The Effects of Rotavirus on the Small Intestinal Epithelium of the Gnotobiotic Lamb.

Frances Grace Hay. B.Sc.(Hons.)

Ph D.

University of Edinburgh.

1983.

I declare that this thesis was composed by myself and that the work described was performed personally while I was a member of a research team.

Acknowledgements.

I wish to thank Dr Anne Ferguson for the encouragement, supervision and advice which she has provided in the last five years. I would also like to thank Dr D Snodgrass for his veterinary assistance, advice and supervision of my experimental animals and Dr S Tzipori who deputised in his absence. In addition I would like to thank my former colleagues, Miss G Paul and Mrs M Gordon for their assistance in the collection of the tissue samples, Mr A Sutherland who prepared all of the histology and Miss J Tocher who processed the scanning electron microscopy material.

Index.

Table of contents.

Publications.

Work described in this thesis is included in the following publications.

Ferguson A, *F Allan, D Al-Thamery. (1980).
Functional maturity of villus enterocytes in states of accelerated cell turnover without villus atrophy. In: Appleton D, JP Sunter, A Watson. eds. Cell Proliferation in the Gastrointestinal Tract. Tunbridge Wells: Pitman Medical. p343.

Snodgrass DR, A Ferguson, *F Allan, KW Angus, B Mitchell. (1979).
Small intestinal morphology and epithelial cell kinetics in lamb rotavirus infections. Gastroenterology. 76:477.

(*Published in my maiden name)

Abbreviations.

B cell.	bone marrow derived lymphocyte.
CCPR.	crypt cell production rate.
CMI.	cell-mediated immunity.
CO_2.	carbon dioxide.
DNA.	Deoxyribonucleic acid.
EM.	electron microscope.
g.	gravity.
3H.	Tritium.
HCl.	Hydrochloric acid.
H&E.	Heamalum and Eosin.
IE.	Intra-epithelial.
Ig.	immunoglobulin.
M.	Molar.
MIF.	Migration inhibition factor.
NCDV.	Nebraska calf diarrhoea virus.
p.	probability.
PBS.	Phosphate buffered saline.
pH	reciprocal of the logarithm to the base 10 of the hydrogen ion concentration.
RNA.	ribonucleic acid.
SEM.	Scanning electron microscope.
S.E.	Standard error.
TEM.	Transmission electron microscope.
TGE.	Transmissible gastroenteritis.
T cell.	Thymus-dependent lymphocyte.

Length.

cm	centimetre
mm	millimetre
µm	micrometre
nm	nanometre

Volume.

l	litre
ml	millilitre
µl	microlitre

Weight.

Kg	kilogram
g	gram
mg	milligram
µg	microgram

Symbols.

<	less than
%	percent
/	per

Summary.

Rotavirus is now recognised as the major cause of viral gastroenteritis in the young of both humans and animals. The disease has been extensively studied by immunofluorescent, histological and electron microscopic methods in several animal species and the pattern of disease is well defined. Susceptibility of individuals varies according to species, age and immune status. In susceptible animals the virus causes a severe diarrhoeal illness associated with pathological lesions of the small intestine. The disease is generally self-limiting and clinical recovery occurs within 5-7 days although fatalities can occur particularly in very young or germfree animals.

The mechanism controlling the diarrhoea, which occurs as a result of infection, is unknown but it is thought to be related either to quantitative or qualitative deficiencies of the villus epithelial cells. The former situation can arise in states of acute cell loss, the latter when increased rates of cell production and migration in the crypts give rise to a villus epithelial cell population composed of undifferentiated, immature cells.

The primary objective of this thesis was to test the hypothesis that rotavirus infection caused an increased cell proliferative response within the small intestinal crypts which in turn led to the formation of a functionally immature villus epithelium and that enzyme abnormalities of these cells was the direct cause of the diarrhoea.

The majority of previous experimental studies have concentrated on the location of virus within the epithelium or the pathological effects of the disease. None have involved detailed time course

experiments from the time of infection to beyond the time of recovery or attempted to correlate the alterations in mucosal architecture and intestinal cell kinetics with the onset of clinical illness and the distribution of virus within the small intestinal enterocytes. In my examination of the small intestinal epithelium of lambs I have used histology, a metaphase-arrest method with microdissection, scanning electron microscopy, immunofluorescence and a lactase assay to provide a comprehensive account of the response of this organ to rotaviral infection. These techniques were also used to compile a description of the small intestinal mucosa in normal gnotobiotic lambs to provide base-line information for the subsequent analysis of the changes associated with viral infection.

The effect of age on the susceptibility and mucosal response of the individual was tested by infecting one group of animals on the second day of life and others on the seventh day of life.

A limited structural study of the follicle-associated epithelium of the Peyer's patches and their response to rotavirus infection was performed.

Clinical signs of illness were produced only in the younger group of animals. The onset of the diarrhoea did not correlate with increased crypt cell production rates, the peak of crypt cell proliferation occurring after the diarrhoea had ceased. Virus was first detected within the villus enterocytes of the small intestine within 6 hours of infection and villus epithelial cell abnormalities were also noticeable at this time. It was concluded that the diarrhoea was more likely to be a direct result of epithelial cell destruction due to viral infection and replication processes rather than a result of enzyme deficiencies of the villus epithelial cells.

The distribution of virus in the enterocytes correlated positively with the development of the pathological lesions.

Villus atrophy was present at all sites of the small intestine in the younger group of animals. The onset and development of the lesions varied according to the site of the small intestine studied and the rate of recovery followed a similar pattern. Villus damage was more severe and persistent in the midgut and ileal regions and was accompanied by massive exfoliation of infected epithelial cells. Animals infected when seven days old showed little evidence of pathological lesions and the distribution and persistence of virus-infected cells within the epithelium was also less.

Examination of the cell kinetic responses in both groups of animals suggested that the stimulus for increased cell proliferation by the crypts involved more than one control mechanism. There was a local feedback mechanism which operated in response to the reduction of the villus epithelial cell population. This mechanism was responsible for the cell proliferative response which was initiated when villus atrophy and exfoliation of epithelial cells occurred. In addition an adaptive control mechanism of crypt cell production was probably involved. There were indications that this control mechanism operated independently of the size of the villus cell population. Evidence for this proposal came from the increase in crypt cell production which occurred in the midgut and ileum of the animals infected on the seventh day of life and showing only minor pathological effects.

The stimulus for the adaptive response was not detected but there were indications that it might be associated with a local CMI reaction to viral invasion. Virus was shown to gain entry to the 'M' cells of the Peyer's patch which are believed to be the antigen

processing structures of the lymphoid follicles of the small intestine.

Chapter 1.

Introduction.

Acute infectious diarrhoeal disease (gastroenteritis) has long been recognised to be a leading cause of morbidity and mortality in many areas of the world. Yet despite sophisticated bacteriological and parasitological investigations, the specific pathogen could be identified in only 50% of cases. The organisms responsible for non-bacteriological gastroenteritis were presumed to be viral (filterable agents) but it has only been within the last two decades that the agents responsible for viral diarrhoeas have been identified, initially by electron microscopy. This thesis concerns rotavirus, the principal agent of acute viral diarrhoeas in man and animals. The organ affected in acute rotavirus infection is the epithelium of the small intestine.

The pathogenesis of rotavirus infections has been studied by immunofluorescence, histology and electron microscopy, with similar findings in calves, piglets and lambs and also in calves infected with human rotavirus. The disease follows a well defined pattern. The virus initially infects the absorptive cells of the villus epithelium and these tall columnar cells are rapidly shed, to be replaced by immature cuboidal epithelial cells. Copious diarrhoea is produced in the first few days after infection, however clinical recovery usually occurs within several days and thereafter the structure of the small intestine appears essentially normal.

The mechanism by which rotavirus infection leads to diarrhoea (net water secretion by the intestine) is still disputed. Current thinking relates the diarrhoea either to a quantitative deficiency of absorptive cells as occurs in villus atrophy (i.e. loss of absorptive surface epithelium) or to a qualitative deficiency of absorption due to the presence of an immature population of

epithelial cells lining the intestine.

The primary objective of the work described in this thesis is to test the hypothesis (which had been formulated as a result of studies of epithelial cell enzymes) that in the course of the rotavirus infection, rapid production of cells from the crypts leads to the formation of a functionally immature villus epithelial cell population and that enzyme abnormalities of these immature enterocytes directly cause the diarrhoea.

The lamb rotavirus was discovered and identified in Edinburgh at the Moredun Institute where a research programme is in progress on the virology, pathology and immunology of the infection. I therefore had the opportunity to test the above hypothesis, using rotavirus infected and control gnotobiotic lambs, in collaboration with the team at Moredun.

For ethical and practical reasons, in that the techniques would be impossible to carry out in man, most of the work on this aspect of rotavirus infection has been done in animals. It has been necessary to use gnotobiotic animals for much of the work on viral enteritides. The main reason for this is that there is considerable variation between animals in anti-rotavirus antibody titres of serum, colostrum and milk which greatly influence the susceptibility of newborn animals. Possible transfer of antibody in colostrum therefore makes it necessary to use colostrum deprived animals. Colostrum is essential for the normal development of the conventionally born and reared calf, however gnotobiotic lambs can develop without colostrum and therefore provide hosts devoid of possible rotavirus immunity. The use of gnotobiotic animals has the further advantage that natural infection with rotavirus and other pathogens is avoided and the unpredictable gut changes associated

with bacterial colonization are excluded from the system.

The additional objectives contained within the study are as follows:

(1) to describe the small intestinal epithelium of the normal gnotobiotic lamb, with specific reference to the effects of maturation during the first few weeks of life, thereby providing base-line information for subsequent experiments.

(2) to describe the small intestinal epithelium of lambs infected on the second day of life with rotavirus, both during clinical illness and after the period of recovery.

(3) to correlate the abnormalities of the small intestinal epithelium with (a) the distribution of virus within the small intestinal epithelial cells and (b) the presence and absence of diarrhoea.

(4) to establish the relative involvement of the jejunum and ileum in rotavirus infection.

(5) to find out if the severity of the lesion was less in the small intestine of animals infected on the seventh day of life.

(6) to examine the appearance of the Follicle-Associated Epithelium in the region of the Peyer's Patch in normal animals and the possible effects of rotavirus infection on this epithelium.

An integrated approach to the investigation of the intestinal epithelium has been used. Epithelial cell morphology examined by histological methods and by scanning electron microscopy in addition to direct measurements of mucosal architecture and epithelial cell proliferation kinetics, have been correlated during the course of infection, at different levels of the gut, with the presence of viral particles within the epithelial cells and with the clinical manifestations of the infection.

Chapter 2.

The small intestinal epithelium.

Introduction.

The epithelium of the small intestine of a typical mammal consists of a continuous sheet of cells which extends from the bases of the crypts to the tips of the villi and is supported by the loose connective tissue of the lamina propria. Villi, which can be of various shapes, extend from the mucosal surface into the lumen of the intestine. The cylindrical crypts of Lieberkuhn (here after referred to as crypts) descend from the bases of the villi towards the muscularis mucosae. The typical structure of the small intestine is illustrated in Fig 2.1. The number of crypts associated with each villus varies with the site of small intestine under observation and between species, Fig 2.2. The epithelium of the small intestine is constantly renewed. Cells divide in the crypts and the absorptive cells migrate up the villi to be shed from the tips (Eastwood, 1977). The lifespan of individual cells within the epithelium varies from two to eight days according to the age and species of the individual and the microbial flora of the intestine under study.

There is a specialised epithelium over the lymphoid nodules in the gut (Peyer's patches); this will be described separately at the end of this section

Mitosis of stem cells located at the base of the crypts gives rise to the four major cell types found in the epithelium of the small intestine (Cheng and Leblond, 1974,c). The majority of these cells are columnar enterocytes whose function is the digestion and absorption of nutrients and the transport of ions and water. The remainder are mucus-producing goblet cells, enteroendocrine cells and Paneth cells. In addition there are many lymphocytes within the epithelium, the majority of which are T-dependent (reviewed by

Ferguson, 1978) and may have a role in the initiation of CMI (cell mediated immunity) responses (Ferguson and Mowat, 1980). Counts of intra-epithelial lymphocytes have been suggested as an indirect index of local CMI (Mowat and Ferguson, 1982) and will not be discussed further.

The compartments of the crypt.

The small intestinal epithelium has a highly ordered structure. The crypts reflect this order by their compartmentalisation into three distinct zones. There is a stem cell zone in the base of the crypt (Bjerknes and Cheng, 1981), a proliferative zone in the mid crypt (Cairnie, Lamerton and Steele, 1965; Cheng, 1974 a,b; Cheng and Leblond, 1974 a,b,c; Quastler and Sherman, 1959) and a maturation zone towards the top of the crypt. These zones are illustrated in Fig 2.3.

The compartments of the small intestinal crypts of the mouse have been extensively studied (Bjerknes and Cheng, 1981, ; Cheng, 1974 a,b; Cheng and Leblond, 1974 a,b,c;). In the normal mouse crypt the stem cells are limited to cell positions 1 to 4 from the crypt base. Stem cells have the capacity to proliferate throughout their life and their proliferation gives rise to both new stem cells and to daughter cells which proceed to differentiate fully. The micro-environment of positions 1 to 4 appears to be free from the signals which cause stem cells to differentiate, however, above position 4 the micro-environment is somehow altered and contains factors which induce differentiation. When, as a result of cell division, a cell moves from position 4 to position 5, these differentiation-inducing factors take effect and the majority of the differentiating cells, including the columnar, mucus and

enteroendocrine cells, migrate in an upward direction to populate the villi. These cells continue to divide as they move up through the proliferative zone until the maturation zone is reached and the cells lose the ability to divide. However all Paneth cells, a few columnar cells and a few enteroendocrine cells migrate downwards into the stem cell zone where they mature.

The epithelium of the gastrointestinal tract undergoes constant rapid renewal. This was first noticed almost a hundred years ago (Bizzozero, 1888) on the basis of mitotic counts, but the exact details of small intestinal cell kinetics were not elaborated until autoradiographic methods in the 1950's (Leblond and Messier, 1958; Leblond and Walker, 1956), confirmed the facts of epithelial cell renewal. The cell cycle, which is fundamental to the subject of cell kinetics, was defined using the intestinal crypt as a model system (Quastler and Sherman, 1959) and subsequently this system has continued to be used extensively in basic and experimental cell kinetic research.

The cell cycle.

The events occuring during the cell cycle are illustrated in Fig 2.4. Cell division is known as MITOSIS which is divided into four phases:- prophase, metaphase, anaphase and telophase. The chromosomes of the cell duplicate immediately prior to mitosis and are not visible in the normal non-dividing cell. During prophase the individual chromosomes become visible and as the cell moves into metaphase the chromosomes line up along the centre of the cell, linked to either end of the cell by a spindle. In anaphase, the chromosomes separate and move towards either end of the cell. In the final phase, telophase, the daughter cells are formed and division

is completed.

The second phase of mitosis, metaphase, is important in relation to this thesis as this is the site of action of the metaphase-arrest drugs used in stathmokinetic experiments to measure cell turnover rates. The drugs act by blocking the formation of the spindle and so prevent the cell from continuing to the next stage of mitosis. Over the period of time between the administration of the drug and the sacrifice of the animal, blocked metaphases accumulate in the crypts, thereby allowing the rate of entry of these cells into mitosis to be calculated. Mitosis is only a small part of the cell cycle, the remainder being composed of two gaps, G.1 and G.2 and a period of D.N.A. synthesis, the S. phase.

G1 is the gap which occurs between mitosis and the onset of S. phase. As G.1 progresses, the cells may either continue in the cycle to mitosis again, enter a resting phase, G.O. before returning to the cycle, or undergo differentiation for their final function. Cells continuing in the cycle synthesise R.N.A. and protein in late G.1. before entering the S. phase. It is during the S. phase when D.N.A. is being synthetised that 3H-Thymidine, used in cell labelling experiments can be incorporated (Johnson and Guthrie, 1974). The cells then enter G.2, the short gap during which R.N.A. and protein are again synthesised.

The rate of renewal in the intestinal epithelium is so rapid that cells do not enter the prolonged interphase, G.O, a feature of more slowly renewing tissues (Cairnie, 1976). The events of the cell renewal cycle are accompanied by great metabolic activity. Proliferating cells have patterns of nucleic acid metabolic enzymes which are characteristic and which alter as the cells differentiate and move out of the proliferative zone. For example, thymidine

kinase levels are high in proliferating crypt cells and the activity of this enzyme falls as the cells mature and migrate upwards onto the villi (Hamilton and Gall, 1982).

The relationship between the cytokinetic parameters of the small intestinal epithelium.

The villus and the whole of its attendant crypt population makes up an "intestinal proliferon" or proliferative unit (Zajicek, 1977) and all parts of this unit must be considered when assessing the process of cell renewal in the small bowel.

Most investigations of small intestinal cell kinetics concentrate almost exclusively on the crypt and factors which influence the crypt cell production rate (CCPR). However, from a functional viewpoint, the most important part of the mucosa is the villus epithelium, which is concerned with both secretion and absorption. Where cell proliferation in the gut is concerned, any control process, whether local or systemic, can elicit a change in the crypt cell production rate, whatever the kinetic mechanism. However, what is essential to the villus cell population is not only the rate of cell production, but also the net influx into the villus population, i.e. the product of the CCPR and the total number of crypts associated with each villus (the crypt-villus ratio), (Clarke, 1970a)

Many of the kinetic parameters involved can be measured accurately and their contribution to hypoproliferative or hyperproliferative mucosal responses assessed, but the controlling mechanisms involved have yet to be established.

The parameters affecting the CCPR and their relationship to the villus cell population are illustrated in Fig 2.5. Each will be

described separately.

In chapter 5, I have considered in detail which techniques of measurement would be appropriate for my project and the factors influencing their selection; in this section I have reviewed the methods available to measure the various factors involved in a study of small intestinal cell kinetics.

1. Duration of the cell cycle.

This can be measured by cell labelling techniques using tritiated thymidine (Leblond and Messier, 1958) or by stathmokinetic techniques (Wright, 1978). A shortening of cell cycle time can cause an increase in crypt cell production and in hyperproliferative states crypt cell production can be doubled, purely by the halving of the cell cycle time (Wright, Appleton, Marks and Watson, 1979; Wright, Watson, Morley, Appleton, Marks and Douglas, 1973). Similarly in the rat, decreases in the cell cycle time lead to increased crypt cell production rates during recovery from irradiation (Lesher and Bauman, 1969), recovery from crypt cell damage with cytotoxic drugs (Al-Dewachi, Wright, Appleton and Watson, 1981) or during the adaptive response to resection (MacDermott and Roundnew, 1976). A lengthening of the cell cycle time would lead to a decrease in the cell production rate, as occurs in the rat after a period of starvation (Al-Dewachi, Wright, Appleton and Watson, 1975)

2. Number of cells in the crypt population.

The crypt cell population can be measured by histological means by counting the number of cells in the crypt circumference (column count) and the number of cells along the length of the crypt (crypt

column), (Al-Dewachi, Wright, Appleton and Watson, 1977). Expansion of the whole crypt could theoretically affect any of the three compartments (i.e. stem, proliferative or maturation). A change in total crypt size is often associated with adaptive cell kinetic responses, such as occur after ileo-jejunal transposition experiments (Rijke, Hanson and Plaisier, 1977).

3. Size of the proliferative compartment.

In the small intestine, it is believed that all cells in the proliferative compartment of the crypt are cycling (Cairnie, 1976) and that none are in the resting G.0 phase. Cell labelling is used to establish whether or not mitosis is taking place at each cell position in the crypt. The position in the crypt where cells no longer exhibit mitosis marks the boundary between the proliferative compartment and the maturation compartment. Expansion or contraction of the proliferative compartment can be considered as a separate phenomenon to that affecting the total crypt as mentioned in the preceding paragraph. Increases in cell production rate mediated by changes in the proliferative compartment occur in many instances, for example in rat crypts during continuous irradiation (Cairnie, 1967) and in the crypts of human small intestinal biopsies showing villus atrophy (Wright, Watson, Morley, Appleton, Marks and Young, 1979); decreases in the proliferative compartment are found after starvation (Clarke, 1972).

4.Crypt cell production rate.

The crypt cell production rate is the central measurement when whole tissue structure and function are under study and gives information about the overall proliferative activity of the whole

crypt. Changes in CCPR may result from a change in cell cycle time, the size of the proliferative compartment, the total size of the crypt population or from a combination of all these factors and it also determines the efflux into the functional compartment. The CCPR is a measure of the number of cells which leave the crypt to move onto the villus in a given time, thereby influencing the number of cells per villus and ultimately the number of functional villus cells in the small intestine.

CCPR is generally measured using stathmokinetic techniques either in histological sections (Cairnie, Lamerton and Steele, 1965; Wright, Morley and Appleton, 1972) or using a microdissection technique. This method was devised by Clarke (1970a).

5. The crypt to villus ratio.

Each villus is supplied by a variable number of crypts, the numbers of which are measured directly by low magnification studies of tissue (Ferguson, Sutherland, McDonald and Allan, 1977; Smith and Jarvis, 1980). The ratio varies according to the site of the gut.

6.Influx: cell input from the crypts.

This is the product of the CCPR and the crypt to villus ratio, i.e. the rate of cell production per villus per hour.

7.Number of cells per villus.

If the subject undergoing study relates to absorption, the size of the villus population is vital although this is rarely measured directly (Zucolato, Wright, Bramble and Record, 1979). Most workers measure the heights of the villi in um or in cells in histological sections although this correlates poorly with absolute villus cell

number (Al-Mukhtar, Polak, Bloom and Wright, 1982) and does not reflect the shape of the villi (Creamer, 1964; Loehry and Creamer, 1969 a,b). Height measurements are useful in microdissected material where no shrinkage occurs (Clarke, 1974a; Ferguson, Sutherland, McDonald and Allan, 1977), particularly if the villi are cylindrical (finger-like) and is still a convenient way to give an estimate of the villus cell population.

8.Rate of cell efflux.

The rate of cell loss from the villus surface can be measured either by perfusion of the lumen and analysis of the subsequent rate of DNA loss (Croft, Loehry, Taylor and Cole, 1968), by counting the numbers of epithelial cells in the perfusate (Pink, Croft and Creamer, 1970), or by trapping the exfoliated cells in the mucus of the gut and counting them (Clarke, 1970b).

Cell labelling methods can also be used to calculate the time taken for villus cells to move along the length of the villus. When using this method it is necessary to make allowances for the cell loss which occurs on the sides of the villi below the tip (Potten and Allen, 1977). The subject of epithelial cell exfoliation has been relatively neglected in the past and it is likely that lumimal pressures such as the flushing action of fluid and the passage of food materials are relevant as well as the stimulus provided by the differentiating crypt cells and the underlying lamina propria.

9. Maturity of the villus cell population.

As the epithelial cells migrate upwards on the villus many changes occur in the enzyme profiles of the cells. Enzyme assays of small intestinal mucosal homogenates can be used to define the

maturity of the villus epithelial cell population. For example, the activity of disaccharidases within the epithelial cells increases and the level of thymidine kinase decreases as the differentiating cells move from the crypt to the villus (Gall, Chapman, Kelly and Hamilton, 1977).

10. Number of villi per defined unit of small intestine.

This is measured directly when the crypt to villus ratio is assessed. This, combined with the villus cell population is the 'real' functional unit of the small intestine.

Regulation of Epithelial Cell Turnover.

Normally there is a dynamic equilibrium between cell production within the crypt and cell loss at the tip of the villus. The regulatory mechanisms which are responsible for the maintenance of the balance between cell production and cell loss are still largely unknown although much work is currently in progress to investigate the factors which may influence the intestinal epithelium.

The functional capacity of the small intestinal epithelium depends primarily on the number of villus cells present. Alterations in the influx of cells from the crypt onto the villi can be influenced either by changes in crypt cell production or in the number of crypts per villus. No change in the crypt to villus ratio has been found under numerous experimental conditions including fasting, experimental-bypass, lactation, transposition or resection (Clarke, 1972; Clarke, 1975; Ecknauer, Clarke and Meyer, 1977; Harding and Cairnie, 1975; Rijke, Plaisier, Hoogeveen, Lamerton and Galjaard, 1975). Therefore it seems likely that it is the crypt cell production rate which determines the size of the villus cell

compartment and thereby the functional state of the villus epithelium.

Cell production in the crypts may change in two ways, reflecting two separate, theoretical control mechanisms (Rijke and Dongen, 1980). These are:

1. An adaptive control mechanism which would alter the total size of the crypt while retaining the normal ratio between the stem, proliferative and maturation compartments. The size of the villus cell population would be regulated by altering the size of the crypt cell population and subsequently the crypt cell production rate. Such an adaptive response occurs after ileo-jejunal transposition (Rijke, Hanson and Plaisier, 1977), during lactation (Harding and Cairnie, 1975), and as a result of partial resection (McDermott and Roundnew, 1976). The response occurs within 2 to 4 days of the stimulus and ceases after approximately 14 days (Hanson, Osborne and Sharpe, 1977; Rijke, Hanson and Plaisier, 1977). The adaptive control mechanism of crypt cell production may act locally (Clarke, 1974b; Rijke, Hanson and Plaisier, 1977; Rijke, Plaisier, Ruiter and Galjaard, 1977), or, systemically (Hanson, Rijke, Plaisier, Ewijk and Osborne, 1977) as is the case after partial resection. The mechanism of crypt enlargement is not known but may involve changes in the cell cycle time of the stem cells at the base of the crypt (Leblond and Cheng, 1976).

2. A feedback control mechanism of crypt cell production, which would regulate the relative size of the proliferative compartment in the crypt. This theory derives from the studies of acute cell loss due to temporary ischaemia (Rijke, Hanson, Plaisier and Osborne, 1976), and the acute stage of coeliac disease (Trier and Browning, 1970). In both of these states, a reduction in the functional villus

population was accompanied by a rise in crypt cell production rate which ceased when the villus population recovered. It is believed that this feedback control is a purely local response to alterations in the villus cell population as experimental procedures known to give rise to a systemic response had no effect (Rijke and Dongen, 1980).

Feedback control and adaptive control mechanisms may act separately or together in situations where epithelial cell renewal is altered. Many factors, extrinsic and intrinsic, have been implicated and proposed and have been recently reviewed, (Williamson, 1978; Eastwood, 1977). They include blood flow, innervation, local hormones such as enteroglucagon, chalones, circadian rhythms and luminal factors. Possible immunological effects due to a cell mediated response have also been proposed (MacDonald and Ferguson, 1977). All these influences are interdependent and are closely interwoven in the creation of the microenvironment of the epithelial cells. No one factor has yet been demonstrated to be totally responsible for the alterations in epithelial cell turnover which occur in the various hyperproliferative and hypoproliferative states which have been observed.

Species Differences and Age Related Differences.

Small bowel cell kinetics have been thoroughly examined in only a few mammalian species. All were found to have a progressive change in histological features and brush border enzymes during the neonatal period and around the time of weaning (Klodovsky, Sunshine and Kretchmer, 1966; Moon, 1971). These changes manifest principally by an increase in the rate of cell turnover and alterations in brush

border enzymes and must be taken into account in any experiment which involves very young animals.

In rats, the number of villi remains constant with increasing age, although the individual villi elongate and widen (Koldovsky, Sunshine and Kretchmer, 1966). In contrast, the intestinal villi of piglets, calves and lambs increase in number but decrease in length during the neonatal period (Moon and Joel, 1975). Studies in rats, pigs, lambs and calves showed slow replacement times in the newborn, with gradual acceleration as the animals matured.

A constant feature of the small intestine of calves, lambs, piglets and rats, was an increase in the number and length of the crypts during the first few weeks of life (Moon, 1971; Koldovsky, Sunshine and Kretchmer, 1966). The net impact of these species specific changes contributes to important functional differences between species.

The Germ Free State.

The influence of the normal intestinal flora on intestinal epithelial cell kinetics has been studied in germfree mice, pigs and cats. Germfree animals had an abnormal small intestine, with short crypts, very long villi and a scant amount of lymphoid tissue. Cell renewal was slow (Abrams, Bauer and Sprinz, 1963; Thake, 1968) in comparison with conventional animals and the apparent stimulatory effects of bacteriological colonization in the gut has been described as "physiological inflammation" (Eastwood, 1977). There was a rapid return to normal appearance on conventionalisation (Kenworthy and Allen, 1966). Animals reared with only one organism in the gut showed a mucosal morphology intermediate between the germfree and conventional states (Creamer, 1967).

The relationship between villus structure and function and crypt cell proliferation.

The size of the villus enterocyte population depends on the height and shape of the villus. Normally, villi are tall, finger or leaf shaped structures, but in disease states, height is often reduced and shape altered, with the formation of ridges or convolutions and in some diseases the villi may be totally absent. It has been shown (Creamer, 1964) that the alteration in villus shape can be related to the number of mature cells on the villus and correlates with the economical use of the available epithelial cells. However, this may be oversimplifying matters as the size and shape of the lamina propria may also play an important part (Marsh and Trier, 1974). Studies of rabbit jejunum have shown that the pericryptal fibroblasts, which form a major part of the structure of the lamina propria, also undergo a rapid turnover similar to that of the epithelial cells (Parker, Barnes and Kaye, 1974). During the course of cytodifferentiation, the intestinal epithelium acquires an intimate connection with the lamina propria and through interaction with its elements the intestinal cell fulfills its function (Sprinz, 1971).

It has been established that many enzymes are synthesised as cells move up the sides of the crypt towards the villus (Both and Plaisier, 1974; Nordstrom and Dahlqvist, 1973). In the neonate, one of the most important of those enzymes is the brush border disaccharidase, lactase, in view of the importance of lactose in the diet of young mammals. It was suggested that when there is an increased rate of cell migration towards the tips of the villi that lactase synthesis may be reduced (Rey, Schmitz, Rey and Jos, 1971).

To support this idea, lactase deficiency, with clinical lactose intolerance may be seen in children recovering from viral enteritis and other enteropathies, i.e. in situations where there is an increased production and migration of enterocytes (Walker-Smith, 1979).

Alterations in the functional capacity of the enterocytes, in disease states, has been relatively ignored until recently, most emphasis being placed on gross histological and morphological appearances. However, it is evident that to define the effects of disease on the small intestinal epithelium it is necessary to look at many aspects of structure, function and cell kinetics.

Follicle-associated epithelium.

Lymphoid cells are distributed all along the mammalian intestinal tract, between the epithelial cells and within the lamina propria. Large aggregates of lymphocytes are organised into follicles beneath the epithelium in the tonsils, appendix and within the Peyer's patches. These lymphoepithelial structures have been recognised for centuries by morphologists, but their significance has only recently become apparent (Parrott, 1976; Owen and Jones, 1974). The gut associated lymphoid tissue constitutes more than one third of the total lymphoid tissue in the body. Although prominent in young animals, in the adult state these lymphoepithelial organs become atrophic.

The Peyer's patches are scattered throughout the small intestine, with the greatest concentration in the distal part of the ileum. The lymphoid aggregates are separated from the lumen by a distinctive follicle-associated epithelium. Patches contain varying

numbers of follicles but each patch is only one follicle thick, thus preserving the close relationship between the follicle and the overlying epithelium. The follicular epithelium has been studied in mice (Chin and Hudson, 1971; Owen and Nemanic, 1978; Sobhon, 1971; Smith, Jarvis and King, 1980; Smith and Peacock, 1980), rabbits (Faulk, McCormick and Godman, 1970; Owen and Nemanic, 1978), swine (Chu, Glock and Ross 1979; Chu, Glock and Ross and Cox, 1979), monkeys, hamsters, dogs and humans (Owen and Nemanic, 1978).

The follicles contain three main components:- a germinal centre (B cells), internodular regions (T cells), and a dome area. The overlying dome epithelium (follicle-associated epithelium) consists of microvillus covered, absorptive, epithelial cells, goblet cells and microfold cells (M. cells), which have a characteristic surface configuration which differs from that of the villus enterocytes. These M. cells have short ridges or microfolds rather than microvilli on their luminal aspect. They form cytoplasmic bridges between the columnar, microvillus covered, epithelial cells and are intimately related to underlying nests of lymphocytes (Owen and Jones, 1974). The M. cells contain a tubulo-vesicular system represented by pits on their luminal surface and this system has been shown to allow the uptake of antigens and particles from the lumen and their transfer to the subjacent lymphoid cells (Owen and Nemanic, 1978).

The anatomical disposition of Peyer's patches and the features of the follicle associated epithelium seem ideally adapted to the sampling of antigenic material from the lumen of the gut. Follicle-associated epithelium has been found in association with all the major gut mucosal lymphoid tissue aggregates studied (Owen and Nemanic, 1978) and would appear to be concerned with the

induction of immune responses to antigens presented via the gut.

The origin of these M. cells is not known. It has been suggested that they may be formed from immature columnar cells and that all stages of the columnar cell cycle, including mitosis, are to be found spread across the entire surface of the follicle (Owen, 1977). It has also been suggested that M. cells are formed by the interaction of fully differentiated enterocytes and migrating lymphocytes within the epithelium of the follicle (Smith and Peacock, 1981).

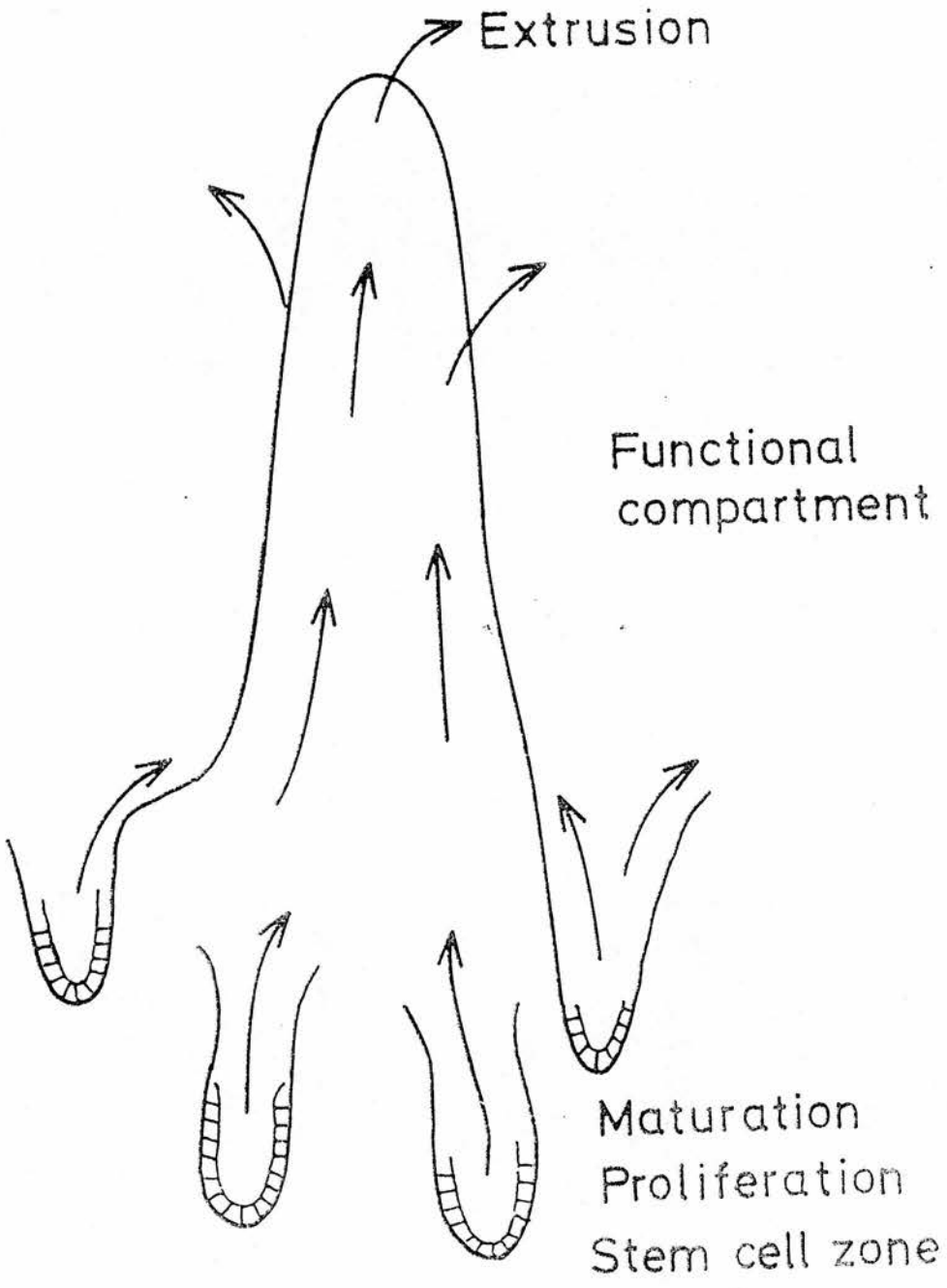

Fig 2.1. A diagrammatic illustration of the structure of the small intestinal mucosa of a typical mammal.

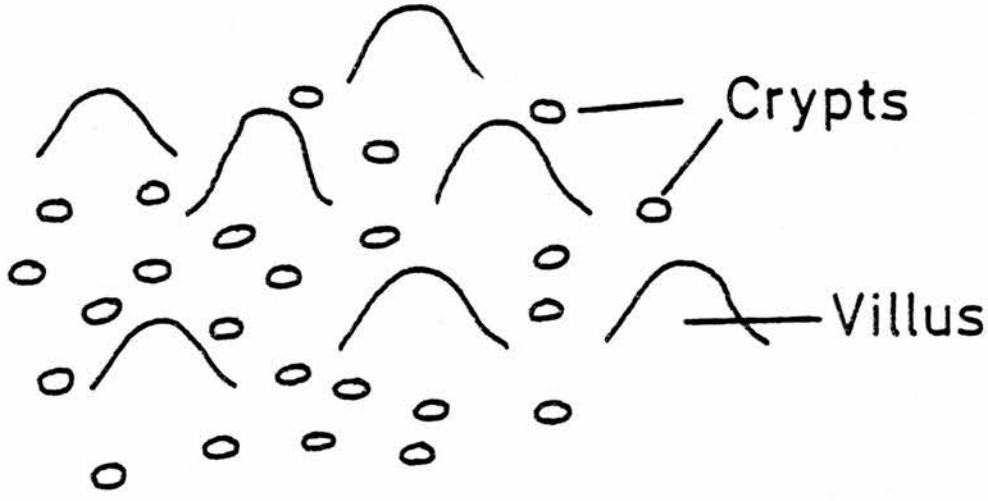

Fig 2.2. The distribution of the villi and crypts in the small intestine of a typical mammal. Each villus is surrounded by a variable number of crypts, depending on the level of the intestine.

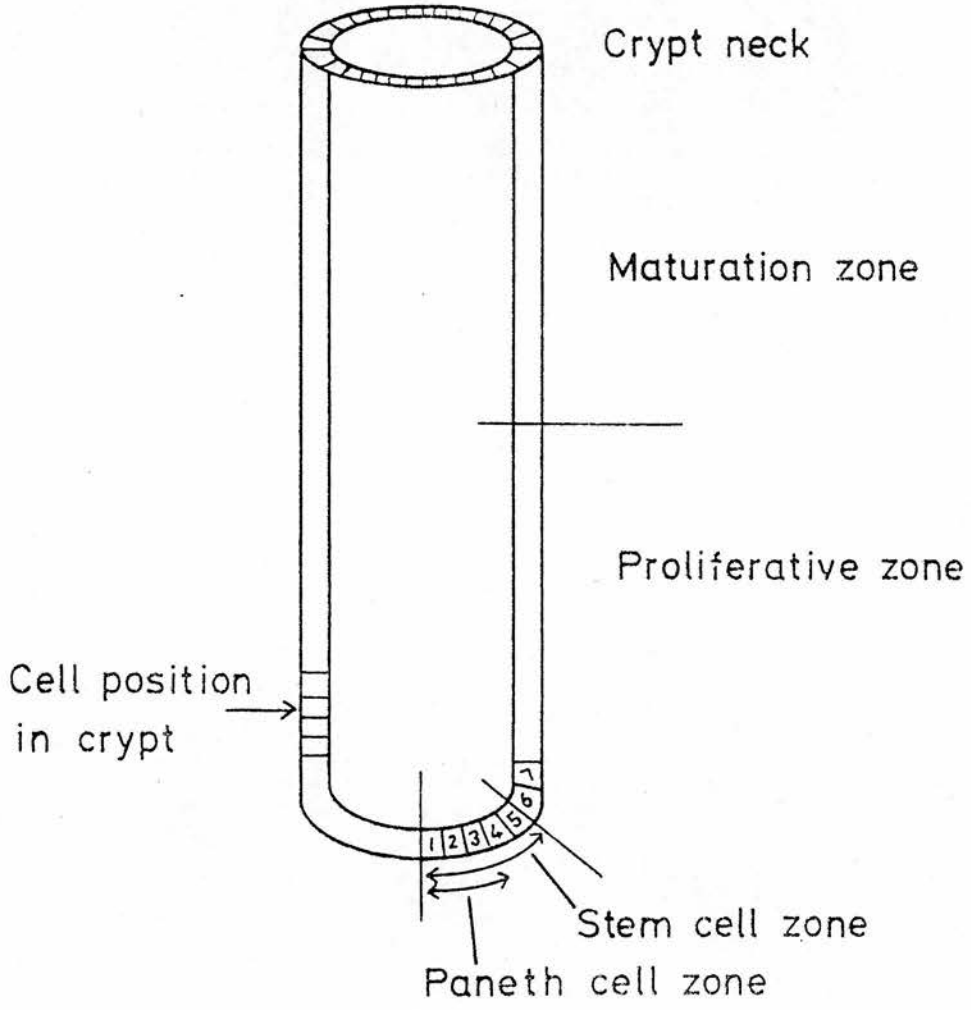

Fig 2.3. The compartments of the small intestinal crypt.

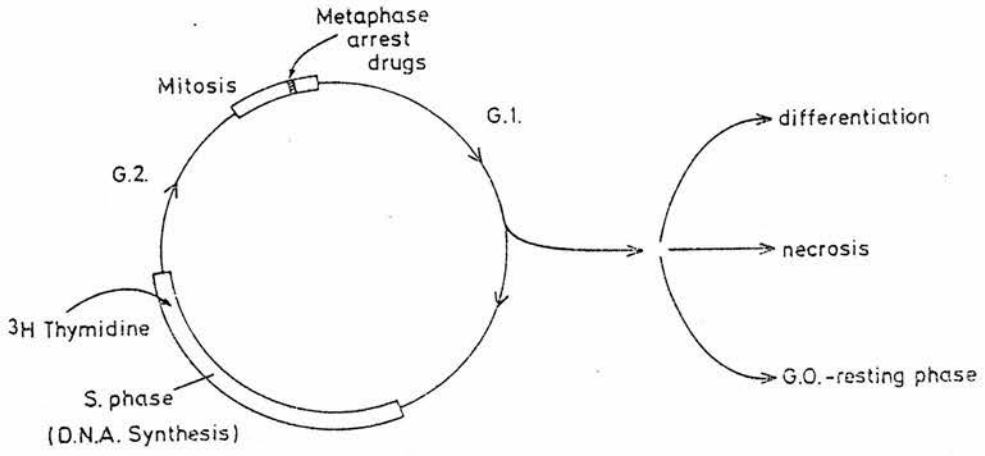

Fig 2.4. A diagrammatic representation of the various stages of the cell cycle in the small intestine.

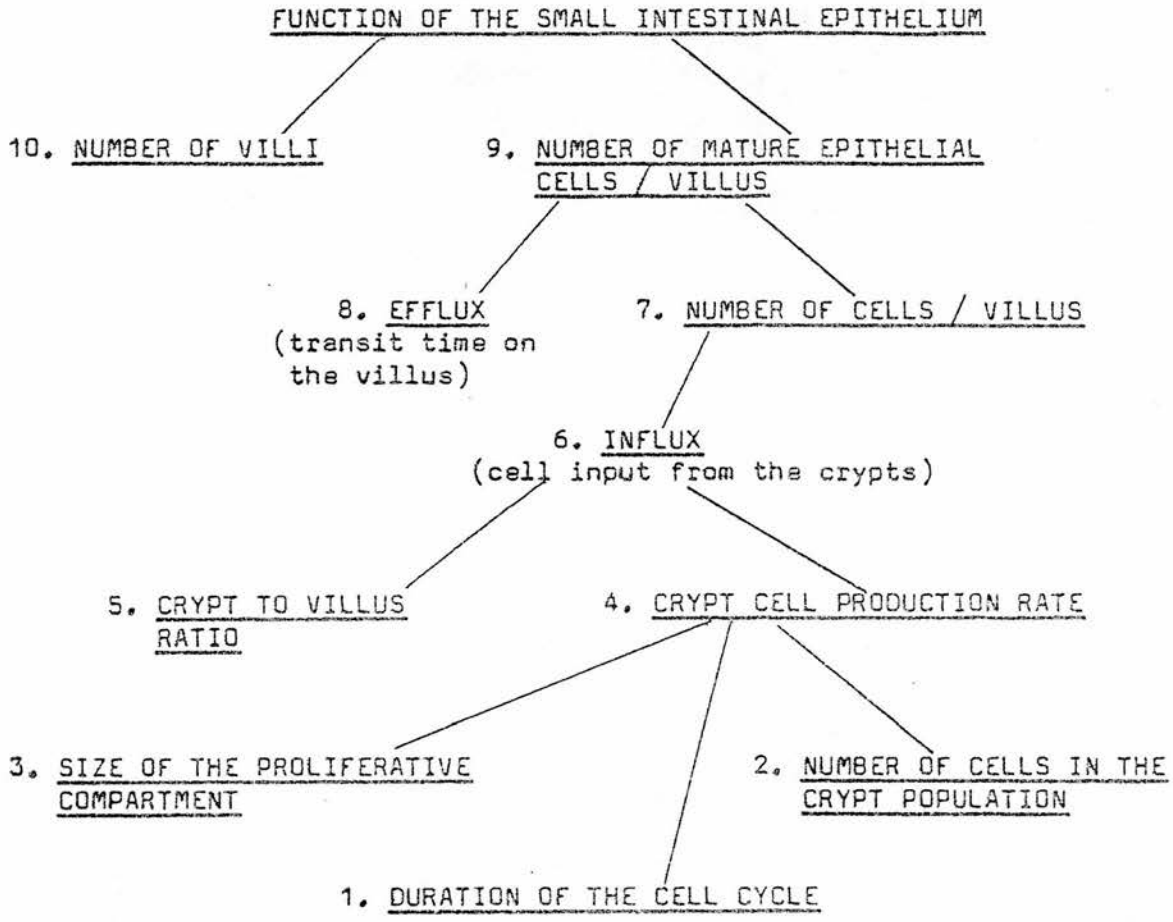

Figure 2.5. Relationships between the cytokinetic parameters of the small intestinal epithelium.

Chapter 3.

Rotavirus enteritis and the small intestinal epithelium.

Introduction.

Infectious gastroenteritis is a major cause of mortality and morbidity in children, particularly in developing countries, and is also a clinical problem in Western society, (Carlson, Middleton, Szymanski, Huber and Petric, 1978; Middleton, Szymanski and Petric, 1977). Several agents contribute to infantile diarrhoea, including bacteria, parasites and viruses. Several aetiological viral agents are known including the Norwalk group, (Thornhill, Wyatt, Kalica, Dolin, Chanock and Kapikian, 1977), astroviruses (Madeley and Cosgrove, 1975), coronoviruses, (Mebus, 1978), calicivirus, (Woode and Bridger, 1978) and adenoviruses, (Flewett, Bryden, Davies and Morris, 1975) but by far the most commonly associated with infantile gastroenteritis is rotavirus (Flewett and Woode, 1978; McNulty, 1978). It has been estimated that between 5 - 18 million childhood deaths per year are attributable to viral gastroenteritis in Asia, Africa and South America, (Elliot, 1976).

Viral gastroenteritis is also an important disease of domestic animals, especially when it affects animals in a commercial situation. Newborn animals are particularly vulnerable and mortality is high in this group. Much of the knowledge which has been gained about the aetiological agents of viral gastroenteritis has come from studies of animal models.

The historical background of rotavirus isolation.

The present era of rotavirus research can be traced back to 1969 when the Nebraska calf diarrhoea virus (NCDV) was isolated (Mebus, Underdahl, Rhodes and Twiehaus, 1969). This was later

identified as a rotavirus. However it is also now recognised that the earlier work in calves involving a diarrhoea induced by an inoculum of human faecal material (Light and Hodes, 1943) and studies in mice (Cheever and Mueller, 1947) also involved rotavirus. All subsequent identification of rotavirus in humans and animals has been confirmed by antigenic and morphological comparisons with NCDV.

Rotaviruses have been isolated from cases of diarrhoea in calves (Woode, Bridger, Hall and Dennis, 1974), lambs (McNulty, Allan, Pearson, McFerran, Curran and McCracken, 1976; Snodgrass, Herring and Gray, 1976), piglets (Lecce, King and Mock, 1976; McNulty, Pearson, McFerran, Collins and Allan, 1976; Woode, Bridger, Hall, Jones and Jackson, 1976), deer (Tzipori, Caple and Butler, 1976), foals (Flewett, Bryden and Davies, 1975; Tzipori and Walker, 1978), monkeys (Malherbe and Strickland-Cholmley, 1967), rabbits (Bryden, Thouless and Flewett, 1976), pronghorn antelope (Reed, Daley and Shane, 1976), kittens (Snodgrass, Angus and Gray, 1979) and dogs (Fulton, Pearson and Woode, 1981). Rotaviruses have also been isolated from avian species (Bergeland, McAdaragh, Reed and Stotz, 1976; McNulty, Allan and Stuart, 1978). Rotavirus was initially identified in man by electron microscopy of duodenal biopsies in 1973 (Bishop, 1973).

From the literature on rotavirus infections it is clear that rotavirus is endemic globally in man, many other mammals and some birds (McNulty, 1978). By the latter half of the 1970's, the pathology and pathogenesis of rotavirus infection had been investigated in several animal species including lambs (Snodgrass, Angus and Gray, 1977), calves (Pearson, McNulty and Logan, 1978; Mebus and Newnam, 1977; Mebus, Stair, Underdahl and Twiehaus, 1971) and pigs (Chasey and Lucas, 1977; Crouche and Woode, 1978;

McAdaragh, Bergeland, Meyer, Johnshoy, Stotz, Benfield and Hammer, 1980; McNulty, Pearson, McFerran, Collins and Allan, 1976; Pearson and McNulty,1979; Theil, Bohl, Cross, Kohler and Agnes, 1978; Torres-Medina and Underdahl, 1980) and mice (Coelho, Bryden, Hall and Flewett, 1981).

Clinical features of rotavirus infection.

Infection generally occurred as a sudden and rapidly spreading epizootic in young animals (Woode and Crouche, 1978). The incubation period varied from 15 - 96 hours and appeared to be dependent on the strength of the inoculum and the age of the animal (Woode, Bridger, Hall, Jones and Jackson, 1976). Depression and listless behaviour was common, vomiting could occur in piglets and profuse watery diarrhoea developed. The diarrhoea usually lasted 5 - 10 days and recovery generally took place without treatment although mortality was high in very young animals. The clinical severity of the disease varied considerably and virus particles were excreted in the stools for several days after clinical recovery. Clinical signs of respiratory disease sometimes occurred and this has also been noted in rotavirus infections of children (Tallet, MacKenzie and Middleton, 1977) although rotavirus particles have not been found in the respiratory tract. Subclinical infections occurred and were common in epidemiological studies of healthy infants and children (Scott, Madeley, Cosgrove and Stanfield, 1979).

Viral infection and replication in the small intestine.

Virus-intestinal cell interactions and viral replication patterns have been studied extensively using immunofluorescence, transmission electron microscopy and histological methods (Coelho,

Bryden, Hall and Flewett, 1981; Crouche and Woode, 1978; Pearson and McNulty, 1979). Infected cells were generally found initially in the proximal small intestine and rapidly spread to the more distal regions of the small intestine. Depending on the species, one or more areas of the small intestine could remain uninvolved (Crouche and Woode, 1978; Pearson and McNulty, 1977). The virus replicated only in the mature epithelial cells on the upper parts of the villi, never in the crypt cells. The epithelial cells were damaged by the virus replication process, the alterations consisted of vacuolization, necrosis, and finally desquamation of the infected villus cells, which in turn led to a progressive shortening of the villi. Early in the infection, infected cells were present on the upper halves of the villi and as the infection progressed the infected cells were shed and only isolated infected cells were then seen at the villus tips. The crypts became elongated and the villi were rapidly repopulated with newly formed crypt cells. The microvilli of these epithelial cells were short and poorly defined and the brush border was altered (Siaf, Theil and Bohl, 1978). At the height of epithelial cell shedding the lamina propria was occasionally devoid of an epithelial cell covering and in direct contact with the intestinal lumen (Mebus, Stair, Rhodes and Twiehaus, 1973; Pearson and McNulty,1977). The localization of the morphologic lesions correlated well with the distribution of virus as established by fluorescence studies (Theil, Bohl, Cross, Kohler and Agnes, 1978).

The extent of the epithelial cell damage varied from species to species and between individuals (Crouche and Woode, 1978), was associated with the infective dose (Hall, Bridger, Chandler and Woode, 1976), the virulence of the virus and the state of

differentiation of the epithelial cells of the villi (Thake, Moon and Lambert, 1973). The length of time during which virus presisted in the epithelium also varied according to the species involved, being present from 24 - 96 hours post-infection in piglets and 12 - 96 hours in lambs (Pearson and McNulty, 1977; Snodgrass, Angus and Gray, 1977), whereas in mice the virus was first detected two days after infection and persisted for sixteen days (Wilsnack, Blackwell and Parker, 1969).

As the course of infection progressed, the number of infected cells increased rapidly and reached a peak early in the infection then dropped as the infected cells were shed, limiting the disease, as the new cells which repopulated the villi were resistant to infection by the virus. The action of immune processes was also thought to have a role in limiting the infection (Porter, 1973).

As the crypt cells were resistant to the virus, the damage due to viral invasion was rapidly repaired by cell migration from the crypt (Pensaert, Haelterman and Hinsman, 1970). The new cells on the villi had the characteristics and enzyme profiles of undifferentiated crypt cells (Davidson, Gall, Petric, Butler and Hamilton, 1977). When the work described in this thesis was begun litle was known of the effects of rotavirus infection on the epithelial cell kinetics of the small intestine. The migration rates of the crypt cells were thought to be increased and the crypts were hyperplastic (Snodgrass, Angus and Gray, 1977).

The effects of rotavirus infection can be summarised as follows:

(1) the disease caused shortening of the villi and some degree of crypt lengthening.

(2) there was a lymphocytic infiltration of the villus lamina

propria.

(3) mitosis of the crypt cells of the small intestine was increased.

(4) there were alterations in the brush border enzyme activities in the epithelial cells of the villi.

(5) the infection was thought to progress along the intestine in a cephalocaudal direction.

(6) the columnar cells of the villi were replaced by cuboidal cells after the infected cells were shed.

(7) the diarrhoea which was present was possibly due to a loss of absorptive capacity of the epithelial cells in the small intestine.

The majority of investigations relating to alterations in the cell kinetics and the maturity of the epithelial cells in viral gastroenteritis were performed in pigs infected with transmissible gastroenteritis (TGE), (Butler, Gall, Kelly and Hamilton, 1974; Moon, Kemeny, Lambert, Stark and Booth, 1975; Thake, Moon and Larbet, 1973).

Transmissible gastroenteritis of pigs.

