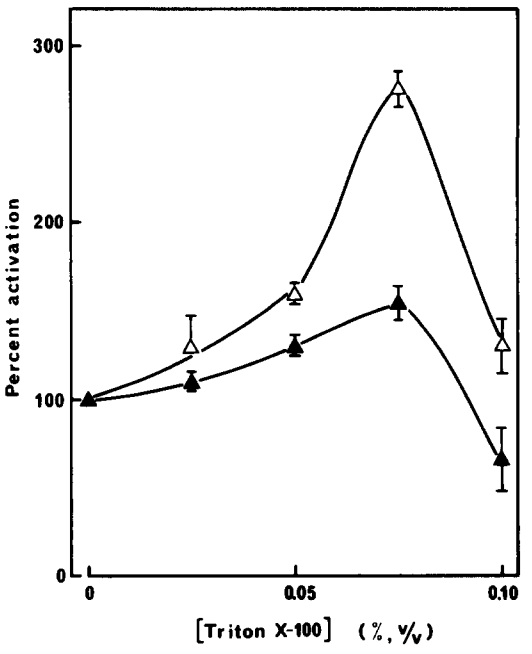

Fig. 2. Activation of $(Na^+ + K^+)$-ATPase activity by Triton X-100. Homogenates (1–2 mg·ml^{-1}) of cells from villus (△) or crypt fractions (▲) were preincubated in the presence of the detergent concentration shown in the abscissa for 30 min followed by assay of the ouabain-inhibitable ATPase activity. All activities are referred to controls that were preincubated in the absence of detergent (100) and are means ± S.E. for four determinations.

the cells have been isolated sequentially from villus tip to crypt base. This was confirmed by microscopic examination of the isolated cells; cells in early samples showed a prominent brush border and elongated shape typical of differentiated enterocytes, while a later sample was enriched in rounded cells devoid of brush border. The ratio of protein to DNA was constant in all fractions (26.6 ± 1.5 μg protein per μg DNA, $n = 12$).

Detergent activation of $(Na^+ + K^+)$-ATPase activity in isolated enterocytes

Specific $(Na^+ + K^+)$-ATPase was assayed by measuring the release of inorganic phosphate from ATP in the presence and absence of ouabain. The difference between these two determinations was taken to be the specific transport-related $(Na^+ + K^+)$-ATPase. It is known that in fresh cell homogenates or in subcellular fractions a major part of the activity is latent. Latent activity was unmasked by incubation with different concentrations of sodium deoxycholate, sodium dodecylsulphate and Triton X-100.

All these detergents had biphasic effects with a maximum activation at 0.6 mg/ml for sodium deoxycholate, 0.025 mg/ml for SDS and 0.075% for Triton X-100. The protein concentration in the activation procedure was kept at 1–2 mg/ml as it is known that the protein/detergent ratio alters the ability of the detergent to unmask latent activity [26,27].

The relative concentrations of detergent needed to produce maximal activation as well as the shape of the activation curves were similar to those reported before for the enzyme from rabbit [26] and guinea-pig [28] kidney (data not shown). In the present case Triton X-100 produced the greatest unmasking of latent activity and was adopted for all subsequent work. To test that detergent activation follows a similar pattern in different cell fractions, detergent-activation curves were constructed for early and late cell fractions. Fig. 2 shows that a similar optimal concentration of Triton X-100 was needed to produce maximal activa-

tion of enzyme activity from villus tip and crypt. Relative increase in activity was higher, in early (villus tip) than in late (crypt) fractions.

($Na^+ + K^+$)-ATPase activity at different locations along the villus

Fig. 3 shows ($Na^+ + K^+$)-ATPase activity as well as Mg^{2+}-ATPase (ouabain-insensitive) activity in enterocytes from different locations along the villus crypt axis. The γ-glutamyltransferase activity is also illustrated to show that discrimination between young and old cells was achieved in these experiments.

No change in the ouabain-insensitive activity was detected but a 2.3-fold increase in ($Na^+ + K^+$)-ATPase was observed in villus as compared with crypt cells. The lower part of Fig. 3 shows the

