REGULATION OF CELL PROLIFERATION AND DIFFERENTIATION IN INTESTINAL EPITHELIUM

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Proefschrift

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CONTENTS

CHAPTER I

GENERAL INTRODUCTION

For a number of cell renewal systems a feedback control by differentiated functional cells on the proliferative activity of early undifferentiated cell stages has been described. In skin, teeth and hemopoietic system the existence of such a feedback regulation can be tested directly by experimental removing of functional cells and subsequent studies on the effect of this removal on cell proliferation and -differentiation in the organ system. In the skin stripping of the superficial epithelial layers results in an increased proliferative activity of the basal cells as found by autoradiography after ^{3}H-thymidine labelling and by determination of the mitotic index. For various types of skin in man increased proliferative activity is observed within 1 - 2 days after removal of the functional cells whereas for the skin of hairless mice the response occurs within 4 - 8 hours (23, 42, 76, 77).

In rat teeth shortening of the incisal end every 48 hr during two weeks was found to result in increased proliferation of the inner enamel epithelium achieved through expansion of the proliferative cell compartment and shortening of the intermitotic time (103).

In the hemopoietic system, the presence of several types of functional cells in the peripheral blood and the mixture of numerous undifferentiated cell types within the blood forming tissues complicate exact studies of the effect of removing one type of functional cells on its proliferating cell precursors. Nevertheless it has been shown that depletion of red blood cells caused by bleeding results in an increase in the number of cell divisions in the early stages of erythropoiesis (39).

A more indirect approach to obtain depletion of functional cells is by a temporary block in cell production using cytotoxic agents or ionizing radiation. In skin after different doses of X-radiation an initial drop in proliferative activity is followed by a temporary overshoot in cell proliferation. For various types of skin the time interval between irradiation and the occurence of increased proliferation corresponds remarkably well with the turnover time of the particular skin epithelium (23). These results also suggest that a depletion of the functional cells in the upper layers of the skin stimulates cell proliferation in the basal layer. Also, in the hemopoietic system the use of ionizing radiation results, after a latent period, in an increased production of early non different-

iated stages of erythropoiesis (5, 6, 7, 50, 90).

Although evidence has been collected for a feedback regulation of cell proliferation in some cell renewal systems little is known about the nature of the regulation mechanism. In a review on the cell kinetics of hemopoiesis Lajtha (51) states that as far as the proliferation control is concerned evidence is present for both local and distant humoral control mechanisms. But apart from erythropoietin which is now known to control the step from the "erythropoietin responsive cell population" to the "hemoglobin synthesizing erythroid cells" nothing is known about the nature of the control mechanisms playing a part in the other steps of cell proliferation and -differentiation.

As far as the regulation of proliferative activity in skin is concerned some authors (16, 45) have postulated the existence of a local growth inhibiting factor ("chalone") which is normally being produced by differentiated cells only. According to their hypothesis the removal of functional cells results in a decreased production of this inhibiting substance and hence in an increased proliferative activity within the region surrounding the zone of depletion. During the last few years the presence of tissue specific growth inhibiting substances for some other cell systems (erythrocytes, granulocytes, sebaceous glands) has also been described (17, 47, 84). However, attempts to isolate the regulating substance have so far been unsuccessful (9).

For another important cell renewal system, the intestinal epithelium, even less is known about the regulation of cell proliferation and -differentiation in the crypts. It is the purpose of the studies described in this thesis to obtain more information about this regulation mechanism. Unfortunately, an experimental depletion of functional villus cells by their direct removal is impossible as this would result in rapid death of the animal. However, as in other cell renewal systems, a temporary reduction of functional villus cells can be induced by blocking the cell production in the crypts by X-irradiation. After relatively low radiation doses Galjaard and Bootsma (35) found that during recovery of rat duodenal epithelium an expansion of the pool of proliferating cells occurs in the crypts. This effect is accompanied by a decrease in the number of non-dividing differentiating crypt cells as determined by histochemical analyses of various enzyme activities (34). From their results these authors suggested a relationship between the turnover time of the intestinal epithelium and the moment of increased proliferative activity during repopulation of the crypt after irradiation.

To test this hypothesis we have studied the influence of a temporary disturbance in cell production by X-irradiation on the processes of cell proliferation and -differentiation of

intestinal epithelium in conventional and germfree rats. These two categories of animals were chosen because the intestinal epithelium was expected to have a different turnover time in both groups of animals (1, 46, 57, 66, 67). The results of these studies on the local regulation of cell proliferation and -differentiation are described in Chapter II.

The existence of local regulation mechanisms has been suggested by Bond (10). This theory was based on the absence of any reaction in the adjacent tissue after local irradiation of the intestine. On the other hand, Loran et al. (61, 62) performed partial resection of small intestine and did find changes in proliferative activity in the remaining parts of the intestine. The same authors also studied the effects of partial resection in pairs of parabiotic rats (63) and postulated the existence of humoral factors influencing cell proliferation in intestinal crypts. For further studies on regulation mechanisms it is of great importance to know whether such distant regulation factors really exist or not. In order to investigate this topic we have studied the effect of various types of partial resection of rat small intestine on the process of cell proliferation and -differentiation in the remaining tissue. The results of these experiments on distant regulation are described in Chapter III.

In Chapter IV the results of the experimental work are discussed.

CHAPTER II

LOCAL REGULATION OF CELL PROLIFERATION AND MATURATION IN INTESTINAL EPITHELIUM

INTRODUCTION

The effects of ionizing radiation on the intestine have been studied quite extensively since it was shown that mammals, including man, exposed to higher doses of whole body irradiation died as a result of gastro-intestinal damage (10, 89).

The group of Quastler (79, 80, 81, 86) demonstrated that irradiation blocks the crypt cell production, which results in a depletion of functional villus cells. A minimum number of functional villus cells is found after 2 - 3 days, i.e. after a period corresponding to the turnover time of the intestinal epithelium (36, 37, 80, 85, 102).

Whether or not a restoration of the villus epithelium occurs depends on whether the proliferative activity in the crypts is restored. This in turn depends on the radiation dose. For rat small intestine radiation doses below 1.000 rads result in a restoration of intestinal morphology whereas higher doses lead to complete denudation of the epithelium followed by "gastro-intestinal death" (10, 79, 89). It is clear that in studies on the regulation of cell proliferation in intestinal epithelium relatively low radiation doses should be used in order to be able to analyze the effects of depletion of villus cells on the restoration of proliferative activity in the crypts.

Williams et al. (96) studied the effect of various doses of X-irradiation on the mitotic activity in rat intestinal crypts and they showed that an initial decrease in mitotic activity was followed by an overshoot in the number and the percentage of dividing cells. The time interval between the irradiation and the beginning of increased mitotic activity was found to be about 2 days and seemed to be independent of the radiation dose. The duration of the overshoot in proliferative activity, however, was found to be longer after higher radiation doses. Studying the effects of low radiation doses Galjaard and Bootsma (35) found that during repopulation of the crypts the pool of proliferating cells increased at the expense of the number of non-dividing differentiating crypt cells. Using autoradiography after ^{3}H-thymidine labelling they found that from 36 - 48 hours after 400 rads X-irradiation nearly all cells in the upper half of the crypt

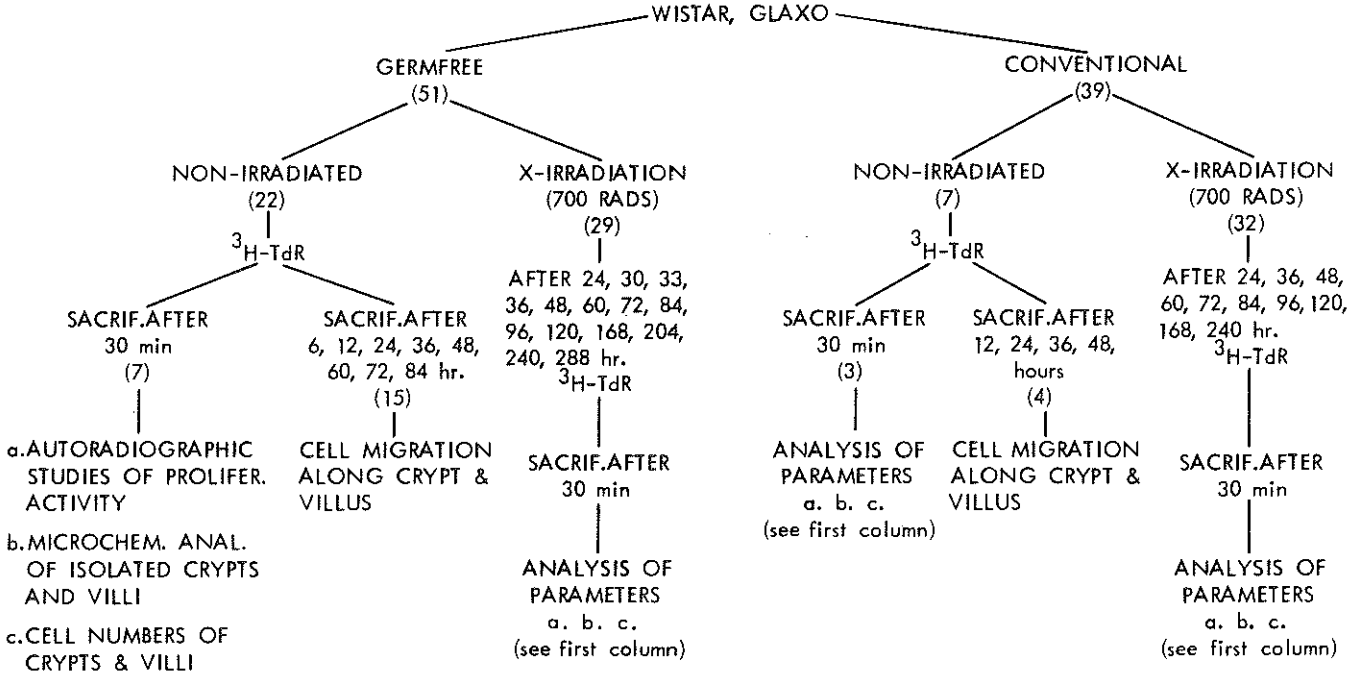
became involved in cell proliferation whereas in non-irradiated controls DNA-synthesis is known to be restricted to cells in the lower half of the crypt (18, 19, 35, 52, 53, 58, 59, 60).

As far as the non-dividing maturing crypt cell compartment is concerned, histochemical and quantitative microchemical analyses revealed that the carboxyl esterase is one of the few (groups of) enzymes of which the activity gradually increases during the process of cell differentiation in the upper half of the crypt. The activity of this enzyme could therefore be used as a parameter for the non-dividing maturing crypt cells (34). After X-irradiation the period of increased proliferative activity was found to be accompanied by a decrease in carboxyl esterase activity (35). This decreased activity occurred between 36 and 48 hours after different radiation doses (50 - 400 rads). The expansion of the proliferative cell compartment in the crypt occurred at the same period after irradiation (35). As the total turn-over time of rat intestinal epithelium is also 36 - 48 hours (23, 44, 53, 54, 60, 81, 102) these results suggest that in the intestine, there is a relationship between the processes of cell proliferation and -differentiation in the crypt and a depletion of functional villus cells.

To test this hypothesis we have made use of the fact that the turnover time of the intestinal epithelium is different in conventional and germfree animals. Several studies indicate that in germfree mice the total turnover time of the intestinal epithelium is some days longer than in conventional animals of the same strain. This has been observed by autoradiography at various time intervals after ^{3}H-thymidine labelling and subsequent localization of labelled cells along crypt and villus (1, 46, 57, 66, 67). Furthermore it has been described that the survival time after relatively high radiation doses is longer in germfree animals than in conventional ones (65, 66, 68, 82, 98, 99, 100). If the functional villus cells were to play a role in the regulation of cell proliferation and -differentiation in the crypt, one would expect a different response of the crypt cells to the same radiation dose in germfree animals compared to conventional animals. In this chapter the effects of 700 rads of X-radiation on conventional and germfree rats will be described. At various time intervals after irradiation, in both groups of animals the proliferative cell compartment has been studied by autoradiography after ^{3}H-thymidine labelling, whereas the pool of non-dividing differentiating crypt cells has been studied by histochemical and quantitative microchemical analyses of carboxyl esterase activity.

FIG. 1

EXPERIMENTAL DESIGN FOR COMPARITIVE STUDIES ON DUODENAL EPITHELIUM FROM CONVENTIONAL AND GERMFREE RATS



MATERIAL AND METHODS

1. Experimental design

The number of germfree and conventional Wistar rats investigated and the scheme of X-irradiation are illustrated in Fig. 1. Also some conventional Wistar rats of the Brofo type were investigated. The germfree rats were provided by the Radiobiological Institute TNO, Rijswijk. These rats are offspring from W(istar)A(lbino)G(laxo) rats born by sectio caesarea under sterile conditions (91), and which were nourished by germfree fostermothers (from the Lobund Institute, Notre Dame University, Indiana). The germfree rats were kept in sterile plastic isolators and fed with Hope farms pellets sterilised by autoclaving for 8 min at 134°C ($1\frac{1}{2}$ Atm.). Tap water sterilised by autoclaving for 30 min at 121°C (1 Atm.) was available ad libitum. Female rats were used for analyses after 24 hour starvation. Irradiation was carried out with a 250 KV Philips X-ray machine (18 mA, 200 KV, 1.5 mm corrected Cu filter, HVL 1.9 mm Cu, dose rate 46 rads/min). The germfree animals were irradiated under sterile conditions. For the study of proliferative activity in the crypts irradiated and non-irradiated animals from both groups were injected intravenously with 100 μC ^{3}H-thymidine (specific activity 2 Ci/mMol Amersham) 30 min before sacrifice. For the study of the migration rate of the epithelial cells along crypt and villus non-irradiated conventional animals were labelled with 100 μC. ^{3}H-thymidine (specific activity 18.9 Ci/mMol) at 12, 24, 36 and 48 hours before sacrifice. Intestinal tissue from non-irradiated germfree rats was investigated 12, 24, 36, 48, 60 and 72 hours after labelling.

