ULTRASTRUCTURAL DEVELOPMENT AND CELLULAR KINETICS IN INTESTINAL EPITHELIUM

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The influence of 400R X-irradiation on the number and the localization of mature and immature goblet cells and Paneth cells in intestinal crypt and villus.
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GENERAL INTRODUCTION

Despite the increased knowledge in the various fields of molecular biology the regulation of cell proliferation and differentiation in higher organisms has remained an intrigueing, complex problem in developmental biology.

In some instances growth during embryonic or postnatal development is characterized by an increase in cell size without an accompanying increase in cell number like for instance in the postnatal development of cerebellum (Leblond, 1964). In most cases, however, growth is accompanied by an increase in cell number and a concurrent production of intercellular substance. In these so-called "expanding cell populations" the ability to divide is not restricted to a certain fraction of the population but nearly all cells are able to proliferate as for instance during development or regeneration of liver.

Cell renewing systems are characterized by an equilibrium between cell production and cell loss. Such systems are not only present during foetal development but they also occur in adult life. Examples are skin, mucous membranes, testis, bone marrow and intestinal epithelium. Such systems generally consist of a proliferative cell compartment, in which stem cells maybe present, a differentiation compartment where specific cellular characteristics are being developed and a functional compartment. These cell renewal systems have frequently been used for the study of regulation processes involved in cell proliferation and differentiation. More insight in these regulatory mechanisms are likely to contribute to the understanding of malignant cell growth.

Autoradiographic studies on the epidermis of the skin and of the keratinizing squamous epithelium of rodent esophagus have shown that the proliferating cells are mainly confined to the one layer thick stratum basale (Leblond and Walker, 1956; Iversen, 1969). Daughter cells loose their proliferative property when they leave this layer and migrate up into the stratum spinosum, where they start the differentiation process of keratinization. Ultimately the keratinized cells are expelled from the system as horny remnants. Cells which remain in the basal layer continue with proliferation and can be considered as the stem cells of the system. The epidermis of the skin has served as a model for regulation studies. By abrasion or destruction of the outer layers of the skin increased proliferation in the basal layer was observed (Pinkus, 1951, 1952). From studies of wound healing Bullough and Laurence (1960) postulated the existence of a mitotic inhibitor substance in the epidermis. These so called chalones would be produced by the differentiating or functional cells and influence the proliferative cell compartment via negative feedback mechanisms. (Bullough and Laurence, 1960; Bullough, 1962, 1975; Iversen et al., 1974). The exact chemical structure and the working mechanisms of these chalones are not yet understood, although more recently some progress has been made (Thornley and Laurence, 1975).

The cell renewal system in the testis is more complex as far as the number of the different cell types is concerned; also meiotic cell divisions occur during gametogenesis. On the other hand specific substances, i.e. hormones are known to affect cell proliferation and differentiation processes in the testis. Under hormonal influence at puberty the thickness of the seminiferous tubules increases due to proliferation of the spermatogonia until the full size is attained. In addition, cell differentiation in the form of spermatogenesis is initiated and

ultimately a steady state is established between cell proliferation and loss of maturing spermatozoa (Steinberger and Steinberger, 1972). Clermont and Mauger (1974) found an inhibitory effect of normal testis homogenates on the proliferative activity of A spermatogonia during recovery after irradiation. An antimitotic chalone like substance had been postulated earlier by studies on spermatogenesis after unilateral castration (Harvey and Clermont, 1964).

Another important cell renewal system is the haemopoietic system. In comparison to the epidermis and testis the haemopoietic system is even more complex, because of the many different cell types and their localization in different structures throughout the body (Lajtha, 1970; 1971). No orderly sequential localization of stem cells, proliferating cells, differentiating cells and mature cells is found. Using the spleen colony technique of Till and McCulloch (1961) in combination with chromosome markers it could be ascertained that there is only one type of stem cell for all different lines of blood cell development (Micklem et al., 1966; Wu et al., 1967). Van Bekkum et al. (1971) were able to concentrate stem cells by means of density gradient centrifugation and they concluded that the stem cells resemble small lymphocytes.

Several factors seem to be involved in the regulation of cell proliferation and the differentiation into the various categories of blood cells. These include the local environment of the cell (McCulloch, 1970; Trentin, 1970), antimitotic, chalone like substances which affect cell proliferation in erythropoiesis and granulopoiesis (Hanna, 1967; Rijtömaa and Kiviniemi, 1968; Kivilaaksoo and Rijtömaa, 1971; Kiger et al., 1972) and finally humoral factors like erythropoietin, granulopoietin and thrombopoietin (Tavassoli, 1975). So far, all evidence is in favor of the hypothesis that in every cell renewal system the various differentiated cell types originate from one type of stem cell.

In the present thesis the epithelial lining of rat small intestine has been used as a model for the study of the relation between cell proliferation and cell differentiation. This cell renewal system has several advantages compared with other tissues. The regular distribution of the various developmental stages of the epithelial cells along crypt and villus has enabled us to perform ultrastructural and microchemical studies of the normal cell differentiation process in relation to the exact localization and age of the epithelial cells. Also it was investigated whether changes in crypt cell proliferation, induced by a low dose of X-irradiation, affect the normal differentiation process. The relatively short cell cycle of the proliferating cells and the rapid turnover time of the intestinal epithelium have the advantage that disturbances in proliferative activity are rapidly propagated through the rest of this cell renewal system.

Autoradiographic studies after administration of ^{3}H-thymidine showed that the proliferative cell compartment is localized in the lower half of the crypts. After a number of cell divisions cells migrate along the upper half of the crypt, where they differentiate in a non proliferative state, and after 10-12 hours differentiated cells migrate onto the villus. Subsequently the cells perform their absorptive function during a 28 - 36 hour period of migration along the villus. Finally the cells are extruded from the tip of the villus into the intestinal lumen (Leblond and Messier, 1958; Quastler and Sherman, 1959; Cairnie et al., 1965a and b; Galjaard and Bootsma, 1969).

In the small intestinal crypt several cell types originate, i.e. goblet cells, Paneth cells and entero-endocrine cells, but in duodenum/jejunum 95% of all

epithelial cells are columnar cells and about 96% of the observed mitotic figures belong to these cells (Cheng and Leblond, 1974). The average length of a longitudinally cut jejunal crypt column in rat is about 32 cells. Columnar cells in cell positions 1 - 18 (counted from the base of the crypt) are all involved in cell proliferation. The cell cycle time of these cells is extremely short and varies from 15.5 hours for the lower cell positions to 10 hours for the higher positions (Cairnie et al., 1965a; Thrasher and Greulich, 1966; Lesher, 1967; Al-Dewachi et al., 1974). When columnar cells arrive in the so-called "critical decision zone" in the middle region of the crypt (cell position 18-22) the percentage of labeled cells after ^{3}H-thymidine incorporation rapidly falls. In normal circumstances cells do not proliferate during subsequent migration along the upper half of the crypt above cell position twenty two (Cairnie et al., 1965a and b).

The ultrastructure of the cells at the lower positions in the crypt is characteristic for primitive proliferating cells, i.e. many free ribosomes and only a few cell organelles. In the upper part of the crypt cells are more mature, i.e. more numerous and longer microvilli, more rough endoplasmic reticulum, and a more elaborate Golgi complex (Padykula, 1962; Taylor and Adamstone, 1964; Toner, 1968; Cheng and Leblond, 1974; van Dongen and Visser, 1974). Also, the biochemical properties of the columnar cells change during migration along the crypt. Microchemical assays of different crypt cell compartments have shown a gradual increase in the activity of esterases (Galjaard and Bootsma, 1969).

At the crypt-villous junction the most prominent changes occur both in terms of ultrastructural and biochemical features. There is a sudden increase in cell size and of nearly all cell organelles (Toner, 1968; Cheng and Leblond, 1974). The capacity of the epithelial cells to absorb fat, amino acids and sugars (Palay and Karlin, 1959; Padykula, 1962; Dowling and Riecken, 1974) is accompanied by changes in the activity of several enzymes involved in intestinal function. Biochemical studies on homogenates of bulk quantities of separated villous cells (Webster and Harrison, 1969), enzyme assays on plane parallel cut frozen sections (Nordström et al., 1968; Moog and Grey, 1968; Imondi et al., 1969; Fortin-Magana et al., 1970) and microchemical assays of microdissected cell compartments (Galjaard et al., 1970, 1972a and b) showed a sudden increase in activity for several disaccharidases, dipeptidases, alkaline phosphatases and esterases at the crypt-villous transition. A number of enzymes which are essential for the maintainance of the so-called "household" of the cell, like many lysosomal and mitochondrial enzymes show a rather constant activity along crypt and villus (Fortin-Magana et al., 1970; Galjaard et al., 1970; 1972b; Webster and Harrison, 1969).

Experimentally induced disturbance of the steady state of the intestinal epithelium by irradiation has contributed to the insight in regulatory mechanisms involved in cell proliferation. Quastler and Sherman (1959) were the first to apply lethal doses of radiation and studies of the resulting damage yielded basic data on the cellular kinetics. Williams et al. (1958) by using lower radiation doses could show that an initial block in mitotic activity in the crypt is followed by a temporary overshoot in proliferative activity and restoration to normal values. Using autoradiography after ^{3}H-thymidine Lesher (1967) found an increase in the number of DNA synthesizing cells during recovery after low radiation doses. Galjaard and Bootsma (1969) demonstrated that this was mainly due to an expansion of the proliferative cell compartment along the upper half of

the crypt. Comparative studies of the recovery after irradiation of intestine in conventional and germfree rats showed that expansion of the proliferative crypt cell compartment was related to a decrease in the villous cell population (Galjaard et al., 1972a; van der Meer-Fièggen, 1973). By experimental reduction of the villous cell compartment, either by radiation or by temporary clamping of the blood supply, Rijke et al. could substantiate the existence of a feedback regulatory mechanism of crypt cell proliferation (Rijke et al., 1974, 1976). It is, however, not yet clear in which way the information of a reduction in the number of villous cells is transferred to the crypt and how a cell "knows" to continue or to stop with proliferation halfway the crypt.

Increased proliferative activity during recovery after irradiation was found to be accompanied by a marked reduction in the activity of several enzymes i.e. esterases, alkaline phosphatase and α-glucosidase, involved in intestinal function (Galjaard et al., 1970, 1972a and b; de Both and Plaisier, 1974; Rijke et al., 1975).

The main question to be answered by the experimental work described in this thesis was whether increased crypt cell proliferation also results in changes of the ultrastructural development of the columnar cell. For this purpose a morphometric electronmicroscopic study was performed for columnar cells at each position along the whole length of the crypt and the lowest positions of the villus. The results of these studies of the normal ultrastructural development were compared with those obtained at 72 hours after irradiation with 400 R, where it is known that nearly all crypt cells are involved in proliferation. The data on ultrastructural differentiation were related with the results of microchemical assays of several enzymes in various cell compartments of crypt and villus. Cytochemical staining at the electron microscope level was performed for esterases and alkaline phosphatase to investigate whether changes in activity of these enzymes during normal differentiation and during increased crypt cell proliferation could be related to changes in specific subcellular organelles.

Finally, it was investigated whether increased proliferation of the columnar cells in the crypt affects the development of two other cell types in the intestinal epithelium, i.e. the goblet cells and Paneth cells. The goblet cells form between 4 and 12% of the crypt cell population depending on the localization in the small intestine (Cheng and Leblond, 1974). They originate in the lower half of the crypt and migrate to the tip of the villus at about the same rate as the columnar cells. The same is true for the relatively rare entero-endocrine cells (Pearse, 1974). The few Paneth cells of which the function is not yet known, remain localized at the bottom of the crypt. After a life span in the order of 20 - 30 days these Paneth cells degenerate and their remnants are phagocytosed by primitive columnar cells at the base of the crypt (Cheng and Leblond, 1974).

As in the blood forming tissues the question arises whether more than one type of stem cell is present for the different cells in the intestinal epithelium. Autoradiographic studies after ^{3}H-thymidine and ultrastructural analyses suggest that the goblet cells, Paneth cells, entero-endocrine cells and the mature columnar epithelial cells are all derived from one type of stem cell at the bottom of the crypt (Moe, 1968; Merzel and Leblond, 1969; Troughton and Trier, 1969; Cairnie, 1970; Cheng and Leblond, 1974). The experiments described at the end of this thesis are aimed to obtain a better understanding of the mechanism by which the formation of the various cell types is regulated.

INTRODUCTION AND DISCUSSION OF EXPERIMENTAL WORK

The main purpose of the experiments described in the present thesis is to investigate the effect of changes in the cellular kinetics of intestinal epithelium on ultrastructural and biochemical differentiation characteristics. In most studies changes in the normal cellular kinetics of the intestinal columnar cells have been induced by exposure of rats to a low dose of X-radiation. After a low radiation dose crypt cell proliferation is temporarily blocked and during the subsequent recovery an overshoot in proliferative activity occurs between 48 and 120 hours after irradiation. In the present studies several cell differentiation processes have been analyzed with light microscopic, electron microscopic and (micro) biochemical techniques both in normal rat jejunum and at 72 hours after 400 R, when nearly all crypt cells are involved in proliferation.

Paper I describes the development of several biochemical and ultrastructural characteristics of the jejunal epithelium during normal migration of the cell along crypt and villus. Microchemical assays of different crypt and villous cell compartments isolated by dissection from freeze-dried cryostat sections enabled us to relate the activity of several enzymes with cell position and cell age. Activity of alkaline phosphatase, a marker for the microvilli and probably involved in absorption processes could not be demonstrated in crypt cells and was found to increase linearly with cell age during subsequent migration from base to tip of the villus. The activity of carboxylesterases, localized mainly in endoplasmic reticulum and involved in hydrolysis of lipids was found to be very low in cells at the bottom of the crypt and to increase gradually during migration along the upper half of the crypt and the lower half of the villus. Microelectrophoretic studies indicated that this increase in activity was not due to any specific changes in the iso-enzyme pattern since this is similar in all cell compartments tested. The activity of the lysosomal enzyme β-N-acetylglucosaminidase and the mitochondrial enzyme monoamine oxidase did not show any significant changes during cell differentiation and cell ageing.

By studying differentiation processes at 72 hours after 400R X-irradiation the effect of increased crypt cell proliferation could be evaluated. At this time interval after radiation it is known from autoradiographic studies after ^{3}H-thymidine (Galjaard and Bootsma, 1969) that the proliferative cell compartment has expanded over the whole length of the crypt whereas normally it is confined to the lower half of the crypt. This implies that cells migrate onto the villus very shortly after their last division and without their normal 12 hour period of maturation in a non-proliferative state during migration along the upper half of the crypt. These changes in cellular kinetics were found to result in a marked decrease of esterase activity both in the upper half of the crypt and in the various parts of the villus. This decrease in esterase activity might be related to the relatively undifferentiated state of the cell at the base of the villus as a result of expansion of the proliferative cell compartment. In this respect it is, however, interesting to note that in irradiated animals the esterase activity levels off halfway the villus like in control animals. One might have expected that in irradiated animals the activity would have increased to attain normal values during subsequent migration of the cell along the upper half of the villus. It seems, however, that the esterase activity is not only related to the age of the cell after its last division but also to the position of the cell along crypt or villus.

When compared with controls at 72 hours after 400R the alkaline phosphatase activity is markedly decreased in all parts of the villi. This finding was somewhat surprising since in normal crypts no activity of this enzyme is demonstrable and yet increased crypt cell proliferation affects the enzyme activity on the villus. This effect seems to be in agreement with the hypothesis that an inactive pro-enzyme is being synthesized during cell differentiation in the upper half of the crypt. Increased proliferative activity at the expense of normal differentiation might then interfere with the synthesis of such a pro-enzyme. Another explanation of the biochemical data would be a specific change of subcellular organelles, where the enzymes are normally localized.

To study the ultrastructure of various stages of crypt cell differentiation qualitative electron microscopic studies were performed on cells at various positions along the crypt and at the base of the villus. A gradual development of various ultrastructural features like microvilli, endoplasmic reticulum, Golgi apparatus, mitochondria was observed during migration of the cell from the base to the tip of the crypt. No specific changes were seen at the "critical decision zone" halfway the normal crypt where cells stop proliferation. A normal pattern of ultrastructural development was observed in crypts where nearly all cells are involved in cell proliferation as is the case at 72 hours after 400R X-irradiation. Contrary to some biochemical differentiation characteristics, the ultrastructural features of the epithelial cell seems not to be significantly affected by a change in cellular kinetics. Major changes in ultrastructural features, i.e. increase of cell size and of nearly all cell organelles as well as some specific structural changes occur at the crypt-villous transition.

Further information about the development of biochemical differentiation characteristics could be obtained by comparing the results of microchemical assays on various cell compartments isolated from small intestine of normal and of germfree rats. The total life span of the villous cells in the latter animals is about 12 hours longer than in conventional rats of the same type. In both experimental groups alkaline phosphatase and carboxylesterases showed a similar development of activities during cell migration and the 30% longer life span of the villous cells in germfree rats was not reflected in higher activities. This indicates that the activity of these enzymes is not exclusively determined by cell age but also by the position along the villus.

Paper II describes experiments on the effect of increased crypt cell proliferation on the activity of a few enzymes involved in the conversion of nucleotides and in nucleic acid synthesis. The activity of hypoxanthine phosphoribosyltransferase and of thymidine kinase was measured in isolated crypt and villous cell compartments both in normal rat intestine and 72 hours after 400R X-irradiation. One of the reasons for this study was to find out whether microchemical analysis of these enzyme activities could serve as a reliable marker for increased proliferative activity. In experimental animals there is no specific need for such a marker since incorporation studies of ^{3}H-thymidine are both simple and reliable. In case of intestinal biopsies from human individuals, however, such studies with radioactive labeled substances cannot be performed. Yet, in the diagnosis of intestinal diseases, like various forms of idiopathic sprue or in follow-up studies of treatment, like in cases of gluten sensitive sprue, it will be interesting for the clinician to obtain information about the proliferative activity in intestinal biopsy material. The microchemical studies on rat jejunum, described in Paper II show that the activity of

thymidine kinase in isolated crypts can be used as a reliable parameter for crypt cell proliferation. This is indicated by the fact that in normal intestine the thymidine kinase activity is about 10 times higher in crypts than in villi. Also, in case of increased crypt cell proliferation, as occurs during recovery after a low radiation dose, the thymidine kinase activity was found to increase about 5 fold.

In the same paper (II) experimental data are provided about the capacity of "de novo" protein synthesis in relation to villous cell ageing. Using autoradiography after intraperitoneal administration of ^{3}H-labeled aminoacids (leucine, methionine and proline) it was observed that all villous cells were labeled except the upper 10 - 20 cell positions at the tip of the villus. A similar pattern was found at 72 hours after 400R radiation which is in agreement with the finding by Rijke et al (1974) that the total life span of a villous cell is not affected by changes in crypt cell proliferation. If, however, labeled aminoacids are administered intraluminally all villous cells show "de novo" protein synthesis. These findings suggest that cells at the tip of the villus mainly incorporate aminoacids derived from the food in the intestinal lumen.

Paper III describes the results of an extensive morphometric electron microscopic study to obtain quantitative data on the development of ultrastructural features of the columnar epithelial cell in small intestine and the way this is affected by increased crypt cell proliferation.

As far as the development of ultrastructural features during cell differentiation is concerned no definite conclusions can be based on qualitative studies of electron micrographs. Morphometric analysis of normal crypt cell differentiation revealed a gradual increase in the absolute size of the cell, cytoplasm, the terminal web and of nearly all cell organelles. Crypt cell migration is not just characterized by growth, i.e. a proportional increase in size of the whole cell and of all organelles, because there is also an increase in the relative size of the cytoplasm, mitochondria, microvilli and endoplasmic reticulum. There is no specific change during migration along cell positions 18- 22 where cell proliferation stops. At the crypt-villous junction, however, there is a 1.5 - 3 fold increase in cell size, cytoplasm, terminal web and of nearly all organelles. Expansion of the proliferative crypt cell compartment does not affect the normal ultrastructural differentiation of the cell. Also the major ultrastructural changes at the crypt-villous junction seem to occur independently of the question whether the cell has just completed its last division or has differentiated during a 12 hours period in a non-proliferative state. Contrary to several biochemical differentiation characteristics, the development of the ultrastructure of the cell seems to be determined only by its localization along crypt and villus.

Electron microscopic cytochemical studies, described in Paper IV try to answer the question whether the decreased activity observed for alkaline phosphatase and esterases after increased crypt cell proliferation can be related to specific changes in their ultrastructural localization. In normal rat jejunum microchemical assays of alkaline phosphatase showed a 4 fold increase in activity from the upper part of the crypt towards the base of the villus and a 20 fold increase towards the tip of the villus. Cytochemical staining at the electron microscopic level shows that there is no alkaline phosphatase activity in the crypt cells and that in the villous cells it is localized in the microvilli, along the lateral cell membranes and in the dense bodies. Morphometric analysis of electron micrographs indicate that the relative size of these compartments does not significantly change during crypt cell differentiation

and at the transition from crypt to villus. Since the (micro)biochemical assays of enzyme activity are expressed per unit dry weight, these data should be compared with the relative size of the various cell structures where alkaline phosphatase is localized. Hence, the activity of alkaline phosphatase in relation with cell age cannot be explained by changes in the particular subcellular compartments where the enzyme is localized. Increased crypt cell proliferation does not affect the relative size of the microvilli, lateral cell membranes and dense bodies in crypt or villous cells. Yet, the activity of alkaline phosphatase in the villous cells is markedly (2 - 3 fold) decreased at 72 hours after 400R X-radiation. Again this decrease cannot be explained on the basis of any specific changes at the ultrastructural level.

All data are in support of the conclusion that the activity of alkaline phosphatase is not proportionally related to the size of subcellular structures where it is localized. Instead its activity seems mainly dependent on the age of the cell in a non-proliferative state (see also Paper I).

As far as the studies on esterases are concerned it was observed that this enzyme is localized in the endoplasmic reticulum, the dense bodies and the perinuclear space. Morphometric analysis showed a 3 fold increase in relative size of these compartments from the bottom of the crypt to the middle of the villus. Microchemical assays of the esterase activity show a 10 fold increase during differentiation. During recovery after irradiation the marked decrease in esterase activity could not be explained by any specific change in its subcellular localization. Increased proliferative activity seems to result in a decrease in the overall activity of all iso-enzyme and no loss of activity in specific subcellular localizations occurs.

In Paper V we report the effect of changes in the cellular kinetics of the columnar epithelial cells on the number and localization of two other epithelial cell types present in intestinal epithelium i.e. the goblet cell and the Paneth cell. In normal rat intestine immature goblet cells are mainly localized in the lower third of the crypt. In the upper half of the crypt only mature goblet cells occur, as is the case on the villus. Shortly after 400R X-irradiation, when proliferation of the columnar epithelial cells is blocked there is an increase in the number and proportion of both immature and mature goblet cells. During recovery after irradiation, at 72 hours, when nearly all columnar crypt cells are involved in proliferation there is mainly an increase in the number of immature goblet cells and these are now also localized along the whole length of the crypt. It seems as if the development of goblet cells is markedly affected by the proliferative pattern of columnar epithelial cells. A close correlation is observed concerning the number and localization of proliferating columnar cells and of immature goblet cells.

No changes were observed in the localization and the number of Paneth cells during recovery after irradiation as would be expected from their long turnover time.

The main conclusions from the experimental work on the proliferation and differentiation of epithelial cells in small intestine are the following:

1. increased proliferative activity in jejunal crypts has no effect on the normal ultrastructural differentiation of the columnar cell in crypt and villus, nor on the activity of enzymes tested which are involved in the normal "house-hold" of a cell.
2. increased crypt cell proliferation results in a marked decrease in the activity of several enzymes involved in specific villous cell functions.

3. these changes in biochemical differentiation characteristics are not due to any specific changes in subcellular organelles nor to effects on specific iso-enzymes.
4. both the normal development of enzymes, involved in intestinal function, and changes in their activity due to increased crypt cell proliferation can be related to the age of the cell in a non proliferative state and to its localization along crypt and villus.
5. there is a close correlation concerning the number and localization of proliferating columnar cells and of immature goblet cells; the formation and differentiation of goblet cells is markedly affected by changes in the proliferative activity in the crypt.
6. in case of disturbed intestinal functions the possibility of changes in crypt cell proliferation should also be considered since these may result in abnormal differentiation and hence in abnormal villous cell characteristics.

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SUMMARY

Regulation mechanisms involved in cell proliferation and differentiation can be studied during embryonic development or in cell renewing systems of adult organisms. The use of small intestinal epithelium as a model has the advantage that the various developmental stages of the epithelial cell are regularly distributed along the crypt and villus. The most frequent cell type (about 89 - 95%) is the columnar epithelial cell which is responsible for the absorptive functions of the villus. These cells originate by cell proliferation in the crypts as do the goblet cells (about 4%-12% of all epithelial cells), the entero-endocrine cells and the Paneth cells. The latter cells remain localized at the base of the crypt, all other cells migrate upwards along the crypt and villus until they are extruded from the tip of the villus into the intestinal lumen.

Another advantage of intestinal epithelium is the rapid turnover time of the epithelium (2 - 4 days) since experimental disturbances in crypt cell kinetics will be readily propagated through the other cell compartments.

Cell proliferation is confined to the lower 18 cell positions of the crypt. During migration of the cells along the so called "critical decision zone" at cell positions 18 - 22 the number of proliferating cells rapidly falls to nearly zero. The columnar epithelial cells then migrate during a 12 hour period along the upper part of the crypt (positions 22 - 32) where they mature in a non proliferative state. After entering onto the villus the columnar cells become involved in absorptive functions whilst they migrate along the villus during a period of 28 - 36 hours.

The experimental work described in the present thesis deals with the study of normal biochemical and ultrastructural differentiation of rat jejunal epithelium. Furthermore the effect of increased crypt cell proliferation on these processes has been investigated. Increased crypt cell proliferation has been experimentally induced by exposing animals to a relatively low dose (400R) of X-irradiation. After an initial block in cell production there is a restoration of proliferative activity with an overshoot during the period of 48 - 120 hours after irradiation. At 72 hours after 400R cell proliferation occurs not only in the lower part but also in the upper part of the crypt at the expense of the maturation cell compartment.

Morphometric electron microscopic studies on the columnar cells were carried out to obtain quantitative data on the development of ultrastructural features during normal crypt cell differentiation and in a situation of increased crypt cell proliferation. In control rats a gradual increase was observed in cell size, cytoplasm, terminal web and nearly all subcellular organelles during cell migration from the bottom to the tip of the crypt. These changes do not just represent growth (proportional increase of the size of all organelles and the whole cell) since the morphometric data also showed an increase in the relative size of several organelles like endoplasmic reticulum, microvilli and Golgi apparatus. No specific ultrastructural changes occur when proliferation stops halfway the crypt nor has any significant effect been observed of increased crypt cell proliferation on the normal ultrastructural differentiation of the cells.

As a parameter for biochemical differentiation the activity of several enzymes was measured in isolated cell compartments of crypts and villi using microchemical techniques. During crypt cell differentiation and progression from crypt to villus no

changes were observed for monoamine oxidase and β-N-acetylglucosaminidase which are not specifically involved in intestinal function. An increase in crypt cell proliferation had very little effect on the ratio of the activities of these enzymes in the crypt and in the villus. Analysis of a number of other enzymes involved in the absorptive function of the villus, however, revealed marked differences at the various developmental stages of crypt and villous columnar cells. A variety of brush border enzymes, such as alkaline phosphatase, various disaccharidases and peptidases only show activity in villous cells. Yet the subcellular structures where these enzymes are to be localized are already present in differentiating crypt cells. Carboxyl esterases, localized in endoplasmic reticulum and dense bodies and possibly involved in fat metabolism, show a low activity in cells at the bottom of the crypt while the activity increases during crypt cell differentiation and migration along the lower half of the villus. Increased crypt cell proliferation results in a marked decrease in the activity of all these enzymes. Microelectrophoretic studies, electron microscopic cytochemistry and investigations of intestinal epithelium with a longer life span derived from germfree animals were carried out to elucidate the background of these changes in the differentiation of biochemical characteristics. The development of esterases and alkaline phosphatase was found to be mainly related to the age of the non proliferating cell and to the localization of the cell along the crypt or villus. The changes in activity during normal development and during altered cellular kinetics are not due to any changes in specific organelles or specific esterase isoenzymes. Cell age and microenvironment seem to determine the overall activity of the enzymes.

Changes in crypt cell proliferation not only affects the activity of several enzymes involved in intestinal function it also has a marked influence on the differentiation of another cell type, the goblet cell. Normally, immature goblet cells originate in the lower half of the crypt, where they differentiate into mature goblet cells, which subsequently migrate along the upper part of the crypt and along the villus. If proliferation of the columnar epithelial cells is blocked, the number of immature and mature goblet cells increases. In case of an expansion of the proliferative cell compartment over the whole length of the crypt, immature goblet cells are observed along the whole crypt as well. This illustrates a close relationship between the columnar epithelial cells and the goblet cells; therefore, these results are in agreement with the hypothesis that goblet cells are derived from primitive columnar cells.

The main conclusions of the experimental work, described in this thesis, are:

- ultrastructural development of the columnar intestinal epithelial cells occurs gradually during migration along crypt and villus and is independent of proliferative activity and maturation time in the crypt.
  The same seems to be true for the activity of a number of enzymes, involved in the maintenance of the cell and not specifically related to its absorptive function.
- the development of enzymes involved in specific villous cell functions is dependent on the age of the non-proliferative cell and also to its position on crypt or villus. Changes in proliferative activity have a marked effect on the overall activity of these enzymes.
- in the evaluation of the microscopic structure of intestinal biopsies in case of disturbed intestinal function the investigator should be aware of the importance of changes in crypt cell kinetics. In case of increased crypt cell proliferation, the development of certain biochemical characteristics will be disturbed and

hence the intestinal function might be affected even though the villous epithelium seems to have a normal microscopic structure.

- microchemical assays of thymidine kinase activity in isolated crypt cells may serve as a useful tool for the determination of the proliferative activity in human intestinal biopsies.
- there is a close correlation between the number and localization of proliferating columnar cells, and immature goblet cells; the development of goblet cells is markedly affected by changes in the proliferative activity in the crypt.

SAMENVATTING

Regulatie mechanismen betrokken bij celproliferatie en differentiatie kunnen bestudeerd worden tijdens de embryonale ontwikkeling, of aan "cell renewal" systemen van het volwassen organisme. De bestudering van het dunne darmepitheel als modelsysteem voor deze processen heeft het voordeel, dat de verschillende ontwikkelingsstadia van de epitheelcel, ruimtelijk gescheiden, regelmatig langs krypt en villus liggen gerangschikt. Het meest voorkomende celtype van alle epitheelcellen in de darm is de cylindrische epitheelcel (89 - 95%), welke verantwoordelijk is voor de absorptieve functie van de villus. Deze cellen ontstaan door celproliferatie in de krypt. Dit laatste is ook het geval voor slijmbekercellen (ongeveer 4 - 12% van alle epitheelcellen), de entero-endocriene cellen en de Paneth cellen. Paneth cellen blijven onder in de krypt liggen, terwijl de andere celtypen naar boven migreren langs krypt en villus, totdat ze aan de top van de villus in het darmlumen worden afgestoten.

Een ander voordeel van het epitheel in de dunne darm is de snelle "turnover" (2 - 4 dagen), waardoor experimenteel geïnduceerde verstoringen in de celkinetiek in de krypt zich snel zullen verbreiden door de andere celcompartimenten.