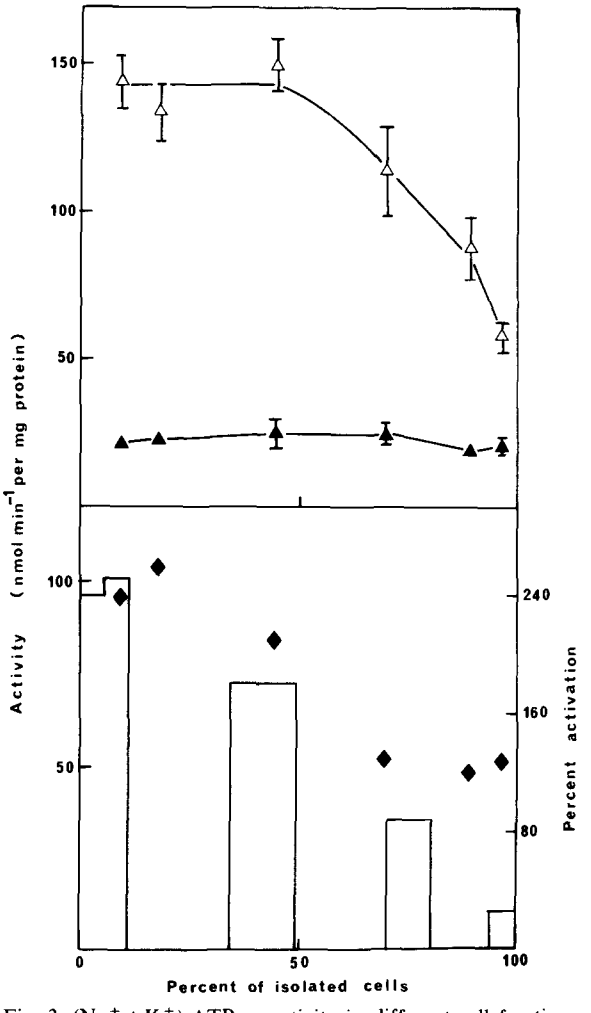

Fig. 3. ($Na^+ + K^+$)-ATPase activity in different cell fractions during enterocyte isolation. The upper panel shows ($Na^+ + K^+$)-ATPase (△) and Mg^{2+}-ATPase (ouabain-insensitive) (▲) and are the means ± S.E. of four different cell isolation experiments. Activities were measured after treatment with 0.075% Triton X-100. The lower panel shows the mean percent detergent-activation of ($Na^+ + K^+$)-ATPase activity (◆, right-hand side axis) observed in each of the assays of the upper panel. The lower panel also shows γ-glutamyltransferase activity in some of the samples (histogram). The abscissa shows percent isolated cells calculated as in Fig. 1.

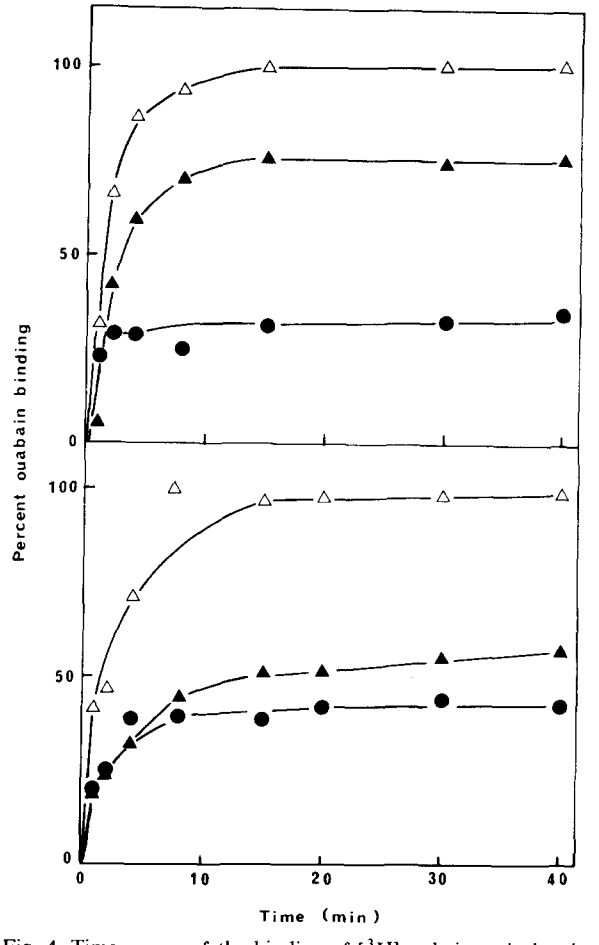

Fig. 4. Time-course of the binding of [^{3}H]ouabain to isolated intestinal cells. Measurements were carried out as described in the text on villus (upper panel) and crypt (lower panel) cells. A constant radioactivity concentration in the incubation medium was used throughout. Measurements were made at 1.1 μM (△) or 100 μM (●) unlabelled ouabain and the results are expressed as percent of the maximal amount of radioactivity associated with cells at 1.1 μM. The difference between binding of radioactivity at the two concentrations is also shown (▲).

percent increase in activity produced by the detergent in the different cell fractions. As suggested, by the result in Fig. 2, latent activity was proportionally higher in homogenates of old enterocytes.