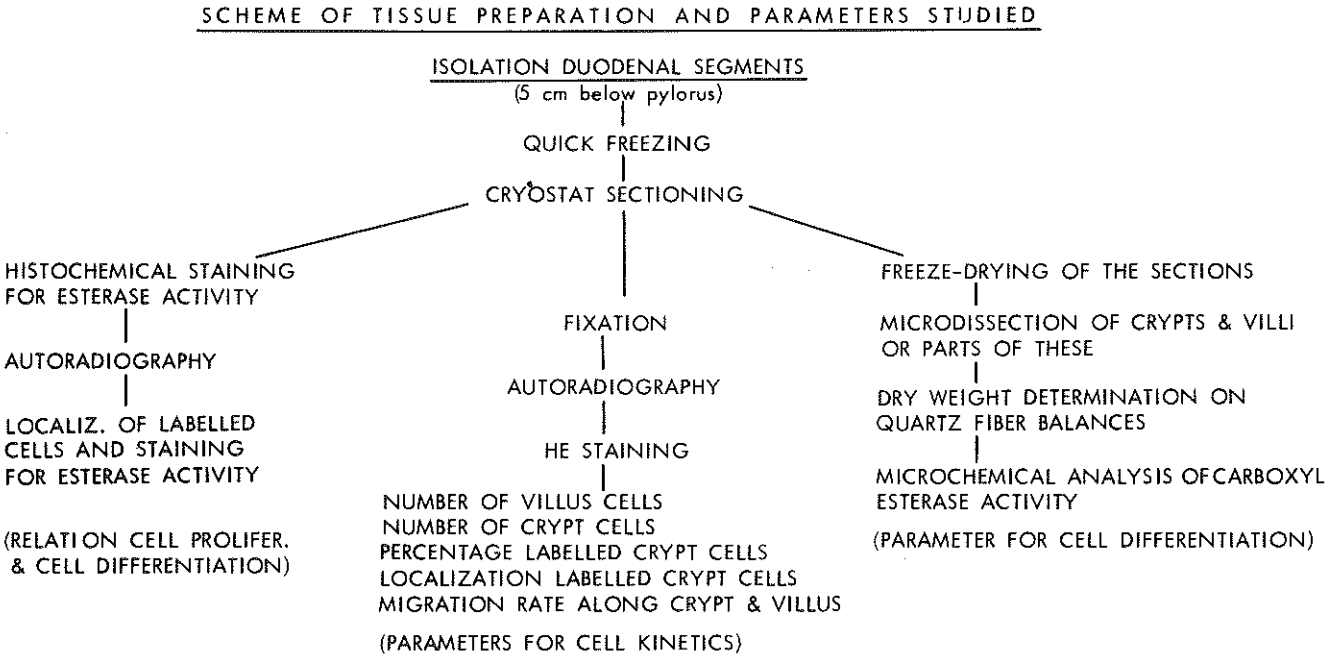
2. Tissue preparation

The analysis of cell proliferation and -differentiation in duodenal epithelium was studied according to the scheme illustrated in Fig. 2.

A segment of duodenum was taken out under general ether anaesthesia at 5 cm below pylorus. The intestine was cut open and then frozen in CO_2 vapour (-70°C) or isopentane cooled in liquid N_2 (-170°C) and cryostat sections of 6 μ and 10 μ thickness were cut at -15°C.

For histochemical staining cryostat sections (6 μ) were fixed during 15 min. at 20°C in a mixture of neutral formalin-calcium-macrodex (1 : 1 : 8 v/v) and subsequently a

FIG. 2


SCHEME OF TISSUE PREPARATION AND PARAMETERS STUDIED
ISOLATION DUODENAL SEGMENTS
(5 cm below pylorus)
QUICK FREEZING
CRYOSTAT SECTIONING
HISTOCHEMICAL STAINING FOR ESTERASE ACTIVITY
AUTORADIOGRAPHY
LOCALIZ. OF LABELLED CELLS AND STAINING FOR ESTERASE ACTIVITY
(RELATION CELL PROLIFER. & CELL DIFFERENTIATION)
FIXATION
AUTORADIOGRAPHY
HE STAINING
NUMBER OF VILLUS CELLS
NUMBER OF CRYPT CELLS
PERCENTAGE LABELLED CRYPT CELLS
LOCALIZATION LABELLED CRYPT CELLS
MIGRATION RATE ALONG CRYPT & VILLUS
(PARAMETERS FOR CELL KINETICS)
FREEZE-DRYING OF THE SECTIONS
MICRODISSECTION OF CRYPTS & VILLI OR PARTS OF THESE
DRY WEIGHT DETERMINATION ON QUARTZ FIBER BALANCES
MICROCHEMICAL ANALYSIS OF CARBOXYL ESTERASE ACTIVITY
(PARAMETER FOR CELL DIFFERENTIATION)

histochemical staining for carboxyl esterase activity was carried out. After the slides were rinsed in running tap water for 10 minutes and briefly in distilled water they were incubated for 60 min at 37°C in 100 ml 0.1 M Tris HCl buffer (pH 7.2) to which was added 5 mM potassiumferricyanide, 5 mM potassiumferrocyanide, 10 mM $CaCl_2$ and as substrate 0.5 mM 5-bromo - 4-chloro-indoxyl acetate solution in 1 ml alcohol 100%. After rinsing in distilled water, the slides were either immersed in glycerine-gelatine for microscopic investigation or they were further processed for autoradiography.

For autoradiography both cryostat sections which were previously stained for carboxyl esterase activity and unstained sections fixed for 15 min in neutral formalin were used. All object slides being used for autoradiography had been coated with 2% gelatin and 0.2% chrome-alum after thorough cleaning. Autoradiography was performed using Kodak AR10 stripping film and after an exposure time of about 6 weeks and development in Kodak D19b the slides were embedded in depex either immediately or after staining with hemotoxylin-eosin.

For quantitative microchemical analysis of carboxyl esterase activity 10 µ cryostat sections from each duodenal segment were freeze-dried according to Lowry (64). Cryostat sections pressed to coverslips were put in copper holders which were placed in a glass tube connected to a high vacuum diffusion pump (Edwards, ES-50). Lyophilization was carried out for 6 - 18 hours at -45°C with a pressure of less than 10^{-3} mm Hg. After freeze-drying the sections were either used immediately for microchemical analysis or they were stored in vacuo at low temperature (-70°).

3. Quantitative microchemical analysis of carboxyl esterase activity in isolated cell compartments from duodenal crypts and villi

Freeze-dried sections were warmed to 20°C in vacuo and further handled in a room of 20°C and 40% relative humidity. Free hand dissection of the various cell compartments was carried out under a dissecting microscope (120x magnification) using fragments of razor blades and hairpoints. After separating the tunica mucosa from the other layers of the gut, longitudinally cut crypts and villi were isolated from each other whereas a small transitional zone (6-8 cells) between crypt and villus was discarded to avoid mutual contamination of both cell compartments. In some instances the proliferating crypt cells and the non-dividing maturing crypt cells were isolated by dissecting the lower and the

upper half of the crypt. The dry weight of the isolated fragments was determined on quartz fiber balances (sensitivity down to 10^{-9} gm) constructed and calibrated according to Lowry et al. (64).

After weighing, the tissue fragments were introduced into small polythene tubes and the activity of carboxyl esterases was measured by a modified method of Doyle et al. (26). Depending on the amount of cell material, incubation was carried out for 20 min at 40°C in 5 - 50 µl 0.1 M Tris HCl buffer (pH 7.4) containing 0.05% bovine serum albumine and 10 - 100 µl 6.6 mM naphthylacetate. After incubation the liberated naphthol was coupled to a diazodye by the addition of 20 - 200 µl diazored ITRN (Dupont) dissolved in 3% laurylsulfonate (4 mg/5 ml). After 15 min at 20°C the absorbance at 550 mµ was measured in 35 - 350 µl final volume using microcuvettes adapted to a Zeiss spectrophotometer. The carboxyl esterase activity has been expressed in µmol α-naphthol liberated per hour per mg dry weight of the isolated fragment.

RESULTS

1. Cell migration in duodenal epithelium from conventional and germfree rats

In both groups of animals the migration of labelled cells along crypt and villus was studied at different time intervals after ^{3}H-thymidine incorporation. For each time interval in various sections from one or two animals the position of the highest labelled cell along 50 - 100 longitudinally cut crypts and villi columns was determined as counted from the base of the crypt. The mean values of the highest labelled cell position at different time intervals after ^{3}H-thymidine injection are shown in Figs. 3 and 4 for conventional and germfree rats. At 1/2 - 1 hour after injection only cells belonging to the proliferative cell compartment in the crypt are labelled. In both groups of animals this involves the first 16 - 17 cell positions in the lower half of the crypt. For 12 hours after their last DNA synthesis in both groups of animals, the crypt cells migrate along the maturation zone in the upper half of the crypt. This is indicated by the the fact that after 12 hours labelled cells are found up to the base of the villus. Subsequently the cells migrate along the villus at about the same rate in both conventional and germfree rats, which is indicated by the slope of the curves for both groups in Fig. 3.

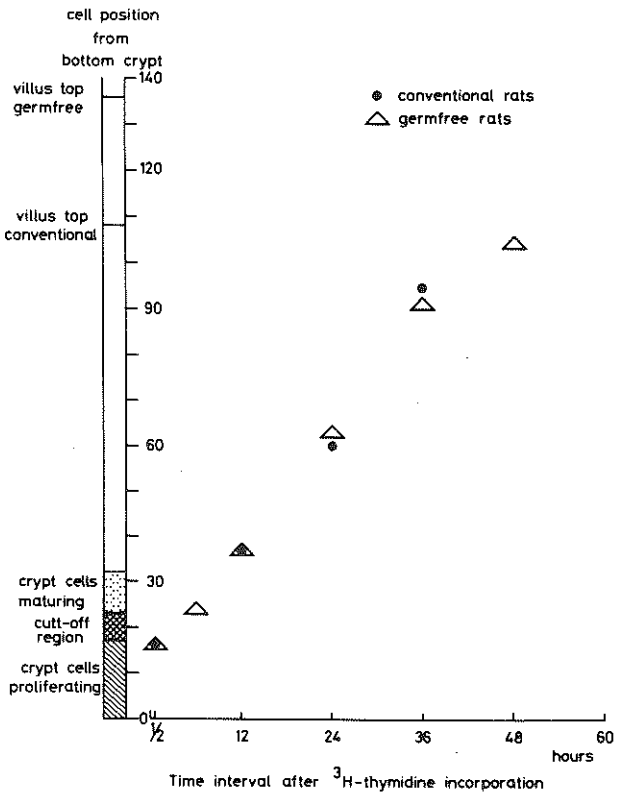


Fig. 3: Cell migration along crypt and villus

In conventional rats labelled cells are observed at the tips of the villi at 36 or 48 hours after labelling (Fig. 5 A and B) depending on the length of the villus. In germfree rats, however, the mean number of villus cells was found to be larger than in conventional rats, and this is reflected in a longer time interval before labelled cells are present at the tips of the villi. In germfree rats in no instance have labelled cells been observed at the tips of the villi before 60 hours after ^{3}H-thymidine incorporation (Fig. 3 and Fig. 5 C - E).

In Fig. 4 the mean cell numbers per crypt and villus column are illustrated as well as the mean cell positions at which labelled cells have been observed at the various time intervals after ^{3}H-thymidine incorporation. From this scheme it is clear that in both germfree and conventional rats after their last DNA synthesis the cells migrate for the same period (12 hours) along the maturation zone at the upper half of the crypt. The

migration is about 1 - 2 cell positions per hour.

After entering the pool of functional cells on the villus migration along the basal part of the villus occurs at a somewhat higher rate (2 cell positions per hour) and in the middle part of the villus the migration rate increases to about 3 cell positions per hour. Towards the top the migration rate seems to slow down again which is most clear in the germfree animals. An exact determination of the cell migration rate at the tips of the villi is difficult because of the variation in total villus length and the fact that the cells are sloughed off into the intestinal lumen.

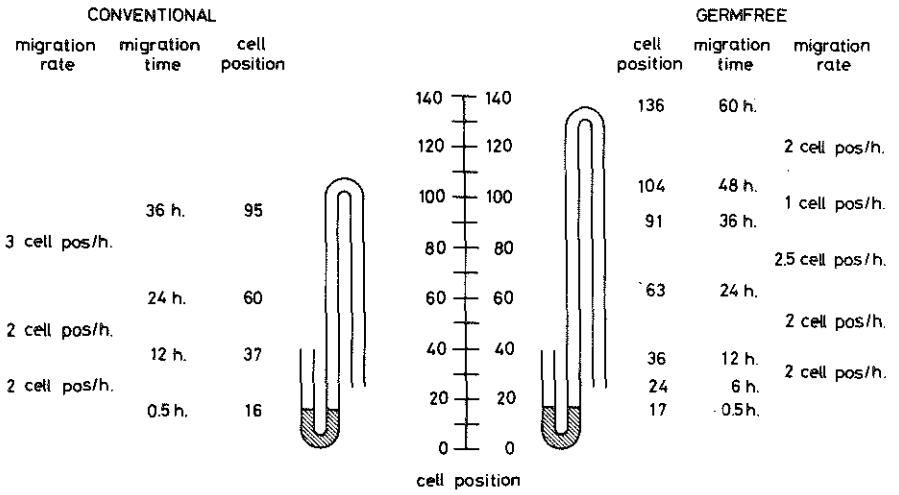


Fig. 4: Scheme of cell migration in duodenum from conventional and germfree rats

The experimental results on cell migration show that in conventional rats the turnover time of the duodenal epithelium cells is between 36 and 48 hours and in germfree rats about 60 hours. The turnover time is defined as the period between the last DNA synthesis of the cell in the crypt and the moment that the cell reaches the top of the villus. The longer turnover time in the germfree rats is due to the longer villi (larger number of villus cells) because the migration rate of the cells was found to be similar in conventional animals. The period of cell maturation in the crypt after the last cell division seems to be equal (12 hours) in germfree and conventional rats.