Studies of the pathogenesis and pathology of TGE indicated that the features of this disease were very similar to those of rotavirus infection. Viral destruction of the villus epithelial cells produced villus atrophy, subsequent crypt hyperplasia and an increased rate of cell migration from the crypts resulted in the repopulation of the villi with immature epithelial cells. The disease was self limiting and recovery was rapid after the infected cells were shed from the villi (Haelterman, 1972; Hamilton, Gall, Butler and Middleton, 1976; McClung, Butler, Kernzer, Gall and Hamilton, 1976; Morin, Morehouse, Solorzano and Olson, 1973).

However there were differences between the mucosal response in

rotavirus infection and that in TGE infection.

- the infected epithelial cells were exfoliated more rapidly in TGE infection.

- crypt cell migration was maximal at the same time as the diarrhoea was most severe but this has not been established in rotavirus infection.

The similarities between TGE and rotavirus infection were explored by Hamilton's group in Canada when they used TGE as a model to investigate the mechanisms of infantile viral diarrhoea (Kelly, Butler and Hamilton, 1972).

The mechanism of diarrhoea in viral enteritis.

The diarrhoea of viral enteritis appeared to be related to the functional and morphological damage which occurred as a result of viral invasion and replication within the villus epithelial cells. The subsequent compensatory response within the crypt was also implicated.

Experimental studies in TGE infected piglets and in a group of pigs infected with human rotavirus (Kelly, Butler and Hamilton, 1972; Davidson, Gall, Petric, Butler and Hamilton, 1977) indicated that the most important effect of viral invasion and replication was damage to the microvillus border of the epithelial cells. The microvillus border is involved in disaccharidase digestion and the synthesis of the glucose sodium carrier and Na+K+ATPase (Gall, Chapman, Kelly and Hamilton, 1977).

The activities of the disaccharidase enzymes, the glucose sodium carrier and Na+K+ATPase were greatly reduced in the jejunum and ileum of piglets following TGE and rotavirus infections and similar results were obtained in calves (Butler, Gall, Kelly and

Hamilton, 1974; Kerzner, Kelly, Gall, Butler and Hamilton, 1977; Phillips and Lewis, 1973; Shepherd, Gall, Butler and Hamilton, 1979).

Two different theories emerged from these observations.

Firstly, that the deficiency in disaccharidases, in particular, lactase, led to the development of lactase intolerance. The osmotically active lactose accumulated in the lumen, increasing the osmotic pressure in the small intestine which in turn caused both decreased absorption and increased secretion. Bacterial fermentation due to the accumulated lactose may also have contributed to the net secretion.

Secondly, the undifferentiated cells from the crypt which migrated upwards to replace the desquamated villus cells had a high secretory activity rather than an absorptive function for sodium and water (Moon, 1971) and it was assumed that the secretory capacity of the epithelium increased in association with the increase in the proportion of crypt-like cells in the epithelial population. The enhanced secretion could also be linked to the observed deficiencies in the glucose stimulated sodium carrier, which could lead to an accumulation of fluid in the small intestinal lumen.

It was the first theory which led to the formation of the hypothesis under test in this thesis, i.e. that rotavirus infection causes exfoliation of the damaged villus cells accompanied by an increase in crypt cell production rates, this leads to the presence of enzyme deficient epithelial cells on the villi and that this deficiency contributes significantly to the resultant diarrhoea.

The severity of the diarrhoea would be expected to vary according to the extent and site of the lesion if enzyme deficiencies and ion transport mechanisms have an important role in

producing the diarrhoea. There is recent evidence that this is in fact the case (Shepherd, Butler, Cutz, Gall and Hamiltom, 1979).

Pathogenesis and pathology of rotavirus infection in lambs.

Several experiments were performed in 1976 to study the pathogenesis and pathological effects of lamb rotavirus infection, involving immunofluorescence, histopathology and electron microscopy (Snodgrass, Angus and Gray, 1977; Snodgrass, Herring and Gray, 1976).

The rotavirus used in these experiments was initially isolated and identified by Dr D Snodgrass and his research team (Snodgrass, Smith, Gray and Herring, 1976) at the Moredun Institute. An outbreak of disease had occurred in a group of specific-pathogen free lambs and rotavirus particles were identified by electron microscopy of the diarrhoeic faeces. Immune-electron microscopy and immunofluorescent examination confirmed the identity of the virus.

Six gnotobiotic lambs were involved in the first experiment, three were infected on the second day of life with rotavirus and three were kept as controls. The lambs were observed for clinical signs of disease and allowed to recover, all six lambs were then infected at twelve days of age (Snodgrass, Herring and Gray, 1976). In the second experiment, ten gnotobiotic lambs were used, eight being infected on the second day of life and two kept as controls. The infected animals were killed 12, 18, 27, 42, 48, 96 and 144 hours after infection. Daily faecal samples were collected and examined for the presence of rotavirus particles by electron microscopy and immunofluorescence, as were the gut contents at post-mortem. Tissue was removed from three sites of the small intestine for histological and ultrastructural examination and for

immunofluorescent staining (Snodgrass, Angus and Gray, 1977).

The results of the preceding experiments.

The animals infected on the second day of life became anorexic and listless 12 - 16 hours after infection, they subsequently developed watery diarrhoea which continued for up to four days. Animals infected when twelve days old developed mild diarrhoea with no anorexia or other clinical features. Rotavirus particles were present in the faeces within 24 hours of infection and continued to be excreted for 6 - 7 days. Rotavirus particles were present in gut contents until 96 hours after infection. Lambs infected at twelve days of age excreted rotavirus particles in the faeces for only 1 or 2 days after infection.

Rotavirus immunofluorescence was present in the epithelial cells of the middle and distal regions of the small intestine from 12 hours after infection until 96 hours after infection.

Pathological changes were present in the middle and distal regions of the small intestine from 12 - 27 hours after infection. These comprised villus shortening, oedema, damage to the epithelial cells and infiltration of the lamina propria with eosinophils.

Elecron microscopic examination demonstrated many villus epithelial cells, in the middle and distal regions of the small intestine, containing virus, 12 - 18 hours after infection. From then until 96 hours after infection only isolated cells contained virus. Infected cells were only present within the anterior site at 18 hours after infection. Virus particles were never found within the crypt cells or in goblet cells. The virus was located within the distended cisternae of the endoplasmic reticulum and formed large vesicles in the cytoplasm. The microvilli of the epithelial cells

were generally undamaged.

It was suggested that rotavirus infection resulted in the rapid replacement of damaged epithelial cells with immature cells from the crypt.

The effects of rotavirus infection in lambs can be summarized as follows:

(1) susceptibility to viral infection varied with the age of the animals.

(2) lamb rotavirus multiplied in the villus epithelial cells of the middle and posterior small intestine.

(3) there were few, virus containing cells in the anterior small intestine.

(4) the presence of virus, identified by electron microscopy and immunofluorescence, correlated with the pathological changes observed in the small intestine.

(5) most damage to the epithelial cells of the small intestine occurred during the incubation period and immediately after the onset of clinical disease.

(6) all signs of disease had disappeared by six days after infection.

(7) infected cells were rapidly replaced by uninfected cells.

(8) there was the possibility of an immature villus cell population having replaced the exfoliated infected cells and this was consistent with the hypothesis that such a situation could lead to decreased absorption in the small intestine.

Chapter 4

Methods

Animals.

All the experiments were performed in gnotobiotic lambs. The animals used in my experiments were produced and maintained at the Moredun Institute in Edinburgh, under the supervision of Dr David Snodgrass.

The gnotobiotic lambs were produced by an established procedure (Hart, Mackay and McVittie, 1971). Briefly, oestrus in a group of ewes was synchronised by the administration of hormones, the date of mating was recorded and at 144 days gestation a caesarean section was performed and the uterus was removed under sterile conditions (Plates 4.1, 4.2). The unopened uterus was then immersed in a trough of antiseptic liquid and introduced into a sterile plastic isolator (Plate 4.3). The uterus was opened and the lambs were removed and rubbed dry (Plates 4.4 and 4.5). The lambs were maintained in pairs in sterile isolators, input and output from the isolators being through the entry port which was sterilised by peracetic acid. The lambs were fed sterilised, evaporated cow's milk, intake of which was recorded daily. Faecal samples were taken from the animals at the time of delivery and at regular intervals thereafter to ensure that the lambs remained gnotobiotic. The lambs remained in the isolators until they were taken to the operating theatre. A total of 45 lambs were used over a period of four years, lambs being available only from January to April each year.

Preparation of the viral inoculum.

The virus was isolated from an outbreak of disease in a group of lambs (Snodgrass, Smith, Gray and Herring, 1976). The virus was passaged once by oral inoculation in a newborn lamb and subsequently by several passages in gnotobiotic lambs. At each passage level,

faeces or intestinal contents were diluted to 20% in distilled water and filtered through a 0.45μm membrane. These filtrates were aliquoted and stored at -70°C. The rotavirus content of the filtrate was checked by direct electron microscopy.

Lambs were infected with 2mls of the filtrate, either orally or intranasally, on the second day of life. The lambs were observed for clinical signs and faecal samples were collected daily and examined by electron microscopy for the presence of rotavirus. Faecal samples for E.M. were prepared, either by making a 10-20% suspension of faeces in distilled water or by further clarification of this suspension by centrifugation at 10,000g for 30 minutes followed by centrifugation of the supernatant fluid at 150,000g for 45 minutes. The pellet was then resuspended in distilled water, a drop of the suspension was transferred to a carbon coated grid, stained with 1% phosphotungstic acid (pH 7.4) and examined. Faecal swabs were taken at intervals during the course of the infection and were cultured for bacteria.

All virology and bacteriology examinations were performed by menbers of staff at Moredun.

Necropsy procedures.

All surgical procedures were performed by veterinary surgeons in an operating theatre. Animals undergoing cell kinetic experiments were weighed and injected with vincristine sulphate (Oncovin, Eli Lilly and Co. Ltd.) at a rate of 1mg/kg body weight by slow intravenous injection. This drug blocked cells in mitosis at the metaphase stage of the cell cycle. The animals were anaesthetised one hour later by inhalation of halothane and nitrous oxide and maintained in this way for a further two hours. Two animals were

operated on simultaneously, an infected lamb and an age matched control. Laparotomy was performed between 9am and 12 noon on each occasion to minimise the effects of diurnal variations on the results of the cell kinetic measurements. The skin of the abdomen was shaved, opened and the small intestine was clamped at the three sampling sites. These were:

Site 1, jejunum, distal to the ligament of Treitz

Site 2, midgut, equidistant from the pylorus and the ileo-caecal junction.

Site 3, ileum (without Peyer's patch).

A piece of ileum containing Peyer's patch was also removed. At three timed intervals over the two hour period, samples were removed for cell kinetic analysis and at the first timed interval samples were also removed for histology, transmission and scanning electron microscopy, immunofluorescent studies and lactase assay.

Samples were treated as follows:-

Microdissection and cell kinetics.

the tissue was slit lengthwise and immediately placed in Clarke's fixative for 24 hours then transferred to 75% ethyl alcohol.

Histology:

the samples were slit open, mounted on card and placed in 10% buffered formalin.

Transmission and scanning electron microscopy:

small cubes of tissue were fixed in 3% gluteraldehyde in phosphate buffer (pH 7.4).

Immunofluorescence:

lengths of small intestine, approximately 10cms, were snap

frozen in a CO_2-isopentane mixture and then stored at -70°C.

Lactase assay:

lengths of small intestine (5cm) were stored in plastic vials at -20°C.

Animals not involved in cell kinetic experiments were deeply anaesthetised with sodium pentobarbitone. Samples were removed from the three sites described previously and treated in the same way.

Four lambs were used for the detailed study of the distribution of the lesions in the intestine. They were sampled comprehensively as before from multiple sites at 30cm. intervals from the pylorus to the ileo-cecal junction. These animals received intravenous injections of Oncovin, 1mg./Kg. body weight, approximately 60 minutes before the samples of small intestine were removed. The precise time interval between the administration of the Oncovin and the removal of the tissue was noted.

Immunofluorescence.

The samples were stored at -70°C. Frozen sections, 5μm thick, were cut, placed on a slide and fixed for 5 minutes in ice cold acetone. The slides were air dried and either stained immediately or stored overnight. They were then placed on racks and stained with convalescent gnotobiotic lamb rotavirus anti-serum at 1/20 dilution in PBS, incubated at 37°C for 30 minutes in a moist box and washed twice in PBS for 10 minutes and 5 minutes respectively. The slides were then stained with rabbit IgG anti-sheep IgG conjugated with fluorescein isothiocyanate (Wellcome) at a dilution of 1/20 in PBS. After incubation at 37°C for 25 minutes in a moist box, the slides

were again washed twice in PBS as before, dried and mounted with buffered glycerol. They were examined with a fluorescence microscope using ultra-violet light.

A PBS control was set up for each slide stained with rotavirus anti-serum and a positive slide from a rotavirus infected lamb was included with each batch of slides tested.

Microdissection and epithelial cell kinetics.

Feulgen staining of samples.

Small pieces of tissue, 2cm square, were cut from the stored material. The staining procedure was as follows:

10 minutes - 50% alcohol

10 minutes - tap water

6 minutes - hydrolysis in 1M HCl at 60°C.

10 minutes - tap water, 3 changes

20 minutes - Schiff reagent (Difco)

Specimens were then stored in tap water until examined.

Dissection method.

The tissue was orientated in a petri dish in water and examined with a dissecting microscope. The muscle layer was removed from the under surface of the tissue with fine forceps. The tissue was then placed flat, villus surface upwards, and using a cateract knife, a thin slice of villi and the associated crypts was cut using firm vertical pressure. The slice was placed on a slide in a drop of 45% acetic acid and a coverslip was placed on top.

Measurement of villus and crypt length.

The length of the villi and of the crypts was measured with a Wild M20 microscope fitted with a calibrated eye-piece. The x10 objective was used to measure the villi and the x40 objective to measure the crypts. At least 10 villi and 10 crypts were measured from each specimen.

The number of blocked metaphases per crypt.

The tissue slice was flattened by gently squashing the cover-slip with an orange stick. The individual crypts dissociated from the lamina propria and the blocked metaphases were readily identified and counted using the x40 objective on the Wild microscope. The number of metaphases in 10 intact crypts were counted and the mean value obtained. The number of cells produced /crypt/hour was calculated from the regression line of accumulated metaphases against time.

The number of crypts per villus.

The remnant of the 2cm square of tissue, processed for the preceding measurements was used. The stained tissue was destained by placing in 45% acetic acid for 24 hours at room temperature. The tissue fragment was placed, villi upwards, on a slide in a drop of 45% acetic acid, covered by a coverslip and examined on a microscope fitted with a squared grid eyepiece. By focusing up and down alternately the number of villi and crypts could be counted in the same area of gut. Six areas in each specimen were counted and the mean values calculated.

Number of cells per villus.

The tissue was initially processed in the same manner as for microdissection but was stained in Schiff reagent for 45 minutes instead of 20 minutes, followed by 10 minutes in tap water with three changes. The tissue was then placed in Geimsa stain (diluted 1 in 10 with water) for 30 minutes, rinsed in tap water to remove excess stain and examined with a dissecting microscope in a petri dish containing water. The muscle layer was removed as before and, using a cateract knife, thin strips, one or two villi thick were cut. Individual villi were separated from the strips with a fine needle, damaged villi were discarded. A single villus was then put in a drop of 45% acetic acid on a slide and covered with a coverslip. The length of the villus was measured and the image of the villus was projected, from a fixed distance, onto a screen and the outline of the villus was drawn on a piece of paper. The projected surface area of the villus was calculated by planimetry.

The villus was then gently pressed under the coverslip with the tip of a needle to spread the cells uniformly. The nuclei of the epithelial cells could be readily differentiated from the nuclei of lymphocytes, plasma cells, fibroblasts and polymorphonuclear cells. The number of epithelial cells was counted using the x40 objective, field by field, until all the fields on the slide had been counted. Total cell counts were performed on ten villi from each specimen.

Histology.

After fixation the tissues were embedded in wax and 5μm longitudinal sections were cut. Sections were stained with Heamalum and Eosin (H&E). Selected sections were specifically stained to demonstrate the presence of plasma cells and eosinophils (methyl

green, pyronin stain).

Villus and crypt length.

Villus and crypt lengths were measured in selected, well orientated sections. Villus length was measured from the tip of the villus to the neck of the crypt and crypt length from the neck of the crypt to the base of the crypt. At least ten measurements of villi and crypts were made in each specimen and the mean was calculated.

Quantification of intestinal lymphoid cells.

The lymphocytes within the epithelium (intra-epithelial lymphocytes) were counted by the method described by Ferguson and Murray, (1971). In well orientated sections the number of epithelial cells in a strip of villus epithelium and the number of associated intra-epithelial lymphocytes in the same strip were counted. This was repeated until approximately 500 epithelial cells had been counted. The number of lymphocytes was expressed per 100 epithelial cells present. The lymphoid cells of the lamina propria were assesed subjectively.

Electron microscopy.

As soon as possible after fixation the sample was divided for transmission electron microscopy and scanning electron microscopy. Tissue for T.E.M was cut into small pieces and allowed to finish fixation. The samples were then processed, embedded in Araldite and sectioned. Areas for ultra-thin sectioning were chosen from Geimsa stained, 1μm thick, semi-thin sections. Ultra-thin sections were examined using a Philip's EM300 electron microscope.

Scanning electron microscopy.

Tissue for S.E.M was pinned flat in wax filled dishes and allowed to finish fixation. The samples were then processed through graded acetones, critical point dried, mounted on specimen holders and coated with gold. This processing was performed by Ms J Tocher of the Teaching and Research Unit, Western General Hospital.

The specimens were examined with an I.S.I 60 scanning electron microscope.

Lactase assay.

The tissue stored at -20°C was weighed and the lactase activity was estimated using a modification of the Dahlqvist method (Dahlqvist, 1964) as follows.

The tissue was homogenised in distilled water, 10mg/1ml, in an ice bucket and centrifuged at 400g for 10 minutes.

Tubes were prepared as follows:-

Reagent blank: 125µl distilled water

Standard: 15µl glucose standard + 100µl distilled water.

Tests: 25µl homogenate + 100µl substrate.

Substrate blanks: 25µl distilled water + 100µl substrate.

The tubes were placed in a 37°C water bath to equilibrate. The reaction was started, timed exactly, by the addition of the substrate buffer to the tests. The reaction was stopped after 60 minutes by adding 1.5 mls of TGO to all tubes, the tubes were left in the 37°C water bath for one more hour and then the optical density was read at 420nm against the reagent blank in glass micro-cuvettes. The results were expressed as millimoles of substrate hydrolised per gram of tissue (wet weight) at 37°C.

List of reagents required for the lactase assay.

Maleate buffer: 0.0625M, pH 6.0, used to prepare substrate solution.

Substrate solution: Lactose, 0.035M: 0.631g in 50mls maleate buffer.

Tris buffer: 0.5M, pH 7.

Peroxidase: 1mg/ml dissolved in water.

O-Dianisidine: 100mg dissolved in 20mls ethanol (96%).

Triton x 100 : 20mls dissolved in 80mls ethanol (96%).

TGO reagent : 4mg glucose oxidase, 2.0 mls O-dianisidine, 0.5mls peroxidase, 1.0mls triton x 100 solution to 100mls of Tris buffer.

Glucose standard : 100mg in 100mls water.

Other solutions used.

Clarke's fixative:

3 parts ethyl alcohol / 1 part glacial acetic acid.

Phosphate buffered saline (PBS).

To 2 litres of distilled water was added:

3.55 g $Na_2PO_4.H_2O$

0.685 g KH_2PO_4

17.532 g NaCl

The pH of the solution was 7.4.

Statistics.

All results of villus and crypt measurements were expressed as the mean +/- 1 standard error (S.E)

The Student's 't' test was used to assess any differences in villus height and crypt depth between groups of animals.

The rate of metaphase accumulation per crypt per hour was calculated from the slope of the line derived from the regression coefficient of the graph of metaphase accumulation against time. Co-varience analysis was used to detect differences in the gradients of the regression lines.

All calculations were performed with a Texas TI-44 calculator.

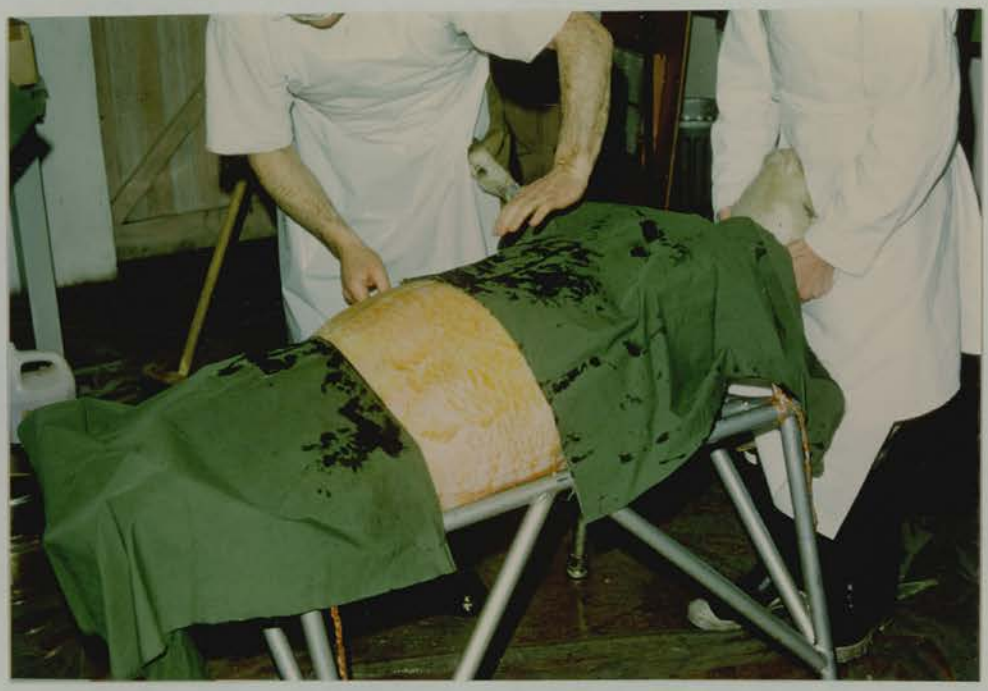

Plate 4.1. A ewe prepared for laparotomy.

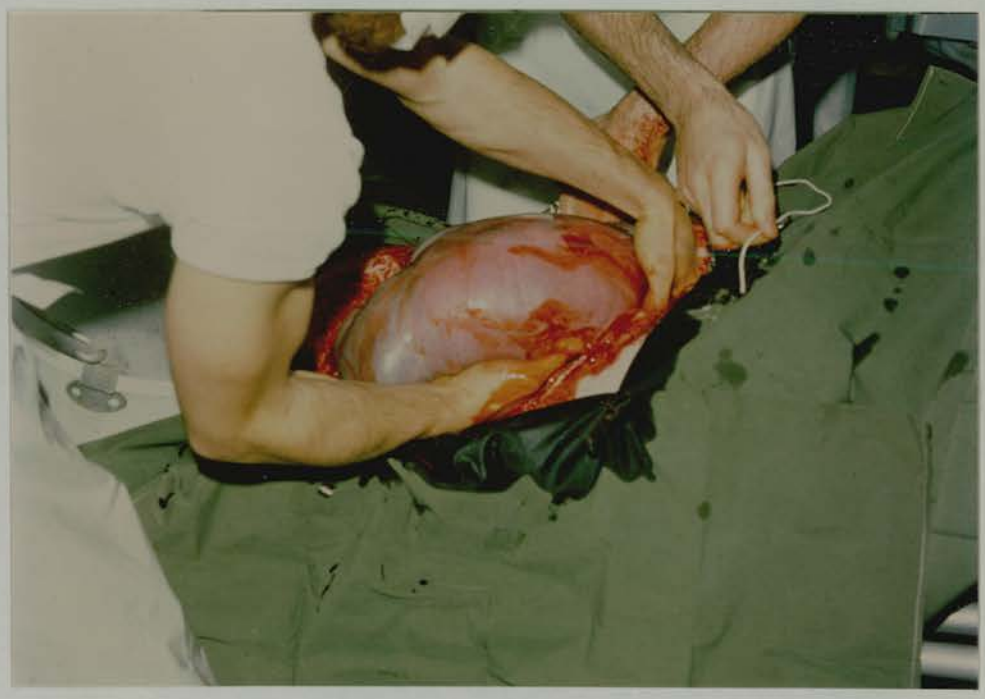

Plate 4.2. The removal of the uterus.

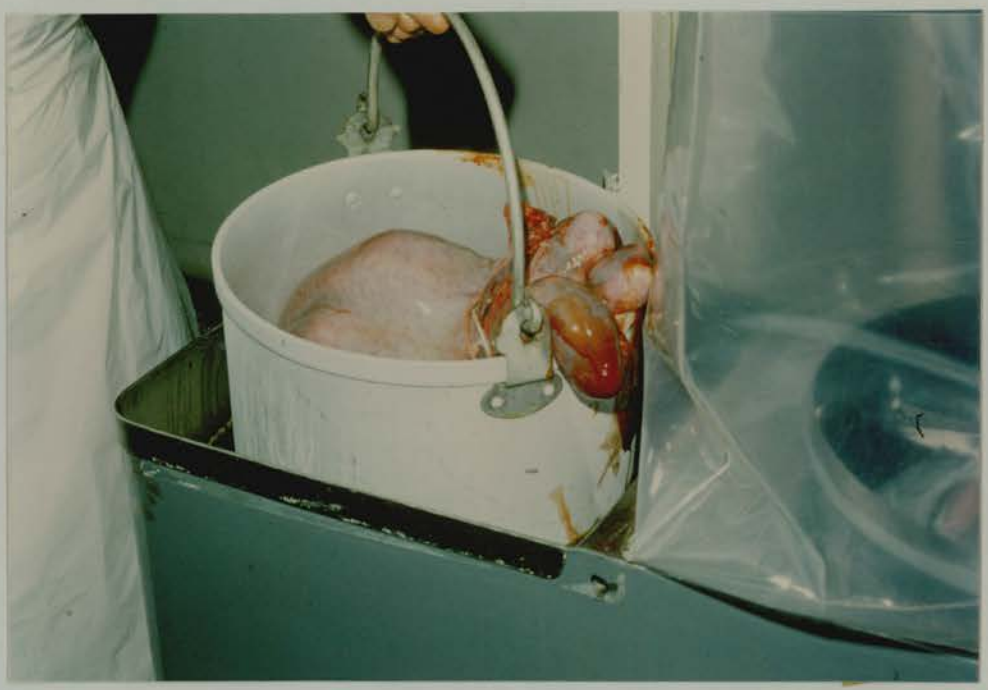

Plate 4.3. Transfer of the unopened uterus to the sterile isolator.

Plate 4.4. Removal of the lamb from the uterus is performed within the dome of the isolator. Note the use of gloved isolator sleeves for all manipulations.

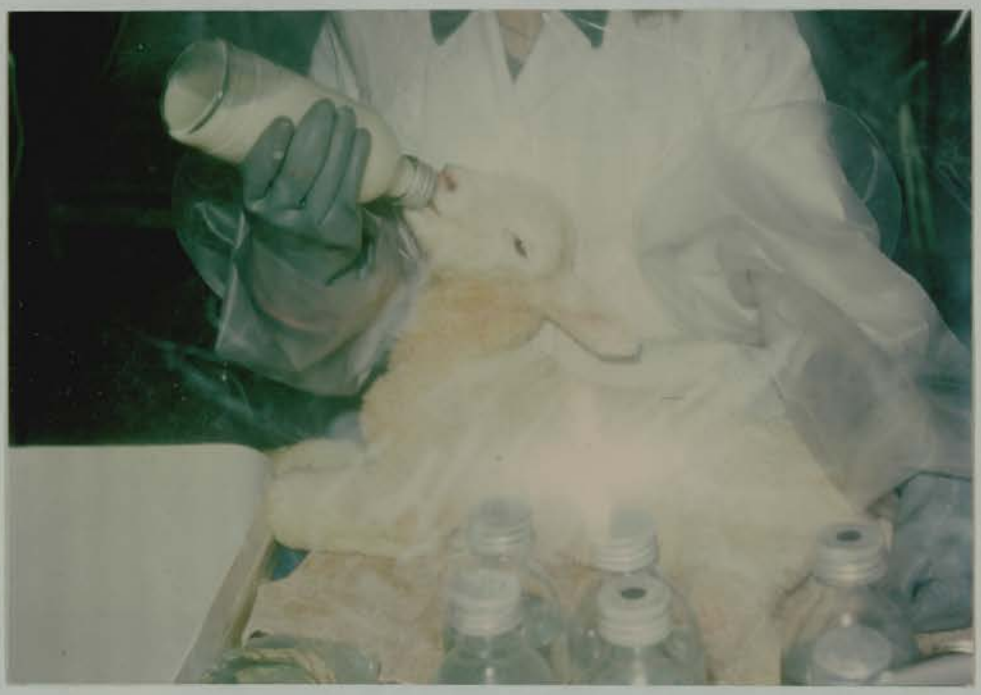

Plate 4.5. A newborn gnotobiotic lamb which has been rubbed dry and is being fed with sterilised evaporated milk.

Chapter 5.

Selection and critical evaluation of the techniques used to study intestinal architecture and epithelial cell kinetics.

Introduction.

To make a complete description of small intestinal architecture and the related epithelial cell kinetics it is necessary to examine a number of different parameters.

The villi (functional unit) and the crypts (proliferative unit) are usually considered separately. The crypt cell production rate is the central measurement in epithelial cell kinetic work but should be complemented by measurements of villus enterocyte function. Alterations in the function of the villus enterocytes are linked to the number and maturity of the cells clothing the villi and while this is to some extent dependent on crypt cell production, villus size and the rate of cell loss from the villus are also involved.

Studies of small intestinal structure and function require measurements of the sizes and shapes of the villi and crypts, the size of the villus and crypt cell populations, the number of crypts associated with each villus, the various compartments of the crypt (stem, proliferative and maturation zones), brush border enzyme levels and crypt cell production rates.

A wide variety of techniques have been developed to determine these parameters. In this chapter the techniques available are listed and from these I have selected a group of methods which were practicable in use and which would give sound results in examination of the lamb intestinal epithelium. To avoid later repetition the experiments on the evaluation of the techniques used are covered in this chapter.

Techniques used to examine the small intestinal mucosa.

These are detailed in Table 5.1 and have been compiled from personal experience and from the experimental and technical literature available. The references cited below are not exhaustive but are representative of the literature on the subject.

Villus and crypt morphology can be measured using histological sections, dissection microscopy and single villus preparations (Clarke, 1973; Clarke, 1974a; Mouwen, 1971; Wimber and Lamerton, 1963).

The number of crypts per villus can be measured by direct visualisation methods using dissection microscopy or microdissected material (Clarke, 1972; Ferguson, Sutherland, MacDonald and Allan, 1977).

There is a vast array of techniques available for the measurement of the various parameters involved in the measurement of cell turnover. The majority of those involve 3H thymidine cell labelling and autoradiographic methods. More recently, stathmokinetic methods have been developed and these provide rapid results in comparison with autoradiographic methods (Al-Dewachi, Appleton, Watson and Wright, 1979; Wright, Al-Dewachi, Appleton and Watson, 1975). The rate of cell efflux can be measured either by perfusion of the lumen and analysis of the subsequent rate of DNA loss (Croft, Loehry, Taylor and Cole, 1968), by counting the cells in the perfusate (Pink, Croft and Creamer, 1970) or by trapping the exfoliated cells in the mucus of the gut and counting them (Clarke, 1970b). Cell labelling methods can also be used to monitor the time taken for villus cells to move along the length of the villus. However this method does not take account of cell loss occurring on

the sides of the villi (Potten and Allen, 1977).

I have combined a stathmokinetic technique with microdissection in my study of small intestinal cell renewal. This method also allows measurement of villus and crypt shapes and sizes, the total epithelial cell numbers of the villus and crypt populations and the crypt to villus ratio and was developed from that described by Clarke (Clarke, 1970a).

Factors which influenced the selection of techniques for this study.

I have already emphasised that the crypt cell production rate is the fundamental measurement in any study of this nature as the whole hypothesis is related to the crypt cell proliferative changes. It was clear from the literature that either 3H thymidine labelling techniques with autoradiography or stathmokinetic techniques would be appropriate to measure the crypt cell production rate in the small intestinal crypts. When considering the choice of methods for my investigation I was aware that, due to our limited resources, the number of animals available was restricted. The general protocol for this sort of experiment was originally devised for experiments using mice, costing approximately £1 each, in contrast my animals cost £250 each. The number of animals used in the experimental design had therefore to be a compromise between cost and the number of animals required to provide valid results. The minimum number of animals was therefore used, although the consistency of the results was checked by duplication of the experimental procedures in a small number of animals. It was impossible to use 3H thymidine labelling techniques in the lambs to monitor the results obtained using the microdissection stathmokinetic technique for reasons involving cost and safety due to the large amounts of radioactive isotope which

would have been required. In addition, in the stathmokinetic techniques used in the past, requiring many animals in each experimental group, the measurements involved were laborious in practice and unsuitable for use in an experiment where many aspects of intestinal damage were to be measured.

A stathmokinetic method using vincristine was devised which allowed the samples required to be obtained from a single animal, instead of the more usual multiple animal sampling, for each time interval after the administration of the metephase arrest drug. The animal was injected with the metaphase arrest drug and one hour later it was anaesthetised and maintained alive for a further two hours. A laparotomy was performed, samples were removed on three accurately timed occasions and the tissue was processed as described in the methods section.

Having decided to use a stathmokinetic method in single animals, we then had to decide whether to use histological methods to measure the crypt cell production rate or to use a microdissection method. The microdissection method was chosen as it has several advantages:

(a) villus and crypt dimensions are not altered by the fixation process and no shrinkage occurs (Ferguson, Sutherland, MacDonald and Allan, 1977) therefore accurate values are obtained.

(b) many of the relevent measurements can be performed in the same piece of tissue.

(c) the crypt to villus ratio can be measured easily.

(d) the mitotic activity of single crypts can be measured.

(e) by slight modification of the technique the villus epithelial cell population can be measured.

It was, of course, necessary to select other more conventional

techniques to allow comparison with the results in the literature, also for the examination of material from earlier pilot studies, to examine the villus epithelial cells in greater detail and to provide valuable additional information about the effects of the rotavirus infection on the small intestinal epithelium.

These included:

Conventional histology; this method of examination is complementary to the microdissection technique, allowing correlation between villus and crypt lengths measured by the two separate methods. In addition studies of non-epithelial aspects of the tissue can be performed. When surveying the literature on rotavirus infection, histology is frequently the only method used to describe the extent of the lesion and therefore histological examination was necessary for comparison with other published results. Finally, histological examination of the tissue is a central technique in any pathological study and allows predictions to be made as to the outcome of other more advanced methods of study.

Scanning electron microscopy; this is another valuable complementary technique when used in conjunction with other methods of study and allows the accurate description of changes in the surface detail of the epithelial cells which are frequently so subtle that they are not apparent by other methods of study.

Transmission electron microscopy; many studies of this nature have been previously performed in rotavirus infection and thus it has only been used in my study to examine selected areas of interest.

Brush border enzyme assay; measurements of the lactase content of the small intestinal epithelial cells were performed for two reasons.

(1) to discover whether or not lactase deficiency was the cause of the diarrhoea produced by the infection.

(2) as a functional marker, showing the maturity of the epithelial cells on the villi and to assess the damage, if any, to the brush borders of the epithelial cells of the small intestine.

Immunofluorescence; this technique was used to monitor the distribution of infected epithelial cells and to correlate the observed lesions with the presence of the virus.

Use of metaphase arrest drugs.

The periwinkle alkaloids, vincristine and vinblastine, are now thought to be superior as agents of choice, although colchicine is useful in certain tissues, for example in rodents, (Tannock, 1967; Clarke, 1971). The arresting action of these compounds within cells in mitosis depends on their interaction with the mitotic spindle or its sub-units, which results in the failure of spindle assembly producing abnormal mitotic figures and preventing the cell from completing the process of mitosis. These blocked metaphases are readily identified instead of normal metaphases. When using metaphase arrest drugs several requirements must be satisfied. These were outlined by Tannock (Tannock, 1967) and have been further reviewed recently by Wright (Wright and Appleton, 1980). An effective stathmokinetic agent should have the following characteristics:-

(a) an optimum dose which arrests all metaphases in the tissue over a certain period of time.

(b) the mitotic arrest properties should not be highly sensitive to the dose.

(c) there should be no effect on non mitotic cells.

(d) the arrested metaphases should remain intact during the time interval over which samples are taken.

(e) the stathmokinetic results should correlate positively with other methods of assessing the proliferative state of the tissue.

If these features are indeed provided by an agent there will be a well defined dose curve and values obtained in accumulation studies will be linear with time.

Assessment of vincristine (Oncovin) as a suitable metaphase arrest agent in the lamb.

The requirements which must be met when using metaphase arrest drugs have been outlined above. Normally, before starting an experimental regime in an animal model using such drugs, a series of experiments would be performed to assess the effects of different doses of the drug on the tissue system of the particular species involved. The purpose of such experiments is to establish the optimum dose of the drug and the collection period over which the accumulation of metaphases will be linear and the blocked metaphase cells will remain stable.

Due to the scarcity and high cost of the animals involved in the experiments it was not feasible to perform a pilot study of this nature. On the advice of Professor Wright, 1mg/kg body weight was chosen as the optimum dose. Careful observations were subsequently carried out throughout the course of the experiments to ensure that:

(1) this dose was adequate to block all cells as the metaphase stage of mitosis was entered.

(2) the dose was not excessive, thereby causing the blocked cells to disintegrate during the period of collection.

(3) the accumulation of blocked cells was linear in form during the

collection period.

In all experimental animals the numbers of metaphases accumulated was plotted against time and the correlation coefficients of the regression lines were calculated. In all cases except one (see chapter 7) the accumulation was linear and the values of the correlation coefficients of the regression lines ranged from 0.86 to 0.99 with a mean of 0.97 and a standard error of 0.004. Cells blocked in metaphase by the action of the drug were intact at the final sampling time and no cells were seen to exhibit telophase escape (i.e. to have passed through metaphase without being arrested by the action of the drug) Plate 5.1.

Typical appearance of microdissected tissue.

It is appropriate to demonstrate in this section the appearance of the microdissected material, its applications and to describe a number of experiments performed to validate the techniques involved.

Plate 5.2 illustrates the typical appearance of a thin strip of mucosa which has been cut and mounted on a slide prior to its use in measuring villus and crypt lengths. In Plate 5.3 the preparation was gently squashed, releasing the individual crypts. The heavily stained, blocked metaphases are easily identified. Plate 5.4 shows a single villus which was mounted on a slide and gently squashed to dissociate the cells to enable the villus epithelial cell population to be counted.

Comparison of histological and microdissection measurements.

Tissue samples from control animals, aged 1 - 16 days, were divided, one half was processed in the standard manner for histology, the other half was processed by microdissection methods.

Villus and crypt lengths were measured in both groups and the results are presented in Fig. 5.1. The measurements of the villi and crypts in histological sections were consistently shorter than those obtained from microdissected material. It was decided to use microdissected material for all measurements of villus and crypt lengths.

Reproducibility of the microdissection technique.

The validity of using only ten measurements of individual villi and crypts to calculate the mean length of the villus and crypt populations was proved several years ago in studies of human small bowel (Ferguson, Sutherland, MacDonald and Allan, 1977). Measurements were made of the lengths of more than 100 villi and crypts from individual specimens. The values were found to be normally distributed and the means obtained from 100 values were within 10% of those obtained from 10 values.

The validity of using the same method for the measurement of the villus and crypt lengths in lamb small intestine was assessed by examining selected samples in triplicate. The values obtained in the case of lamb 124 are detailed in Table 5.2. By the Student's 't' test there was no difference between the three groups, $P > 0.05$. Tissue samples were also re-examined at intervals ranging from one week to one year after fixation and again the results were consistent.

Similar checks were carried out to assess the consistency of the method for counting the blocked metaphases in the dissociated crypts. Counts were performed in more than 100 individual crypts, the values were normally distributed and counts in 10 intact crypts were chosen as a suitable sample under experimental conditions.

The correlation between villus epithelial cell number and villus length.

Samples of small intestine from an infected lamb and an age matched control were used. The sites chosen were representative of all areas of the small intestine and included areas having both finger and leaf shaped villi. Villus length, villus area and villus epithelial cell numbers were measured and the regression lines of villus length / number of villus epithelial cells and villus area / number of villus epithelial cells were drawn and the correlation coefficients were calculated. The results are presented in Table 5.3.

There was a good, positive correlation between the villus lengths and the number of villus epithelial cells at all sites. A positive correlation between villus area and villus epithelial cell numbers occurred only where the villi were leaf shaped.

Distribution of blocked metaphase cells within the crypt.

Studies employing radioactive labelling techniques to define the boundries of the proliferative zone and the maturation zone of the crypt were not feasible within the confines of my experimental procedures. A limited study was performed to assess the relative positions of blocked metaphase cells within the crypts of an infected animal and a control animal. It was hoped that this study would provide a general indication of the changes occurring in the relative sizes of these zones at the time of increased cell production within the small intestine.

The experimental animal chosen for this study was a lamb which had been infected with rotavirus for eight days. At this time the

level of cell production in the crypts was at its peak in the middle region of the small intestine and normal elsewhere. An age matched control was also studied. Samples were taken from areas which represented 23%, 38%, 62% and 85% of the distance along the small intestine from the duodenum to the caecum. The samples were processed in the routine manner for microdissection examination. Thin strips of villi and associated crypts were cut, mounted on a slide and the tissue was gently squashed under a cover-slip until the crypts dissociated from the lamina propria. A projection microscope was used to project the image of single crypts onto a piece of paper at a fixed distance from the microscope. The outline of the crypt was drawn and the positions of the blocked metaphase cells were plotted within the outline of the crypt. These positions were then expressed as a percentage of the total length of the crypt. Fig. 5.2 shows the frequency with which the blocked metaphases occurred in the different areas of the crypt. In the control lamb, cells were in mitosis in 80% of the crypt with few being present in the bottom 10% of the crypt. This suggested that the proliferative zone accounted for approximately 80% of the crypt and that the maturation zone was confined to the upper 10% of the crypt.

In the animal which had been infected with rotavirus for 8 days mitotic cells were also present in 80% of the length of the crypt in the jejunum and ileum. In the middle regions of the small intestine, mitotic figures were present only in the lower 70% of the crypt but the numbers of arrested metaphases were greater. It was possible that this represented a change in the relative sizes of the zones within the crypt as a result of infection but the results were not conclusive.

Conclusions.

The stathmokinetic method satisfied the criteria laid down for such experiments and provided consistent results.

The microdissection method, when applied to assess villus and crypt lengths, gave accurate results without the problems associated with shrinkage which occurred in histological sections and the results were reproducible.

Villus length correlated well with villus epithelial cell number.

It was not satisfactory to use the distribution of blocked metaphase cells within the crypt to determine the various crypt compartments.

Item to be measured	Technique	Advantages	Disadvantages
Villus morphology			
length	paraffin sections.	readily available.	inaccurate due to tissue changes during fixation.
'length' in number of cells.	paraffin sections.		no indication of actual size of villus.
length	microdissection.	very accurate, no tissue shrinkage.	
shape	dissecting microscopy.	fast, no processing required.	subjective, difficult to see whole villus.
	histology.		few villi examined, distortion during processing.
	scanning electron microscopy.	large area assessed easily.	fixation artifacts may be produced.
	microdissection and projection.	accurate.	
area	histology (height x width at base).		does not give a good correlation with cell numbers.
total villus cell number	microdissection.	accurate.	time consuming.
	histology		inaccurate, especially in situations where the villi are abnormal.

Table 5.1.

(continued)

Item to be measured.	Technique	Advantages	Disadvantages
Crypt morphology			
length	histology.		inaccurate, changes due to fixation process.
	microdissection.	very accurate.	
'length' in number of cells	histology.	accurate.	requires well orientated sections.
shape	microdissection.	accurate.	
total number of cells/ crypt	histology, crypt column counts.	accurate.	requires sections in two planes.
crypt compartments	cell labelling	accurate, reproducible.	involves radioactive materials.
	microdissection of crypts + 3H thymidine labelling.	correlates well with other methods.	technically difficult.
Cell turnover			
duration of the cell cycle	fraction of labelled mitosis	accurate.	radiation hazards.
Mitotic Index per cell position	histology sections.	accurate.	technically difficult.

Table 5.1.

(continued)

Item to be measured	Technique	Advantages	Disadvantages
Crypt cell production rate	Stathmokinetic techniques:		
	histology		poor estimation of mitotic duration.
	autoradiography	suitable for use with small animals.	requires large numbers of animals and special housing conditions.
	microdissection	accurate, rapid results.	
Crypt to villus ratio	direct examination	illustrates the relationship between the villi and the crypts.	
	scanning electron microscopy		inaccurate.
Cell efflux from the villi	DNA estimations of lumenal perfusate		result depends on the rate of perfusion.
	cell trapping	correlates well with the crypt cell production rate.	
	direct observation by electron microscopy	the various cell types are identified.	

Table 5.1. Summary of the techniques which are commonly used to study the small intestinal epithelium.

Villus length(μm)			Crypt length(μm)		
974	1183	1044	143	207	173
858	1122	1044	174	174	171
1055	1114	928	151	196	168
1032	939	974	210	151	165
1114	1018	881	193	179	168
1032	1148	928	182	184	168
1148	1183	1287	184	179	171
974	1067	1299	168	168	165
1102	1079	986	154	168	165
1056	1090	1009	168	157	168
mean	mean	mean	mean	mean	mean
1034	1094	1038	172•7	176•3	168•2
±26•5	±23•7	±45•5	±6•4	±5•3	±0•9

Table 5.2. Villus and crypt lengths in the small intestine of a gnotobiotic lamb aged 3 days. Site 2. Microdissection measurements on three separate occasions. (μm ± S.E.)

8 days after infection

Site and villus shape	Villus length(μm)	Villus Area	Epithelial cell number	V.L./cells (correlation	V.A./cells coefficient)
1	1276	6•6	2784		
finger villi	1450	4•9	3164		
	835	2•5	1166	*0•995	0•13
2	1276	7•7	2728		
finger villi	1543	6•8	3778		
	1218	3•9	2948	*0•933	0•094
2	1148	5•1	1510		
leaf villi	986	4•5	1443		
	695	2•4	1029	*0•973	*0•996
3	696	3•0	715		
leaf villi	754	3•5	850		
	754	3•7	1010	*0•840	*0•812
Age matched control					
1	1160	2•7	3439		
finger villi	1044	4•4	2868		
	1508	3•4	4550	*0•99	-0•4
2	1160	4•2	3329		
finger villi	835	1•7	2225		
	812	3•5	1620	*0•956	0•413
3	1334	2•4	4145		
finger and leaf villi	835	2•8	2121		
	766	2•1	1842	*1•00	0•128
3	986	1•8	2467		
leaf villi	568	1•5	1032		
	835	1•7	1823	*0•996	*0•98

Table 5.3. Villus length, villus area and epithelial cell number at selected sites in the small intestine of a gnotobiotic lamb, 8 days after infection with rotavirus, and an age matched control.
* - positive correlation.

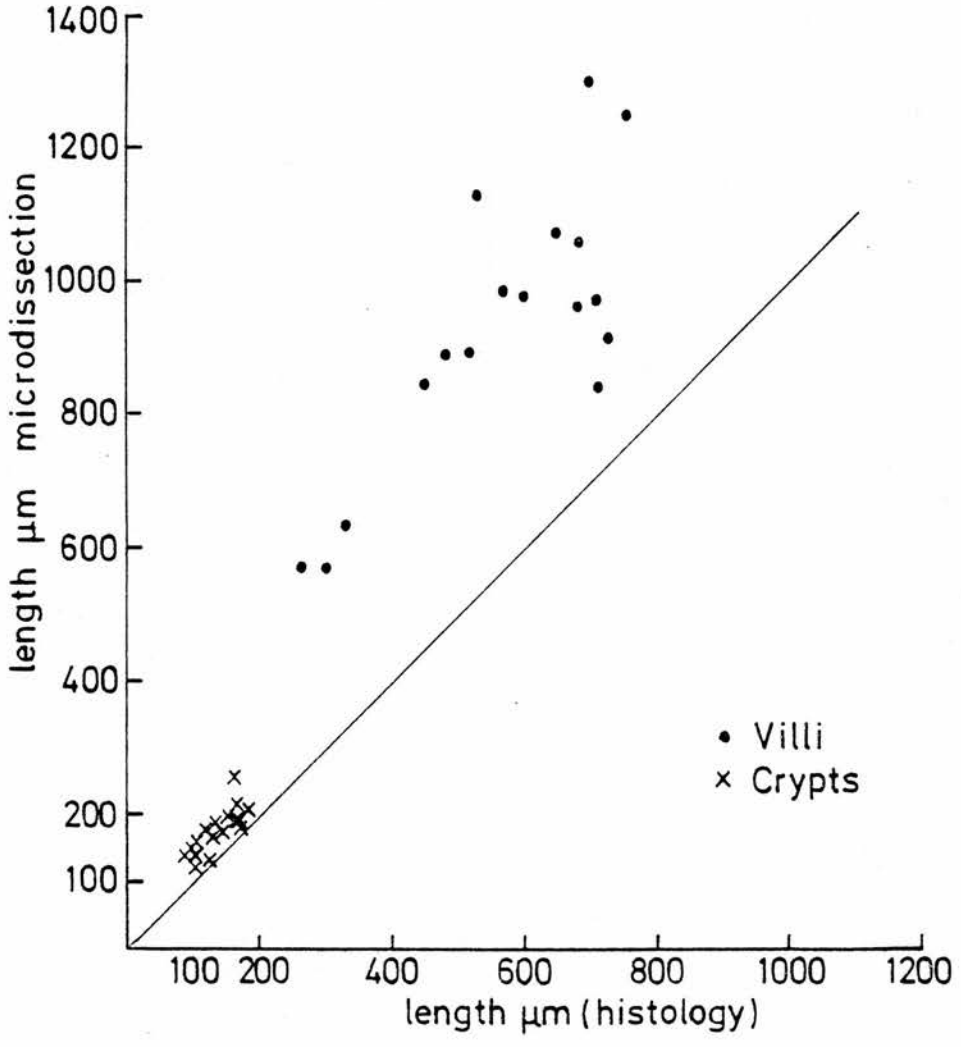

Fig 5.1. Relationship between the villus and crypt lengths measured in histological material and microdissected material.

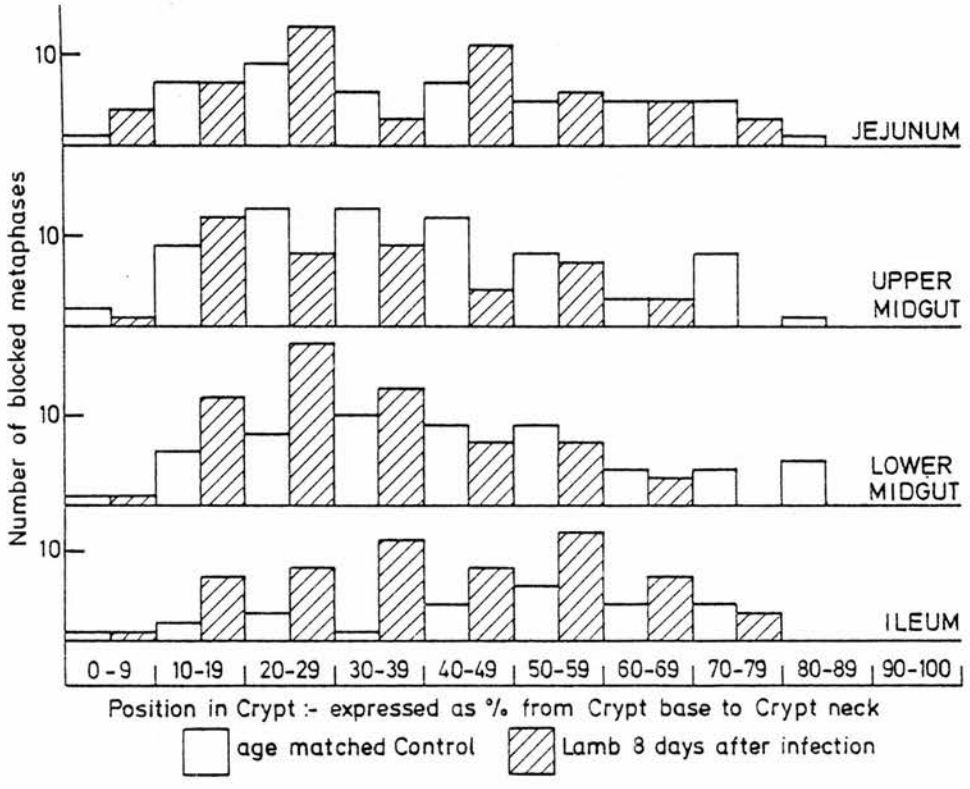

Fig 5.2. The distribution of blocked metaphases in the crypts. Relative positions from the base of the crypt to the neck. Small intestinal sites from the proximal to the distal regions of the small bowel. Measurements in a gnotobiotic lamb, 8 days after infection with rotavirus on the second day of life and an age matched control.