De celproliferatie is beperkt tot de laagste 18 celposities in de krypt. Gedurende de migratie van de cellen door de zgn. "critical decision zone", van celpositie 18 - 22, neemt het aantal prolifererende cellen snel af tot bijna nul. De cylindrische epitheelcellen migreren vervolgens tijdens een periode van 12 uur door het bovenste deel van de krypt (positie 22 - 32), waar ze rijpen zonder verder nog te delen. Nadat deze cellen de villus hebben bereikt wordt de absorptieve functie uitgeoefend gedurende een periode van 28 - 36 uur, waarin de cellen langs de villus migreren.

Door middel van het experimentele werk, beschreven in dit proefschrift, werd de normale biochemische en ultrastructurele differentiatie van het epitheel van het jejunum van de rat onderzocht. Bovendien werd de invloed bestudeerd van verhoogde celproliferatie in de krypt op deze processen. Deze verhoogde proliferatie werd experimenteel bewerkstelligd door dieren bloot te stellen aan een relatief lage dosis (400R) Röntgen straling. Na een tijdelijke blokkade van de celproliferatie treedt een herstel van de proliferatieve activitieit op, gepaard gaande met een overcompensatie tijdens de periode van 48- 120 uur na bestraling. Op 72 uur na 400R vindt celproliferatie niet alleen in het onderste deel van de krypt plaats, maar ook in de bovenste helft ten koste van het maturatie celcompartiment.

Met behulp van morphometrie op electronenmicroscopisch niveau werden quantitatieve gegevens verkregen over de ultrastructurele ontwikkeling van de cylindrische epitheelcel tijdens de normale differentiatie in de krypt en gedurende verhoogde proliferatieve activiteit na bestraling. Een geleidelijke toename van de grootte van de cel, van het cytoplasma, "terminal web" en van bijna alle celorganellen werd waargenomen gedurende celmigratie van de basis naar de top van de krypt in controle ratten. Deze veranderingen berusten niet op groei (proportionele toename van alle celorganellen en gehele cel), aangezien de morphometrische resultaten ook een toename laten zien in de relatieve grootte van verscheidene organellen zoals endoplasmatisch reticulum, microvilli en Golgi apparaat. Er zijn geen plotselinge ultrastructurele veranderingen waarneembaar als de celproliferatie halverwege de krypt ophoudt, noch werd enige invloed waargenomen van verhoogde cel-

proliferatie in de krypt op de normale ultrastructurele differentiatie van de cellen.

Als parameter voor biochemische differentiatie werd de activiteit van verschillende enzymen gemeten in geïsoleerde celcompartimenten van krypt en villus met behulp van microchemische technieken. Gedurende de differentiatie in de krypt en bij de overgang van krypt naar villus, werden geen veranderingen waargenomen voor monoamine-oxidase en β-N-acetylglucosaminidase, enzymen die niet betrokken zijn bij de specifieke darmfunctie. Verhoogde proliferatieve activiteit in de krypt beinvloedt de verhouding van de activiteiten van deze enzymen in krypt en villus nauwelijks.

Een analyse van een aantal andere enzymen, die wel betrokken zijn bij de absorptieve functie van de villus, toonde echter duidelijke verschillen in activiteit aan in de diverse ontwikkelingsstadia van de cylindrische cel in krypt en villus. Verschillende enzymen, gelocaliseerd in de borstelzoom, zoals alkalische phosphatase, disaccharidasen en peptidasen vertonen alleen een duidelijke activiteit in de villuscel. De subcellulaire structuren hiervoor zijn echter al aanwezig in de differentierende kryptcellen. Carboxylesterasen, gelocaliseerd in het endoplasmatisch reticulum en in "dense bodies" en mogelijk betrokken bij het vetmetabolisme, vertonen een lage activiteit in cellen onder in de krypt. De activiteit neemt toe tijdens de differentiatie in de krypt en gedurende de migratie in de onderste helft van de villus. Een toename in de proliferatieve activiteit van de cellen in de krypt heeft een aanzienlijke verlaging in de activiteit van al deze enzymen tot gevolg. Bestudering van iso-enzymen met behulp van microelectrophorese, toepassing van cytochemie op electronenmicroscopisch niveau en onderzoek van het darmepitheel van kiemvrije dieren, waar de darmcel een langere levensduur heeft, hebben meer informatie opgeleverd over deze veranderingen van de biochemische parameters. Het bleek, dat de ontwikkeling van de esterasen en van alkalische phosphatase voornamelijk bepaald werd door de leeftijd van de niet delende cel en door de localisatie van de cel in krypt en villus. Veranderingen in de activiteit van deze enzymen tijdens de normale ontwikkeling van het dunne darmepitheel en gedurende veranderingen in de celkinetiek o.i.v. bestraling zijn niet toe te schrijven aan veranderingen van specifieke organellen of specifieke esterase isoenzymen. De leeftijd van de cel en de micro-omgeving lijken de algehele activiteit van deze enzymen te bepalen.

Veranderingen in de celproliferatie in de krypt beinvloeden niet alleen de activiteit van verscheidene enzymen betrokken bij de darmfunctie, maar hebben ook een duidelijk effect op de differentiatie van een ander celtype, n.l. de slijmbekercel. Onder normale omstandigheden ontstaan onrijpe slijmbekercellen in de onderste helft van de krypt, waar ze tot rijpe slijmbekercellen differentiëren, welke vervolgens langs de bovenste helft van de krypt en langs de villus migreren. Als de proliferatie van de cylindrische epitheelcellen wordt geblokkeerd, neemt het aantal rijpe en onrijpe slijmbekercellen toe. Bij een uitbreiding van het proliferatieve celcompartiment over de gehele lengte van de krypt, worden ook onrijpe slijmbekercellen langs de gehele krypt waargenomen. Dit illustreert de nauwe relatie tussen de cylindrische epitheelcellen en de slijmbekercellen. Deze resultaten zijn daarom in overeenstemming met de hypothese, dat slijmbekercellen afkomstig zijn van primitieve cylindrische cellen.

De belangrijkste conclusies van het in dit proefschrift beschreven experimentele werk zijn:

- de ultrastructurele ontwikkeling van de cylindrische epitheelcellen in de dunne darm vindt geleidelijk plaats tijdens migratie langs krypt en villus en is onafhankelijk van de proliferatieve activiteit en van de maturatietijd in de krypt. Dit lijkt ook het geval te zijn voor de activiteit van een aantal enzymen betrokken bij enige algemene functies van de cel en niet bij de specifieke absorptiefunctie.
- de ontwikkeling van enzymen, betrokken bij specifieke functies van de villus cel, is afhankelijk van de leeftijd van de niet delende cel en ook van de positie in krypt of villus. Veranderingen in proliferatieve activiteit hebben een duidelijke invloed op de activiteit van deze enzymen.
- bij de evaluatie van de microscopische structuur van darm biopten in gevallen van een gestoorde darmfunctie, moet de onderzoeker zich bewust zijn van het belang van veranderingen in de celkinetiek in de krypt. Bij verhoogde celproliferatie in de krypt zal de ontwikkeling van bepaalde biochemische eigenschappen gestoord zijn en daardoor wellicht ook de darmfunctie, ook al vertoond het villusepitheel een normale microscopische structuur.
- microchemische bepalingen van de activiteit van thymidinekinase in geïsoleerde kryptcellen kunnen als een nuttig hulpmiddel dienen bij het bepalen van de proliferatieve activiteit in darmbiopten van de mens.
- er bestaat een nauw verband tussen het aantal delende cylindrische cellen en hun localisatie, en onrijpe slijmbekercellen. De ontwikkeling van slijmbekercellen wordt sterk beïnvloed door veranderingen in de proliferatieve activiteit in de krypt.

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Lieve Nel, jij bent steeds mijn steun en toeverlaat geweest tijdens het werk, dat tot dit proefschrift heeft geleid.

CURRICULUM VITAE

In 1933 ben ik in Breda geboren, maar al snel werd verhuisd naar Delden (Ov.), alwaar ik van 1938 - 1939 de kleuterschool bezocht en mij de eerste beginselen van het fröbelen en knutselen werden bijgebracht.

Gedurende mijn verdere opleidingen heb ik deze basistechnieken verfijnd en geleidelijk op een hoger niveau gebracht.

Na een genoeglijke lagere school begon het zwaardere werk op het gymnasium te Hengelo en vervolgens te Leiden, waar in 1953 het eindexamen werd behaald. Door een kortstondige tweejarige militaire carriere ging veel van de verworven kennis verloren. Vervolgens werd in Leiden met de opleiding biologie begonnen, welke in 1964 werd bekroond met het doctoraalexamen met als hoofdvak plantenfysiologie en als bijvakken biofysica en histologie.

Tijdens mijn studie gaf ik biologie les aan leerlingen van het St. Liduina Lyceum te Schiedam.

Na het doctoraalexamen was ik tot 1968 verbonden aan de mikrobiologische afdeling van het Unilever Research Laboratorium te Vlaardingen, waarna ik in dienst trad van de afdeling Celbiologie van de Medische Faculteit te Rotterdam, waar ik tot op heden nog werkzaam ben. Binnen deze afdeling werd het onderzoek verricht, dat in dit proefschrift is beschreven.

APPENDIX : PUBLICATIONS I - V

DEVELOPMENTAL BIOLOGY 38, 119–137 (1974)

PUBLICATION I

The Influence of Various Cell Kinetic Conditions on Functional Differentiation in the Small Intestine of the Rat

A Study of Enzymes Bound to Subcellular Organelles

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The process of cell maturation and cell ageing of absorptive epithelial cells was investigated in normal rat duodenum. The development of a number of enzymes bound to subcellular organelles was studied by using microchemical analyses on various cell compartments dissected from crypts and villi from freeze-dried cryostat sections. The development of the ultrastructural features of the absorptive epithelium was investigated by electron microscopy of various cell positions along the whole length of the crypt and the base of the villus. The data obtained were related to cell position along the crypt and villus and to cell age during migration from the bottom of the crypt to the tip of the villus.

The influence of changes in the life-span of the cells and of increasing proliferative activity was studied by comparing normal rat duodenum with that from germfree rats and rats recovering from low radiation doses (72 hr after 400 R).

Our data show that the specific activity of nonspecific esterases mainly localized in the endoplasmic reticulum increases when the cells migrate along the upper half of the crypt and the basal part of the villus. Activity of alkaline phosphatase, measured as a marker for the microvilli, is absent in the crypt, but increases linearly from the base of the villus to the tip. The longer life-span of villus cells in germfree animals does not result in a higher activity of these enzymes than in normal animals. An increased proliferative activity in the crypt, as present 72 hr after X-irradiation, is accompanied by a decreased activity of both enzymes but the pattern of activity during cell migration remains the same. The specific activity of enzymes bound to mitochondria or lysosomes (monoamineoxidase and β-*N*-acetylglucosaminidase) are not affected by changing crypt cell kinetics.

Electrophoretic analyses of isolated cell compartments showed that the increase during normal differentiation or the decrease after X-irradiation of esterase activity is due to changes in overall activity, not to the appearance or disappearance of specific isoenzymes. Electron microscopy showed that in the normal intestine there is a gradual development of ultrastructural features during migration of the cell along the crypt while the most drastic changes in cell structure occur at the moment the cell enters the villus. Contrary to our expectation, the ultrastructural development was not influenced by increased proliferative activity in the crypt 72 hr after irradiation, and hence the decrease in enzyme activity found cannot be related to changes in ultrastructure.

INTRODUCTION

In the study of cell differentiation the intestinal epithelium has some advantages compared to other cell renewal systems. The turnover time of the epithelial cells is very short (about 48 hours) and the proliferating, differentiating and functional cells are regularly distributed along the crypts and villi (Leblond and Messier, 1958). Both cell kinetic studies using autoradiography after ^{3}H-thymidine incorporation and (micro)biochemical analyses of cells isolated at successive stages of their differentiation and ageing can be done (Moog and Grey, 1967, Fortin-Magana *et al.*, 1970; Galjaard *et al.*, 1970). After a number of divisions in the lower half of the crypt, the epithelial cell differentiates during a

12-hr period while migrating along the upper half of the crypt. After entering the functional compartment on the villus the cells migrate for about 36 hr toward the tip of the villus where they are extruded into the intestinal lumen.

The functional villus cell is characterized by a number of specific ultrastructural features, and it shows a high activity of enzymes that are involved in absorption, breakdown, and resynthesis of food constituents (Nordström *et al.*, 1968). Part of these characteristics develop during maturation in the crypt. Some others first become apparent after the cell has migrated onto the villus (Padykula *et al.*, 1961; Palay and Karlin, 1959); for example, a rather extensive apical smooth endoplasmic reticulum, an increased rough endoplasmic reticulum, a well developed Golgi apparatus, and numerous long microvilli.

It has been shown that during recovery of intestinal crypts from relatively low doses of X-irradiation an expansion of the compartment of proliferating cells occurs at the expense of the nondividing maturing crypt cells (Williams *et al.*, 1958). Histochemical studies and quantitative microchemical analyses showed that increased proliferative activity in duodenal crypts resulted in a decrease of the activity of some enzymes which normally are active in the upper half of the crypt. These changes in crypt cell maturation are also reflected in the functional villus cells (Galjaard and Bootsma, 1969; Galjaard *et al.*, 1970).

It was the purpose of the present study to investigate the activity of a number of enzymes bound to subcellular particles during differentiation and to analyse the effect of increased crypt cell proliferation on the activity of these enzymes. Moreover, it was investigated whether the enzyme activities are correlated with the development of the ultrastructural features.

Using microchemical techniques, different parts of intestinal villi could be analysed, which makes it also possible to relate the data obtained to villus cell ageing. Part of the analyses has also been carried out on intestine from germfree rats to study the effect of a longer life-span of the epithelial cells (Abrams *et al.*, 1963; Lesher *et al.* 1964). Since during organogenesis in other systems (Lagnada, 1967; Holmes and Masters, 1967a, b; Kingsburry and Masters, 1971; O'Hara *et al.*, 1971) certain isoenzymes show a successive appearance or disappearance, the increase of esterase activity on the crypt-villus transition has been analysed for its isoenzyme pattern.

MATERIALS AND METHODS

Tissue preparation. The experiments were carried out with Glaxo-Wistar rats (about 8 weeks old and 250 g in weight). Germfree animals of the same strain were kindly provided by the Radiobiological Laboratories TNO at Rijswijk. Some of the conventional and germfree animals were exposed to 400 Roentgen from a Philips X-ray machine (200 kV, 13 mA, added filtration of 1.6 mm Cu filter, H.V.L. 1.9 mm Cu, dose rate 20 R/min) and sacrified 72 hr after irradiation.

Control and irradiated animals were starved 24 hr before sacrifice. A segment of duodenum 5 cm below the pyloris was removed under ether anaesthesia and immediately frozen with CO_2 vapor. Longitudinal cryostat sections were cut at $-20°C$ and freeze-dried during 15 hr at $-40°C$ and 10^{-4} mm Hg. Microdissection, weighing, and microchemical analyses were carried out according to Lowry, 1953. In the present study the crypts were dissected into two parts containing the proliferating and maturing compartment. The villi were cut into three parts, representing various stages of cell ageing. The longer duodenal villi of germfree animals were further dissected into four parts.

Microchemical assays. The protein content of pooled isolated fragments was measured according to Lowry *et al.*, 1951, using bovine serum albumin as a standard. The DNA content of isolated fragments was

determined fluorometrically according to Kissane and Robins, 1957 as modified by Robertson and Tait, 1971, using herring sperm DNA as reference.

Alkaline phosphatase was determined spectrophotometrically using *p*-nitrophenylphosphate-disodium salt (conc. 2 m*M*) as a substrate in an incubation volume of 100 µl of 0.1 *M* AMP (aminomethylpropanediol) buffer (pH 9.3) containing 10 m*M* $MgCl_2$. After 20 min incubation at 36°C, 200 µl of 0.1 *N* NaOH was added and *p*-nitrophenol was measured at 405 nm in microcuvettes.

Nonspecific esterases were measured according to a modified method described earlier (Doyle *et al.*, 1959), using α-naphthylacetate (conc. 7 m*M*) as a substrate in a final volume of 350 µl 0.05 *M* Tris HCl pH 7.2. After 20 min incubation 200 µl 0.8% Diazo-Red in 3% sodium lauryl sulfate was added and the extinction measured at 550 nm.

Monoamineoxidase was measured fluorometrically according to Weissbach *et al.*, 1960, using kynuramine as substrate (conc. 0.1 m*M*) in 0.1 *M* borax HCl, pH 8.2. Incubation in a volume of 450 µl was carried out for 30 min at 36°C, and the reaction was stopped with 50 µl of 5 *N* NaOH. Fluorescence was measured in 250-µl cuvettes at 386 nm (exc. 318 nm).

β-N-Acetylglucosaminidase was determined fluorometrically using 1 m*M* methylumbelliferyl-β-*N*-acetylglucosamine as a substrate in 0.1 *M* citrate buffer (pH 5.0). After 20 min incubation the reaction was stopped with 1.5 ml of 0.5 *M* sodium bicarbonate buffer pH 10.5, and the fluorescence was measured at 448 nm (exc. 360 nm).

Analysis of the Data

All enzyme activities are expressed as specific activities in micromoles of substrate converted per hour per milligram of protein or of DNA. Each value expressed in the graphs is the average of data from 3–5 animals. Per animal 6–10 samples of dissected fragments from crypts and villi were analysed. The standard deviation calculated from all the data obtained varied from about 5–15% and is plotted in the graphs.

Electrophoresis

Polyacrylamide electrophoresis of nonspecific esterase isoenzymes was carried out in tubes 5 mm in diameter according to Maurer (1967). The use of an upper gel proved to be unnecessary provided the sharpness of the boundary layer was sufficient. Equivalent dry weight amounts of dissected freeze-dried crypts and villi were homogenized in 0.25 *M* sucrose containing 0.1% Triton X-100 and applied in samples of 10 µl.

Electrophoresis was carried out with 120 V and 4 mA per tube, using bromophenol blue as tracking dye. After electrophoresis for about 90 min, the gels were removed and incubated with α-naphthylacetate (30 m*M*) in 0.1 *M* Tris-HCl at pH 7.2. Fast blue BB (35 mg/50 ml) was used as coupling and staining agent (Markert and Hunter, 1959; Allen *et al.*, 1965). After 5 min incubation, the gels were transferred for fixation to 7% acetic acid and photographed. The gels were scanned with an accessory attachment of a Gilford spectrophotometer. Part of the electrophoresis has been carried out on a discontinuous flat band gel varying from 4.5 to 8% w/v acrylamide.

Electron Microscopy

The intestine of conventional Wistar-Glaxo rats was fixed for 5 minutes by means of a retrograde perfusion technique. The perfusion needle was inserted into the aortal bifurcation, and a ligature was placed around the aorta between the arteria coeliaca and arteria mesenterica superior.

The fixative could leak out via a cut in a liver lobe. The fixative used was 1.5% glutaraldehyde (50% Fischer), 25 m*M* $CaCl_2$, 4% PVP in 0.1 *M* cacodylate buffer

pH 7.2, 37°C. After perfusion fixation, small pieces of intestine 4–5 cm distal to the pyloris were isolated and rinsed for 24 hr in 0.1 *M* cacodylate with 25 m*M* $CaCl_2$, pH 7.2, at 4°C. After a 2-hr rinse in 0.1 *M* cacodylate, the tissue pieces were postfixed with a tricomplex fixation technique (de Bruyn, 1969). Ultrathin sections were cut longitudinally through crypt and villus using the mesatechnique (de Bruyn and McGee-Russell, 1966). The sections were contrasted with venable and examined with a Philips EM 300 electron microscope.

Cell Position and Cell Age

Duodenal segments of conventional, germfree, and X-irradiated rats were fixed in Bouin and stained with haematoxylin eosine. The number of epithelial cells of 50 crypts and villi were counted from the bottom of the crypts to the tip of the villi in longitudinally cut columns. From these data the average cell position of the dissected fragments could be determined. The microchemical analyses could be related to cell age by using data obtained by our group with autoradiography after different time intervals after ^{3}H-thymidine incorporation (Galjaard *et al.*, 1972).

RESULTS

Microchemical Analyses

The cell kinetic data, which were used to relate the enzyme activities to cell position or to cell age, are summarized in Fig. 1. The epithelial cells originate in the lower half of the crypt and differentiate in about 10–12 hr in the upper half of the crypt. As functional absorptive cells they migrate to the top of the villi during 36 hr in normal animals and 48 hr in germfree animals.

Alkaline Phosphatase

Quantitative microchemical measurements in conventional rats show that the activity of alkaline phosphatase is negligible in the crypts. The enzyme activity increases as soon as the cells reach the base

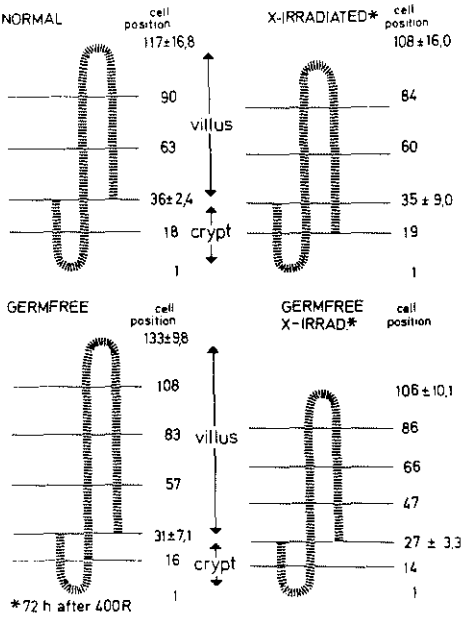


FIG. 1. Data on cell positions under various cell kinetic conditions (n = 50). Lines indicate borders of dissected compartments. Values without standard deviations are interpolated.

of the villus. This increase continues linearly towards the tip. Three days after irradiation with 400 R, when the proliferative compartment is extended to the base of the villus, the activity of alkaline phosphatase is decreased over the whole length of the villus. Now the gradual increase of activity begins in the middle of the villus and continues towards the tip (Fig. 2a).

In germfree animals the villi show a similar linear increase of activity, but although the villi are longer than in conventional animals, the maximal enzyme activities are even lower. Irradiation of the germfree animals results in a pattern of decreased activity similar to that observed in the conventional animals.

When the enzyme activity in conventional and germfree animals is expressed in relation to cell age (Fig. 2b), a linear increase is observed during ageing of the villus cell. Although the life-span of cells in the germfree animals is about 12 hr longer

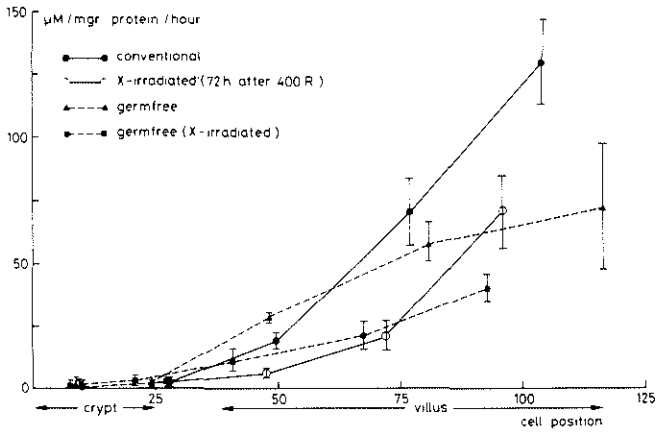


FIG. 2a. Alkaline phosphatase activity in different dissected compartments of crypts and villi, expressed per average cell position. Vertical bars indicate standard deviations. Each point is the average of data of 3–5 animals. Per animal 6–10 samples were analysed.

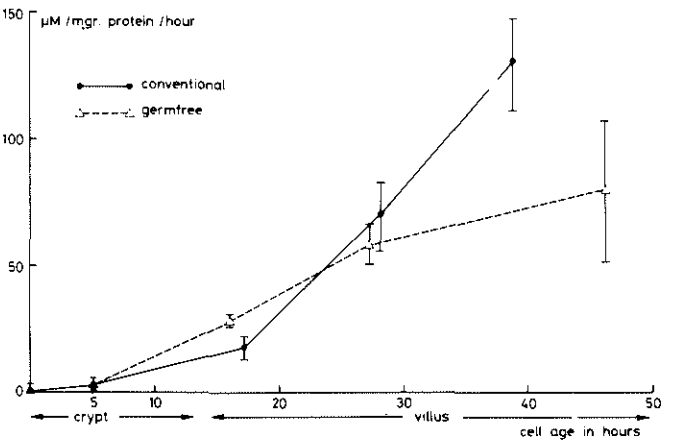


FIG. 2b. As for Fig. 2a, expressed per average cell age.

than in conventional rats, this does not result in a further increase of enzyme activity. At the top of the villi alkaline phosphatase activity is still lower than in conventional animals.

Nonspecific Esterases

For these enzymes which are mainly located in the endoplasmic reticulum a different pattern of activity is found. The esterase activity is low in the proliferation zone in the lower half of the crypt and a gradual increase can be observed during maturation in the upper-half of the crypt (Fig. 3a). This increase continues in the basal and middle part of the villus. In the upper part of the villus, however, a plateau is reached. In germfree animals a similar pattern was observed.

During recovery after X-irradiation the esterase activity is considerably reduced along the whole crypt, which is now completely involved in cell proliferation. There is an increase in enzyme activity in the basal and middle part of the villus. In the germfree rats a plateau seems to be

reached already at the basal part of the villus. However, the esterase activities in the villi remain lower after irradiation when compared to normal controls. When the enzyme activities are related to cell age (Fig. 3b) the comparison with the data based on the relation with cell position (Fig. 3a) indicate that the pattern of esterase activity seems to be more dependent on cell age than on cell position, since the curves for conventional and germfree animals coincide in Fig. 3b to a greater extent than in Fig. 3a.

To investigate whether or not the increase in esterase activity during cell differentiation and the decrease in esterase activity observed after irradiation are due to changes in the number and intensity of specific isoenzymes electrophoresis of isolated crypts and villi was carried out. The results are represented in Fig. 4. It can be seen that when equal quantities of dissected freeze-dried material are applied to the starting zone, this results in much less activity of the individual bands in the crypt compared to the villus. However, the

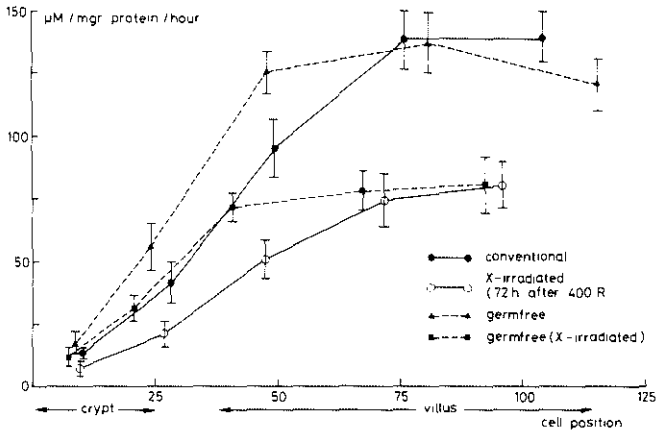


FIG. 3a. Nonspecific esterase activity in different dissected compartments of crypts and villi, expressed per average cell position. Vertical bars indicate standard deviations. Each point is the average of data of 3–5 animals. Per animal 6–10 samples were analysed.

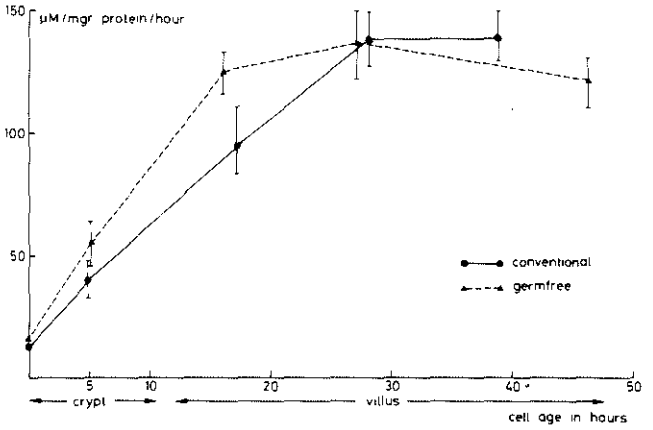


FIG. 3b. As for Fig. 3a. expressed per average cell age.

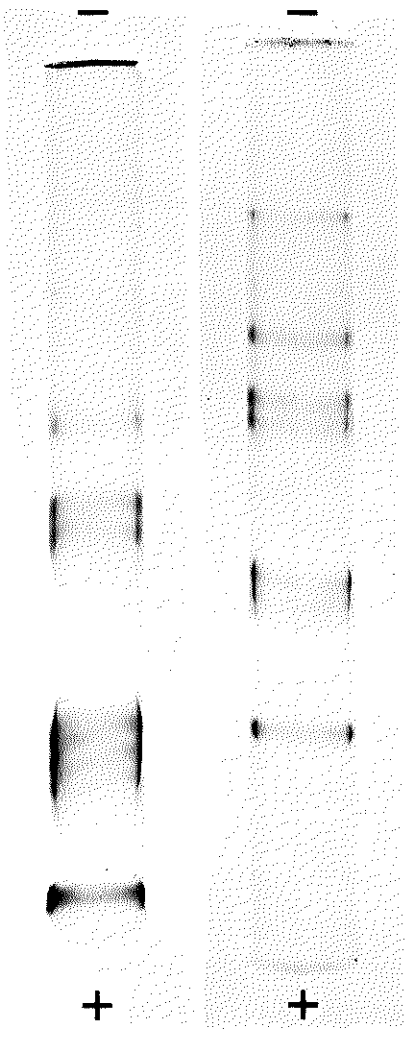


FIG. 4. Isoenzyme pattern of nonspecific esterase in dissected crypts (right) and villi (left).

number of isoenzymes is the same for crypt and villus. The curves obtained by scanning the gels at 485 nm are shown in Fig. 5 a and b. In the isolated villi the relative intensity of the first to the sixth bands predominates over the slow moving bands. The last bands are difficult to detect in the villi. On the contrary, in the crypts the intensity of these two bands seems to increase, while the fast moving bands decrease in intensity. The decrease in esterase activity after recovery from low doses of X-irradiation is not caused by a selective disappearance of certain isoenzymes, but by a decrease in activity of all isoenzymes. In Fig. 6 the isoenzyme pattern of normal and X-irradiated animals is illustrated after electrophoresis of whole freeze-dried tissue sections. If germfree animals are compared with conventional ones, no differences between the number and migration rate of the esterase isoenzymes during electrophoresis can be detected. Neither increase of cell age nor lack of intestinal flora has any influence on these isoenzymes.

Monoamine oxidase was investigated as an enzyme marker for the outer membrane of the mitochondria. The activity is a factor 10^3 lower than that of the two enzymes described earlier. As shown in Table 1 the activity in the villus is slightly higher than in the crypt. For this reason the crypts and villi were not further dissected into separate compartments. No clear effect of irradiation was observed. Similar data were obtained when the enzyme activity was expressed per unit dry weight or per milligram of protein. When the enzyme activity is expressed per milligram of DNA, lower crypt/villus ratios were found.

β-N-Acetylglucosaminidase activity was studied as a marker for lysosomes in dissected crypts and villi. As can be seen from Table 2 the activity in the crypts is somewhat higher than in villi. When expressed per milligram of DNA, the difference in enzyme activity between crypt and villi are no longer present. During recovery after X-irradiation, the glucosaminidase activity decreases about 40% in the crypts and in the villi, but the crypt/villus ratio is not markedly changed.