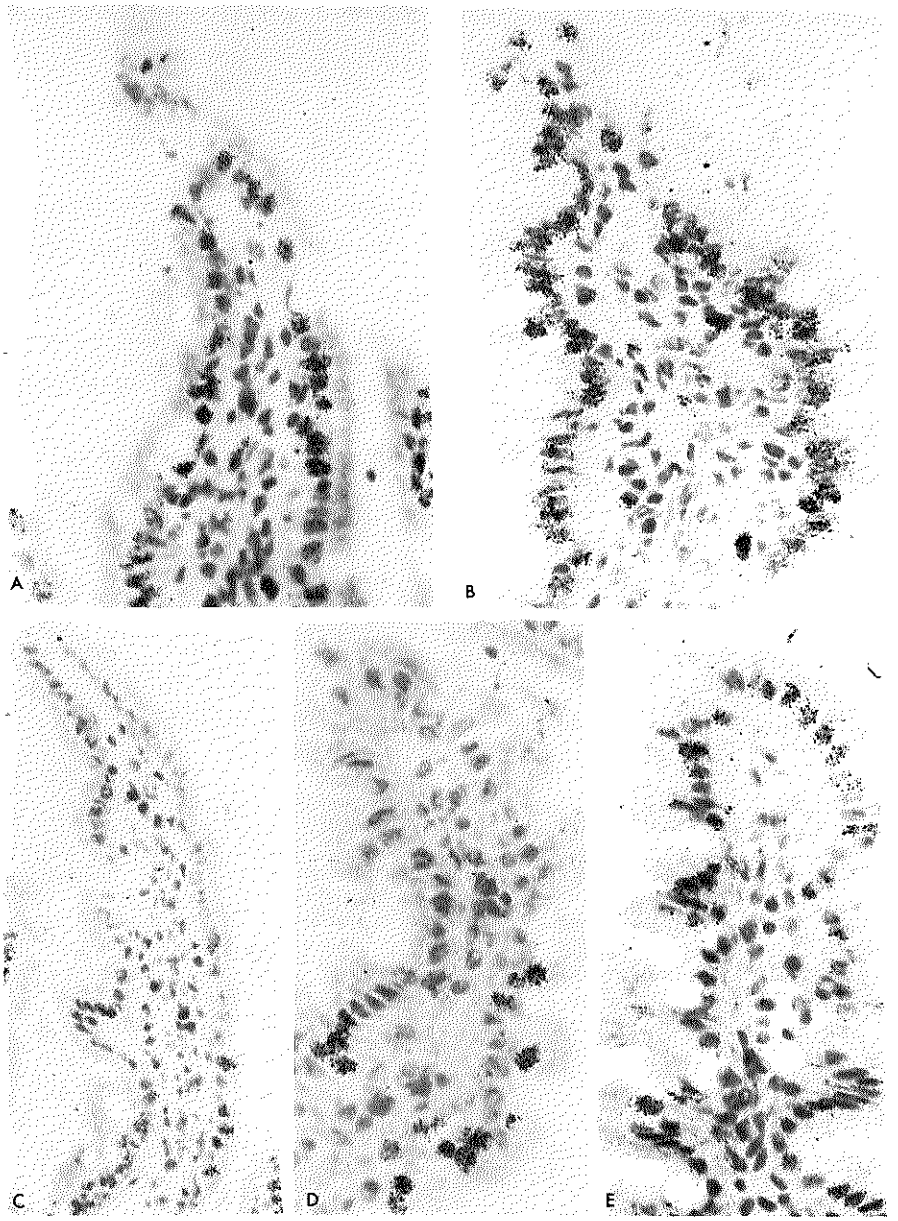


Fig. 5: Cell migration in duodenum from germfree and conventional rats. Cryostat sections (6 µ), stripping film autoradiography and HE staining, obj. 40x. Conventional rats at 36 h (A) and 48 h (B) after ^{3}H-TdR injection; germfree rats 36 h (C), 48 h (D) and 60 h (E) after ^{3}H-TdR incorporation

2. Cell proliferation in duodenal crypts during recovery after X-irradiation in conventional and germfree rats

a. Percentage labelled crypt cells

The total number of crypt cells and the total number of labelled cells at 30 min after ^{3}H-thymidine incorporation were determined in 50 - 100 longitudinally cut crypt columns from 2 - 4 animals at various time intervals after 700 rads X-irradiation. The results for conventional and germfree rats were compared with those of the respective non-irradiated controls. The mean values of the total number of crypt cells and the percentages of labelled cells are presented in Table I.

TABLE I

CELL NUMBER AND PERCENTAGE OF LABELLED CELLS 30 MIN AFTER ^{3}H-THYMIDINE INCORPORATION IN DUODENAL CRYPTS FROM GERMFREE AND CONVENTIONAL RATS AFTER 700 RADS X-IRRADIATION*

	AVERAGE NUMBER OF CELLS PER CRYPT COLUMN		PERCENTAGE OF LABELLED CELLS					
			Conventional			Germfree		
Hours after X-irradiation	Conventional	Germfree	%	sd	n**	%	sd	n**
Control	32	31	26	8	85	25	9	230
24	19	18	13	6	88	4	4	100
36	15	14	23	11	70	19	11	200
48	13	13	47	21	75	24	12	100
60	17	19	52	18	92	42	17	168
72	24	25	--	--	--	54	16	150
84	32	31	50	14	72	56	15	127
96	33	32	43	14	95	55	13	50
120	33	32	32	10	86	35	9	100
168	32	32	35	8	82	--	--	---
204	--	32	--	--	--	30	7	100
240	--	32	--	--	--	30	10	100

* Cell number and percentage labelled cells are calculated per longitudinally cut crypt column. The mean values of 50 - 230 crypt columns from 2-4 animals per time interval are presented.

** n = number of crypt columns counted.

In the non-irradiated control animals the mean values of the total number of cells per crypt column were found to be similar in conventional and in germfree rats ($\bar{x}$ = 32 and $\bar{x}$ = 31 respectively with a standard deviation = 1). Also the percentages of labelled cells, indicating the proliferative activity, showed similar values, i.e. 26% in the conventional rats and 25% in the germfree rats.

Shortly after X-irradiation a block in proliferative activity occurs which is reflected in a decrease of the percentage of labelled cells at 24 hours after irradiation. As cell migration from the crypt into the villus is known to continue after irradiation the temporary block in cell proliferation results in a depopulation of the crypt. This is shown by the decreased number of total cells per crypt column until 84 hours after irradiation. Cell depletion in the crypts follows the same pattern in the duodenum from conventional and germfree rats (Table 1). After a dose of 700 rads, the proliferative activity, i.e. a recurrence of DNA-synthesis, occurs in both groups of animals from 24 hours after irradiation on. This results in a repopulation of the crypt which is completed at about 84 hours after irradiation in both groups of animals. However, even before complete repopulation of the crypts has occurred the percentage of labelled cells per crypt column is found to exceed the control values. Here, a difference is observed between the two groups of animals. In the conventional rats at 48 hours after irradiation about 50% of the crypt cells were found to be labelled (indicating that all cells proliferate, as the duration of the DNA synthesis is about 50% of the total intermitotic time of the crypt cells) whereas in the germfree rats this is the case between 60 and 72 hours after irradiation. The period that all cells are involved in proliferation exceeds that required for a complete repopulation of the crypt. In both groups of animals from about 4 to 9 days after irradiation the percentage of labelled cells gradually decreases to control values.

The main conclusion from these results is that during repopulation of the crypt after irradiation the percentage of labelled crypt cells markedly exceeds the control values both in conventional and germfree rats, but the peak occurs at different time intervals.

b. Localization of labelled cells

To localize the labelled cells within the crypt, cell positions have been numbered along longitudinally cut columns from 1 at the bottom of the crypt to 40 at the top. The localization of each labelled cell was scored and the frequency of labelling at any crypt cell position was determined. The same categories of animals and the same

number of crypts as mentioned in the preceeding paragraph were analyzed.

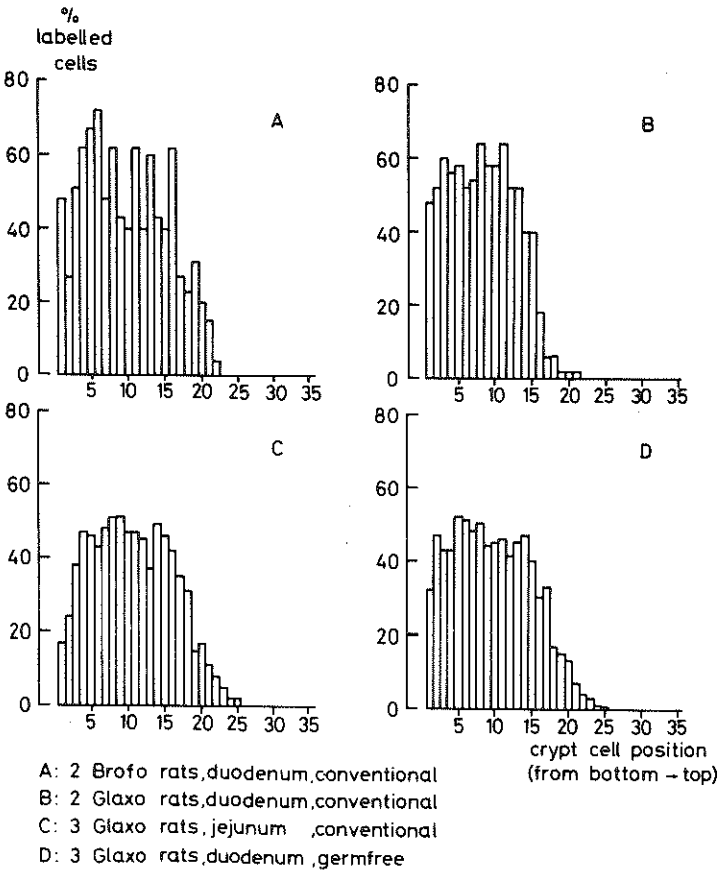


Fig. 6: Distribution of labelled cells in crypts of non-irradiated conventional and germfree rats (the distribution of labelled cells is scored 30 min. after ^{3}H-TdR injection

The variation in the localization of labelled cells in crypts from non-irradiated conventional rats and germfree rats is illustrated by the results of 4 experiments (Fig. 6). Along the abcissa the various crypt cell positions are indicated and on the ordinate the frequency of labelled cells. In all instances the mean number of cells per crypt column was 31 or 32. Fig. 6 A represents the labelling pattern of 25 - 50 crypt columns in duodenum from 2 Wistar rats of the Brofo type. Fig. 6 B shows this pattern of 50 - 100 crypt columns in duodenum from two Wistar rats of the Glaxo type, Fig. 6 C represents

the analyses on 50 - 100 columns in jejunum from three Glaxo rats. Even in different experiments on various types of animals and different parts of the small intestine the localization of labelled cells shows little variation. 50 - 60% labelling can be used as a criterion for a completely proliferative cell compartment in conventional rats and 40 - 50% in the germfree, as this is the ratio between duration of the DNA synthesis and that of the intermitotic time of crypt cells (18, 53, 58). In the non-irradiated conventional controls the proliferating cell compartment is localized in the lower 15 - 17 cell positions of the crypt. Between cell position 16 to 25 the percentage of labelled cells rapidly decreases and in position 25 - 32 no cell proliferation occurs. As shown in Fig. 6 D the distribution of labelled cells in duodenum as obtained from 230 crypt columns from three germfree Glaxo rats does not differ significantly from that in the various types of conventional animals. The lower percentages of labelled cells found for the cell positions in the lower half of the crypt in jejunum from conventional rats and in duodenum from germfree rats are probably due to a larger intermitotic time compared to that in duodenum from conventional rats.

The study of the localization of labelled cells at various time intervals after X-irradiation is complicated by the fact that shortly after irradiation a temporary depopulation of the crypt occurs, followed by a repopulation which is completed at about 84 hours after irradiation. Hence, at different time intervals after irradiation the crypt consists of a varying number of cells which complicates a comparison of the positions with those in control animals. To overcome these problems differences in crypt length have been normalized by positioning the labelled cells in fractional distances between bottom and top of the crypt according to Cairnie (20). For non-irradiated controls and for these time intervals after irradiation, where complete repopulation of the crypt has occurred, the distribution pattern of labelled cells is of course independent of the way of expressing the localization (compare Fig. 6 B, D and Fig. 7 A).

The localization of labelled cells at various time intervals after irradiation has been compared for conventional and germfree rats. The results in Fig. 7 illustrate the frequencies of labelled cells as found for the various fractional distances between bottom and top of the crypt. Fig. 7A confirms that there is little difference in the localization pattern between non-irradiated conventional and germfree rats. At 24 hours after irradiation (Fig. 7 B) incorporation of ^{3}H-thymidine into DNA is decreased markedly in the cells of the proliferative compartment at the lower half of the crypt. After 36 hours in both groups the proliferative activity recovers and in the conventional rats some

cells at the upper half of the crypt are already involved in cell proliferation whereas in the control, cells at these positions were never found to proliferate. After 48 hours this effect is much more pronounced and in the conventional rats for nearly all crypt cell positions labelling percentages of 40 - 60% are being observed (Fig. 7 D). In the germfree animals however, this effect occurs after a longer time interval i.e. at about 60 hours after irradiation (Fig. 7 D, E). After 84 hours, when repopulation of the crypt is completed (Table I) in both germfree and conventional animals, most cell positions at the upper half of the crypt are still involved in cell proliferation (Fig. 7 F). Subsequently the distribution of labelled cells is restored to normal from about 4 - 10 days after irradiation.

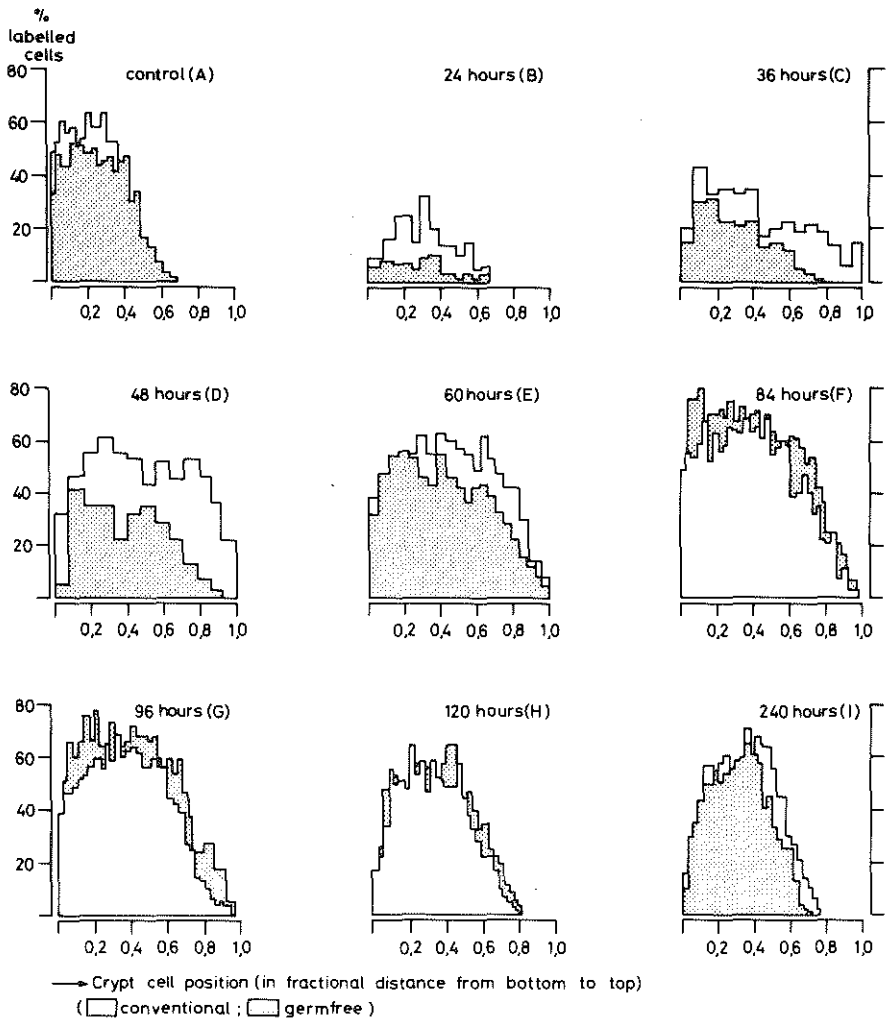


Fig. 7: The distribution of labelled cells in duodenal crypts from germfree and conventional rats at various time intervals after X-irradiation (700 Rads)

The percentages of labelled cells in the proliferating cell compartment is somewhat lower in non-irradiated germfree rats than in conventional ones and this is also true shortly after irradiation. However, during subsequent recovery of the duodenal epithelium in the germfree rats, the labelling percentages increase above the control values, mainly at 84 - 96 hours after irradiation. This effect is not observed in conventional rats.