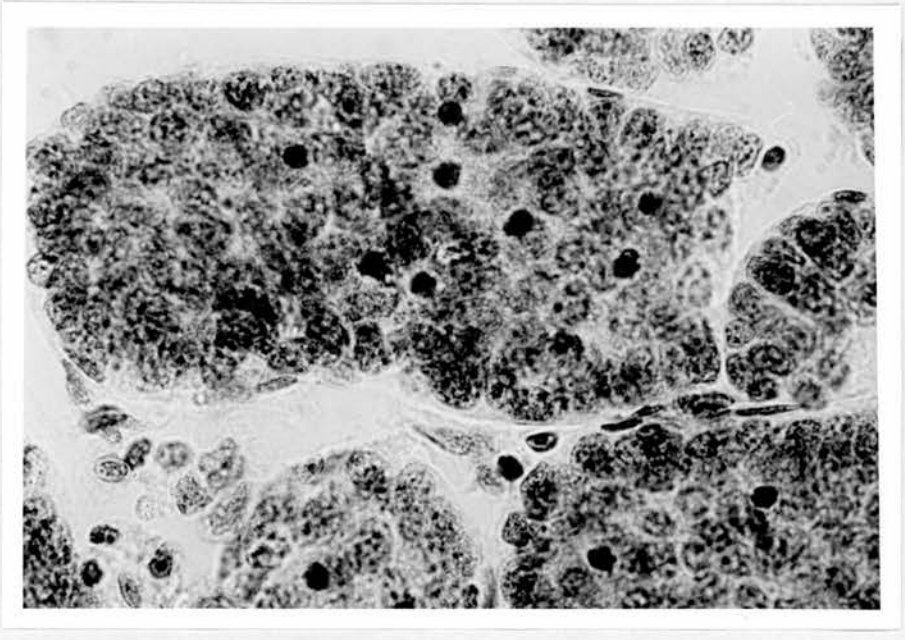

Plate 5.1. A squashed crypt with blocked metaphases. Collection period of 100 minutes. Feulgen X 500

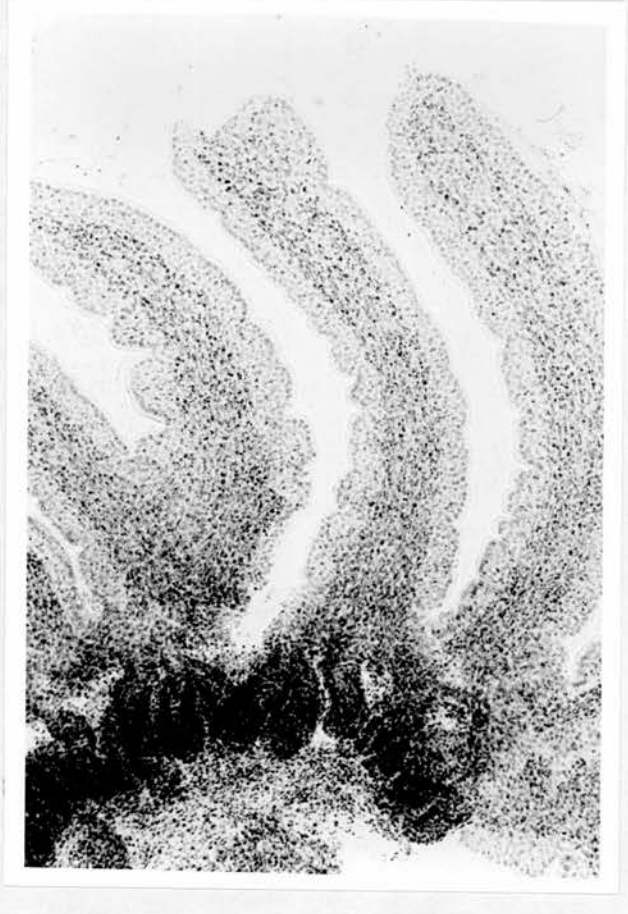

Plate 5.2. The typical appearance of the small intestinal mucosa after microdissection. Feulgen X 50

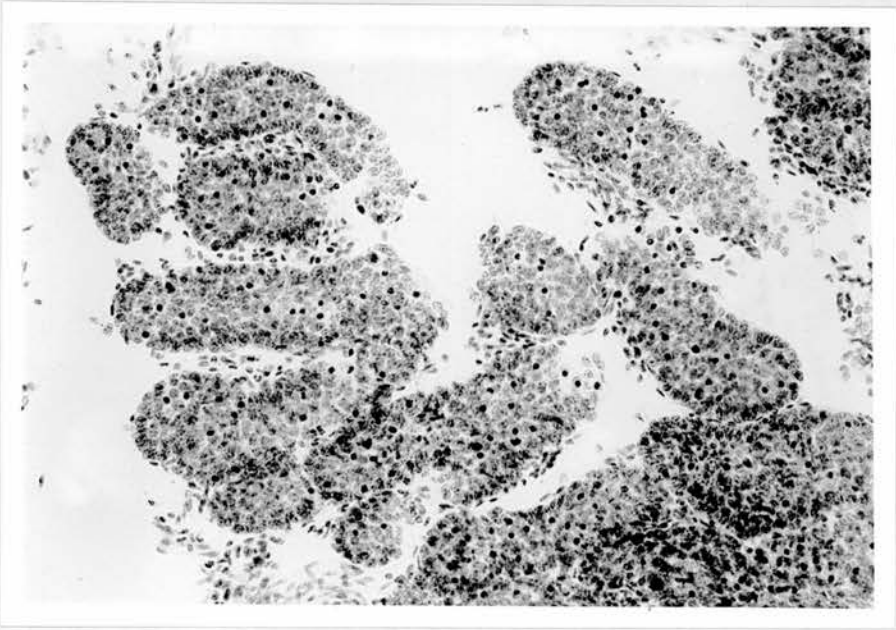

Plate 5.3. A preparation of squashed crypts. The heavily stained blocked metaphases are easily distinguished and counted. Feulgen X 125

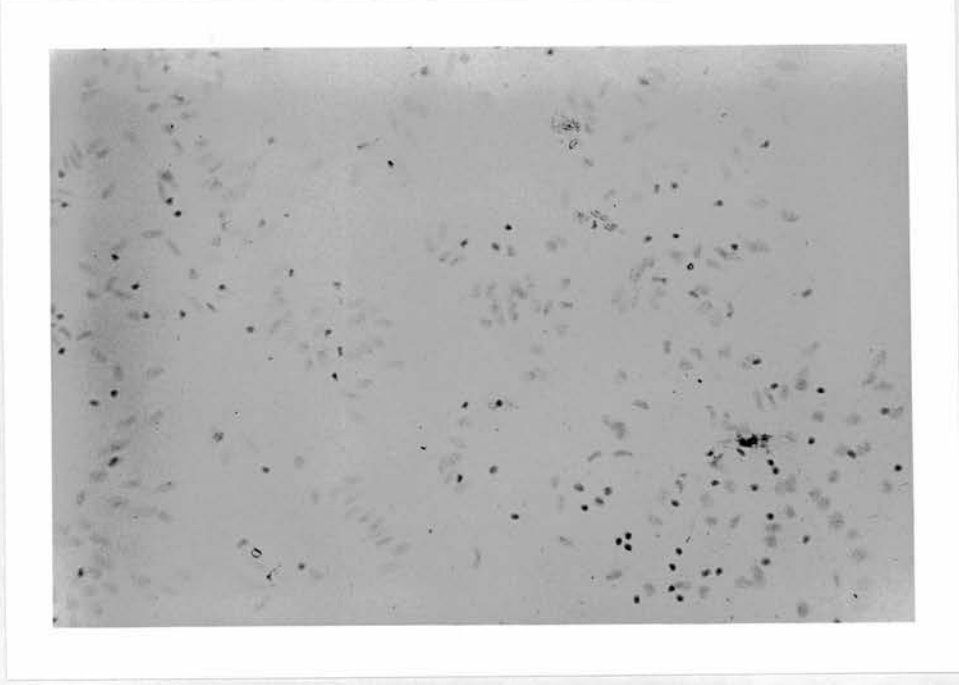

Plate 5.4. A preparation of a single villus squashed to allow the villus cell population to be measured. Feulgen/Geimsa X 500

Chapter 6.

The normal small intestine of gnotobiotic neonatal lambs.

Introduction.

In all species, the period following birth is associated with rapid growth and changes of structure and function in the small intestine. These changes are partly intrinsic, in that the development is controlled genetically and hormonally, superimposed on this orderly pattern are the effects of extrinsic stimuli such as food and the microbial flora which are present in the lumen of the gut. In gnotobiotic animals the interpretation of the histological changes, brought about by experimental means, is simplified by the absence of changes associated with microbial colonization.

It was essential to define the morphology and histological appearance of the small intestine of the normal gnotobiotic lamb, since there were no detailed accounts of this in the literature.

One of the most obvious changes of intestinal function in the period after birth relates to colostrum absorption. Lambs absorb colostrum and immunoglobulin molecules pinocytotically, via specialised vacuolated cells located within the epithelium of the small intestinal villi. These cells are gradually replaced as new cells clothe the villi as part of the normal process of cell renewal, the new cells are non-vacuolated and do not have a pinocytotic function.

Thus, the aims of the work described in this chapter were:
(1) to describe the epithelium of the small intestine of the normal gnotobiotic lamb, with particular emphasis on the distribution of the vacuolated absorptive cells.
(2) to examine the effects of age on mucosal architecture, cell

kinetics, lymphoid cell populations and tissue lactase levels in the small intestine.

Details of the animals used in this work are given in Table 6.1.

Mucosal architecture in lambs aged 1-20 days.

Villus lengths and crypt lengths were measured in histological sections and in Feulgen stained, microdissected material. The values obtained are contained in Tables 6.2 and 6.3. In general the villi of the jejunum and the midgut were longer than those in the ileum. Shrinkage, which occurred during histological processing, was responsible for the histological values being only 50-60% of those obtained from microdissected material. By both techniques, it was clear that villus length was influenced by age only in the ileum. Until day 5, the villi of the ileum were similar in length to those in the more proximal regions of the small intestine, in microdissected material being 834-907μm, whereas after day 9, the villi in the ileum measured 573-637μm. There was also a change in villus shape at this time from finger to leaf form.

At all sites, longer crypts were found in older animals. The shape of the crypts changed during the first two weeks of life; at first the majority of the crypts were bifid or branched (Plate 6.1) but later the crypts became longer and cylindrical, (Plate 6.2).

The small intestinal epithelium and the associated mucosa.

The appearance of the surface epithelium was examined using histological sections and scanning electron microscopy. The mucosa was examined by histological sections.

Site 1.

Long finger villi were present from 1-20 days of age (Plates 6.1, 6.3). The villus surfaces showed crenations (ridging patterns); these were shallow, annular and of a regular distribution (Plates 6.4, 6.5). The cell boundries were well marked and illustrated the polygonal shape of the microvillus-covered cells. There was a well defined extrusion zone at the villus tip (Plate 6.5); occasionally a few cells were noted to be in the process of exfoliation. No absorptive vacuoles were seen at the anterior site (Plates 6.1, 6.4).

Site 2.

The villi were initially long, thin and finger-like (Plates 6.6, 6.7). However, by 7 days of age the villi had thickened but remained cylindrical (Plates 6.8, 6.9). The crenations on the villus surface became deeper with age but retained their annular pattern (Plate 6.10). The margins of the regularly shaped, microvillus-covered cells were generally visible (Plate 6.9). Absorptive vacuoles were present within the villus epithelial cells from 1 - 5 days of age (Plates 6.11, 6.12), and were confined to the upper parts of the villi on day 5 (Plate 6.12). Goblet cells were present in the epithelium.

Site 3.

In the first week of life the villus population consisted of a mixture of finger and leaf types (Plate 6.13) which were relatively long. By 8 days of age, the villi had shortened and become conical and triangular (Plates 6.14). The depth of the crenations increased with age (compare Plates 6.13 and 6.14), and the goblet cell

openings were obvious. Cell boundaries were easily distinguished in younger animals (Plate 6.15), however as the depth of the crenations on the villi increased, they became difficult to see. Vacuolated epithelial cells were present over most of the surface of the villus at day 2 (Plate 6.13), thereafter only the epithelial cells on the upper parts of the villi were vacuolated (Plate 6.16, 6.17). By day 5, normal columnar epithelium was present on the lower part of the villi and this gradually replaced the vacuolated epithelium.

All sites.

No Paneth cells were observed within the small intestinal mucosa from birth to 20 days of age. The cellularity of the lamina propria increased with age in all areas (Plates 6.6, 6.10), with the increase in lymphoid cells being greatest after 8 days of age. Many eosinophils were present in the lamina propria, the greatest concentrations being in the area at the bases of the crypts (Plate 6.2). The numbers of intra-epithelial lymphocytes in the epithelium increased with age, Table 6.4.

Small intestinal epithelial cell kinetics.

Feulgen stained material from metaphase-arrested tissue was used to examine the cell production rate in the crypts. The results are given in Table 6.5. The number of cells produced per crypt per hour tended to increase with age at all three sites. The value obtained in the lamb aged 3 days, at site 1, appeared to be abnormally low. The reason for this may have been that the blood supply to site 1 was obstructed during the course of the laparotomy and thereby prevented metaphase accumulation from occurring. The action of the metaphase-arrest drug was not impaired at the other

two sites of sampling and the values of the accumulated metaphases against time were linear.

Number of crypts per villus.

The crypt to villus ratio was measured in Feulgen stained material. The results are presented in Table 6.6. The number of crypts associated with each villus did not differ consistently from 2 days to 16 days of age.

Tissue lactase levels in the small intestinal epithelium.

The results of the lactase assays are shown in Table 6.7. Highest lactase levels were present at site 1, the range being 5.6 to 14.5 units/gram. At site 2, levels of lactase were lower, the range being 2.9 to 7.3 units/gram. In both these sites the levels did not vary greatly with the age of the animal. However at site 3, lactase levels were similar to those at sites 1 and 2 until 5 days of age, thereafter, the observed lactase levels were very low, being less than 1 unit/gram in all cases.

Conclusions.

Alterations in villus and crypt length and shape, crypt cell production rates, mucosal tissue lactase content and the mucosal lymphoid cell population were influenced by the age of the animal. The resultant differences indicated that age matched controls were necessary in the design of further experiments.

Absorptive vacuolated cells were a feature of the small intestinal epithelium at sites 2 and 3 and were lost between 5 and 9 days of age.

Technique.	Age of animals used, (days).								
	1	2	3	5	8	9	12	16	20
Histology.	1	2	1	2	-	1	1	1	1
Microdissection.	-	1	1	1	-	1	1	1	-
Scanning electron microscopy.	1	2	-	1	1	1	-	1	1
Lactase assay.	1	1	1	1	-	1	1	1	-
Intra-epithelial lymphocytes.	1	2	1	2	-	1	1	1	1

Table 6.1. The numbers of animals used to define the features of the normal small intestine of the gnotobiotic lamb from 1 - 20 days of age.
- indicates no animal used.

Age(days)	Site 1		Site 2		Site 3	
	Villus length	Crypt length	Villus length	Crypt length	Villus length	Crypt length
1	655±17	126±3	690±38	143±6	631±20	142±2
2	649±18	129±4	562±41	126±8	737±22	127±3
2	560±19	113±3	491±15	85±4	557±20	84±3
3	707±22	101±5	750±22	105±4	709±36	98±4
5	531±12	136±4	454±14	131±6	688±34	91±3
5	734±27	159±4	574±25	162±5	386±21	91±6
9	682±18	119±2	513±9	162±6	261±18	162±8
12	594±36	166±9	448±15	169±6	327±20	159±6
16	713±22	164±9	695±11	174±5	360±13	134±7
20	709±35	170±10	643±41	204±9	243±27	131±8

Table 6.2. Villus and crypt lengths in 3 sites of small intestine of normal, gnotobiotic lambs, aged 1 - 20 days. (μm, mean ± S.E.)
Measurements of histological sections.

Age(days)	Site 1		Site 2		Site 3	
	Villus length	Crypt length	Villus length	Crypt length	Villus length	Crypt length
2	1069±21	172±3	980±39	180±5	907±23	132±2
3	1308±50	151±8	1247±44	120±2	834±27	135±6
5	1131±36	171±5	843±37	170±5	954±66	133±6
9	1053±31	158±3	890±24	191±5	573±22	173±3
12	976±28	199±7	979±25	209±4	637±18	181±3
16	972±17	262±5	897±24	206±5	562±19	193±8

Table 6.3. Villus and crypt lengths in 3 sites of small intestine of normal, gnotobiotic lambs aged 2 - 16 days. (μm, mean ± S.E.)
Measurements of microdissected material.

	Intra-epithelial lymphocytes / 100 epithelial cells		
Age(days)	Site 1	Site 2	Site 3
1	6•8	8•9	6•4
2	11•7	8•8	16•9
2	11•5	11•1	7•1
3	14•0	14•2	12•0
5	11•9	11•6	15•6
5	12•2	12•6	12•2
9	12•1	13•1	21•1
12	10•4	12•8	13•6
16	12•5	13•0	17•3
20	14•4	22•1	18•3

Table 6.4. I.E. lymphocytes / 100 epithelial cells in 3 sites of small intestine of normal, gnotobiotic lambs aged 1 - 20 days.

	Cells / crypt / hour.		
Age(days)	Site 1	Site 2	Site 3
2	8•9	2•8	4•3
3	1•0	2•8	3•1
5	2•2	5•6	3•0
9	10•1	3•2	8•5
12	18•8	8•7	15•5
16	28•6	15•1	25•7

Table 6.5. Crypt cell production rates in 3 sites of small intestine of normal, gnotobiotic lambs aged 2 – 16 days. (CCPR expressed as the number of cells produced per crypt per hour).

	The number of crypts per villus		
Age(days)	Site 1	Site 2	Site 3
2	6•5	3•0	4•1
3	5•1	2•9	4•0
5	4•0	2•5	3•5
9	2•8	3•4	2•4
12	3•2	4•2	3•0
16	3•0	4•1	3•3

Table 6.6. The crypt to villus ratio in 3 sites of small intestine of normal, gnotobiotic lambs aged 2 – 16 days.

Age(days)	Site 1	Site 2	Site 3
1	5•5	5•6	9•9
2	7•4	4•4	1•9
3	9•1	7•3	3•1
5	14•5	6•5	7•9
9	9•3	2•9	0•6
12	13•6	4•8	0•5
16	10•3	4•8	0•2

Table 6•7. Tissue lactase activity in 3 sites of small intestine of normal, gnotobiotic lambs aged 1 - 16 days. Activity is expressed as units of activity/ gram wet weight of tissue. (Unit being 1μmole of substrate hydrolysed per minute at 37^{o}C)

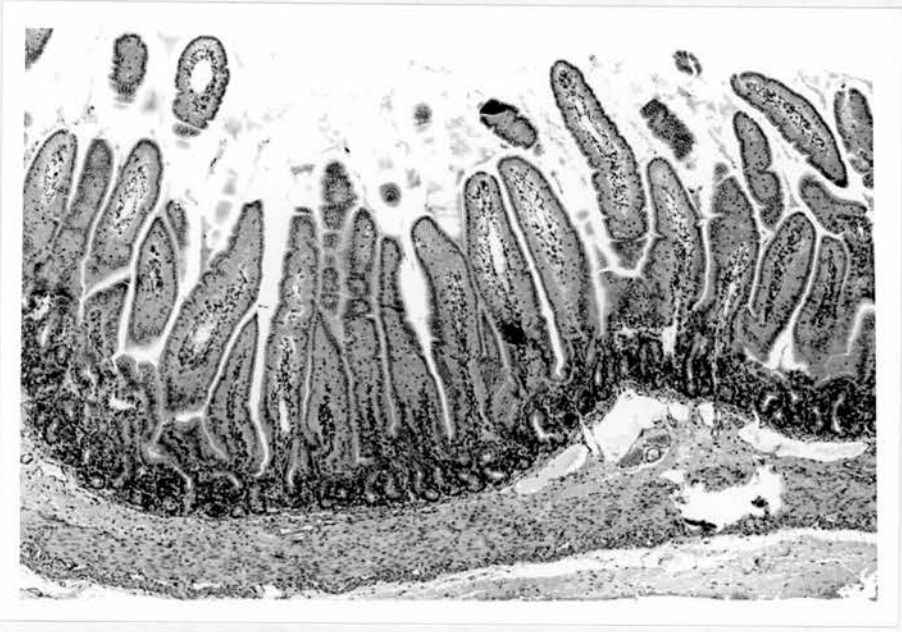

Plate 6.1. The small intestinal mucosa of an uninfected, gnotobiotic lamb, aged 2 days. Site 1. The villi are long and finger-like, the crypts are short and some have a bifid form. The epithelial cells are non-vacuolated. H & E. X 50.

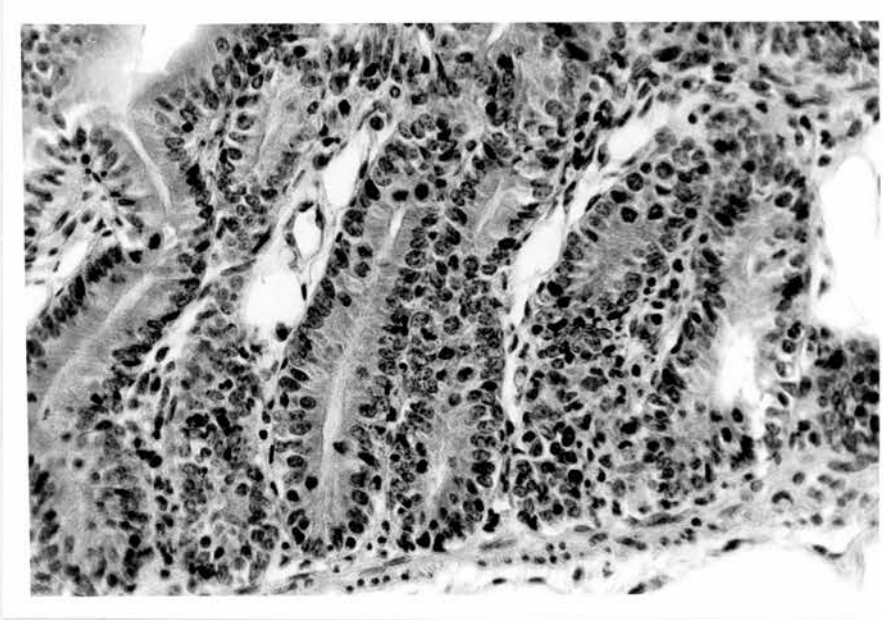

Plate 6.2. The crypt region of the small intestine of an uninfected, gnotobiotic lamb aged 9 days. Site 2. Note the long crypts. Many eosinophils are present in the lamina propria. H & E. X 320.

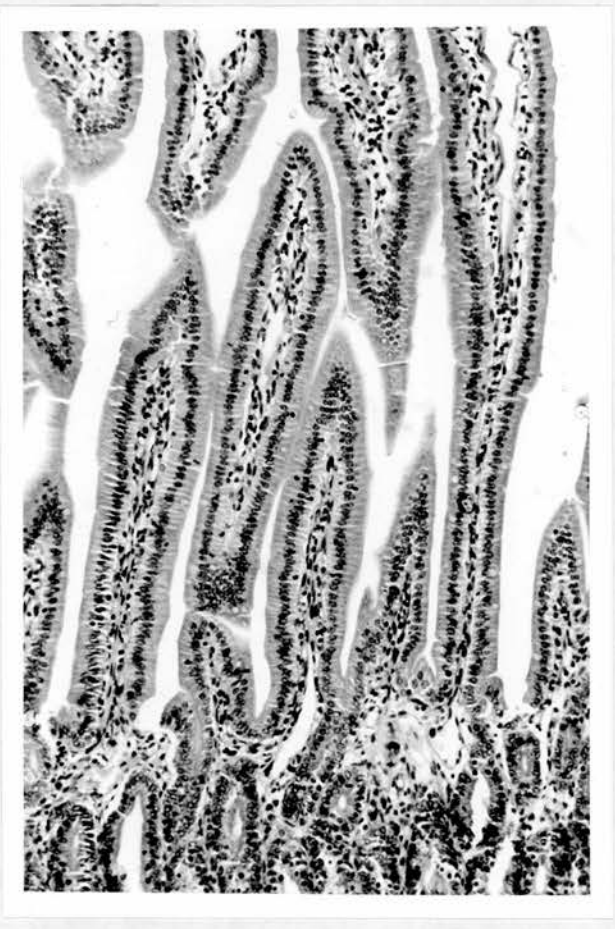

Plate 6.3. The small intestinal mucosa of an uninfected, gnotobiotic lamb aged 12 days. Site 1. The villi are long and thin and clothed by columnar epithelial cells. H & E. X 125.

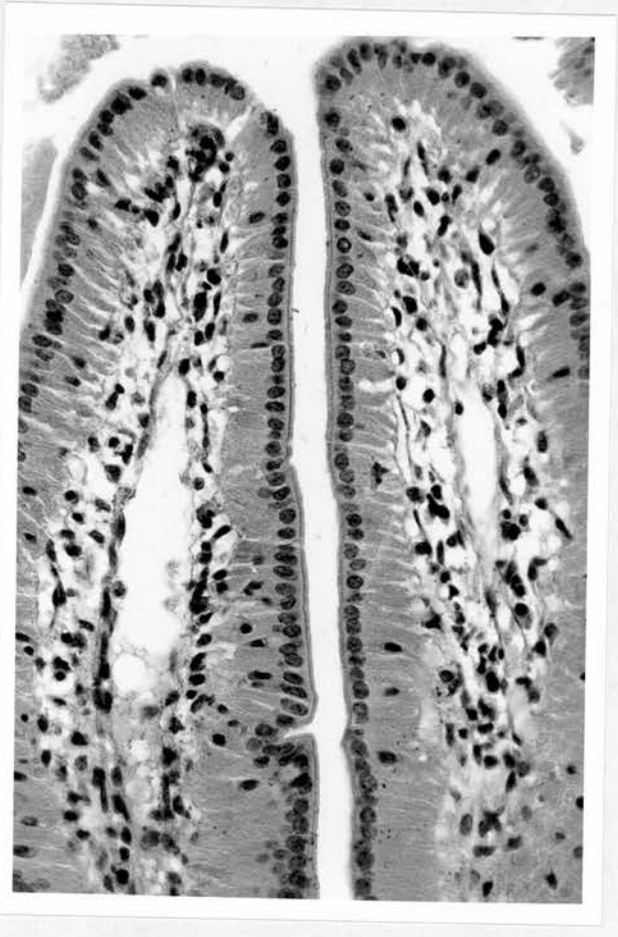

Plate 6.4. The villus epithelium of the small intestine of an uninfected, gnotobiotic lamb aged 2 days. Site 1. There are I.E. lymphocytes between the regular columnar epithelial cells and the annular crenations are shallow. H & E. X 320.

Plate 6.5. The small intestinal mucosa of a gnotobiotic lamb aged 2 days. Note the shallow annular crenations and the extrusion zone at the villus tip. SEM. X 200.

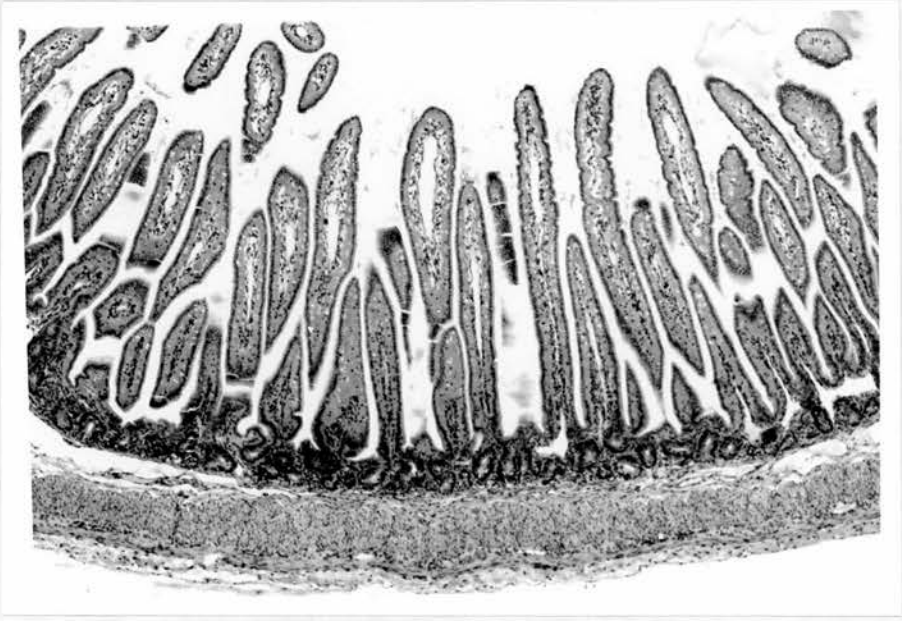

Plate 6.6. The small intestinal mucosa of an uninfected, gnotobiotic lamb aged 2 days. Site 2. The villi are long and thin with shallow annular crenations. Absorptive vacuoles are present within the epithelial cells. H & E. X50.

Plate 6.7. The small intestinal villi of an uninfected, gnotobiotic lamb aged 5 days. Site 2. The villi are long and thin with annular crenations. Several crypt mouths are visible at the bases of the villi. SEM. X 200.

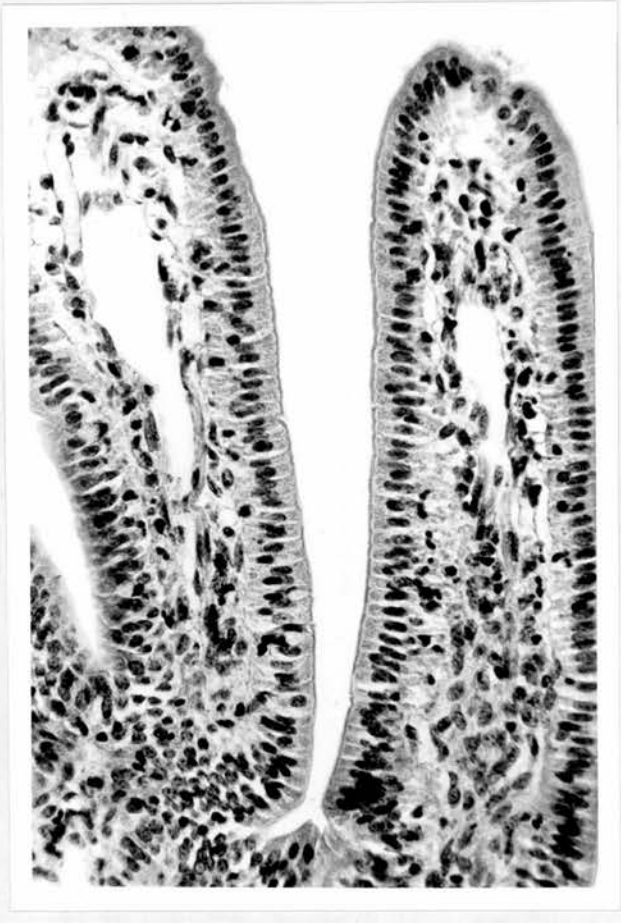

Plate 6.8. The villus epithelium of the small intestine of an uninfected, gnotobiotic lamb aged 9 days. Site 2. The villi are wide and cylindrical in shape and there are many cells in the lamina propria. H & E. X 320.

Plate 6.9. The small intestinal villi in an uninfected, gnotobiotic lamb aged 7 days. Site 2. The villus shape is wider but remains cylidrical. SEM. X200.

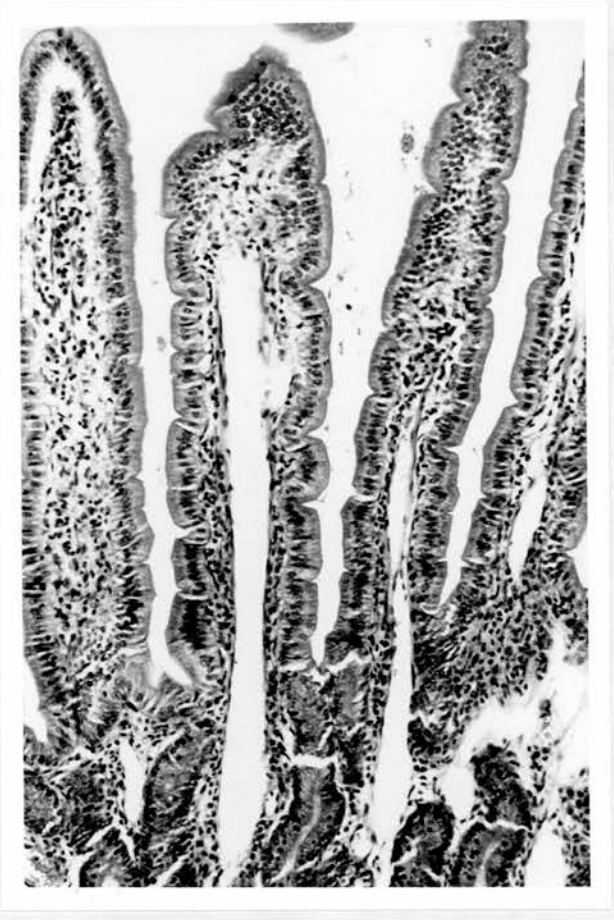

Plate 6.10. The small intestinal mucosa of an uninfected, gnotobiotic lamb aged 20 days. Site 2. The villi are long with deep, annular crenations and there are many lymphoid cells in the lamina propria. H & E. X 125.

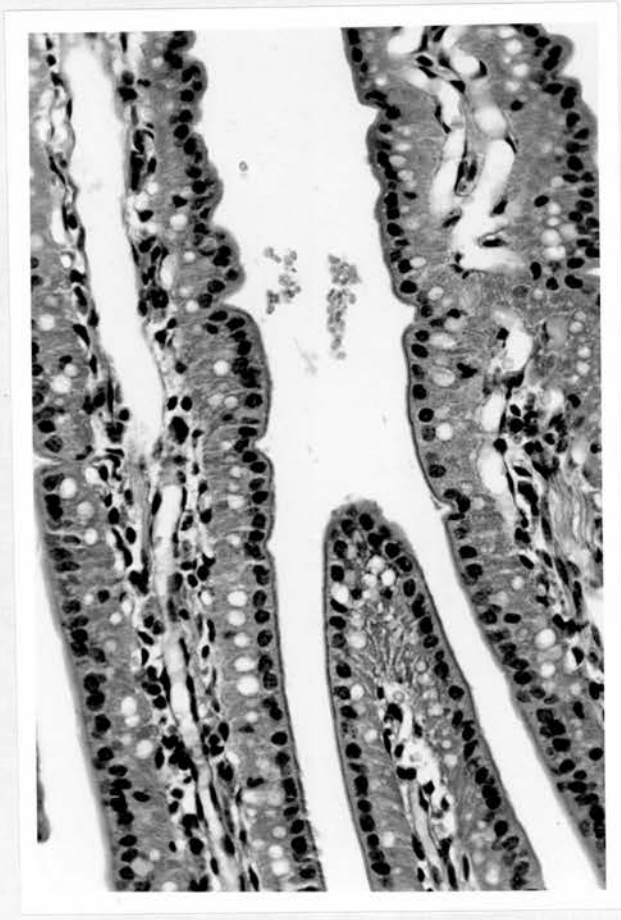

Plate 6.11. The villus epithelium of the small intestine of an uninfected, gnotobiotic lamb aged 2 days. Site 2. Note the absorptive vacuoles within the villus epithelial cells. H & E. X 320.

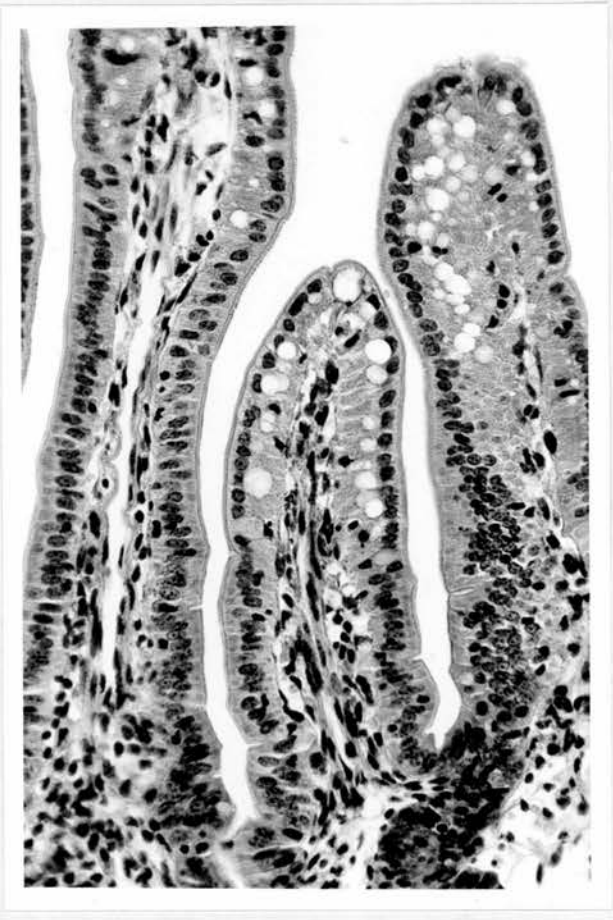

Plate 6.12. The villus epithelium of the small intestine of an uninfected, gnotobiotic lamb aged 5 days, Site 2. The absorptive vacuoles are confined to the upper part of the villus. H & E. X 320.

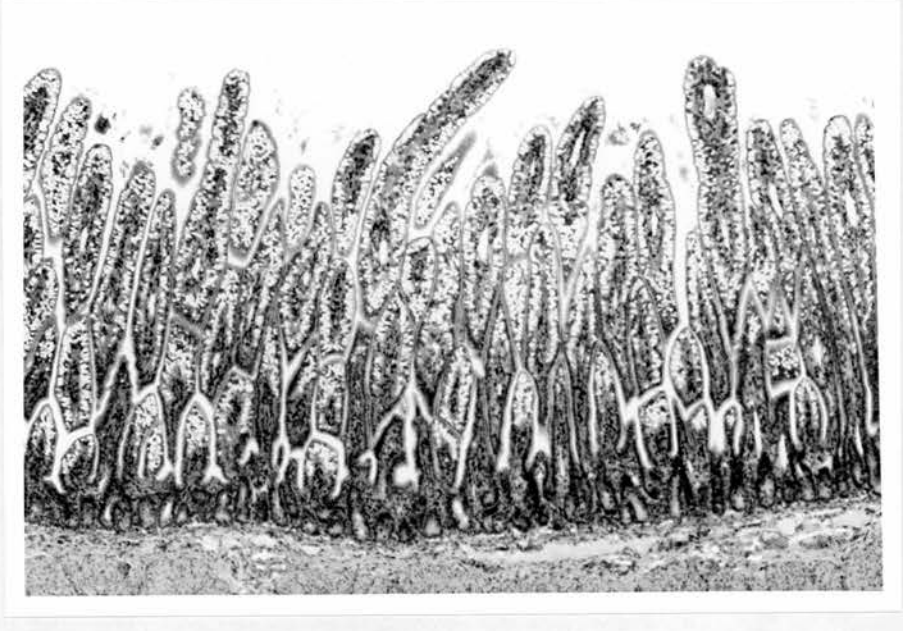

Plate 6.13. The small intestinal mucosa of an uninfected, gnotobiotic lamb aged 2 days. Site 3. The villi are a mixture of fingers and leaves, the crenations are shallow and vacuolated absorptive cells are present over the majority of the villus surface. H & E. X 50.

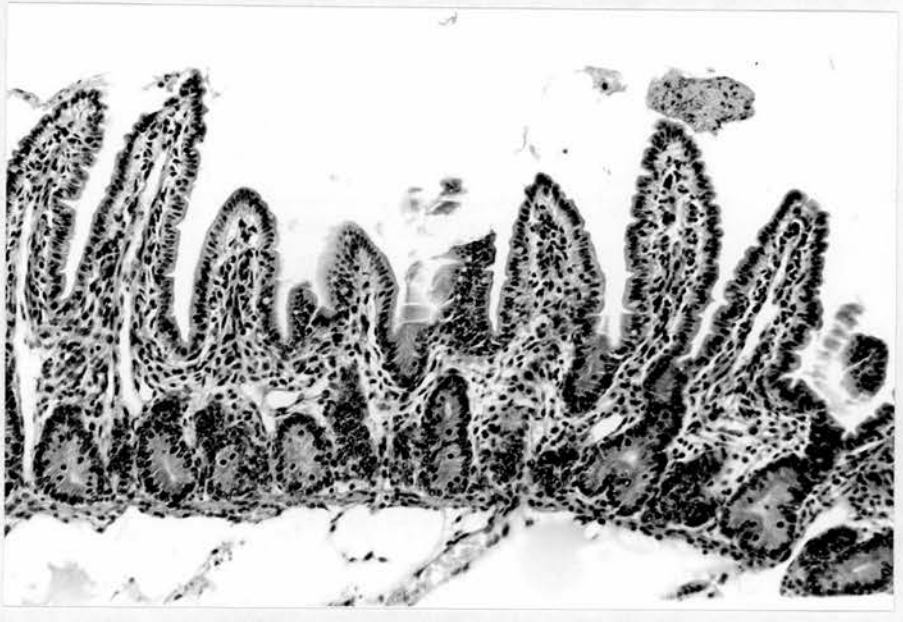

Plate 6.14. The small intestinal mucosa of an uninfected, gnotobiotic lamb aged 8 days. Site 3. The villi are leaf and conical shaped and deeply crenated. H & E. X 125.

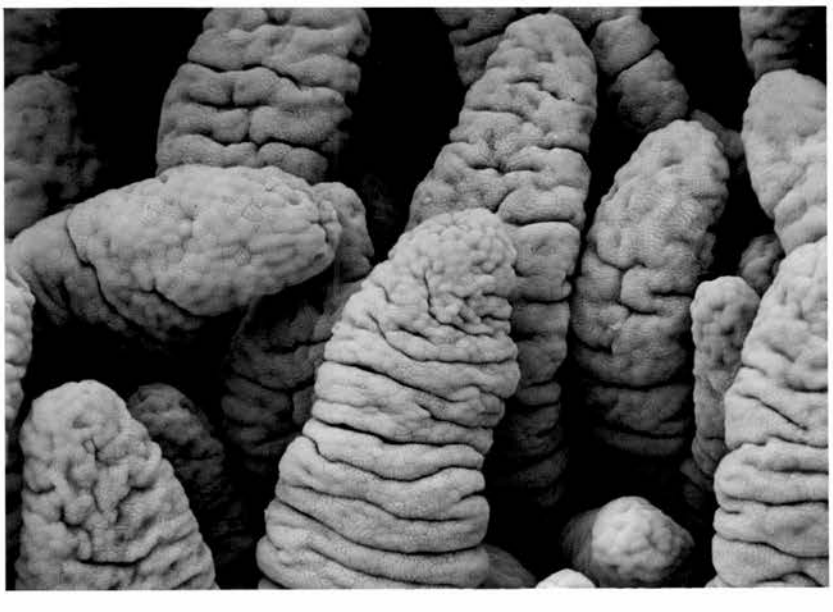

Plate 6.15. The villus epithelium in the small intestine of an uninfected, gnotobiotic lamb aged 5 days. Site 3. Note the polygonal epithelial cells which are easily distinguished. SEM. X 200.

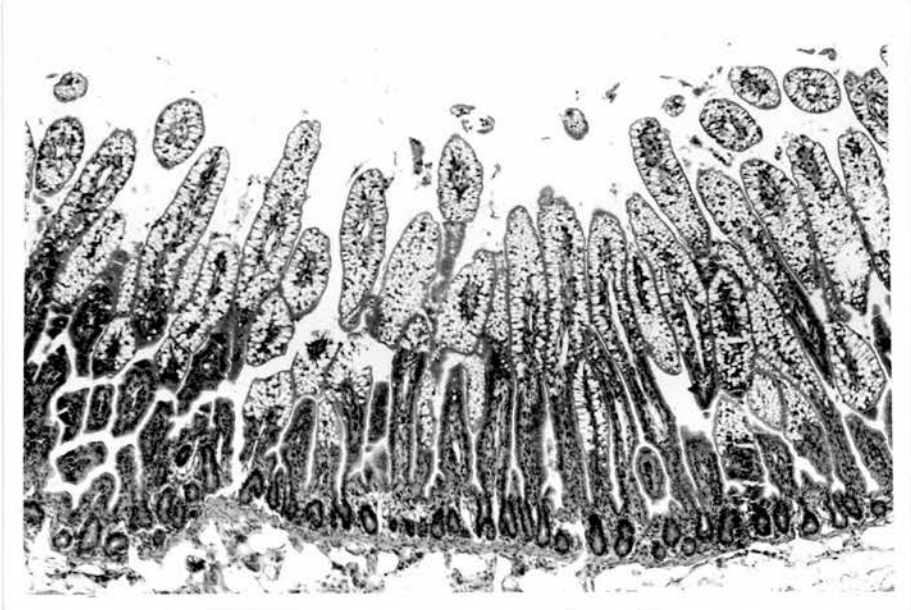

Plate 6.16. The small intestinal mucosa of an uninfected, gnotobiotic lamb aged 5 days. Site 3. Absorptive vacuoles are present on the upper parts of the villi only. H & E. X 50.

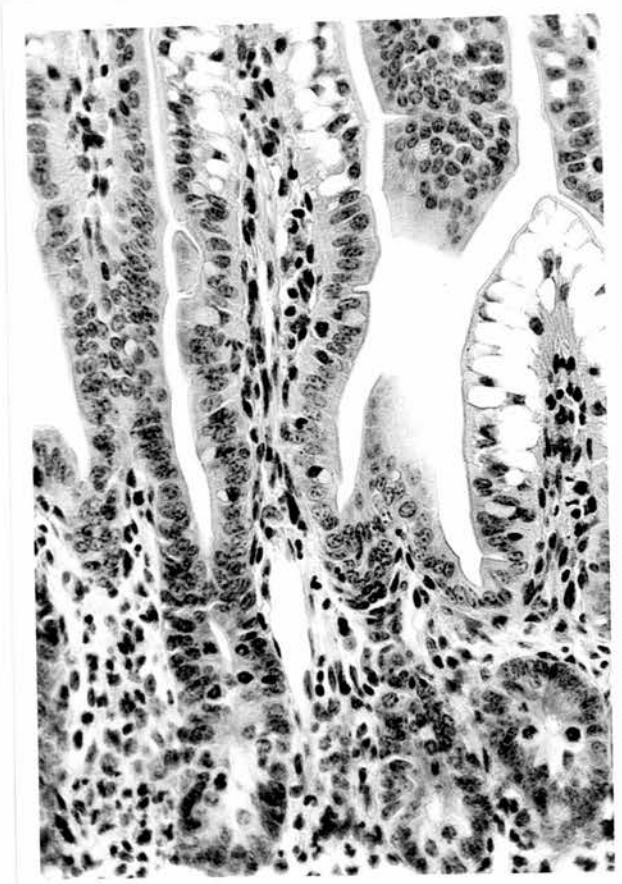

Plate 6.17. The villus epithelium of the small intestine of an uninfected, gnotobiotic lamb aged 5 days. Site 3. Detail of the absorptive vacuolated cells on the upper parts of the villi. H & E. X 320.

Introduction.

In a previous section, I have described the current knowledge of the effects of rotavirus infection on the intestinal epithelium. These studies were based on viral immunofluorescence, histology and electron microscopy, in piglets, calves and lambs. From these studies, and experiments involving transmissible gastroenteritis of swine, (TGE), came the theory that infected cells were sloughed off quickly, the villi became atrophied and were rapidly reclad by undifferentiated cells from the crypts, which in turn caused deficiencies of the brush border enzymes in the intestinal epithelial cells in the vicinity of the lesion. In fact, lactose intolerance was clearly stated in two reviews as an important mechanism of rotavirus diarrhoea (Flewett and Woode, 1978; Walker-Smith, 1978). However, it now appears that changes in the sodium transport systems are more relevant (Shepherd, Gall, Butler and Hamilton, 1979).

The aims of the experiment described in this chapter were:

(1) to describe the effect of rotavirus infection on villus and crypt structure, from initial infection until after clinical recovery.

(2) to establish whether lactase deficiency occurred as a result of the presence of immature, undifferentiated enterocytes and could this be attributed to rapid cell production in the crypts.

(3) to discover if there was a correlation between the observed diarrhoea and the crypt cell production rate.

Animals used.

Twelve gnotobiotic lambs were involved, six were infected on the second day of life with rotavirus and six were kept as age matched controls. These controls were included in the description in Chapter 6. At each of 1, 2, 4, 8, 11 and 15 days after infection, an infected lamb and an age matched control were sampled for cell kinetic measurements, microdissection, histology and lactase assay. Additional material was obtained from a different group of lambs, similarly infected with rotavirus, for scanning electron microscopic examination. Details of the animals and the techniques used are shown in Table 7.1.

Clinical and virological response.

The infected lambs were dull and listless in their behaviour and some showed evidence of abdominal discomfort. The infected animals drank considerably less milk than the controls in the first 48 hours after infection, Fig 7.1. The diarrhoea, which developed 15-20 hours after infection, continued for 3 - 4 days, was watery and varied from brown to green in colour. The animals were clinically well after the fourth day.

Examination of the faeces by electron microscopy confirmed the presence of rotavirus in infected animals. Virus particles continued to be shed in the faeces until 6 days after infection. The control lambs did not excrete rotavirus.

Immunofluorescence.

Samples from these animals were not available for examination.

Villus length and crypt length.

Marked changes in villus and crypt length were seen at sites 2 and 3. Villus shortening was present 1 and 2 days after infection at site 2, and from 1 - 4 days after infection at site 3. The crypts were elongated at sites 2 and 3 on day 2 after infection, whereas there was no change at site 1. Thereafter, there was no difference from the age matched control values, Fig 7.2. Although single animals were used for the comparisons, multiple villus and crypt measurements were performed. The measurements for groups of villi and crypts in infected animals were then compared against similar groups of villi and crypts in control animals and the 'p' values confirmed the observed differences between the two groups to be significant.

Histology.

Site 1.

On day 1 after infection the villi were finger shaped with crenations on the upper halves. Columnar epithelium covered the villi and the epithelial cells at the villus tips were damaged. By 2 days after infection the columnar cells at the villus tip were disordered and vacuoles, containing dense, granular material, were present within these apical epithelial cells (Plate 7.1). These vacuoles were still present 4 days after infection. From day 8 until day 15 after infection, the villi were of normal appearance (Plate 7.2).

From days 1 - 8 after infection, the lamina propria was densely packed with lymphoid cells and fibroblasts and large numbers of eosinophils were present. The number of intra-epithelial lymphocytes

did not vary greatly from normal levels, Table 7.2.

Site 2.

On day 1 after infection, the villi were short, covered with cuboidal epithelial cells and the nuclei of the epithelial cells were closely packed. The villus surface appeared rough and bumpy, especially at the villus tip (Plate 7.3) and the epithelial cells were distended (Plate 7.4). The appearance was similar 2 days after infection, but the cuboidal cells on the lower parts of the villi were replaced by columnar cells (Plate 7.5). Vacuoles filled with dense, granular material were present within the epithelial cells of the villus tips (Plate 7.6). By 4 days after infection, the villus shape was normal but deeply crenated at the tips and the epithelial cells at the villus tips were disordered. The lower parts of the villi were covered by columnar cells. From day 8 after infection the villi were completely normal (Plate 7.7). The lamina propria was densely filled with cells from 1 - 8 days after infection (Plates 7.4, 7.7). The intra-epithelial lymphocytes were similar in number to those found in control animals, Table 7.2.

Site 3.

On the first and second days after infection, the villi were short and clothed with cuboidal epithelial cells and in places the epithelial cell layer was disrupted, exposing the lamina propria. Vacuoles, filled with dense, granular material, were present in the epithelial cells at the villus tip (Plate 7.8). From day 4 after infection, the villi looked normal, with columnar epithelium, slight crenations and no vacuoles in the epithelial cells (Plate 7.9).

Scanning electron microscopy.

Site 1.

The surfaces of the villi were wrinkled and irregular by 1 day after infection and the tips of the villi were abnormal (Plate 9.18) and by 2 days after infection the cells covering the villus tip were disordered and loose (refer to Plate 8.11). However, by day 3 after infection, the villi were long and finger-like in shape with shallow annular crenations and generally resembled the villi of control animals. The exceptions were a few villi which had a bifid form (Plate 7.10). By 8 days after infection the villi appeared completely normal.

Site 2.

The villi were short and clad with swollen, damaged epithelial cells by 1 day after infection (Plate 7.11), in some instances the lamina propria was exposed (Plate 7.11). There were a few 'blebs' (blister-like structures) on the surfaces of the epithelial cells (Plate 7.12). By 3 days after infection the villi were still short and damaged and clothed with collapsed epithelial cells which were detaching from both the tips and the sides of the villi. At 8 days after infection the villus structure and the associated epithelium were normal in appearance.

Site 3.

The surfaces of the upper halves of the villi were crinkled with evidence of fusion at the tips and the epithelial cells in this area were swollen, 1 day after infection (Plate 7.13). By 2 days after infection the villi appeared considerably shortened, with jumbled, distended epithelial cells present on the apical part of the villus

(refer to Plate 8.11). The regular crenations were replaced by irregular folds. The annular crenations were again present 8 days after infection and by 15 days after infection the villi appeared normal.

Epithelial cell kinetics.

Site 1.

The crypt cell production rate was increased at site 1 only on day 2 after infection. Thereafter, values were comparable to those in age matched controls, Table 7.3.

Site 2.

There was a marked change in the crypt cell production rate at site 2. The rate of cell production increased by 2 days after infection and continued to rise to a maximum of 21.2 cells/crypt/hour on the eighth day after infection, compared to a control rate of 3.2 cells/crypt/hour. By day 11 after infection the crypt cell production rate was approaching control levels, Table 7.3.

Site 3.

The crypt cell production rate was increased by day 2 after infection, remained raised 4 days after infection and had returned to normal levels by 8 days after infection, Table 7.3.

Crypt to villus ratio.

The crypt to villus ratio was not significantly changed by rotavirus infection, Table 7.4.

Tissue lactase levels.

Site 1.

Lactase levels were lower than those of the control animals throughout the course of the experiment in the anterior site, Fig. 7.3.

Site 2.

Lactase levels were reduced from day 1 to day 4 after infection, Fig. 7.3.

Site 3.

Lactase levels were lower than normal, 1 - 4 days after infection, Fig. 7.3.

Conclusions.

The jejunum was least severely damaged by rotavirus infection, no villus shortening being apparent from histological examination of the tissues and only slight abnormalities appearing on scanning electron microscopic examination. In the more distal regions of the small intestine, villus atrophy was present from 1 to 4 days after infection.

The onset of the increase in crypt cell proliferation and the elongation of the crypts appeared to be associated with the appearance of villus atrophy. However at site 2, the increase in

crypt cell production was maintained for a considerable period after the villus structure had returned to normal.

Changes in lactase levels also appeared to be associated with the size of the villi and not with changes in crypt length. The reduction in lactase levels did not correlate with the increased crypt cell production rate. The diarrhoea did not seem to be related to the increase in crypt cell production rate observed in infected animals as the highest levels of crypt cell production were present after the diarrhoea had ceased, on the 8th day after infection.

There were considerable differences between the 3 sites, even within the same animal; it was therefore important to complement this work with studies at multiple sites to examine specific transition points. The points chosen were day 2 after infection (peak of villus atrophy) and day 8 after infection (essentially normal villi, peak of crypt cell production at site 2).

Furthermore, by 24 hours after infection, substantial abnormalities were already present; it was necessary to examine the earlier stages of the infection to find out what, if any, effects preceded or accompanied the onset of diarrhoea. Details of these additional experiments are contained in chapters 8 and 9.

Technique	Time after infection(days)						
	1	2	3	4	8	11	15
Histology.	1	1	1	1	1	1	1
Microdissection.	1	1	-	1	1	1	1
Scanning electron microscopy.	2	2	1	-	-	-	1
Lactase assay.	1	1	-	1	1	1	1

Table 7.1. Numbers of gnotobiotic lambs used to study the effects of rotavirus infection administered on the second day of life, on the villus and crypt epithelium of the small intestine.

- indicates no animal used.

Time after infection(days)	Intra-epithelial lymphocytes / 100 epithelial cells		
	Site 1	Site 2	Site 3
1	16•5	13•2	14•4
2	9•2	8•7	11•1
4	9•4	10•1	10•1
8	13•6	12•7	15•4
11	16•3	17•3	14•0
15	20•5	18•9	15•1

Table 7.2. I.E. lymphocytes / 100 epithelial cells in 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the second day of life.

Time after infection(days)	Cells / crypt / hour. Site 1	Site 2	Site 3
1	12•5	3•8	11•1
2	17•2	13•8	12•8
4	6•5	16•6	10•7
8	5•3	21•2	9•1
11	11•1	12•3	9•6
15	38•2	9•3	12•4

Table 7.3. Crypt cell production rates in 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the second day of life.
The CCPR is expressed as the number of cells produced per crypt per hour.

Time after infection(days)	The number of crypts per villus		
	Site 1	Site 2	Site 3
1	4•1	2•1	2•2
2	4•0	3•5	2•7
4	4•1	2•9	3•0
8	3•8	2•8	3•1
11	4•6	3•5	4•3
15	4•3	3•2	4•2

Table 7.4. The crypt to villus ratio in 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the second day of life.