Subcellular Structure

In the present study the description of ultrastructural features in relation to changes in proliferative activity in the

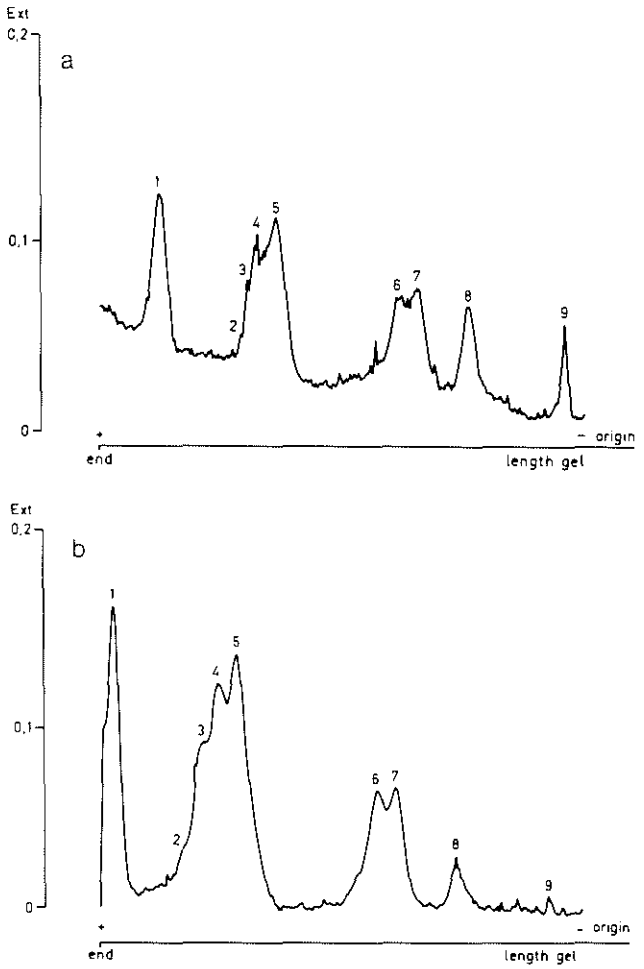


FIG. 5. Scanning pattern of nonspecific esterase isoenzymes in dissected crypts (a) and villi (b).

crypt will be restricted to the absorbing villus cell and its developmental stages in the crypt.

The developing chief cells in the lower part of the crypts have few and very small projections on their luminal surface, which probably develop into microvilli.

The terminal web, the rough endoplasmic reticulum and the Golgi apparatus are poorly developed. Many small apical vesicles can be seen, which are probably involved in the production of the glycocalix. The nucleus is localized in the basal part of the cells with very little underlying cytoplasm. Many free ribosomes are present and mitochondria are predominantly localized in the supranuclear region of the cells. Only few interdigitations between the lower crypt cells have been observed (Fig. 7).

In the differentiation compartment at the upper half of the crypt, an increasing number of microvilli is present although they are relatively short. A terminal web is present and the Golgi apparatus is more developed than in the basal part of the crypts. There seems to be more rough endoplasmic reticulum and an increase in the number of mitochondria per cell. The smooth endoplasmic reticulum is still ab-

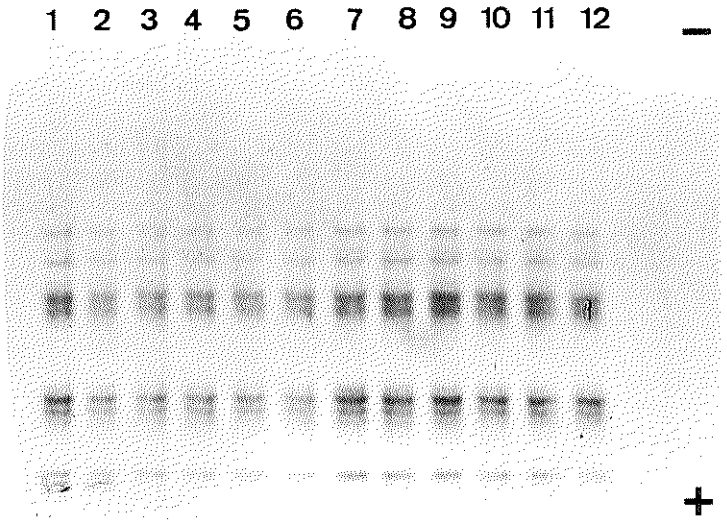


FIG. 6. Comparison of intestinal esterase isoenzymes of freeze-dried sections (crypts + villi) in X-irradiated (1–6) and in normal rats (7–12). Flat-band gel consisting of discontinuous gradient 4.5–6–8% w/v of polyacrylamide (Ortec Inc.).

TABLE 1

MONOAMINE OXIDASE ACTIVITY IN DISSECTED CRYPTS AND VILLI

	Crypt	Villus	Ratio
Normal			
nMoles/mg dry weight/hr	10.8 ± 0.5 n = 30	12.0 ± 1.6 n = 30	0.90
nMoles/mg protein/hr	15.3	17.9	0.85
nMoles/mg DNA/hr	162.4	242.4	0.67
X-irradiated (72 hr after 400 R)			
nMoles/mg dry weight/hr	12.7 ± 3.7 n = 20	13.8 ± 2.3 n = 20	0.92
nMoles/mg protein/hr	18.0	20.6	0.87
nMoles/mg DNA/hr	214.1	288.7	0.74

TABLE 2

β-*N*-ACETYL-GLUCOSAMINIDASE ACTIVITY IN DISSECTED CRYPTS AND VILLI

	Crypt	Villus	Ratio
Normal			
μMoles/mg dry weight/hr	1.12 ± 1.25 n = 40	0.81 ± 0.15 n = 40	1.38
μMoles/mg protein/hr	1.58	1.21	1.31
μMoles/mg DNA/hr	16.8	16.3	1.03
X-irradiated (72 hr after 400 R)			
μMoles/mg dry weight/hr	0.69 ± 0.17 n = 20	0.46 ± 0.08 n = 20	1.50
μMoles/mg protein/hr	0.87	0.61	1.42
μMoles/mg DNA/hr	11.63	9.62	1.21

sent. Many free ribosomes can be seen and more interdigitations between cells are present than in the lower part of the crypt (Fig. 8).

The development of the ultrastructural characteristics of the epithelial cell occurs gradually during migration of the cells along the crypt. There is no indication of

sudden changes when the proliferating cells enter into the nondividing differentiation compartment. As soon as the cells enter the functional compartment on the villus striking changes in ultrastructure occur.

The villus cells are much larger then those of the crypts, there is much more cytoplasm localized under the nuclei and the microvilli are longer and more numerous. The terminal web and the Golgi apparatus are better developed and an elaborate smooth endoplasmic reticulum is present in the apical part of the cell. More rough endoplasmic reticulum and many mitochondria are present both in the apical region and in the basal part of the cell (Figs. 9–12).

Cell kinetic studies have shown that at 72 hr after irradiation with 400 R, the proliferative compartment occupies the whole length of the crypt. However, this does not much affect the development of the ultrastructural features of the crypt cells. Apart from mitotic figures which are found in the highest positions of the crypt, and apart from a more elaborate Golgi apparatus in the lower part of the crypt, the same gradual changes in ultrastructure occur as described for controls (Figs. 13 and 14).

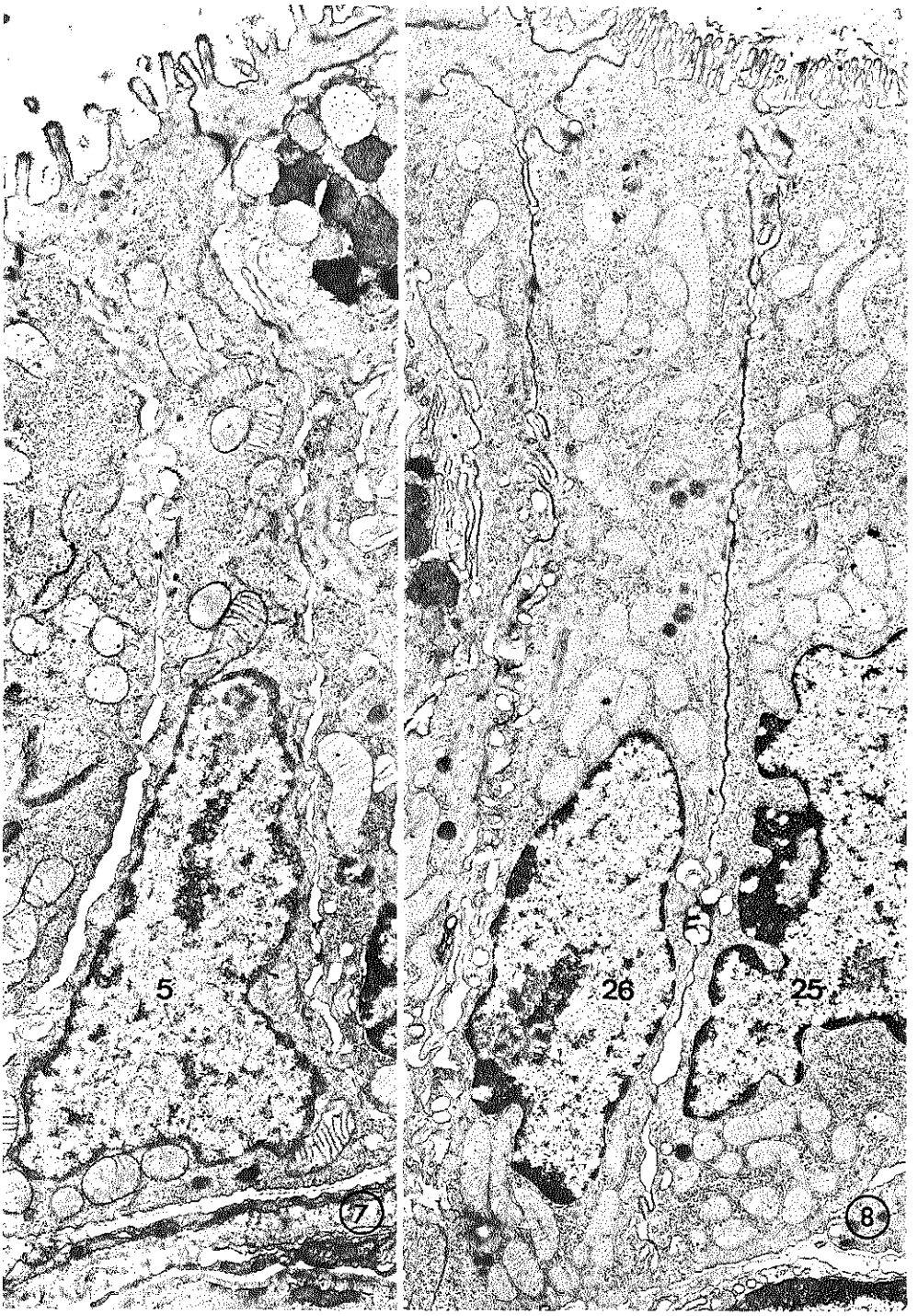
The same sudden changes in submicroscopic structures also occur in the irradiated animals, when the cell migrates onto the villus. Despite the many changes that have been detected in activity of enzymes bound to subcellular particles at 72 hr after 400 R, the ultrastructure of crypt and villus is nearly the same as that for unirradiated animals.

DISCUSSION

Cytochemical staining procedures have the advantage of accurate localization on the cellular level but specific reactions are only available for a limited number of enzymes, and no quantitative data can be obtained. Different methods have been used to obtain biochemical information in relation to cell differentiation and cell ageing of the epithelial cells along crypts and villi from the small intestine. Quantitative analyses can be carried out on cell fractions isolated by cutting cryostat sections parallel to the surface of the intestinal mucosa (Moog and Grey, 1967; Nordström *et al.*, 1968; Fortin-Magana, 1970). However, this isolation procedure or that of mechanical separation of viable intestinal epithelial cells (Harrison and Webster, 1969) does not enable such accurate fractionation of various cell compartments along crypt and villus as Lowry's procedure (1953) of dissection under direct microscopic control from freeze-dried cryostat sections. For this reason, the latter procedure has been applied for the present analysis of certain organelle-bound enzymes in relation to cell localization and cell age in normal rat intestine. The same parameters were studied after experimental changes in the kinetics of the epithelial cells. The use of germfree rats enabled the study of the effect of prolonged life-span (Abrams *et al.*, 1963; Lesher *et al.*, 1964). Earlier investigations showed that in this type of animal the duodenal villi contain about 25% more cells and that the total life-span is prolonged to 60 hr compared to 48 hr in conventional rats (Galjaard *et al.*, 1972). Experimental changes in the proliferative activity of the crypt cells were produced by low doses of X-irradiation. Such doses were found to result in a temporary block in crypt cell proliferation followed by an overshoot in proliferative activity to restore the loss of cells caused by continuing migration along the villus (Williams *et al.*,

FIG. 7. Chief cell in the proliferation compartment of a crypt of a nonirradiated animal. Cell position 5, counted from the bottom of the crypt. x 16,400.

FIG. 8. Chief cells in the differentiation compartment of a crypt of a nonirradiated animal. Positions 25,26. x 12,200.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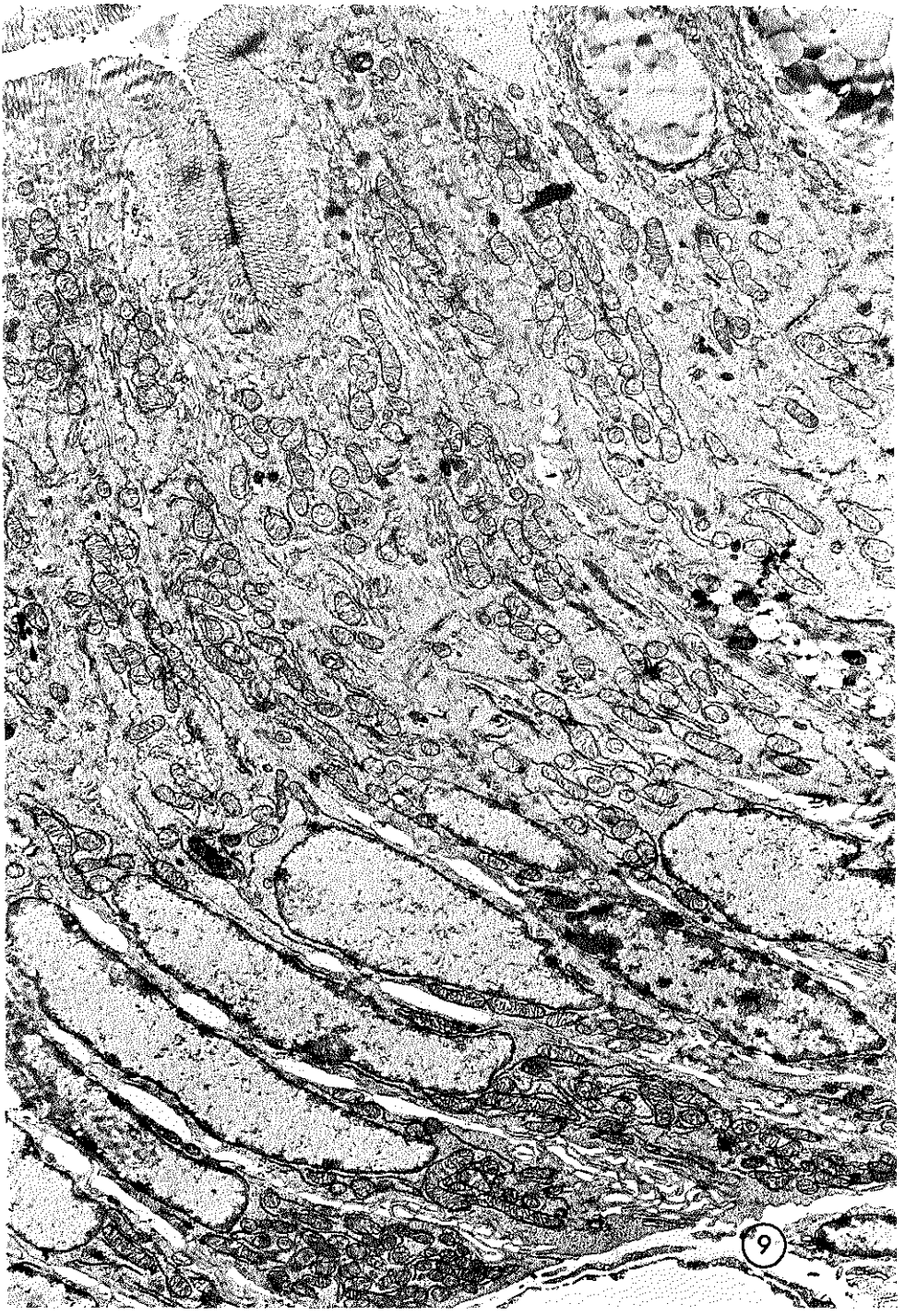
1958; Lesher *et al.*, 1964; Galjaard and Bootsma, 1969). From these studies it has been shown that at about 72 hr after 400 R the normal crypt is recovered and that an expansion of the proliferation zone has occurred, resulting in proliferating cells along the whole length of the crypt. In this situation an epithelial cell entering the functional compartment on the villus has just finished its last cell division and has had much less time to mature in a non-proliferative state as is the case in the normal intestine where the cells mature during 12 hr during migration in the upper half of the crypt.

As is shown in Figs. 2 and 3 the activity of alkaline phosphatase and esterases are affected by increased proliferation in the crypt. As regards alkaline phosphatase activity which shows a maximal activity in the duodenum (Harrison and Webster, 1971) and which is localized in the microvilli, hardly any activity is observed during cell maturation in the crypt. Once the cell enters the villus alkaline phosphatase activity becomes clearly apparent and during subsequent migration towards the tip of the villus the activity increases linearly, which confirms other studies (Nordström *et al.*, 1968; Grey and Lecount, 1970). A prolonged life-span of the cells, as in germ-free animals, also results in a linear increase of alkaline phosphatase activity. However, the maximal activity at the top does not exceed the activity in conventional animals. Further studies on the background of the increasing activity in terms of protein synthesis on the villus (Das and Gray, 1969) and on the turnover of these proteins are required (James *et al.*, 1971).

During restoration of the villus epithelium at 72 hr after irradiation a lower activity of the villus cells is observed. In the basal part hardly any alkaline phosphatase can be detected, but a linear increase starts, when the cell migrates along the middle part of the villus. However, at the top of the villus the activity remains lower than in controls. Insufficient maturation of cells present at about 36–48 hr after irradiation in the crypt probably leads to a decrease in enzyme activity on the villus. The nonspecific esterases which are assumed to be localized in the endoplasmic reticulum (Underhay *et al.*, 1956; Holt and Hicks, 1966; Bernsohn *et al.*, 1966; Deimling and Madreiter, 1972) show already increasing activity in the normal intestine during cell maturation in the crypt and subsequent migration along the lower half of the villus. No further increase in activity occurs when the cell moves along the upper half of the villus. The longer life-span of the villus cells in germ-free rats does not lead to a different pattern of esterase activity (Fig. 3b). Expansion of the proliferation zone during recovery after irradiation results in a markedly decreased esterase activity in the upper half of the crypt and although there is an increasing activity during subsequent migration along the lower half of the villus the normal activities just as those of alkaline phosphatase are not restored. In the upper part of the villus again the activity levels off. If there is no possibility for previous maturation after X-irradiation in the upper half of the crypt, a decreased enzyme activity can be observed at 72 hr post-irradiation which cannot be restored during the time these cells remain on the villus. It seems that the increase of esterase activity is dependent on the age of the epithelial cells (measured as transit time after its last cell division), but that in the middle part of the villus the increase in activity stops and becomes independent of increasing cell age (Fig. 3 a and b).

Our electrophoretic studies on isolated cell compartment did not show any difference in the isoenzyme pattern between

FIG. 9. Absorbing cells in the basal part of a villus of a nonirradiated animal. × 8500.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crypts and villi nor between normal and irradiated rat intestine. This indicates that increase in esterase activity during differentiation and the decrease in activity 72 hr after X-irradiation is not due to the specific appearance or disappearance of esterase isoenzymes. Contrary to findings for other developing systems (Holmes and Masters, 1967a, b; Beckendorf and Stephen, 1970; Kingsburry and Masters, 1971), our data suggest that during the development of the epithelial cell in the intestine all genes responsible for the isoenzymes of nonspecific esterases are activated at the same time. The lower esterase activity after X-irradiation is the result of a decrease in the activity of all the isoenzymes present.

The activity of two particle bound enzymes which are probably less related to absorption do not show any apparent differences in cells from the various cell compartments from crypt and villus (see Tables 1 and 2). Monoamine oxidase, localized in the outer membrane of mitochondria (Schnaitman *et al.*, 1967) which is supposed to be assembled in the crypts (Iemhoff and Hülsmann, 1971) was found to be equally active in crypt and in villus cells. No marked differences are observed when the cells migrate along the villus. However, when expressed per mg DNA the enzyme activity increases on the villus. Changes in cellular kinetics do not influence the activity of this enzyme.

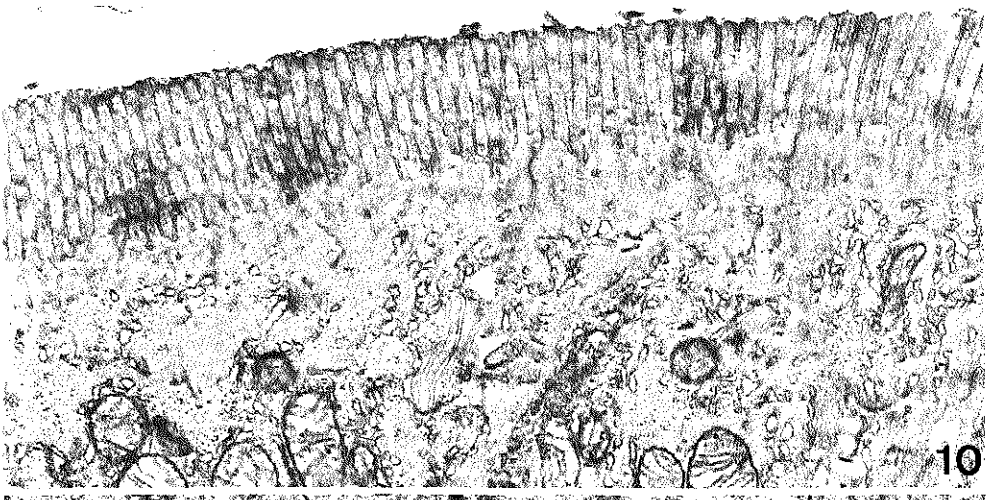
The same is true for β-*N*-acetylglucosaminidase as a lysosomal marker. This is in agreement with findings for other lysosomal enzymes (Fortin-Magana *et al.*, 1970). Lysosomes are present in rather abundant numbers in the few Paneth cells at the bottom of the crypt (Behnke and Moe, 1964), but few are present in the epithelial cells along crypt and villus. Again the activity of this enzyme was not found to be affected by low doses of X-irradiation in contrast to those enzymes which are directly involved in intestinal function.

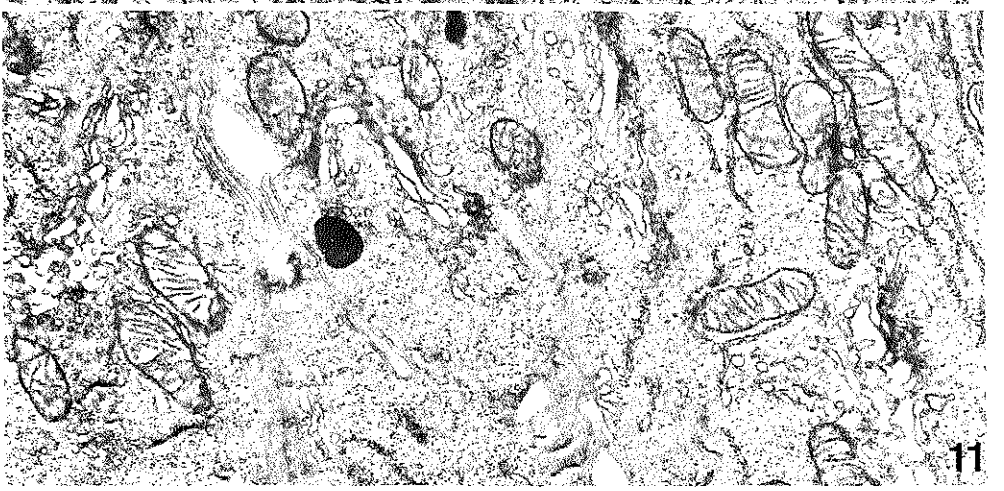
It is well known that considerable differences exist between the ultrastructure of crypt and villus cells (Padykula, 1961; Palay and Karlin, 1959). The present study shows a gradual development of the ultrastructure of cells at different positions in the crypt. No indication was found for any sudden structural change in the zone halfway the crypt where cell proliferation stops and the cell enters the maturation zone. Ultrastructural features which develop gradually during migration of the cell along the crypt are mainly the endoplasmic reticulum and the microvilli. This development does not seem to be affected by an expansion of the proliferative compartment at the expense of the maturation zone at the upper half of the crypt. In the normal intestine the most striking structural changes occur when the cell leaves the crypt and enter the villus. The cell increases considerably in length, and the microvilli, the rough endoplasmic reticulum, and the Golgi system become highly developed. An extensive smooth endoplasmic reticulum appears, and many mitochondria are seen below the nucleus. It is quite remarkable that such large structural changes occur within the few hours the cells migrate along the transitional zone between crypt and villus. Although at 72 hr after irradiation proliferating cells are found at the very top of the crypt, the ultrastructure of the cells at the base of the villus seems to be the same as in controls.

FIG. 10. Apical part of absorbing cells in the basal part of a villus of a nonirradiated animal. The smooth endoplasmic reticulum and a terminal web are well developed. x 13,300.

FIG. 11. Mid-region of absorbing cells in the basal part of a villus of a nonirradiated animal. The Golgi apparatus is well developed. x 13,300.

FIG. 12. Basal part of absorbing cells in the lower part of a villus of a nonirradiated animal. There is much cytoplasm present under the nuclei, and many interdigitations can be seen. x 13,300.


10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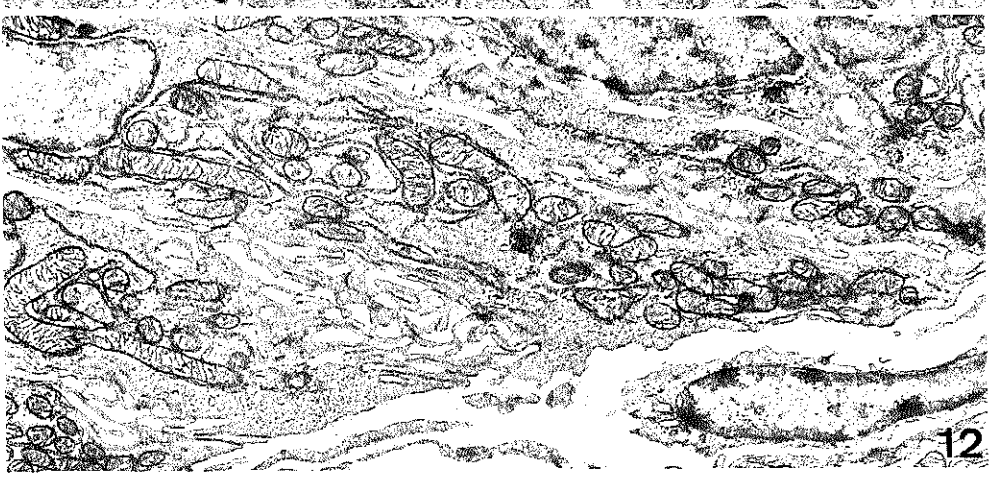
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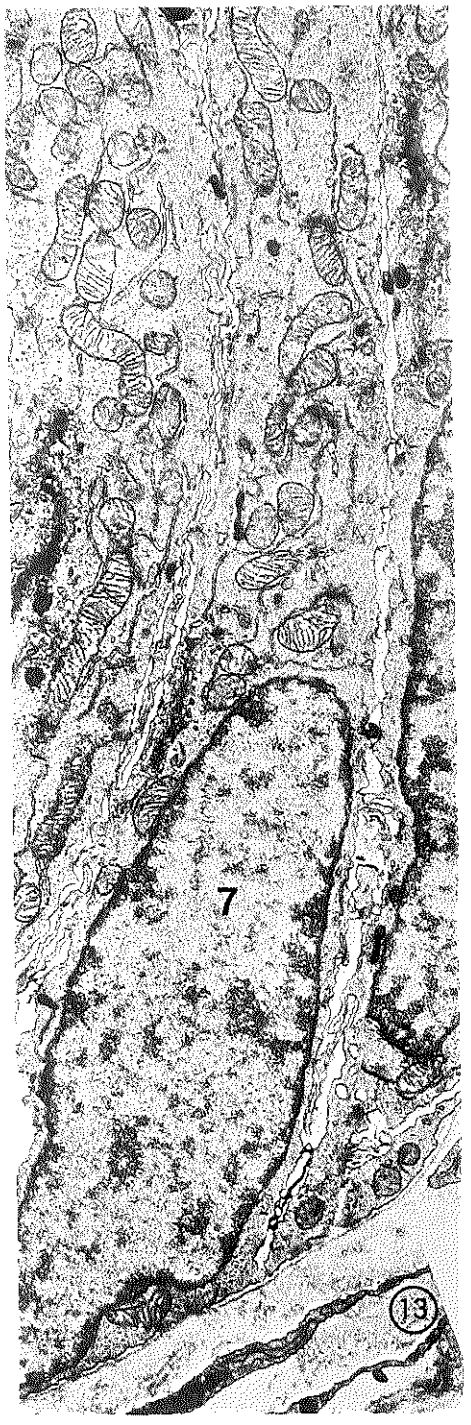


This implies that after its last division at the top of the crypt the epithelial cell is able to change its ultrastructure into that of an apparently normal absorptive villus cell. This is in contrast with the decrease in activity of nonspecific esterase localized in the endoplasmic reticulum and that of alkaline phosphatase in the microvilli.