The results presented indicate that the overshoot in percentages of labelled cells during recovery after irradiation (Table I) is mainly based on an increase in the number of crypt cells that become involved in DNA synthesis. An expansion of the normal proliferative cell compartment in the crypt seems to occur at the expense of the non-dividing maturing cells that normally occupy the upper half of the crypt. However, this expansion of the proliferating cell compartment starts between 36 - 48 hours after irradiation in conventional rats and after about 60 hours in germfree animals.

3. The maturing crypt cell compartment during recovery after irradiation in germfree and conventional rats.

TABLE II

MICROCHEMICAL ANALYSIS OF CARBOXYL ESTERASE ACTIVITY IN ISOLATED DUODENAL CRYPTS FROM A NON-IRRADIATED CONVENTIONAL GLAXO RAT

NUMBER OF SAMPLE	DRY WEIGHT $x.10^{-6}$ gm	EXTINCTION (550 mμ)	ENZYME ACTIVITY*
1	0.496	0.206	19.8
2	0.682	0.213	15.0
3	0.816	0.300	18.0
4	0.924	0.296	15.6
5	0.612	0.243	18.9
6	1.108	0.445	19.5
7	0.520	0.182	17.1
8	0.848	0.361	24.3
9	1.130	0.395	17.1
10	0.578	0.206	17.1
			mean 18.3 $\pm$ 2.7

*μmol naphthol / hour / mg dry weight.

Microchemical analyses of the carboxyl esterase activity in isolated crypts and villi or parts of these were carried out to study the effects of recovery after irradiation on the non-dividing maturing cell compartment.

For each animal the carboxyl esterase activity was analyzed in 10 samples of the cell compartment concerned, dissected from various freeze-dried cryostat sections. Table II gives an example of such analyses on isolated duodenal crypts from a non-irradiated conventional rat. The dry weight of the crypt samples varied from 0.496 - 1.130. 10^{-6} gm, the extinction values were between 0.206 - 0.445 in a final volume of 350 µl. The variation among the samples was found to be about 10% for the determination of esterase activity in isolated crypts. The mean enzyme activities in different control animals as illustrated in Table III appeared to be relatively constant.

TABLE III

VARIATIONS IN CARBOXYL ESTERASE ACTIVITY IN ISOLATED DUODENAL CRYPTS FROM DIFFERENT NON-IRRADIATED CONVENTIONAL RATS

NUMBER OF ANIMAL	MEAN ENZYME ACTIVITY* (µmol naphthol / hour / mg dry weight)
Control 1	18.3 ± 2.7
2	20.7 ± 2.4
3	19.5 ± 2.4
4	16.8 ± 2.4
5	18.3 ± 1.2
6	19.2 ± 1.5
7	18.9 ± 0.9
	mean activity: 18.8 ± 1.9

* Each value is the mean of analyses from 10 samples.

The mean values of carboxyl esterase activity in duodenal crypts from 2 - 8 non-irradiated germfree and conventional rats are illustrated in Fig. 8. The enzyme activity in isolated crypts shows no clear differences between duodenum from conventional rats (18.8 µmol/hr/mg) and that from germfree animals (20.4). The esterase activity in the proliferating cell compartment has been determined after dissection of the lower half of the crypt, whereas the activity for the non-dividing maturing crypt cells was obtained by analyses of the upper half of the crypt (see Fig. 8).

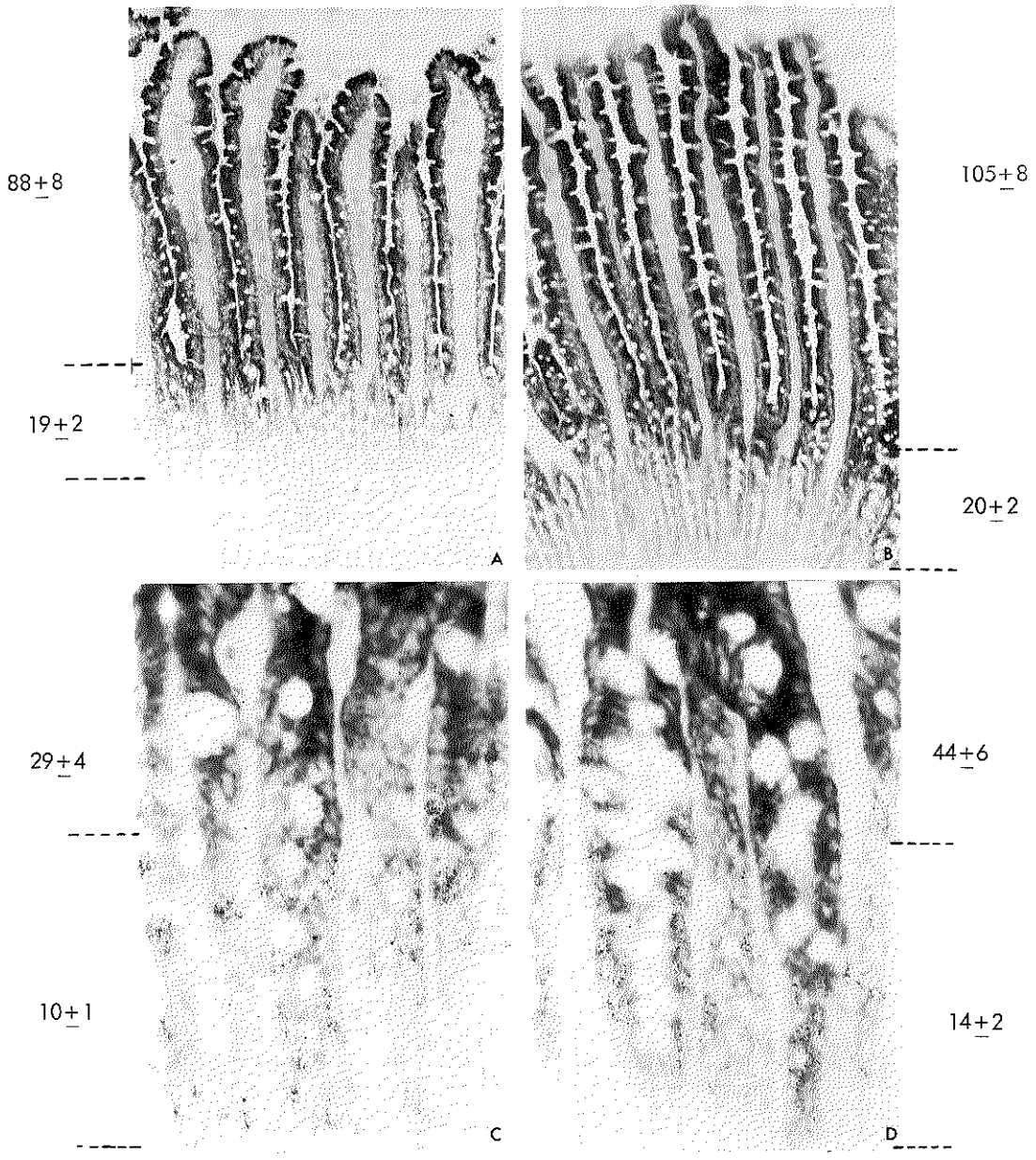


Fig. 8: The proliferative activity and cell maturation process in duodenum from normal (A,C) and germfree (B,D). Combined autoradiography (30 min. after ^{3}H-TdR incorporation) and enzyme histochemistry (non-specific esterase activity)on duodenal mucosa (A,B,obj. 10x) and details of the crypts (C,D,obj. 40x). The data represent the mean and standard deviation of quantitative crypts and villi (A, B) and in the proliferative and maturing zone of the crypt (C,D)

The results of these quantitative analyses are illustrated in Fig. 8 where a comparison can be made with the results of a qualitative histochemical staining for non-specific esterase activity. Both in conventional and germfree rats the esterase activity is extremely low in the proliferating crypt cell compartment and an increase in activity occurs during migration of the cells along the maturation zone at the upper half of the crypt.

Subsequently the carboxyl esterase activity in isolated duodenal crypts from conventional and germfree animals was compared at various time intervals after X-irradiation. The results of these analyses are presented in Table IV.

TABLE IV

CARBOXYL ESTERASE ACTIVITY* IN ISOLATED DUODENAL CRYPTS FROM CONVENTIONAL AND GERMFREE RATS AT VARIOUS TIME INTERVALS AFTER X-IRRADIATION (700 RADS)

	ENZYME ACTIVITY	
HOURS AFTER X-IRRAD.	CONVENTIONAL	GERMFREE
Control	18.8 ± 1.9	20.4 ± 1.2
24	18.0 ± 1.7	21.0 ± 3.8
30	-	20.4 ± 2.1
36	10.2 ± 2.3	17.1 ± 2.4
48	6.9 ± 1.1	13.8 ± 1.8
60	5.4 ± 1.3	6.6 ± 1.5
72	3.6 ± 0.4	4.2 ± 1.2
84	4.2 ± 1.2	5.7 ± 1.1
96	5.4 ± 1.0	7.8 ± 1.2
120	11.1 ± 2.4	12.0 ± 1.4
168	12.0 ± 1.8	16.2 ± 2.5
204	-	16.8 ± 2.3
240	15.9 ± 2.7	20.7 ± 2.6

* Enzyme activities are expressed in µmol naphthol / hour / mg dry weight. Each value is the mean of 10 samples per animal and from 2 - 8 animals per group.

In conventional rats a marked decrease in esterase activity (maturing crypt cell compartment) is observed between 36 - 48 hours after radiation, whereas in the germfree animals this occurs after 48 - 60 hours. In the period from 96 - 240 hours the esterase activity is gradually restored to normal values. From these data it is clear that the carboxyl esterase activity in isolated duodenal crypts, indicative for the presence of non-dividing maturing crypt cells, decreases about 12 hours later in germfree animals than in conventional ones after the same radiation dose.

4. Relation between functional villus cells and the process of cell proliferation and differentiation in the crypt

The present study was undertaken to determine whether the turnover time of the intestinal epithelium is related to the response of cell proliferation and -differentiation in the crypt during recovery after irradiation. The initial depopulation and subsequent repopulation of the crypt after irradiation was found to follow a similar pattern in germfree and conventional rats (Table I).
However, the increase in proliferative activity during recovery after irradiation as expressed by the percentage of labelled cells per crypt column occurs about 12 hours later in germfree rats than in conventional animals (Table I). The same is true for the decrease in carboxyl esterase activity in the crypt which is indicative for the non-dividing maturing crypt cell compartment (Table IV).

It appears from Table V, first two columns, that the mean cell number per longitudinally cut villus column is higher in the germfree (105 cells) than in the conventional animals (76 cells). After irradiation a temporary block in crypt cell production results in a decrease in the number of villus cells, but minimal numbers of villus cells are found about 12 hours earlier in the conventional rats (48 - 84 hr) than in the germfree animals (60 - 84 hr). This is in agreement with the difference in villus transit time (Figs. 3 and 4). In the last two columns of Table V the esterase activities of isolated villi are shown. In the non-irradiated controls the activity is about 20% higher in the germfree rats than in the conventional ones.

After irradiation a decrease of esterase activity occurs in the villus, which is probably the result of insufficient cell maturation in the crypt during the repopulation period. Again, minimal enzyme activities in the villi are found about 12 hours later in the germfree rats than in the conventional ones.

TABLE V

CELL NUMBER AND CARBOXYL ESTERASE ACTIVITY OF DUODENAL VILLI IN CONVENTIONAL AND GERMFREE RATS AT VARIOUS TIME INTERVALS AFTER X-IRRADIATION (700 RADS)

Hours after X-Irradiation	NUMBER OF VILLUS CELLS*		CARBOXYL ESTERASE ACTIVITY**	
	Conventional	Germfree	Conventional	Germfree
Control	76	105	88.2 ± 6.4	105.0 ± 8.4
24	80	85	89.4 ± 4.2	99.3 ± 8.4
30	--	77	--	105.6 ± 6.0
36	60	68	62.1 ± 6.6	87.9 ± 6.9
48	45	58	41.4 ± 6.0	80.4 ± 6.9
60	35	41	34.8 ± 6.0	68.1 ± 5.1
72	36	35	22.2 ± 2.4	46.2 ± 6.6
84	41	32	31.5 ± 3.9	35.7 ± 6.0
96	61	56	44.7 ± 4.5	52.2 ± 6.3
120	75	80	55.2 ± 7.5	78.6 ± 7.8
168	75	80	70.2 ± 6.9	97.5 ± 8.7
204	--	95	--	98.4 ± 3.9
240	--	95	69.3 ± 11.1	102.6 ± 5.1

* Each value represents the mean of 20 - 100 villi. Standard deviations vary from 7 - 15.

** In μmol naphthol/hour/mg dry weight.

To test a possible relationship between cell density of the villus and the moment of expansion of the proliferating crypt cell compartment, the number of villus cells and the percentage of labelled crypt cells at various time intervals after irradiation were plotted. The results in Fig. 9 show an inverse relationship between the number of villus cells and the proliferative activity in the crypt for both germfree and conventional rats. In both groups of animals the increase in the pool of proliferating crypt cells compared with control values occurs at the moment of a decrease in the number of villus cells and maximal proliferative activities are observed when minimal cell densities on the villi are recorded. However, these events occur about 12 hours later in germfree rats than in conventional rats. In both groups of animals the subsequent rise in the number of villus cells is accompanied by a decrease in the percentage of labelled cells in the crypt.