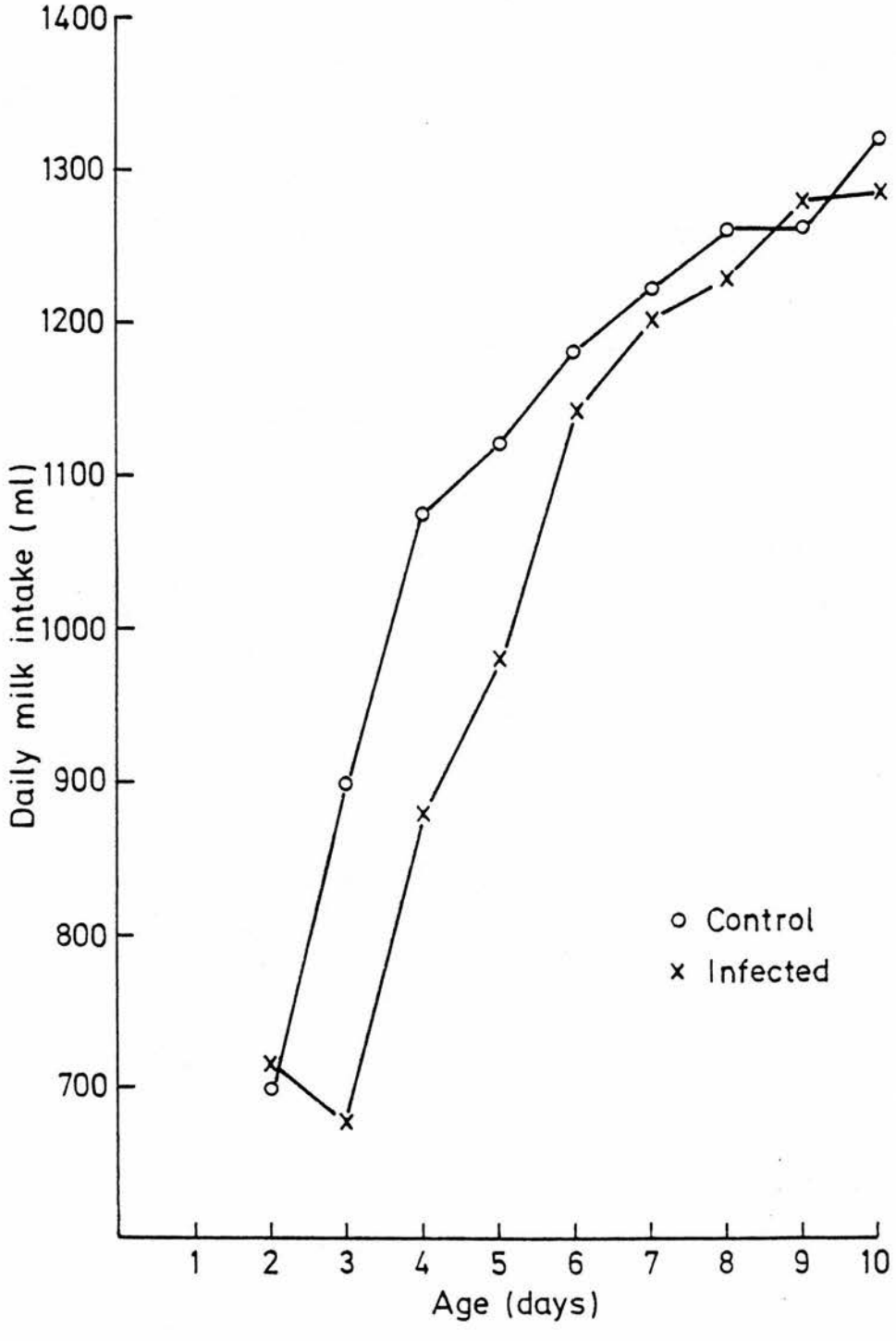

Fig 7.1. Daily milk intake in gnotobiotic lambs infected with rotavirus on the second day of life and age matched controls.

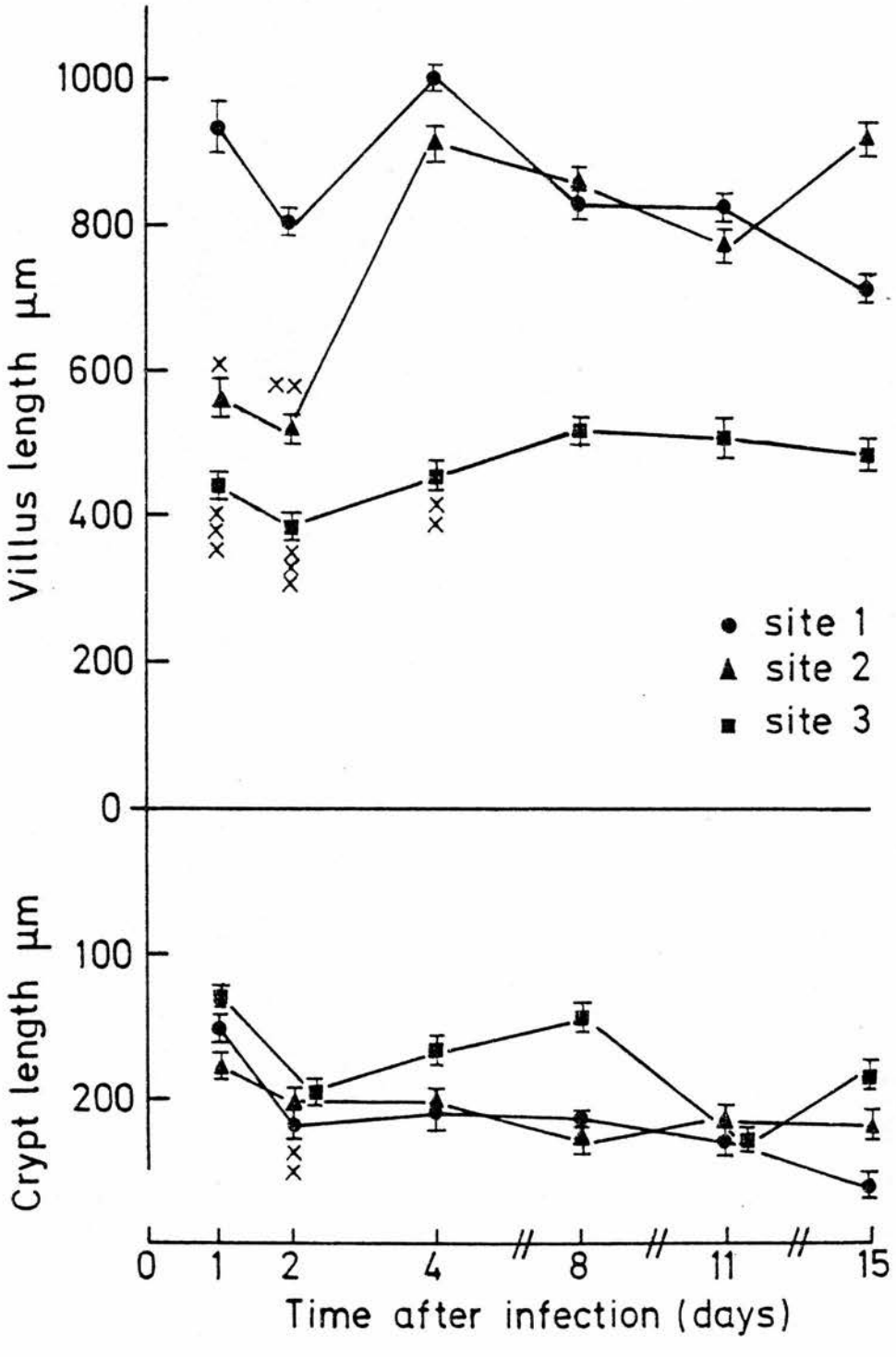

Fig 7.2. Villus and crypt lengths at 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the second day of life. Microdissection measurements.

x, xx and xxx denote level of significance of difference from controls. x : p less than 0·05 to xxx : p less than 0·001

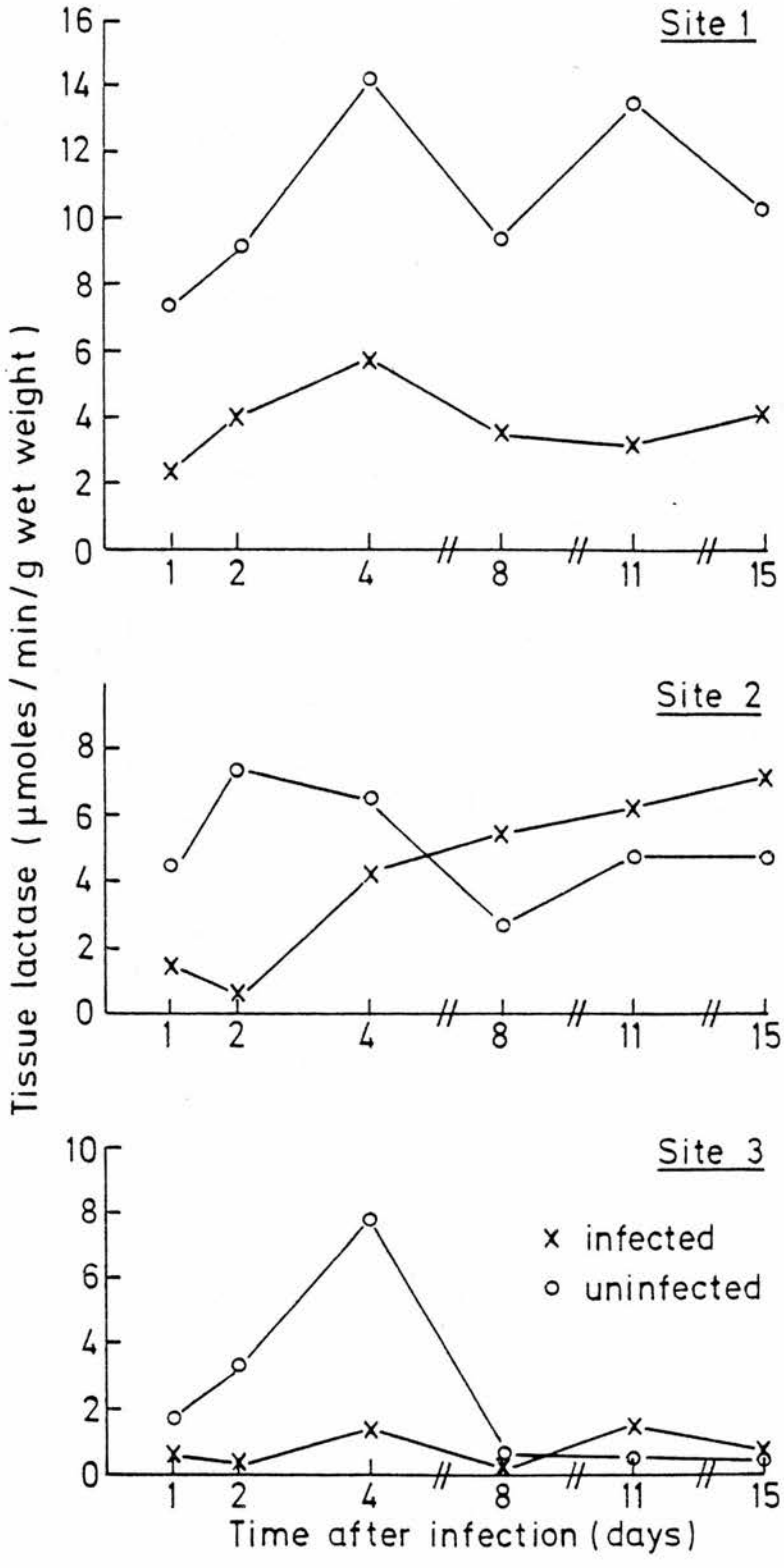

Fig 7.3. Tissue lactase levels at 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the second day of life.

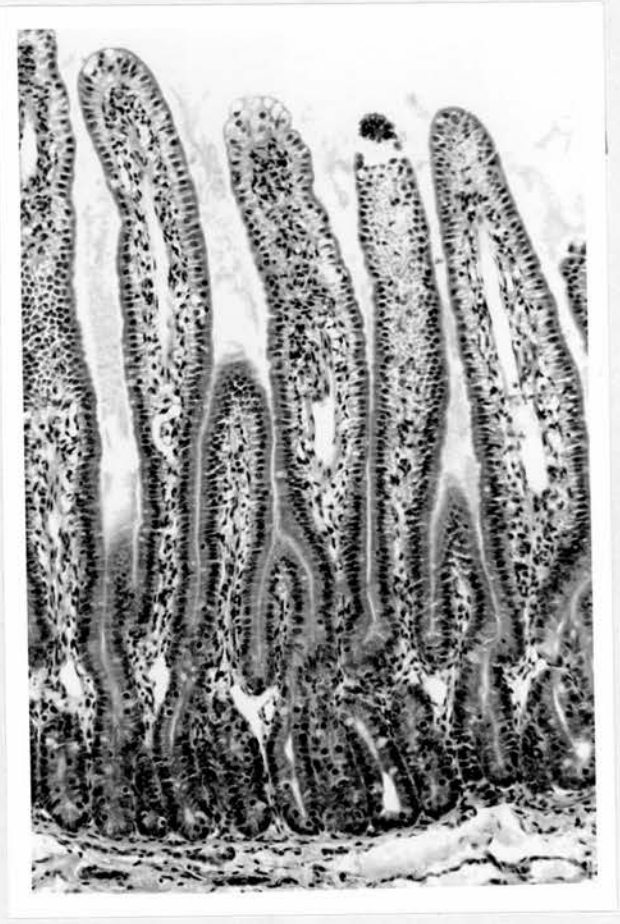

Plate 7.1. The small intestinal mucosa of a gnotobiotic lamb, 2 days after infection with rotavirus on the second day of life. The villus cells at the villus tips are disarranged and dense, granular material is present within vacuolated cells. H & E. X 125.

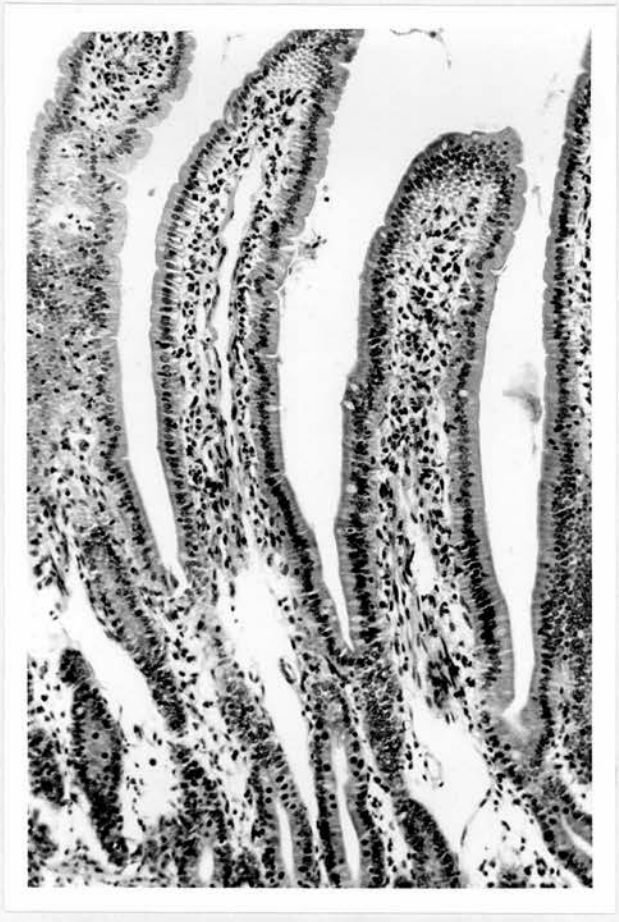

Plate 7.2. The small intestinal mucosa of a gnotobiotic lamb, 8 days after infection with rotavirus on the second day of life. Site 1. The tissue has a normal appearance. H & E. X 125.

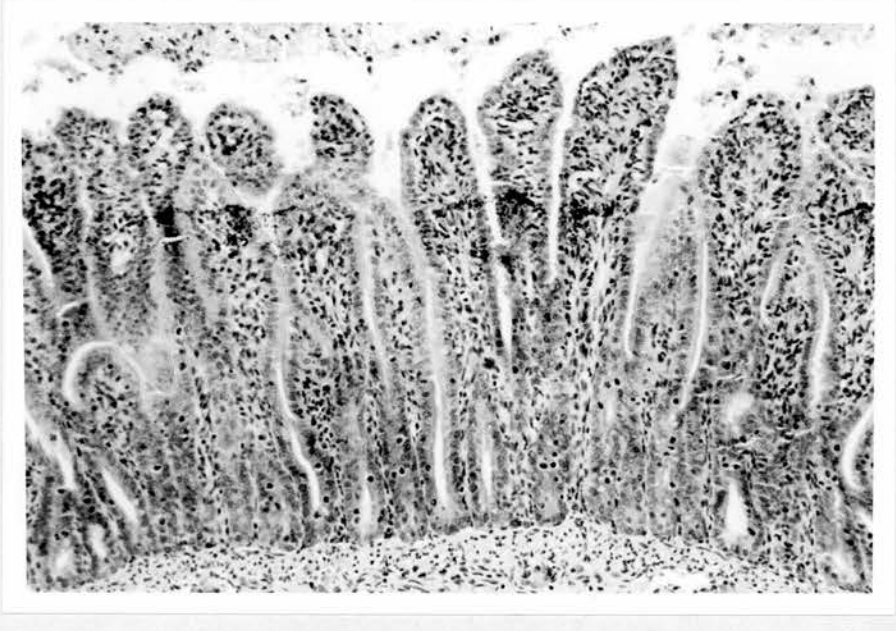

Plate 7.3. The small intestinal mucosa of a gnotobiotic lamb, 1 day after infection with rotavirus on the second day of life. Site 2. The villi are short and covered by swollen cuboidal epithelial cells. H & E. X 125.

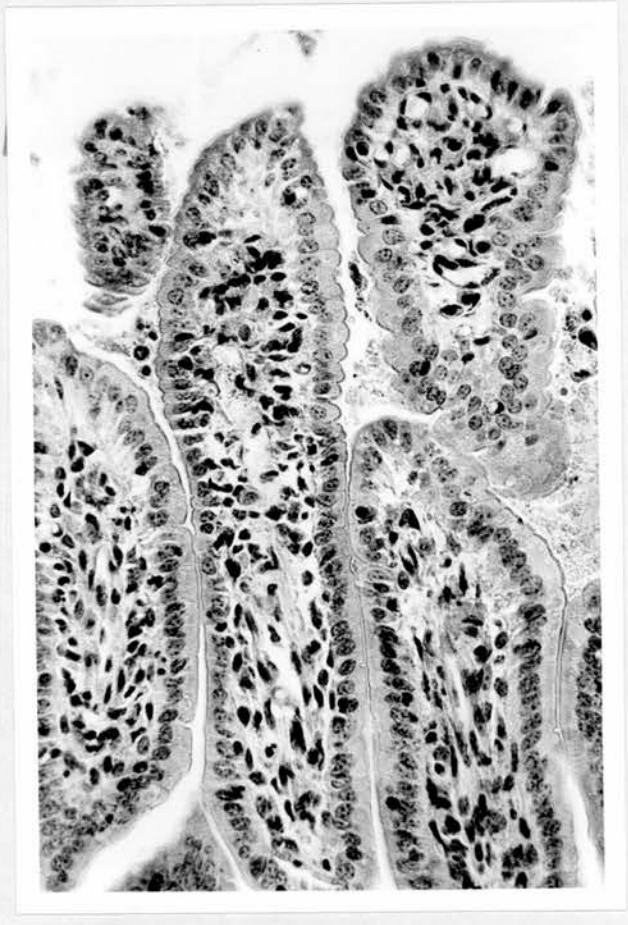

Plate 7.4. The villus epithelium of a gnotobiotic lamb, 1 day after infection with rotavirus on the second day of life. Site 2. The epithelial cells at the villus tip are swollen. H & E. X 320.

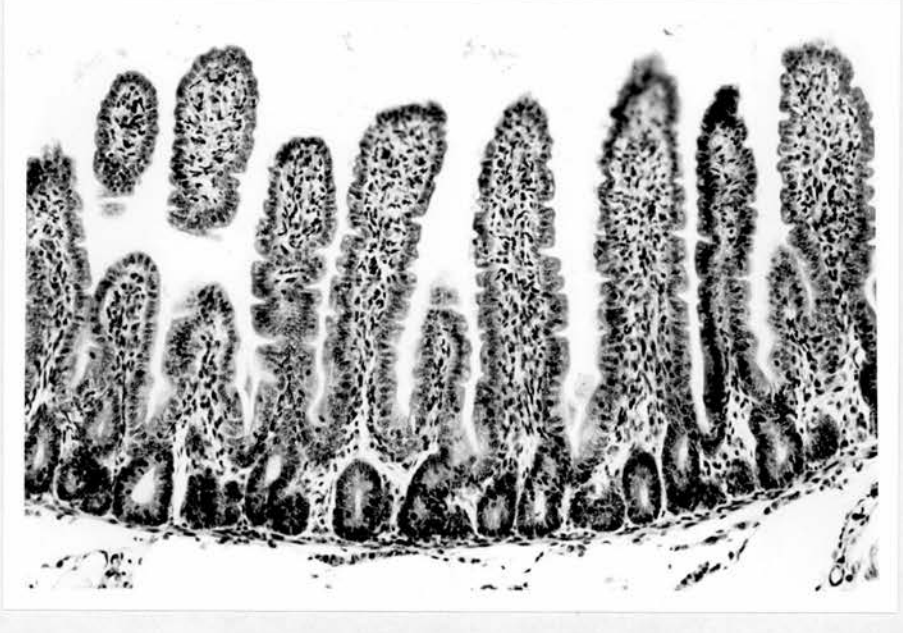

Plate 7.5. The small intestinal mucosa of a gnotobiotic lamb, 2 days after infection with rotavirus on the second day of life. Site 2. The cuboidal epithelium of the villi is gradually replaced by columnar cells as the villi recover from the infection. H & E. X 125.

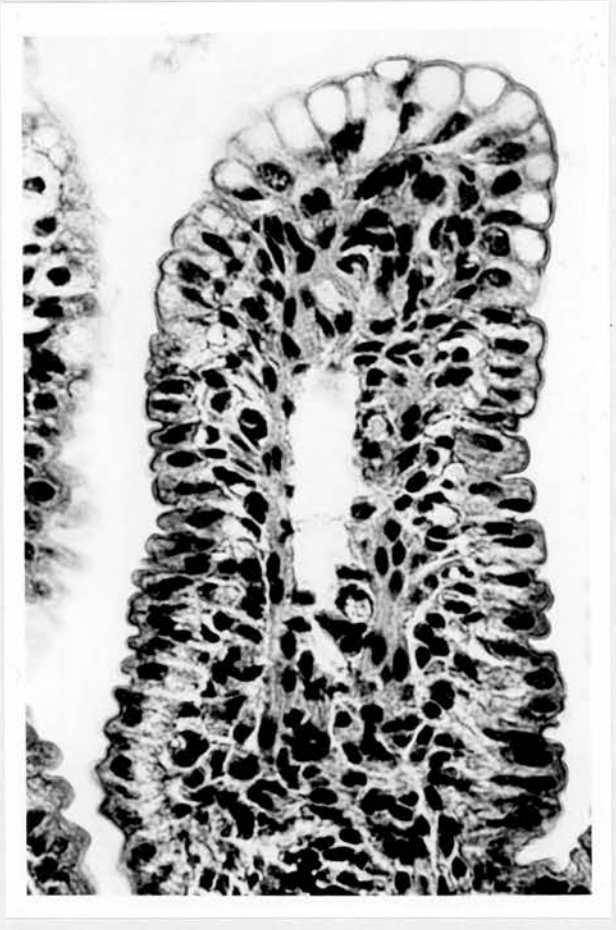

Plate 7.6. High magnification of the vacuoles filled with dense, granular material which are present at the villus tips. Small intestine of a gnotobiotic lamb, 2 days after infection with rotavirus on the second day of life. Site 2. H & E. X 500.

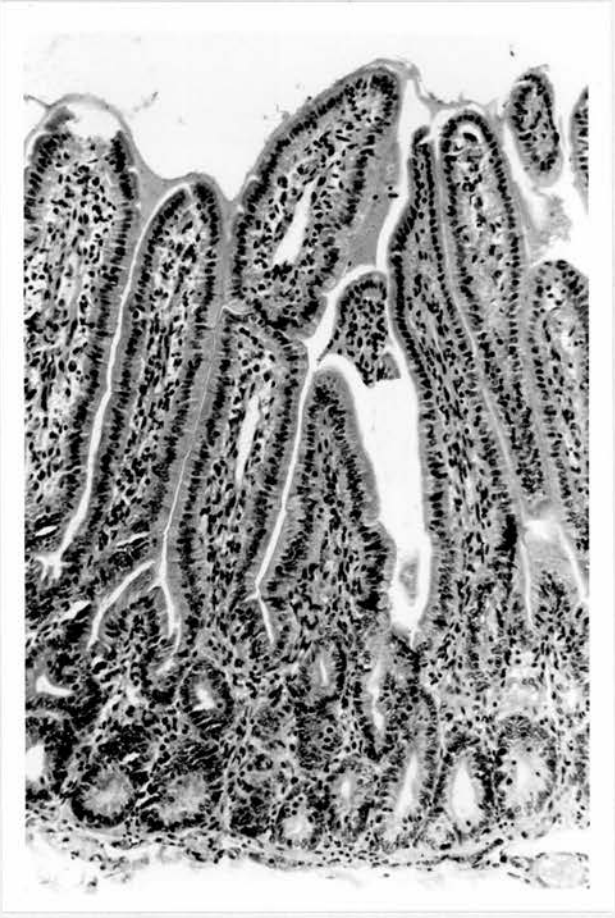

Plate 7.7. The small intestinal mucosa of a gnotobiotic lamb, 8 days after infection with rotavirus on the second day of life. Site 2. The villi are normal.
H & E. X 125.

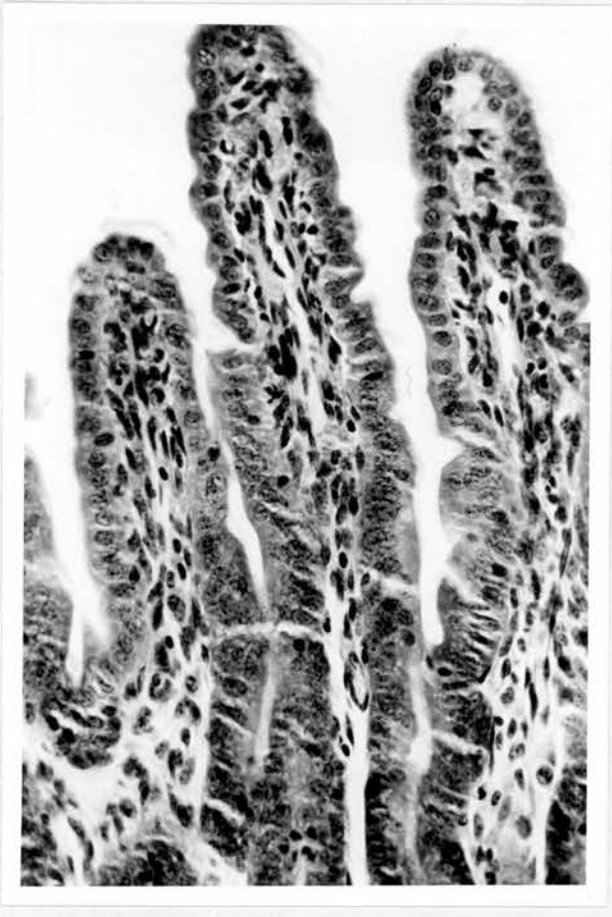

Plate 7.8. The small intestinal mucosa of a gnotobiotic lamb, 2 days after infection with rotavirus on the second day of life. Site 3. The epithelial cells on the upper part of the villus are cuboidal and contain vacuoles filled with dense granular material. H & E. X 125.

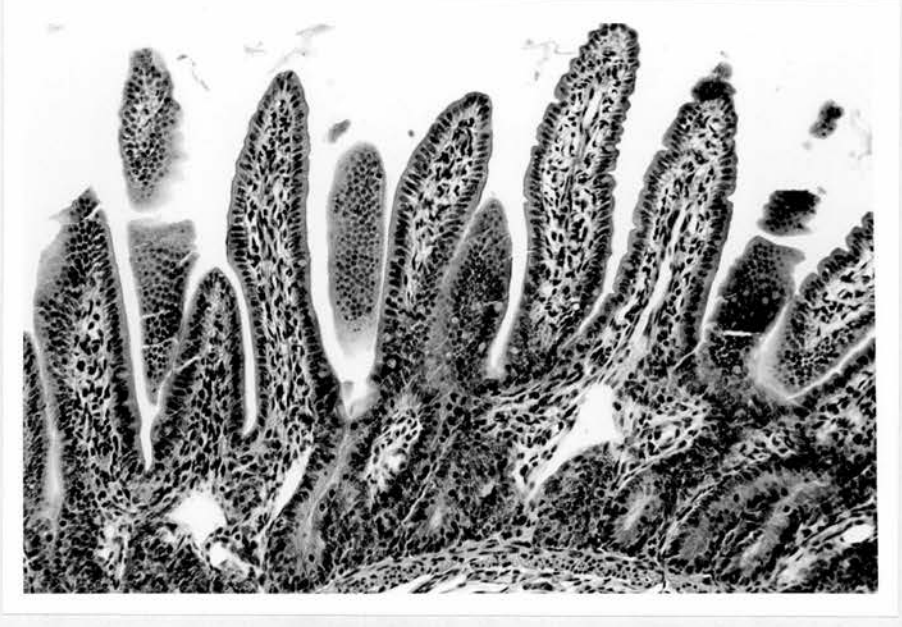

Plate 7.9. The small intestinal mucosa of a gnotobiotic lamb, 4 days after infection with rotavirus on the second day of life. Site 3. The villi are essentially normal. H & E. X 125.

Plate 7.10. Villus structure in the small intestine of a gnotobiotic lamb, 3 days after infection with rotavirus on the second day of life. Site 1. Bifid villus. SEM X 200.

Plate 7.11. The villus epithelium in the small intestine of a gnotobiotic lamb, 24 hours after infection with rotavirus on the second day of life. Site 2. The epithelial cells are swollen and damaged and the lamina propria is exposed. SEM. X 500.

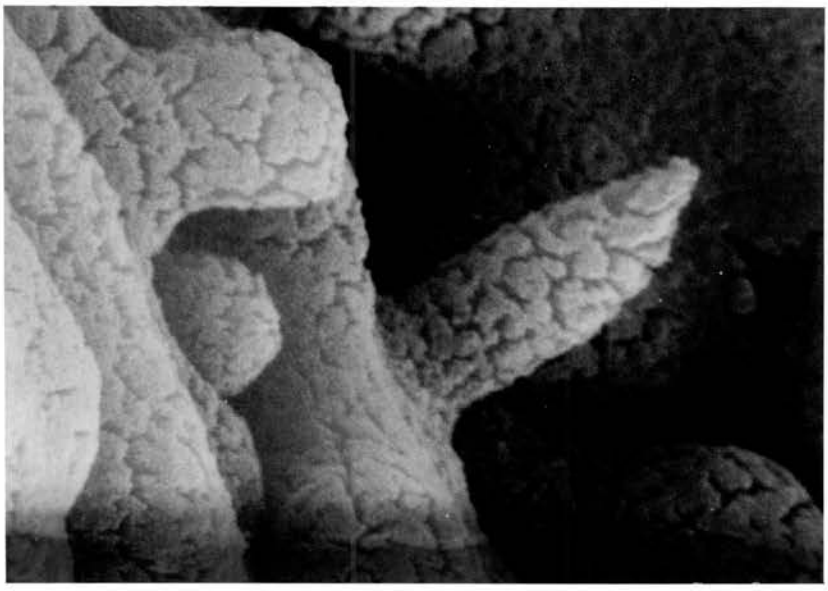

Plate 7.12. The villus epithelium of the small intestine of a gnotobiotic lamb, 2 days after infection with rotavirus on the second day of life. Site 2. The formation of a 'bleb' in an epithelial cell. SEM. X 10,000.

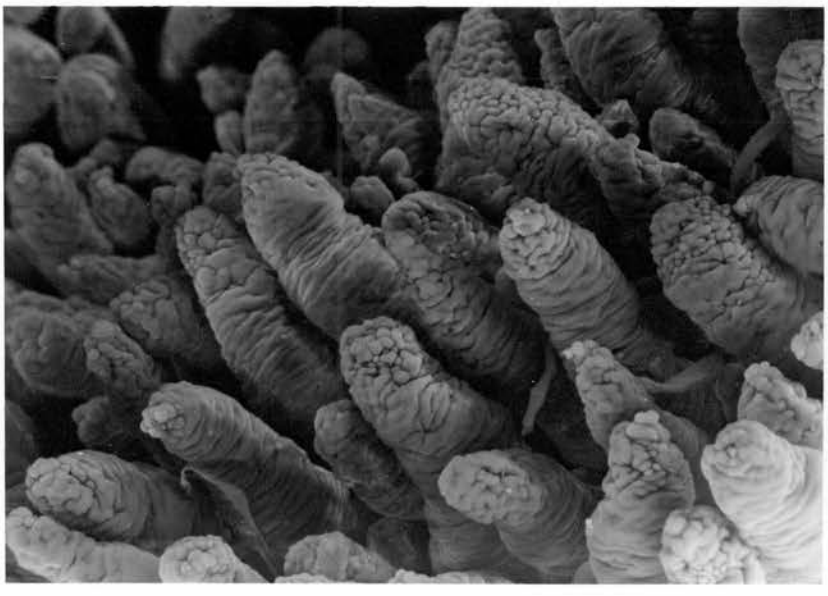

Plate 7.13. Small intestinal villi in a gnotobiotic lamb, 24 hours after infection with rotavirus on the second day of life. Site 3. The villi are short and the epithelial cells on the upper half of the villi are swollen and disordered. SEM. X 200.

Chapter 8.

The effects of rotavirus infection on the small intestinal epithelium at 30cm intervals along the small intestine of gnotobiotic lambs infected on the second day of life

Introduction.

Dr Snodgrass and his colleagues had selected three sites of small intestine for their previous experiments describing the pathology of rotavirus infection. These sites were proximal, mid and distal small intestine, as described in chapter 4. Other workers have often studied only one site, that which was known to be affected, (i.e. the ileum of the pig).

The experiment described in chapter 7 was performed on the same sites of small intestine as had been used previously. In order to confirm that the three sites previously chosen did in fact give a true sample, and were representative of the lesions reported, additional studies were performed in 4 lambs, 2 infected and 2 controls. An added advantage of this experiment was the duplication of experimental animals at two important stages in the pathology of the disease. Samples were taken at 30cm intervals along the total length of the small intestine.

Thus the aims of this experiment were:

(1) to describe the lesion at 30cm intervals along the small intestine.

(2) to ascertain whether the three sites of sampling in general use, were representative of the lesion throughout the small intestine.

Animals used.

Four gnotobiotic lambs were involved in this experiment. Two were infected on the second day of life with rotavirus, two were retained as age matched controls.

At 2 and 8 days after infection, samples from an infected lamb and the age matched control were removed from the small intestine at

30cm intervals and examined by immunofluorescence, histology, microdissection, tissue lactase assay and scanning electron microscopy.

Immunofluorescence.

There was distinct, bright fluorescence, 2 days after infection, in the epithelial cells at all sites of the small intestine (Plate 8.1). The greatest concentration of infected cells was present in the middle region of the small intestine, Table 8.1. By 8 days after infection, there was little evidence of virus infected, epithelial cells in the tissue samples, with only a few single cells at the villus tips in the middle region of the small intestine exhibiting fluorescence.

Villus and crypt length.

The age matched control animals had the same pattern of small intestinal architecture as the control animals in chapter 6. In the youngest control animal (4 days of age), the villi of the first site (duodenum) and the distal sites (ileum) were shorter than those present in the middle regions. This proximal to distal variation in length was most obvious in the older control animal. Crypt lengths were consistent throughout the small intestine and increased in length as the age of the animal increased, Tables 8.2 and 8.3.

When the infected lamb, 2 days after infection, was compared with the control, a significant reduction in villus length was apparent in nine out of the twelve sites ($p < 0.05$). Crypt length was also significantly increased distal to site 4, with all but one of the biopsies having longer crypts ($p < 0.05$), Table 8.2 and Fig. 8.1.

Villus and crypt lengths did not differ significantly between the lamb killed 8 days after infection and the age matched control, Table 8.3.

Histology.

Control animals.

The histological appearance of the small intestine of the two controls was very similar to that described in chapter 6. In the younger animal, the villi were long and finger shaped with shallow, annular crenations. The epithelial cells were columnar in type and absorptive vacuoles were present only at the distal sites, (Plates 8.2, 8.3). The older control animal had finger villi in the anterior and middle regions of the small intestine, whereas those in the distal regions were generally leaf shaped with a few finger shaped villi interspersed among them, (Plates 8.4, 8.5). The finger villi had shallow, annular crenations and those on the leaf villi were deeper and irregular in form. The villus epithelial cells were columnar and there were many goblet cells in the distal region. No absorptive vacuoles were present within the epithelial cells. Many eosinophils were present in the lamina propria, especially in the crypt region and there appeared to be more lymphoid cells in the lamina propria of the older animal.

Infected animals.

There were abnormal histological features at all sites, 2 days after infection, (Plates 8.6, 8.7). The villi were shortened with deep crenations and the epithelial cell layer had lost its regular appearance. The epithelial cells were densely packed, jumbled and swollen, especially at the tips of the villi. In the mid region of

the small intestine there were vacuolated cells filled with dense, granular material, at the tips of the villi, (Plate 8.8). The lamina propria was cellular with increased numbers of lymphoid cells present. By 8 days after infection the small intestine appeared normal, except in the mid region, where the epithelial cells at the tips of the villi contained granular material (Plate 8.9).

The number of intra-epithelial lymphocytes within the epithelium at the two stages of the rotavirus infection was measured with interesting results. In the animal examined 2 days after infection, the number of intra-epithelial lymphocytes was less than the control value in the mid and distal regions of the small intestine. In contrast, the numbers of intra-epithelial lymphocytes present in the small intestine of the animal which had been infected for 8 days were higher than control values in the mid and distal regions, Fig 8.2.

Scanning electron microscopy.

Control animals.

The control animals showed the same features, on scanning electron microscopic examination, as the other normal gnotobiotic lambs of the same age described previously in chapter 6.

Infected animals.

The surface of the small intestine was abnormal 2 days after infection. The villi were short throughout the small intestine (Plate 8.10, 8.11), the epithelial cells on the villus surfaces were swollen and distended (Plate 8.11) and some of the epithelial cells had burst (Plate 8.12) and were detaching from the villus surface, exposing the lamina propria. By 8 days after infection, the villi were still not completely normal. In the anterior part of the small

intestine, the villi appeared similar to those of control animals, being long and finger like with shallow, annular crenations. However, in the mid and distal regions, the villi seemed wider than was normal, with very pronounced crenations in a convoluted rather than an annular pattern, (Plate 8.13). A small proportion of the villi were fused. In general, the changes were as already described in chapter 7 and were consistent within adjacent areas of the gut.

Epithelial cell kinetics.

The number of blocked metaphases accumulated in the 2 day post infection lamb was much higher than that in the control animal, being 11.7 - 19.4/crypt/hour in the infected animal against 5.5 - 7/crypt/hour in the control lamb. The rate of metaphase accumulation was uniformly raised at all the sample sites in the infected animal, Fig.8.3. The lamb killed 8 days after infection also showed raised metaphase accumulation levels, ranging from 8.3 - 18.8/crypt/hour with peak values in the mid region of the small bowel, compared to 7.5 - 11.5/crypt/hour in the control. These results were similar to those demonstrated in chapter 7, using material from three sample sites.

Single, timed samples after inoculation with a metaphase arrest agent, do not give accurate indications of crypt cell production rates, however they do show the general trend of the response to the infection. This experiment confirmed the observation of a prolonged increase in crypt cell production rate at a time when all other clinical and histological features were apparently normal.

The crypt to villus ratios in all four animals were not different.

Tissue lactase levels.

Control lambs had highest levels of lactase activity in the anterior and mid regions of the small intestine with lower levels being present in the distal region.

Lactase levels were dramatically lowered in the lamb studied 2 days after infection, with none being detected in the ileum. Levels in the anterior and mid regions were reduced to approximately one quarter of that present in normal animals, Fig. 8.4. Lactase levels, 8 days after infection, were similar to those in the control animal.

Conclusions.

The lesions found at the multiple sampling sites were similar to those previously described for the three standard sites.

The lesions were present throughout the small intestine, although the severity of the lesion and its duration varied according to the site studied and also between individuals.

The effects of the rotavirus infection were least striking in the jejunum and most severe and long lasting in the mid region.

% distance along the small intestine.	2 days after infection.	8 days after infection.
8	+	-ve
16	+	-ve
23	++	-ve
31	++	-ve
38	+++	-ve
46	+++	+
54	+++	+
62	+++	-ve
69	+++	-ve
77	+++	-ve
85	+	-ve
92	+	-ve

Table 8.1. Presence of rotavirus fluorescence at multiple sites of small intestine of gnotobiotic lambs; 2 days and 8 days after infection with rotavirus on the second day of life.

+ single fluorescent cells.

++ clumps of fluorescent cells.

+++ confluent fluorescence over at least half of each villus.

-ve no fluorescence demonstrated.

Control			2 days after infection		
% distance along the small intestine	Villus length	Crypt length	% distance along the small intestine	Villus length	Crypt length
10	884±19	130±3	8	734±22	157±6
20	1100±34	133±5	16	**662±17	179±6
30	1229±29	117±3	23	**588±13	165±4
40	1233±35	132±3	31	**644±18	**187±5
50	996±38	122±3	38	**660±21	**187±5
60	1214±31	116±4	46	660±11	**201±4
70	1148±40	126±1	54	**523±22	**169±3
80	962±24	103±3	62	**534±23	**184±5
90	972±21	123±8	69	**515±23	151±7
			77	**601±31	**168±3
			85	623±24	**166±4
			92	**452±26	**160±5

Table 8.2. Villus and crypt lengths at multiple sites of small intestine of a gnotobiotic lamb infected with rotavirus on the second day of life and an age matched control. (μm, mean ± S.E.) Microdissection measurements. ** : $p<0.01$ by Student's 't' test.

Control			8 days after infection		
% distance along the small intestine	Villus length	Crypt length	% distance along the small intestine	Villus length	Crypt length
8	1057±16	170±6	8	1070±33	192±8
16	1071±22	159±4	16	900±25	163±4
23	1169±23	187±4	23	1057±21	167±5
31	863±35	168±7	31	889±31	144±5
38	935±18	184±5	38	885±29	161±3
46	986±26	170±6	46	1087±24	179±4
54	902±32	163±5	54	945±30	175±8
62	956±28	188±6	62	901±15	176±6
69	712±16	153±6	69	863±17	178±3
77	Peyer's patch		77	831±39	178±5
85	635±19	162±3	85	637±14	152±4
92	Peyer's patch		92	465±18	137±4

Table 8.3. Villus and crypt lengths at multiple sites of small intestine of a gnotobiotic lamb infected with rotavirus on the second day of life and an age matched control. (μm, mean ± S.E.) Microdissection measurements.

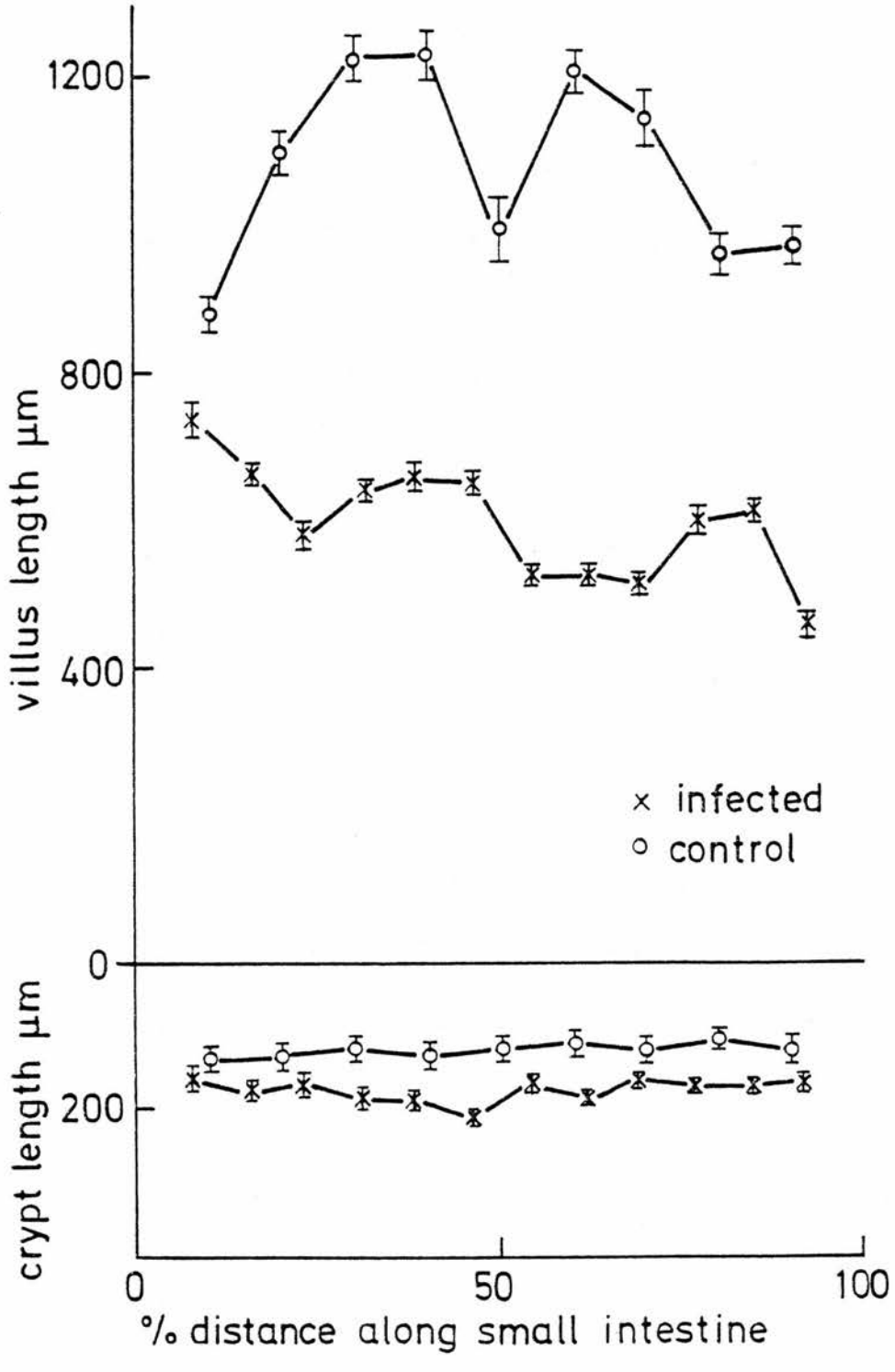

Fig 8.1. Villus and crypt lengths in the small intestine of a gnotobiotic lamb, 2 days after infection with rotavirus on the second day of life and an age matched control. Microdissection measurements.

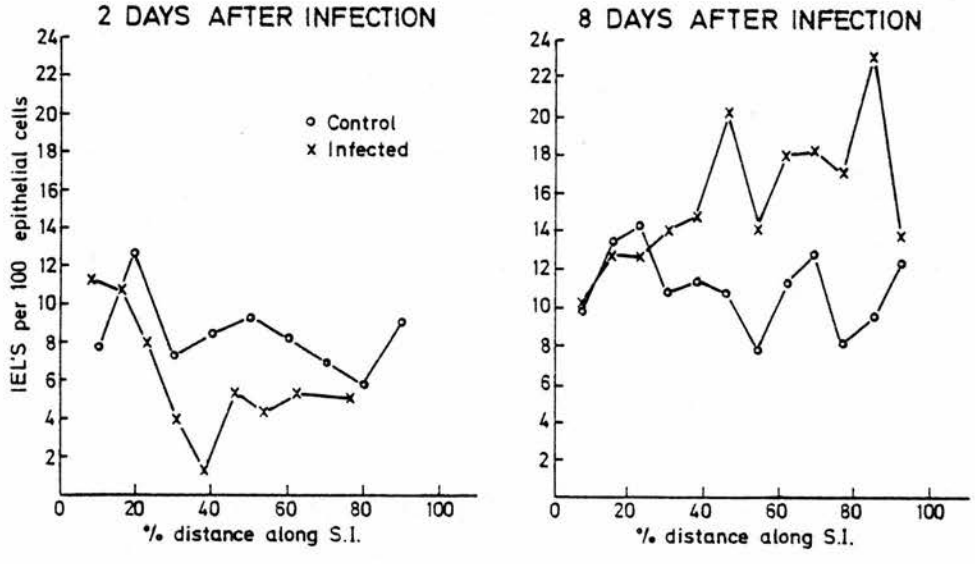

Fig 8.2. Numbers of I.E. lymphocytes / 100 epithelial cells in the small intestine of gnotobiotic lambs, 2 days and 8 days after infection with rotavirus on the second day of life and age matched controls.

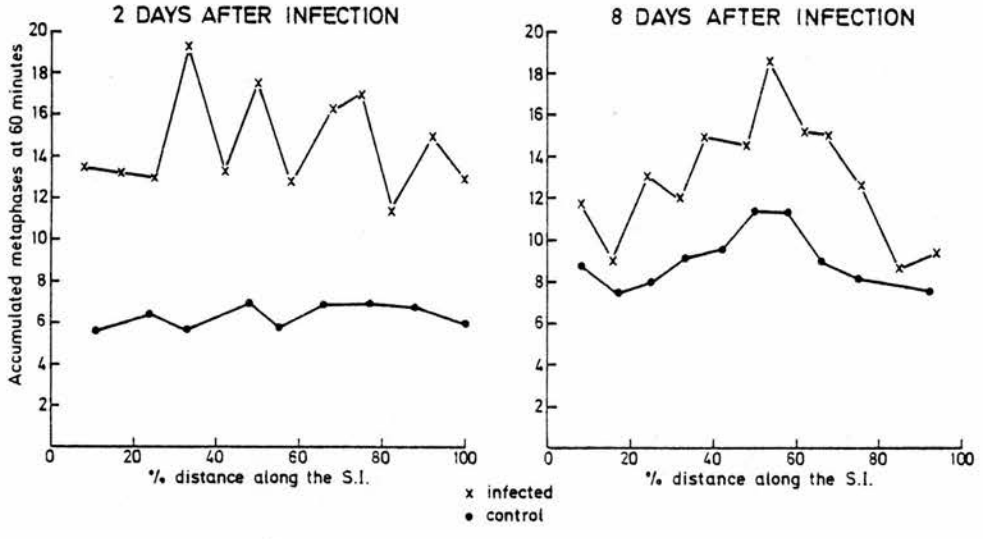

Fig 8.3. The rate of metaphase accumulation in the small intestine of gnotobiotic lambs, 2 days and 8 days after infection with rotavirus on the second day of life and age matched controls.

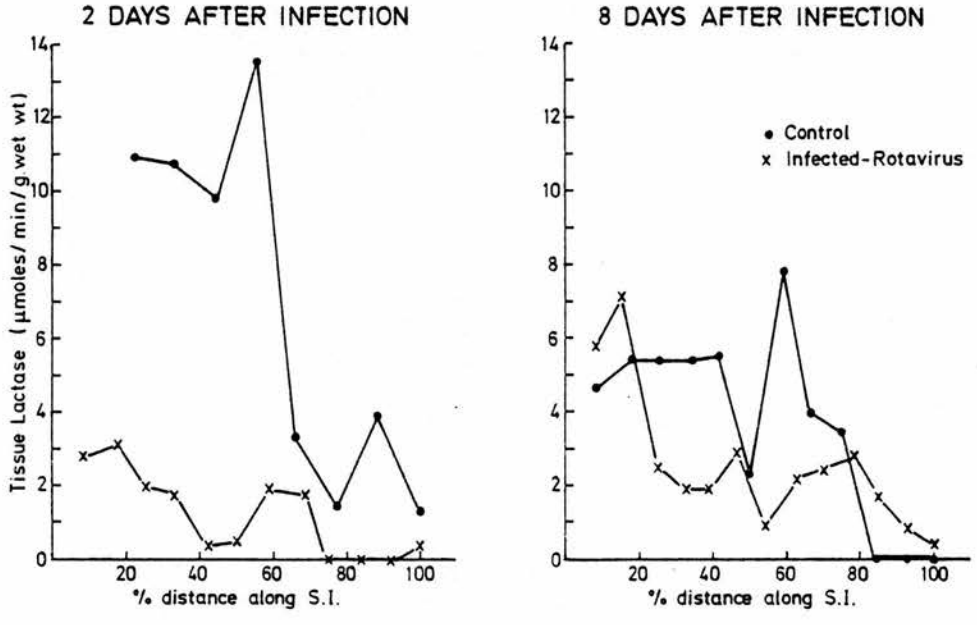

Fig 8.4. Tissue lactase levels in the small intestine of gnotobiotic lambs, 2 days and 8 days after infection with rotavirus on the second day of life and age matched controls.

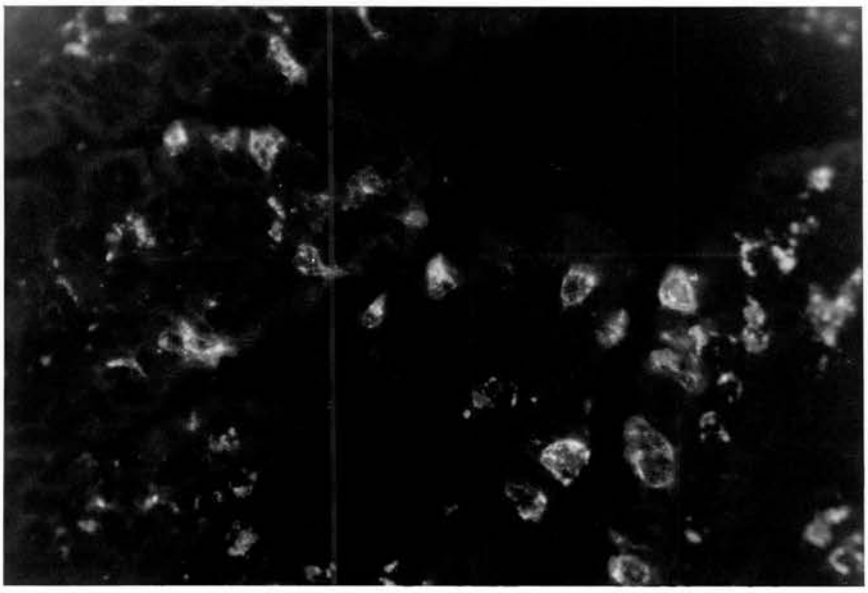

Plate 8.1. Rotavirus infected epithelial cells within the villus epithelium of the small intestine of a gnotobiotic lamb, 2 days after infection with rotavirus on the second day of life. Immunofluorescence. X 300.