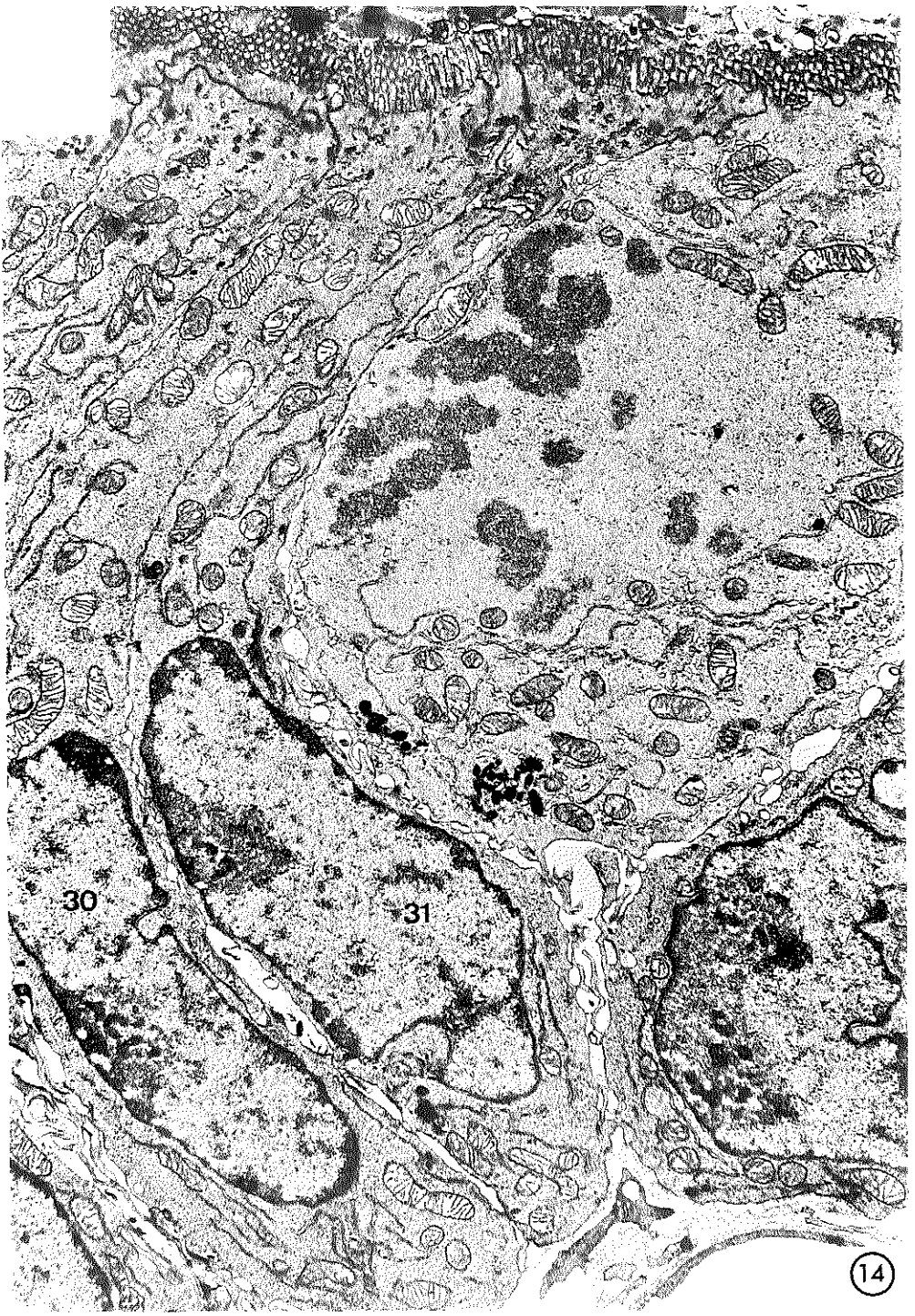
On the electron micrographs mitochondria are present in larger numbers in villus cells. This correlates with the crypt/villus ratio of monoamine-oxidase activity expressed per mg DNA. The presence of lysosomes both in crypts and villi seems to correlate with the activity of acid β-*N*-acetylglucosaminidase, although morphometric measurements are required for a more quantitative comparison (Weibel, 1969).

Contrary to our expectations based on changes in activities of some enzymes bound to subcellular structures, the development of the ultrastructural features of the absorptive villus does not seem to be affected by changes in the crypt cell kinetics.

Germfree rats were kindly supplied by the Radiobiological Institute TNO, Rijswijk. Miss L. Abels is thanked for her technical assistance and Miss M. van Duuren and Mr. T. van Os are responsible for preparation of the illustrations. Mrs. R. van den Hoek and Miss M. H. J. van Rijn kindly typed the manuscript.

FIG. 13. Chief cell in the basal part of a crypt 72 hr after irradiation. Position 7 counted from the base of the crypt. x 12,900.

FIG. 14. Chief cells in the upper part of a crypt 72 hr after irradiation. Positions 30–31. x 14,200.


30
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The Influence of Changing Crypt Cell-Kinetics on Functional Differentiation in the Small Intestine of the Rat

Nucleotide and Protein Synthesis

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In the intestinal epithelium, cell production and cell differentiation take place in the crypts and require both the synthesis of (deoxy)nucleotides for the assembly of DNA + RNA polymers and the synthesis of structural proteins and enzymes. These processes were studied in isolated cell compartments in normal animals and in animals recovering from radiation damage in which the proliferation zone becomes extended to the base of the villus.

The correlation of DNA + RNA synthesis and enzymes involved in nucleotide synthesis was investigated. Hypoxanthine phosphoribosyl transferase was measured as an enzyme which is involved in the conversion of nucleotides. The activity of HPRT was found to be twice as high in the villus than in the crypt cells. Thymidine-kinase, in contrast, which is assumed to be specific for proliferating tissues showed a much higher activity in crypt than in villus cells. TK activity increases when cell production increases during regeneration of the epithelium after X-irradiation. However, 5'-nucleotidase activity which is found to be localised ultrastructurally in the same place as alkaline phosphatase, interferes with the assay of thymidine kinase.

Protein synthesis was investigated by intraperitoneal and intraluminal administration of ^{3}H-labelled amino acids. The incorporation in crypt and villus cells was analysed by autoradiography. All three of the ^{3}H-labelled amino acids. i.e., L-leucine, L-methionine and L-proline were intensively incorporated in the crypt and the lower half of the villus after intraperitoneal injection. The cells of the upper 20 cell positions beneath the tip of the villus did not show grains. Normal and irradiated animals showed a similar pattern of incorporation. When the labelled amino acids were administered intraluminally, grains could also be observed at the tip of the villi, implying protein synthesis in cells immediately before their extrusion into the lumen. This suggests that protein synthesis at the tip is only possible with amino acids absorbed from the intestinal lumen.

Introduction

The continuous production of cells in the bottom of the crypts of the intestinal epithelium requires a continual DNA synthesis. Some of the new cells migrate along the upper half of the crypt and differentiate into cells specialised in the absorption of low molecular substances. During differentiation the cells form more rough endoplasmatic reticulum [1, 2] containing large amounts of RNA. Consequently for both processes an intensive nucleotide synthesis has to take place in the crypt. This is illustrated by the fact that ^{3}H-thymidine is exclusively incorporated in the lower half of the crypt and ^{3}H-uridine is reported to be mainly incorporated in the crypts and lower third of the villus [3]. During migration of crypt cells into the villus a considerable increase in activity of membrane-bound enzymes has been observed. For most enzymes involved in absorption this increase continues to the middle of the villus [1, 4, 5] which correlates with the *de novo* protein synthesis on the villi [6, 7].

The normal cell-kinetic conditions can be modified by the application of low doses of X-irradiation. During the

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subsequent regeneration of the villus three days after 400 Röntgens whole-body irradiation the proliferation zone expands over the whole crypt [8, 9]. Then the increase in activity of hydrolytic enzymes takes place on the base of the villus instead of in the upper half of the crypt as has been shown by microchemical analysis on isolated crypt and villus compartments [1, 5]. In the present study the pattern of protein synthesis in normal and regenerating epithelium was investigated autoradiographically after labelling with different ^{3}H-amino acids. Since it has recently been reported that the specific activity of labelled proteins synthesised in the intestinal mucosa, measured after TCA precipitation, differs considerably, when the amino acids were applied intraperitoneally or intraluminally [10], both routes of administration were investigated.

A number of the enzymes involved in nucleotide synthesis are known to be active in the conversion of nucleotides taken up from the intestinal lumen [11, 12]. Most of the enzymes involved in nucleotide metabolism are therefore not specific for proliferating and/or differentiating intestinal cells. To verify whether the activity of such enzymes correlates with the localisation of the proliferative and maturing crypt cell compartment thymidine-kinase was measured as marker enzyme for nucleotide synthesis. Hypoxanthine phosphoribosyltransferase and 5′-nucleotidase were measured as enzymes involved in nucleotide conversion and degradation. The activity of these enzymes was also determined to investigate the possible influence of the extension of the proliferating compartment during recovery from X-irradiation.

In addition the localisation of 5′-nucleotidase and alkaline phosphatase was determined ultrastructurally.

Methods

Glaxo-Wistar rats were irradiated with a Philips X-ray machine (200 kV · 13 mA, added filtration 1.6 mm Cu filter, H. V. L. 1.9 mm Cu, dose rate 20 R/min). Three days after a dose of 400 Röntgens total-body irradiation, the animals were killed and duodenal segments of the intestine were analysed. Control and irradiated animals had been fasted for 24 h before killing. Protein synthesis was measured autoradiographically after intraperitoneal injection of rats with 100 μC ^{3}H-L-leucine (S.A. 390 mCi/mM), ^{3}H-L-methionine (S.A. 250 mCi/mM and ^{3}H-L-proline (S.A. 6800 mCi/mM). For intraluminal administration 12.5–50 μCi label in 1 ml physiological salt solution was used, which was aborally applied to the stomach. Segments of duodenum were removed half an hour after injection and fixed in formol 4% for 24 h. After sectioning liquid emulsion was used for autoradiography. It was necessary to wash the sections in a 5% solution of non-labelled amino acids before dipping in order to remove the non-incorporated absorbed label completely. After 5 weeks exposure time the sections were developed and stained with haematoxylin-eosin. The position of labelled cells along longitudinally cut sections of crypt and villi was recorded.

Isolation, freezing, sectioning and freeze-drying of duodenal segments were carried out for (micro)biochemical analyses as previously reported [1, 5, 9]. Crypts and villi were dissected from freeze-dried sections and weighed on quartz fiber balances [13]. Fresh cell suspensions of crypts and villi were prepared according to Harrison and Webster [14] and used for assay of those enzymes whose activity is too low to be measured on dissected freeze-dried sections with radioactive substrates. *Hypoxanthine phosphoribosyltransferase* was measured with ^{3}H-hypoxanthine (5 μC, S.A. 1 Ci/mM) as substrate (conc. 72 μM) in an incubation volume of 70 μl consisting of 0.14 M Tris-HCl pH. 8.0, 14 mM $MgCl_2$, 0.28 mM EDTA di-Na salt and 0.55 mM phosphoribosylpyrophosphate Na-salt.

After 30 min incubation the reaction was stopped with 10 μl 20% TCA. Inosine-5′-monophosphate, formed during the reaction, was separated from the non-converted hypoxanthine by thin-layer chromatography with a mixture of 1 M NH_4-acetate and alcohol 100% (3 : 7) on DEAE cellulose plates (Baker). The method was preferred to disc-washing techniques [15, 16] which has certain disadvantages which may be critical in microchemical analysis [17]. With nonlabelled hypoxanthine and inosine monophosphate as carriers the spots were identified under UV light of 254 nm. The spots were scraped off the plastic plates and collected in scintillation vials. 1 ml of 0.25 M Na_2HPO_4 was added to remove the nucleotides from the DEAE cellulose. For counting in a liquid scintillation spectrophotometer 15 ml BRAY solution was added, consisting of 100 g naphtalen, 7 g PPO and 0.3 g POPOP/litre dioxane. Disintegrations per min (DPM) were calculated from the counts per min by the computer of the Heart Centre, Medical Faculty Rotterdam. Since the specific activity is known, the amount of ^{3}H-inosine-monophosphate/mg protein/h can be calculated. *Thymidine-kinase* was measured with a modification of the method of Masui and Garren [18] on cell suspensions of crypt and villi. The incubation medium of 70 μl consisted of 0.14 M Tris-HCl pH 8.0, 14 mM $MgCl_2$, 0.28 mM EDTA di-Na salt, 3 mM ATP di-Na salt and 4 μC ^{3}H-methyl thymidine (S.A. 2 Ci/mM) as substrate (conc. 28.5 μM). The reaction was stopped 20 min after incubation by 10 μl 20% TCA. The phosphorylated ^{3}H-thymidine was separated and counted as described above. Since TMP is required for the formation of di- and trinucleodite the contribution of TDP and TTP to the total amount of phosphorylated ^{3}H-TdR does not interfere with the thymidine kinase assay.

5′-nucleotidase was measured using 0.1 μC ^{14}C thymidine monophosphate (S.A. 43 mCi/mM) as substrate (conc. 33.3 μM) under identical conditions as the thymidine kinase assay. As a second substrate 4 μC ^{3}H-inosinemonophosphate (S.A. 5.5 Ci/mM) was used (conc. 13.4 μM) and the enzyme activity was measured under conditions similar to the HPRT assay. All radiochemicals were derived from Radiochemical Centre Amersham (United Kingdom).

Alkaline phosphatase was measured as described earlier [1]. The protein content of pooled dissected fragments and of homogenates was measured according to Lowry *et al.* [19].

Enzyme activities are expressed as mμmoles substrate converted/mg dry weight or protein/h. Eight measurements were carried out per fraction obtained from each animal. Four animals were used for each experiment. Standard errors of the mean of the data are indicated in the tables.

Electron Microscopic Localization of Alkaline Phosphatase and 5′-nucleotidases

Small pieces of rat intestine 4–5 cm distal to the pylorus were isolated under ether anaesthesia and fixed in 2% glutaraldehyde in 0.067 M cacodylate buffer, pH 7.2, 0° C, for 3 h. After rinsing in 0.25 M sucrose

in 0.067 M cacodylate buffer, pH 7.2, 0° C, 50 u cryosections were cut. For alkaline phosphatase these sections were incubated in Millonig's medium consisting of: 3 ml 0.2 M Tris-HCl pH 8.0, 0.2 ml 0.03 M β-glycerophosphate, 0.3 ml 0.1 M tri-sodium-citrate and 0.3 ml 0.09 M Pb-nitrate. The pH was adjusted to 8 instead of Millonig's value of 9.2 to approximate the condition of the thymidine kinase assay.

For the localisation of 5′-nucleotidase the β-glycerophosphate in Millonig's medium was replaced by thymidine monophosphate or inosine monophosphate with the same final concentration as for β-glycerophosphate. As controls sections were heated during 5 min at 90° C before incubation. Also sections were incubated without substrate. For all the enzymes incubation at 4° C lasted 12 min. After incubation the sections were rinsed 3 times for 10 min in the same rinsing fluid as described above and were post-fixed in 1% OsO_4 in 0.14 M cacodylate buffer 7.2 for 1 h at 4° C.

After dehydration *via* an ethanol series the thick sections were embedded on flat-topped small epon cylinders. Thin sections were cut with a Reichert microtome and studied with a Philips E. M. 300.

Results

Enzymes Involved in Nucleotide Metabolism

In pooled dissected fragments of crypts and villi a number of enzymes involved in nucleotide metabolism is measured to verify if the activity can be correlated to DNA and RNA synthesis in normal and regenerating epithelium.

The results of the enzyme measurements show that the activity of hypoxanthine-phosphoribosyltransferase (HPRT) lies in the range of nanomoles/mg dry weight/h. The activity in the villus cells is about twice as high as in the crypt cells (Table 1). Three days after X-irradiation the activity in the crypts is increased and becomes equal to that of the villi. The activity of the villus cells is the same in controls and after irradiation. The ratio of crypt to villus activity changes from 0.41 to 1.0.

These differences are not due to different amounts of protein since the crypt/villus ratio of protein content/mg dry weight is 1.05. The ratio for irradiated and normal crypts is 1.11, for villi 1.13.

Table 1. Comparison of the activity of hypoxanthine phosphoribosyl transferase and 5′-nucleotidase (mu moles/mg dry weight/h) in isolated crypts and villi

	HPRT		5′-Nucleotidase[a]
	Normal	X-irrad.[b]	Normal
crypt $n = 8$	2.44 ± 0.32	5.12 ± 0.57	19.5 ± 4.36
villus $n = 8$	5.98 ± 0.57	5.96 ± 0.78	46.4 ± 5.85
crypt/villus	0.41	1.0	0.43

[a] Inosine-5′-monophosphate as substrate.
[b] 72 h after 400 Röntgens.

The product formed by the phosphorylation of hypoxanthine can be degraded to inosine by 5′-nucleotidase. In Table 1 the activities of the enzymes are compared. The 5′-nucleotidase activity is 10 times higher than that of HPRT, but the ratio between crypt and villus activity is similar.

Thymidine-kinase shows a completely different pattern. The activity is in the order of picomoles/mg protein, which makes it impossible to measure the enzyme activity in dissected freeze-dried fragments by a radiochemical assay. For this reason the enzyme was assayed in crypt and villus cell suspensions isolated according to Harrison and Webster [14]. In these suspensions the yield of cells is sufficient to measure thymidine-kinase, although the separation of crypt and villus is not as reliable as that obtained by micro-dissection under direct microscopic control.

The results in Table 2 show that thymidine-kinase has a 10 times higher activity in crypt cell suspensions than in villus cells. During the recovery after low doses of X-irradiation the proliferation zone is extended to the base of the villus. The thymidine-kinase activity is increased five-fold compared to controls. The crypt/villus ratio increases from 9 to a value of 28. The product formed by the reaction in this assay, thymidine-5′-monophosphate, is, like inosine-5′-monophosphate a substrate for 5′-nucleotidase. Incubation of the cell suspensions with ^{14}C-thymidine-monophosphate establishes that the reverse reaction

Table 2. Thymidine-kinase activity in crypt and villus cell suspensions of normal and X-irradiated rats (uu moles/mg protein/h)

	Normal	X-irrad.
crypt $n = 8$	48 ± 1.1	256 ± 7.8
villus $n = 10$	5 ± 1.2	9 ± 2.5
crypt/villus	9.6	28.5

Table 3. Comparison of thymidine-kinase, 5′-nucleotidase and alkaline phosphatase activity in crypt and villus cell suspensions (mu moles/mg protein/h)

	Thymidine-kinase	5′-nucleotidase[a]	Alkaline phosphatase
crypt $n = 8$	0.048 ± 0.001	5.440 ± 0.28	15274 ± 1.06
villus $n = 8$	0.005 ± 0.001	15.324 ± 2.2	37983 ± 1.03
crypt/villus	9.6	0.36	0.40

[a] Thymidine-monophosphate as substrate.

can take place under the assay conditions for thymidine-kinase (Table 3). Under these conditions the nucleotidase activity is three times higher in the villus cells than in the crypt cells. The crypt/villus ratios of the activity of 5′-nucleotidase and thymidine-kinase differ from 0.36 to 9.6. When the alkaline phosphatase is measured again under the same conditions with p-nitrophenolphosphate as substrate, the enzyme activity is more than a factor 10^3 higher than that for 5′-nucleotidase. However, the crypt/villus ratios for both enzymes are similar. In the assay for thymidine-kinase the measured activity has in fact to be corrected for a higher 5′-nucleotidase activity on the villus, since the product formed by the first enzyme can immediately be converted by the second.

With the light microscope as well as with the electron microscope (Figs. 1–3) it could be demonstrated that the localisation of the activities of alkaline phosphatase and the 5′-nucleotidases were identical. The enzyme activities could be demonstrated on the brush border and the lateral membranes of the villus cells, while in the crypts activity of these enzymes could only be demonstrated at the highest crypt positions. When both substrates were used, i.e. inosine-5′-monophosphate or thymidine-5′-monophosphate, the ultrastructural distribution was identical. The controls of all enzyme localisations did not show any activity.

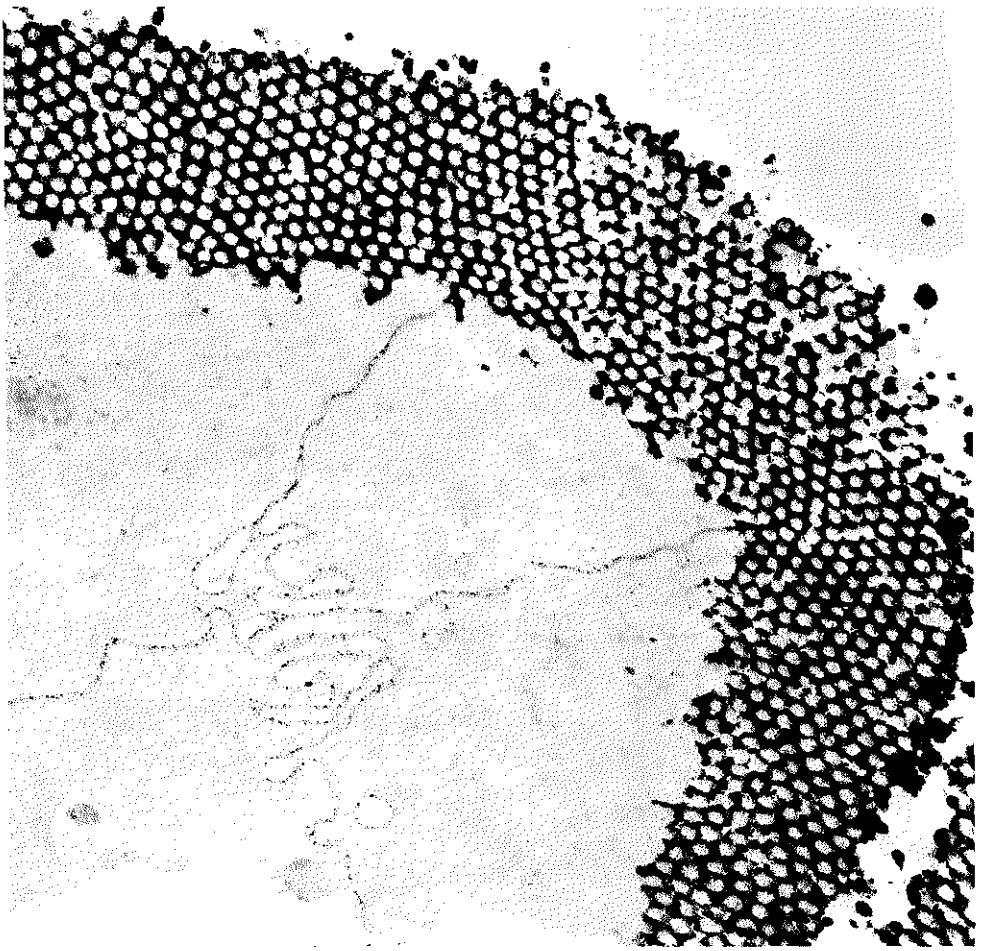

Fig. 1. Ultrastructural localisation of alkaline phosphatase in the brush border and lateral membranes of absorbing villus cells (10500 ×)

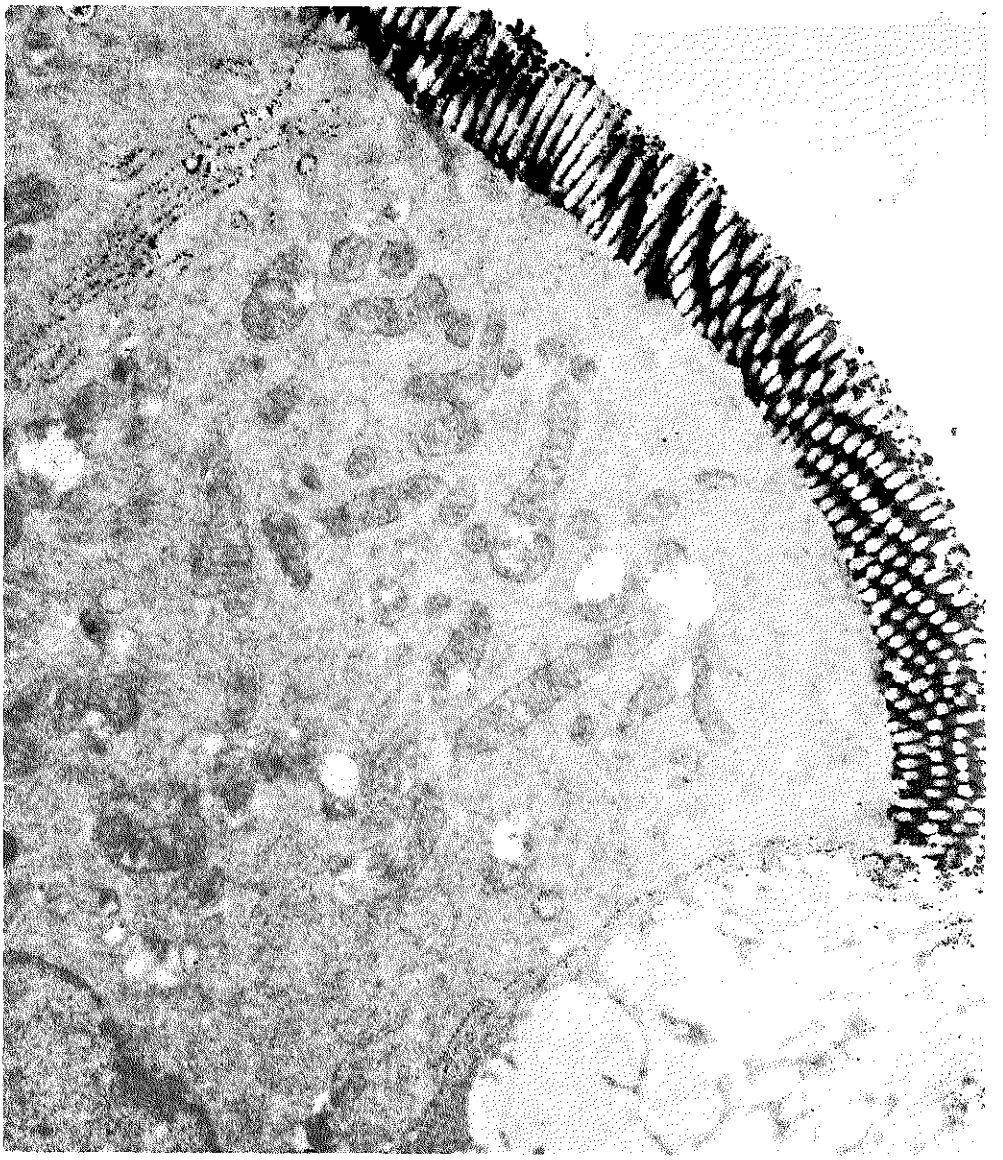

Fig. 2. Ultrastructural localisation of 5'-nucleotidase in absorbing villus cells (11400 ×)

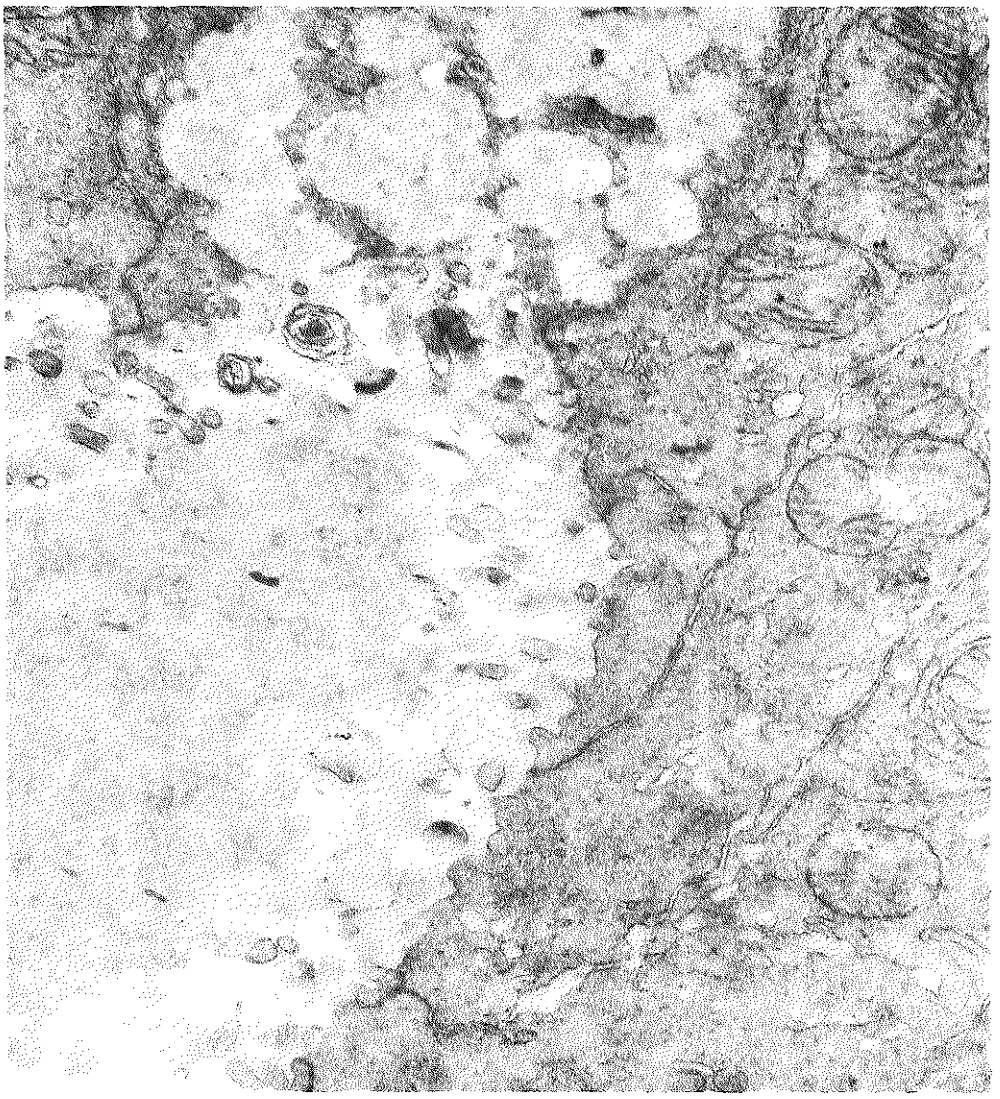

Fig. 3. Absence of 5′-nucleotidase in crypt cells (26300 ×)

The Localisation of Protein Synthesis was determined in autoradiographs after injection of ^{3}H-L-leucine, ^{3}H-L-methionine and ^{3}H-L-proline. The pattern of incorporation along various cell positions of crypt and villus was found to differ considerably when different methods of administration were used.

After intraperitoneal injection of labelled amino acids the highest labelled cell position (counted from the base of the villus) appeared between cell position 56 and 64 in normal animals (Table 4 and Figs. 4a and b). These positions correspond to the middle of the villus. In all autoradiographs a rather sudden absence of grains can be detected in cells above this area. No marked differences in the number of grains along the lenght of the villus were observed.

Three days after 400 Röntgens the border between labelled and unlabelled cells lies between cell positions 53 and 55 on the villus. Since regeneration of the villi is not yet finished at this time, the villi are about 10 cell positions shorter than normal. The fraction of labelled villus cells is therefore larger than in non-irradiated animals but again the cells at the villus tip remain unlabelled. After intraluminal administration the ^{3}H-labelled amino acids were also incorporated in the cells of the villus tip (Fig. 5). Grains were almost absent in the apical part of the goblet cells. Apparently this can be explained by a too short incubation time, since within 30 min only the perinuclear zone of the goblet cells is labelled.

Table 4. Incorporation of ^{3}H-labelled amino acids in intestinal epithelium of the rat[a]. Highest labelled cell position of the villus[b]

	Normal duodenum $n = 20$	After X-irradiation $n = 20$
^{3}H-L-leucine (SA 390 mCi/mM)	56 ± 3	55 ± 3
^{3}H-L-methionine (SA 250 mCi/mM)	64 ± 2	54 ± 4
^{3}H-L-proline (SA 6800 mCi/mM)	61 ± 2	53 ± 3

[a] Killed 30 min after intraperitoneal injection of 100 µC ^{3}H-labelled amino acids.

[b] Counted from the base of villus (total villus length corresponds with 81 in normal and 73 cell positions 72 h after 400 Röntgens).