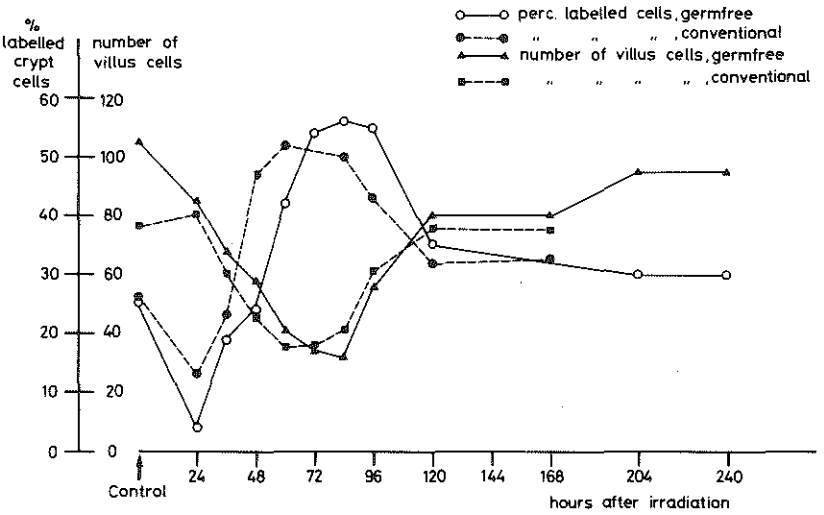


Fig. 9: Relation between number of functional villus cells and proliferation in the crypt

These data clearly indicate a relationship between the number of villus cells and the processes of cell proliferation and -differentiation in the crypt.

CHAPTER III

DISTANT REGULATION OF CELL PROLIFERATION AND MATURATION IN INTESTINAL EPITHELIUM

INTRODUCTION

In Chapter II experimental evidence has been described for a regulation of cell proliferation and maturation in the intestinal crypts via a feedback signal from the functional cells on the villus. The next question to be answered is whether this feedback information to the crypt cells is of a local origin or whether distant regulatory factors are involved. Is the regulation of proliferation mediated through a chemical substance, of, for instance, a hormone-like nature circulating in the blood, one would expect that a local destruction of villus epithelium would affect cell proliferation in the crypts in more distant parts of the intestine, not merely in the part where the villi are damaged.
Williams et al. (96) exteriorised a loop of gut and protected this with a lead shield from X-rays during whole body irradiation. Although they found definite alterations in the proliferation pattern of irradiated gut, the proliferative activity in the non-irradiated loop was normal. On the other hand evidence for a regulatory mechanism extending to more distant parts of the intestine than the particular villus-crypt unit, has been obtained by Loran et al. (61, 62). After resection of 10% of the distal ileum in rats they found an increased proliferative activity in the crypts, a higher migration rate along crypt and villus and an elongation of the villi in the remaining small intestine. Loran and Carbone (63) also used parabiotic pairs of rats and subjected one of the animals to a 10% resection of the distal ileum. They found definite alterations in the cell proliferation pattern in both animals of the parabiotic pairs 1 - 2 months after resection, whether the resection preceded the parabiosis or vice versa. The proliferation in the intestinal crypts had increased and the intermitotic time had decreased.

After separation of the parabiotic pairs in the non-resected animal the increase in the proliferative activity remained whereas the intermitotic time returned to normal. From these results Loran and Carbone postulated the existence of two distinct humoral factors circulating after resection. Many investigators have studied the influence of resection on the cell population kinetics in the intestine of the rat (8, 12, 25, 40, 49, 61, 62, 63, 73, 74, 88, 95), but unfortunately the results are in conflict. According to some

authors the number of labelled crypt cells is increased (40, 62, 63, 73), but others find the labelling index unaltered (40, 49). A normal (62, 63) and an increased (40) length of the crypt have been reported, and a lengthening (8, 25, 61, 88, 95) or a shortening (74) of the villi, and in some but not all cases a hypertrophy of the layers of the gut.

According to Loran et al. and Hanson et al. the intermitotic time of the proliferating crypt cells is decreased and the migration rate of the cell is increased (40, 61, 62). Experimental work on dogs (22, 27, 30, 48, 97) showed in most cases a hypertrophy of all layers of the gut. The length of the villi remained unchanged in the work of Knudtson et al. (48), but a lengthening is described in the work of Wilmore (97). The percentage of labelled cells in the crypt was found to increase after resection in dogs (48) whereas the same author found no change in experiments with rats.

In man relatively few cell kinetic studies have been performed (78) and most of these are concerned with the function of the gut after resection (2, 3, 24, 43) and the treatment of the general condition (15, 87).

In general the restoration of absorption in the rat seems to be faster and more complete after proximal resection than after distal resection (71, 72, 94). After studying the results of all authors mentioned it seems clear that nobody has as yet been able to prove Loran and Carbone's theory of the existence of humoral factors regulating cell proliferation in the intestinal crypt.

However, for future research in this field it is very important to know whether there is a local or a distant regulation of cell proliferation in the gut.
To get a clearer insight into this matter the present work, which involves studies on cell population kinetics at various time intervals after 10% or 40% resection of various parts of the small intestine, has been undertaken. In addition the process of cell maturation has been studied under these various conditions of resection by microchemical analyses of isolated crypts and villi. Apart from the longer time intervals after resection (30 and 60 days), for which distant regulation has been described by Loran et al., in the present investigation short time intervals(2 and 6 days) have also been included. The reasons for this are the short life span of the intestinal epithelial cells (36 - 48 hours) and the fact that an increased proliferative activity was found very soon (36 - 48 hours) after X-irradiation (Chapter II).

MATERIAL AND METHODS

1. Experimental design

64 Young adult male Wistar rats (170 - 240 g) were operated on (by Dr. L. Poulakos) according to the scheme presented in Table VI and Fig. 10. The rats were starved for 24 hours prior to surgery and anaesthesia was carried out by intraperitoneal injection of nembutal (40 mg per kg of body weight). A midline laparotomy was performed and the intestine was exteriorized from the ligament of Treitz to the cecum. The intestine was kept moist with warm physiological saline. As the length of the jejunum and ileum in these rats was found to be about 90 cm, 10% or 40% resection was obtained by removing 9 cm or 36 cm of intestine respectively. Entero-entero-stomy by the Connell method (serosa to serosa) was performed by means of a 6/0 silk suture and the incision was closed in two layers: muscles and peritoneum with 3/0 silk thread and the skin with wound clips.

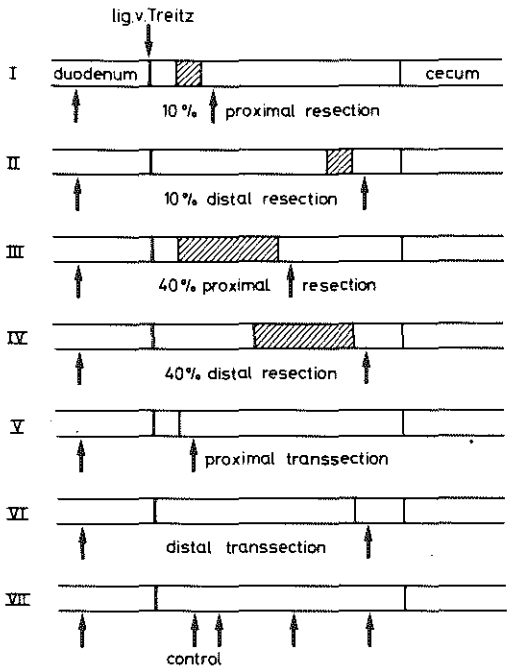


Fig. 10: Localization of analyzed intestinal segments

TABLE VI

SCHEME OF INTESTINAL RESECTION [3]

Number of Animals	Surgical group	Prox. incision of gut	Distal incision of gut	Resected cm	Resected %	Category	Post-operat. death
10	I	10 cm distal to A[1]	19 cm distal to A	9 cm	10%	Prox. resection	2
10	II	19 cm prox. to B[2]	10 cm prox. to B	9 cm	10%	Distal "	2
18	III	10 cm distal to A	47 cm distal to A	36 cm	40%	Prox. "	5
10	IV	46 cm prox. to B	10 cm prox. to B	36 cm	40%	Distal "	0
8	V	10 cm distal to A				Prox.transsect.	0
8	VI	10 cm prox. to B				Distal "	0

1) A = ligament of Treitz
2) B = cecum
3) 10 non-resected animals served as controls (group VII).

10 Wistar rats of the same age and weight served as controls. The animals received no solid food for the first post-operative day; 5 percent sucrose drinking water was available ad libitum. On the second post-operative day, each animal received some pellets (Hope farms, standard rat chow) and sucrose-containing drinking water. From then on all animals were fed a normal diet and tap water ad libitum.

2. Tissue preparation

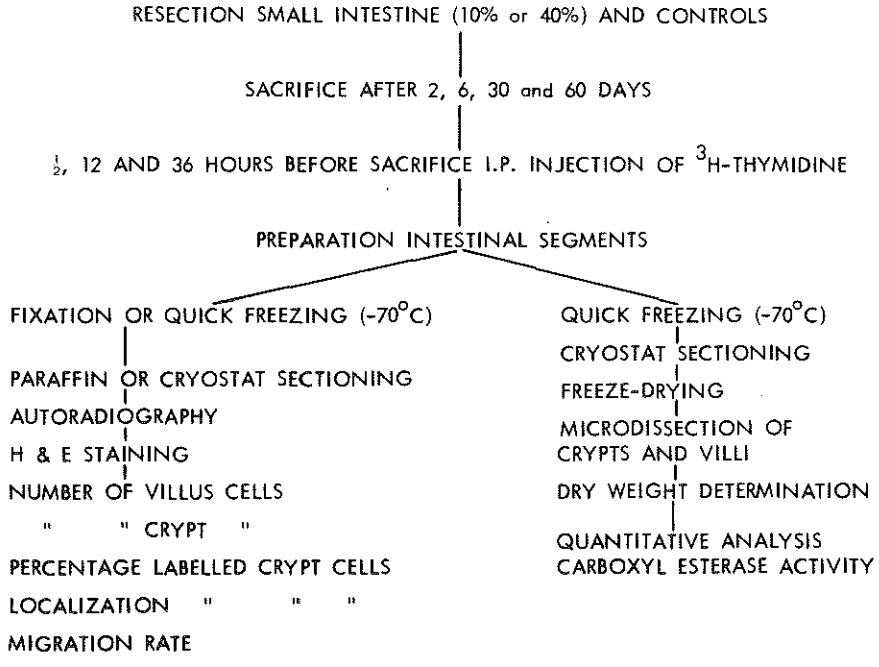
2, 6, 30 and 60 days after resection, two animals from each surgical group, together with 2 control animals, were injected intraperitoneally with 100 μ Ci ^{3}H-thymidine specific activity 2 Ci/mmol (Amersham, England) for studies on the proliferative activity in the intestinal crypts. After 30 minutes the animals were anaesthesized with ether and intestinal segments were taken according to the scheme in Fig. 10. As the distal incision of the gut varies with respect to the place of resection and the amount of intestine resected (see Table VI and Fig. 10), the localization of the intestinal segments taken at 1 cm distal to the resection suture is different for the different surgical groups. After 10% proximal resection the intestinal segment investigated corresponds with a sample 20 cm distal to the ligament of Treitz in the control. After 40% proximal resection this segment was originally situated at 47 cm distal to the ligament of Treitz and after 10% and 40% distal resection at 80 cm distal to the ligament of Treitz. Two controls and six animals from group III were injected with 100 μC ^{3}H-thymidine (spec. activity 15 Ci/mmol) 36 and 12 hours prior to sacrifice for studies on the migration rate of the intestinal epithelial cells. Intestinal segments were handled for further studies according to the experimental design given in Fig. 11.

From each site of the intestine indicated in Fig. 10 pieces of tissue were quickly frozen in CO_2 vapour (-70°C) and cryostat sections were further prepared and handled as described in Chapter II. Adjacent pieces of intestine were cut open and fixed for at least 48 hours in 10% (v/v) neutral formalin. After dehydration and embedding in paraffin, sections (4 μ) were cut on a Minot rotary microtome. The sections were then deparaffinized and either stained with hematoxylin-eosin or processed for autoradiography.

Autoradiography was carried out by dipping in Ilford K_2 emulsion and storing in light tight boxes at 18°C. After 5 weeks the slides were developed in Kodak D-19 developer for 5 min. at 18°C, fixed, rinsed, dried, stained with H & E and embedded in gelatin. In the resection experiments, the cryostat sections were also processed for autoradiography by the "dipping method" in contrast to the procedure described in Chapter II.

FIG. 11

EXPERIMENTAL DESIGN FOR THE STUDY OF PARTIAL RESECTION IN SMALL INTESTINE



3. Quantitative analyses on carboxyl esterase activity

Quantitative analyses on carboxyl esterase activity in isolated intestinal crypts and villi were carried out as described in Chapter II. From each intestinal segment, ten crypts and villi were dissected from various freeze-dried cryostat sections.

4. Determination of parameters to study the effect of resection

The total number of crypt and villus cells was counted in both paraffin and cryostat sections from at least two animals per group. Only longitudinally cut crypts and villi were selected for counting and the number of cells was expressed per crypt or villus column in about 50 - 100 columns from various sections.

To study the proliferative activity in the crypts the percentage of labelled cells was determined per crypt, using a minimum of five grains over the nucleus as a criterion for labelling. The localization of labelled cells was scored by determining the cell position from the bottom of the crypt in about 50 crypts for each animal.
An impression of the migration rate of the intestinal epithelial cells was obtained by the estimation of the highest cell position showing radioactive labelling at 12 and 36 hours after ^{3}H-thymidine incorporation.

As a parameter for crypt cell differentiation the carboxyl esterase activity of isolated crypts was used. Additional information about the functional villus cells was obtained by estimation of this enzyme activity in isolated villi. For each animal the enzyme activities were determined for a total of ten samples of crypts or villi and expressed per unit dry weight of the tissue fragments.

RESULTS

1. Cell migration in duodenum and jejunum from normal and resected rats

In Chapter II the migration rate of duodenal epithelial cells from conventional and germfree rats along crypt and villus was determined by the maximal cell position at which labelled cells were observed at various time intervals ($\frac{1}{2}$ h - 84 h) after injection

of ^{3}H-thymidine. In this chapter data are presented of the migration of duodenal and jejunal epithelial cells along crypt and villus at ½ h, 12 h or 36 h after ^{3}H-thymidine incorporation from rats who were subjected to a 40% proximal resection of intestine 2, 6 or 30 days prior to investigation. These time intervals after ^{3}H-thymidine injection were chosen as check points in order to get an impression whether or not there are consistent alterations in the migration rate after resection. For each time interval, the position of the highest labelled cells along 25 - 50 longitudinally cut crypt and villus columns was determined, (counted from the base of the crypt) for various sections from one animal. The mean values of the highest labelled cell position in duodenum, at ½ h, 12 h or 36 h after ^{3}H-thymidine injection, was determined 2, 6 or 30 days after 40% proximal resection. This was also done for the control animals. The results are presented in Fig. 12. At ½ h after injection, only cells belonging to the proliferative cell compartment in the crypt are labelled (cell positions 1 to 17). In the duodenum no expansion of the proliferation pool is observed at different time intervals after 40% resection (Fig. 12).