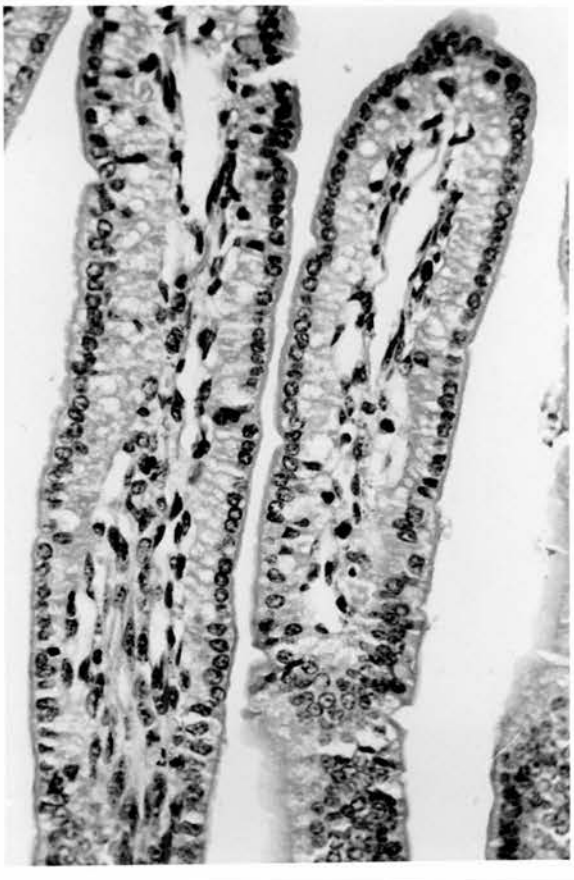

Plate 8.2. The small intestinal villi in the proximal ileum of an uninfected gnotobiotic lamb aged 3 days. The villi are long with shallow annular crenations. Absorptive vacuolated cells are present within the columnar epithelium. H & E. X 320.

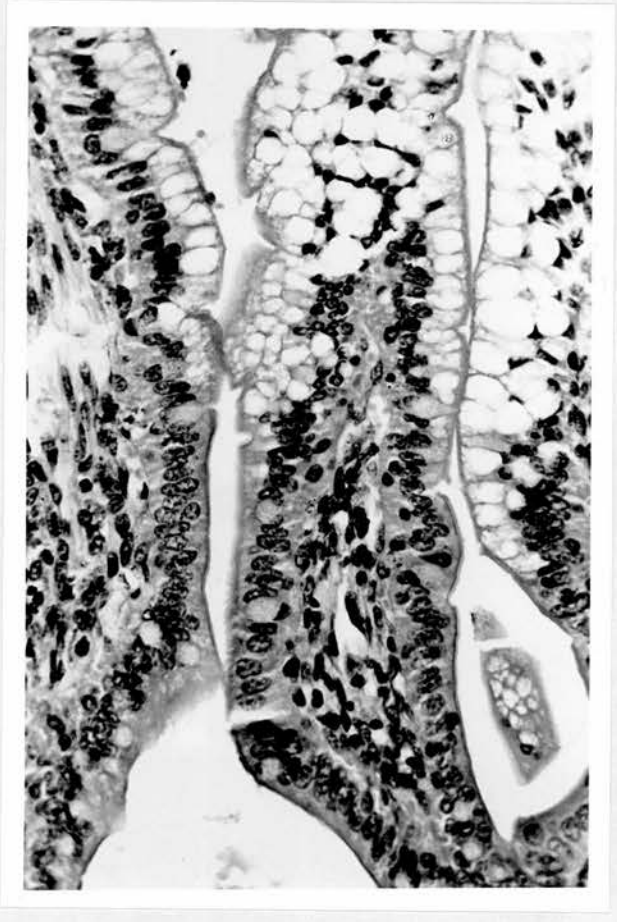

Plate 8.3. The villus epithelium in the ileum of an uninfected gnotobiotic lamb aged 3 days. The absorptive vacuolated cells are confined to the upper regions of the villi. H & E. X 320.

Plate 8.4. The small intestinal mucosa in the midgut of a gnotobiotic lamb aged 10 days. The villi are long with deep annular crenations. No absorptive vacuolated cells are present. H & E. X 125.

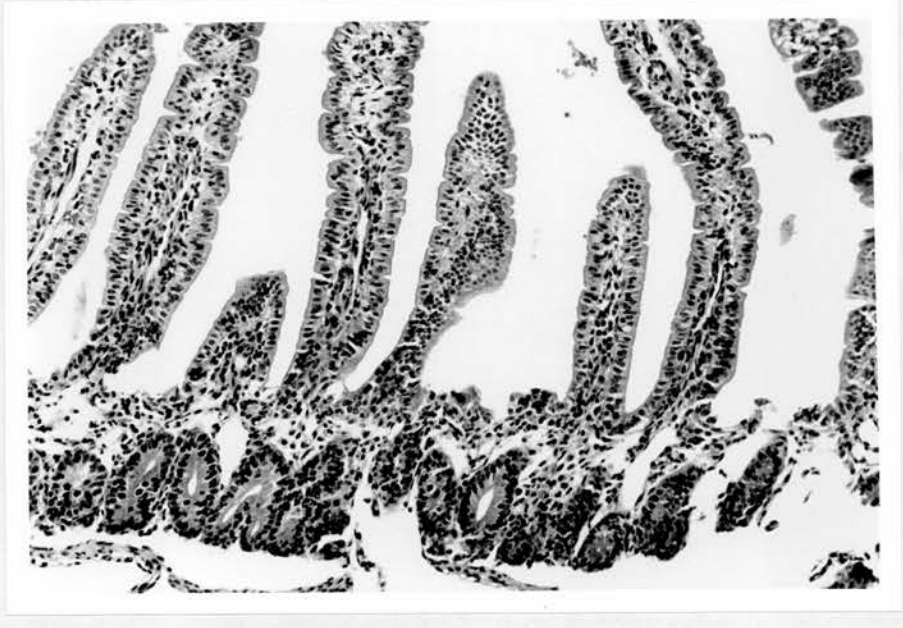

Plate 8.5. The small intestinal mucosa in the ileum of a gnotobiotic lamb aged 10 days. There are a mixture of leaf and finger shaped villi and there are many eosinophils in the crypt region of the lamina propria. H & E. X 125.

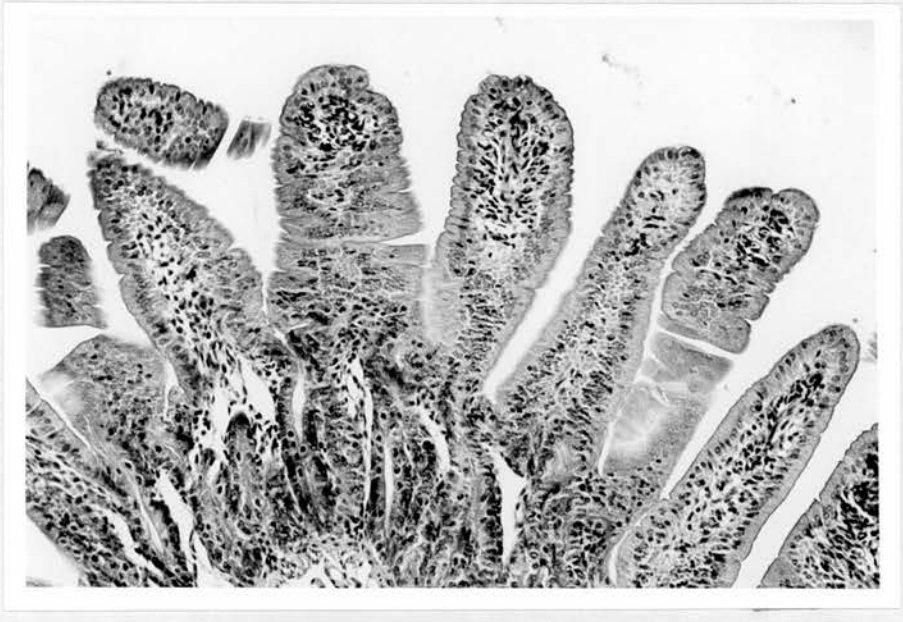

Plate 8.6. The small intestinal mucosa of a gnotobiotic lamb, 2 days after infection with rotavirus on the second day of life. Jejunum. The villi are short and deeply crenated while the crypts are long. The epithelial cell layer is abnormal. H & E. X 125.

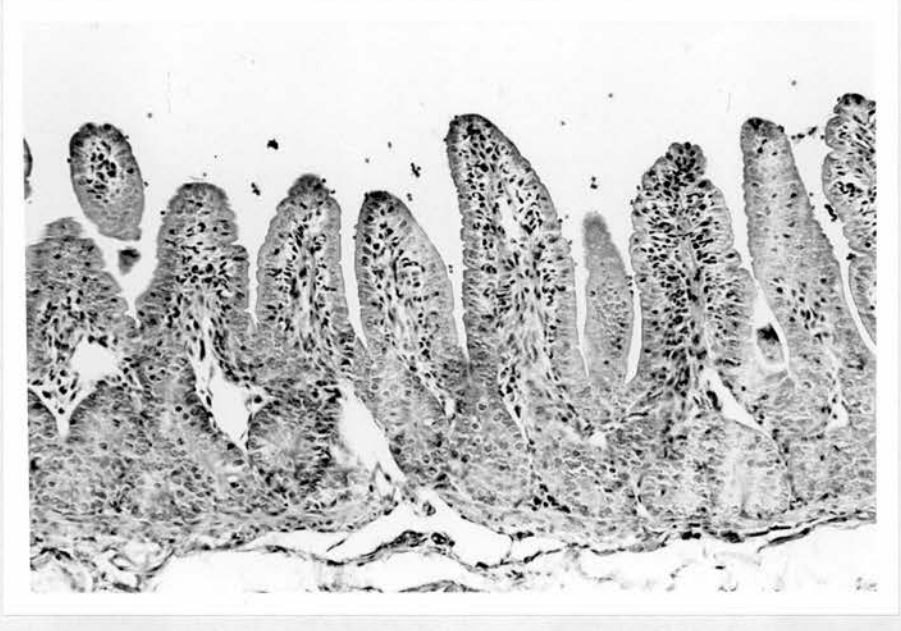

Plate 8.7. The small intestinal mucosa of a gnotobiotic lamb, 2 days after infection on the second day of life. Ileum. The villi are short and the epithelial cells are disordered. H & E. X 125.

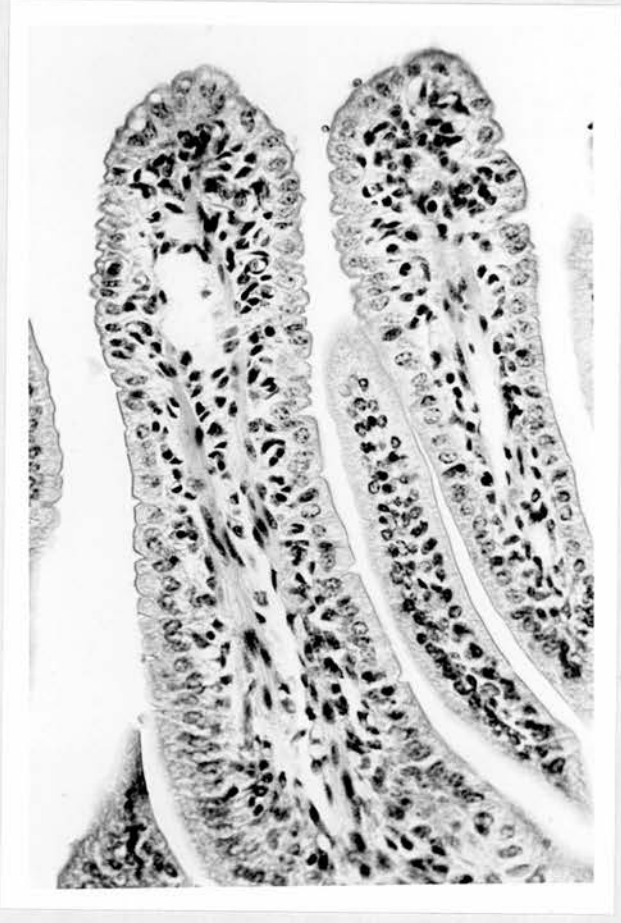

Plate 8.8. The small intestinal mucosa of a gnotobiotic lamb, 2 days after infection with rotavirus on the second day of life. Midgut. There are vacuolated cells filled with dense material at the tips of the villi and the upper half of the villus is abnormal. H & E. X 320.

Plate 8.9. The small intestinal mucosa of a gnotobiotic lamb, 8 days after infection with rotavirus on the second day of life. Midgut. The villi are of normal length but there are numerous vacuoles at the villus tips containing granular material. H & E. X125.

Plate 8.10. The small intestinal villi in the jejunum of a gnotobiotic lamb, 2 days after infection on the second day of life. The villi are very short and many of the epithelial cells have been lost, exposing the lamina propria. SEM. X 200.

Plate 8.11. The small intestinal villi of the ileum of a gnotobiotic lamb, 2 days after infection on the second day of life. The villi are short and the villus epithelial cells are swollen and rounded. SEM . X 500.

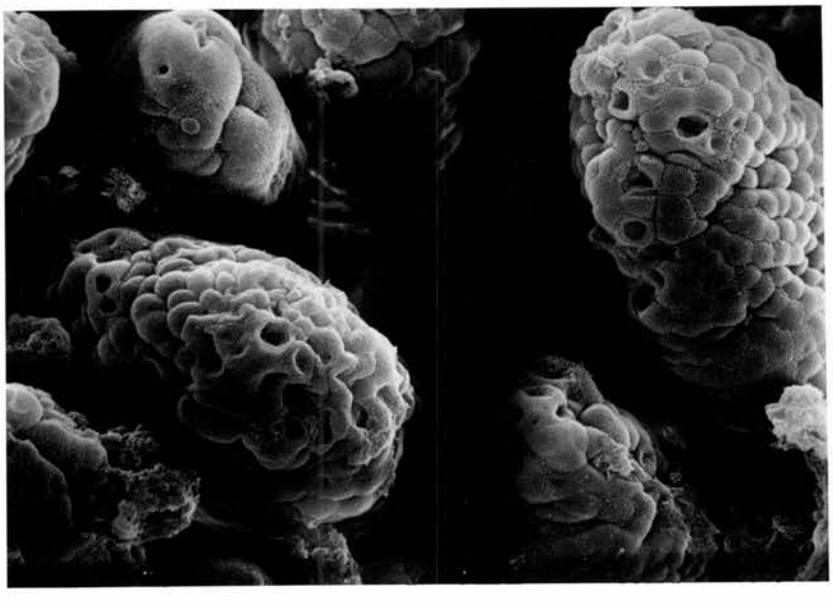

Plate 8.12 The villus epithelium of the small intestine, midgut, of a gnotobiotic lamb, 2 days after infection on the second day of life with rotavirus. There are many damaged epithelial cells on the tips and sides of the villi. SEM. X 500.

Plate 8.13. The small intestinal villi in the ileum of a gnotobiotic lamb, 8 days after infection with rotavirus on the second day of life. The villi are conical with convoluted crenations. SEM. X 200.

Chapter 9.

The effect of rotavirus infection on the villus and crypt epithelium of the small intestine of gnotobiotic lambs: The 24 hour period after infection on the second day of life.

Introduction.

In the first experiment, where the effects of rotavirus infection were studied from 1 - 15 days after infection, lesions were already visible in the first animal sampled at 24 hours after infection. These lesions were confined to sites 2 and 3. In the earlier study of the pathogenesis of rotavirus infection (Snodgrass, Angus and Gray, 1977), virus was shown to be present at all sites by 12 hours after infection and histological lesions were present at this time at sites 2 and 3.

It was therefore decided to study the events occurring in the small intestinal epithelium during the first 24 hours after infection at short time intervals.

The aims of this study were:-

(1) to discover whether villus atrophy occurred at any site prior to 24 hours after infection.

(2) to show when the virus could first be identified within the intestinal epithelial cells by immunofluorescence.

(3) to establish whether or not all levels of the small intestine were similarly affected.

(4) to see if any changes were apparent by scanning electron microscopy which were not noticeable by histological examination.

(5) to monitor tissue lactase levels during the first 24 hours after infection.

Animals used.

Eight gnotobiotic lambs were used in this experiment. All were infected on the second day of life. At each of 2, 4, 6, 9, 12, 18, and 24 hours after infection, samples were removed for cell kinetic

measurements, microdissection, histology, tissue lactase assay, immunofluorescence and scanning electron microscopy. Material obtained from various other uninfected animals at other times served as the controls. These controls were aged 1-3 days.

Details of the animals used in this chapter are shown in table 9.1.

Clinical and Virological response.

Diarrhoea developed at 15 and 16 hours respectively in the lambs killed 18 and 24 hours after infection. These two lambs were unwell, apathetic and anorexic. The control lamb passed firm brown faeces throughout.

Rotavirus was detected in the faeces of lambs with diarrhoea by E.M. examination. No rotavirus was detected in the faeces of any of the lambs before infection or in the control lambs.

Immunofluorescence.

Site 1.

Specific rotavirus fluorescence was first seen in the epithelial cells of site 1, 6 hours after infection. Single cells were stained on the upper parts of the villi (Plate 9.1). By 12 hours after infection clumps of cells were fluorescent at the tips of the villi (Plate 9.2). Fluorescence was again sparse 18 hours after infection.

Site 2.

Specific fluorescence was first observed 6 hours after infection, both single cells and clumps of fluorescent cells were present at the villus tips. The fluorescence was confluent over the

upper halves of the villi, 12 - 18 hours after infection (Plate 9.3).

Site 3.

Fluorescence was sparse 6 - 9 hours after infection (Plate 9.4) and from 12 - 18 hours after infection, there was widespread fluorescence in the epithelial cells on the upper halves of the villi (Plate 9.5).

The distribution of the rotavirus fluorescence is illustrated in Table 9.2.

Villus length and crypt length.

Site 1.

The villi were significantly shortened 4 - 6 hours after infection. The crypts were short initially but significant lengthening was observed by 12 hours after infection, Table 9.3. This was only apparent in one individual and may have been due to individual variation.

Site 2.

Significant shortening of the villi occurred 18 and 24 hours after infection, villus lengths being 636μm and 556μm respectively, compared with a control value of 1093μm, Table 9.3. The crypts did not vary from normal.

Site 3.

Significant shortening of the villi occurred from 12 hours after infection. The crypts were elongated at 18 hours after infection, Table 9.3.

Histology.

Site 1.

From 2 - 9 hours after infection the villi were long with annular crenations. The epithelial cells were columnar in type and vacuoles were present in the lower parts of the epithelial cells (Plates 9.6, 9.7), and within 4 hours of infection the upper halves of the villi were distended. By 12 hours after infection the villi looked wider across the base and at 18 hours after infection the epithelial cells were irregularly arranged with cuboidal cells covering the lower parts of the villi. By 24 hours after infection these cuboidal cells completely clothed the villi. The density of the lamina propria was greater than normal throughout, with many eosinophils, especially in the crypt region. From 2-12 hours after infection, the damage to the villi was confined to the upper half to two thirds of the villi (Plate 9,6, 9.8), whereas 18-24 hours after infection the whole villus was affected. No absorptive vacuoles were seen within the epithelial cells.

Site 2.

Villus length remained normal until 18 hours after infection when significant shortening occurred. The annular crenations were deep and by 12 hours after infection, were confined to the upper halves of the villi (Plate 9.9). The tips of the villi were distended from 4 hours after infection and the cells were irregularly arranged (Plate 9.10) and gaps were apparent between the epithelial cells from 9 hours after infection. The upper half of the villus showed obvious damage from 9 hours after infection and the whole villus was affected by 24 hours after infection (refer to

Plate 7.3). The absorptive vacuoles, characteristic of this area of the gut, were present in the epithelial cells until 6 hours after infection (Plate 9.10), after which they disappeared although present in control animals. The lamina propria showed an increase in cellularity from 9 hours after infection onwards.

Site 3.

Villus shortening began 9 hours after infection. The annular crenations disappeared at this time. Villus tip distension was present from 4 hours after infection (Plate 9.11), and many vacuoles were present on the upper part of the villi. These were not absorptive vacuoles (Plate 9.12, 9.13). Cuboidal epithelial cells had replaced the columnar epithelial cells by 24 hours after infection and these cells had a very disordered arrangement. Absorptive vacuoles were present in the epithelial cells until 18 hours after infection. From 4 to 18 hours after infection, the upper halves of the villi showed obvious damage (Plates 9.11, 9.13, 9.14) and by 24 hours the whole villus was abnormal. The lamina propria was densely packed with cells.

Scanning electron microscopy.

Site 1.

The villi were finger shaped and long, with annular crenations until 12 hours after infection (Plates 9.15, 9.16), although some damage was apparent in one of the animals sampled 6 hours after infection (Plate 9.17). By 18 hours after infection the villi were shorter with jumbled epithelial cells at the villus tip and 24 hours after infection the annular crenations had disappeared and the villus surface was very convoluted (Plate 9.18).

Site 2.

From 2 - 4 hours after infection, the villi were long with annular crenations and rounded epithelial cells at the villus tip (Plate 9.19). By 6 hours after infection the villi had a wider shape, the epithelial cells were loosely arranged on the surface of the upper villus (Plate 9.20). By 9 hours after infection, some bifid villi were present and the cells on the villus looked swollen. From 12 hours after infection onwards, the villi were short and broad with abnormal tips (Plate 9.21). There were blebs on the epithelial cells of the upper halves of the villi (Plate 9.22). Distinct areas of damage were present 18 hours after infection (Plate 9.23). At 24 hours after infection the upper half of the villus was very abnormal with blister-like projections from the epithelial cells. (Plate 9.24), the villi had lost numerous epithelial cells and the lamina propria was exposed in several places (Plate 9.25).

Site 3.

Within 2 hours of infection the epithelial cell arrangement was abnormal (Plate 9.26), the surface having a bulging swollen appearance and throughout the 24 hour period the villi appeared abnormal. By 6 hours after infection the epithelial cells were damaged and full of holes (Plate 9.27) and by 9 hours after infection fused villi were present. Most of the damage to the villi caused by cellular exfoliation occurred at the tips of the villi but some cells had also become detached from the sides of the villi (Plate 9.28) and by 24 hours post infection the villi were very short (refer to Plate 7.13).

Epithelial cell kinetics.

Crypt cell production rates at all sites did not differ from control values, Fig. 9.1. The time of administration of the infection in these animals was adjusted so that all animals were sampled between 9am and noon to minimise the effects of circadian rhythms.

Crypt to villus ratio.

The number of crypts associated with each villus did not vary at any site during the first 24 hours after infection, Table 9.4.

Tissue lactase activity.

During the first 12 hours after infection tissue lactase activity at all three sites was similar to that of control animals. By 24 hours after infection, tissue lactase activity in all three sites was much lower than that observed in control tissue, Table 9.5. The levels of lactase were not measured in the 18 hour post infection lamb.

Conclusions.

Villus atrophy occurred in the jejunum, 4 - 6 hours after infection, from 18 hours after infection at site 2 and from 12 hours after infecton at site 3. The crypts showed no change throughout the first 24 hours after infection. The effect on villus architecture noted at site 1 was present before sites 2 and 3 were affected and recovery was rapid.

Virus was present in the epithelial cells of the villi at all sites from 6 hours after infection.

Abnormalities of the epithelial cells were present very early in the course of the infection and were more easily demonstrated by scanning electron microscopy than by histological means.

Tissue lactase levels were not affected until later than 12 hours after infection.

The crypt cell production rate was not affected in the initial 2 - 24 hour period after infection.

Technique	Time after infection (hours)						
	2	4	6	9	12	18	24
Histology.	1	1	1	1	1	1	1
Microdissection.	1	1	1	1	1	1	1
Scanning electron Microscopy.	1	1	1	1	1	1	1
Lactase assay.	1	1	1	1	1	-	1
Immunofluorescence.	1	1	1	1	1	1	-

Table 9.1. Numbers of gnotobiotic lambs used to study the effects of rotavirus infection administered on the second day of life, on the villus and crypt epithelium of the small intestine.
The period from 2 - 24 hours after infection.

Time after infection(hours)	Site 1	Site 2	Site 3
2	-ve	-ve	-ve
4	-ve	-ve	-ve
6	+	++	+
9	not done	+	+
12	++	+++	+++
18	+	++	+++

Table 9.2. Presence of rotavirus fluorescence in 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the second day of life.

+	single fluorescent cells.
++	clumps of fluorescent cells.
+++	confluent fluorescence over at least half of each villus.
-ve	no fluorescence demonstrated.

Time after infection(hours)	Site 1		Site 2		Site 3	
	Villus length	Crypt length	Villus length	Crypt length	Villus length	Crypt length
2	1099±32	*130±2	1049±25	154±4	1049±32	118±3
4	*927±21	*131±3	1173±37	130±2	1005±25	110±2
6	*860±18	*132±2	1091±19	113±2	941±22	111±2
9	1099±24	143±3	1035±23	138±2	1192±33	126±2
12	1033±25	*179±2	962±18	118±2	688±12	117±3
18	1211±46	149±3	*636±22	147±3	**348±5	**164±3
24	932±31	151±1	**556±15	*180±7	**441±17	126±2
Control values for 3 lambs aged 24 - 48 hours.	1069±33	156±1	1093±31	145±4	941±23	131±2

Table 9.3. Villus and crypt lengths at 3 sites of small intestine of gnotobiotic lambs, 2 - 24 hours after infection with rotavirus on the second day of life. (μm, mean ± S.E.) Microdissection measurements. Values marked by * or ** denotes a significant difference from the age matched control value, assessed by Student's 't' test. * p less than 0·05, ** p less than 0·01.

Time after infection(hours)	The number of crypts per villus		
	Site 1	Site 2	Site 3
2	5•2	3•0	4•5
4	4•4	2•9	4•9
6	5•6	4•0	5•1
9	4•3	3•4	4•8
12	5•9	3•6	3•7
18	4•4	4•8	3•8
24	4•1	2•1	2•2

Table 9.4. The crypt to villus ratio in 3 sites of small intestine of gnotobiotic lambs, 2 - 24 hours after infection with rotavirus on the second day of life.

Time after infection(hours)	Site 1	Site 2	Site 3
2	2•3	4•6	3•6
4	7•2	5•7	3•1
6	3•8	2•9	3•2
9	10•6	1•8	6•2
12	6•1	4•8	3•4
24	2•3	1•5	0.6
Control	5•6	5•6	9•9

Table 9.5. Tissue lactase activity in 3 sites of small intestine of gnotobiotic lambs, 2 - 24 hours after infection on the second day of life with rotavirus. Activity is expressed as units of activity / gram wet weight of tissue. (Unit being 1μmole of substrate hydrolysed per minute at $37^{o}C$)

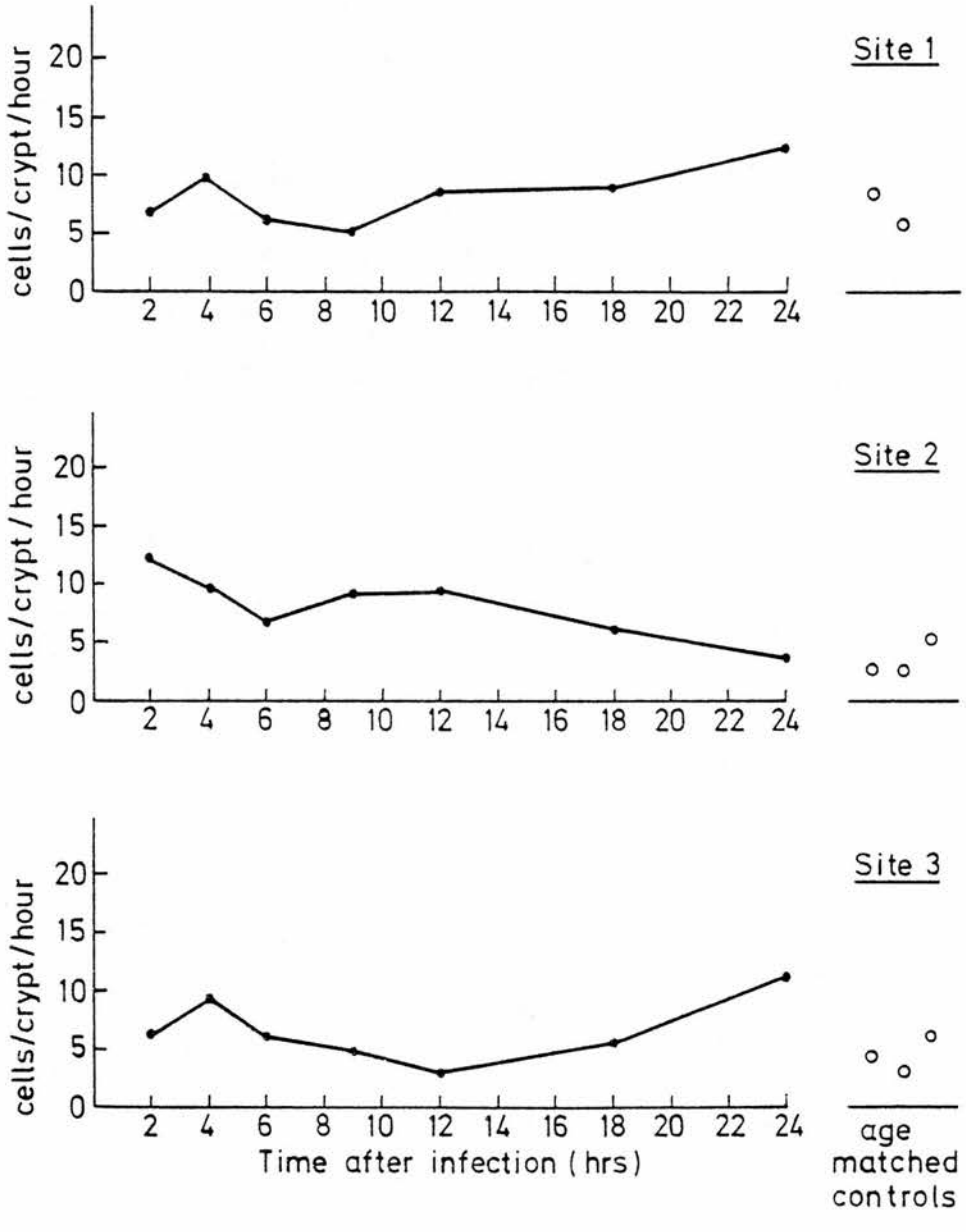

Fig 9.1. Crypt cell production rates at 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the second day of life. The period from 2 - 24 hours after infection.

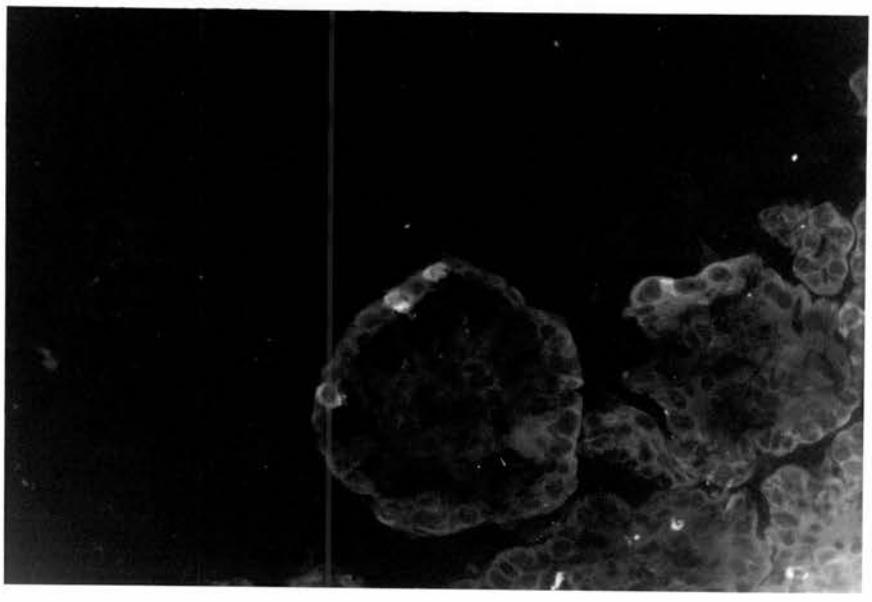

Plate 9.1. Single fluorescent cells at the tips of the villi Site 1. The small intestine of a gnotobiotic lamb, 6 hours after infection with rotavirus on the second day of life. Immunofluorescence. X 300.

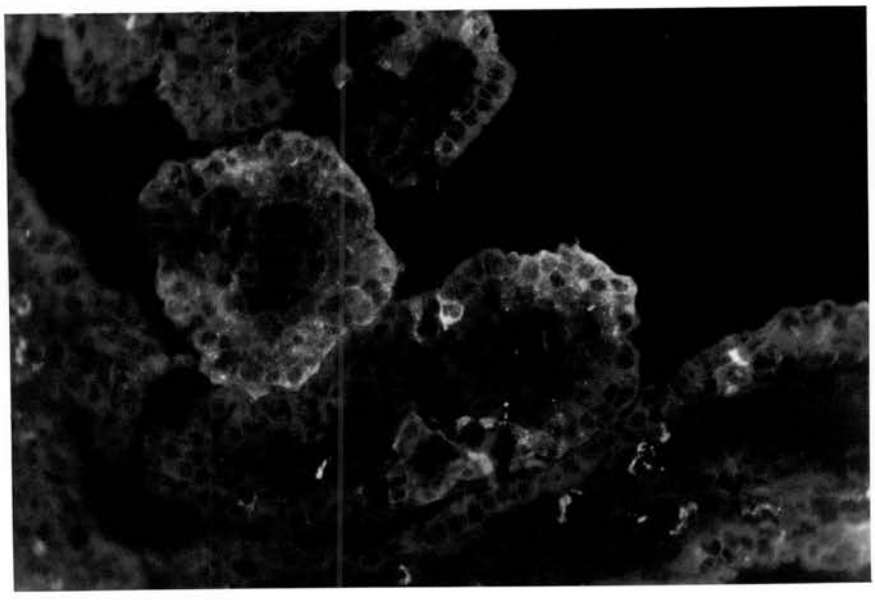

Plate 9.2. Clumps of fluorescent cells at the tips of the villi. Site 1. The small intestine of a gnotobiotic lamb, 12 hours after infection with rotavirus on the second day of life. Immunofluorescence. X 300.

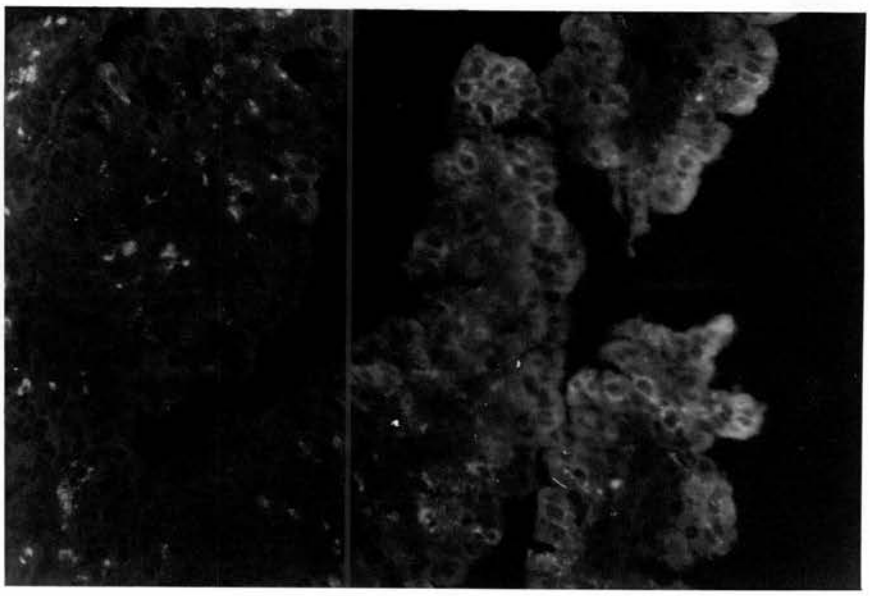

Plate 9.3. Confluent fluorescence on the upper halves of the villi. Site 2. The small intestine of a gnotobiotic lamb, 18 hours after infection with rotavirus on the second day of life. Immunofluorescence. X 150.

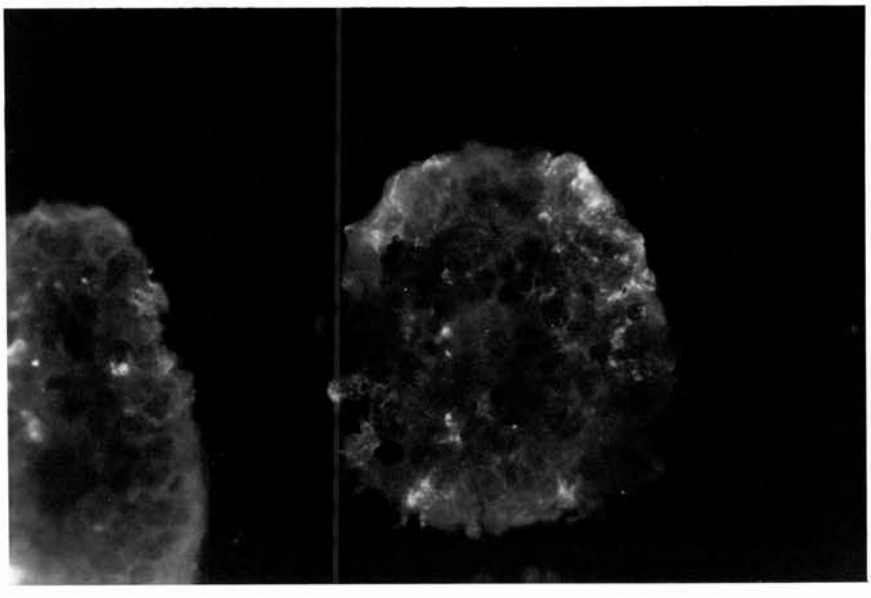

Plate 9.4. Sparse fluorescence at the tips of the villi. Site 3. The small intestine of a gnotobiotic lamb, 9 hours after infection with rotavirus on the second day of life. Immunofluorescence. X 300.

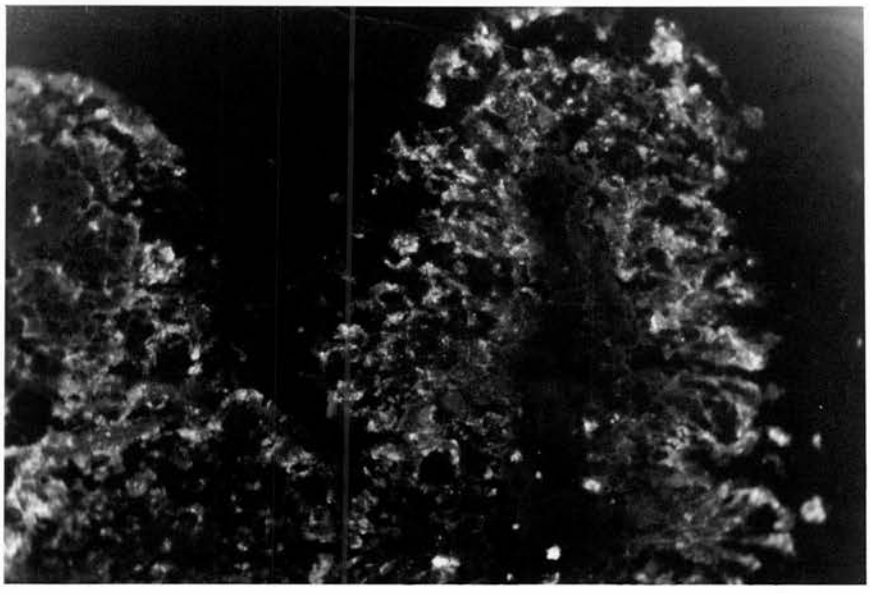

Plate 9.5. Widespread fluorescence on the upper half of the villus. Site 3. The small intestinal epithelium of a gnotobiotic lamb, 12 hours after infection with rotavirus on the second day of life. Immunofluorescence. X 300.

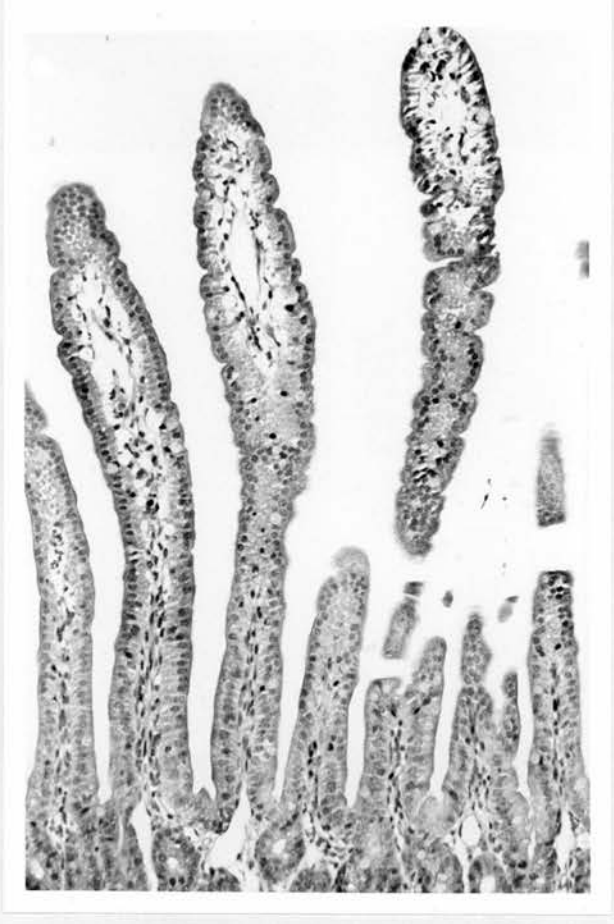

Plate 9.6. The small intestinal mucosa of a gnotobiotic lamb, 4 hours after infection with rotavirus on the second day of life. Site 1. The epithelial cells at the villus tip seem abnormal. H & E. X 125.

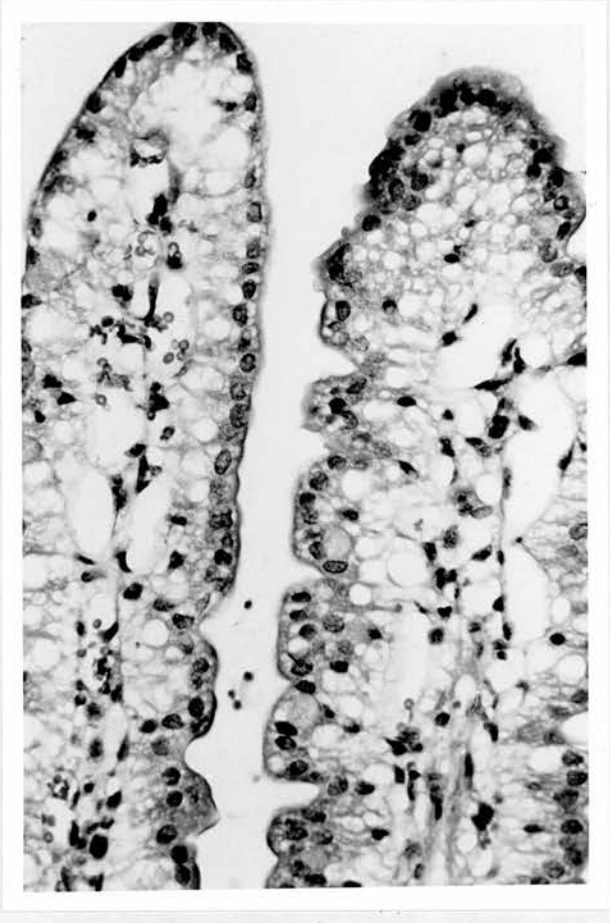

Plate 9.7. The small intestinal epithelium of a gnotobiotic lamb 2 hours after infection with rotavirus on the second day of life. Site 1. The epithelial cells are heavily vacuolated. H & E. X 320.

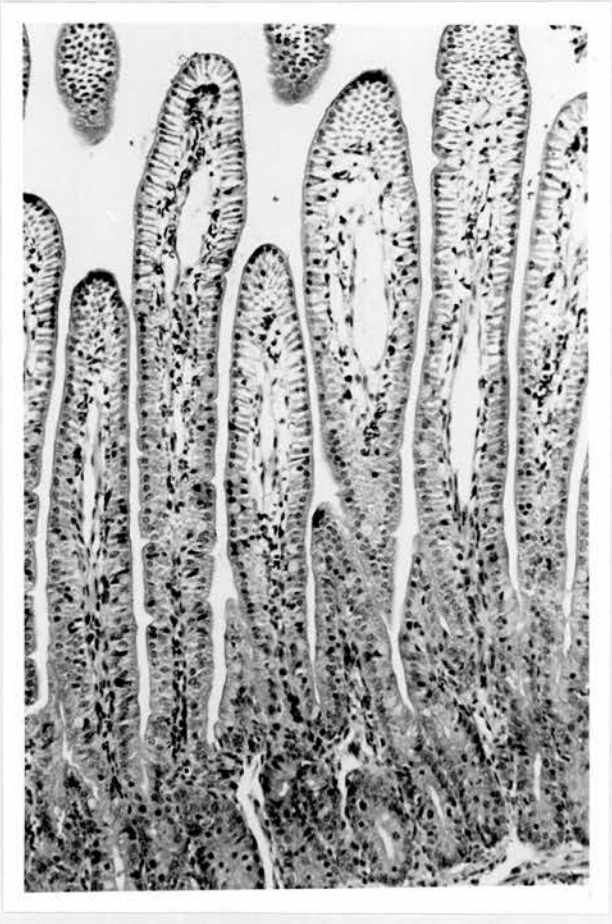

Plate 9.8. The small intestinal mucosa of a gnotobiotic lamb 9 hours after infection with rotavirus on the second day of life. Sits 1. The upper half of the villus is distended and the arrangement of the epithelial cells is unusual. H & E. X125.

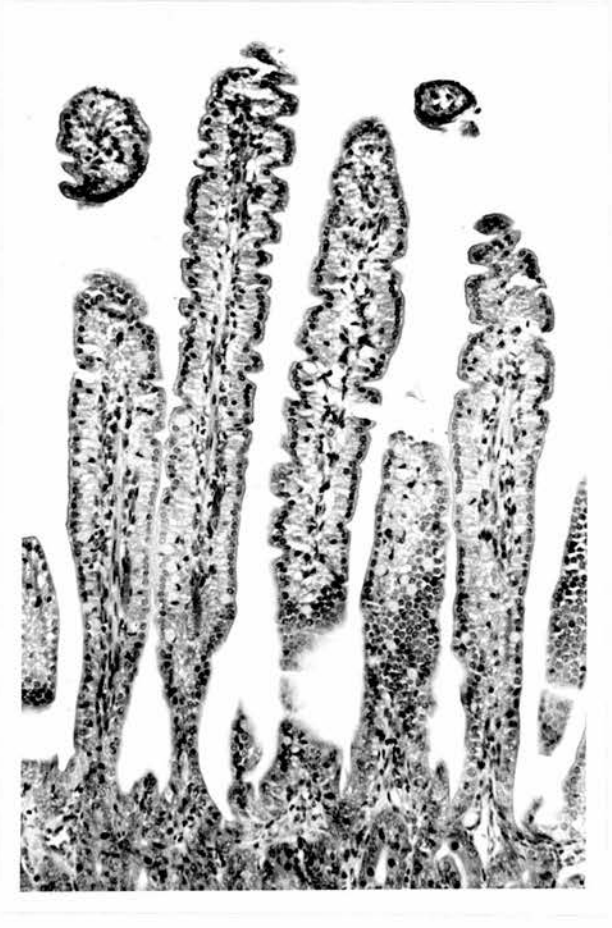

Plate 9.9. The small intestinal mucosa of a gnotobiotic lamb, 12 hours after infection with rotavirus on the second day of life. Site 2. Deep circular crenations on the upper halves of the villi. H & E. X 125.

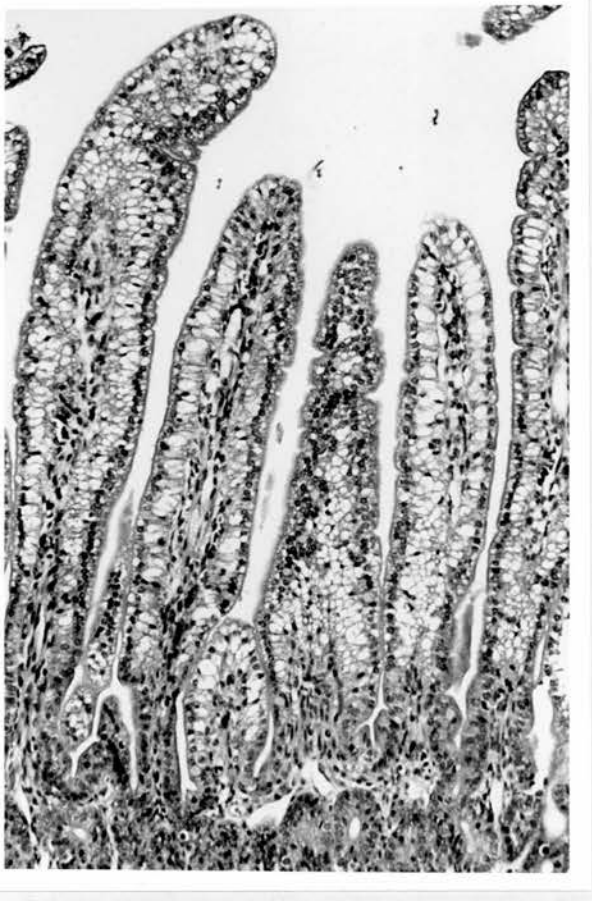

Plate 9.10. The small intestinal mucosa of a gnotobiotic lamb, 6 hours after infection with rotavirus on the second day of life. Site 2. The villus tips are distended, there is extensive vacuolisation of the villus epithelial cells in addition to the presence of the absorptive vacuoles in the cells. H & E. X 125.

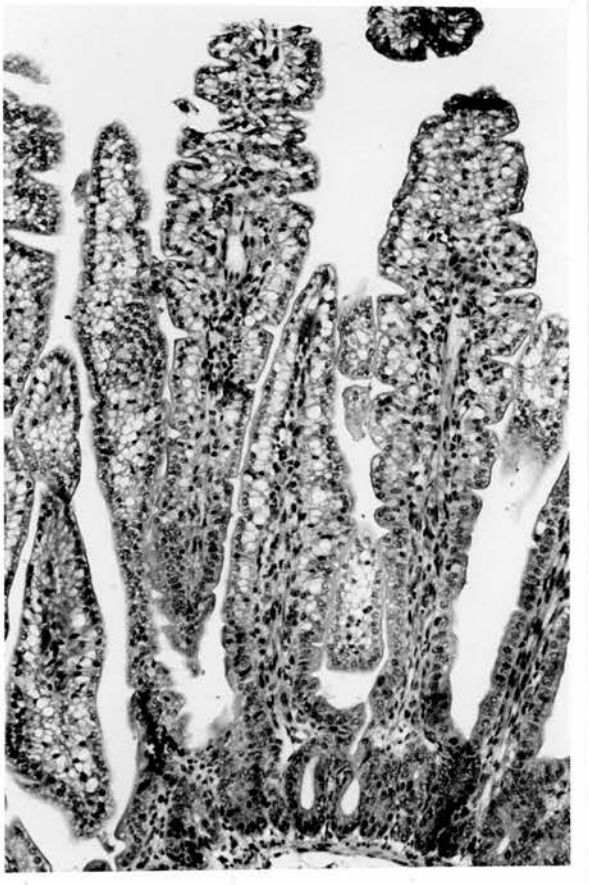

Plate 9.11. The small intestinal mucosa of a gnotobiotic lamb, 4 hours after infection with rotavirus on the second day of life. Site 3. The villi are deeply crenated and are vacuolated on the upper halves. H & E. X 125.

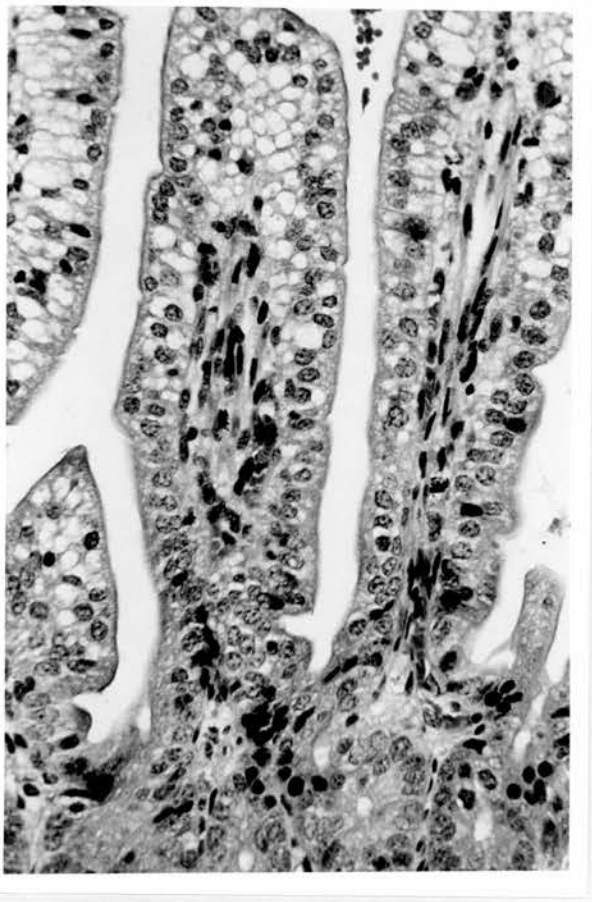

Plate 9.12. The villus epithelium of the small intestine of a gnotobiotic lamb, 2 hours after infection with rotavirus on the second day of life. Site 3.
There are many vacuoles within the epithelial cells.
H & E. X 320.

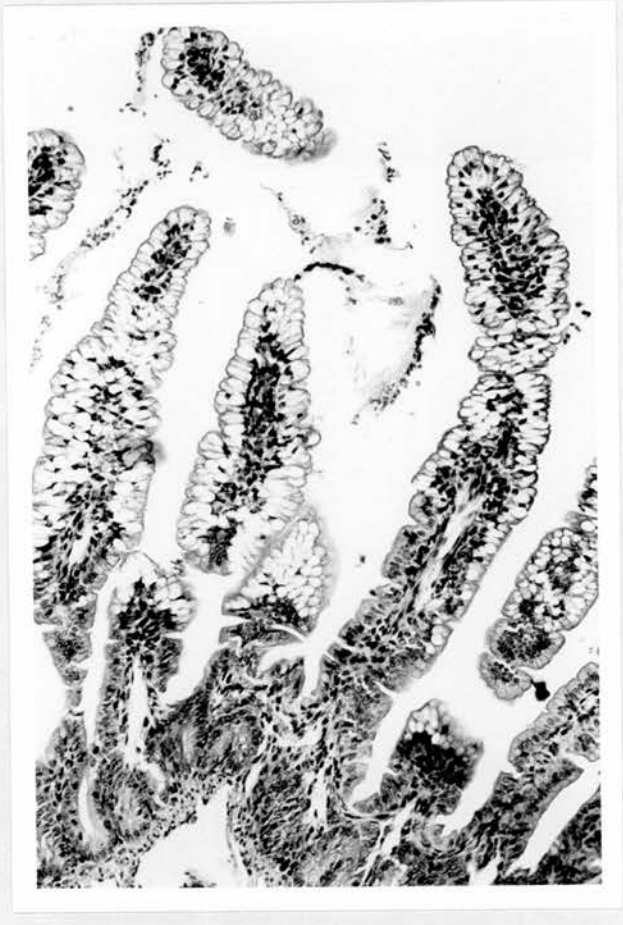

Plate 9.13. The small intestinal mucosa of a gnotobiotic lamb, 18 hours after infection with rotavirus on the second day of life. Note the heavily vacuolated epithelial cells. Site 3. H & E. X 125.