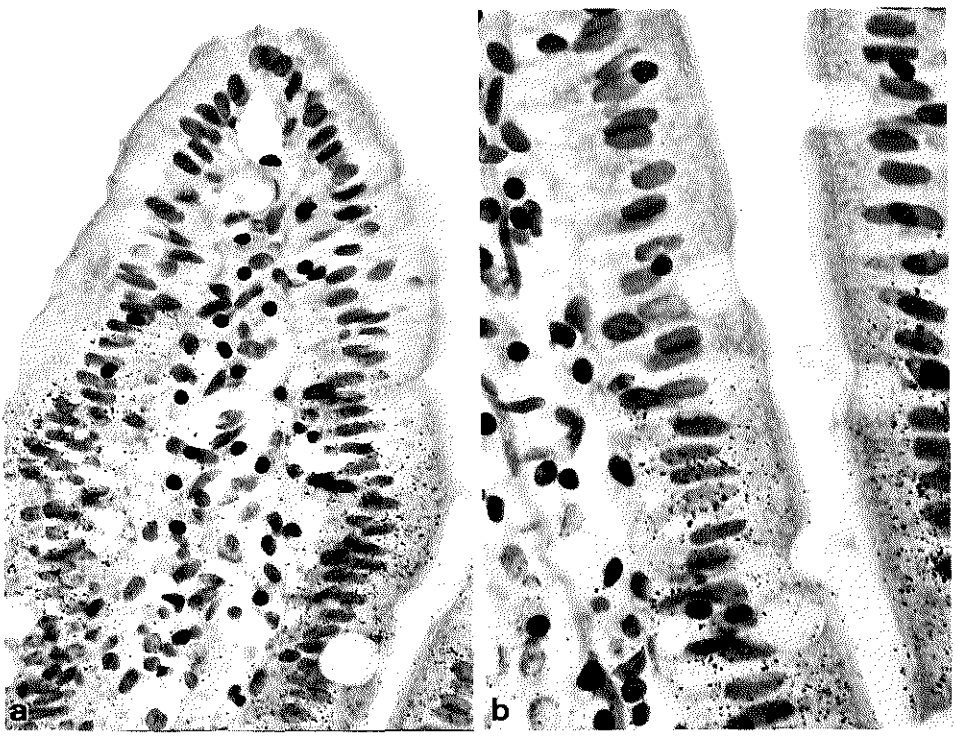

Fig. 4. a) Autoradiograph of the intestinal epithelium after intraperitoneal administration of 100 µC ^{3}H-L-proline. b) As (a); detail of transition between labelled and non-labelled epithelial cells

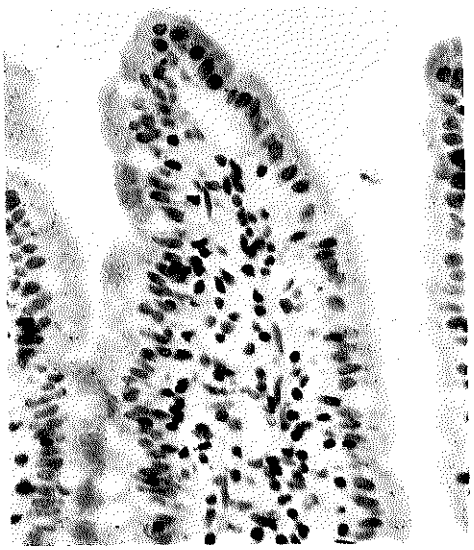

Fig. 5. Autoradiograph of intestinal epithelium after intraluminal administration of 50 uC ^{3}H-L-proline

Discussion

In previous studies it has been shown that non-specific esterase, alkaline phosphatase, α-glucosidase and lactate dehydrogenase increase considerably in activity when epithelial cells differentiate in the upper half of the crypt and migrate to the villus. This increase continues for most enzymes up to the middle part of the villus. For alkaline phosphatase, however, the increase in activity is linear till the cells reach the tip of the villus [1, 4]. During recovery three days after X-irradiation, the proliferation zone is extended to the base of the villus, while the villus has not yet regenerated to its original length [1, 9]. The activity of non-specific esterases, alkaline phosphatase and a-glucosidase is decreased although the activity curves have a similar slope as those in non-irradiated animals [1, 5].

In contrast to enzymes involved in absorption, enzymes involved in nucleotide synthesis have a double function in the intestine. They are necessary for the conversion of nucleotides, absorbed from the lumen, as well as for the synthesis of DNA and RNA during proliferation and differentiation. Many of these enzymes are reported to be active in both crypt and villus cells [11]. The present study confirms this for HPRT and 5'-nucleotidase which were found to be active both in isolated crypt and villus cells. For HPRT no alterations were detected in the villus after X-irradiation. The higher activity in the crypts is probably related to increased DNA synthesis during recovery [8, 9]. Of the enzymes involved in nucleotide metabolism only thymidine-kinase is reported to be specific for cells present in proliferating tissues [11, 20, 21]. For instance during liver development [22] or liver regeneration [23] the activity of this enzyme increases significantly. The enzyme is claimed to be present in a membrane-bound form in the mitochondria and in a soluble cytoplasmatic form [24]. Regulation of its activity can take place by feedback inhibition [16] or by a selective increase of the cytoplasmatic form during development [25] and after hormonal treatment [18]. As is shown in Table 2 the thymidine-kinase activity is considerably higher in crypt cell than in villus cell suspensions, which confirms studies with other methods [11, 20]. Three days after X-irradiation, when the pool of proliferating cells is extended [8], thymidine-kinase activity is strongly increased. Therefore thymidine-kinase activity is a likely parameter for proliferation. The increase of its activity corresponds to the extension of the proliferation compartment as measured by autoradiographic procedures [9]. It remains unknown in how far differences in uptake of thymidine correlate with the observed differences in thymidine-kinase activity.

A change in cell-kinetic conditions similar to regeneration after X-irradiation occurs in the intestinal epithelium in non-tropical sprue. This disease is characterised by atrophic villi and by an enlarged proliferation compartment in the crypts, accompanied by higher levels of aspartic transcarbamylase and dehydroorotate dehydrogenase [26]. No reports on thymidine-kinase activity in biopses of these patients are available.

In the present paper the problem to which degree 5'-nucleotidase interferes with the HPRT and thymidine-kinase assays was also investigated by using ^{14}C-thymidine-5'-monophosphate and ^{3}H-inosine 5'-monophosphate as substrates under the same conditions. The ratios between crypt and villus cells of HPRT activity and hydrolysis of inosine-5'-monophosphate are similar. When the phosphorylation of thymidine and the conversion of thymidine-5'-monophosphate to thymidine were compared, the crypt/villus ratios of both enzymes are even reciprocal (Table 3).

Consequently the low activity of thymidine-kinase for the villus cell suspension [11, 20] should be corrected for 5'-nucleotidase activity, especially since it has been shown recently that thymidine-kinase is present in villus epithelium and can be stimulated by phospholipase C [27]. Similar ratios between crypt and villus activity for 5'-nucleotidase and alkaline phosphatase were found.

In the electron microscopic photographs alkaline-phosphatase is localised in the microvilli of the brush border and the lateral membranes of the villus cells as has been shown in the present study and by others [28, 29]. A similar localisation has been found for 5'-nucleotidase which is in agreement with its presence in the plasma membrane in intestinal [30] and liver cells [31]. This suggests that the enzymes are identical in the intestine as has been proposed for liver [32].

However, results obtained by mixing the two enzymes or by the addition of high concentrations of β-glycerophosphate in the 5'-nucleotidase assay seem to support the existence of two separate enzymes [30]. Moreover, in other systems such as human bone the enzymes seem to vary independently in activity [33].

Provided that villi are sectioned perfectly, the incorporation of intraperitoneally applied different ^{3}H-amino acids shows an identical picture in the autoradiographs. The grains per cell are rather numerous up to the middle of the villus. However, as the cells migrate to the top, a sudden stop of the incorporation of labelled amino acids takes place. No grains can be detected in this area half an hour after injection of the label. A steady state between the injected labelled and the pool of unlabelled amino acids seems to be established within a few minutes [34], after which the amino acids can be incorporated from the extracellular pool [35]. A similar pattern was obtained using scraping techniques and TCA precipitation of proteins [35]. Three days after 400R the proliferation zone is extended during recovery, which results in a higher amount of incorporated ^{3}H-thymidine. No changes in the pattern of incorporation of ^{3}H-labelled amino acids were found. In both experimental series the localisation of incorporation of amino acids corresponds with the increase of hydrolytic enzyme activity [1, 4, 5].

However, after intraluminal administration of labelled amino acids the cells at the villus tip also contained radioactivity which implies that in these cells *de novo* protein synthesis takes place with amino acids derived from the lumen, confirming studies with other techniques [10, 37]. Whether part of this incorporation is due to the turnover of protein in the villus cells, as is suggested on the basis of negative evidence by James and others [38], remains unsolved, since both processes cannot be separated with the method used.

Acknowledgements: Drs. H. R. de Jonge of the Department of Biochemistry is acknowledged for the supply of crypt and villus cell suspensions of normal and irradiated rats. P. Clotscher is acknowledged for help in programming the computer of the Heart Centre of the Faculty.

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Cell and Tissue Research PUBLICATION III

THE RELATION BETWEEN CELL PROLIFERATION, DIFFERENTIATION AND ULTRASTRUCTURAL DEVELOPMENT IN RAT INTESTINAL EPITHELIUM

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SUMMARY

The ultrastructural development of the principal cells in rat small intestine was studied by morphometric analyses in relation to the exact cell position along crypt and villus. From the bottom to the tip of the crypt, a gradual increase occurred in absolute size of the total cell, the cytoplasm, the terminal web and of nearly all cell organelles. Also, the relative size of the cytoplasm, mitochondria, microvilli and endoplasmic reticulum increased during crypt cell differentiation. No sudden changes in ultrastructure were observed in the so-called "critical decision zone", normally located halfway up the crypt where the proliferative activity ceases. At the crypt-villous junction a 1.4 - 3 fold increase in cell size, cytoplasm, terminal web and of most organelles was noted. Expansion of the proliferative cell compartment over the total length of the crypt as occurs during recovery after a low X-irradiation dose (72 hours after 400R) does not affect the normal development of cellular ultrastructure. These findings are discussed in relation to biochemical and cell kinetic data.

Key words: Rat small intestine - Differentiation - Ultrastructure - Morphometry - X-irradiation.

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INTRODUCTION

Small intestinal epithelium has a number of advantages as a model for studies on cell proliferation and differentiation and the relation of these processes with cell

function. Morphologically, there is a regular distribution of the proliferating-, maturing- and functional cells along crypt and villus. This fact and the rapid turnover time of the intestinal epithelium has enabled detailed studies on cell population kinetics (for reviews, see Lipkin, 1973; Cheng and Leblond, 1974). Also, the use of cytochemical staining techniques and of (micro) biochemical assays of isolated crypt and villous cell compartments has provided interesting data on cell differentiation processes (Padykula et al., 1961; Jervis, 1963; Moog, 1967; Nordström et al., 1968; Webster and Harrison, 1969; Imondi et al., 1969; Fortin-Magana et al., 1970; Galjaard et al., 1970; Iemhoff and Hülsmann, 1971; de Both et al., 1974a; de Jonge, 1975).

The relative easy access to the intestine and the possibility of isolating viable parts of the gut enable investigation of the effect of pathological or experimental conditions on the structure and biochemistry of the absorbing villous cell (Palay and Karlin, 1959a and b; Jasper and Bronk, 1968; Porter, 1969; Osborne et al., 1970; Clarke, 1972; Ginsel et al., 1973; Riecken and Dowling, 1974). For a correct interpretation of such studies it is necessary to know whether these conditions affect primarily the functional villous cells or whether they alter crypt proliferation and differentiation which secondarily affect the absorbing villous cells.

In the early days of radiobiology it was shown that inhibition of crypt cell proliferation resulted in structural and functional disturbances of small intestinal villi. Later on it was established that increased crypt cell proliferation occurred during recovery after a relatively low dose of X-irradiation (Williams et al., 1958; Lesher, 1967). This overshoot in normal proliferative activity was shown to be accompanied by marked changes in the normal development of the activity of several enzymes during crypt cell differentiation. A decreased activity of those enzymes which play a role in the specific villous cell function may, of course, impair intestinal function (Galjaard and Bootsma, 1969; Galjaard et al., 1970; de Both et al., 1974a).

The purpose of the present study was to investigate whether an increased proliferative activity in jejunal crypts also affects the normal ultrastructural development of the absorbing villous cell. Morphometric analyses (Weibel, 1969) were carried out on electron micrographs taken from cells at known positions in different parts of the crypt and the base of the villus. A comparison of data from the jejunum of normal animals and animals 72 hours after 400R X-irradiation was undertaken. In the former proliferation was restricted to cells in the lower half of the crypt (Cairnie et al., 1965), whereas in the irradiated animals all crypt cells were involved in proliferation (Lesher, 1967; Galjaard and Bootsma, 1969). By studying the ultrastructure of cells at known positions in crypt and villus, the morphometric data can be related to data of cellular kinetics and biochemical studies (de Both et al., 1974a).

MATERIAL AND METHODS

Male Glaxo Wistar rats 8 weeks of age, weighing 250 g were used for the experiments. Eight animals served as controls and the same number was exposed to 400 Roentgens whole body X-irradiation from a Philips X-ray machine (200 kV, 13 mA, added filtration of 1.6 mm Cu filter, H.V.L. 1.9 mm Cu, dose rate 20R/min). Control

and irradiated animals were starved 24 hours before sacrifice.

To localize the proliferative cell compartment two controls and two irradiated animals were injected intraperitoneally 72 hours after irradiation with 100 μC ^{3}H-thymidine; 30 min thereafter a segment of the small intestine (5 cm below the pylorus) was dissected out under ether anesthesia. The tissue pieces were washed in a cold 5% solution of non-labeled thymidine in order to remove non-incorporated label. Fixation was performed in 4% formaldehyde for 24 hours. After embedding in paraffine and sectioning, autoradiography was carried out using K2-Ilford liquid emulsion.

ELECTRON MICROSCOPY

For electron microscopy the intestines of six controls and of six animals 72 hours after irradiation were fixed in situ by a perfusion technique (de Both et al., 1974a). Small pieces of intestine, about 5 cm distal to the pylorus, were then isolated and fixed by immersion fixation. These tissue pieces were embedded in Epon and ultrathin sections were cut longitudinally through crypts and villi by means of the "mesa-technique" (de Bruyn and McGee-Russel, 1966). The sections were contrasted with 7% uranyl acetate and lead citrate according to Venable and Coggeshall (1965).

Cells at each position in the crypt and the basal part of the villus were selected only when cut through their median axis as shown by the presence of their basal membrane, the nucleus and microvilli. Cells in mitosis did not fulfil these criteria.

The cell positions in the crypt were numbered from the bottom of the crypt and on the villus from the crypt/villous junction. In electron micrographs, selected in this way, the area of the whole cell, the cytoplasm, terminal web and the different cell organelles was obtained by point counting planimetry using a square lattice test system according to Weibel (1969). The number of points on the lattice scored for the whole cell, the nucleus, the cytoplasm, the terminal web, microvilli, mitochondria, Golgi system and the rough and smooth endoplasmic reticulum were used as a measure for the absolute areas of these structures. The mean absolute area of the whole cell, the cytoplasm, terminal web and the different cell organelles for each cell position was calculated from values of 2 to 14 cells in each particular cell position. Regression lines were calculated for these areas as a function of the position in the crypt. Also, histograms were made for the mean absolute areas in groups of four cell positions in the crypts and for cells at the lowest twenty positions on the villus.

The relative area was calculated from the number of points on the lattice test system scored for the different cell structures divided by those scored for the total cell. Regression lines and histograms were made as described above.

In addition, data were collected about the mean number of mitochondria per cell. The relative number of mitochondria was defined as the number of mitochondria per cell divided by the number of points scored for the total cell.

All observations and measurements for both experimental groups were carried out at the same magnification.

RESULTS

In normal jejunal crypts the proliferative cell compartment is located in the lower half of the crypt (Fig. 1a). At 72 hours after 400R of X-irradiation the proliferative compartment has expanded over the whole length of the crypt (Fig. 1b).

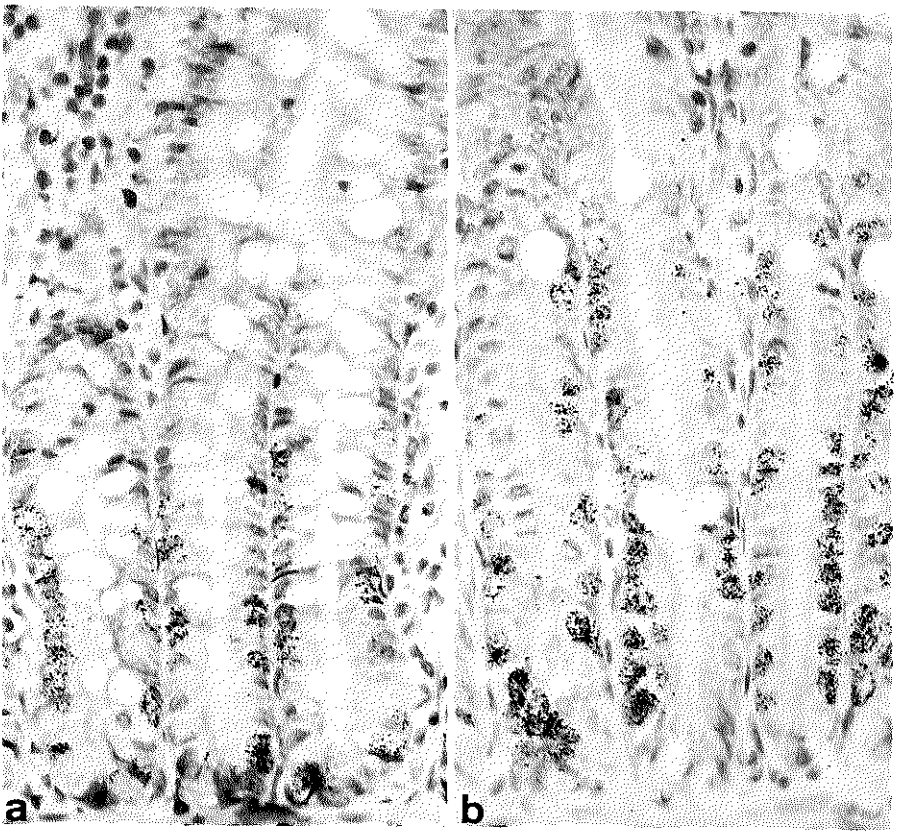
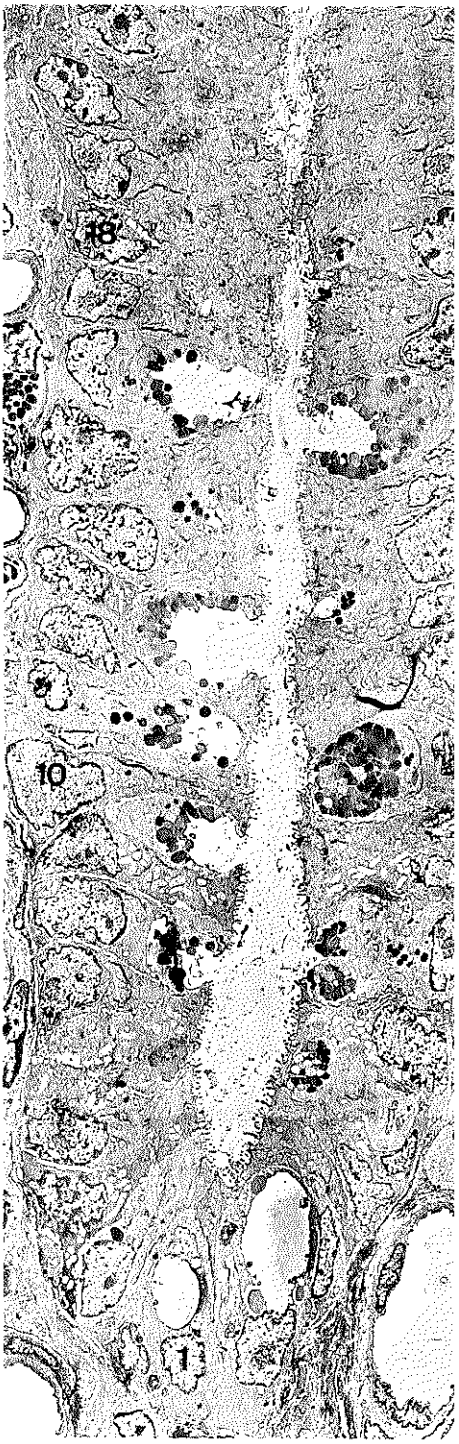


Fig. 1 . Localization of the proliferative compartment in the crypts of controls (a) and of animals 72 hours after 400 R X-irradiation (b) . x140.

For an analysis of the ultrastructure of principal cells in relation to the position in the crypt and the lowest part of the villus, longitudinally cut crypts as illustrated in Fig. 2 must be selected. Principal cells of crypts sectioned in this manner from normal animals were chosen for detailed ultrastructural analysis.



QUALITIVE OBSERVATIONS

In control animals the principal cells in the lower half of the crypt (Fig. 3) posses a few, short microvilli; the Golgi apparatus and terminal web are not well-developed, although the latter already contains many apical vesicles. Many free ribosomes and a few strands of rough endoplasmic reticulum are present, but no smooth endoplasmic reticulum. Most of the mitochondria are located in the apical part of the cell, whereas the nucleus is situated in the basal part with a small amount of subnuclear cytoplasm. A junctional complex is already present. In principal cells in the mid-region of the crypt (position 18-22) more and longer microvilli can be observed and the Golgi complex becomes larger.

In the upper part of the crypt, the so-called maturation compartment (Fig. 4), nearly all cell organelles have increased. An increase in the length and number of microvilli are found, the terminal web and the Golgi system are better developed and there seems to be an increase in the rough endoplasmic reticulum (RER). Smooth endoplasmic reticulum, however, is still absent. The number of mitochondria has increased and many free ribosomes are still present.

From these qualitative studies it appears as if the ultrastructure of the principal cells develops gradually as the cells migrate from the bottom towards the tip of the crypt. The transition from a proliferating cell into a non dividing, maturing cell midway up the crypt is not accompanied by any sudden changes in ultrastructure.

When the crypt cells enter the functional compartment of the villus, a sudden change

Fig. 2 . Reconstruction of a longitudinally cut crypt of a control animal . ×1600. Numbers in the cells indicate their exact position from the bottom of the crypt.

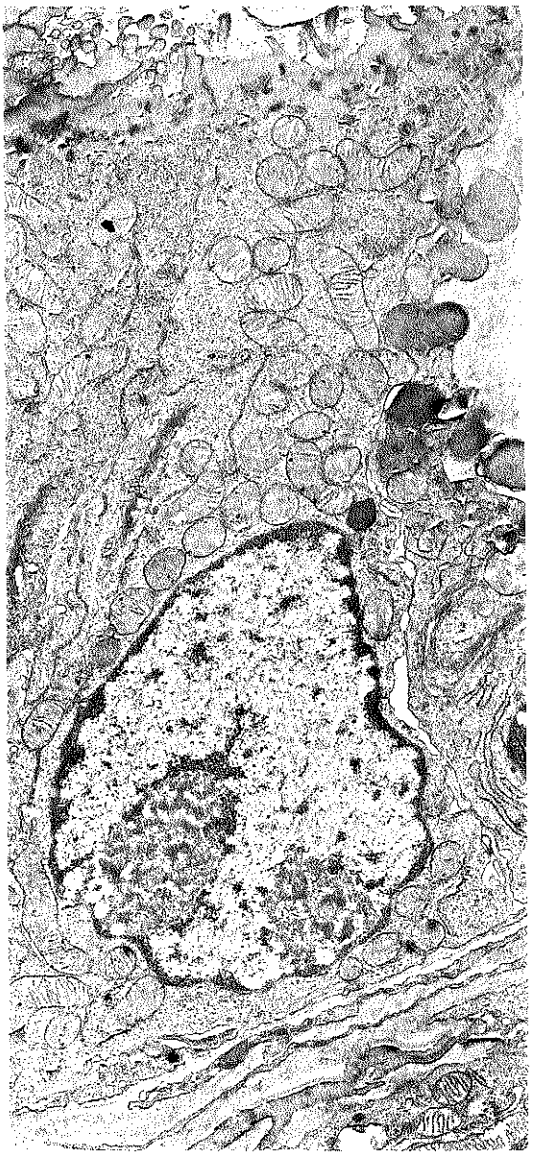


Fig. 3 Principal cells in the lower half of the crypt at cell position 8 of a control animal (x 9700).

in ultrastructure is found (Fig. 5). Many long and slender microvilli, a distinct terminal web, a complex Golgi system and many strands of rough endoplasmic reticulum are found. In contrast to crypt cells, a smooth endoplasmic reticulum is present in the apical part of the villous cell. More mitochondria can be seen and the nucleus is located towards the middle of the cell with a large area of subnuclear cytoplasm, containing mitochondria and strands of RER.

From the study of electron micrographs of crypt and villous cells of irradiated animals, it appears that the ultrastructural development of the principal cell is not affected, although the proliferative activity in the crypts has changed considerably 72 hours after irradiation.

MORPHOMETRY

To substantiate these qualitative findings a morphometric analysis was carried out. Fig. 6 shows the mean absolute areas of the principal cells and their different components. The absolute areas of the total cell, cytoplasm, terminal web, microvilli, mitochondria, endoplasmic reticulum and Golgi apparatus all show a gradual increase as the cells migrate from the bottom to the tip of the crypt. This is true both for the controls and irradiated animals. In the latter, the area of the total cell, the cytoplasm, terminal web and nucleus is generally greater than in the controls. The area of the nucleus remains the same during normal crypt cell differentiation, but it increases in the irradiated animals.

When the cell leaves the crypt and enters the villus, a sudden increase of the absolute area of the total cell, the cytoplasm, the terminal web and of all cell organelles measured was found for controls, but also for irradiated animals even when proliferating cells are present up to the tip of the crypt (Fig. 6).

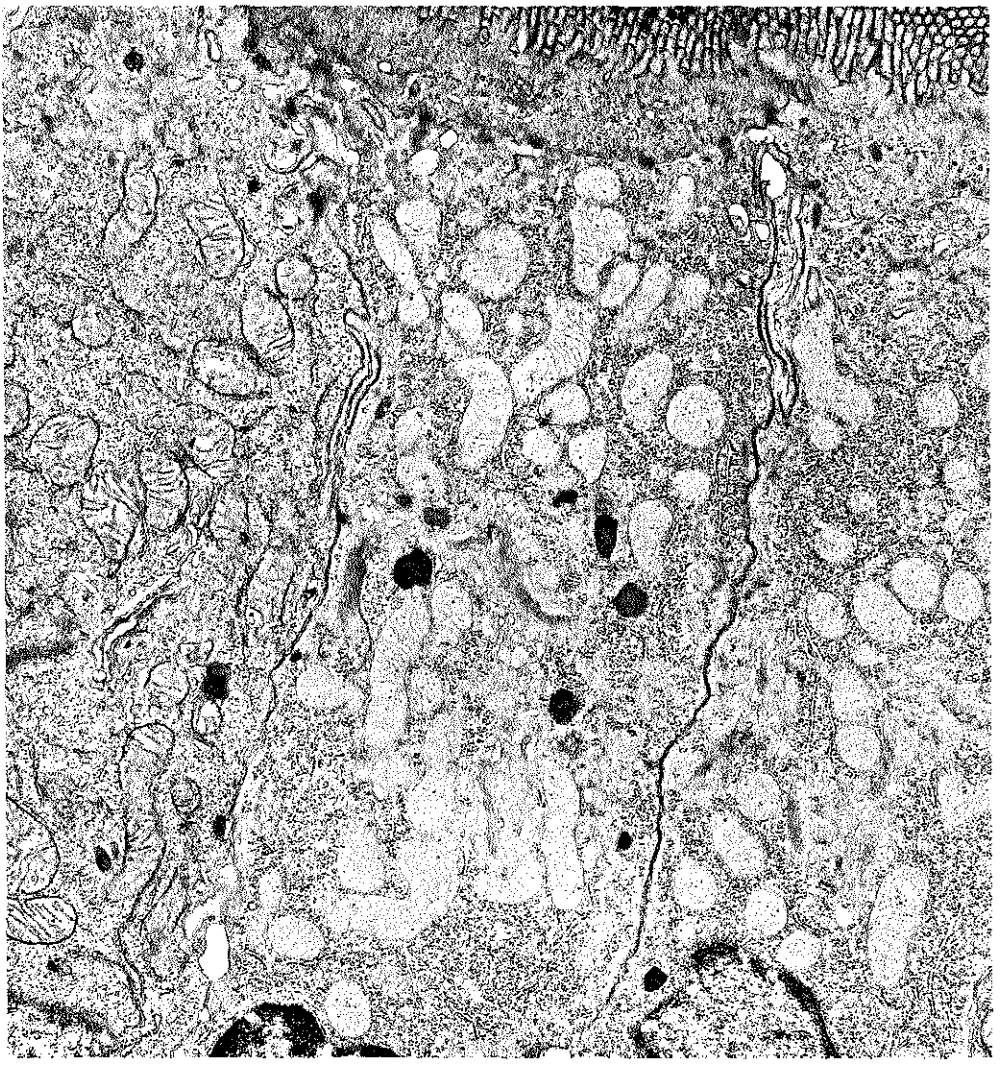


Fig. 4. Principal cell in the upper-part of a crypt of a control animal. Cell position 27. × 11700.

To obtain exact data on the relation between ultrastructure and crypt cell position, regression lines were calculated as described above. The values of the correlation and regression coefficients of the regression lines for the absolute areas of all cell parameters and for the number of mitochondria per cell for both experimental groups are given in Table I. Except for the nucleus, significant positive correlation coefficients are found for the absolute area of the total cell, the cytoplasm, terminal web and all cell organelles of both experimental groups. Also, the mean number of mitochondria per cell increases during crypt cell migration.

These quantitative data show that the increase in the size of the cell and of the various cell components is not markedly affected by the alteration in proliferat-

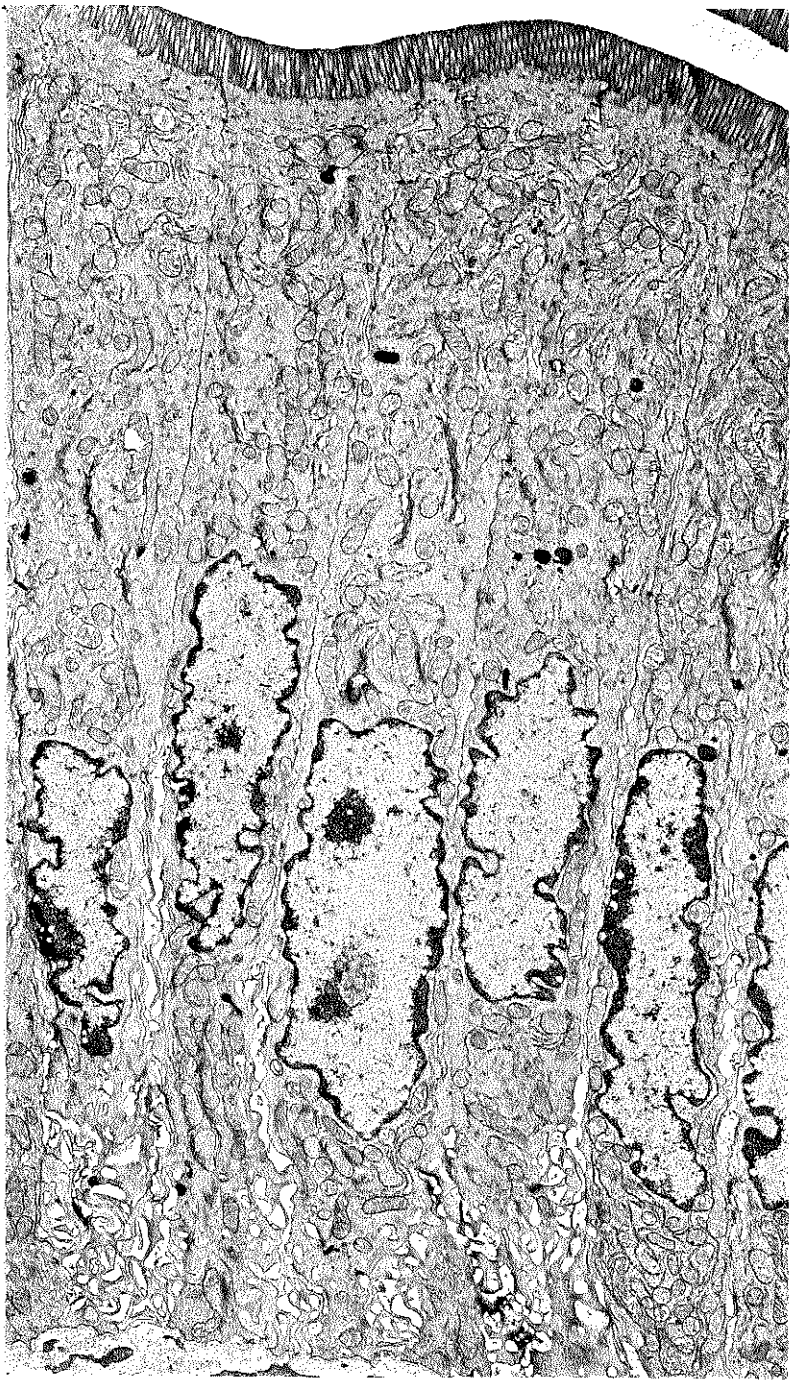


Fig. 5. Absorbing cells at the base of a villus of a control animal. x 4900.