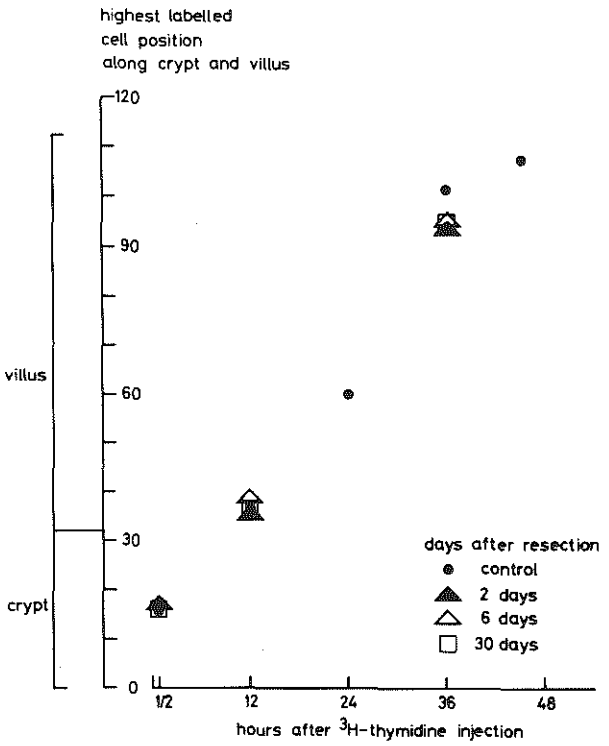


Fig. 12: Cell migration in duodenal epithelium from rats at different time intervals after 40% proximal jejunal resection

In the duodenum the migration rate along the maturation zone in the upper half of the crypt and along the functional cell compartment of the villus is about the same in the controls and the resected animals, as indicated by the slope of the curves in Fig. 12.

The same is true for the migration rate of the epithelial cells in the jejunum, which is illustrated in Fig. 13. The mean values of the highest labelled cell position at ½ h, 12 h or 36 h after ^{3}H-thymidine injection are shown for 2, 6 or 30 days after 40% proximal jejunal resection.

The turnover time, defined as the period between the last DNA synthesis of the cell in the crypt and the moment that the cell is shed off from the tip of the villus has not been determined in the present experiments, but is known to be between 36 and 48 hours for duodenum (Chapter II).

From the results of these experiments it may be concluded that the migration rate along crypt and villus in duodenum and jejunum is not influenced at 2, 6 or 30 days after 40% proximal jejunal resection.

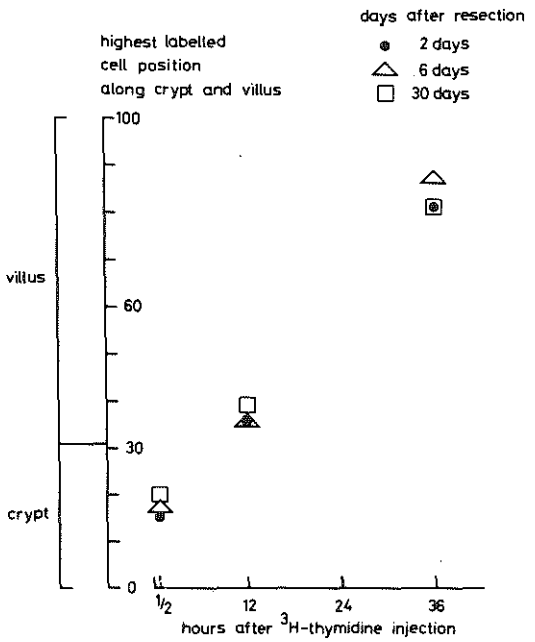


Fig. 13: Cell migration in jejunal epithelium from rats at different time intervals after 40% proximal jejunal resection

2. Cell proliferation in intestinal crypts at different time intervals after intestinal resection

a. Percentage labelled crypt cells

The total number of crypt cells and the percentage of labelled cells at 30 min. after ^{3}H-thymidine incorporation were determined in 50 - 100 longitudinally cut crypt columns from 1 - 3 animals at various time intervals after 10% or 40% proximal or distal intestinal resection.

The mean values of the total number of crypt cells and the percentage of labelled cells of the non operated controls at different sites in the small intestine are presented in Table VII.

In the control animals the total number of cells per crypt shows little variation at different sites in small intestine ($\bar{x}$ = 29 - 32) and the same is true for the percentages of labelled cells, indicative of the proliferative activity ($\bar{x}$ = 25 - 28%).

TABLE VII

CELL NUMBER AND PERCENTAGE OF LABELLED CELLS IN CRYPTS AT DIFFERENT LOCALIZATIONS IN CONTROL INTESTINE (AFTER ^{3}H-THYMIDINE INCORPORATION)

LOCALIZATION	NUMBER OF CRYPT CELLS			PERCENTAGE OF LABELLED CELLS		
	$\bar{x}$	s.d.	n	$\bar{x}$	s.d.	n
duodenum	32	1	85	26	8	85
20 cm distal to ligament of Treitz (jejunum)	30	2	50	27	7	50
47 cm distal to ligament of Treitz (jejunum)	29	1	167	28	9	167
80 cm distal to ligament of Treitz (ileum)	31	1	100	25	8	100

$\bar{x}$ = mean value, s.d. = standard deviation, n = number of estimations.

In Table VIII data are presented for the proliferative activity in jejunum or ileum at 2 or 6 days after 10% or 40% proximal or distal resection. The results are compared with those of non operated controls and those of rats where a distal transsection was carried out without resection of an intestinal segment.

These results indicate that shortly after intestinal resection the total number of crypt cells and the percentage of labelled cells in jejunum and ileum are not changed as a result of 10% or 40% resection of the proximal or distal part of the small intestine.

TABLE VIII

CELL NUMBER AND PERCENTAGE OF LABELLED CELLS IN CRYPTS FROM JEJUNUM OR ILEUM AT 2 AND 6 DAYS AFTER INTESTINAL RESECTION

	NUMBER OF CRYPT CELLS			PERCENTAGE OF LABELLED CELLS		
	$\bar{x}$	s.d.	n	$\bar{x}$	s.d.	n
Control	29 - 31			25 - 28		
2 days after 10% prox. resection	30	-	5	24	-	5
10% distal "	31	1	64	25	10	64
40% prox. "	31	1	50	21	12	50
40% distal "	31	1	53	27	11	52
6 days after 10% prox. resection	30	2	50	27	11	50
10% distal "	32	2	50	29	10	50
40% prox. "	30	2	20	29	10	20
40% distal "	33	1	38	34	13	38
distal transsection	32	1	100	22	12	100

$\bar{x}$ = mean value, s.d. = standard deviation, n = number of estimations.

Subsequently the total number of crypt cells and the percentage of labelled cells were determined at longer time intervals after 40% proximal or distal resection and compared with the values in non operated controls. The results are presented in Table IX.

After 40% proximal resection no differences were found in the mean values of the total number of crypt cells and the percentage of labelled crypt cells, even at longer time intervals after operation. After 40% distal resection a slight elongation of the crypt column was observed at 30 and 60 days after operation, but the percentages of labelled cells showed similar values to those in the controls.

TABLE IX

NUMBER OF CRYPT CELLS AND PERCENTAGE OF LABELLED CELLS IN JEJUNUM OR ILEUM AT VARIOUS TIME INTERVALS AFTER 40% INTESTINAL RESECTION

	NUMBER OF CELLS			PERCENTAGE OF LABELLED CELLS		
	$\bar{x}$	sd	n	$\bar{x}$	sd	n
Control jejunum	29	1	167	28	9	167
2 days after 40% prox. resection	31	1	50	21	12	50
6 " " 40% " "	30	2	20	29	10	20
30 " " 40% " "	33	2	100	30	11	100
60 " " 40% " "	32	2	29	26	6	29
Control ileum	31	1	100	25	9	100
2 days after 40% distal resection	31	1	53	27	11	52
6 " " 40% " "	33	1	38	34	13	38
30 " " 40% " "	35	2	100	26	10	100
60 " " 40% " "	34	1	50	28	9	50

$\bar{x}$ = mean value, sd = standard deviation, n = number of estimations.

A possible influence of partial resection of jejunum or ileum on the duodenal part of the intestine has also been investigated by determining the proliferative activity in the crypts. The results presented in Table X indicate that a 40% resection has no effect at all on the proliferative activity of the duodenum.

The results described in this paragraph indicate that there are no alterations in the proliferative activity in the remaining crypts after 10% or 40% intestinal resection, both after short time intervals and after longer periods.

TABLE X

NUMBER OF CRYPT CELLS AND PERCENTAGE OF LABELLED CELLS IN DUODENUM AT VARIOUS TIME INTERVALS AFTER 40% INTESTINAL RESECTION

	NUMBER OF CRYPT CELLS			PERCENTAGE OF LABELLED CELLS		
	$\bar{x}$	s.d.	n	$\bar{x}$	s.d.	n
Control	32	1	85	26	8	85
2 days after 40% prox. resection	33	2	50	24	8	50
6 " " 40% " "	33	2	25	-	-	-
30 " " 40% " "	32	1	50	27	6	50
60 " " 40% " "	31	2	50	25	7	50

$\bar{x}$ = mean value, s.d. = standard deviation, n = number of estimations

b. Localization of labelled crypt cells

To investigate whether partial intestinal resection leads to an expansion of the proliferation pool in the crypt the positions of the labelled cells were determined.

As the number of crypt cells at different time intervals after resection showed only minor variations, the localization of labelled cells were scored by positioning the cells from 1 at the bottom to 30 - 40 at the top of the crypt. The localizations of labelled cells in jejunum at different times after 40% proximal resection are illustrated in Fig. 14. Along the abcissa the various crypt cell positions are indicated and on the ordinate the percentages of labelled cells for each position in the crypt. In comparing the data after 40% proximal resection it must be taken into account that for 2, 6 and 60 days after operation only one animal and a limited number of crypts have been investigated. Nevertheless from the results obtained it seems unlikely that there are considerable differences in the distribution pattern of the labelled cells between the different groups of animals.

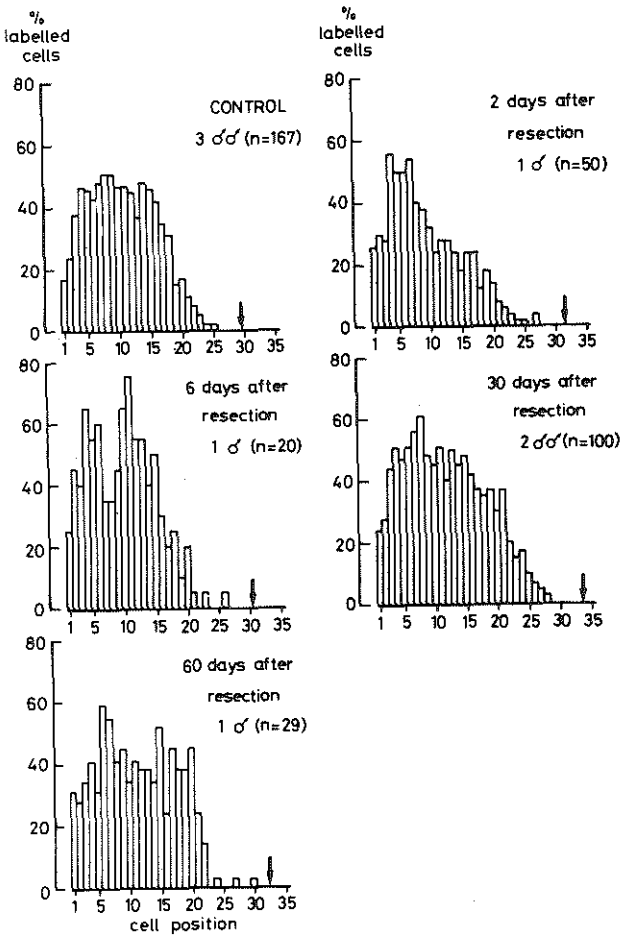


Fig. 14: Jejunum after 40% prox. resection (↓=crypt-villus junction)

The localization of labelled cells in the ileum at different time intervals after 40% distal resection is illustrated in Fig. 15. Here again the data indicate no expansion of the pool of proliferative crypt cells after partial resection of the intestine.

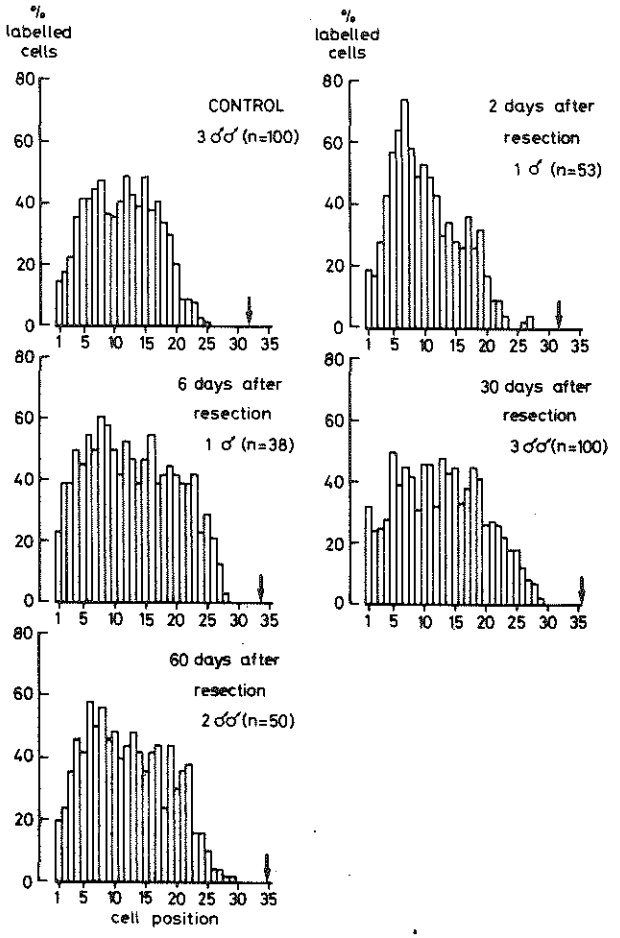


Fig. 15: Ileum after 40% distal resection (↓=crypt-villus junction)

3. The maturing crypt cell compartment at different times after intestinal resection

The previous results of the determination of percentage and localization of labelled crypt cells showed no significant changes in the proliferative activity after 10% or 40% intestinal resection. The investigation of the effect of resection on the non-dividing maturing crypt cells is described in this paragraph. As the activity of the non-specific esterases in isolated duodenal crypts proved to be a reliable parameter for this cell compartment microchemical analyses of carboxyl esterase activity in isolated crypts were carried out at various time intervals after 10% or 40% intestinal resection.