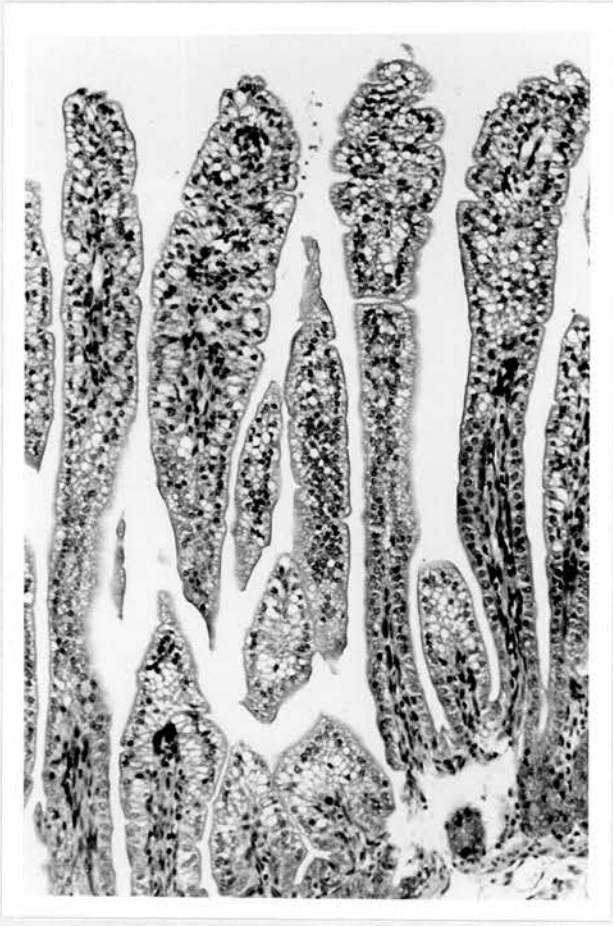

Plate 9.14. The small intestinal mucosa of a gnotobiotic lamb, 12 hours after infection with rotavirus on the second day of life. Site 3. The upper half of the villus is abnormal and the epithelial cells are vacuolated. H & E. X 125.

Plate 9.15. The small intestinal villi of a gnotobiotic lamb, 2 hours after infection on the second day of life, with rotavirus. Site 1. Long finger villi with shallow annular crenations. SEM. X 200.

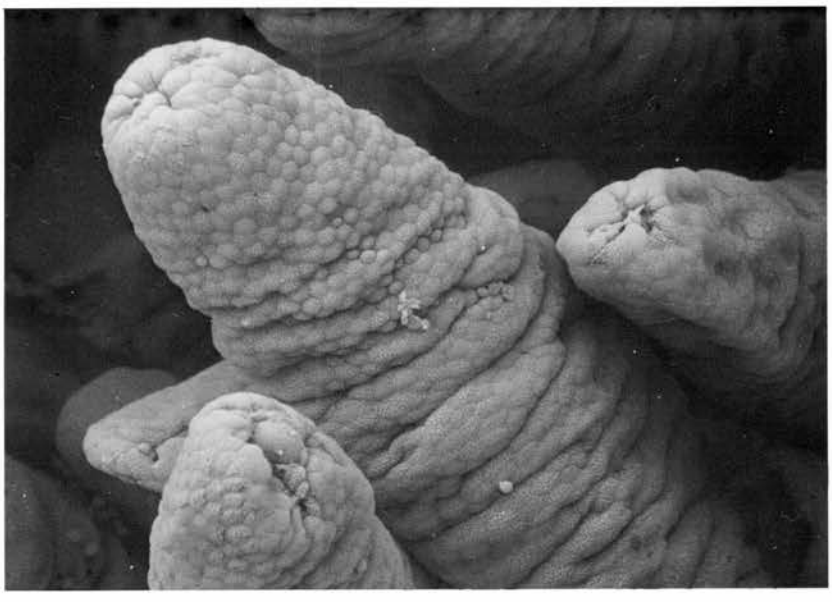

Plate 9.16. The small intestinal epithelial cells in a gnotobiotic lamb, 12 hours after infection with rotavirus on the second day of life. Site 1. SEM. X 500.

Plate 9.17. The small intestinal villi of a gnotobiotic lamb, 6 hours after infection with rotavirus on the second day of life. Site 1. Damage to the extrusion zone of the villus. SEM. X 160.

Plate 9. 18. The small intestinal villi of a gnotobiotic lamb, 24 hours after infection with rotavirus on the second day of life. Site 1. The villi are short and the crenations of the villus surface have a convoluted appearance. SEM X 200.

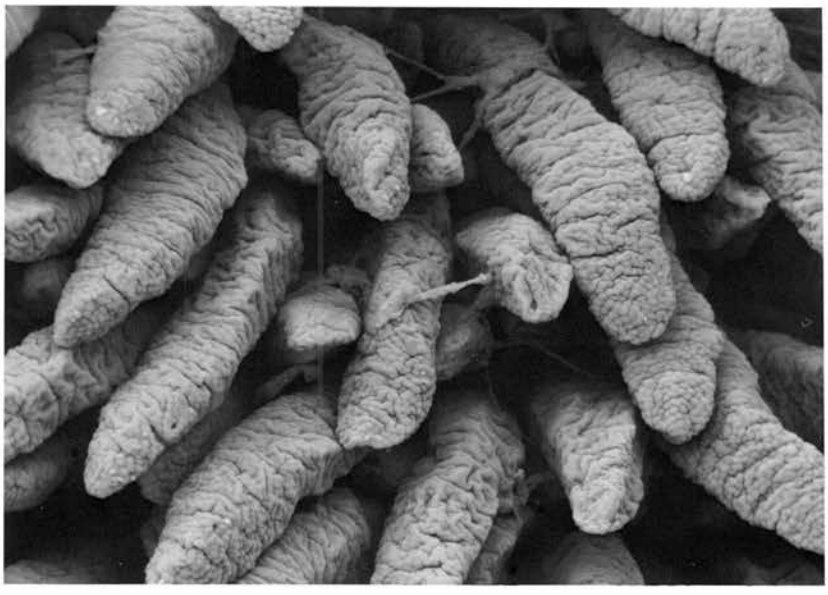

Plate 9.19. The small intestinal villi of a gnotobiotic lamb, 2 hours after infection with rotavirus on the second day of life. Site 2. The epithelial cells at the villus tip are swollen and appear rounded. SEM. X 200.

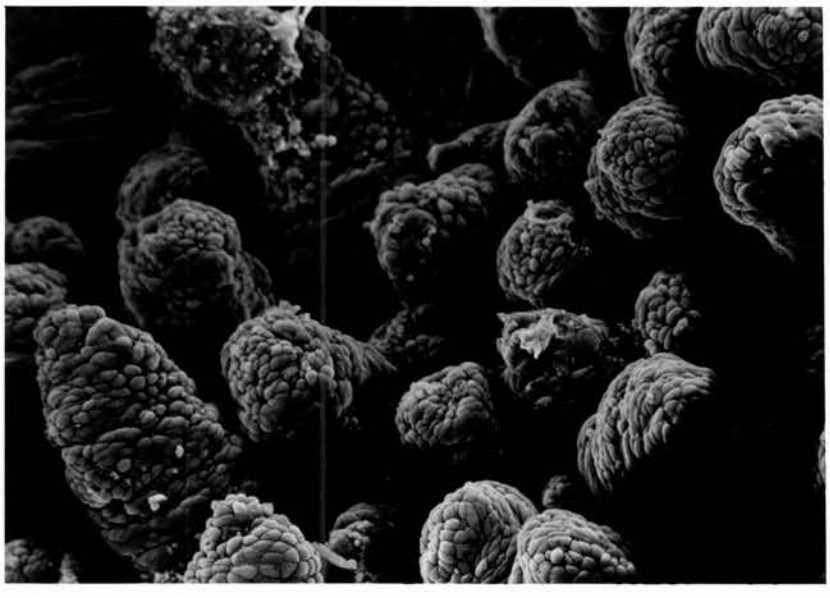

Plate 9. 20. The small intestinal villi of a gnotobiotic lamb, 6 hours after infection with rotavirus on the second day of life. Site 2. The villi are shortened and the epithelial cells are swollen and loosely arranged. SEM. X 200.

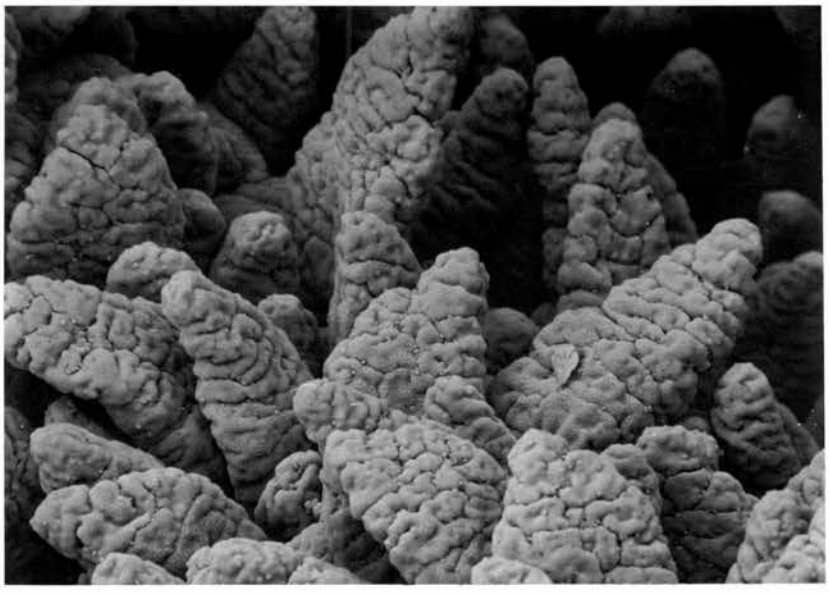

Plate 9.21. The villus epithelium of a gnotobiotic lamb, 12 hours after infection with rotavirus on the second day of life. Site 2. Leaf shaped villi with 'blebs' developing on the upper halves of the villi. SEM. X200.

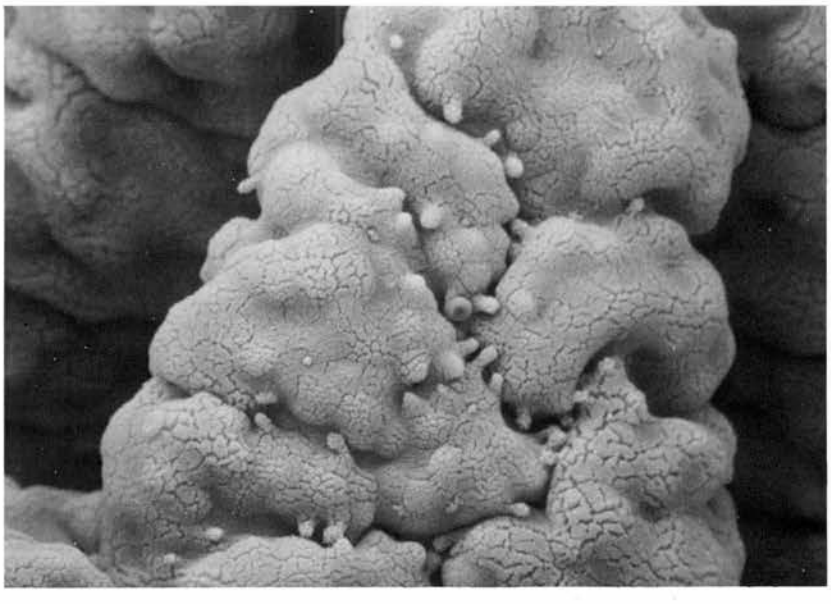

Plate 9.22. The villus epithelium of the small intestine in a gnotobiotic lamb, 12 hours after infection with rotavirus on the second day of life. Site 2. 'Blebs' on the epithelial cells. SEM. X1,000.

Plate 9.23. The small intestinal villi of a gnotobiotic lamb, 18 hours after infection with rotavirus on the second day of life. Site 2. The upper halves of the villi are damaged with swollen epithelial cells present. SEM. X 150.

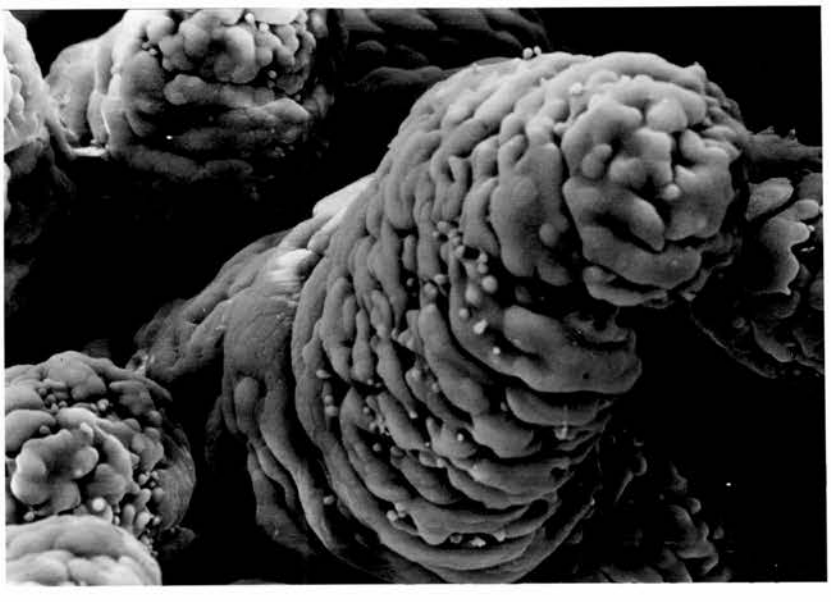

Plate 9.24. The villus epithelium of the small intestine of a gnotobiotic lamb, 24 hours after infection with rotavirus on the second day of life. Site 2. The villus tip is abnormal and 'blebs' are present. SEM. X 500.

Plate 9.25. The small intestinal villi of a gnotobiotic lamb, 24 hours after infection with rotavirus on the second day of life. Site 2. Exposure of the lamina propria due to epithelial cell damage. SEM. X 500.

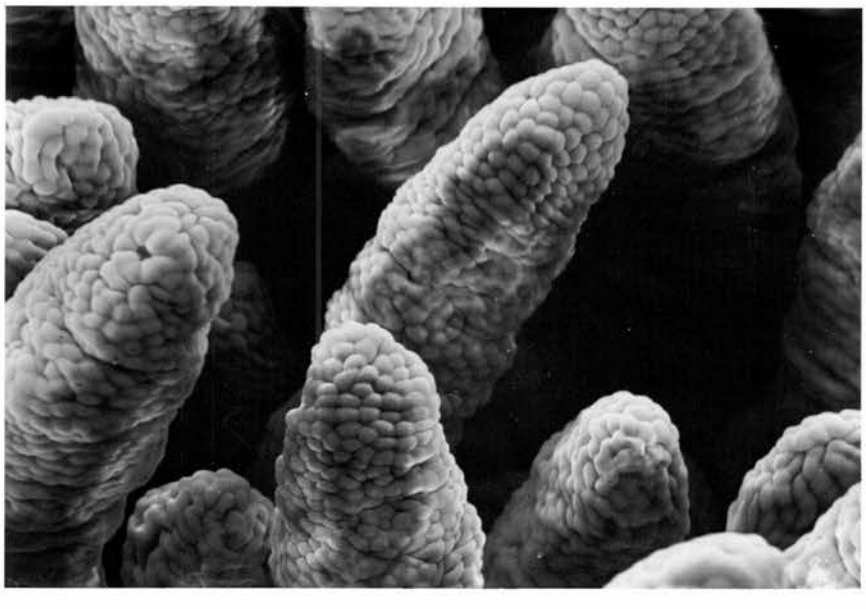

Plate 9.26. The small intestinal villi of a gnotobiotic lamb, 2 hours after infection with rotavirus on the second day of life. Site 3. The epithelial cells on the upper parts of the villi are swollen and the arrangement of the cells is unusual. SEM. X500.

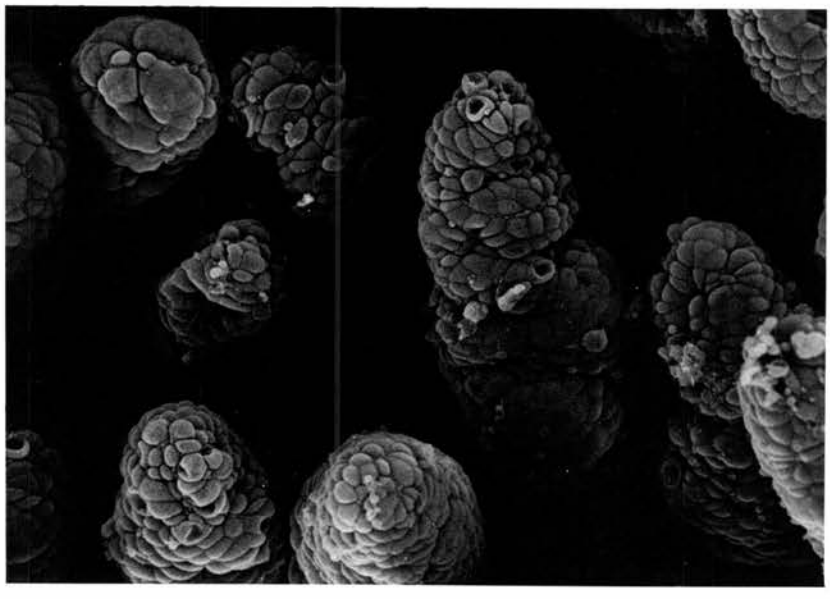

Plate 9. 27. The small intestinal villi of a gnotobiotic lamb, 6 hours after infection with rotavirus on the second day of life. Site 3. Severely damaged villus epithelial cells. SEM. X 300.

Plate 9.28. The small intestinal villi of a gnotobiotic lamb, 18 hours after infection with rotavirus on the second day of life. Site 3. Cell loss from the sides of the villi. SEM. X 350.

Chapter 10.

The effect of rotavirus infection on the villus and crypt epithelium of the small intestine of gnotobiotic lambs: infection on the seventh day of life.

Introduction.

It is known that older lambs are less susceptible to the effects of rotavirus infection (Snodgrass, Herring and Gray, 1976). Twelve day old lambs were only mildly affected by the virus, showing no anorexia and shedding virus in the faeces for only a short period of time compared to animals infected soon after birth. However, detailed studies were not performed in these animals, investigations being confined to clinical observation, electron microscopy of faecal samples and serological studies.

The aims of the experiments described in this chapter were:
(1) to discover whether the pathological effects of rotavirus infection of lambs on the seventh day of life correlated with the mild clinical effects observed.
(2) to describe the duration and extent of any abnormalities occuring in the small intestinal epithelium of infected animals.
(3) to measure any change in the epithelial cell kinetics of the small intestine of infected animals.

Animals used.

A total of seven gnotobiotic lambs were involved in this experiment. The lambs were infected on the seventh day of life. Investigations were performed using the same range of techniques as in the previous experiments. The lambs were sampled at intervals from 15 hours to 12 days after infection. The animals and investigations are detailed in Table 10.1.

Clinical and virological response.

The infected lambs showed no signs of disease, they remained lively, there was no diarrhoea and no reduction in milk intake. Virus was present in the faeces for 3 - 4 days after infection.

Immunofluorescence.

Tissue from the lamb killed 1 day after infection was not available.

Site 1.

Specific fluorescent staining was present only in isolated epithelial cells at the tips of the villi, 2 - 2.5 days after infection (Plate 10.1). At all other time intervals the tissue was negative.

Site 2.

Specific fluorescence was present in single epithelial cells at the villus tips, 15 hours and 2 days after infection. The epithelial cells of the entire villus were positive 2.5 days after infection (Plate 10.2), this being reduced to isolated cells on the tips of the villi, 4 days after infection. The tissue was negative 7 and 12 days after infection.

Site 3.

Single epithelial cells were fluorescent at the tips of the villi, 15 hours after infection. By 2 days after infection, the upper parts of the villi contained clumps of fluorescent epithelial cells (Plate 10.3), and 4 days after infection the fluorescence was confluent over the upper halves of the villi (Plate 10.4). At later

time intervals no virus was present.

The distribution of the fluorescence is detailed in Table 10.2.

Villus length and crypt length.

Site 1.

The villi did not differ from normal at this site. The crypts were elongated 1 and 4 days after infection, having mean values of 210μm and 301μm compared with 158μm and 199μm respectively in controls, (Fig 10.1).

Site 2.

The villi were short 4 days after infection (Fig 10.1), thereafter the villus lengths were within the normal range. The crypts were elongated 1 day after infection.

Site 3.

The lengths of the villi and of the crypts did not differ from those of control animals of the same age (Fig 10.1).

Histology.

Site 1.

From 15 hours to 2 days after infection, the villi were long with shallow, annular crenations. They were clothed by columnar cells (Plate 10.5). By 2.5 days after infection, the villi appeared shorter and wider with deep crenations. The epithelial cells were closely packed together and those at the villus tips showed signs of damage (10.6). From 4 days after infection onwards, the villi were normal. The lamina propria was densely filled with lymphoid cells

during the first 4 days after infection.

Site 2.

The villi were of normal appearance, 15 hours and 1 day after infection having shallow annular crenations. Villus shape remained normal 2 days after infection but vacuoles containing dense, granular material were present within the epithelial cells at the tips of the villi (see Plate 10.9). At 2.5 days after infection the epithelial cells of the villi were closely packed although the villi were otherwise normal (Plate 10.7) and remained so until the end of the experiment. The cellularity of the lamina propria was increased until 4 days after infection.

Site 3.

The villi were long with deep crenations 15 hours after infection and by 1 day after infection the epithelial cells at the tips of the villi were disordered (Plate 10.8). Vacuoles containing granular material were present within the epithelial cells at the villus tips and persisted until 2.5 days after infection (Plate 10.9). From 4 days after infection onwards the villi were leaf like, very crenated and similiar to those found in the control group (Plate 10.10). The lamina propria was densely filled with cells.

Scanning electron microscopy.

Site 1.

The epithelial cells at the villus tips looked abnormal 15 hours after infection (Plate 10.11). For the remainder of the experiment the villi were normal.

Site2.

From 15 hours to 2 days after infection, the villi appeared shortened, with deep convolutions on their surfaces. Cells were exfoliated from the tips and sides of the villi. From 2.5 to 12 days after infection, the villi had deeply convoluted crenations but the structure of the epithelial cells appeared normal (Plate 10.12).

Site 3.

Throughout the course of the experiment the villi were leaf or conical shaped with deep, convoluted crenations (Plate 10.13). This was similar to the appearance of the villi in normal gnotobiotic lambs of the older age group. Epithelial cells were exfoliated 1 day after infection but this was probably part of the normal extrusion process as the tip of the villus did not show signs of damage.

Epithelial cell kinetics.

Site 1.

The crypt cell production rates were similar to those observed in normal animals.

Site 2.

The crypt cell production rate was increased by 2 days after infection and reached a peak of 29.2 cells/crypt/hour by 2.5 days after infection. Control values at this time ranged between 3.2 and 8.7 cells/crypt/hour. Crypt cell production rates had returned to normal by 4 days after infection (Fig 10.2).

Site 3.

The crypt cell production rate increased sharply, 2.5 days after infection, to 21.2 cells/crypt/hour as opposed to control values between 8.5 and 15.5 cells/crypt/hour (Fig 10.2) before reverting to normal.

Crypt to villus ratio.

The number of crypts associated with each villus did not vary from the values observed in normal animals of the same age, Table 10.3.

Tissue lactase levels.

Tissue lactase levels were not affected throughout the course of the infection. The sharp drop observed in the lactase levels in the ileum of normal gnotobiotic lambs, between 5 and 9 days of age, occurred at a comparable time in those lambs infected on the seventh day of life. Table 10.4.

Conclusions.

The lambs infected with rotavirus on the seventh day of life developed very mild changes associated with the infection.

Clinical signs, histological changes and tissue lactase changes were minimal.

Cell kinetic changes were present, with a large if transient increase in cell production, 2 - 2.5 days after infection in the middle and distal regions of the small bowel.

Technique	Time after infection (days)						
	0·6	1	2	2·5	4	7	12
Histology.	1	1	1	1	1	1	1
Microdissection.	1	1	1	1	1	1	1
Scanning electron microscopy.	1	1	1	1	1	1	1
Lactase assay.	1	1	1	1	1	1	1
Immunofluorescence.	1	1	1	1	1	1	1

Table 10.1. Numbers of gnotobiotic lambs used to study the effects of rotavirus infection administered on the seventh day of life, on the villus and crypt epithelium of the small intestine.

Time after infection(days)	Site 1	Site 2	Site 3
0•6	-ve	+	+
1	not done	not done	not done
2	+	+	++
2•5	+	++	++
4	-ve	+	+++
7	-ve	-ve	-ve
12	-ve	-ve	-ve

Table 10.2. Presence of rotavirus fluorescence in 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the seventh day of life.

+ single fluorescent cells.

++ clumps of fluorescent cells.

+++ confluent fluorescence over at least half of each villus.

-ve no fluorescence demonstrated.

Time after infection(days)	The number of crypts per villus		
	Site 1	Site 2	Site 3
0•6	3•7	2•6	2•9
1	4•2	3•0	2•8
2	4•1	2•9	2•7
2•5	5•6	2•7	3•5
4	6•0	5•0	3•4
7	5•9	5•8	4•5
12	6•0	5•5	4•8

Table 10•3. The crypt to villus ratio in 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the seventh day of life.

Time after infection(days)	Site 1	Site 2	Site 3
0•6	12•2	3•0	15•5
1	10•9	3•4	14•0
2	14•9	10•9	1•1
2•5	9•6	6•9	1•3
4	4•2	2•2	0•3
7	19•3	0•6	0•7
12	9•1	8•8	2•3

Table 10.4. Tissue lactase activity in 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the seventh day of life. Activity is expressed as units of activity / gram wet weight of tissue. (Unit being 1μmole of substrate hydrolysed per minute at 37°C)

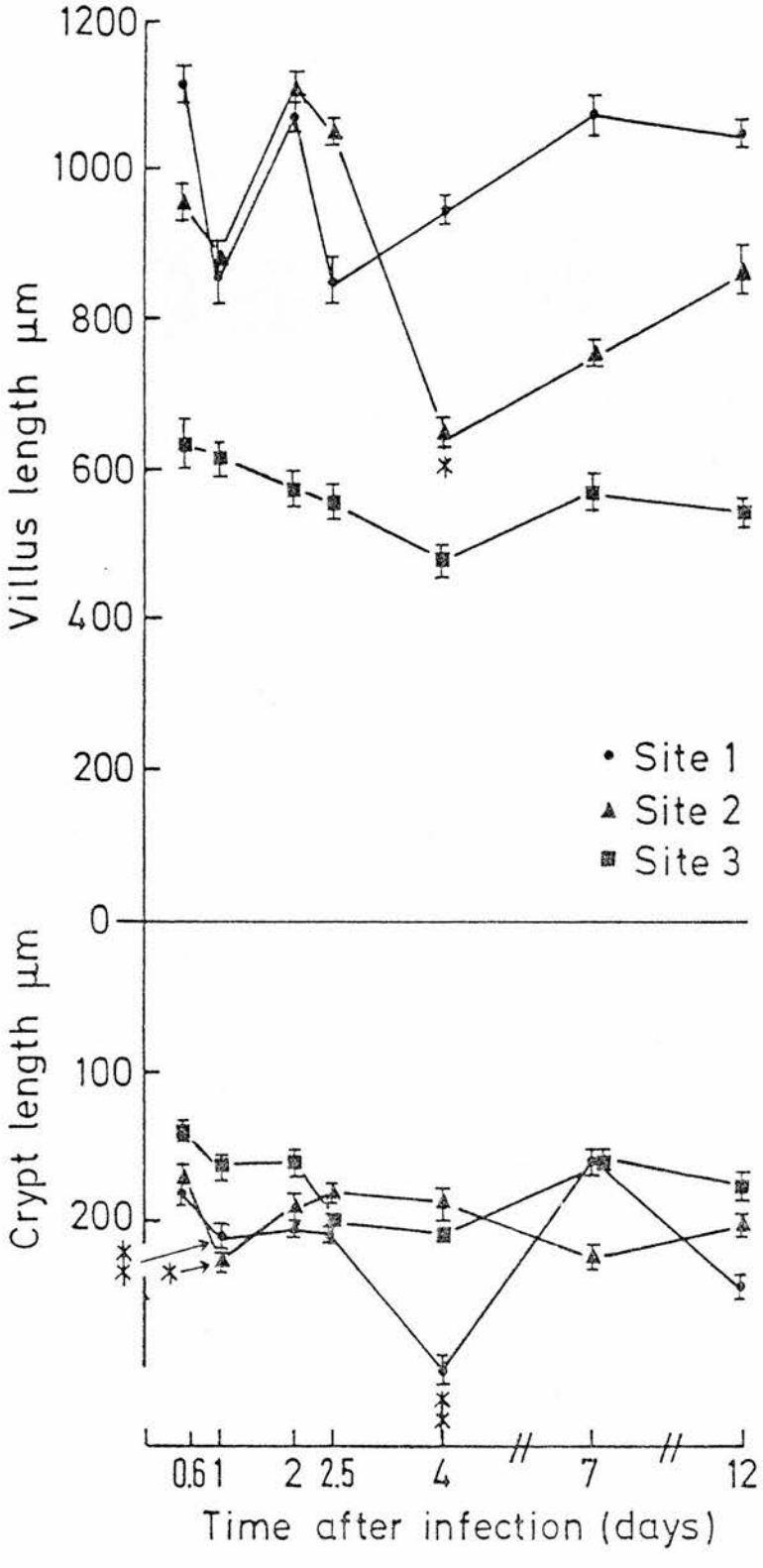

Fig 10.1. Villus and crypt lengths at 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the seventh day of life. Microdissection measurements (µm, mean ± S.E) * p less than 0·05, ** p less than 0·01 by Student's 't' test.

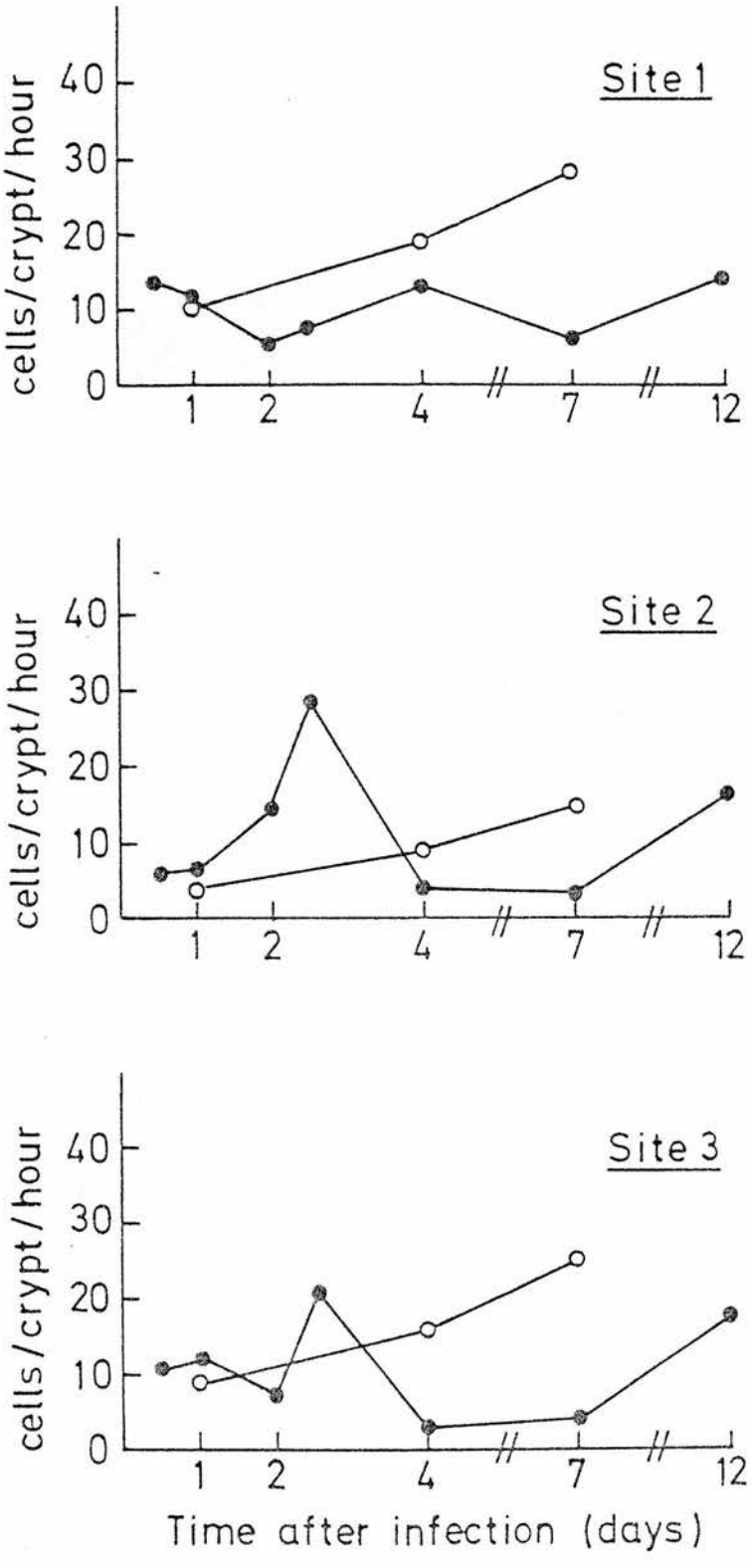

Fig 10.2. Crypt cell production rates at 3 sites of small intestine of gnotobiotic lambs infected with rotavirus on the seventh day of life and age matched controls.

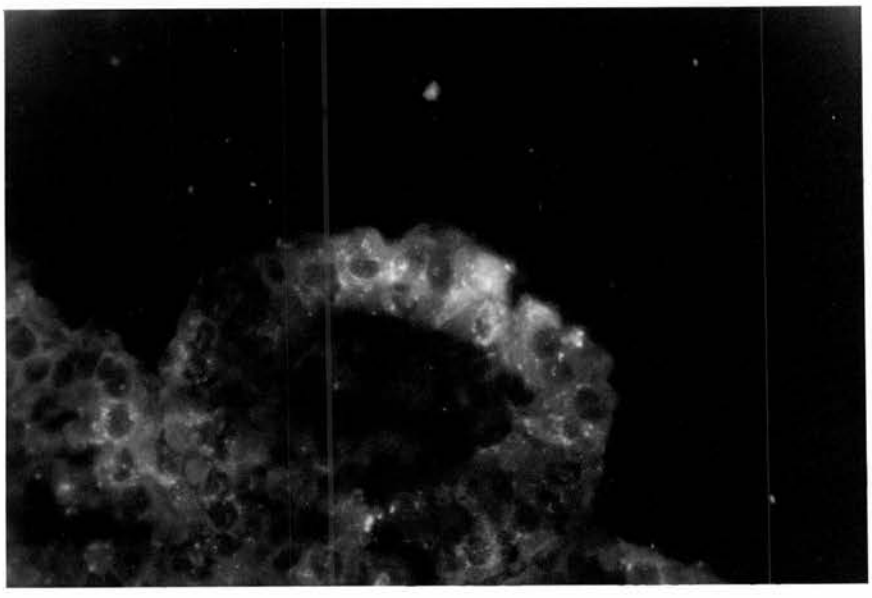

Plate 10.1. Positive rotavirus fluorescence within the villus epithelial cells of a gnotobiotic lamb, 2 days after infection with rotavirus on the seventh day of life. Site 1. Immunofluoresecence. X 300.

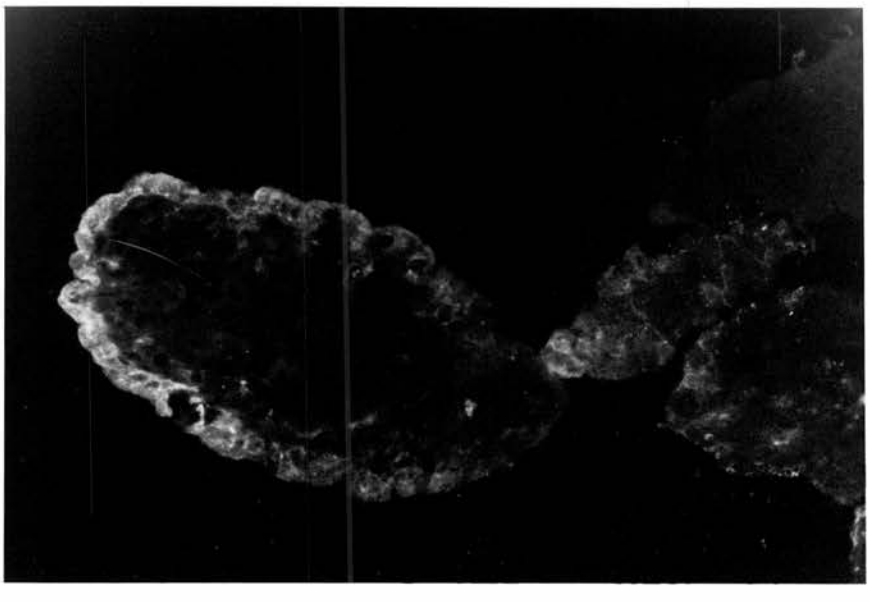

Plate 10.2. Confluent rotavirus fluorescence within the epithelial cells of the small intestine of a lamb, 2·5 days after infection with rotavirus on the seventh day of life. Site 2. Immunofluorescence. X 125.

Plate 10.3. Rotavirus infected cells on the upper parts of the villi of the small intestine of a gnotobiotic lamb, 2 days after infection with rotavirus on the seventh day of life. Site 3. Immunofluorescence. X 300.

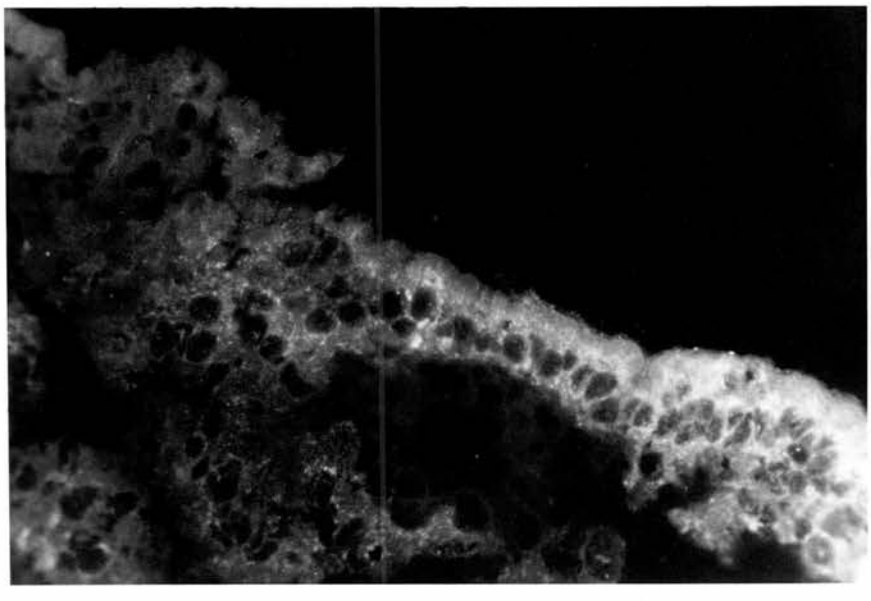

Plate 10.4. Confluent rotavirus fluorescence on the upper halves of the villi of a gnotobiotic lamb, 4 days after infection with rotavirus on the seventh day of life. Site 3. Immunofluorescence. X 125.

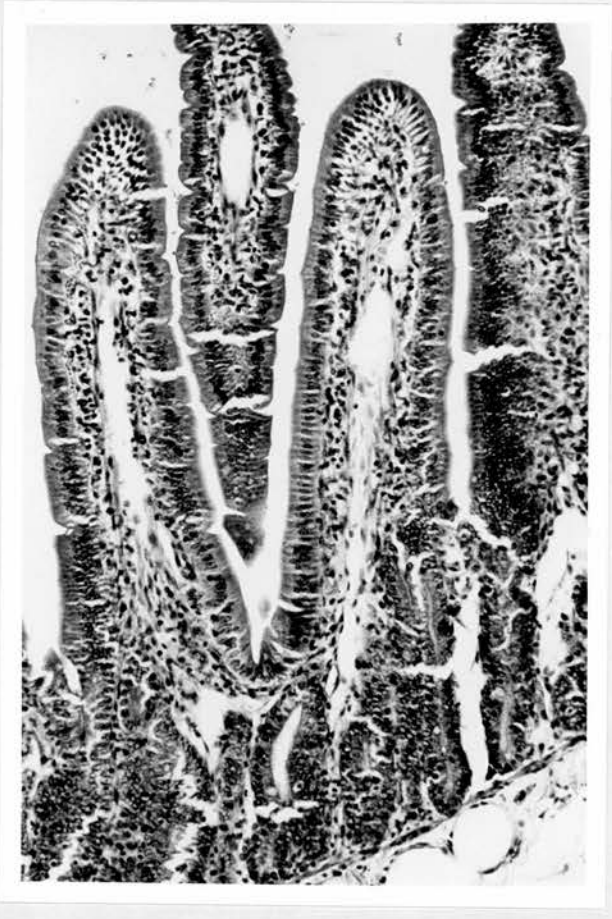

Plate 10.5. The small intestinal mucosa of a gnotobiotic lamb, 15 hours after infection with rotavirus on the seventh day of life. Site 1. The villi appear normal. H & E. X 125.

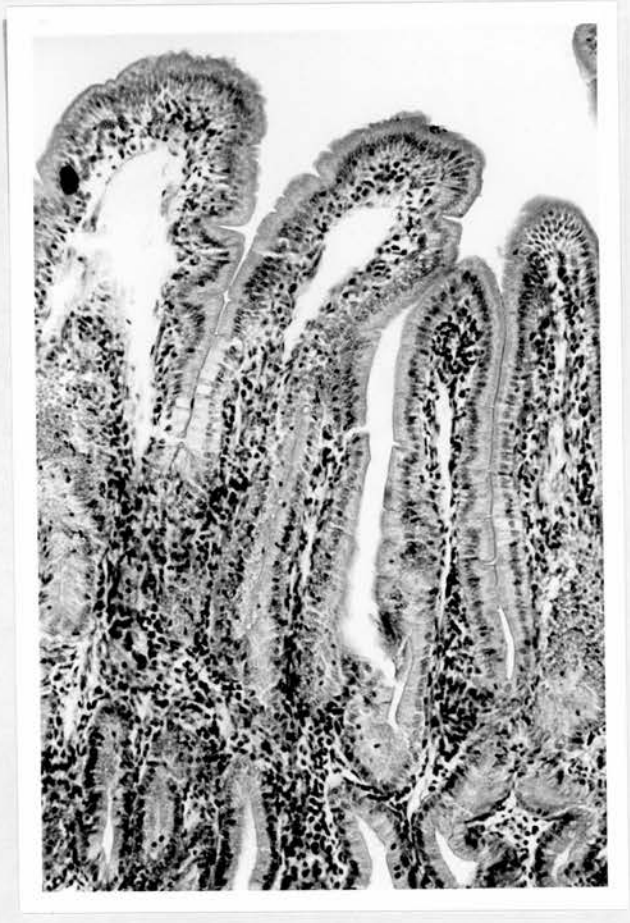

Plate 10.6. The small intestinal mucosa of a gnotobiotic lamb, 2.5 days after infection with rotavirus on the seventh day of life. Site 1. The epithelial cells are densely packed. H & E. X 125.

Plate 10.7. The small intestinal mucosa of a gnotobiotic lamb, 2·5 days after infection with rotavirus on the seventh day of life. Site 2. The villi are normal in shape although the epithelial cells are closely packed. H & E. X 125.

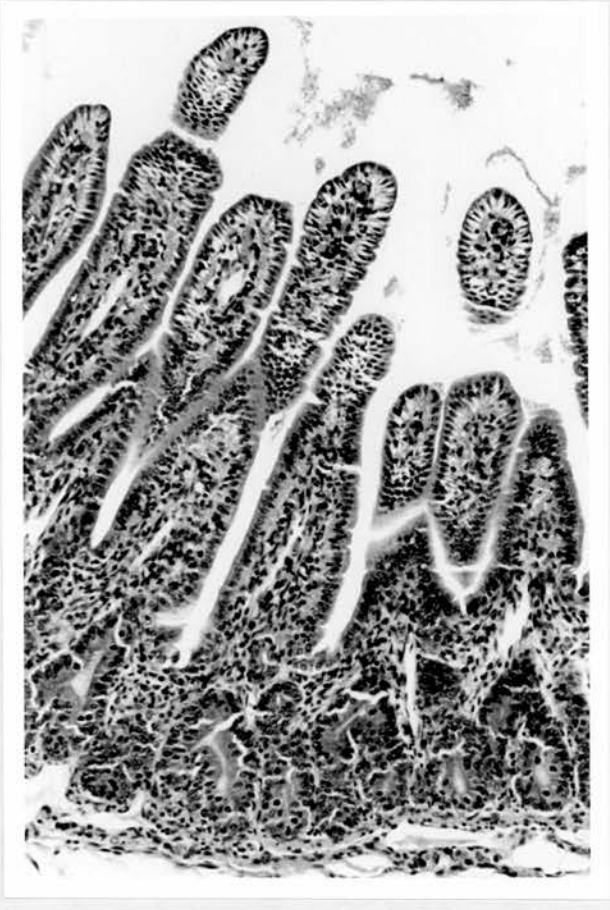

Plate 10.8. The villus epithelium of the small intestine of a gnotobiotic lamb, 1 day after infection with rotavirus on the seventh day of life. Site 3. The cells at the villus tip are disordered. H & E. X 125.

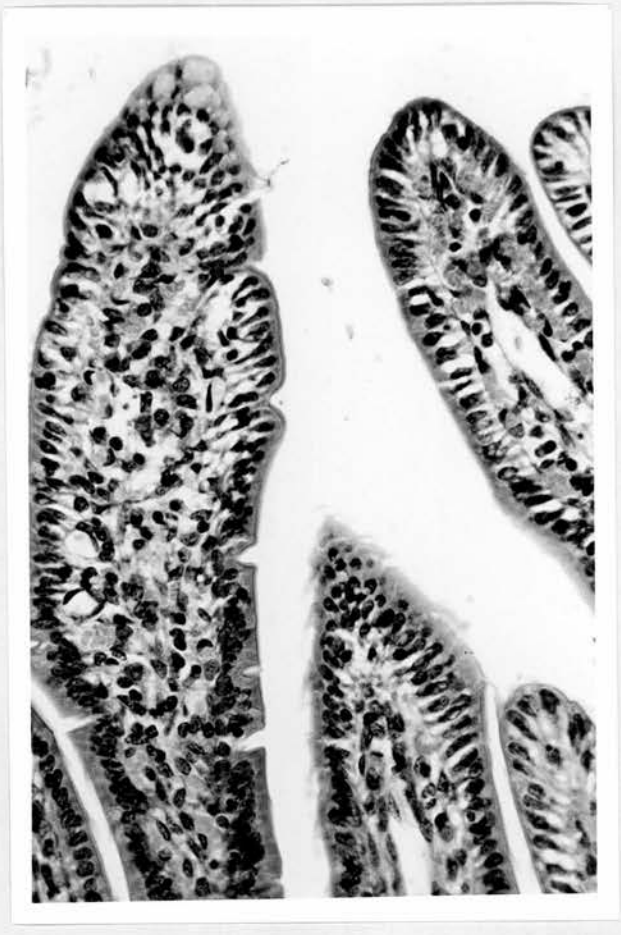

Plate 10.9. The small intestinal villus of a gnotobiotic lamb, 2·5 days after infection with rotavirus on the seventh day of life. Site 3. The epithelial cells at the tips of the villi are vacuolated and contain dense granular material. H & E. X 320.

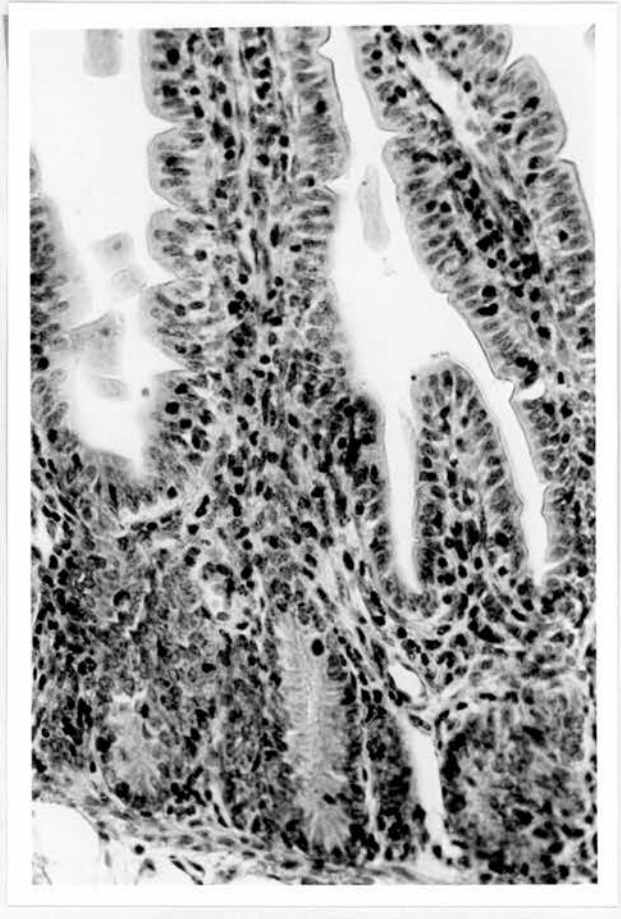

Plate 10. 10. The small intestinal mucosa of a gnotobiotic lamb, 4 days after infection with rotavirus on the seventh day of life. Site 3. The structure of the tissue is similar to that of the control animal. H & E. X 320.

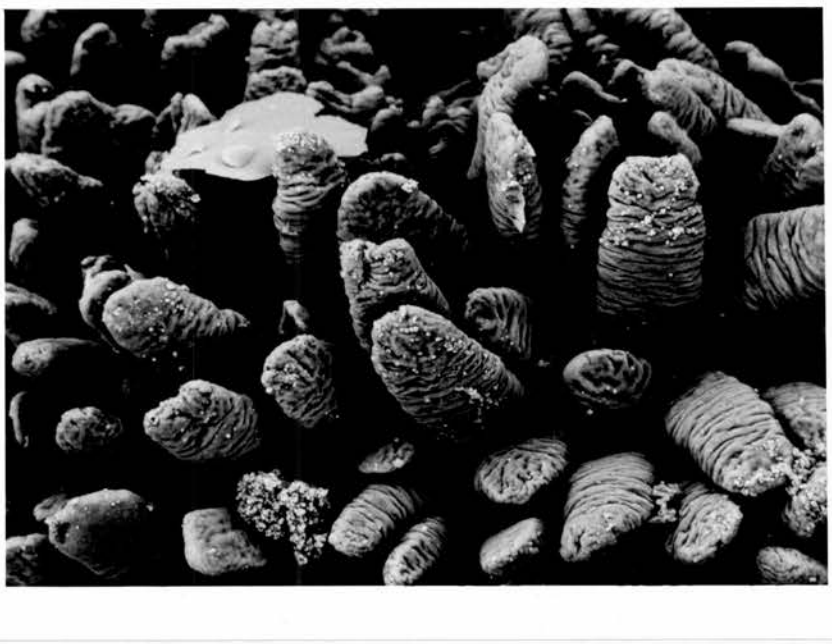

Plate 10.11. The small intestinal villi of a gnotobiotic lamb, 15 hours after infection with rotavirus on the seventh day of life. Site 1. The epithelial cells at the villus tips are slightly swollen. SEM. X 100.

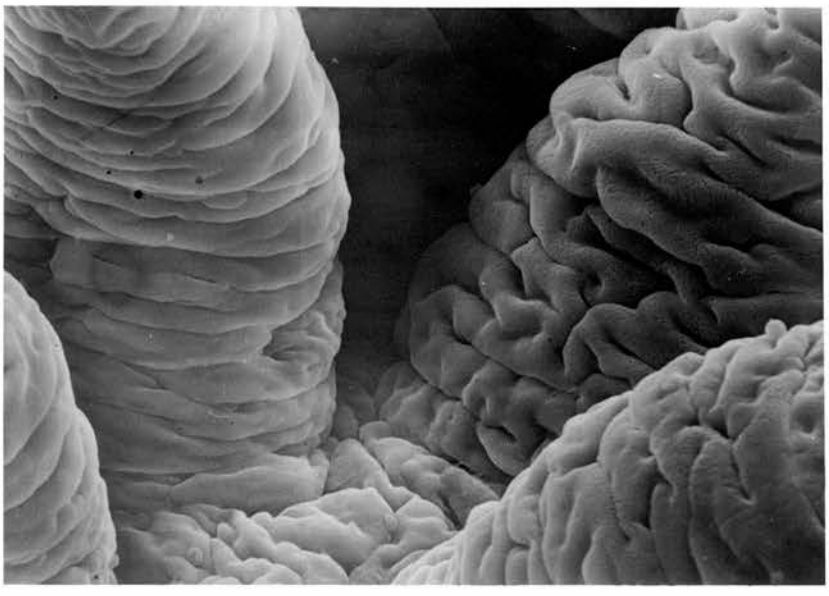

Plate 10.12. The villus epithelium of the small intestinal mucosa of a gnotobiotic lamb, 4 days after infection with rotavirus on the seventh day of life. Site 2. The villi are normal with deeply convoluted crenations. SEM. X 500.

Plate 10.13. The small intestinal villi of a gnotobiotic lamb, 15 hours after infection with rotavirus on the seventh day of life. Site 3. The villi are leaf or conical shaped with deep crenations. SEM. X 200.

Chapter 11.

Comparison between rotavirus infections administered on the second and seventh days of life. The effects on the small intestinal epithelium of gnotobiotic lambs.

Introduction.

The clinical effects of rotavirus infection were minimal in animals infected on the seventh day of life and contrasted with the severe diarrhoeal illness produced in lambs infected on the second day of life. The results of the studies in these two groups of animals have been presented in the preceding chapters. In this chapter the effects of the infection on mucosal architecture, epithelial cell kinetics, tissue lactase levels and the distribution of the infected cells on the villi are compared and contrasted.

The animals involved are detailed in Table 11.1.
The two groups of animals will be referred to as Group 1 and Group 2 throughout.

Group 1 :- animals infected on the second day of life.

Group 2 :- animals infected on the seventh day of life.

Distribution of virus-infected cells within the villus epithelium.

Fluorescent cells were present on the villi in both groups soon after infection, being first observed in Group 1, 6 hours after infection at all sites and in Group 2, at sites 2 and 3, 15 hours after infection. However, the immunofluorescence persisted much longer in Group 1, still being present 8 days after infection, whereas no fluorescence was seen beyond 4 days after infection in Group 2. This agreed with the reduced period of virus shedding in the stool observed in this group of animals.

Villus and crypt lengths.

Site 1.

Villus atrophy was only present 4-6 hours after infection in Group 1. In Group 2 the villi remained normal. Crypt lengthening was present 12 hours and 4 days after infection in Group 1 and also 4 days after infection in Group 2, (Fig 11.1a).

Site 2.

Villus atrophy was severe from 18 hours to 2 days after infection in Group 1, whereas villus shortening was only present 4 days after infection in Group 2, (Fig. 11.1b). The crypts were lengthened 2 days after infection in Group 1 and 1 day after infection in Group 2.

Site 3.

Villus atrophy occurred from 12 hours to 4 days after infection in Group 1 and was mild, 1 day after infection in Group 2. The crypts were lengthened 18 hours and 2 days after infection in Group 1 but not at all in Group 2, (Fig. 11.1c).

The preceding results illustrate the definite, prolonged mucosal response which was observed in Group 1, especially at sites 2 and 3. The reduction in villus length was prolonged in the mid and distal regions of the small intestine and persisted in the ileum until at least 4 days after infection. The crypt hyperplasia observed was confined to sites 2 and 3 and ceased 2 - 4 days after infection in Group 1. In contrast, the mucosal changes observed in the animals in Group 2 were negligible.