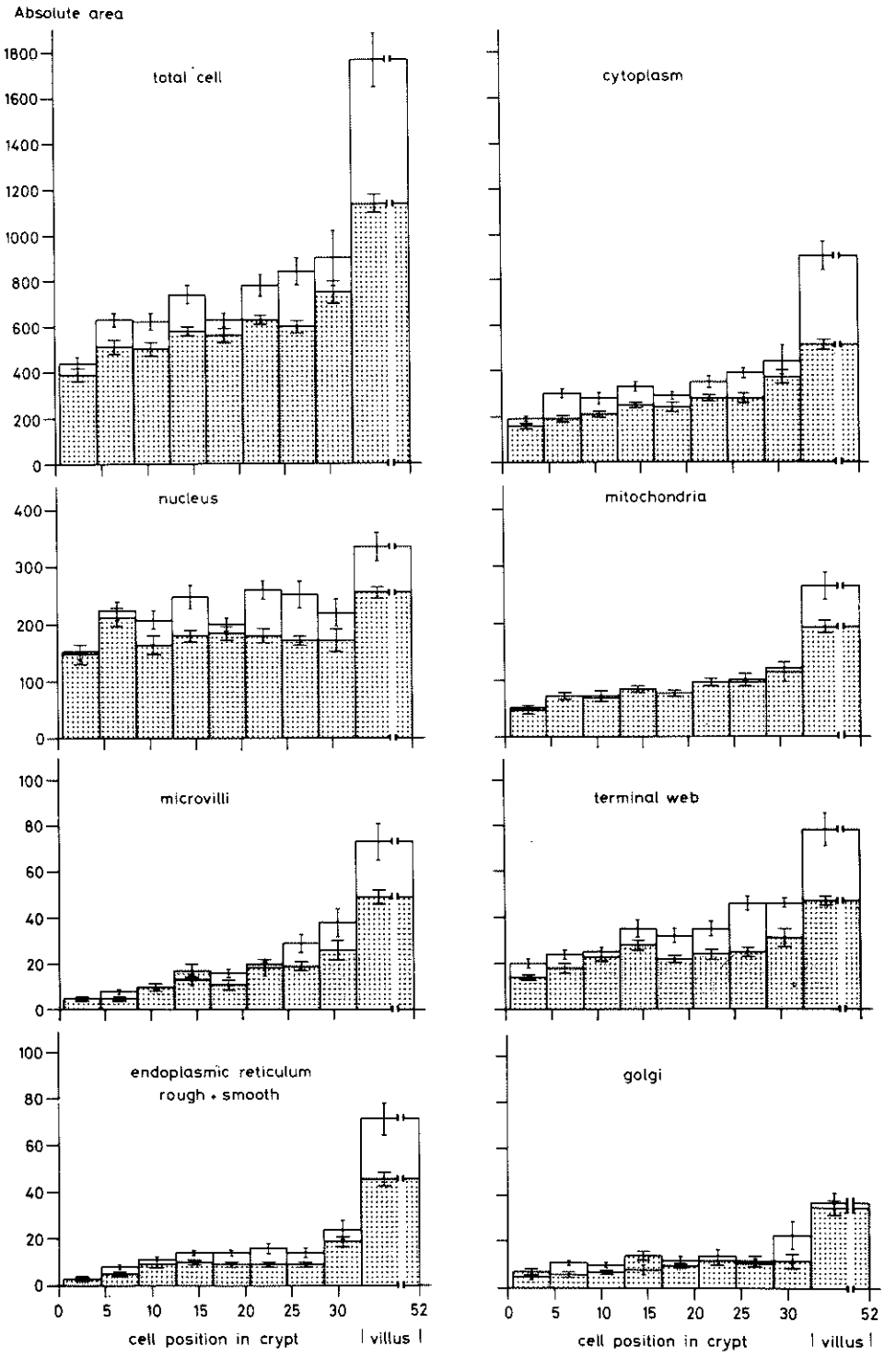


Fig. 6. The absolute areas, expressed as number of points of the test system scored, of the whole cell, the cytoplasm, the terminal web and the different cell organelles for controls (dotted) and irradiated animals (white) as a function of the cell position. Standard errors of the mean are indicated.

ive activity present 72 hours after irradiation.

To investigate whether the ultrastructural development of the principal cells in the crypt merely consists of growth, the relative area of the various cell components was determined quantitatively as well. In Fig. 7 and Table II histograms and data on the regression lines of the relative areas as functions of the cell position are illustrated. The relative area of the nucleus in both experimental groups clearly decreases during crypt cell migration. The relative area of the cytoplasm, mitochondria, microvilli and endoplasmic reticulum in the control animals shows a significant increase during crypt cell migration. The expansion of the proliferative cell compartment during recovery after irradiation seems to prevent the normal increase of the relative area of cytoplasm and mitochondria and to result in a significant increase in the relative area of the terminal web.

Table I

RELATION BETWEEN ULTRASTRUCTURAL DEVELOPMENT AND CELL POSITION IN JEJUNAL CRYPT

	ABSOLUTE AREA			
	CONTROLS		IRRADIATED*	
CELL ORGANELLE	CORRELATION COEFFICIENT	REGRESSION COEFFICIENT	CORRELATION COEFFICIENT	REGRESSION COEFFICIENT
TOTAL CELLS	0.81	10.28	0.74	15.68
CYTOPLASM	0.84	6.77	0.80	8.26
NUCLEUS	-0.05	- 0.17	0.36	2.37
MITOCHONDRIA	0.83	2.29	0.76	1.80
NUMBER OF MITOCHONDRIA	0.77	0.58	0.70	0.46
TERMINAL WEB	0.64	0.43	0.84	0.92
MICROVILLI	0.80	0.64	0.85	1.16
ROUGH AND SMOOTH ENDOPLASMIC RETICULUM	0.69	0.35	0.85	0.56
GOLGI APPARATUS	0.54	0.26	0.63	0.39

Regression lines are based on 27 - 31 points

*72 hours after 400 R. X-irradiation

The relative number of mitochondria remains constant during crypt cell differentiation and at the transition of the principal cells from crypt to villus (Fig. 7 and Table II). The number of mitochondria per cell increases twofold from the bottom to the tip of the crypt for controls and 1.6 fold for irradiated animals. At the transition from crypt to villus there is another 1.5 fold increase in controls and a twofold increase in irradiated animals.

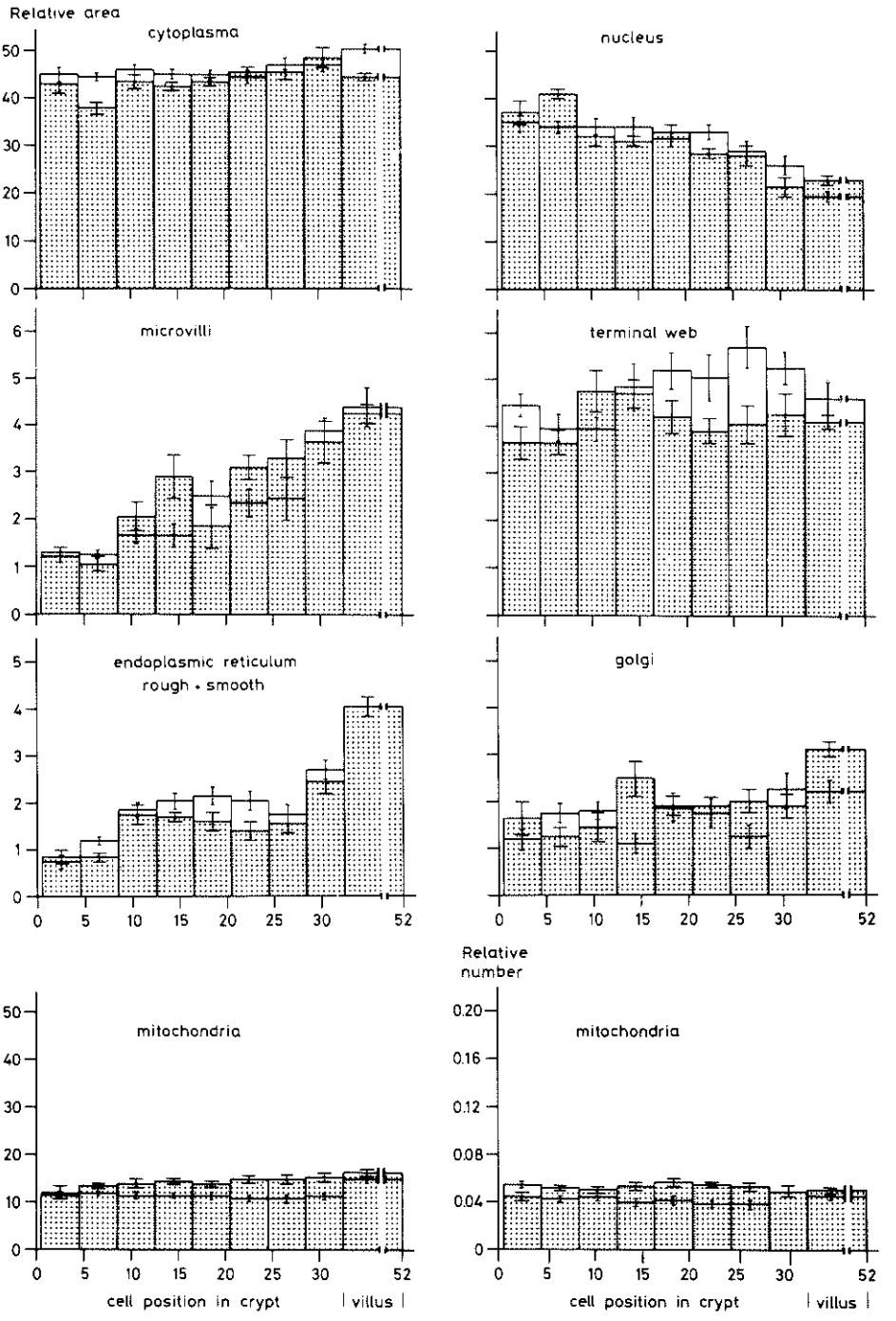


Fig. 7. The relative area, in percentages, of the cytoplasm, terminal web and the different cell organelles and the relative number of mitochondria as a function of the cell position for controls (dotted) and for irradiated animals (white). Standard errors of the mean are indicated.

Table II

RELATION BETWEEN ULTRASTRUCTURAL DEVELOPMENT AND CELL POSITION IN JEJUNAL CRYPT

	RELATIVE AREA			
	CONTROLS		IRRADIATED*	
CELL ORGANELLE	CORRELATION COEFFICIENT	REGRESSION COEFFICIENT	CORRELATION COEFFICIENT	REGRESSION COEFFICIENT
CYTOPLASM	0.60	0.23	0.28	0.06
NUCLEUS	-0.81	-0.53	-0.56	-0.26
MITOCHONDRIA	0.64	0.15	0.11	0.02
RELATIVE NUMBER OF MITOCHONDRIA	0.20	0.0001	-0.26	-0.0002
TERMINAL WEB	0.08	0.006	0.45	0.04
MICROVILLI	0.77	0.07	0.81	0.09
ROUGH AND SMOOTH ENDOPLASMIC RETICULUM	0.51	0.03	0.70	0.05
GOLGI APPARATUS	0.21	0.01	0.10	0.006

Regression lines are based on 29 - 31 points

* 72 hours after 400 R. X-irradiation

DISCUSSION

Following the initial studies of Zetterquist (1956) on the ultrastructural organization of the absorbing cells in the mouse jejunum, many electron microscopic investigations on the intestines of various animals have been carried out. Some of these studies were mainly concerned with the relation between ultrastructure and intestinal function (Palay and Karlin, 1959a and b; Trier and Rubin, 1965, Trier, 1967; Jasper and Bronk, 1968; Toner, 1968; Porter, 1969). Others have studied ultrastructural changes in intestinal villi under pathological conditions (Padykula, 1961, 1962; Rubin et al., 1966; Loehry and Creamer, 1969; Ginsel et al., 1973). Although several papers have dealt with the electron microscopy of the crypt cell (Padykula, 1961; Trier, 1963; Taylor and Adamstone, 1964; Toner, 1968), it is only recently that qualitative studies of the ultrastructure have been related to various crypt cell positions during differentiation (de Both et al., 1974a; Cheng and Leblond, 1974).

For an evaluation of ultrastructural changes during crypt cell differentiation, the position of the cells must be accurately determined. Many crypts are more or less convoluted and most of them are not located in the same longitudinal plane as the adjacent villus. This necessitates screening of many sections to obtain sufficient electron micrographs at each crypt cell position for the qualitative and morphometric analyses.

From electron micrographs of cells in various positions along the crypt of normal rat jejunum and from the quantitative data in Fig. 6 and Table I, it became clear that the ultrastructural changes during crypt cell migration are of a gradual nature. Yet, in normal crypts the proliferative cell compartment is confined to the lower 18 cell positions. In the so-called "critical decision zone" between cell position 18 and 22, cells cease DNA synthesis (Cairnie et al., 1965; Galjaard and Bootsma,

1969). During the 12 hours of subsequent migration from cell position 22 to 32 at the tip of the crypt, cell maturation occurs without cell proliferation. According to the present study, the transition of a proliferating cell into a non dividing, maturing cell halfway up the crypt is not accompanied by any sudden changes in ultrastructure.

The ultrastructural differentiation of principal cells from the bottom to the tip of the crypt in control animals is characterized by a gradual increase in the absolute areas of the total cell, the cytoplasm, the terminal web and nearly all cell organelles. This gradual increase of the cell components cannot merely be defined as growth, since there is also a relative increase of the cytoplasm, mitochondria, microvilli, endoplasmic reticulum and a relative decrease of the nucleus.

During migration from the tip of the crypt into the base of the villus, which probably occurs within a few hours, quite marked structural changes occur. The absolute area of the total cell increases considerably, especially that of the subnuclear region. Smooth endoplasmic reticulum also appears. Most organelles increase proportionally with the cell size, whereas the Golgi apparatus and the rough and smooth endoplasmic reticulum also show a clear relative increase as can be seen in Fig. 7.

Expansion of the proliferative pool of cells along the whole length of the crypt as a compensatory reaction for previous cell loss, e.g. shortly after X-irradiation, was found earlier by other investigators.

In these studies changes in the mitotic index and the labeling index per cell position were studied at different times after irradiation (Williams, et al., 1958; Lesher, 1967; Galjaard and Bootsma, 1969).

In the present study labeled cells were found at the highest positions of the crypt 72 hours after 400R X-irradiation (Fig. 1b). This is in accordance with the studies of Galjaard and Bootsma (1969) who found a maximum compensatory reaction of the proliferative pool in the crypts also 72 hours after 400 R.

This expansion of the proliferative cell compartment along the whole length of the crypt does not result in marked changes in the ultrastructural differentiation of the crypt and villous cells. This finding is in contrast to the behavior of certain biochemical features of the intestinal epithelium, indicating that increased proliferative activity results in a marked decrease in the activity of several enzymes involved in villous function (Galjaard et al., 1970, 1972; de Both et al., 1974a and b; Rijke et al., 1975).

During crypt cell differentiation a gradual increase in the area of microvilli is observed (Brown, 1962; Taylor and Adamstone, 1964), which was found here to be both an absolute and a relative increase (Fig. 6, 7; Table I, II). Biochemical analysis of isolated crypt and villous cells showed that activity of several brush border enzymes, such as alkaline phosphatase, leucine aminopeptidase, neutral α-glucosidase and guanylate cyclase, is present only in the epithelium of the villus and the tip of the crypt (Moog, 1967; Nordström et al., 1968; Galjaard et al., 1970, 1972; de Both et al., 1974a and b; de Jonge, 1975; Rijke, 1975).

Hence, the structural development of the microvilli and the appearance of brush border enzyme activity do not occur simultaneously. It is possible, however, that during crypt cell differentiation a gradual synthesis of pro-enzymes occurs, whereas nzyme activity appears at the crypt-villous transition zone i.e. when membrane nsport function is required (Wachsmuth and Torhorst, 1974). Indirect support for s supposition is the fact that increased crypt cell proliferation results in a reased activity of several brush border enzymes in villous cells (Galjaard et al., 72; de Both et al., 1974a; Rijke et al., 1975). This could be explained by a

reduced synthesis of the enzyme proteins in the crypt cells as a result of increased proliferation.

The Golgi apparatus is poorly developed in crypt cells and becomes prominent during migration along the upper part of the crypt and onto the villus. Towards the tip of the villus it decreases again (Josephson and Altmann, 1972). In view of the work of Bennett (1970) this would indicate that synthesis and transport of glycoproteins for the cell coat mainly occur in the upper part of the crypt and in the basal and middle part of the villus. Changes in crypt cell proliferative activity are not likely to affect this process in view of the fact that the ultrastructure of the Golgi apparatus is not affected by irradiation. Studies on enzymes involved in the function of the Golgi apparatus should, however, be carried out to substantiate this hypothesis.

A gradual increase in the absolute and relative area of the rough endoplasmic reticulum from the bottom to the tip of the crypt occurs. Smooth endoplasmic reticulum becomes visible only at the crypt-villous junction (for review, see Cheng and Leblond, 1974). Using histochemical staining procedures and quantitative biochemical analyses of isolated cell compartments, a gradual increase of non-specific esterase activity has been observed from the bottom of the crypt towards the middle part of the villus (Padykula et al., 1961; Eder, 1964; Galjaard et al., 1969, 1970; de Both et al., 1974a). At the transition from crypt to villus esterase activity increases 2 - 3 fold (Galjaard et al., 1972). In this case there seems to be a simultaneous development of the ultrastructure and enzymic activity during differentiation from crypt to villous cell. As in the case of microvilli, increased proliferative activity does not affect the ultrastructure of the endoplasmic reticulum but it does decrease the activity of the non-specific esterases, one of the groups of enzymes which are localized in this structure (Galjaard et al., 1972). Using microelectrophoresis de Both et al. (1974a) could demonstrate that this decrease in esterase activity is not caused by a loss of specific iso-enzymes.

During normal crypt cell differentiation there is an increase in the absolute and relative area occupied by the mitochondria. The number of mitochondria per cell increases about two-fold from the bottom to the tip of the crypt and another 1.5 fold increase at the transition onto the villus. A remarkable constancy in the number of mitochondria per unit cellular area has been observed (Fig. 7 and Table II). The increase in cell size at the crypt-villous junction is also accompanied by a proportional increase in the number of mitochondria, which, however, does not occur at the expense of the size of each individual mitochondrium. Also, the activity of several mitochondrial enzymes was found to be constant in different isolated crypt and villous cell compartments (de Both et al., 1974a; de Jonge, 1975). A good correlation between ultrastructural and biochemical data exists for this organelle.

Increased proliferative activity in the crypt during recovery after X-irradiation has no marked effect on the ultrastructure of the mitochondria nor on the activity of several mitochondrial enzymes (de Both, 1974a; de Jonge, 1975). Again, the number of mitochondria per unit cellular area remains constant during cell differentiation, which is in agreement with the finding of Jeynes and Altmann (1975) that no direct relationship exists between crypt cell division and division of mitochondria. The change in the subcellular localization of mitochondria at the crypt-villous junction might be related to the fact that villous cells become involved in the absorption process (Gonzalez-Licea, 1970). During recovery after irradiation number of mitochondria in villous cells was found to be higher than in control

animals. This is probably related to the larger size of the epithelial cells, as the number of mitochondria per unit cellular area was shown to be constant under various conditions. The increased villous cell size during recovery after irradiation might be the result of the previous block in cell proliferation in the crypt at the time of irradiation (Williams et al., 1958).

In conclusion, the present electron microscopic studies reveal that the gradual ultrastructural development during crypt cell differentiation not merely consists of growth but also of a specific increase of several cell components. These processes are not markedly affected by experimentally increased cell proliferation. In controls there appears to be a simultaneous development of ultrastructure and enzyme activity in certain organelles such as the endoplasmic reticulum and mitochondria. The ultrastructure of microvilli is, however, apparent at an earlier developmental stage than the activity of some brush border enzymes. The decreased activity of several enzymes which occurs after experimentally increased proliferation remains as yet unexplained. The present studies indicate, however, that these effects are not due to changes in the ultrastructure of the organelles concerned.

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Histochemical Journal PUBLICATION IV

THE EFFECT OF INCREASED CRYPT CELL PROLIFERATION ON THE ACTIVITY AND SUBCELLULAR LOCALIZATION OF ESTERASES AND ALKALINE PHOSPHATASE IN RAT SMALL INTESTINE

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SYNOPSIS

The activity and ultrastructural localization of alkaline phosphatase and of esterase has been studied in normal rat intestine and after the increased crypt cell proliferation which occurs during recovery after 400R X-irradiation.

Alkaline phosphatase activity is not present in crypt cells of normal intestine, but becomes apparent after the cell has migrated on to the villus. The enzyme is localized in the microvilli, along the lateral cell membranes and in dense bodies. Its activity increases 10 - 15 fold from the base to the tip of the villus. Morphometric analysis of the cell structures where this enzyme is localized reveals no marked changes in their relative proportions during crypt cell development.

The expansion of the proliferative cell compartment along the whole length of the crypt which occurs during recovery after irradiation (72 hours after 400R X-irradiation) results in a marked reduction of alkaline phosphatase activity in the lower 10-15 cell positions at the base of the villus. During subsequent migration of these cells the activity increases with cell age but normal values are not attained. Morphometric analysis shows that the ultrastructural development is similar to that in controls. These results suggest that during cell maturation normal values for alkaline phosphatase activity are only attained after a 10 - 12 hour period of maturation in a non proliferative state and only after the cell has migrated on to the functional villus compartment.

In normal intestine, esterase activity shows a 3 fold increase from the bottom to the tip of the crypt and a 3 - 4 fold increase during migration up to the middle of the villus. Enzyme activity is localized in the endoplasmic reticulum, the dense bodies and the perinuclear space. Morphometric analyses show a 2 - 3 fold increase in the absolute size of these subcellular compartments during crypt cell differentiation and a 2-fold increase at the crypt-villus junction. The relative sizes increase 1.5 fold during crypt cell differentiation and at the time of transition of the cells

on to the villus.

Increased crypt cell proliferation after irradiation leads to a marked decrease in esterase activity both in crypts and villi. Morphometric analyses of electron micrographs indicate that these changes in activity are not related to any changes in the subcellular structures in which the enzyme is localized. It appears that the normal development of esterase activity depends both on the functional state of the cell and its localization in the crypt or villus.

INTRODUCTION

Small intestinal epithelium is an attractive model for studies of cell proliferation and cell differentiation because of the sequential distribution of the various developmental stages of the cell along the crypts and villi. Autoradiography and scintillation counting after ^{3}H-thymidine incorporation has provided much information about the cellular kinetics of this cell renewal system (Cairnie et al., 1965a, b; Hagemann et al., 1970; Cheng and Leblond, 1974). So have studies of the effect of low doses of X-radiation, which result in a block of crypt cell proliferation, followed by a recovery and even a temporary expansion of the proliferative cell compartment along the whole length of the crypt (Williams et al., 1958; Lesher, 1967; Galjaard and Bootsma, 1969).

Qualitative information about the presence of specific proteins and enzyme activities during cell differentiation has resulted from the use of cytochemical staining methods (Padykula, 1962; Jervis, 1963; Galjaard et al., 1970; Pearse, 1974). Biochemical analyses on homogenates of isolated cell populations (Webster and Harrison, 1969; de Jonge, 1975) and microchemical assays on various cell groups dissected from frozen sections have provided data on changes in enzyme activity during differentiation (Moog and Grey, 1968; Nordström et al., 1968; Galjaard et al., 1970; de Both et al., 1974). In addition, quantitative analysis during recovery after low radiation doses has shown that increased crypt cell proliferation is accompanied by decreased activity of several enzymes involved in intestinal function (Galjaard and Bootsma, 1969; Galjaard et al., 1970; de Both et al., 1974; Rijke et al., 1975).

The purpose of the present study was to investigate the activity and subcellular localization of alkaline phosphatase and esterases during the normal development of the columnar cells using quantitative microchemical assays and ultrastructural cytochemistry. Morphometric analyses of electron micrographs of these cells at different stages of development were also carried out to investigate the development of the subcellular structures in which these enzymes are localized. Finally, the effect of increased cell proliferation on these parameters was studied by microchemical analysis and electron microscopy 72 hours after 400R X-irradiation.

EXPERIMENTAL

Male Glaxo Wistar rats about 8 weeks old and 250 g in weight were used for these experiments. Some of the animals were exposed to 400R whole body radiation from a Philips X-ray machine (200 kV, 12 mA, added filtration 1.6 mm Cu filter, HVL

1.9 mm Cu, dose rate 20R/min). Intestinal tissue was taken from the animals 72 hours after irradiation, and also from control animals, both groups having been starved for 24 hours beforehand.

QUANTITATIVE MICROCHEMISTRY

Segments (2 cm long) of intestine 5 cm distal to the pylorus of control and irradiated rats were removed under ether anesthesia, cut into smaller pieces and then immediately frozen in isopentane cooled in liquid nitrogen. Cryostat sections (7 μm) were cut longitudinally at -20°C and these were freeze dried in vacuo (15 hr at -45°C and 10^{-4} mm Hg pressure). By microdissection of the freeze-dried sections crypts were subdivided into two equal parts and the villi into three parts. After weighing on a quartz fibre balance (Lowry, 1953), the fragments of crypts and villi were separately collected and microchemical analyses for alkaline phosphatase and esterases were carried out according to procedures described earlier (Galjaard et al., 1970; de Both et al., 1974). Eight to twelve assays per animal and 3 - 10 animals were used for each experimental group.

ELECTRON MICROSCOPIC CYTOCHEMISTRY

Small pieces of intestine from both normal and irradiated rats isolated as described above were fixed in 2 - 4% glutaraldehyde in 0.067 M cacodylate buffer, pH 7.3, at 0° C for 2 hr. After rinsing in 0.25 M sucrose in 0.067 M cacodylate buffer, pH 7.3, at 0° C for 3 hr, frozen sections (50 μm) were cut from the fixed tissue at -25° C and collected in the rinsing fluid. For the localization of alkaline phosphatase the sections were incubated in a medium consisting of 3 ml 0.2 M Tris pH 9.2; 0.2 ml 0.03 M β-glycerophosphate; 0.3 ml 0.1 M tri-sodium citrate and 0.3 ml 0.09 M lead nitrate (Millonig, 1973a). The pH was adjusted with acetic acid to pH 9.2 if necessary. Incubation was carried out for 10 min. at 4° C.

For the localization of the esterases the 50 μm sections were incubated for 2 hr at 4°C in the substrate described by Holt and Hicks (1966), consisting of 0.03 M citrate buffer, 1 mM indoxylacetate, 1 mM hexazotised pararosaniline at pH 6.0, with the addition of formaldehyde to a concentration of 0.3M (Holt; unpublished).

After both incubations the sections were washed in the rinsing fluid for 2 hr at room temperature and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate at pH7 for alkaline phosphatase and in 0.1 M phosphate (pH7) for esterases.

After dehydration in graded strengths of ethanol, sections were embedded in Epon. Ultrathin sections were cut from these larger sections with a Reichert microtome and studied without further staining in a Philips EM 300 electron microscope. As controls for both enzymes 50 μm thick sections were heated for 5 min. at 90°C before incubation; another control consisted of incubation in media without the appropriate substrates.

LIGHT MICROSCOPIC HISTOCHEMISTRY

For the light microscopic localization of alkaline phosphatase 1 μm thick sections were cut from the 50 μm sections embedded in Epon. These sections were treated with a 2% ammonium sulfide solution and examined by phase-contrast microscopy.

MORPHOMETRY OF ELECTRON MICROGRAPHS

To study the development of the subcellular structures in which the two groups of enzymes are localized in relation to the cell position, a morphometric study was carried out after isolation of rat intestine and processing for electron microscopy as described earlier (de Both et al., 1974; van Dongen et al., 1976).

Ultrathin sections were cut longitudinally through crypt and villus to enable exact localization of the columnar cells. All cell positions in the crypts (counted from the bottom of the crypt), were studied; on the villus only cells at the lowest twenty positions were investigated.

For subcellular structures where alkaline phosphatase is localized ("alkaline phosphatase compartment"), the total length of the membranes (expressed in dm) of the microvilli and of the lateral cell membranes was determined in large electron micrographs (50 x 60 cm), using an electronic tracing device (Computec).

Point-counting planimetry by means of a lattice test system (Weibel, 1969) superimposed on the electron micrographs of cells at every cell position was used to count the number of points scored by all those structures in which esterases are localized ("esterase compartment").

All the above measurements were corrected for differences in magnification. These measurements yielded the absolute sizes of both compartments. In addition, their relative size was calculated for every cell, by dividing the absolute values by the number of points of the lattice test system scored for the whole cell.

Histograms of the means of the absolute and relative values for the two compartments were made for successive groups of four cells.

RESULTS

1. QUANTITATIVE MICROCHEMICAL ANALYSIS

Table I presents the results of the microchemical analysis of the two groups of enzymes in the various crypt and villus cell compartments, both for control tissue and that taken 72 hours after 400R irradiation.

For alkaline phosphatase, almost no activity is found in the lower half of the crypts of control intestine. However, a slight increase is observed during cellular migration along the upper part of the crypt. Once the cell enters the villus the activity suddenly increases to high levels and this increase is continued until the cell reaches the tip of the villus.

During recovery after irradiation, again, very little alkaline phosphatase activity can be demonstrated in the crypts. In the lowest one third of the villi, the activity reaches a value comparable to that in the upper half of the crypt of control animals. In the irradiated animal the alkaline phosphatase activity increases 4-fold during cell migration from the base of the villus to its middle. This increase is comparable to that found from the upper part of the crypt to the basal part of the villus in control animals.

In control animals a very low esterase activity is found in cells in the lower half of the crypt, but this activity increases 2 - 3 fold during their passage through the upper half of the crypt. At the crypt to villus transition another 2-fold increase is observed. During subsequent migration along the villus a further

increase in activity occurs up to the middle part of the villus. At 72 hr after X-irradiation, when the proliferative compartment extends over the whole length of the crypt, the esterase activity in crypt and villus is markedly reduced. However, these changes in esterase activity from the base of the crypt to the tip of the villus follow the same pattern as in controls.