Time-course of ouabain binding

Ouabain associated rapidly with isolated cells at 37°C as shown in Fig. 4 for early and late collections. The binding at 1.1 or 100 μM was half-maximal after about 2 min of incubation, reached equilibrium at about 15 min and remained stable for up to 40 min. If it is assumed that the binding observed at high concentration represents non-specific interaction of the cardiac glycoside with the cells (see below) specific binding time course curves can be constructed from the difference between values at 1.1 and 100 μM. These were fitted to increasing exponentials with coefficients of -0.32 and -0.55 min^{-1} for villus and crypt cells, respectively, in the experiments shown in Fig. 4.

Concentration dependence of ouabain binding

Fig. 5 shows the binding of ouabain to cells from both early and late fractions measured over a wide range of concentration (15 nM–2 mM). In both cases there was evidence for two components to total binding. One that saturated at about 0.5–1.0 μM ouabain and a second that seemed linear with concentrations at least up to 2 mM ouabain. This sort of behaviour has been reported in other cells and tissues [29] where it has been demonstrated that the saturable portion represents the specific combination of ouabain with Na^+-pumping sites, while the non-saturable component, apparently unrelated to the pump, represents the non-specific interaction of the glycoside with the plasma membrane. In order to study the specific binding in more detail cells were incubated in the presence of a range of low ouabain concentrations (15 nM–200 nM) either in the absence (total binding) or presence (non-specific binding) of a large

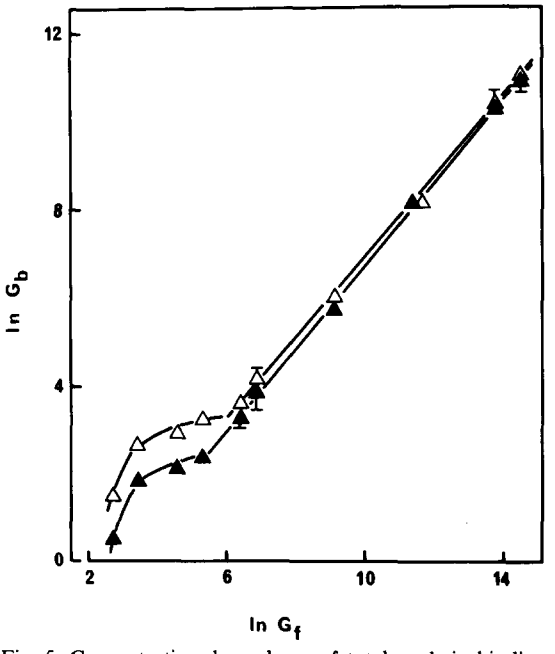

Fig. 5. Concentration-dependence of total ouabain binding to isolated intestinal cells. Binding of ouabain to villus (△) or crypt (▲) cells was measured as described in the text. G_b is amount of ouabain bound in pmoles per mg of cell DNA and G_f is the concentration of free cardiac glycoside in nM. The results are means ± S.E. of four experiments. Note that logarithms are plotted.

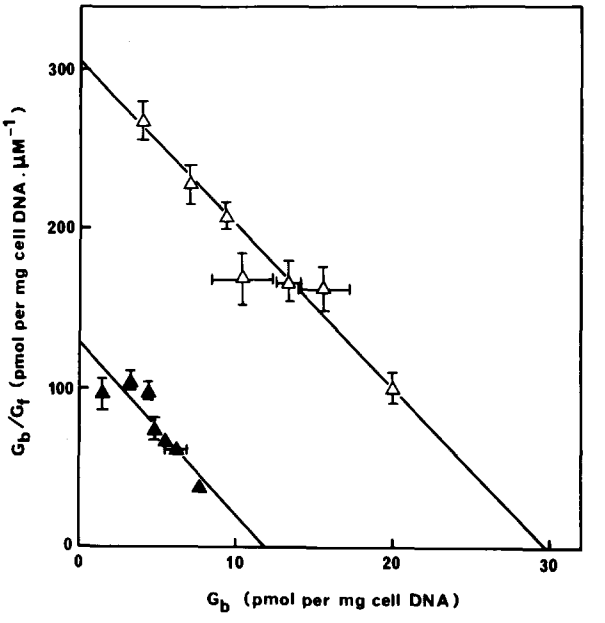

Fig. 6. Concentration-dependence of the specific ouabain binding to intestinal cells. Scatchard plots of the concentration dependence of ouabain binding to villus (△) and crypt (▲) cells are shown. At each concentration of ouabain used (15–200 nM) a parallel tube was run which contained in addition 1 mM non-radioactive ouabain, results (means ± S.E., $n = 4$) are calculated after subtracting the radioactivity associated with the cells at the high glycoside concentration. Lines shown were obtained by least-squares analysis.