Histology and scanning electron microscopy.

The pathological lesions occurring as a result of rotavirus infection were severe and persistent in Group 1. These were in accord with the comparisons noted regarding villus and crypt lengths, lactase levels, epithelial cell kinetics and distribution of virus within the villus epithelial cells.

In Group 2, only slight alterations from normal occurred and these did not persist beyond 4 days after the time of infection. In Group 1 the evidence of infection was generally confined to sites 2 and 3, with mild lesions present briefly in the proximal site, and the lesions were noticeable from 4 hours to 4 days after infection by histological examination and from 4 hours to 8 days after infection by scanning electron microscopy.

Epithelial cell kinetics.

Site 1.

group 1 exhibited a rise in CCPR, 1-2 days after infection, recorded values being 12.6 - 17.2 cells/crypt/hour compared to control values of 8.8 cells/crypt/hour. The values in Group 2 did not vary from these of age matched controls (Fig 11.2a).

Site 2.

The CCPR in Group 1 was consistently raised from 2 days to 11 days after infection, with a peak value of 21.2 cells/crypt/hour at 8 days after infection. In Group 2, there was a rise in CCPR of short duration, 2.5 days after infection with a value of 29.2 cells/crypt/hour being recorded compared to 3.2 - 8.6 cells/crypt/hour in control animals (Fig 11.2b).

Site 3.

There was an increase in CCPR in both groups, 1 - 4 days after infection in Group 1 and 2.5 days after infection in Group 2 (Fig 11.2c).

The sustained increase in CCPR with crypt hyperplasia observed at site 2 in Group 1 was not repeated in the animals in Group 2.

The induction of a cell kinetic response in both groups of animals, one group suffering from severe villus atrophy and epithelial cell damage, the other not, suggested that the stimulus for the induction of the cell proliferative response was made up of two separate components. The first could be linked to the size of the villus cell population and operated in conjunction with the viral induced destruction of the villus epithelial cells. The other component of the proliferative response was caused by different factors, as yet unknown.

Tissue lactase levels.

Site 1.

Lactase levels were consistently less in the epithelial cells of the villi of the animals in Group 1, from 1 day after infection until the end of the experimental period. The lactase levels were not altered in Group 2 (Fig 11.3a).

Site 2.

Group 1 had lowered lactase levels from 6 hours to 4 days after infection, whereas no alteration occurred in Group 2 (Fig 11.3b).

Site 3.

Lactase levels were reduced in Group 1 during the early stages

of the infection whereas Group 2 showed no alteration. The reduction which occurred in the lactase levels of the ileum between 5 and 9 days of age, Table 6.7, as part of the normal maturation process, was not influenced by either regime of infection.

Conclusions.

The susceptibilities of the two groups of animals to rotavirus infection were very different.

Virus was detected for a longer period of time in animals infected on the second day of life, both in the epithelial cells of the small intestine and in the faeces.

Villus atrophy and crypt lengthening was minimal in animals infected on the seventh day of life whereas villus atrophy was pronounced in the group of animals infected on the second day of life.

Histological and scanning electron microscopic examination indicated that few changes occurred in the structure of the small intestinal epithelium of animals infected on the seventh day of life, and on purely subjective assessment, these animals would be classed as normal. This was not the case for animals infected on the second day of life, the differences between age matched control tissue and that from infected animals being obvious, especially at sites 2 and 3.

Group 1

Technique	Controls	Time after infection(days)							
		0 - 0•75	1	2	3	4	8	11	15
Histology.	6	7	3	3	1	1	1	1	2
Microdissection.	6	7	1	1	-	1	1	1	1
Scanning electron microscopy.	5	7	2	2	1	-	-	-	1
Lactase assay.	7	6	1	1	-	1	1	1	2
Immunofluorescence.	7	6	-	-	-	-	-	-	-

Group 2

Technique	Controls	Time after infection(days)						
		0•6	1	2	2•5	4	7	12
Histology.	3	1	1	1	1	1	1	1
Microdissection.	3	1	1	1	1	1	1	1
Scanning electron microscopy.	4	1	1	1	1	1	1	1
Lactase assay.	3	1	1	1	1	1	1	1
Immunofluorescence.	3	1	1	1	1	1	1	1

Table 11.1 Numbers of gnotobiotic lambs used to compare the effects of rotavirus infections given on the second and seventh days of life.

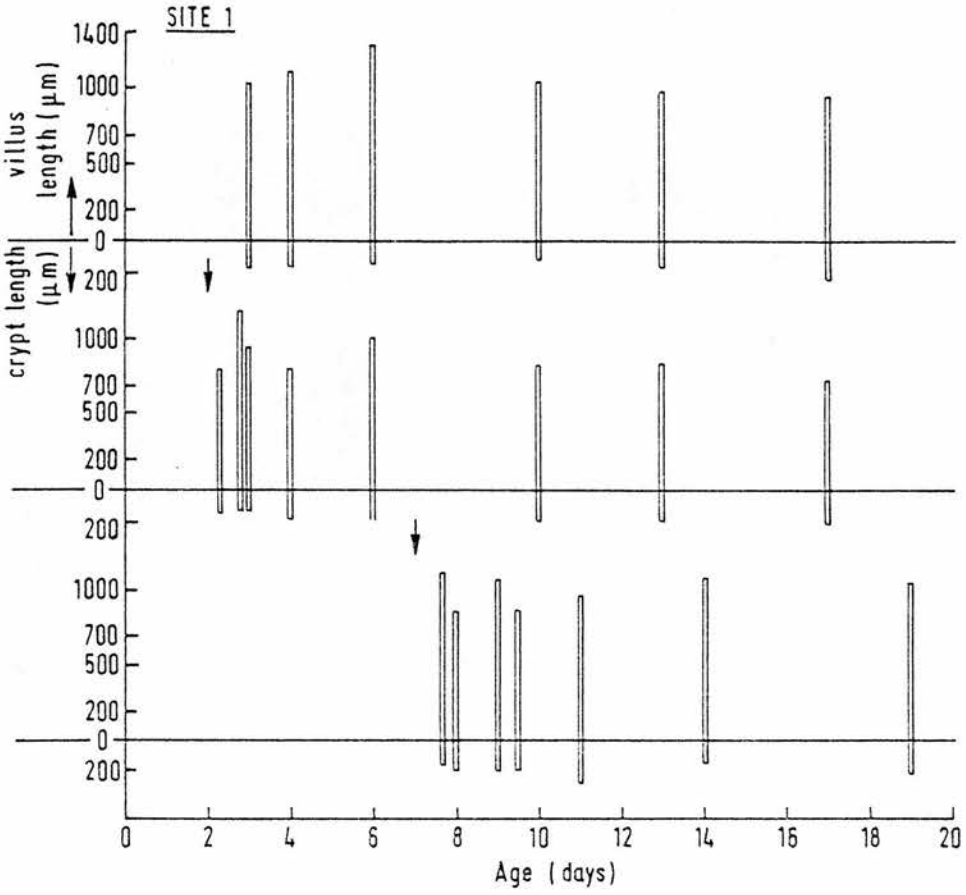

Fig 11.1a. Villus and crypt lengths at Site 1 of the small intestine of gnotobiotic lambs infected with rotavirus on the second and seventh days of life and a group of age matched controls. Microdissection measurements. Arrows indicate the administration of rotavirus.

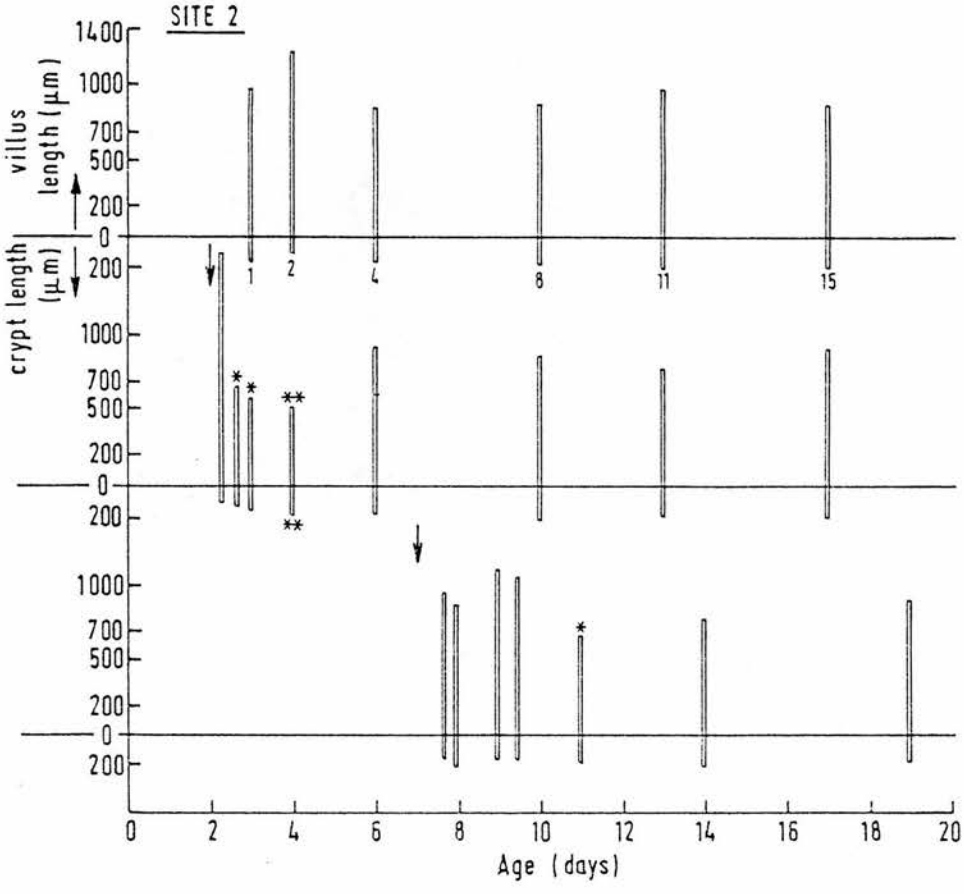

Fig 11.1b. Villus and crypt lengths at Site 2 of the small intestine of gnotobiotic lambs infected with rotavirus on the second and seventh days of life and a group of age matched controls. Microdissection measurements. Arrows indicate the administration of rotavirus.

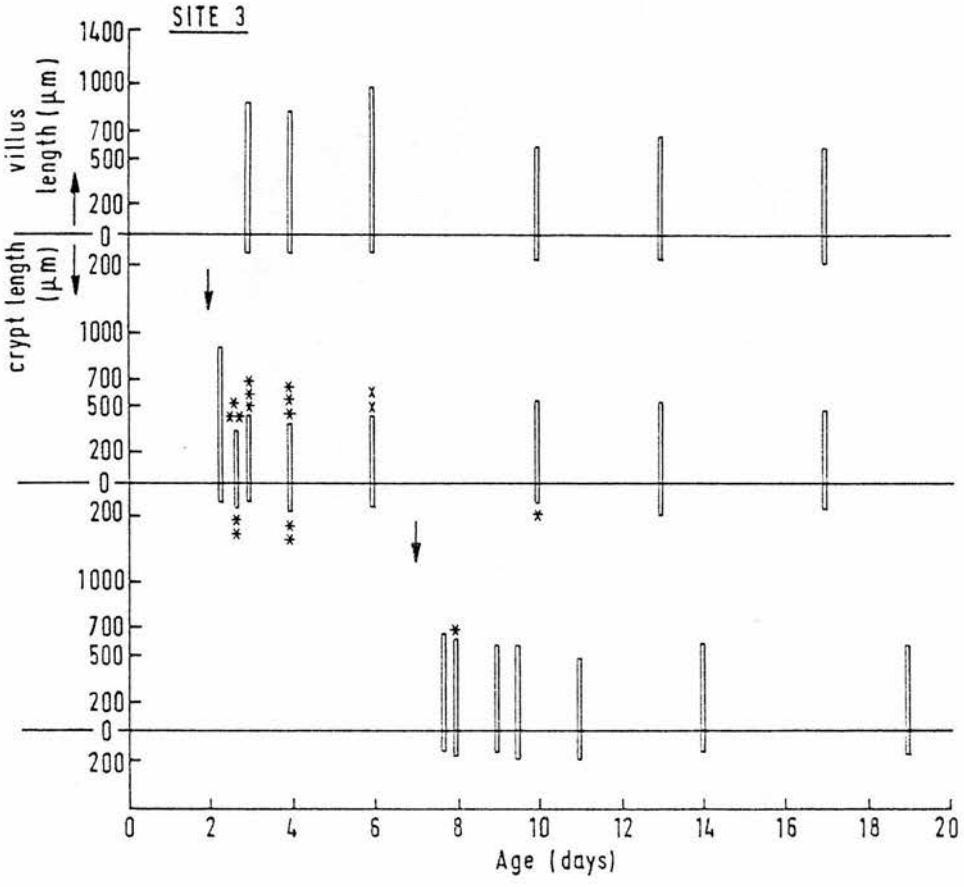

Fig 11. 1c. Villus and crypt lengths at Site 3 of the small intestine of gnotobiotic lambs infected with rotavirus on the second and seventh days of life and a group of age matched controls. Microdissection measurements. Arrows indicate the administration of rotavirus.

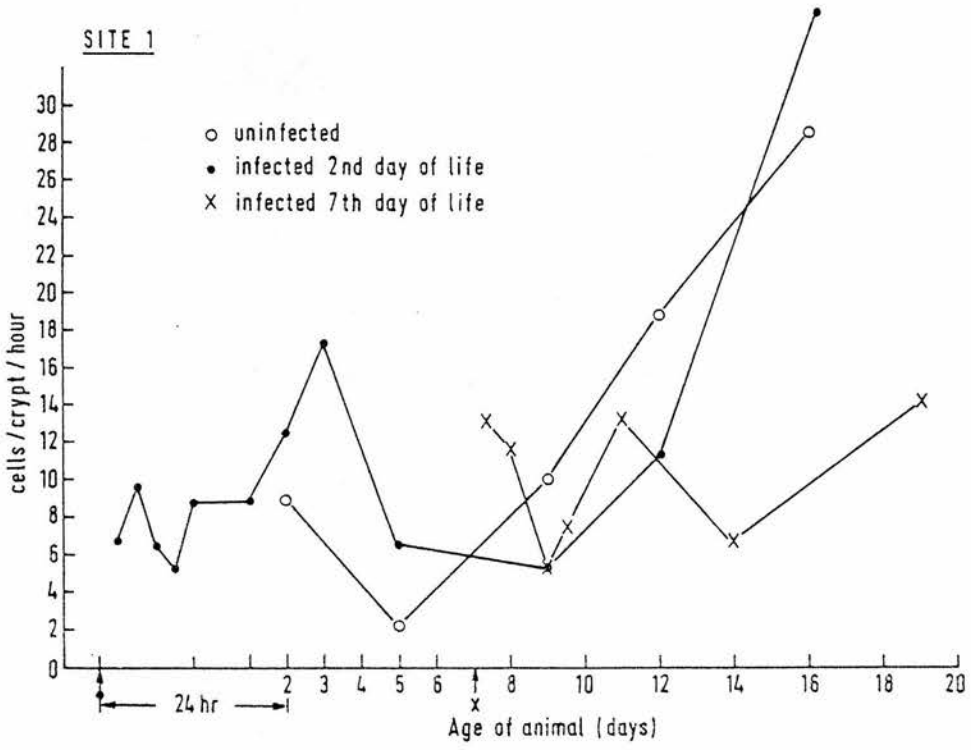

Fig 11.2a. Crypt cell production rates at Site 1 of the small intestine of gnotobiotic lambs infected with rotavirus on the second and seventh days of life and a group of age matched controls. The arrows indicate the administration of rotavirus.

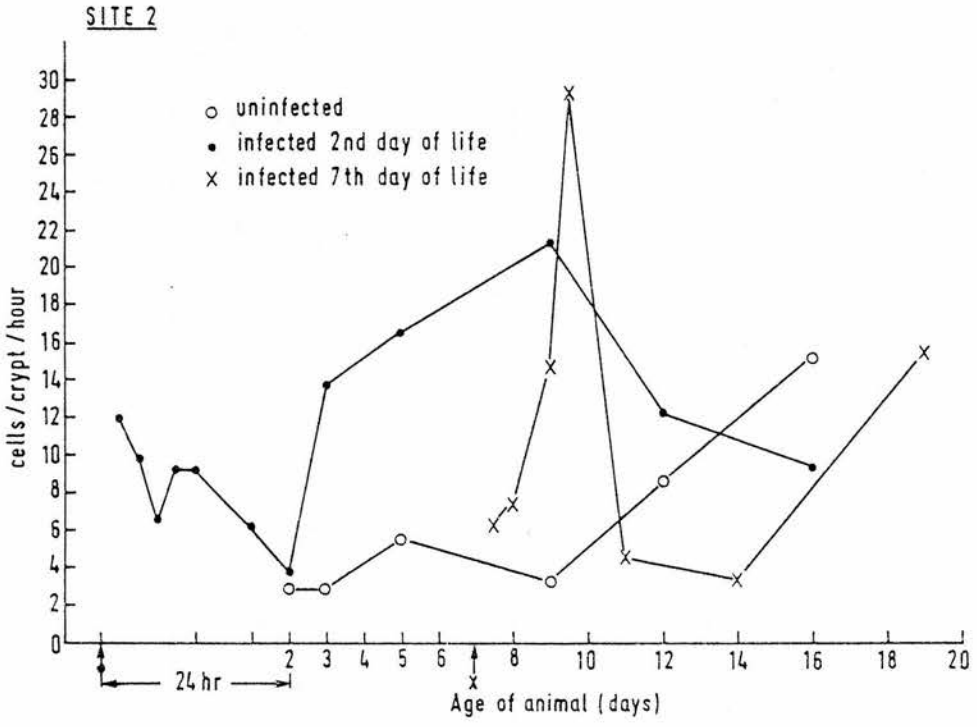

Fig 11.2b. Crypt cell production rates at Site 2 of the small intestine of gnotobiotic lambs infected with rotavirus on the second and seventh days of life and a group of age matched controls. The arrows indicate the administration of rotavirus.

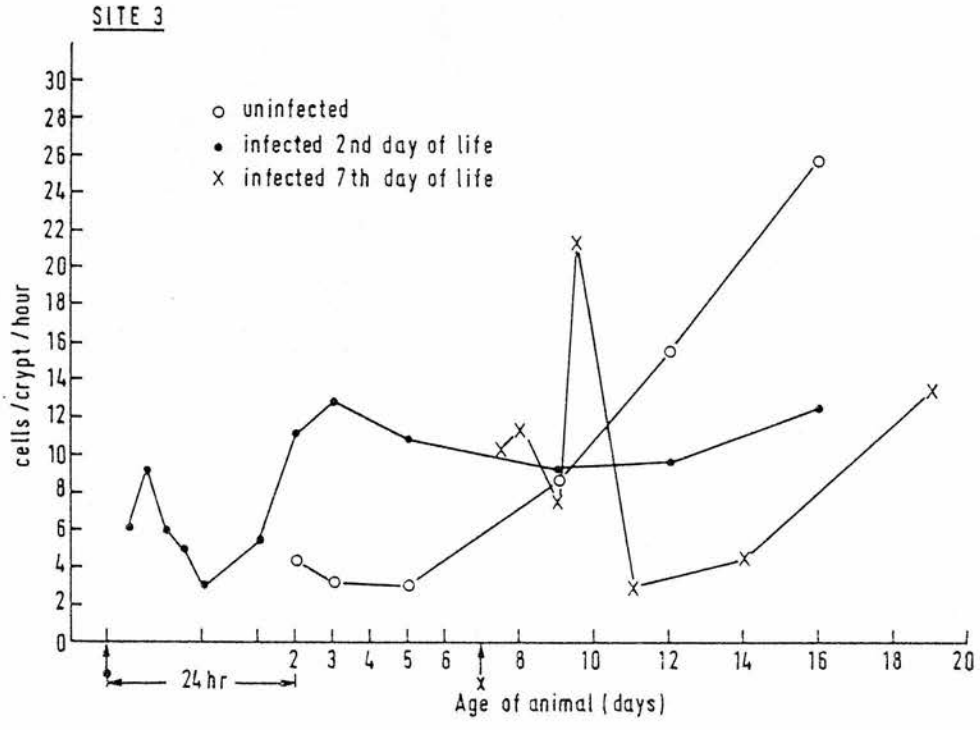

Fig 11.2c. Crypt cell production rates at Site 3 of the small intestine of gnotobiotic lambs infected with rotavirus on the second and seventh days of life and a group of age matched controls. The arrows indicate the administration of rotavirus.

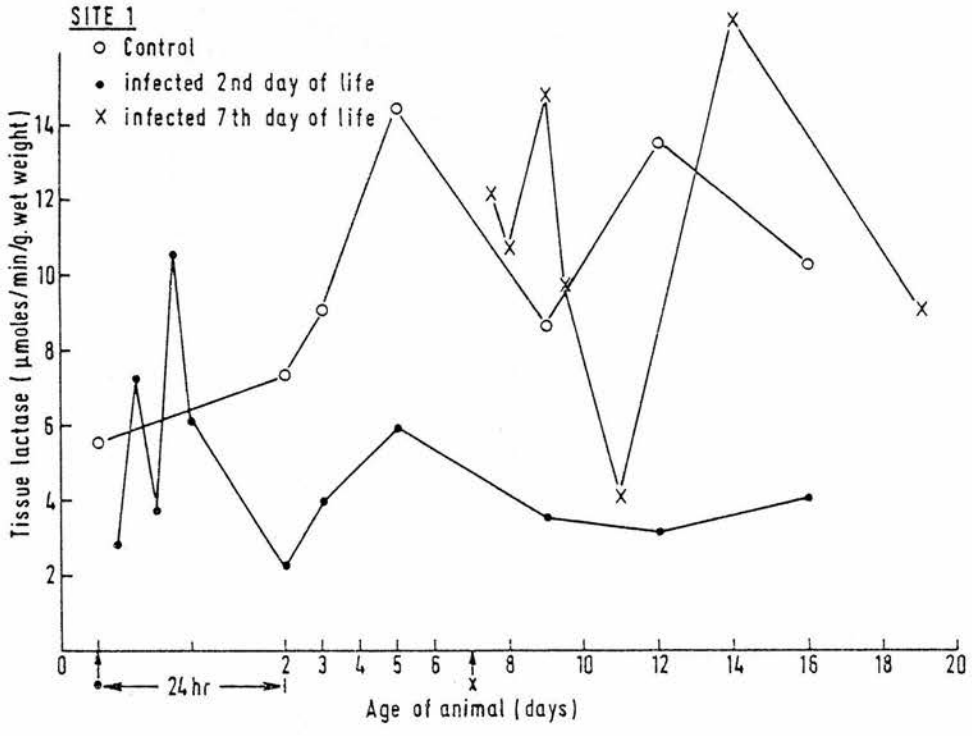

Fig 11.3a. Tissue lactase levels at Site 1 of the small intestine of gnotobiotic lambs infected with rotavirus on the second and seventh days of lifa and a group of age matched controls. The arrows indicate the administration of rotavirus.

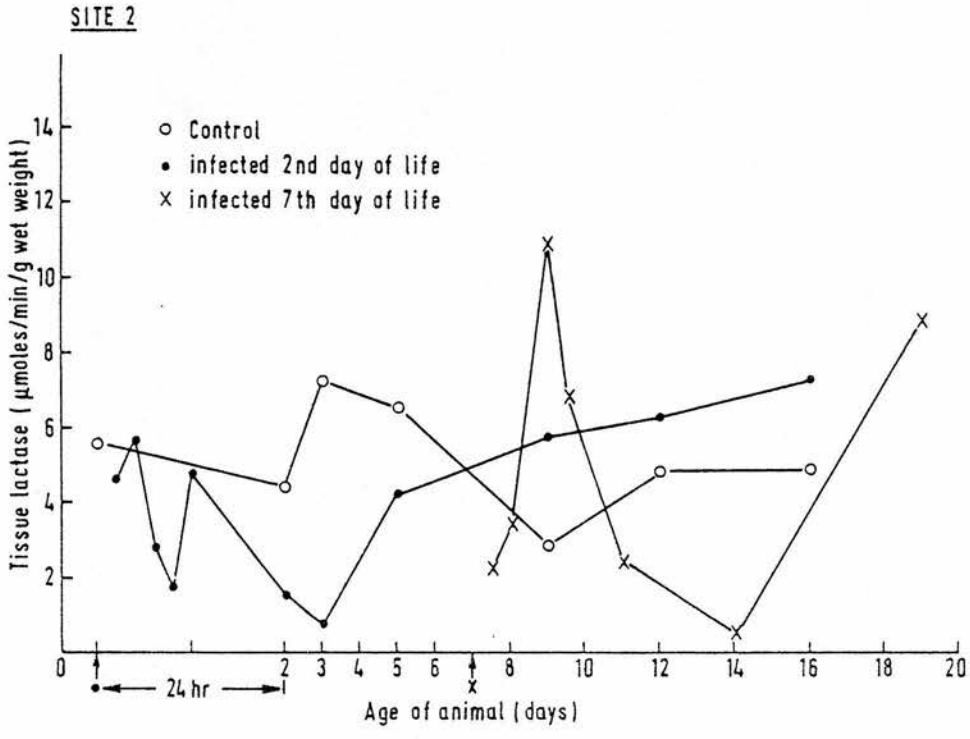

Fig 11.3b. Tissue lactase levels at Site 2 of the small intestine of gnotobiotic lambs infected with rotavirus on the second and seventh days of life and a group of age matched controls. The arrows indicate the administration of rotavirus.

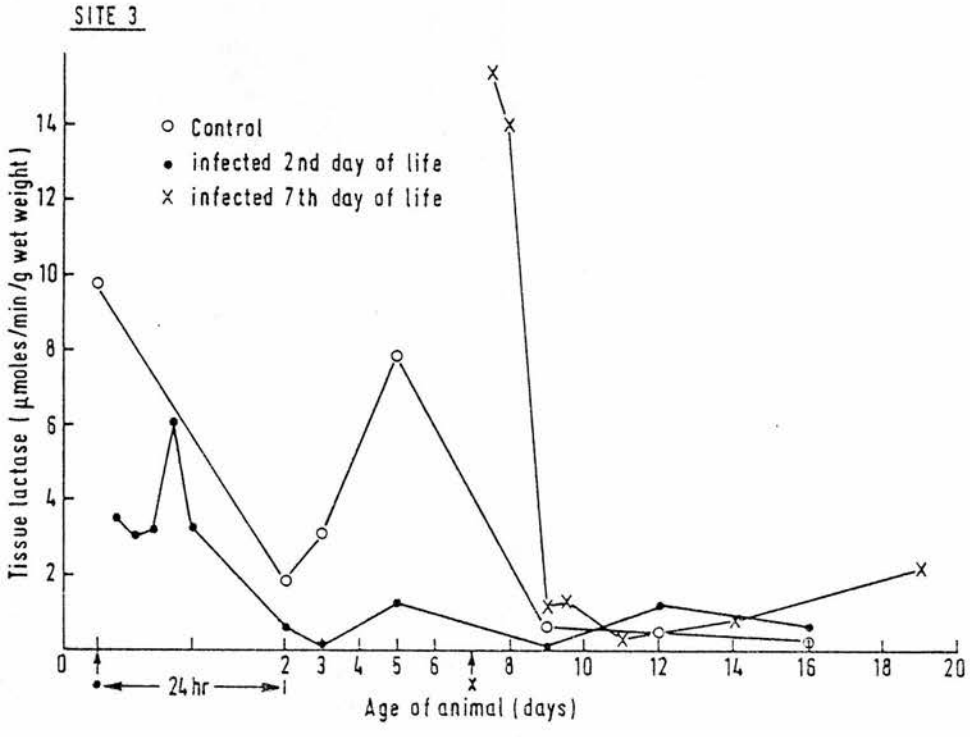

Fig 11.3c. Tissue lactase levels at Site 3 of the small intestine of gnotobiotic lambs infected with rotavirus on the second and seventh days of life and a group of age matched controls. The arrows indicate the administration of rotavirus.

Chapter 12.

The follicle-associated epithelium of the Peyer's patches in gnotobiotic lambs infected with rotavirus.

Introduction.

In the lamb, the Peyer's patches occupy a substantial proportion of the distal ileum, covering approximately 75% of the luminal surface of the ileum. Each lymphoid follicle within the patch is topped by a 'dome' which is surrounded by villi which are distinct from the ileal villi in non-patch areas. The epithelium of the dome is produced in the same way as the villus epithelium, by cell division in the crypts. The epithelium lining the crypts forms a continuous sheet with the epithelium of the dome (Smith and Peacock, 1980). The epithelial surface of the dome consists of columnar epithelial cells (enterocytes), which are covered with dense microvilli, interspersed with cells known as 'M' cells (microfold cells). These 'M' cells have been implicated in the presentation of antigen to the immune system via the gut, with the subsequent induction of an immune response (Owen and Nemanic, 1978).

In view of the distinctive characteristics and important immunological role of the follicle-associated epithelium, samples of ileum containing Peyer's patch were examined.

Only a limited structural study of this tissue in rotavirus infected lambs and control animals was performed. The study was essentially descriptive in nature, primarily involving scanning electron microscopy with limited correlative studies employing transmission electron microscopy, light microscopy and immunofluorescence.

The aims of this study were:

(1) to describe the mucosal architecture of the Peyer's patch region of the small intestine of the gnotobiotic lamb, with particular

emphasis on the follicular epithelium.

(2) to describe the effects of rotavirus infection on the Peyer's patch epithelium.

(3) to study the distribution of viral particles within the follicular epithelium.

Animals used.

Samples of small intestine were taken from areas of distal ileum known to contain Peyer's patches. Animals examined included uninfected lambs aged 1 to 20 days, 11 lambs infected with rotavirus on the second day of life and 7 lambs infected with rotavirus on the seventh day of life. The animals involved were selected from the lambs detailed in chapters 6 to 10 and are listed in the tables of animals used at the start of each of these chapters.

Control lambs.

In the youngest uninfected lambs, 1 to 2 days of age, the villi in the Peyer's patch region were long and finger-like with absorptive vacuoles present in the epithelial cells of the upper parts of the villi. There were numerous follicles present but the domes of these follicles were not easily seen with the scanning electron microscope due to the dense distribution of the surrounding villi and the shortness of the domes (Plate 12.1). The domes were occasionally seen where a few villi had broken off during processing (Plate 12.2). In animals aged 7, 16 and 20 days the domes were easily seen among the villi (Plate 12.3) and their greater elevation above the crypt neck was confirmed by light microscopy (Plate 12.4).

The villi in the Peyer's patch region were shorter and

leaf-like in older animals and this paralleled the change in ileal structure, associated with the age of the animal, as noted in chapter 6. By 16 days of age the follicles of the Peyer's patch were well developed and the gross appearance of the tissue was as shown in Plate 12.5. The leaf shaped villi were deeply crenated, the domes of the individual follicles protruded between the villi and each follicle was a distinct, separate structure. The dome was conical in shape (Plate 12.6). The epithelium of the dome consisted of columnar epithelial cells densely covered by microvilli and other cells ('M' cells) whose surfaces were formed by low ridges or microfolds (Plate 12.7). Light microscopic examination showed the presence of lymphocytes within the epithelial cell layer (Plate 12.8).

Lambs infected with rotavirus on the second day of life.

Animals sampled during the first 12 hours after infection had long, deeply crenated villi, creating difficulties in the examination of the dome surfaces. The presence of the domes was confirmed by light microscopy; long villi with absorptive vacuoles on their upper parts and low domes lying between the villi were observed (Plate 12.9). Lymphoid cells were present within the dome epithelial layer.

By 24 hours after infection, the ileal villi showed abnormalities and many of the villus epithelial cells had small out-pushings on their surfaces. The villi were short, squat and deeply crenated (Plate 12.10) and the absorptive vacuoles were absent. This feature was more pronounced in animals examined 48 hours after infection (Plates 12.11, 12.12). The domes were prominent among the villi, the epithelial cells of the villi had a very irregular appearance, with obvious bleb-like structures which

were clearly extrusions of the epithelial cell walls (Plate 12.13). Previous studies have mentioned similar structures (Coelho, Bryden, Hall and Flewett, 1981; Pearson and McNulty, 1979) and it has been suggested that the 'blebs' may be evidence of vesicles containing virus particles within the epithelial cells. These 'blebs' were not present beyond 3 days post infection. The light microscopic appearance of a 'bleb' covered ileal villus is shown in Plate 12.14.

The surface epithelium of the dome after infection differed from that of control animals. The epithelial cells were rounded in appearance and the area covered by the 'M' cells seemed enlarged, suggesting that the epithelial cells were crowded by the 'M' cells. The complexity of the microfold surface of the 'M' cell is shown in Plate 12.15. 'M' cells were not visible by light microscopy but the rounded, distended appearance of the epithelial cells was obvious (Plate 12.16). Large numbers of lymphoid cells were present in the epithelial cell layer.

Transmission electron microscopic studies of the dome epithelium in a lamb 2 days after infection showed epithelial cells with long microvilli on either side of 'M' cells. Lymphocytes were present within the confines of the 'M' cell (Plate 12.17). Immunofluorescence studies demonstrated the presence of virus within the dome epithelium, 2 days after infection (Plate 12.19).

The domes were still prominent 8 days after infection, with the epithelial cells of the dome separated by 'M' cells (Plate 12.20). The villi were leaf-shaped and of normal appearance. The domes were less prominent 15 days after infection and the tissue resembled that of the age matched control (Plate 12.21).

Lambs infected on the seventh day of life.

The ileal villi were leaf-shaped 15 hours after infection, and deeply crenated with prominent domes lying between them (Plate 12.22). The dome epithelium consisted of flat epithelial cells surrounded by 'M' cells with low microfolds which occupied a sparse area between the epithelial cells. By 24 hours after infection, the villi over the Peyer's patch were wedge shaped and some were fused to form ridges (Plate 12.23), the domes were prominent and the epithelial cells of the dome were relatively widely separated by the 'M' cells. The epithelial cells looked swollen (Plate 12.24).

From 2 to 7 days after infection the villi were conical with deep crenations. The domes were easily seen and the epithelial cells of the dome were widely separated by 'M' cells (Plate 12.25).

The appearance of the tissue 12 days after infection was similar to that of the age matched control (Plate 12.26).

Conclusions.

During a rotavirus infection the domes of the follicles increased in size in relation to the ileal villi and became prominent.

The epithelium of the dome changed in response to the rotavirus infection. The overall volume of the 'M' cells appeared to increase, the microfolds became more numerous and deeper and the epithelial cells appeared to be constricted in some way by the 'M' cells. The number of lymphocytes present in the epithelial layer of the dome appeared greater.

Viral particles were identified by transmission electron microscopy and immunofluorescence within the epithelium of the dome,

48 hours after infection and 6, 9 and 12 hours after infection by immunofluorescence alone. Viral fluorescence was present 4 days after infection in the lambs infected on the seventh day of life.

Maximum increases in the volume of the dome and the associated 'M' cells occurred approximately 48 hours after infection and were present both in animals which showed clinical signs of illness and those which did not.

As a result of this preliminary investigation it is clear that studies of the responses of the Peyer's patch epithelium during viral enteritis infection could be rewarding. The type of investigations which could possibly be performed are those relating to the uptake of particles by the 'M' cells and the induction of immune responses due to antigen sampling by the 'M' cells.

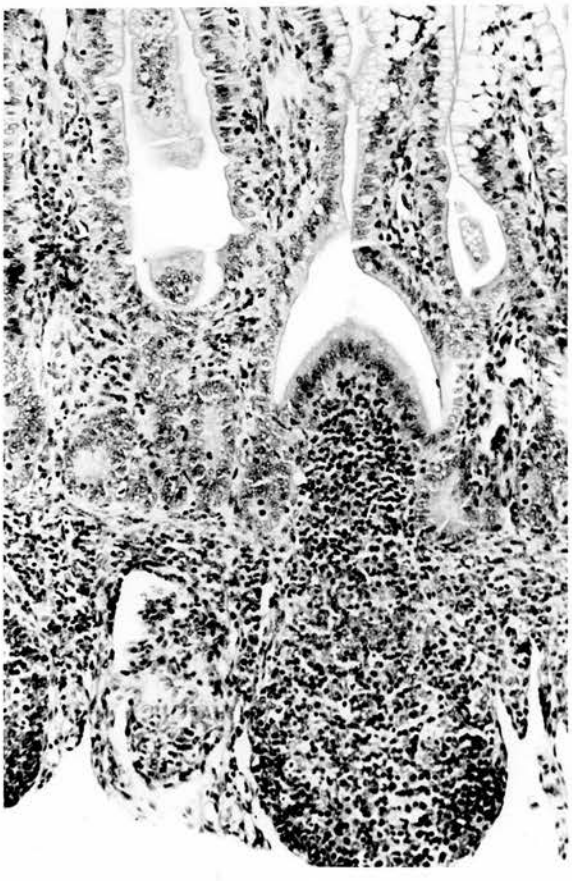

Plate 12.1. The small intestinal mucosa of an uninfected gnotobiotic lamb aged 1 day. Peyer's patch. A lymphoid follicle with associated dome epithelium and ileal villi. Note the absorptive vacuoles in the villus epithelium. H & E. X 125.

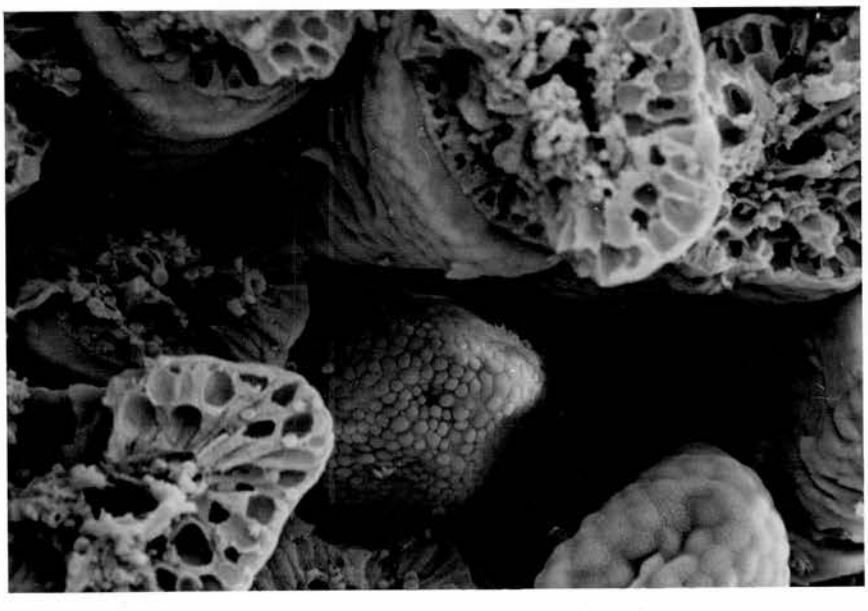

Plate 12.2 The dome epithelium of a lymphoid follicle in a Peyer's patch of a gnotobiotic lamb aged 2 days. SEM. X 500.

Plate 12.3. The domes of the lymphoid follicles lying between the ileal villi in the Peyer's patch region of a gnotobiotic lamb aged 7 days. SEM. X 207.

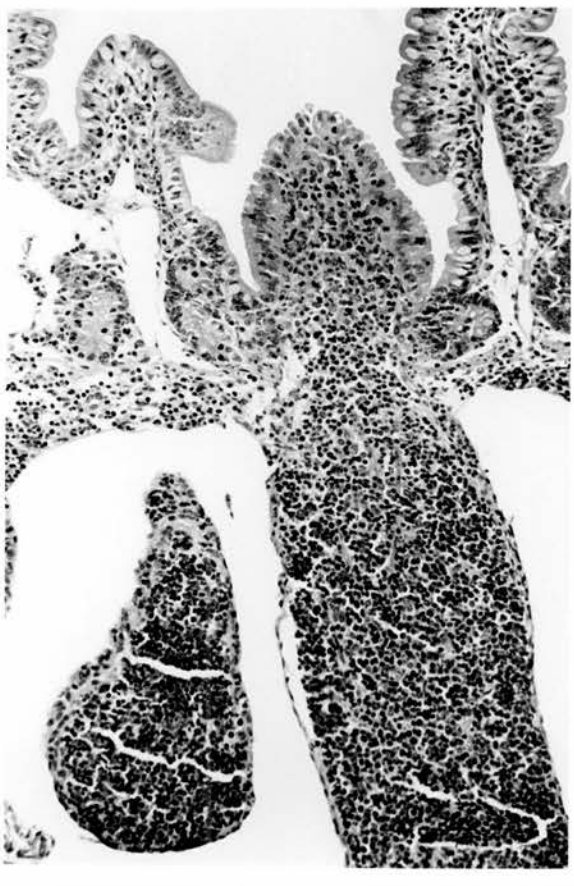

Plate 12.4. The Peyer's patch follicle showing the dome and the leaf shaped ileal villi. The dome protrudes further into the lumen in older animals (compare with Plate 12.1) H & E. X 125.

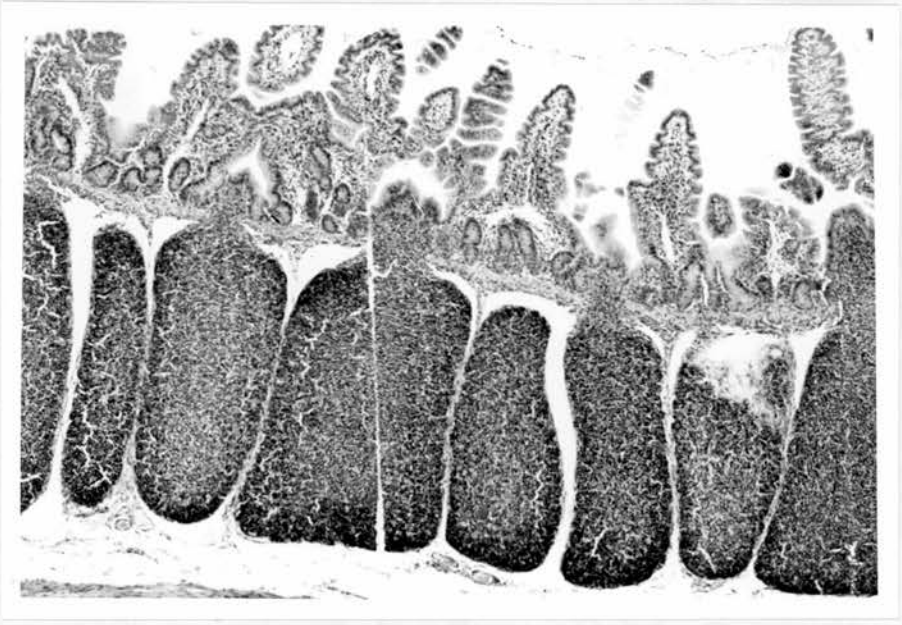

Plate 12.5. The small intestinal mucosa in a gnotobiotic lamb aged 16 days. Gross view of the lymphoid follicles, domes and deeply crenated ileal villi. H & E. X 50.

Plate 12.6. The dome above a lymphoid follicle in the Peyer's patch region of the small intestine of a gnotobiotic lamb aged 9 days. SEM. X 320.

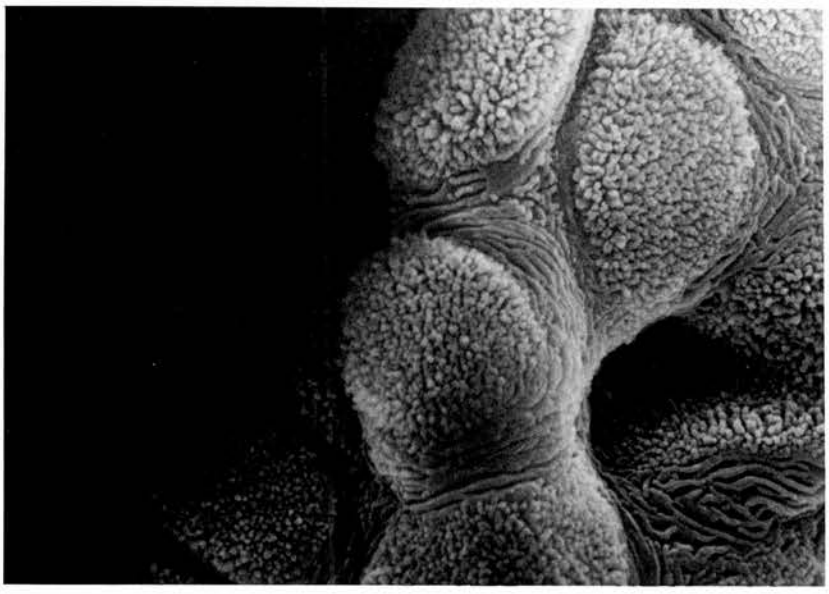

Plate 12.7. M. cells and columnar epithelial cells which make up the dome epithelium of the lymphoid follicles in the Peyer's patch region of the small intestine in the gnotobiotic lamb aged 16 days. SEM. X 5200.

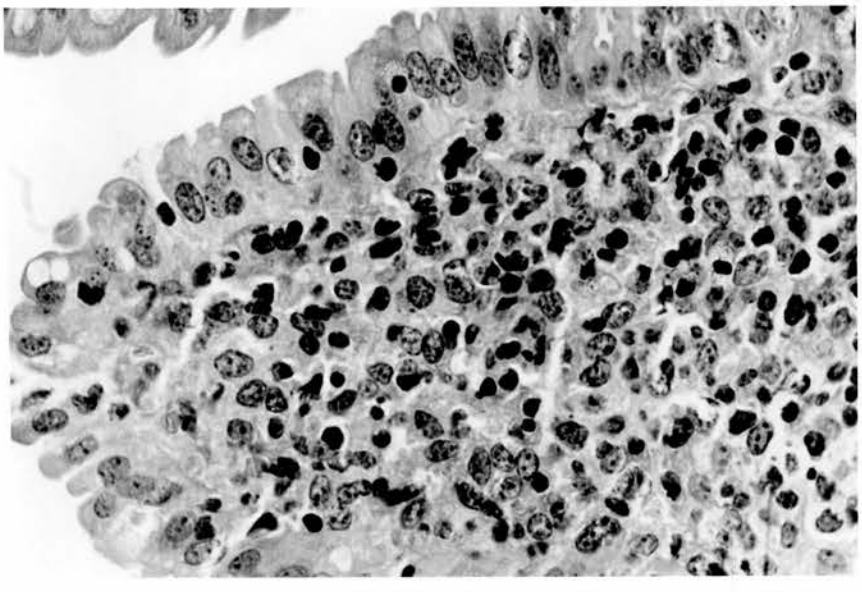

Plate 12.8. The dome epithelium of the Peyer's patch in a gnotobiotic lamb aged 8 days. Note the lymphocytes within the epithelial cell layer. H & E. X 500.

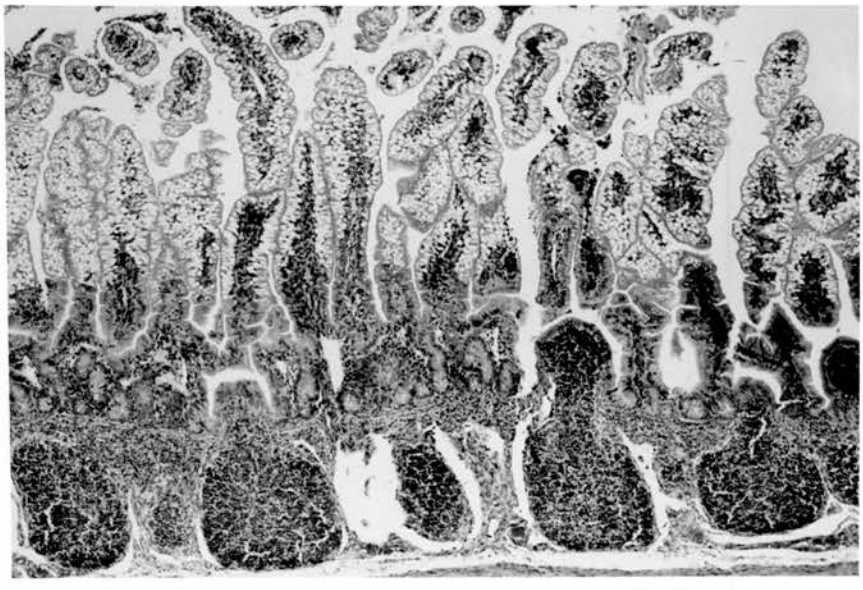

Plate 12.9. The small intestinal mucosa in the Peyer's patch region of a gnotobiotic lamb, 18 hours after infection with rotavirus on the second day of life. The ileal villi are heavily vacuolated. H & E. X 50.

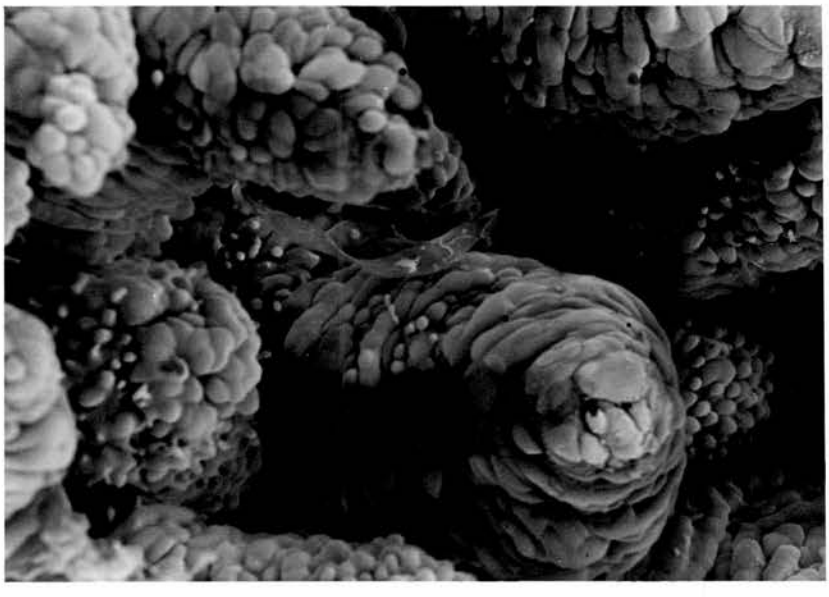

Plate 12.10. The luminal surface of the small intestine in the region of the Peyer's patch in a gnotobiotic lamb 24 hours after infection with rotavirus on the second day of life. Note the 'blebs' on the villus surfaces and the irregular appearance of the epithelial cells. SEM. X 550.

Plate 12.11. Prominent domes between the villi in the Peyer's patch region of a gnotobiotic lamb, 48 hours after infection with rotavirus on the second day of life. SEM. X 200.

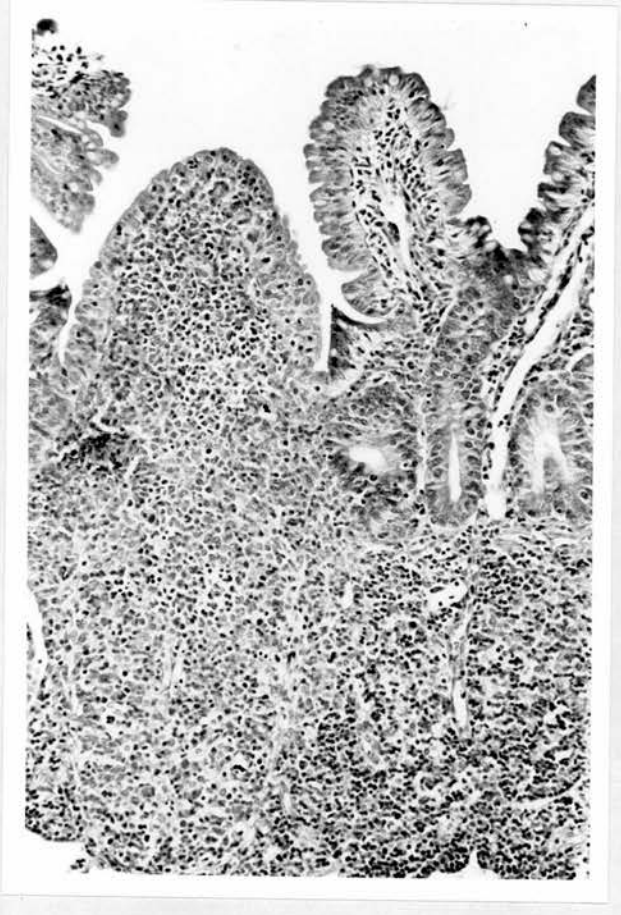

Plate 12.12. The prominent dome and the associated ileal villi in the Peyer's patch region of the small intestine of a gnotobiotic lamb, 48 hours after infection with rotavirus on the second day of life. H & E. X 125.

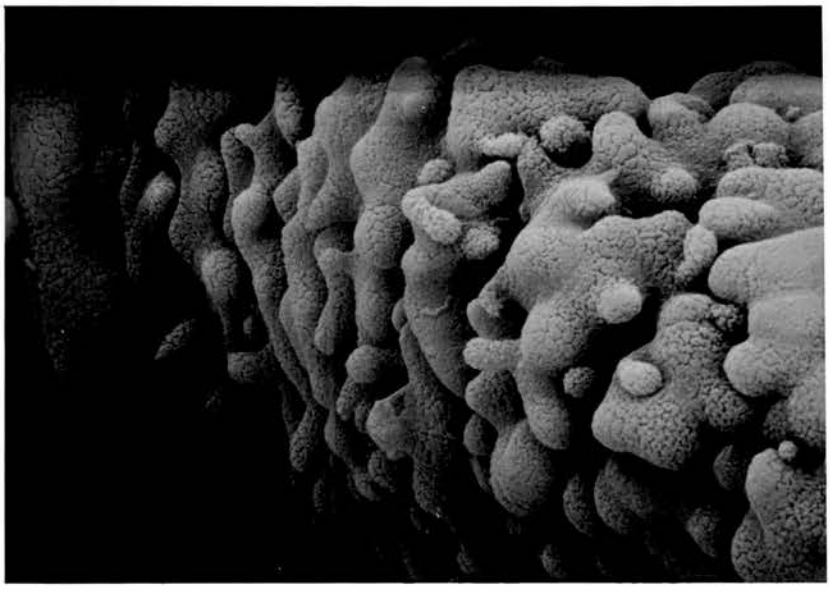

Plate 12.13. 'Blebs' on the surface of the epithelial cells of the ileal villi in the Peyer's patch region of the small intestine of a gnotobiotic lamb, 48 hours after infection with rotavirus on the second day of life. SEM. X 2000.