TABLE XI

CARBOXYL ESTERASE ACTIVITY* IN DUODENAL CRYPTS AFTER INTESTINAL RESECTION

	NUMBER OF ANIMALS	ESTERASE ACTIVITY CRYPT µmol / mg / hour $\bar{x}$ ± s.d.
Control	11	21.8 ± 3.1
2 days after 10% prox. resection	1	15.9 ± 4.8
2 " " 10% distal "	2	18.8 ± 4.2
2 " " 40% prox. "	2	27.9 ± 6.0
2 " " 40% distal "	2	33.3 ± 6.8
6 " " 40% prox. "	2	25.2 ± 7.7
6 " " prox. transsection	1	26.7 ± 3.9
6 " " distal "	1	13.5 ± 4.2

* Each value represents the mean of the results of 10 analyses per animal for various numbers of animals.

In Table XI the results of such analyses are presented for duodenal crypts at 2 or 6 days after resection.

In order to obtain the proper controls for the maturing cell compartment after resection of various parts of the small intestine the carboxyl esterase activity was analysed in isolated crypts from normal non-operated Glaxo rats at different localizations in the gut. In Table XII the results of these analyses are presented. The carboxyl esterase activity seems to decrease from the duodenum and upper jejunum downwards to the ileum. The analyses at 20 cm distal to the ligament of Treitz were only carried out on 10 samples from one single animal and are therefore of limited value.

Comparison of the data of Table VII and XII shows clearly that in the small intestine the proliferative activity proceeds independently of the carboxyl esterase activity. The proliferative activity is the same at different localizations in the small intestine whereas the process of crypt cell maturation, as judged by its carboxyl esterase activity, decreases from proximal to distal.

TABLE XII

CARBOXYL ESTERASE ACTIVITY IN INTESTINAL CRYPTS AT DIFFERENT LOCALIZATIONS IN THE GUT

	ESTERASE ACTIVITY* IN DIFFERENT ANIMALS µmol / mg / hour
duodenum	21.8 ± 3.1 **
jejunum 20 cm distal to the ligament of Treitz	27.9 ± 9.3
jejunum 47 cm distal to the ligament of Treitz	20.7 ± 4.2
	14.7 ± 4.2
	17.4 ± 5.4
	19.8 ± 6.0
ileum 80 cm distal to the ligament of Treitz	10.2 ± 1.8
	7.8 ± 2.1
	7.2 ± 1.8

*Each value represents the mean of 10 analyses from various sections per animal.

**This value is the mean of the results from 11 animals (10 samples per animal).

Now that the normal variation in carboxyl esterase activity along the gut was known, the next step was to evaluate the effect of proximal or distal intestinal resection on the segments 1 cm distal to the point of resection. In Table XIII the results of the determinations 2, 6 and 30 days after proximal resection are presented, and in Table XIV those at 2 and 6 days after distal resection. Although the variations among the different animals within the same surgical group are rather large, there seem to be no consistent effects on the maturing crypt cell compartment comparable to those found during recovery after irradiation (Chapter II).

TABLE XIII

EFFECT OF PROXIMAL INTESTINAL RESECTION ON CARBOXYL ESTERASE ACTIVITY IN JEJUNAL CRYPTS

	ESTERASE ACTIVITY μmol / mg / hour
Control 10% prox. resection (n =1)	27.9 ± 9.3
6 days after 10% prox. resection	16.5 ± 6.3
	21.3 ± 9.3
Control 40% prox. resection (n=4)	18.2 ± 5.0
2 days after 40% prox. resection	6.6 ± 3.0
	12.9 ± 3.6
	18.4 ± 4.8
6 days after 40% prox. resection	17.1 ± 2.1
	21.3 ± 5.4
30 days after 40% prox. resection	12.0 ± 4.2
	11.1 ± 3.3
	11.7 ± 3.9
	20.7 ± 5.4

The same is true for the effect of distal resection illustrated in Table XIV. If there is an effect, this is limited to the intestinal segments near the site of operation, but there is no indication of any long distance effect of resection on the remaining parts of jejunum or ileum.

TABLE XIV

EFFECT OF DISTAL INTESTINAL RESECTION ON CARBOXYL ESTERASE ACTIVITY IN ILEAL CRYPTS

	ESTERASE ACTIVITY μmol / mg / hr
Control 10% and 40% distal resection (n=3)	8.4 ± 1.9
2 days after 10% distal resection	11.1 ± 2.4
2 days after 40% distal resection	6.3 ± 1.5
	4.8 ± 1.8
6 days after 10% distal resection	4.8 ± 1.2

4. The functional villus cell compartment after intestinal resection

The functional villus cell compartment was studied in two different ways. The total numbers of cells were determined in 50 - 100 longitudinally cut villus columns from 1 or 2 animals at different time intervals after intestinal resection. Secondly, the carboxyl esterase activity in isolated villi was estimated at different time intervals after resection. For each animal the analysis was carried out in 10 samples dissected from various freeze-dried sections. To analyse the normal variation along the small intestine the lengths of the villi and their carboxyl esterase activity were determined at different localizations in the gut. The results of these determinations are presented in Table XV. Neither for the number of villus cells nor for the esterase activity was any difference found between the duodenum and the proximal part of the jejunum. However, from the ligament of Treitz downwards to the ileo-cecal valve the total number of cells per villus decreases and this also applies to the carboxyl esterase activity of the villus epithelium. As shown in Table XVI none of the different groups of resection exhibit a significant distant effect after 2 and 6 days on the carboxyl esterase activity in duodenal villi.

TABLE XV

CELL NUMBER AND CARBOXYL ESTERASE ACTIVITY OF VILLI AT DIFFERENT LOCALIZATIONS IN THE GUT

	NUMBER OF VILLUS CELLS				ESTERASE ACTIVITY IN VILLUS		
	number of animals	$\bar{x}$	s.d.	n	number of animals	µmol / mg / hr. $\bar{x}$	s.d.
duodenum	2	80	14	100	11	77.2	6.0
jejunum 20 cm distal to the ligament of Treitz	3	79	9	65	1	73.5	9.0
jejunum 47 cm distal to the ligament of Treitz	3	69	8	50	4	67.3	9.7
ileum 80 cm distal to the ligament of Treitz	3	58	6	100	3	31.6	4.6

TABLE XVI

CARBOXYL ESTERASE ACTIVITY* IN DUODENUM AFTER INTESTINAL RESECTION

	NUMBER OF ANIMALS	ESTERASE ACTIVITY VILLUS µmol / mg / hour $\bar{X}$ ± s.d.
Control	11	77.2 ± 6.0
2 days after 10% prox. resection	1	70.2 ± 9.9
2 " " 10% distal "	2	78.2 ± 9.5
2 " " 40% prox. "	2	89.1 ± 8.6
2 " " 40% distal "	2	99.6 ± 14.9
6 " " 40% prox. "	2	67.8 ± 10.2
6 " " prox. transsection	1	78.6 ± 9.3
6 " " distal "	1	72.9 ± 13.8

*Each value represents the mean of the results of 10 analyses per animal for various numbers of animals.

Subsequently the effect of partial resection was studied at different time intervals on intestinal segments 1 - 2 cm distal to the resection suture (compare Table VI and Fig. 10). The results of these analyses are presented in Table XVII and suggest a slight increase in the number of villus cells and some decrease in esterase activity at longer time intervals (30 - 60 days) after resection. No indication of any compensatory increase in enzyme activity in the remaining parts of the intestine after resection could be detected.

TABLE XVII

EFFECT OF INTESTINAL RESECTION ON CELL NUMBER AND CARBOXYL ESTERASE ACTIVITY OF THE VILLI IN REMAINING TISSUE AT 2 CM DISTAL TO THE RESECTION SUTURE

	NUMBER OF VILLUS CELLS*			ESTERASE ACTIVITY µmol / mg / hour			
	$\bar{x}$	±	sd	$\bar{x}$	±	sd	n**
Control 10% prox. resect.	79	±	9	73.5	±	9.0	1
2 days after 10% prox. resect.	69	±	7				
6 " " 10% " "				81.5	±	11.1	2
Control 40% prox. resect.	69	±	8	67.3	±	9.7	1
2 days after 40% prox. resect.	66	±	7	67.3	±	6.6	3
6 " " 40% " "	77	±	13	60.8	±	8.1	2
30 " " 40% " "	82	±	11	46.6	±	5.1	4
60 " " 40% " "	79	±	8	-		-	
Control 10% and 40% distal resect.	58	±	6	31.6	±	4.6	1
2 days after 10% distal resect.	-		-	46.5	±	5.4	1
2 " " 40% " "	64	±	10	35.0	±	6.9	2
6 " " 40% " "	56	±	7	-		-	-
30 " " 40% " "	75	±	10	-		-	-
60 " " 40% " "	63	±	7	-		-	-

* The mean values of the villus cell number are based on counting of 25 - 150 columns.

** n = number of animals.

CHAPTER IV.

DISCUSSION

The intestinal epithelium provides an attractive model for studies on regulation processes because of the regular microscopic distribution of proliferating, maturing, and functional cells along the length of crypt and villus.
This allows both cell kinetic studies, using autoradiography after ^{3}H-TdR incorporation, and histochemical and biochemical analysis in relation to cell age.

Cairnie et al., (18, 19) developed a model of the intestinal crypt, where all cells in the lower part of the crypt are involved in cell proliferation and each division gives rise to two proliferating cells. The non-dividing maturing crypt cells are localized in the upper third of the crypt and, according to this model, in between these two compartments there is a transitional zone where the cell has to make a critical decision to stop proliferation.

In our experiments this "critical decision zone" was found to be localized between cell positions 15 - 24, counted from the bottom of the crypt (see Fig. 6). A number of investigators found an increase in proliferative activity after radiation doses varying from 150 - 1250 rads, using different methods of investigation like mitotic counting (Williams, 96), autoradiography after ^{3}HTdR-incorporation (Lesher, 58, Matsuzawa and Tsubouchi, 68), or cell cloning (Withers and Elkind, 101).
Although some of the increased proliferative activity is supposed to be due to a shortening of the intermitotic time of the crypt cells (32, 58, 59) such an effect was not clearly apparent from the labelling indices in the lower crypt cell population during repopulation after 700 rads (Fig. 7). Moreover, shortening of the intermitotic time only occurs shortly after irradiation and the same is true for a possible synchronization of the crypt cell population. Galjaard and Bootsma (35) demonstrated that the increased proliferative activity after irradiation with 400 rads is based mainly on an expansion of the proliferative cell compartment at the expense of the non-dividing crypt cell compartment. Such an expansion was found to occur between 36 - 48 hours after whole body irradiation with 400 rads, whereas in our experiments the same time interval was found using 700 rads, (see Fig. 7 and Table I).
This suggests that the information for a crypt cell in the critical decision zone to

continue DNA synthesis is obtained after a time interval which is independent of the radiation dose. A similar conclusion can be drawn from the results of the histochemical studies, which showed that a reduction in the maturing crypt cell compartment occurred 36 - 48 hr after 50 - 400 rads (34).
This time interval corresponds remarkably well to the turnover time of intestinal epithelium as found for a variety of specimens (33).

In this connection it is interesting to quote Fry in the same review article "comparative data of the time at which recovery of the crypt population starts following irradiation in two species with markedly different turnover rates might provide a means of studying the problem of whether damage to the crypt population alone triggers repopulation, or, whether feedback from a depleted adult population is necessary".

In our experiments a comparison of cell proliferation and -differentiation in the crypt during recovery after the same radiation dose in germfree and conventional rat intestine was undertaken. This was based on the fact, discovered by various authors (1, 46, 57, 67), that a marked difference exists in the turnover times of the intestinal epithelium for germfree and conventional mice of the same strain. In all instances the turnover time in germfree mice proved to be about twice as long, which was caused mainly by a slower migration of the cells along crypt and villus. Some authors also found a larger number of villus cells in germfree animals. These differences could well be caused by variations in villus length and migration rate in various segments of the intestine (Matsuzawa, personal communication).
In our studies on rats the migration of the duodenal epithelial cells along crypt and villus was found to be similar in both germfree and conventional animals (Figs. 3 and 4), but in the former the villi were about 25 cells longer (Fig. 4). The difference in total turnover time between the two groups of rats (12 hr, Fig. 5) is thus considerably smaller than that observed in mouse intestine.

Our results in Table I indicate that during recovery after the same radiation dose the proliferative activity, as expressed by the percentage of labelled crypt cells, 12 hours later exceeds control values in the germfree rats. From the same table it is clear that this difference in response is not related to the cell density in the crypt, as the number of crypt cells in both groups is similar at all time intervals after irradiation. The distribution pattern of the labelled cells (Fig. 7) shows that the increased proliferative activity is based mainly on an expansion of the pool of proliferative cells toward the top of the crypt. In non-irradiated controls the percentage of labelled cells in the lower half of

the crypt is higher in conventional than in germfree animals. This difference is probably due to a different ratio between the duration of the S-phase and the intermitotic time (18, 19, 57, 68). During recovery after irradiation this percentage of labelled cells remains about the same in conventional rats but increases from about 50% to 70% after 84 - 96 hours in germfree animals. This increase could be explained by a shortening of the intermitotic time in germfree animals, whereas the shorter intermitotic time of crypt cells from conventional animals (57, 68) would offer little scope for a further reduction (51, 53). The main feature during recovery after irradiation in both groups is, however, the expansion of the proliferative cell compartment (Fig. 7). As a criterion for a completely proliferating crypt cell population we have used a labelling index of 50 - 60%. This is based on our results illustrated in Fig. 7 A which are in agreement with the ratio between the duration of the S-phase (6-8 hr) and that of the intermitotic period (10 - 14 hr) (18, 19, 57, 68). Using this criterion an expansion of the proliferating cell compartment was found to occur 36 - 48 hours after irradiation in conventional rat duodenum and 12 hours later in germfree animals.

The results of our microchemical analyses of the esterase activity as one of the parameters for crypt cell differentiation after irradiation (Table IV) also showed a decreased activity after 36 - 48 hours in conventional rats and 12 hours later in the germfree animals.