excess of unlabelled ouabain (1 mM). Non-specific binding at 200 nM ouabain concentration, accounted for 23 ± 5% and 36 ± 10% of total binding in villus and crypt cells, respectively. The specific ouabain binding, calculated as the difference between total and nonspecific binding, was a saturable process; the Scatchard analysis, shown in Fig. 6, suggests that in both crypt and villus cells the cardiac glycoside binds to a single population of binding sites. The equilibrium constant for the dissociation in four replicates was (mean ± S.E.) 109 ± 22 nM and 99 ± 26 nM for villus and crypt cells, respectively; corresponding maximum binding capacities were 290 ± 20 and 125 ± 14 pmoles per mg of cell DNA, respectively.

Discussion

The results in the present report show that differentiation of rabbit enterocytes is accompanied by a 2.3-fold increase in the specific activity of the ouabain-sensitive $(Na^+ + K^+)$-ATPase with no change in the non-specific Mg^{2+}-ATPase. The observed change in $(Na^+ + K^+)$-ATPase activity was evident only after activation of latent activity with Triton X-100. As discussed by Jørgensen and Skou [26] and Jørgensen [16] this is probably due to the formation of membrane vesicles during homogenisation. The activity of the enzyme needs the presence of K^+ (and ouabain for its specific inhibition) on the outside of the cell plasma membrane, and the presence of Na^+, Mg^{2+} and ATP on the cytosolic side. The demasking by detergents has been explained as opening of the vesicles resulting in free access of substrates and activators to their respective sites in the membrane. A similar explanation could account for the observations in the present report. The fact that latent activity is proportionally higher in homogenates of old enterocytes provides an explanation for the discrepancy between the results of Charney et al. [11] and those of Gratecos et al. [12]. The first group of workers detected a higher $(Na^+ + K^+)$-ATPase activity in villus compared with crypt cells in the presence of deoxycholate, while Gratecos et al. [12] found no difference in activity, but did not attempt any demasking procedure.

In the present work rabbit intestine has been used. The reason for this choice is the higher sensitivity of rabbit intestinal $(Na^+ + K^+)$-ATPase to ouabain compared with rat or mouse [30]. This property is used here to distinguish between specific $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase in cell homogenates.

It is usually assumed that the specific activity of an enzyme under appropriate conditions is proportional to the amount of enzyme. This was tested by ouabain binding measurements. These measurements, when performed in intact cells, are devoid of the problems of latency because the ouabain site is extracellular.

As reported for other cells [29] equilibrium binding of ouabain follows a complex kinetics with a large linear component that becomes dominant at high concentrations of the cardiac glycoside (between 1 μM and 2 mM). Correction for the linear component reveals a saturable binding kinetics. Scatchard analysis is consistent with the presence of a single family of binding sites in both villus and crypt cells. The increase in binding capacity with age of the cell suggests that the similar increase in $(Na^+ + K^+)$-ATPase activity is due to a change in the number of pump sites. The molecular activity (transfer of inorganic phosphate per min per enzyme molecule) can be calculated on the assumption that only one ouabain molecule binds to each pump [8,16]. The values obtained here are $1.31 \cdot 10^4$ and $1.24 \cdot 10^4$ min^{-1} for upper villus and crypt cell populations, respectively. These are very similar to those measured for the enzyme in rabbit kidney medulla ($1.3 \cdot 10^4$ min^{-1}, [26]), guinea-pig brain ($1.3 \cdot 10^4$ min^{-1}, [31]) and ox brain ($0.8 \cdot 10^4$ min^{-1}, [32]).

The Na^+ pump is involved in the control of the cellular ionic balance and hence in the control of the electrical potential difference, and in the maintenance of cellular volume. In addition the Na^+ pump, being electrogenic, contributes directly to the membrane potential [33]. It has been shown recently [34] that there is an increase in membrane potential and intracellular K^+ activity as intestinal epithelial cells migrate from crypt to villus tip. An increase in the number of Na^+ pumps, and consequently in the capacity to take up K^+, could contribute to provide an explanation for both the electrical potential and the K^+ activity changes as epithelial cells migrate up the villus.

It is also known that Na^+-coupled amino acid

and sugar transepithelial transport occurs in the upper villus [5,6]. This must increase the traffic of Na^+ through the cells' cytoplasm. The increase in pumping capacity observed here might be an adaptation of the nonpolarised crypt cells to their absorptive function.

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