Table 1

QUANTITATIVE MICROCHEMICAL ENZYME ANALYSIS IN ISOLATED CRYPT AND VILLUS CELL COMPARTMENTS IN CONTROL INTESTINE AND AFTER INCREASED CRYPT CELL PROLIFERATION

control	carboxyl-esterase	alkaline phosphatase	irradiated	carboxyl-esterase	alkaline phosphatase
	91±7	145±15		58±6	70±18
	92±9	84±12		58±7	31±8
	66±9	28±8		40±5	8±3
	28±4	7±1		16±4	1.5±0.6
	9±1	1±0.6		6±1	1±0.1

Enzyme activities are expressed in 10^{-6} moles substrate hydrolyzed / hour / mg dry weight; mean values and standard deviations are derived from 8-12 assays per animal and 3-10 animals. Increased proliferation occurs during recovery after X-irradiation (localization of proliferating crypt cells is indicated by ▨)

2. LIGHT AND ELECTRON MICROSCOPIC CYTOCHEMISTRY

Alkaline Phosphatase

With the light microscope no alkaline phosphatase activity was found in the intestinal crypts of either experimental group (Fig. 1). In control animals a positive reaction was observed in the brush border of all absorptive cells. In irradiated animals hardly any alkaline phosphatase activity could be observed in cells at lower positions on the villus.

Using the electron microscope, again no activity was detected in the crypts of either group of animals (Fig. 2a), whereas in control animals alkaline phosphatase was present in all absorptive villus cells, the activity being localized on the surface of the microvilli and of the lateral cell membranes and also in dense bodies (Fig. 2b). No activity was found in the Golgi complex.

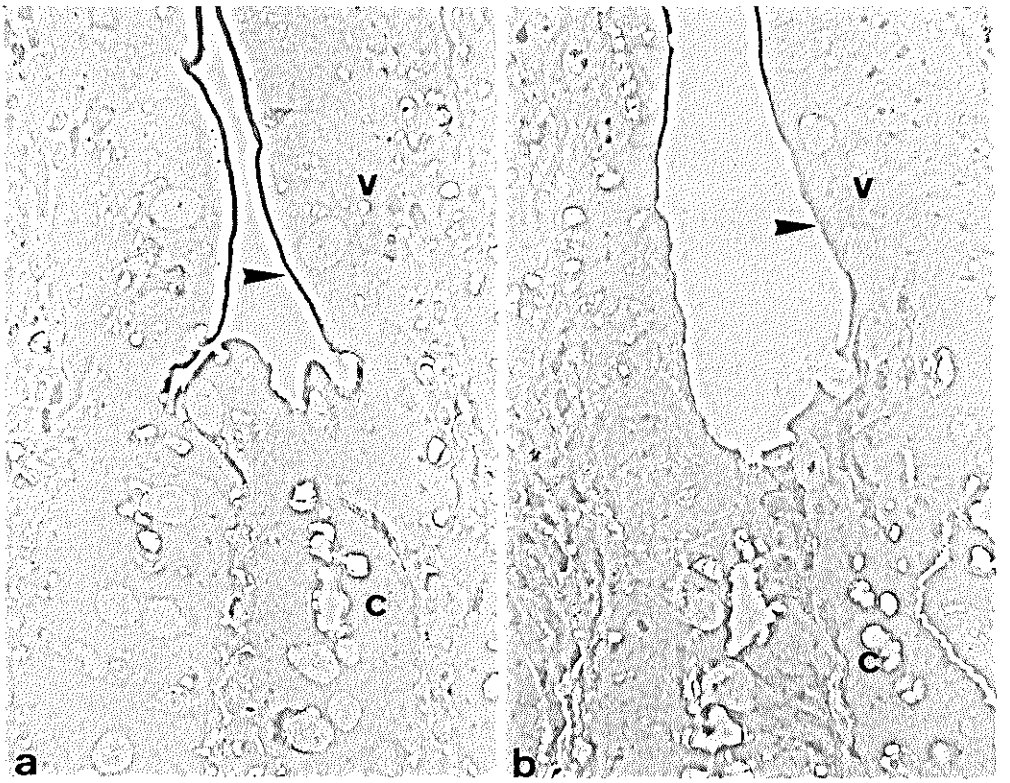

Fig. 1. Localization of alkaline phosphatase activity in small intestine of controls (a) and of animals 72 hr after 400R X-irradiation (b). No activity is present in the crypt cells (c). A positive reaction is found in the brush border of absorptive cells at the lower positions of the villi (v) of the controls, whereas nearly any activity is localized in the same region of the irradiated animals (see arrows). X 152.

In irradiated animals the lower absorptive cells of the villi showed hardly any alkaline phosphatase activity (Fig. 3a). When the cells migrate to the middle of the villus a gradually stronger reaction is found in the microvilli and the lateral cell membranes (Fig. 3b). The cells at the upper half of the villus show even stronger reactions (Fig. 3c).

Esterases

The esterases of the absorptive villus cells of control animals are localized in the rough and smooth endoplasmic reticulum, in the perinuclear space and in dense bodies (Fig. 4a and b). Esterase activity is also present in the principal cell of the crypts, even in cells at lower positions. This activity is localized in the same organelles as in the villus cells, except for the smooth endoplasmic reticulum which is not present in crypt cells. Figs. 4c - d show esterase activity at various stages of crypt cell differentiation. In irradiated animals the localization of esterases was found to be similar to that in controls (Fig. 5a and b). In control tissues, which were incubated in media without substrate, or in which the enzyme was inactivated, no staining was observed for either group of enzymes.

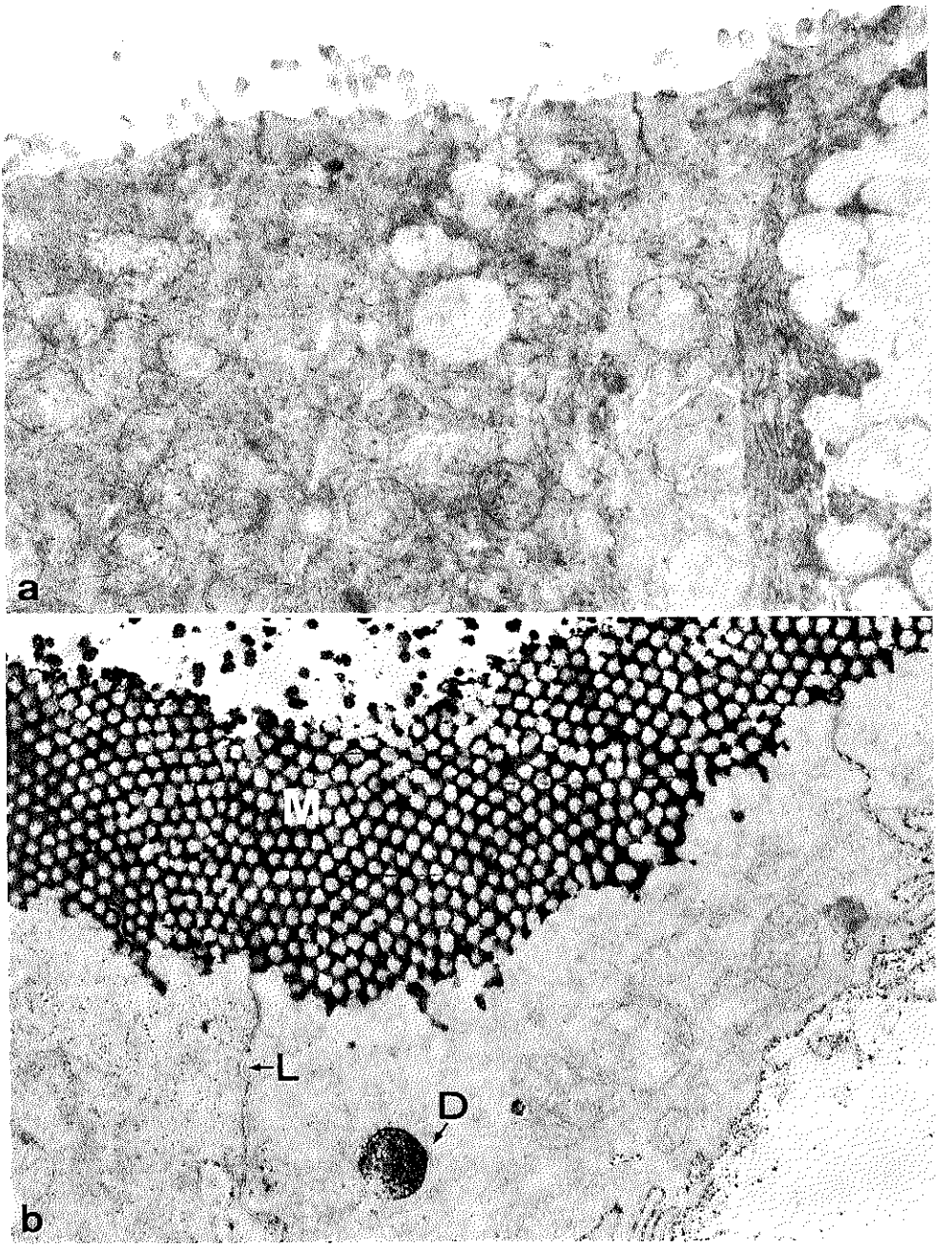

Fig. 2 . Ultrastructural localization of alkaline phosphatase activity in the columnar cells of small intestine of control animals. In crypt cells no reaction product can be seen (a). Alkaline phosphatase activity is shown to be present on the surface of the microvilli (M) and the lateral membranes (L) and in dense bodies (D) of the absorptive (columnar) cells in the lower half of the villus (b). X 16,500 .

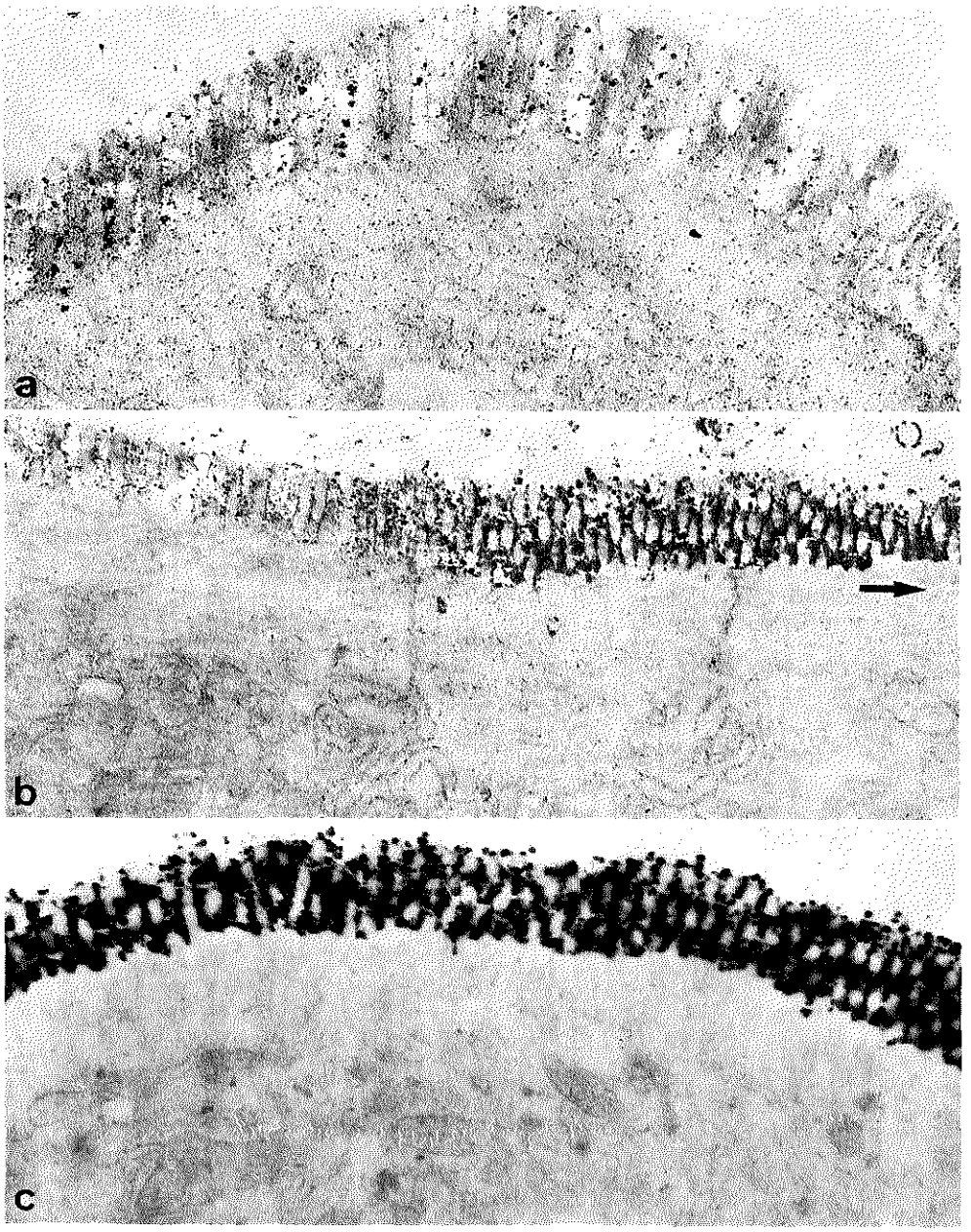

Fig. 3 . Ultrastructural localization of alkaline phosphatase activity in the columnar villus cells of rat small intestine at 72 hr after 400R X-irradiation. Hardly any reaction can be seen in the absorptive (columnar) cells at the lowest positions of the villus (a). However, an ever increasing activity of alkaline phosphatase is found, when the absorptive cells migrate to the higher cell positions (b) until the upper half of the villus is reached (c). The enzyme is localized in the same organelles as in control animals. Arrow indicates position of villus tip. X 16,500 .

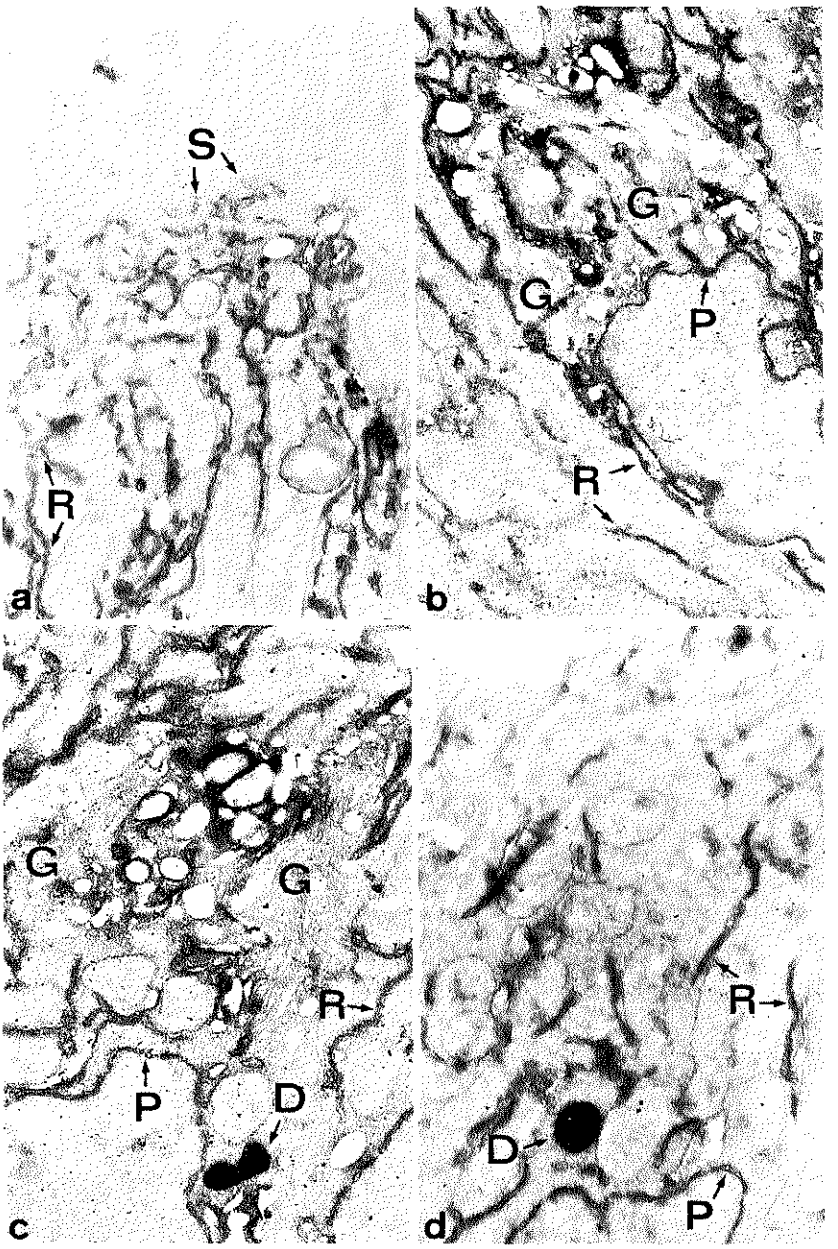

Fig. 4. Ultrastructural localization of esterase activity in the columnar cells of the small intestine of control animals. The esterase activity is present in the smooth (S) and rough (R) endoplasmic reticulum and in the perinuclear space (P) of absorptive (columnar) cells in the lower half of the villus (Fig. 4a, X 12,000; Fig. 4b, X 5,000). No activity is present in the Golgi apparatus (G). Also, esterase activity is localized in columnar cells in the upper (Fig. 4c, X 14,000) and lower (Fig. 4d, X 12,000) half of the crypts, in the same organelles as in the absorptive villus cells. Dense bodies (D) in crypt and villus columnar cells show a positive reaction.

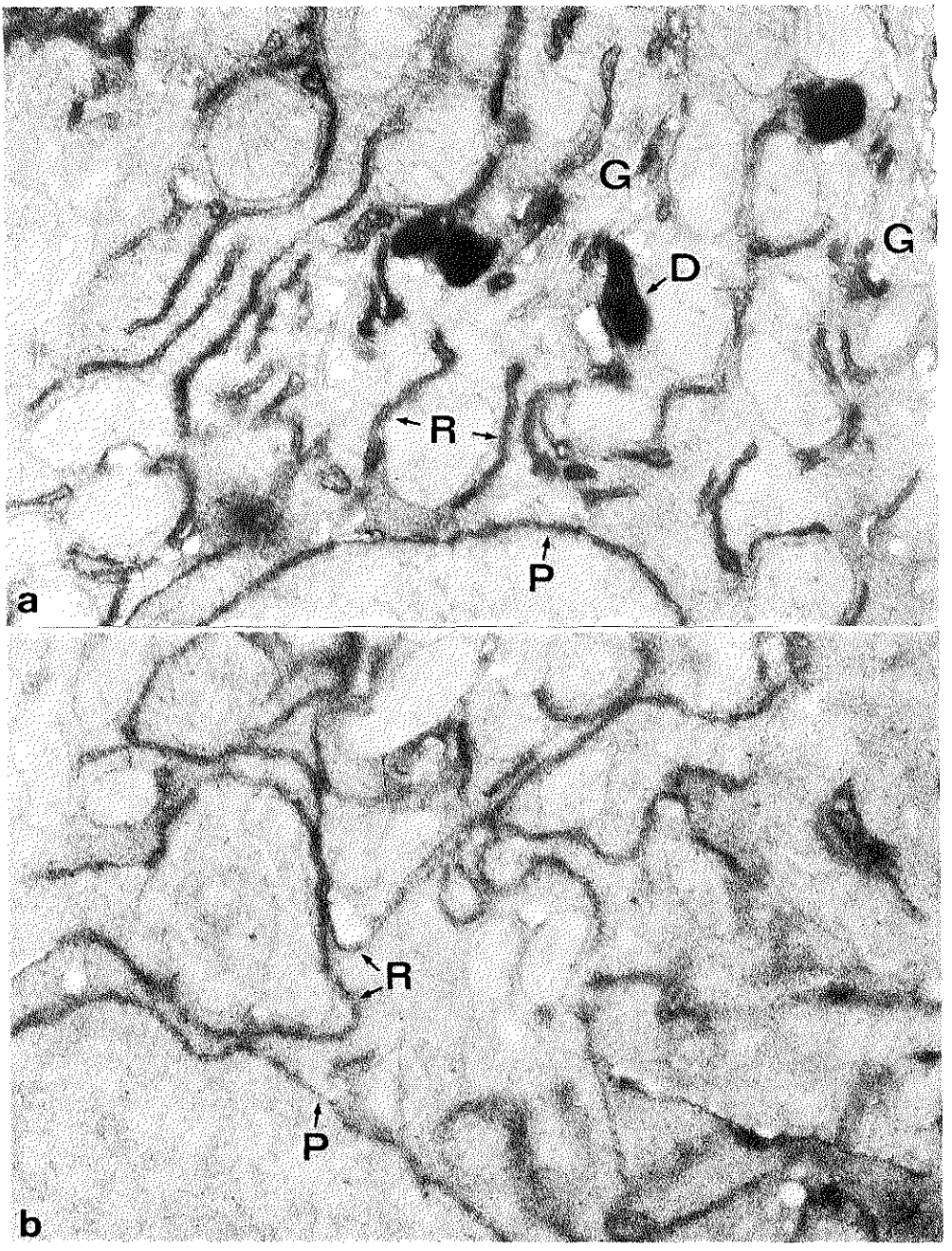

Fig. 5. Ultrastructural localization of esterase activity in columnar cells in the lower half of a villus (Fig. 5a) and crypt (Fig. 5b) in rat small intestine at 72 hr after 400R X-irradiation. The esterase activity is present in the rough endoplasmic reticulum (R), the perinuclear space (P) and in dense bodies (D). No activity is found in the Golgi complex (G). X 23,500.

3. MORPHOMETRY

Alkaline Phosphatase

The length of the membranes of the microvilli, plus that of the lateral cell membranes, was measured as a parameter for the "alkaline phosphatase compartment". This compartment gradually increases from the bottom to the tip of the crypt, both in controls and in irradiated animals (Fig. 6). A sudden increase at the transition from crypt to villus is found in both experimental groups.

The relative size of the "alkaline phosphatase compartment" in the crypts remains about the same during crypt cell differentiation and during transition from crypt to villus. This is true for both experimental groups (Fig. 6).

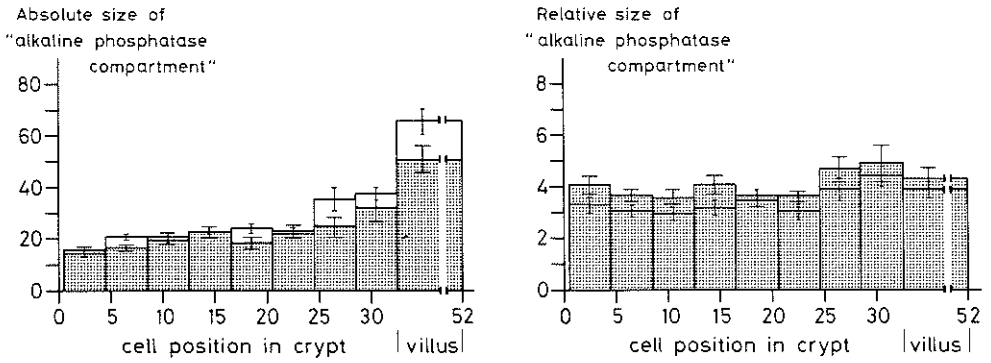

Fig. 6. This figure gives the absolute size, expressed in dm, and the relative size, expressed as the absolute size divided by the number of points of the test system scored by the whole cell, of the "phosphatase compartment" in columnar cells of crypts and villi in rat small intestine. The values for control animals (dotted) and for animals 72 hr after 400R X-irradiation (white) are represented as a function of the position in crypt and villus of the columnar cells. Standard errors of the mean are indicated.

Esterases

For both experimental groups, the absolute size of all organelles in which esterases are localized is illustrated in Fig. 7 for the different cell positions in the crypt and for the lowest 20 cell positions on the villus. In control animals the "esterase compartment" gradually increases in size as the columnar cells migrate from the bottom to the tip of the crypts. At the transition from crypt to villus a 2 - 3 fold increase is observed. In the irradiated animals a similar pattern is found (Fig. 7). The relative size of the "esterase compartment" increases about 1.5 fold from bottom to tip of the crypt; a similar increase occurs at the crypt-villus transition.

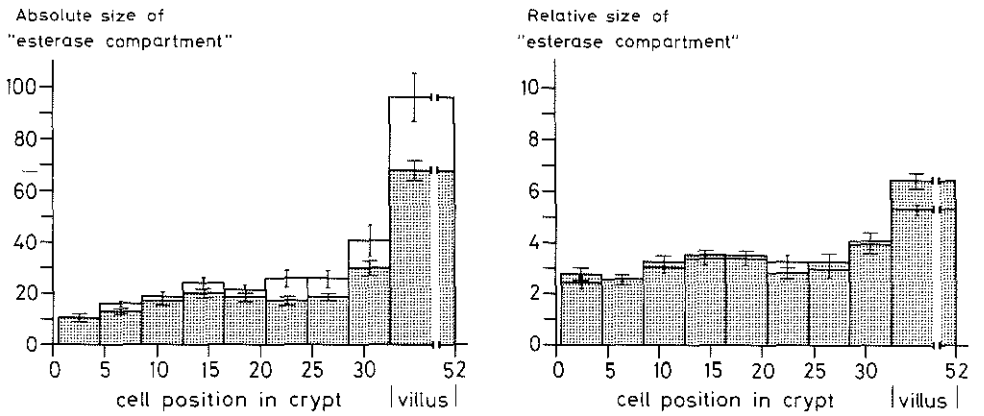

Fig. 7. This figure gives the absolute size, expressed as the number of points of the test system scored, and the relative size, expressed as percentage, of the "esterase compartment" in columnar cells of crypts and villi in rat small intestine. The values for control animals (dotted) and for animals 72 hr after 400R X-irradiation (white) are represented as a function of the position in crypt and villus of the columnar cells. Standard errors of the mean are indicated.

DISCUSSION

This study shows that alkaline phosphatase activity is localized along the membranes of the microvilli and the lateral cell membranes and in dense bodies. The latter were not included in the morphometric analysis because of their minor contribution to the overall membrane activity of the cells. This subcellular localization is in agreement with studies by other investigators (Hugon and Borgers, 1967; Mayahara et al., 1967). The presence or absence of alkaline phosphatase activity in the Golgi apparatus seems to be mainly dependent on the method used (Millonig, 1973b).

Several electron microscopic studies (Padykula, 1961; Brown, 1962; Cheng and Leblond, 1974; van Dongen et al., 1976) have shown that there is a gradual development of microvilli from the bottom of the crypt to the tip of the villus. Undifferentiated cells at the bottom of the crypt show relatively few microvilli which are short and broad. During migration along the crypt, and subsequently from the base to the tip of the villus, the microvilli become longer and thinner and their number per cell increases 6 - 8 fold (Brown, 1962). Morphometric analysis (van Dongen et al., 1976) shows that the total size of the microvillus compartment of the cell increases 5-fold from bottom to tip of the crypt and another 2-fold increase occurs at the crypt-villus junction. However, if the values are expressed as a percentage of the total cell size there is only a small increase in the relative size during differentiation.

The total length of the microvillus membrane, and of the lateral cell membranes where alkaline phosphatase activity is localized, has now been found to increase about 2-fold from the bottom to the tip of the crypt and another 1.6 fold increase

occurs when the cell moves to the base of the villus. The relative length, however, does not change during crypt cell differentiation, nor during migration from the crypt to the villus.

In normal intestine alkaline phosphatase activity in the crypts is absent, in spite of the presence of the appropriate subcellular structures. Also, the sudden increase in enzyme activity at the crypt-villus junction cannot be correlated with the abrupt changes which occur in the ultrastructure of the microvilli and lateral cell membranes, since the relative compartment does not increase in size.

During recovery after low radiation doses the whole crypt is temporarily involved in cell proliferation (Williams et al., 1958; Galjaard and Bootsma, 1969). It is now shown that this expansion of the proliferative cell compartment does not affect the normal ultrastructural development of the microvilli and lateral cell membranes. However, there is a marked decrease of the alkaline phosphatase activity in the basal part of the villus which is not restored to normal values during subsequent migration towards the tip of the villus (see Table I). The cytochemical studies at 72 hours after irradiation show that hardly any alkaline phosphatase activity is present on the microvilli and lateral cell membranes of the lower cell positions of the villus (Fig. 1). This indicates that the changing environment during migration of the cell from crypt to villus is not alone sufficient to achieve normal enzyme activity. These findings also support the idea that the alkaline phosphatase activity is not merely dependent on the presence of the subcellular structures in which this enzyme is normally localized. In fact, the appearance of alkaline phosphatase activity in normal villus cells seems to be related to the age of the cell in the non-proliferating state and in some way to the cellular changes which occur during transition from crypt to villus.

The results of our cytochemical studies and those of the quantitative assays indicate that after irradiation alkaline phosphatase activity reaches control values after migration along the lower 10 - 20 cell positions of the villus, corresponding to a migration period of about 10 hours (Meer-Fieggen, 1973; Rijke et al., 1974). This suggests that a columnar cell in the small intestine requires a period of 10 - 12 hours in a non-dividing state before normal alkaline phosphatase activities are found. The immuno fluorescence studies by Wachsmuth et al., (1974a, b) suggest that this period may be involved in the synthesis of a precursor protein, which, in normal intestine is being synthesized in the crypt, and which becomes hydrolytically active once the cell migrates on to the villus.

During recovery after irradiation it may well be that the expansion of the proliferative crypt cell compartment in some way interferes with the synthesis of this precursor enzyme in the crypt. Instead, the precursor appears to be synthesized predominantly during migration of the cells along the lower part of the villus.

In the present work, esterase activities were measured using α-naphthyl acetate as substrate instead of the method of Barrow & Holt (1971) which uses the histochemical substrate, indoxyl acetate, used here for the ultrastructural localization of esterases. However, it has already been shown (Hobbiger, 1957; Underhay, 1957) that esterase/substrate specificity is almost identical when using either indoxyl acetate or α-naphthyl acetate.

Unlike alkaline phosphatase, esterase activity can readily be demonstrated in crypt cells. As was shown by light microscopic staining methods and by quantitative assays in normal intestine, the esterase activity increases during crypt cell migration and at the crypt-villus transition (Galjaard and Bootsma, 1969; de Both et al.,

1974). During subsequent migration from the base to the tip of the villus a further increase in activity is found (Table 1).

In several tissues, this group of enzymes appears to be localized in the endoplasmic reticulum, in dense bodies (lysosomes) and in the perinuclear space (Holt and Hicks, 1966; Bernsohn et al., 1966; Deimling et al., 1972; Barrow and Holt, 1971). The same structures are, of course, present in the proliferating cells of the intestine and in the non-dividing maturing cells in the crypt, as well as in the absorptive villus cells. The relative size of the "esterase compartment" (endoplasmic reticulum, the dense bodies and the perinuclear space) shows a 1.4 fold increase from the bottom to the tip of the crypt and another 1.6 fold increase at the crypt-villus junction. The quantitative results given in Table 1 show that the concomitant activity of the esterases increases by a factor of 10 from the bottom of the crypt to the middle of the villus. This increase in activity is not associated with a proportional development of the subcellular structures in which the enzymes are localized.

The present studies show that the relative size of the "esterase compartment" is not influenced by irradiation. Also that the ultrastructural localization of the esterases is the same as in controls. The decrease in the overall esterase activity, as observed during recovery after irradiation, can thus not be explained by changes in the subcellular structure of the cells, nor by differences in the ultrastructural localization of the enzyme. For this group of enzymes, increased proliferative activity in the crypt following irradiation is associated with an overall decrease of the normal esterase activity. Although there is an increase of esterase activity during subsequent migration of the cell on to the villus, there is no complete restoration to normal values. The development of the esterase activities thus seems to depend both on the developmental state of the cell and on its position along crypt and villus.