Using these two independent methods of investigation, an increase in proliferative activity and a reduction of the maturation zone are found to occur at different time intervals after irradiation, corresponding to the turnover time of the respective intestinal epithelium. The results in Fig. 9 show an inverse relationship between proliferative activity in the crypt and the number of villus cells. In both groups of animals the proliferative activity exceeds control values when the number of villus cells is reduced to 40 - 50 cells. Comparing the recovery after different radiation doses in conventional rats Rijke (83) found the expansion of the proliferative zone to occur independently of the radiation dose at the moment the villus length was reduced to the same number of 40 - 50 cells.

The intermitotic time of the crypt cells is 10 - 14 hours and the time of migration of the cells along the maturation zone in the upper half of the crypt is 12 hours (Fig. 4). A reduction in the number of villus cells therefore can result only in one additional cell cycle of the crypt cell, when repopulation of the crypt has taken place. The question remains as to the way in which a feedback signal from the villus cells

influences the critical decision zone in the crypt.

For further investigations on the nature of the feedback regulation it is of great importance to know whether local or distant regulatory mechanisms are involved. Various experiments with partial irradiation of the small intestine showed no alteration in the proliferative activity of the non-irradiated part of the intestine (11, 96, Osborne 1970, personal communication). Van Dongen and de Both (1972, personal communication) did not find any changes in proliferative activity in the intestine of the non-irradiated rat of a parabiotic pair after irradiation of the other rat. These facts are in conflict with the existence of a distant regulatory mechanism of the proliferative activity in intestinal crypts. The results of studies on the effect of resection on the remaining intestine are not in agreement with each other (40, 48, 49, 62, 63, 73). Loran's group is the only one that described a clear increase in proliferative activity after partial resection in the intestine of the non-resected partner in parabiotic pairs of rats. Other arguments for a distant regulation could be changes in proliferative activity after hypophysectomy, thyroidectomy, and lactation (20, 55), however, these effects might also influence crypt cell proliferation via the functional compartment (21, 31, 41, 92).

From the data in Chapter III it is clear that resection of different parts of the small intestine does not influence the proliferative activity or the process of cell maturation in the crypts of the remaining intestine.
These results are thus in agreement with the concept of a local regulation mechanism which has also been suggested by Bond (11), who concluded that: "the minimal functional unit in the bowel appears to be the villus and its associated crypts. Each unit appears to be essentially independent with little or no mechanism to alter or improve the functional state of even rather closely adjoining units".

The results described in Chapter II clearly show that an increased proliferative activity is accompanied by a decrease of at least one differentiation characteristic, i.e. the activity of carboxyl esterases (Table IV). This effect is also reflected in the functional cells of the villus (Fig. 8).
A decrease in the activity of other enzymes normally becoming active during cell maturation (34, 70, 75, 93) has also been described (13, 14, 38). Hence an increase in proliferative activity does not seem to be the best compensation for a reduction in total functional activity of the intestine. The concept of the mutual relationship between the proliferative activity in the crypt and the functional cells on the villus may also contribute to better understanding of intestinal diseases. Further studies on other enzymes involved

in intestinal function are required in order to be able to predict compensatory mechanisms after therapeutic intestinal resection in man.

Most authors accept that in non-tropical sprue the functional villus cells seem to be damaged as a result of hypersensitivity to the polypeptide gliadine. Others raise the question as to whether the primary defect is also extended to the crypt cells. According to our hypothesis, a reduction in the number of villus cells results in increased proliferative activity in the crypt, which in turn leads to insufficient cell maturation and cell function. On the other hand, studies on biopsy material from such patients could increase our knowledge about the regulation of cell proliferation and -differentiation in intestinal epithelium.

SUMMARY

For some cell renewal systems (skin, hemopoietic tissue, teeth) a feedback control by functional cells on the proliferative activity and the maturation process of early cell stages has been described. The aim of the present investigations was to study the mechanisms involved in the regulation of cell proliferation and -differentiation in another important cell renewal system, the intestinal epithelium. This tissue has the advantage of a regular microscopic distribution of proliferating, differentiating and ageing functional cells along the crypts and villi, which not only permits accurate cell kinetic studies but, using microtechniques, also enables biochemical analyses of the epithelial cells during their successive stages of differentiation. Furthermore, the short turnover time of the intestinal epithelium (36 - 48 hours) has the advantage that experimentally induced changes in the crypt cell kinetics will rapidly be reflected in the differentiating crypt cells and the functional cell compartment on the villus.

In the first part of this study (Chapter II) low doses of X-irradiation were used to reduce indirectly the number of functional villus cells via a temporary block in crypt cell proliferation. The effect of this was investigated in duodenal epithelium from both conventional and germfree rats. Using autoradiography at various time intervals after ^{3}H-thymidine incorporation we found that the turnover time of the intestinal epithelium in germfree animals was about 12 hours longer than in conventional rats of the same strain. This difference was based mainly on a longer transit time of the cells on the villus, which in turn was due to a larger number of cells lining the villus, whereas the cell migration rate was found to be similar in both categories of animals.

Irradiation with 700 rads resulted in a temporary reduction in the proliferative activity in the crypt followed by an overshoot. Autoradiography after ^{3}H-thymidine incorporation at different time intervals after irradiation revealed that during repopulation of the crypt a temporary expansion of the proliferative cell compartment occurs between 36 - 48 hours in conventional rats and after about 60 hours in germfree animals. Subsequently the proliferative activity gradually decreased to control values between 5 - 10 days after irradiation.

The expansion of the proliferative cell compartment in the crypt was found to occur at the expense of the number of non-dividing maturing crypt cells which normally migrate during 12 hours along the upper half of the crypt. This could be demonstrated by microchemical analyses of the carboxyl esterase activity, which was known to be a good

marker for the process of cell maturation in intestinal epithelium. Crypts and villi or parts of these were dissected from freeze-dried sections and, after determination of their dry weight on quartz fiber balances, the enzyme activity was measured using micro-volumes. After irradiation the esterase activity in isolated crypts was found to decrease after 36 - 48 hours in conventional rats and after 60 hours in the germfree animals.

By using these two independent methods of investigation after the same radiation dose an expansion of the proliferative cell compartment and a reduction in the maturation zone of the crypt were found to start 12 hours later in germfree rats than in conventional rats. These effects could be related to a reduction in the number of functional villus cells which is compatible with the hypothesis that there is a feedback control by villus cells on cell proliferation and -differentiation in the crypt.

The experiments described in Chapter III were designed to find out whether a local regulation or a distant regulation (for instance, via humoral factors) is involved. For this purpose partial resection of various parts of rat small intestine was carried out and the effects of this reduction in total number of functional cells on the cell proliferation and -maturation in the remaining intestinal segments were studied. Using the same techniques as in Chapter II, no changes in the number and localization of the proliferating cells in the crypts were observed at 2, 6, 30 or 60 days after 10% or 40% proximal or distal resection. Also, no indication was observed of changes in the process of cell maturation, as measured by the esterase activity in isolated crypt cells at various time intervals after the various resections.

These results are in agreement with the hypothesis that the regulation of cell proliferation and -maturation is of a local character. The villi and its associated crypts appear to be independent and show little or no alterations after changes in even closely adjoining segments of the intestine. The importance of sufficient knowledge about the cell kinetics and the process of cell maturation and -ageing for the understanding of the background of intestinal disease is discussed.

The main conclusions so far can be summarized as follows.

1. In the normal intestinal epithelium after a number of cell divisions the cell stops DNA synthesis half way along the crypt and proceeds with cell maturation during a 12 hour migration period along the upper half of the crypt.
2. Under "stress conditions" this decision to stop DNA synthesis can be postponed and the crypt cell is able to go through one extra cell cycle during migration along the

upper half of the crypt.

3. The performance of one extra cell cycle in the upper half of the crypt is accompanied by an insufficient development of at least some differentiation characteristics (i.e. the development of normal activities for some enzymes involved in intestinal function).

4. Insufficient cell differentiation is not made up during subsequent migration of the cell into the functional cell compartment along the villus.

5. The information to stop or to continue with cell proliferation in the crypt is probably regulated via feedback control by the functional villus cells.

6. The control mechanisms for cell proliferation and -differentiation are of a local character, and there are no indications that the villi and their associated crypts are influenced by changes in even closely adjoining segments of the intestine.

SAMENVATTING

De in dit proefschrift beschreven experimenten hadden ten doel de regulatie van celdeling en -differentiatie van een belangrijk "cell renewal system" , het darmepitheel te bestuderen. Voor andere "cell renewal systems" (huid, beenmerg, tanden) is een feedback-regulatie door de functionele cellen op delingsactiviteit en rijping van vroege celstadia beschreven. Het darmepitheel heeft een regelmatige rangschikking van delende, rijpende en functionele cellen langs crypt en villus, waardoor niet alleen de celkinetiek gemakkelijk kan worden bestudeerd, maar eveneens een biochemische analyse van de opeenvolgende stadia van differentiatie mogelijk is. Bovendien heeft het epitheel het voordeel van een korte "turnover"-tijd (36 tot 48 uur) waardoor experimenteel veroorzaakte veranderingen in de delingsactiviteit van de cryptcellen snel een weerspiegeling vinden in de gedifferentieerde cryptcellen en de functionele celpopulatie op de villus.

In het eerste deel van dit onderzoek (Hoofdstuk II) zijn lage doses X-straling gebruikt om het aantal functionele villuscellen indirekt te verminderen via een tijdelijke remming van de celdelingen in de crypt. De invloed op het duodenum van conventionele en kiemvrije ratten is vergeleken. De turnover-tijd van duodenumepitheel werd d.m.v. autoradiografisch onderzoek op verschillende tijden na inspuiting van ^{3}H-thymidine bepaald; deze bleek bij kiemvrije dieren ongeveer 12 uur langer te zijn dan bij conventionele ratten van dezelfde stam. Dit verschil werd veroorzaakt door een langer verblijf op de villus ten gevolge van een groter aantal villuscellen bij de kiemvrije dieren; de migratiesnelheid van de epitheelcellen was bij beide groepen dieren dezelfde.

Bestraling met 700 rads resulteerde in een vermindering van de delingsactiviteit in de crypt, gevolgd door een verhoogde proliferatieve activiteit. Op verschillende tijden na bestraling werd 30 min. na inspuiting van ^{3}H-thymidine het darmepitheel autoradiografisch onderzocht: tijdens de repopulatie van de crypt vond een tijdelijke uitbreiding van het compartiment delende cellen plaats en wel na 36 - 48 uur bij conventionele en na 60 uur bij kiemvrije ratten. Vervolgens daalde de proliferatieve activiteit geleidelijk weer tot normale waarden binnen 5 à 10 dagen na bestraling.

De uitbreiding van het compartiment delende cellen in de crypt vond plaats ten koste van de "pool" van niet delende, rijpende cryptcellen die normaliter in 12 uur vanuit het bovenste deel van de crypt opschuiven naar de villus. Dit werd aangetoond door microchemische analyse van de carboxylesterase-activiteit waarvan eerder was gevonden, dat het een goede maat is voor de celdifferentiatie in duodenumepitheel. Crypten en

villi of delen hiervan werden uit gevriesdroogde cryostaat-coupes gedissecteerd en na bepaling van het drooggewicht op kwartsdraad-balansen, werd de enzymactiviteit in microvolumina bepaald. Bij conventionele ratten bleek de esterase-activiteit in de geïsoleerde crypten 36-48 uur na bestraling te dalen, terwijl dit bij kiemvrije dieren pas na 60 uur het geval was.

Door gebruik te maken van 2 onafhankelijke methodieken werd dus gevonden dat na dezelfde stralingsdosis de uitbreiding van het compartiment delende cellen en de inkrimping van de maturatie-zône bij kiemvrije ratten 12 uur later begint dan bij conventionele dieren. Deze effecten konden worden gerelateerd aan een vermindering van het aantal villuscellen en dit is te verenigen met de hypothese dat er een feedback regulatie door de villuscel op celdeling en celdifferentiatie in de crypt bestaat.

De in hoofdstuk III beschreven experimenten hadden ten doel na te gaan of de regulatie lokaal of ook op afstand (b.v. via humorale faktoren) plaatsvindt. Hiertoe werd partiële resectie van verschillende delen van jejunum en ileum uitgevoerd en de invloed van deze vermindering van het totaal aantal functionele epitheelcellen op celdeling en -differentiatie in de resterende delen van de dunne darm bestudeerd. Met behulp van dezelfde technieken als beschreven in hoofdstuk II werden geen veranderingen in aantal en lokalisatie van de delende cellen in de crypt 2, 6, 30 of 60 dagen na 10% of 40% proximale of distale resectie waargenomen; eveneens werd geen aanduiding gevonden voor veranderingen in het proces van celdifferentiatie gemeten aan de esterase-activiteit in geïsoleerde crypten op verschillende tijdstippen na 10% of 40% proximale of distale resectie.

Deze resultaten zijn een steun voor de hypothese dat de regulatie van celdeling en -differentiatie op lokaal niveau plaatsvindt. De villi en bijbehorende crypten worden nauwelijks of in het geheel niet beïnvloed door veranderingen in aangrenzende segmenten van de darm. Het belang van voldoende kennis omtrent de celkinetiek en het proces van celdifferentiatie en veroudering voor een beter begrip van de achtergronden van darmziekten, is besproken.

De voornaamste conclusies kunnen als volgt worden samengevat.

1. In het normale darmepitheel stopt de cel na een aantal celdelingen, halverwege de de crypt, met DNA synthese en rijpt verder gedurende een periode van 12 uur waarin de cel opschuift naar de overgang van crypt naar villus.

2. Onder "stress" toestanden kan deze beslissing om de DNA synthese te stoppen worden uitgesteld en kan de cryptcel één extra celcyclus doormaken tijdens de migratie in de bovenste helft van de crypt.

3. Het volbrengen van een extra celdeling in de bovenste helft van de crypt gaat gepaard met een onvoldoende ontwikkeling van bepaalde differentiatie-kenmerken (i.e. de ontwikkeling van een normale activiteit van sommige enzymen die bij de darmfunctie betrokken zijn).

4. Onvoldoende celdifferentiatie wordt niet hersteld tijdens de migratie van de cellen in het functionele celcompartiment op de villus.

5. De informatie om in de crypt te stoppen of door te gaan met DNA synthese wordt waarschijnlijk via een feedback-mechanisme door de functionele villuscellen gereguleerd.

6. De regulatie mechanismen voor celdeling en celdifferentiatie zijn van lokale aard en er zijn geen aanwijzingen dat de villi en de bijbehorende crypten worden beïnvloed door veranderingen in aangrenzende darmsegmenten.

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