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Cell Tissue Kinet. (1976) **9**, 65–75. PUBLICATION V

THE INFLUENCE OF 400 R X-IRRADIATION ON THE NUMBER AND THE LOCALIZATION OF MATURE AND IMMATURE GOBLET CELLS AND PANETH CELLS IN INTESTINAL CRYPT AND VILLUS

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ABSTRACT

The influence of 400 R X-irradiation on the localization and the number of mature and immature goblet cells and Paneth cells in rat duodenal epithelium has been studied. At short times after irradiation, when the total proliferative activity in the crypts of Lieberkühn is reduced, the proportion of mature and immature goblet cells of the total number of crypt cells was increased; also an absolute increase in the number of goblet cells in the crypts was found. The immature goblet cells were localized in the lower half of the crypt as in control animals, whereas the number of the mature cells increased over the whole crypt length. When the proliferative activity of the crypt cells increases again from 12 to 48 hr after irradiation the number of both types of goblet cells decreases. Between 48 and 72 hr, when the whole crypt is involved in proliferation, a second increase of both types of goblet cells was found. However, the localization of the immature goblet cells is no longer restricted to the lower half of the crypt but they also appear at the higher cell positions.

On the villus no immature goblet cells were found and the changes in the numbers of mature goblet cells do reflect the changes induced by irradiation in the goblet cell population in the crypt. The absolute number and localization of Paneth cells did not change under the experimental conditions. The findings are discussed in relation to cell proliferation and differentiation processes in intestinal crypts.

INTRODUCTION

The intestinal villus cell is characterized by a number of specific biochemical and ultrastructural features, which in part are developed during maturation in the crypt. It has been shown that relatively low doses of X-irradiation result in a temporary decrease in crypt cell proliferation, which is followed by an overshoot in proliferative activity, during which

all crypt cells are involved in proliferation. Subsequently, the proliferative cell compartment is reduced to its normal localization in the lower half of the crypt (Williams *et al.*, 1958; Galjaard & Bootsma, 1969).

During the period of expansion of the proliferative crypt cell compartment the normal development of certain biochemical features is inhibited. For instance, the activity of certain enzymes involved in intestinal function is decreased in crypt and/or villus during recovery after low radiation doses (Galjaard & Bootsma, 1969; Galjaard *et al.*, 1970; de Both *et al.*, 1974). On the other hand, the gradual development of the ultrastructure of the principal cells is not affected by changes in the proliferative activity in the crypt (van Dongen & Visser, 1974; de Both *et al.*, 1974).

The purpose of the present investigation is to study whether changes in the proliferative pattern of the principal cells in the intestinal crypt do affect the processes of proliferation and differentiation of other cell types in the crypt.

Several studies indicate that the goblet cells probably develop from the same proliferating cells as the 'absorbing' principal cells. Throughton & Trier (1969) found transition forms between goblet cells and proliferating principal cells. Based on autoradiographic studies Cairnie (1970) postulated precursor cells for the goblet cells, that would develop from the proliferating principal cells. These precursor cells are at least in part the same as the proliferating oligomucous cells described by Merzel & Leblond (1969) and Cheng (1974a), the proliferating goblet cells described by Thrasher & Greulich (1966), the goblet cells with small thecae mentioned by Moe (1968) and the transition forms from the study by Throughton & Trier (1969). These so-called immature goblet cells are localized in the lower half of the crypt, where they differentiate into mature goblet cells (Moe, 1968; Throughton & Trier, 1969; Merzel & Leblond, 1969; Cairnie, 1970; Cheng, 1974a). While the goblet cells migrate at about the same rate as the maturing principal cells (Merzel & Leblond, 1969; Cairnie, 1970) the Paneth cells form a very stable cell population, which remains localized at the bottom of the crypt. Their turnover time is very long and their origin is probably the same as that for goblet cells and absorbing principal cells (Throughton & Trier, 1969; Devik & Iversen, 1970; Cairnie, 1970; Cheng, 1974b).

In the present study the number and the localization of immature and mature goblet cells and of Paneth cells in small intestinal epithelium was investigated at various time intervals after a relatively low dose of X-irradiation.

MATERIALS AND METHODS

The experiments were carried out with Glaxo Wistar rats (about 8 weeks old and 200 g in weight). All animals were starved 24 hr before they were killed under ether anaesthesia. To study the influence of total body X-irradiation twenty-seven rats were exposed to 400 Roentgens from a Philips X-ray machine (200 kV, 15 mA, added filtration of 1·6 Cu filter, HVL 1·9 mm Cu, dose rate 20 R/min).

At 12, 24, 36, 48, 60, 72, 84, 96 and 168 hr after irradiation and for controls (plotted at zero time in the figures) three rats were anaesthetized by ether and small segments of intestine 5 cm distal to the pylorus were isolated and fixed in 1·5% glutaraldehyde in 0·1 M cacodylate buffer pH 7·3 for 2 hr at 0°C. After rinsing in the same buffer (2 × 15 min at 0°C) the tissue was postfixed in 1% OSO_4 in 0·1 M cacodylate for 2 hr at 0°C, dehydrated in acetone and

embedded in Epon. Serial sections of 1 μm thickness were cut with a Reichert microtome. These sections were stained with a periodic acid–Schiff–toluidine blue staining (PAS) procedure (Cardno & Steiner, 1965).

The distinction with the light microscope between immature and mature goblet cells was based on the small number of PAS positive secretion granules and a rather weak basophilic reaction of the cytoplasm in immature goblet cells as compared to the large number of PAS positive granules and the stronger basophilic reaction of the cytoplasm in mature goblet cells. Paneth cells can be recognized by their large specific secretion granules.

The mean number and localization of mature and immature goblet cells, Paneth cells and principal cells in the intestinal crypts were determined for every experimental group by studying 100 longitudinally cut crypt columns or fifty longitudinally cut crypts from various sections. The percentage of goblet cells in the lowest twenty positions of the villus was determined for all the experimental groups by counting 100 villus columns.

For electronmicroscopy the intestine was fixed by a perfusion fixation technique. After fixation small pieces of intestine 4–5 cm distal to the pylorus were isolated and further prepared for electronmicroscopy as described earlier (de Both *et al.*, 1974).

RESULTS

At the electron microscope level various kinds of immature goblet cells can be seen indicating a gradual development. In Fig. 1 an undifferentiated principal crypt cell and an immature goblet cell in the lower crypt half are illustrated. In comparison with the undifferentiated principal cells the immature goblet cell shows a more extensive Golgi apparatus and rough endoplasmic reticulum with widened cysternae and also a variable small number of secretion granules. The development of the Golgi apparatus, endoplasmic reticulum and of secretion granules will continue until the stage of the mature goblet cell has been reached. Part of these changes in ultrastructure are visible in the light microscope, such as the steady increase in basophilia of the cytoplasm and the increasing number of secretion granules. This enables the distinction between mature and immature goblet cells with the light microscope, although in some instances such a distinction is somewhat arbitrary. At least part of the immature goblet cells are able to divide as is illustrated in Fig. 2. For the control group and for animals at 72 hr after irradiation the mean number of secretion granules in twenty-five electronmicrographs of immature goblet cells was determined. For the control group the mean number of secretion granules amounted to 14·1 with a standard error of the mean of 1·0 and for the experimental group at 72 hr the values were 11·6 and 0·9. In Fig. 3 the mean numbers of mature and immature goblet cells, principal cells and Paneth cells per crypt section, i.e. in two longitudinally cut crypt columns, are illustrated at different time intervals after 400 R X-irradiation. The Paneth cells do not show any change after irradiation, but the number of goblet cells and principal cells clearly fluctuates. At 12 hr after irradiation both the mature and immature goblet cells are increased in number whereas the number of principal cells is reduced. From 12 to 48–60 hr the number of mature and immature goblet cells decreases, whereas that of the principal cells increases. The latter reaches a maximum at 60 hr. From 48 hr on the number of immature goblet cells increases and at a later stage (from 72 to 84 hr) an increase of mature goblet cells occurs. There seems to be a second peak in the formation of immature goblet cells as also is indicated by the fact that the number of immature goblet cells decreases at 84 hr after irradiation, whereas the number of mature cells steadily increases

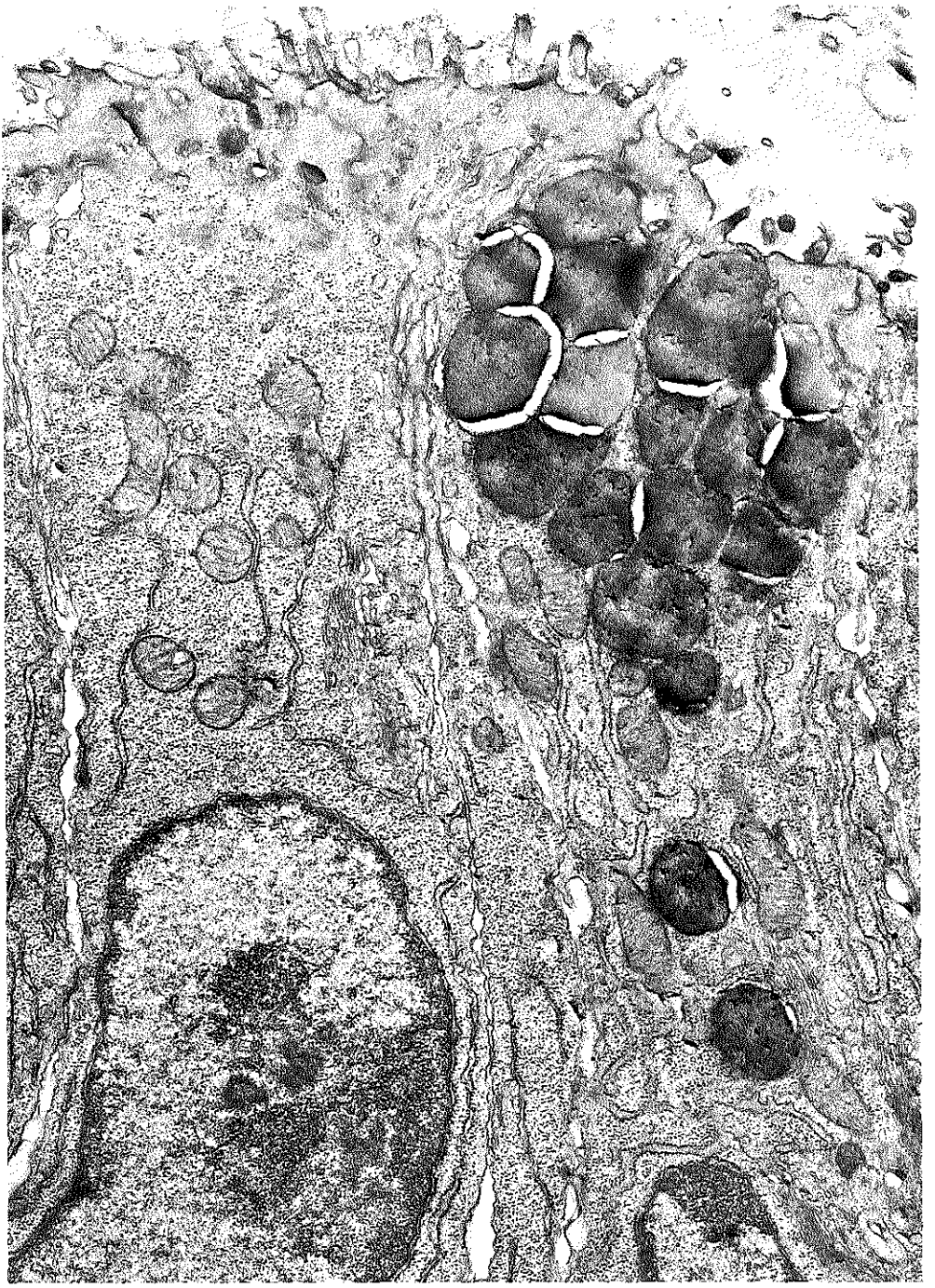

FIG. 1. Principal cell (left) and immature goblet cell (right) in the proliferation compartment of the duodenal crypt of a control animal. ×17,000.

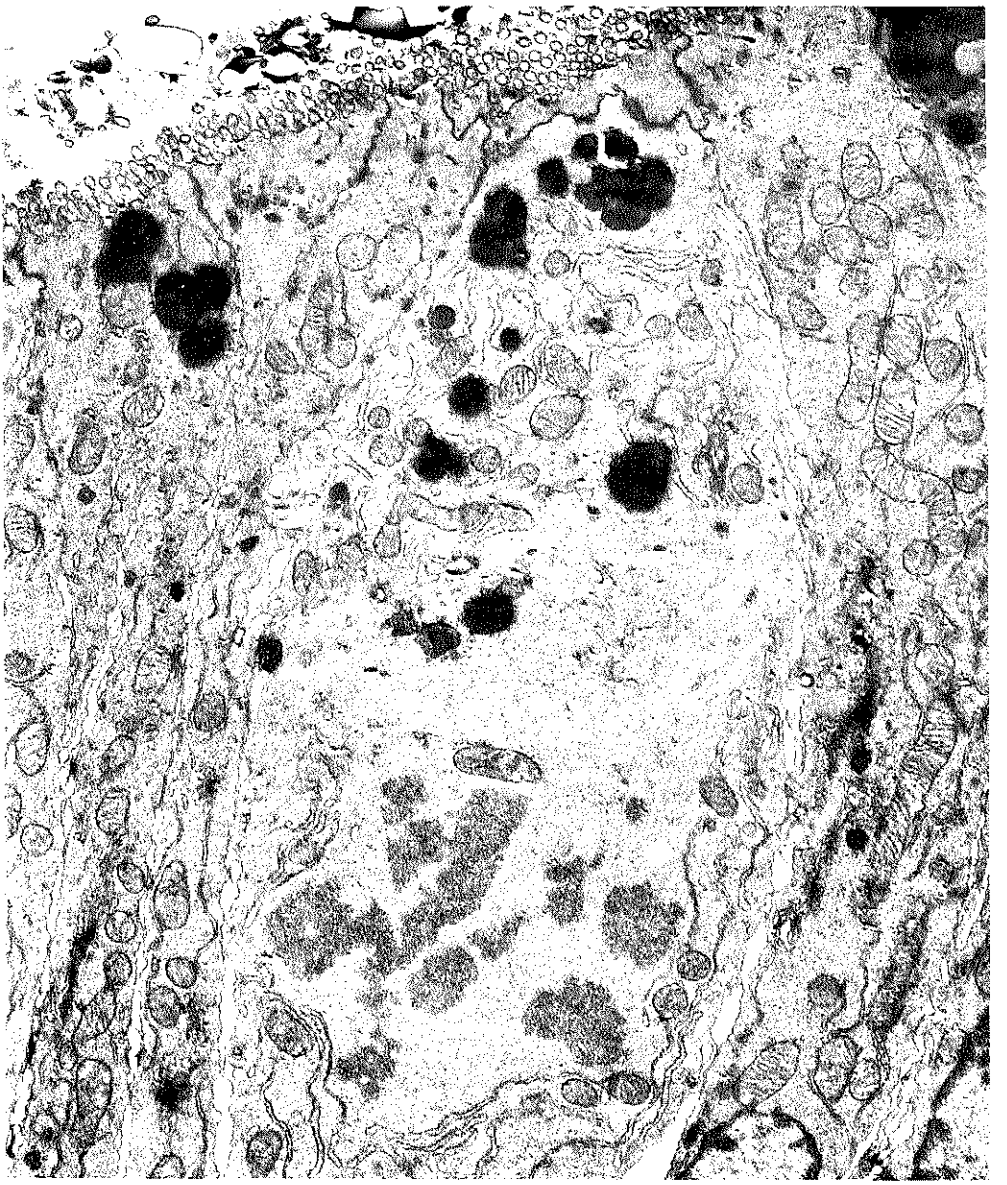

FIG. 2. Dividing immature goblet cell in the proliferation compartment of the duodenal crypt of a control animal. ×11,000.

until control values have been attained. After this radiation dose the number of principal cells, after reaching a maximum value at 60 hr, gradually decreases to control values.

In Fig. 4 the mean ratio of the number of goblet cells (mature and immature) and of the principal cells per crypt are illustrated for the different time intervals after irradiation. This

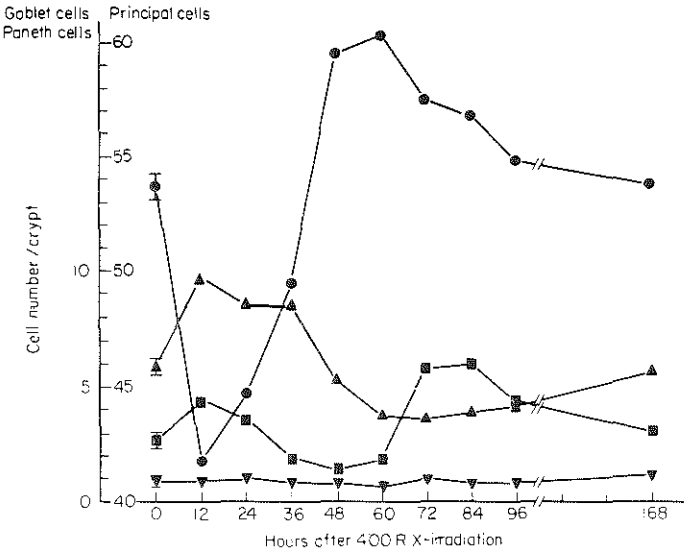

FIG. 3. The mean number of mature and immature goblet cells, principal cells and Paneth cells in duodenal crypt at different time intervals after 400 R X-irradiation. ▲, Mature goblet cells; ■, immature goblet cells; ●, principal cells; ▼, Paneth cells. The values for the standard error of the mean as indicated for the control values are in the same order of magnitude for all other time intervals.

ratio shows the same fluctuations after X-irradiation as observed for the absolute number of mature and immature goblet cells (compare with Fig. 3).

As it is known that the proliferative crypt cell compartment expands during recovery after X-irradiation, we have also studied the localization in the crypt of Paneth cells and of

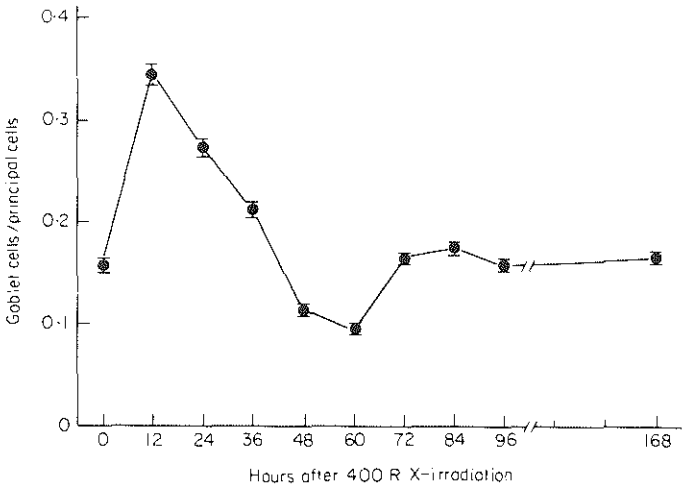

FIG. 4. The mean ratio of the number of goblet cells (mature + immature) and principal cells in duodenal crypts at different time intervals after 400 R X-irradiation. Vertical bars indicate the standard error of the mean.

mature and immature goblet cells at different time intervals after irradiation. The Paneth cells did not show any change in their localization and are always found at the lowest positions in the crypt. The localization of mature and immature goblet cells in the crypts does show variations at different time intervals after irradiation (Fig. 5). In control animals the

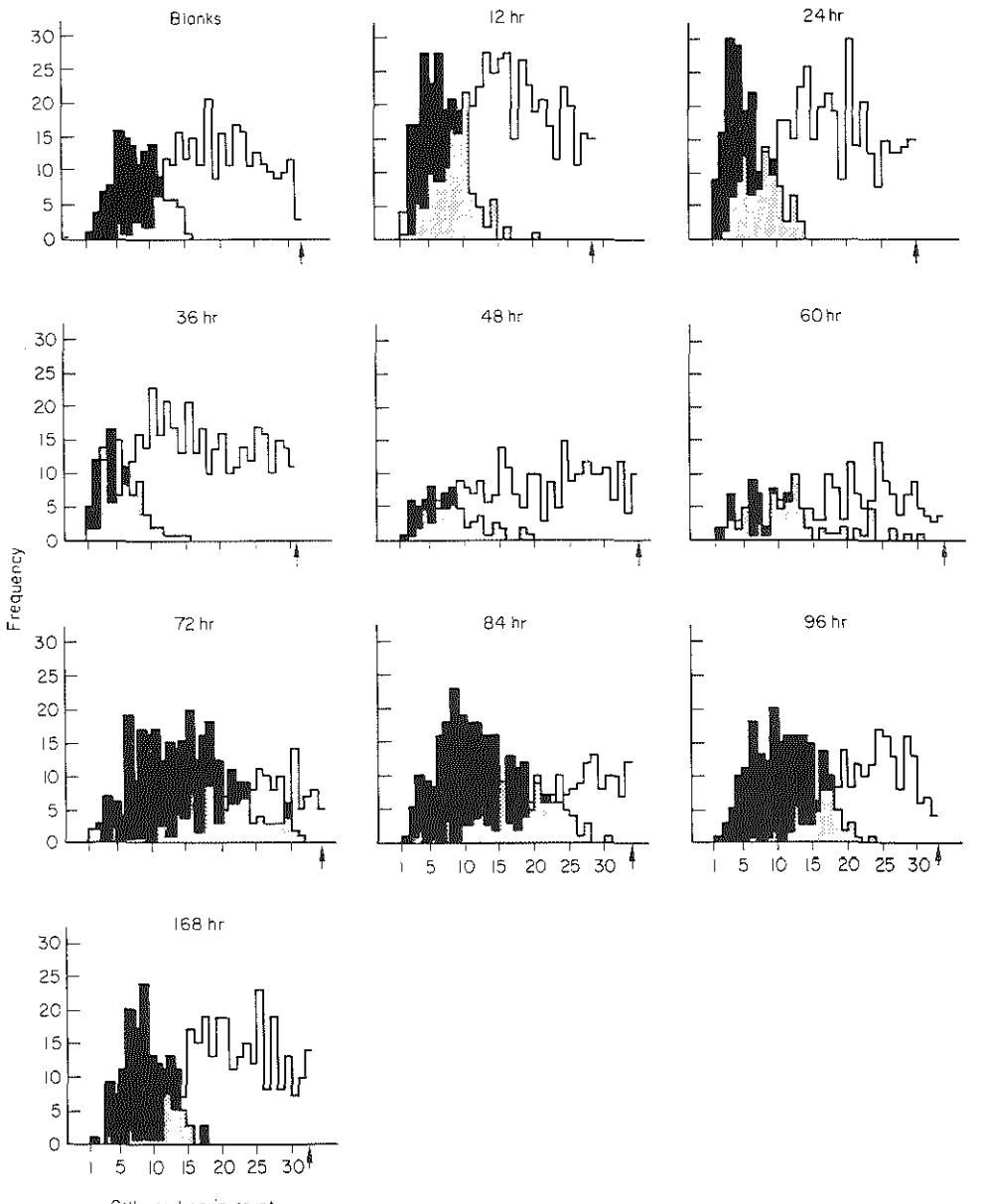

FIG. 5. Distribution of mature and immature goblet cells in duodenal crypts at different time intervals after 400 R X-irradiation. Solid: immature goblet cells; stippled: mature and immature goblet cells; open: mature goblet cells. Arrows indicate the mean total number of cells per crypt column.

immature goblet cells are restricted to the lower half of the crypts, whereas most of the mature ones are found in the upper half. At 12 hr after irradiation there is an increase of immature goblet cells in the lower half of the crypt and of mature goblet cells over the whole length of the crypt. The latter is also true for 24–48 hr after irradiation when both cell types decrease in number at all crypt cell positions. At 60 hr the mature goblet cell population has decreased further and there is a slight increase in immature goblet cells. However, these immature goblet cells are no longer restricted to the lower half of the crypt but are also found at higher positions in the crypt. At 72–84 hr this extension of the pool of immature goblet cells is even more clear as there is also an increase in their number at nearly all cell positions in the crypt. The mature goblet cell population remains about the same, except for a slight shift to the higher positions. During the period 96–168 hr the localization for both types of the goblet cells gradually approaches normal values.

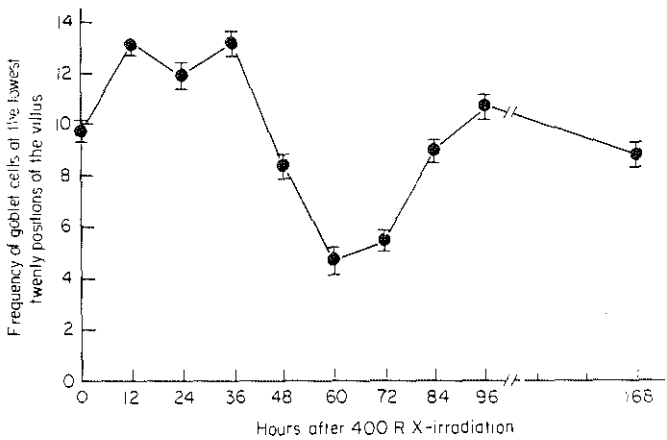

FIG. 6. The percentage of goblet cells at the lowest twenty cell positions of the villus is presented at different time intervals after 400 R X-irradiation; the mean percentages for 100 villus columns are given. Vertical bars indicate the standard error of the mean.

On the villus no immature goblet cells were found, even when they were present at the highest positions in the crypts during the period of 60–84 hr after irradiation. The number of goblet cells at the twenty lowest positions on the villus (Fig. 6) as well as their localization along the whole length of the villus accurately reflect the changes described for the crypt at the different time intervals after 400 R X-irradiation.

DISCUSSION

The number and localization of Paneth cells was not affected by changes in crypt cell kinetics during recovery after irradiation. This is in agreement with the long turnover time of these cells and with the fact no migration occurs (Throughton & Trier, 1969; Devik & Iversen, 1970; Cairnie, 1970; Cheng, 1974b). The population of immature and mature goblet cells, however, does show considerable changes during recovery after irradiation. Our electron-microscopic studies showed that at least part of the immature goblet cells containing secretion granules are able to divide (Fig. 2). The non-dividing pregoblet cells described by

Goblet and Paneth cells in crypt and villus

Cairnie (1970) are a later developmental stage of the goblet cells. As the earliest stages of the immature goblet cells probably arise from dividing principal cells in the lower half of the crypt (Merzel & Leblond, 1969; Throughton & Trier, 1969; Cairnie, 1970; Cheng, 1974a) there may also be dividing immature goblet cells that do not yet contain secretion granules. A distinction between these latter two cell types with the light microscope is impossible but with the electron microscope the dividing immature goblet cells might show a more elaborate Golgi apparatus and rough endoplasmic reticulum than the dividing principal cells. In this respect it is interesting to note that the only change in the ultrastructure of principal cells in the lower half of the crypt occurring at 72 hr after 400 R consists of an elaboration of the Golgi apparatus (de Both *et al.*, 1974; van Dongen & Visser, 1974). At the same time interval after irradiation we also observed an increase in the number of immature goblet cells (Fig. 3). This might suggest that part of the principal cells with a more extensive Golgi apparatus in the lower half of the crypt are in fact early stages of goblet precursor cells without secretion granules.

The population of both immature and mature goblet cells shows considerable changes at various time intervals after irradiation. After 12 hr the number of principal cells is decreased whereas the number of mature and immature goblet cells is increased. The latter increase applies both for the absolute number of goblet cells and for their ratio to principal cells (Fig. 4). These observations do not agree with the results by Cooper (1974) who found after 600 R an increase in the relative number of goblet cells only. However, Wiernik & Plant (1971) did find an increase both in the absolute and in the relative number of goblet cells after different radiation doses. The number of mature goblet cells at the base of the villus is also increased already at 12 hr after irradiation (Fig. 6).

It is generally accepted that goblet cells on the villus originate from immature goblet cells in the lower half of the crypt. Therefore changes in the number of goblet cells on the villus must reflect changes which took place earlier in immature goblet cells in the lower half of the crypt.

About 9–12 hr after labelling with ^{3}H-thymidine labelled principal and goblet cells are found at the base of the villus (Cairnie, Lamerton & Steel, 1965; Galjaard & Bootsma, 1969; Merzel & Leblond, 1969). If we assume that $G_2 + M$ of the cell cycle for both cell types is about 2 hr (Quastler & Sherman, 1959; Cairnie *et al.*, 1965; Thrasher & Greulich, 1966) the cells normally should migrate through the upper half of the crypt for 7–10 hr after their last cell division.

The time for migration through a certain cell compartment does not change after low doses of X-irradiation (Rijke, van der Meer-Fieggen & Galjaard, 1974). This was also suggested by our finding that there was an increase of goblet cells at 12 hr after irradiation at the villus base, while the crypt length did not change significantly (Fig. 5). Therefore changes in the immature goblet cell population in the lower half of the crypt, which are responsible for the changes seen at 12 hr after irradiation at the base of the villus, must take place within a few hours after irradiation. This time interval is too short for an additional cell cycle of the goblet precursor cells to produce extra mature and immature goblet cells since the cell cycle of these cells is about the same as for dividing principal cells (15 hr) (Thrasher & Greulich, 1966). Furthermore at 12 hr after irradiation the proliferative activity of crypt cells is decreased (Williams *et al.*, 1958; Galjaard *et al.*, 1969, 1970). On the basis of these data it can be concluded that the increased formation of goblet cells shortly after irradiation is not the result of increased proliferation.

Also these data cannot wholly be explained by a higher sensitivity to radiation of the principal cells in comparison with the goblet cells resulting in a higher cell death of principal cells. In that case only a relative increase of the goblet cell population would be seen and not an absolute increase as is found here.

The increased production of goblet cells might be due to an acceleration of the cell differentiation process of dividing precursor cells and of non-dividing pregoblet cells. Early developmental stages of the goblet cells without secretion granules, which were counted as principal cells, would more rapidly synthesize these granules and be recognized as immature goblet cells. Another possibility is that more immature goblet cells are formed from early developmental stages of principal cells if the proliferation is temporarily blocked by irradiation.

In the subsequent period after irradiation (12–48 hr) the number of principal cells in the crypt increases whereas the number of mature and immature goblet cells decreases. This could be explained by an exhaustion of immature goblet cells by the changes described above and by the migration of mature goblet cells from crypt to villus. Furthermore the restoration of proliferative activity of principal cells initially results in an increase of cells without secretion granules, which will be scored as principal cells, although part of these cells will develop into goblet cells later on.

In the period after 48 hr the proliferative cell compartment expands over the whole length of the crypt and then probably reaches a normal localization after 1 week. During the period of expansion of the proliferative cell compartment the number of immature goblet cells increases again. However, part of these cells are now being found in positions at the upper half of the crypt (Fig. 5). In controls cell proliferation is restricted to the lower half of the crypt and immature goblet cells are observed only in this cell compartment since evidently differentiation of precursor cells into goblet cells in the lower half of the crypt proceeds more rapidly than cell migration. When proliferative activity expands over the whole length of the crypt immature goblet cells apparently can also develop from proliferating cells without secretion granules in the upper half of the crypt. If this is true we expect to find a reduction of the mean number of secretion granules in the immature goblet cells at 72 hr after irradiation, when proliferation has expanded over the whole crypt length. Proliferating cells without secretion granules in the upper part of the crypts probably contribute to the formation of immature goblet cells. This decrease of secretion granules was actually found.

If immature goblet cells are formed from dividing cells in the upper half of the crypt during recovery after irradiation one would expect that in some instances immature goblet cells would be found at the base of the villus. Since we have never seen immature goblet cells on the villus the formation of goblet cells in the upper half of the crypt must be completed faster than has been described for the differentiation process in the lower half of the crypt (Cairnie, 1970).

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