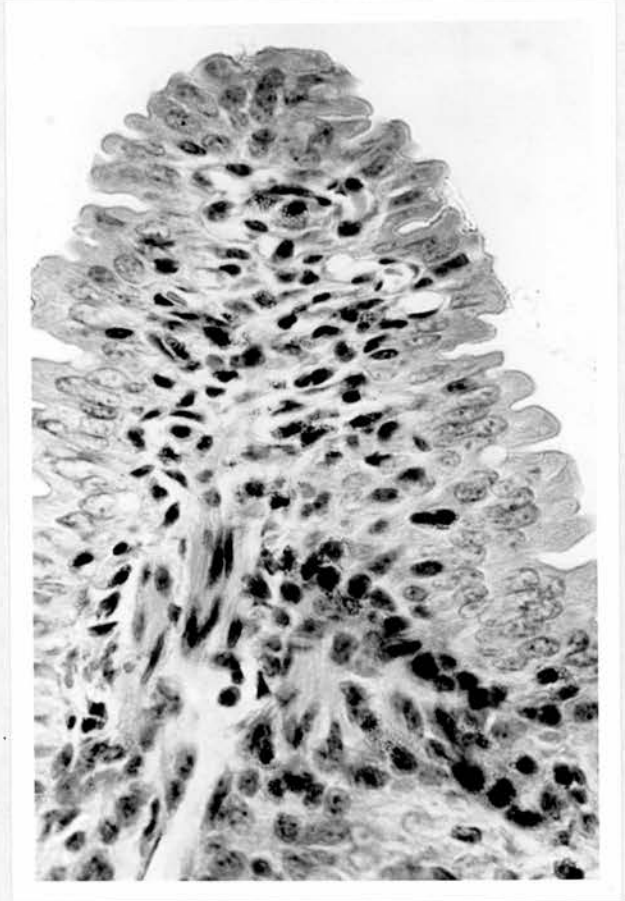

Plate 12.14. The epithelium of an ileal villus in the Peyer's patch region of the small intestine of a gnotobiotic lamb, 48 hours after infection. The irregular epithelial surface is due to the prescence of the 'blebs'. H & E. X 500.

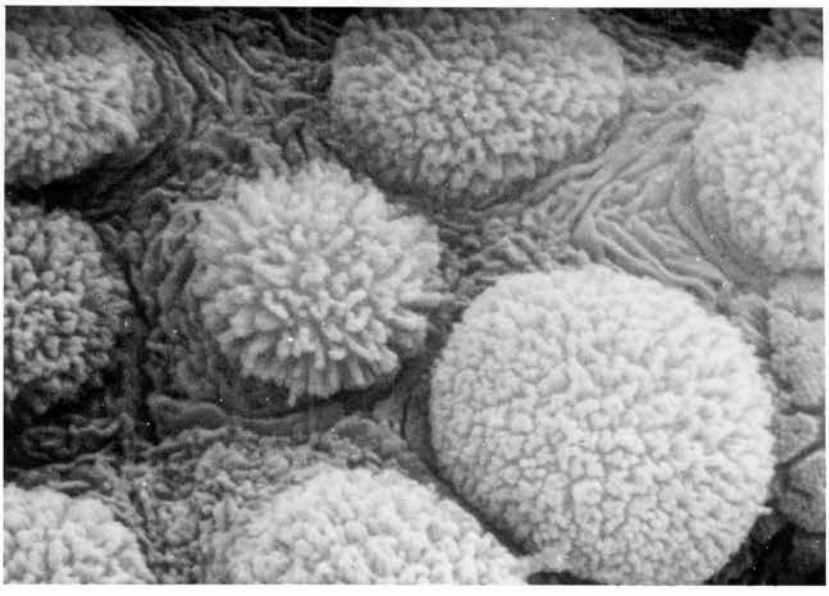

Plate 12.15. The dome epithelium of a gnotobiotic lamb, 48 hours after infection with rotavirus on the second day of life. Note the rounded epithelial cells and the ridges of the M cells. SEM. X 10,000.

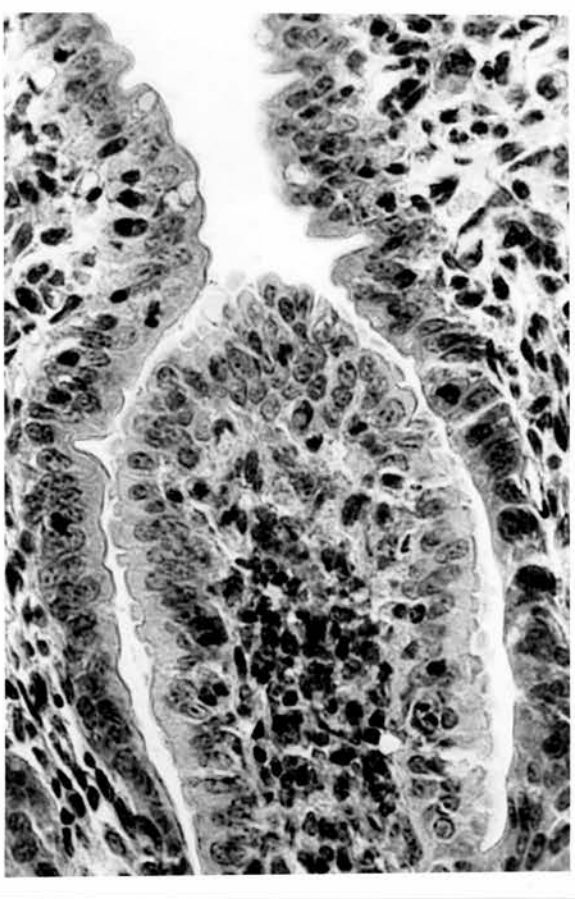

Plate 12.16. High magnification of the dome epithelium in the small intestine of a gnotobiotic lamb, 48 hours after infection with rotavirus on the second day of life. Lymphoid cells are present within the epithelial layer. H & E. X 500,

Plate 12.17. The dome epithelium of the small intestine of a gnotobiotic lamb, 48 hours after infection with rotavirus on the second day of life. Two epithelial cells with an M cell in the middle. Note the lymphoid cells within the boundary of the M cell. TEM. X 10,000.

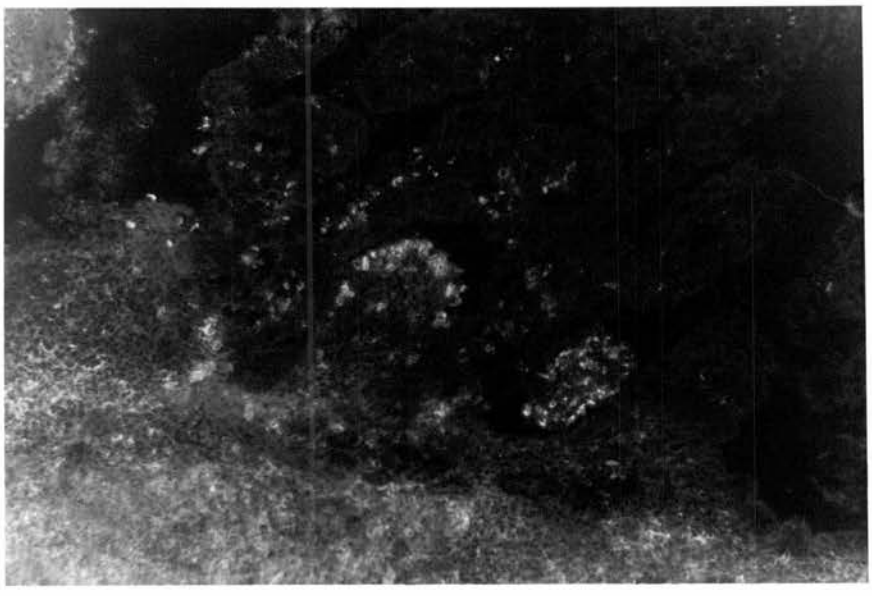

Plate 12.19. Immunofluorescence against rotavirus particles in the dome epithelium of the small intestine of a gnotobiotic lamb, 48 hours after infection with rotavirus on the second day of life. Immunofluorescence X 50.

Plate 12.20. The appearance of the domes and the Peyer's patch villi in the small intestine of a gnotobiotic lamb, 8 days after infection with rotavirus on the second day of life. SEM. X 200.

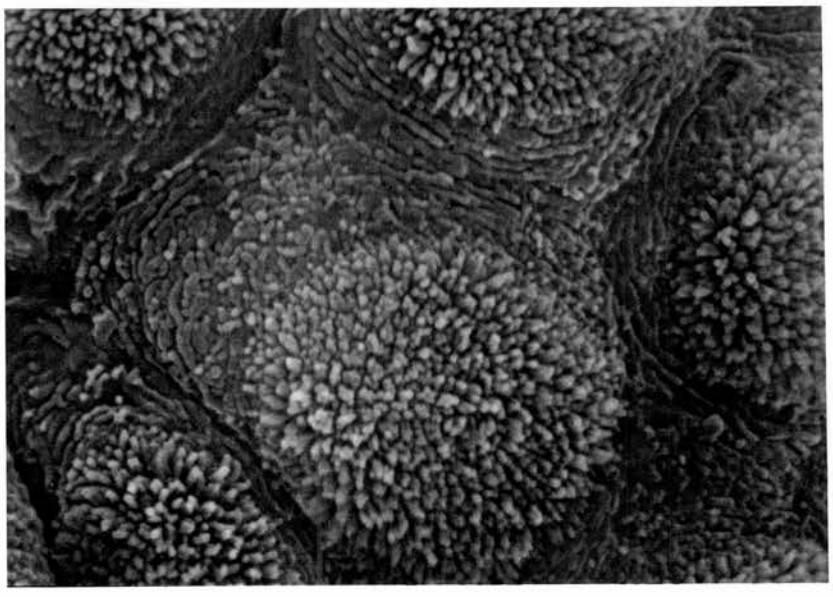

Plate 12.21. The epithelial cells and the M cells of the dome epithelium in the small intestine of a gnotobiotic lamb, 15 days after infection with rotavirus. The structure of the dome epithelium is similar to that found in control animals. SEM. X 10,000.

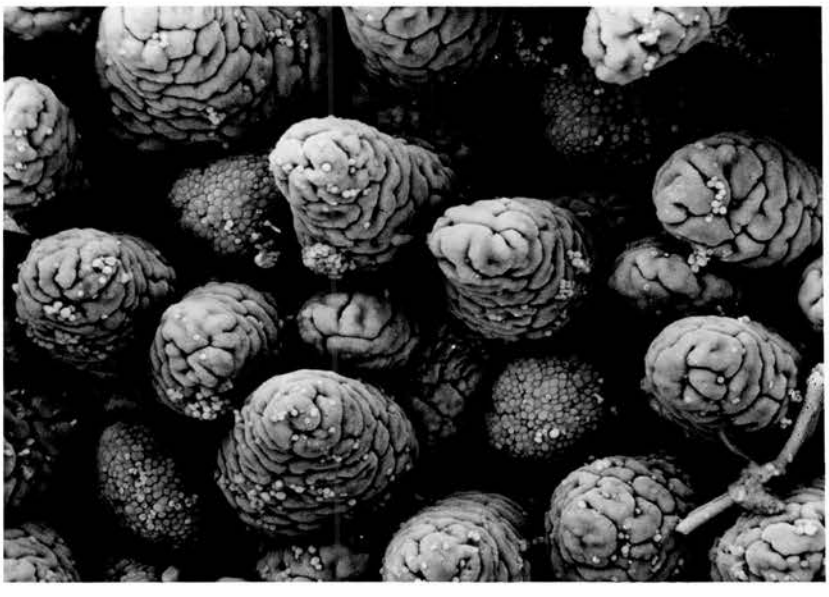

Plate 12.22. Prominent domes between very convoluted ileal villi in the small intestine of a gnotobiotic lamb, 15 hours after infection with rotavirus on the seventh day of life. SEM. X 200.

Plate 12.23. Villi and domes in the small intestine of gnotobiotic lambs, 24 hours after infection with rotavirus on the seventh day of life. Note fused villi and prominance of the domes. SEM. X 200.

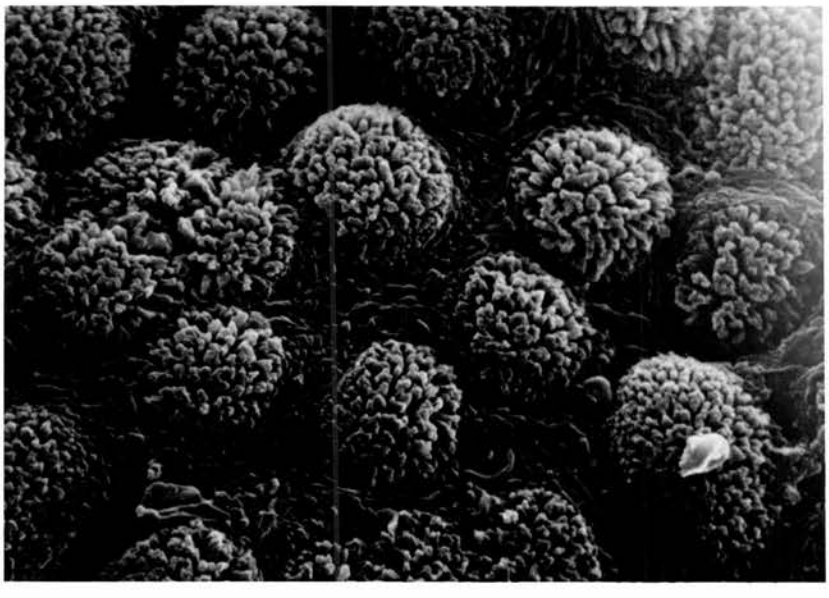

Plate 12.24. The epithelial cells and M cells in the dome epithelium of the small intestine of a gnotobiotic lamb, 24 hours after infection with rotavirus on the seventh day of life. The epithelial cells are rounded up and the M cells are increased in area. SEM. X 5,000.

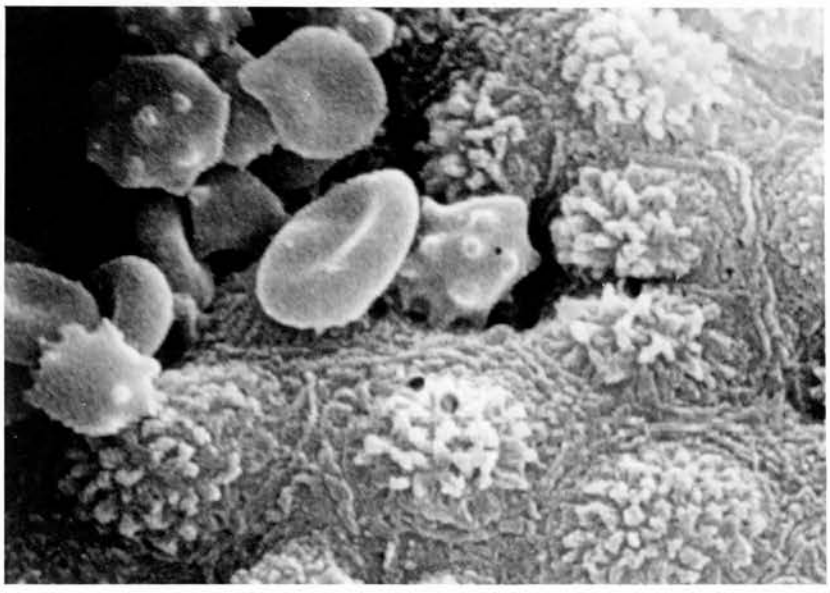

Plate 12.25. Epithelial cells of the dome epithelium widely separated by the M cells. The small intestine of a gnotobiotic lamb 7 days after infection on the seventh day of life. SEM. X 2,000.

Plate 12.26. The domes and ileal villi of the small intestinal mucosa of a gnotobiotic lamb, 12 days after infection with rotavirus on the seventh day of life. The tissue appears normal. SEM. X200.

Chapter 13.

General discussion.

From the work presented in this thesis it is possible to describe in detail the events occurring in the small intestinal epithelium of the lamb as a result of rotavirus infection. Virus was demonstrated within the epithelial cells of the small intestine within hours of infection and diarrhoea developed soon after this. Villus atrophy, of varying duration and severity, was observed throughout the small intestine in association with alterations in small intestinal epithelial cell kinetics. The susceptibility of the animals was found to be related to age and detailed studies of control animals highlighted possible reasons for this. Studies of the early phase of the infection provided insight into the mechanisms responsible for the diarrhoea and allowed correlations to be drawn concerning the onset of clinical illness, the distribution of virus and the development of the enteropathy. The time course experiments indicated that the cell kinetic response may involve both local feedback and adaptive mechanisms for the control of cell proliferation.

The small intestinal mucosa of the normal gnotobiotic lamb.

The investigations performed in Chapter 6, to define the normal structure of the small intestinal mucosa in gnotobiotic lambs, confirmed that many alterations relating to structure and function occurred within a few days of birth. These differences were extremely important in the establishment of base-line control values and must be considered when analysing the effects of rotavirus infection on the small intestinal epithelium of animals infected soon after birth. Villus and crypt shape and length, cell proliferation kinetics and brush border enzyme distribution were all

involved in the changes associated with the maturation of the small intestinal mucosa. The most important of these occurred approximately one week after birth, they included the establishment of a pronounced, proximal to distal gradient of villus length from the jejunum to the ileum, a decrease in the concentration of lactase in the epithelial cells in the ileal region, the disappearance of the specialised, vacuolated, absorptive, epithelial cells from the middle and posterior regions of the small intestine (gut closure) and a generalised increase in crypt cell production rates throughout the small intestine with increasing age. It is possible that the observed differences in susceptibility and the pattern of disease produced in various age groups of animals of the same species are related to these changes.

Validity of the animal model.

The use of a gnotobiotic host provided the opportunity to study the effects of rotavirus infection in a system with a controlled gut flora and free from maternal antibody and naturally occurring rotavirus infection. However such experimental conditions are somewhat different to those involved in naturally occurring field infections and their validity in relation to the real-life situation must be defended before being used as the basis for the experiments described in this thesis.

The clinical and immunological pattern of infection produced by the viral inoculum used in this animal model was consistent throughout the numerous experiments performed by Dr Snodgrass, relating to virological and immunological studies (Snodgrass, Herring and Gray, 1976; Snodgrass, Madeley, Wells and Angus, 1977; Snodgrass and Wells, 1976; Snodgrass and Wells, 1978).

The pattern of infection and the resultant lesions observed in my experiments were also consistent throughout the course of infection and generally agreed with reports of the disease in other species (Mebus, Stair, Underdahl and Twiehaus, 1971; McAdaragh, Bergeland, Meyer, Johnshoy, Stotz, Benfield and Hammer, 1980).

The effects of rotavirus infection in conventional animals were very similar to those observed in germ-free animals and colostrum deprived animals although the severity of the disease produced was variable. Studies included germfree and conventional piglets (Middleton, Petric and Szymanski, 1975), conventional pigs (Chasey and Lucas, 1977), conventional mice (Coelho, Bryden, Hall and Flewett,1981), and calves (Pearson, McNulty and Logan, 1978). However no studies have included both germfree and conventional animals of the same age and it is therefore impossible to make direct comparisons Although conventional animals were susceptible to infection, either natural or experimental, the effects of the disease were not generally as severe as that observed in gnotobiotic or colostrum - deprived animals and recovery was rapid. The cessation of the clinical signs related to the regeneration rate of the functional villus epithelium (Theil, Bohl, Cross, Kohler and Agnes, 1978) and the influence of the difference in the rates of crypt cell production between conventional and gnotobiotic individuals was thought to be the cause. The role of absorbed colostrum in the reduced susceptibility of conventional animals was proposed (Mebus, White, Bass and Twiehaus, 1973) and it has been shown that the presence of colostrum must be continuous within the gut lumen to afford protection (Snodgrass and Wells, 1978) and this may indicate that factors other than antibodies are involved in the protective effect of colostrum.

Although the general pattern of the disease was similar in conventional and germfree animals there are important differences between these two catagories of animals, quite apart from the possible transfer of immunity to rotavirus in colostrum or milk in conventional animals.

The whole structure of the small intestinal mucosa is different in gnotobiotic animals due to the 'arrested development' occurring as a result of the abnormal conditions the animals are raised under. The regeneration rate of the epithelium is less in gnotobiotic animals (Moon, Kohler and Whipp, 1973) giving rise to long villi which are clothed by relatively old cells. The lamina propria of the villi in such animals is sparse and there are few lymphoid cells present in the intestinal epithelium. The gut flora of gnotobiotic animals is also strictly controlled and this has both advantages and disadvantages. Conventionally reared animals have a varied gut flora present in the lumen some of which is harmful and some beneficial. It has been shown that concurrent infection with other potentially pathogenic agents can severely affect the pattern of disease produced. For example, calves infected with both E. coli and rotavirus are more severely affected than animals with only a single infection of either agent (Gouet, Contrepois, Dubourgier, Riou, Scherrer, Laporte, Vacherot, Cohen and L'Heridon, 1978; Mebus, Stair, Underdahl and Twiehaus, 1971). Some of the microbial flora naturally present in the gut lumen have a protective effect and combine with mucus to render the epithelium less susceptible to attack by bacteria, viruses and various parasitological organisms. It is thought that the absence of these commensal organisms and the under development of the small intestinal mucosa contribute to the increased susceptibility of gnotobiotic animals. However,

conventional animals are not kept in such rigorously controlled environments as gnotobiotic animals and exposure to cold temperatures and other forms of environmental stress may increase the severity of the infection.

These differences must be borne in mind before the results of experiments performed in gnotobiotic animal models can be extrapolated and applied to the disease which occurs naturally in conventional animals.

Techniques used to study the small intestinal epithelium.

Many studies of the effects of rotavirus infection on the small intestine have previously been performed in several animal species. However, none has combined detailed studies of viral distribution within the epithelium, the pathological lesions of the disease, the crypt cell kinetic responses of the small intestine and the clinical signs of disease. Most studies have concentrated on the former two aspects of the disease. The techniques used in this study are all complementary to one another and were selected to provide as complete a description as possible of the effects of the disease on the epithelium and the cell proliferation kinetics of the small intestine. The investigative techniques used to study mucosal architecture, viral distribution, brush border lactase patterns and cell renewal rates combine to illustrate the complete sequence of events occurring during the course of the infection.

Scanning electron microscopy proved to be a particularly valuable technique for the study of the villus epithelium and the contours of the mucosal surface, being especially helpful in detecting subtle abnormalities of the epithelial cells early in the course of the infection and not detected by light microscopy. This

method of study also illustrated the desquamation of the infected epithelial cells, demonstrating the presence of a well defined demarcation zone between the damaged epithelial cells and the newly formed cells which replaced them (Plate 9.23). One of the critisisms of the use of scanning electron microscopy to describe lesions of the small intestine relates to the difficulties of differentiating between artifacts and the real effects of the disease. Artifacts are usually caused by faulty fixation processes or autolysis and strict measures were observed to ensure that this did not occur. Samples were removed from the animals prior to death and fixed immediately. The variations encountered in the literature on the scanning electron microscopic appearance of rotavirus infected intestine were often associated with the failure to remove the samples before the death of the animal (McAdaragh, Bergeland, Meyer, Johnshoy, Stotz, Benfield and Hammer, 1980; Torres-Medina and Underdahl, 1980). Artifacts were not necessarily present but there was a risk of their occurrence in such situations. Scanning electron microscopy should ideally be complemented by transmission electron microscopic studies. However, a full ultrastructural investigation was performed in the previous lamb experiment (Snodgrass, Angus and Gray, 1977) to describe the effects of rotavirus infection on the villus epithelial cells. In the experiments contained in this thesis only limited TEM studies were performed to provide correlative information in the SEM investigation of the follicle-associated epithelium. TEM was not used routinely to monitor the distribution of the viral particles within the epithelium, immunofluorescence gave adequate evidence of viral presence and the observed distribution and persistence of infected cells correlated well with the other signs of rotavirus infection.

The majority of the samples were processed and examined without knowing the status of the donor. The exception was the retrospective histopathological studies performed after the results of the scanning electron microscopic studies were examined.

There are some minor gaps in the information obtained, particularly in the electron microscopy and immunofluorescence studies. Samples were removed for immunofluorescent staining from the animals involved in the experiment described in chapter 7 but were inadvertently discarded when a deep freeze was cleared out. In the case of the scanning electron microscopy, this technique was only used in the second part of the investigation after preliminary studies had shown the value of this method of study and access to a scanning electron microscope became available.

In the past when designing stathmokinetic experiments it was usual to have a group of animals for each sampling period after the administration of the metaphase arrest agent. However, for reasons already discussed, it was necessary to design an experimental procedure in which all the stathmokinetic measurements could be performed in a single animal.

As it is now known that there is individual variation in the response of animals to the same dose of rotavirus inoculum (Crouche and Woode, 1978), care must be exercised in the conclusions drawn about the effects of the disease when small numbers of animals are used. The reason for this individual variation in the response to a particular dose of virus may be related to the stage of differentiation of the enterocytes in the small intestine. The enterocytes usually regenerate by a continuous process but various stages of cellular development can be detected at different times and for this reason the number of susceptible cells at the time of

inoculation may vary between individual animals within a particular age group (Moon and Joel, 1975).

However the results clearly show the pattern of the progress of the various stages of the disease despite the small numbers of animals used. In addition the detailed studies carried out in chapter 8 at two important stages of the disease complemented the main experiment and provided extra material for examination as did the histological samples which were available from the previous lamb rotavirus experiment. Despite the criticisms of various aspects of the experimental design, no previous stathmokinetic studies have been performed in animals infected with rotavirus and the results presented in this thesis provide valuable insight into the effects of this disease on the small intestinal epithelium.

It would have been very interesting to have performed a parallel investigation of the enzyme levels and ion transport mechanisms of the enterocytes. This would have enabled a complete enzyme profile to be prepared for the small intestinal cells and may have provided information concerning alterations in the transport mechanisms of the small intestine. A similar study has recently been performed in pigs infected with TGE (Kerzner, Kelly, Gall, Butler and Hamilton, 1977).

When the project was begun lactase deficiency was believed to play a major role in the production of the rotavirus diarrhoea. For this reason and because a lactase assay was already in use in our laboratory, the lactase activity of the small intestinal mucosa in the experimental animals was measured.

Distribution of virus.

There is considerable disagreement in the literature concerning the location of virus infected enterocytes and the persistence of these infected cells in the intestine. Some authors (Coelho, Bryden, Hall and Flewett, 1981; McAdaragh, Bergeland, Meyer, Johnshoy, Stotz, Benfield and Hammer, 1980; Theil, Bohl, Cross, Kohler and Agnes, 1978) have found as I have, that viral fluorescence to rotavirus was present throughout the small intestine from a few hours after infection. Others, (Crouche and Woode, 1978; Pearson and McNulty, 1977; Snodgrass, Angus and Gray, 1977; Torres-Medina and Underdahl, 1980) did not detect evidence of rotavirus particles prior to 24 hours after infection and generally found virus only in the mid and distal regions of the small intestine. There was also a theory which proposed that the infection progressed like a wave from the proximal to the distal regions (Mebus, Wyatt and Kapikian, 1977).

In my studies of lamb rotavirus, the virus was initially detected within the epithelial cells of the apical two thirds of the villi, 6 hours after infection, at all three sites examined. In the jejunum the fluorescent staining was very patchy and continued to be present until 48 hours after infection. In the midgut and ileum, the fluorescence was confluent over the upper halves of the villi by 12 hours after infection and remained so until at least 48 hours after infection, with the greatest concentration of virus present in the midgut. Single cells continued to show signs of rotavirus fluorescence 8 days after infection in the mid region of the small intestine. In relation to the clinical signs of illness, the virus was present in the epithelial cells of the villi at least 6 hours before the onset of the diarrhoea, continued to be present in

substantial amounts when the diarrhoea was at a peak and traces of virus were still present in the epithelial cells of the villi after the animals had clinically recovered.

In animals which were older when infected virus was present only in the mid and distal regions of the small intestine and only until 4 days after infection.

The persistence of the virus within the epithelium will be influenced by the ability of the infected cells to support the process of viral replication. The virus was shed most rapidly from the epithelial cells of the jejunum and the distribution of the infected cells suggested that replication did not occur within the proximal enterocytes. Viral replication was also thought to be less in animals which were infected when older from the pattern of the distribution of infected cells and the density of the fluorescence in these animals.

There are many reasons for the inconsistencies which have been reported. The literature includes studies involving germfree animals, colostrum-deprived animals and conventional animals of various species and differing ages. The age of the animal at the time of infection appeared to affect the susceptibility of the individual to the infection and differed with the species involved, for example, one day old gnotobiotic lambs survived an infection whereas piglets of the same age did not (Crouche and Woode, 1978). The inoculum also varied in virulence and was from varied sources. There was no standarization of experimental design, most authors concentrated on the period after the onset of diarrhoea and possibly missed the time of the first appearance of viral immunofluorescence.

The pathological lesion in rotavirus infection.

There were also many inconsistencies in the descriptions of the enteropathy caused by rotavirus. Again the results in the literature were divided between total small intestinal involvement (Coelho, Bryden, Hall and Flewett, 1981; Mebus and Newman, 1977; Pearson and McNulty, 1977; Theil, Bohl, Cross, Kohler and Agnes, 1978; Torres-Medina and Underdahl, 1980) and restriction of the lesion to specific areas of the gut (McAdaragh, Bergeland, Meyer, Johnshoy, Stotz, Benfield and Hammer, 1980; Mebus, Stair, Underdahl and Twiehaus, 1971;) generally the mid and ileal regions.

Having carried out a detailed study of rotavirus infection, from a few hours after infection until more than a week after clinical recovery, using a wide variety of techniques to investigate the effects of the disease on the small intestinal epithelium, it is possible to explain some of the inconsistencies which appear in the literature

The first signs of pathological damage in lambs occurred 4 hours after infection at all sites, the villi were distended at the tips and villus atrophy was present in the jejunum for several hours. Villus shortening occurred later in the lower regions of the small intestine being present 12 hours after infection in the ileum and 18 hours after infection in the mid region and was accompanied by the rapid exfoliation of infected epithelial cells. The mid region of the small bowel had returned to normal by 4 days after infection but the ileum remained abnormal for a longer period.

Severe damage to the epithelial cells was apparent in the mid and posterior regions of the small intestine and vacuoles containing granular material were seen in the epithelial cells in positions

corresponding to those where virus was known to be present by immunofluorescence. These vacuoles were also reported in mice (Coelho, Bryden, Hall and Flewett, 1981) and in pigs (Pearson and McNulty, 1977) but at a later stage of the infection. The distribution of the enteropathy correlated well with the distribution of virus as observed by immunofluorescence.

The description of the enteropathy in rotavirus infections in the literature followed a similar pattern to that described for the distribution of the virus. In the cases where the clinical effects were mild (Middleton, Petric and Szymanski, 1975) and no ileal involvement was observed (Davidson, Gall, Petric, Butler and Hamilton, 1977) the animals were either sampled after the onset of diarrhoea or were older when infected. This was similiar to the results obtained in my animals which were infected on the seventh day of life, with little clinical evidence of disease and mild lesions only in the middle regions of the small intestine. The majority of the literature, mainly involving very young, germfree or colostrum - deprived animals of various species, indicated that the most severe lesions and greatest concentrations of virus occurred in the middle and distal regions of the small bowel. In the 'wave' theory of infection, (Mebus, Wyatt and Kapikian, 1977), the entire small intestine was found to be affected at different times after infection. The proximal small intestine was affected first and the infection progressed rapidly in a distal direction. Restoration of normal epithelium followed the same pattern although the time scale varied. The sequence of events described in this thesis as a consequence of rotavirus infection tended to support the wave theory. Lesions were first observed in the proximal small intestine and occurred several hours later in the more distal regions,

recovery of the proximal small intestine occurring rapidly with the lesion having a longer duration in the middle and ileal regions. However, on careful examination of the literature in cases where no proximal involvement was reported, mild lesions were in fact present in the proximal sites but they were discounted (McAdaragh, Bergeland, Meyer, Johnshoy, Stotz, Benfield and Hammer, 1980) or the animals were not sampled in the early stages of infection before the onset of diarrhoea (Mebus, Stair, Underdahl and Twiehaus, 1971)

It is not yet known whether the distal regions of the small intestine are more susceptible to rotavirus infection or if there are differences in the abilities of the epithelial cells of the proximal region and the distal regions to support viral replication. The reduced susceptibility of older animals may be of importance in eliciting the reason when the differences which develop between the ileum and jejunum, with age, are considered.

Many factors are likely to be implicated in the variable susceptibility to infection of the epithelial cells of the different regions of the small intestine. These include the luminal environment, the state of differentiation of the epithelial cells, the functional capacities of the epithelial cells, the presence of viral receptors, the ability of the epithelial cells to support viral replication, the regenerative capacity of the epithelium and the presence of antiviral factors within the mucosa. A number of these factors are altered according to the age of the animal, including the state of differentiation of the epithelial cells, the rate of cell renewal and the ability of the animal to mount immune and anti-viral responses.

The brush border enzyme, lactase has been proposed as a receptor for rotavirus. Studies of rotavirus infection in children

were confined to the proximal small intestine and there was evidence of pathological lesions in this region. Because of the high concentrations of lactase present in these cells it was proposed that lactase was a receptor for rotavirus (Holmes, Rodger, Schnagl, Ruck, Gust, Bishop and Barnes, 1976) and facilitated the entry of the virus into the epithelial cells. It is possible that the enzyme profile of the epithelial cells is involved in their susceptibility, evidence for this comes from the knowledge that rotavirus will infect kidney cells in cultures but these cells do not readily permit further multiplication. The microvilli of kidney cells contain similar enzymes to those of the gut and it is possible that one or more of these enzymes is a receptor for rotavirus. Later, more detailed studies in animals illustrated that the midgut and ileum were also severely affected during rotavirus infection. Lactase is probably not the prime factor influencing the susceptibility of the animal but it may play a role in the reduced susceptibility observed in animals infected at an older age. Lactase levels are generally lower in the ileum of older animals and this area showed a reduced level of infection (see chapter 10). As yet, no studies have been performed in animals which have been rendered lactase deficient so it is not possible to define the role of lactase as a viral receptor.

The luminal environment of the epithelial cells has been shown to influence the infectivity of rotavirus. Pre-treatment with trypsin and pancreatic enzymes was necessary for the infectivity of virus cultured in vitro (Theil, Bohl and Agnes, 1977). Various inhibitory agents have been found to be present in the lumen, these include interferon (Welch and Twiehaus,1973) and an unnamed inhibitory factor found in the small bowel of mice (Piazza, 1967).

In addition to the various inhibitory factors which may be present in the lumen and play a role in influencing the infectivity of the virus, there is also the potential for an IgA response to be produced by the many plasma cells in the small intestine.

Functionally the jejunum and the ileum are very different. The jejunum is involved in the digestion and absorption of nutrients whereas the ileum is concerned with fluid absorption and ion transport. The structure and brush border enzyme profiles of the fully differentiated epithelial cells in the two areas of the small intestine reflect these differences. These enzyme profiles do not become established until several days after birth, in the case of lambs this period is approximately seven days (refer to Chapter 6). This may be related to the phenomenon known as gut closure, when the specialised, vacuolated, epithelial cells concerned with the non-selective uptake of macromolecules (Lecce, Matrone and Morgan, 1961), are replaced by normal epithelial cells.

The presence, in the midgut and ileum, of these specialised vacuolated, absorptive cells which absorb macromolecules non-selectively in the first few days after birth may also influence the susceptibility of the animals to viral infection and the resultant distribution of the viral particles within the epithelium. Certainly, in animals infected close to birth, the distribution of virus infected cells and subsequent viral replication correlated positively with the distibution of the vacuolated, absorptive cells. In contrast, the level of infection in animals infected on the seventh day of life and lacking these specialised absorptive cells was much reduced.

The virus is known to infect only mature, fully differentiated cells on the villi and there is a direct relationship between virus

production and cell age (Moon, Kemeny, Lambert, Stark and Booth, 1975), replication being greater in older cells than in new ones. The inability of new cells to support viral replication is believed to be involved in limiting the disease as the infected cells are replaced by new cells from the crypts. The reduced susceptibility of older pigs to the effects of TGE has been related to the rate of regeneration of the villus epithelium, newborn pigs requiring 7-10 days for the replacement of the villus epithelium as opposed to 2-4 days in three week old animals (Moon, 1971), suggesting that the cells are replaced before they become mature enough to support viral replication.

Immune processes may also play a part in limiting the infection, active immunity being present within four days in pigs (Porter, 1973) and it is not clear if this provides additional protection for the newly differentiated enterocytes or whether their inability to support viral replication is sufficient to prevent reinfection.

The age of the animal also influences the rate of recovery from the infection. It has been demonstrated that older gnotobiotic animals (Chapter 10,) and conventionally reared animals recover rapidly from rotavirus infection. This has been linked to the increased rate of cell production of the crypt cells in both groups, (Moon, Kemney, Lambert, Stark and Booth, 1975) the theory being that the infected cells are shed rapidly from the villi and that restoration of the villus epithelium is rapidly accomplished.

The ability of the epithelial cells to support viral replication also influences the course of the disease. It is possible that the transient presence of viral particles within the epithelial cells of the jejunum and the lack of development of

pathological lesions at this site was due to non-replication of the virus in these cells. It is known that it is the viral replication process which causes destruction of the epithelial cells. It also appears likely that replication of virus is less in older animals, this has been shown to be the case in pigs infected with TGE (Moon, Kemney, Lambert, Stark and Booth, 1975) but this has not been proved in rotavirus infections.

There are also differences in the rate at which the different regions of the small intestine recover from the effects of the infection. In severe infections where virus and lesions have been demonstrated at all sites, the proximal region recovered rapidly whereas the ileal region remained abnormal for a substantially longer period. Again these differences may be associated with the replication rates of the virus within the epithelial cells.

Pathogenesis of the villus and crypt changes and alterations in crypt cell production rates.

The effects of rotavirus infection on the small intestine of the lamb can be divided into four stages although each stage will not necessarily apply to all sites at the same time.

(1) Villus atrophy is present without a change in crypt cell production rate and diarrhoea is usually present.

(2) Villus atrophy and crypt hyperplasia are present, there is an associated increase in crypt cell proliferation and the diarrhoea continues.

(3) The villi are normal, crypt cell production rates are increased and the diarrhoea has ceased.

(4) The villus structure is normal and the crypt cell production rates are normal.

The villus atrophy present at stage 1 is probably caused by viral infection and subsequent replication in the villus epithelial cells which leads to the exfoliation of large numbers of cells. The immature epithelial cells present on the lower villus surface migrate upwards and stretch to provide an intact covering for the villus, leading to the formation of a cuboidal epithelium and a continuing insufficiency of cells will lead to villus atrophy (Creamer, 1964). The onset of diarrhoea has been linked to the epithelial cell damage occurring as a result of viral invasion and replication (Theil, Bohl, Cross, Kohler and Agnes, 1978).

At the onset of stage 2, the reduction in the number of cells in the villus epithelium provides a local stimulus for increased proliferation within the crypt cells. At this time virus was still present in the villus epithelial cells and the diarrhoea continued.

By stage 3, the villus length is restored to normal and the cessation of the diarrhoea has been linked to the regeneration of the villi (Theil, Bohl, Cross, Kohler and Agnes, 1978). However, the crypt cell production rate remained raised for several days after the villi had returned to normal and this was suggestive of an adaptive control mechanism of crypt cell production rather than a local feedback mechanism linked to the state of the villus cell population (Rijke and Dongen, 1980). The increased crypt cell proliferation rate did not lead to lactase deficiency but it is very likely that the epithelial cell transit time on the villus was reduced, producing enterocytes with a reduced lifespan which were resistant to reinfection by the virus (Moon, Kemney, Lambert, Stark and Booth, 1975).

At stage 4 recovery is complete with all aspects of the epithelium having returned to normal.

As previously noted this pattern does not appear concurrently throughout the entire length of the small intestine. For example, in the lambs infected soon after birth the proximal small intestine had recovered totally by 4 days after infection whereas the mid and distal regions did not return to normal until at least 11 days after infection. The same general pattern of events occurs but there are differences related to the age of the animal at infection and the site of the gut studied. The variation is probably related in part to the differences observed between the various sites of the gut in uninfected animals and the changes which take place as a consequence of the maturation process of the small intestinal epithelium.

In the absence of detailed studies of the cell kinetic response during rotavirus infection, it was assumed that the loss of a large proportion of the villus cell population due to viral destruction, was the cause of the villus atrophy and that a local feedback response by the crypts to the depleted villus cell population rapidly repaired the damage. However, the persistence of a strong proliferative response in the crypts in the midgut and to a lesser extent in the ileum, long after the villus structure was repaired, showed that the stimulus for cell proliferation involved an adaptive response in addition to the local feedback stimulus provided by the loss of the villus cells. The sustained cell kinetic response occurred within 2 days of infection and persisted until at least 11 days after infection and this was in accordance with previous descriptions of adaptive responses (Hanson, Osborne and Sharpe, 1977; Rijke, Hanson and Plaisier, 1977). Further evidence for the operation of an adaptive response was provided by the increased cell proliferation which occurred, in the absence of a reduction in villus cell population, in the midgut and ileum of lambs infected

when seven days old. One must be cautious in stating the reasons for the lack of an adaptive response in the jejunum but this may be associated with the differences in function and distribution of factors such as gut hormones, for example, enteroglucagon, between the various areas of the gut.

The factors which can give rise to an adaptive response were discussed in detail in Chapter 2. They include mucosal injury, changes in gut hormones, the production of humoral factors and cell mediated immune responses produced by T cells.

There was the possibility that the severe mucosal injury and the resultant products of cell degradation from the viral damage may have provided a proliferative stimulus but such products were not found to have any influence on cell proliferation (Tutton, 1973).

No investigations have been performed to monitor CMI reactions to rotavirus infections. Studies of this nature in pigs infected with TGE (Brundage, Darbyshire and Wilkie, 1980) demonstrated only a weak and localised CMI response although migration inhibition factor (MIF) and cytotoxicity have been demonstrated in the lamina propria lymphocytes of pigs infected with TGE (Frederick and Bohl, 1976). The limited structural study of the response of the follicle-associated epithelium of the Peyer's patch in lamb rotavirus infections demonstrated that viral particles did have access to the M-cells of the follicle epithelium. This method of entry to the lymphoid system of the animal could have resulted in the production of of a CMI response. It is known from studies of graft-versus-host disease (Ferguson and Mowat, 1980) that the CMI response produced in this situation has an effect on crypt cell kinetics, leading to a sustained increase in crypt cell production rates. This response was characterised by crypt hyperplasia and an

increase in the numbers of intra-epithelial lymphocytes in the affected segment of small intestine (Mowat and Ferguson, 1982). Similar features were observed in the lambs infected with rotavirus.

Further studies of the role of the immune response in rotavirus infection and its possible involvement in the development of the post gastroenteritis syndrome (Walker-Smith, 1979), frequently observed in children after rotavirus infection are indicated. This syndrome is characterised by chronic or intermittent diarrhoea and failure to thrive. There may be evidence of milk intolerance and biopsy may demonstrate partial villus atrophy but there is often little histological damage. In addition to investigations of the immune response in rotavirus infection, studies of the response of the Peyer's patch epithelium during infection may be rewarding, in particular the role of the M cells in the uptake of viral particles and presentation of them to the immune system.

Recently, the gut hormone enteroglucagon has been extensively studied in relation to the epithelial cell kinetics of the intestine. Enteroglucagon is a peptide released from endocrine cells in the distal small intestine and the large bowel and there is considerable evidence that it has a major role as a growth hormone in the small intestine (Jacobs, Polak, Bloom and Dowling, 1975). The levels of enteroglucagon in the ileum were increased by a factor of three in acute infective diarrhoea (Besterman, Welsby, Christofides, Sarson and Bloom, 1979) and the levels of enteroglucagon were also elevated in a variety of other G.I conditions where an adaptive cell kinetic response had been demonstrated (Besterman, Bloom, Sarson, Blackburn, Johnston, Patel, Stewart, Modigliani, Geurin and Mallinson, 1978; Besterman, Cook, Sarson, Christofides, Bryant, Gregor and Bloom, 1979; Adrian, McKieran, Johnston, Hiller, Vyas,

Sarson and Bloom, 1980).

The results of my investigations concerning the crypt cell proliferative response to rotavirus infection suggest that in addition to the feedback mechanism operating as a direct result of damage to the villus cell population, there is also an adaptive mechanism operating in the middle and distal areas of the small intestine. The factors causing the adaptive response have not been discovered but further investigation of the CMI responses within the small intestine to rotavirus infection and studies of gut hormones may be helpful.

Validity of the hypothesis.

The original hypothesis stated that an increase in crypt cell production leads to the presence of immature, enzyme deficient, epithelial cells on the villi which in turn causes the rotavirus diarrhoea.

On consultation of the literature regarding the relationship between immature enzyme deficient epithelial cells and the production of diarrhoea, there are at least two mechanisms which have been proposed. One relates to alterations in sodium transport mechanisms in the enterocytes leading to increased secretion or a failure of absorption, the other to lactose intolerance (Shepherd, Gall, Butler and Hamilton, 1979).

The results presented in this thesis confirm by direct measurement that there is an increase in crypt cell proliferation but disprove the hypothesis that it is this cell kinetic abnormality which is the direct cause of the diarrhoea. The onset of the diarrhoea occurred within 24 hours of infection. This was before the crypt cell proliferative response was detected and the initial

diarrhoea was almost certainly caused by direct viral damage to the enterocytes. As the infected cells were shed, lactase levels decreased and remained low for only a limited period after the crypt cell response was initiated. Lactase tolerance tests did not provide any evidence that the lowered lactase levels present in the small intestine contributed to lactose intolerance even at the height of the diarrhoea (Ferguson, Paul and Snodgrass, 1981). From the detailed studies of the course of infection in the lamb it was evident that the damage sustained by the proximal small bowel, the major site of lactose digestion, was transient and less severe than that suffered by the distal regions. It seemed likely that sufficient absorptive capacity was retained by the epithelium to prevent lactose intolerance from occurring. It was therefore most unlikely that the abnormalities of the cell proliferation kinetics directly produced the diarrhoeal illness. In addition the peak of the cell kinetic response did not occur until 8 days after infection, by which time the diarrhoea had ceased and the villus structure had returned to normal.

The hypothesis arose from experimental studies of TGE in pigs. At the time it was formulated, rotavirus and TGE were believed to be similar diseases but it is now known that important differences exist.

TGE has a maximal effect in the proximal small intestine whereas rotavirus tends to affect the mid and ileal regions. When the diarrhoea is maximal in TGE, mitotic activity in the crypts is also increased and levels of the brush border enzymes are similar to those in immature crypt cells, suggesting increased levels of cellular migration from the crypts. This is not the case in rotavirus infections. The virus is shed from the epithelium very

rapidly in TGE infections and the deficiencies in the disaccharidases occur after the virus has been cleared, again this is not the case in rotavirus infection.

It is now known that ion transport abnormalities in the ileum, involving the glucose stimulated sodium transport mechanisms, are a major cause of the diarrhoea which occurs as a result of TGE infection (Gall, Chapman, Kelly and Hamilton, 1977; Shepherd, Gall, Butler and Hamilton, 1979). The glucose stimulated sodium transport mechanism deteriorated in parallel with the disaccharidase activity of the small intestinal epithelial cells of the villi. It is not known whether or not a similar mechanism operates in rotavirus infection. It would possibly be rewarding to perform measurements of brush border enzymes and ion transport in conjunction with cell kinetic studies in rotavirus infections. Detailed studies of brush border enzymes and ion transport mechanisms have been performed in pigs infected with TGE (Kerzner, Kelly, Gall, Butler and Hamilton, 1977; Shepherd, Gall, Butler and Hamilton, 1979) but detailed analysis of the proliferative response in the crypt was not performed.

The results of the experiments on lamb rotavirus infection have proved that the assumption that there was increased mitotic activity in the crypts during the course of the infection was correct and that this increase was associated with an increased crypt cell production rate. However, the observed decrease in the lactase levels of the enterocytes did not correlate with this increased crypt cell production. The decreased levels of lactase were more likely to be caused by the rapid exfoliation of the intestinal epithelium as a result of viral damage. The persistence of the raised crypt cell proliferative response long beyond the time of

clinical recovery has shown that this is not a major cause of diarrhoea in infected animals and it may not be involved at all.

The consequences of the sustained cell kinetic response in the small intestine have not been investigated and it remains to be shown whether this state of accelerated cell proliferation has any adverse effects; if for example, mucosal permeability to macromolecules is less efficient than normal, causing malabsorption, or whether the susceptibility to further infection is increased or alternatively; increased resistance of the small intestinal cells develops

The lesions observed in the small intestine of the lamb by microdissection, histology and scanning electron microscopy correlated positively with the distribution of virus and epithelial abnormalities were present prior to the onset of diarrhoea. The relatively mild and transient nature of the lesion in the jejunum was inportant. Studies of the lesions of rotavirus in children are generally confined to duodenal and jejunal biopsies and it is easy to see how evidence of infection could be missed in such conditions.

The results of the experiments presented in this thesis highlight the necessity for detailed time course experiments from the time of infection until a considerable period after clinical recovery, involving many different methods of study, in order to prepare an accurate description of the sequence of events which occurs as a consequence of rotavirus infection. It is also clear

that in any study of gastrointestinal infections it is important to consider the total structure and function of the target organ rather than to concentrate on separate aspects of the disease in isolation.

Abrams GD, H Bauer, H Sprinz. (1963).
Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. Laboratory Investigation. 12:355.

Adrian TE, J McKieran, DI Johnstone, EJ Hiller, H Vyas, DL Sarson, SR Bloom. (1980).
Hormonal abnormalities of the pancreas and gut in cystic fibrosis. Gastroenterology. 79:460.

Al-Dewachi HS, DR Appleton, AJ Watson and NA Wright. (1979).
Variation in the cell cycle time in the crypts of Lieberkuhn of the mouse. Virchows Archives. B. Cell Pathology. 31:37.

Al-Dewachi HS, NA Wright, DR Appleton, AJ Watson. (1975)
The effect of starvation and refeeding on cell population kinetics in the rat small bowel mucosa. Journal of Anatomy. 119:105.

Al-Dewachi HS, NA Wright, DR Appleton, AJ Watson. (1977).
The effects of a single injection of hydoxyurea on cell population kinetics in the small bowel mucosa of the rat. Cell and Tissue Kinetics. 10:203.

Al-Dewachi HS, NA Wright, DR Appleton, AJ Watson. (1981).
The effects of a single injection of cytosine arabinoside on cell population kinetics in the mouse small intestine. Virchows Archives. (Cell Pathology) 34:299.

Al-Mukhtar MYT, JM Polak, SR Bloom, NA Wright. (1982).
The search for appropriate measurements of proliferative and morphological status in studies on intestinal adaptation. in Robinson JWL, RH Dowling, EO Riecken eds. Mechanisms of Intestinal Adaptation. Falk Symposium, 30. MTP Press Ltd.

Bergeland ME, JP McAdaragh, DE Reed, I Stotz. (1976).

A diarrhoea syndrome in young turkey poults. 129-130. Proceedings of the 27th Annual North Central Poultry Disease Conferences. p68.

Besterman HS, SR Bloom, DL Sarson, AM Blackburn, DI Johnston, HR Patel, JS Stewart, R Modigiliani, S Gevrin, CN Mallison. (1978)

Gut hormone profile in coeliac disease. Lancet. 1:785.

Besterman HS, GC Cook, DL Sarson, ND Cristofides, MG Bryant, M Gregor, SR Bloom. (1979).

Gut hormones in tropical malabsorption. British Medical Journal. 1:1252.

Besterman HS, PD Welsby, ND Christofides, DL Sarson, SR Bloom. (1979).

Gut hormones in acute diarrhoea. Gut. 20:A455.

Bishop RF, GP Davidson, IH Holmes. BJ Ruck. (1973).

Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. Lancet. 2:1281.

Bizzozero G. (1888).

Uber die Regeneration der Elemente der schlauformingen Drusen und des Epithels des Magendarmkanals. Anat Anz 3:781.

Bjerknes M, H Cheng. (1981).

The stem cell zone of the small intestinal epithelium. 3. Evidence from columnar, enteroendocrine and mucous cells in the adult mouse. American Journal of Anatomy, 160:77.

Both NJ, HM Plaisier. (1974).

The influence of changing cell kinetics on functional differentiation in the small intestine of the rat. A study of enzymes involved in carbohydrate metabolism. Journal of Histochemistry and Cytochemistry. 22:352.

Brundage LJ, JB Derbyshire, BN Wilkie. (1980).

Cell-mediated responses in a porcine enterovirus infection in piglets. Canadian Journal of Comparative Medicine. 44:61.

Bryden AS, ME Thouless, TH Flewett. (1977)

A rabbit rotavirus. Veterinary Record. 99:323.

Butler DG, DG Gall, MH Kelly, JR Hamilton. (1974).

Transmissible gastroenteritis. Mechanisms responsible for diarrhoea in an acute viral enteritis in piglets. Journal of Clinical Investigation. 53:1335.

Cairnie AB,(1967).

Cell proliferation studies in the intestinal epithelium of the rat; response to continuous irradiation. Radiation Research. 32:240.

Cairnie AB.(1976).

Homeostasis in the small intestine. in Cairnie AB, PK Lala, DG Osmond. eds. Stem cells of renewing cell populations. New York and London. Academic Press. p67.

Cairnie AB, LF Lamerton, GG Steele. (1965).

Cell proliferation studies in the intestinal epithelium of the rat. 1. Determination of the kinetic parameters. Experimental cell research. 39:528.

Carlson JAK, PJ Middleton, MT Szymanski, J Huber,
M Petric. (1978)
Fatal rotavirus gastroenteritis: An analysis of 21 cases.
American Journal of Diseases of Childhood. 132:477

Chasey D, M Lucas. (1977).
Detection of rotavirus in experimentally infected piglets.
Research in Veterinary Science. 22:124.

Cheever FS, JH Mueller. (1947).
Epidemic diarrhoeal disease of suckling mice. 1. Manifestation,
epidemiology and attempts to transmit the disease. Journal of
Experimental Medicine. 85:405.

Cheng H. (1974a).
Origin, differentiation and renewal of the four main epithelial
cell types in the mouse small intestine. 2. Mucous cells.
American Journal of Anatomy. 141:481.

Cheng H. (1974b).
Origin differentiation and renewal of the four main epithelial
cell types in the mouse small intestine. 4. Paneth cells.
American Journal of Anatomy. 141:521.

Cheng H. CP Leblond. (1974,a).
Origin, differentiation and renewal of the four main epithelial
cell types in the mouse small intestine. 1. Columnar cells.
American Journal of Anatomy. 141:481.

Cheng H, CP Leblond. (1974b).
Origin, differentiation and renewal of the four main epithelial
cell types in the mouse small intestine. 3. Enteroendocrine
cells. American Journal of Anatomy. 141:503.

Cheng H, CP Leblond. (1974,c).

Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. 5.Unitarian theory of the origin of the four epithelial cell types. American Journal of Anatomy, 141:537.

Chin KN, G Hudson. (1971).

Ultrastructure of Peyer's Patches in the normal mouse. Acta Anatomy. 78:306.

Chu RM, RD Glock, RF Ross. (1979).

Gut-associated lymphoid tissues of young swine with emphasis on dome epithelium of aggregated lymph nodules (Peyer's patches) of the small intestine. American Journal of Veterinary Research. 40:1720.

Chu RM, RD Glock, RF Ross, DF Cox. (1979).

Lymphoid tissues of the small intestine of swine from birth to one month of age. American Journal of Veterinary Research. 40:1713.

Clarke RM. (1970a).

Mucosal architecture and epithelial cell production rate in the small intestine of the albino rat. Journal of Anatomy. 107:519.

Clarke RM. (1970b).

A new method of measuring the rate of shedding of epithelial cells from the intestinal villus of the rat. Gut.11:1015:

Clarke RM. (1971).

A comparison of metaphase arresting agents and tritiated thymidine in measurement of the rate of entry into mitosis in the crypts of Lieberkuhn of the rat. Cell and Tissue Kinetics. 4:263.

Clarke RM. (1972).

The effect of growth and of fasting on the number of villi and crypts in the small intestine of the albino rat. Journal of Anatomy. 112:27.

Clarke RM. (1973).

Progress in measuring epithelial cell turnover in the villus of the small intestine. Digestion. 8:161.

Clarke RM. (1974a).

Morphological description of intestinal adaptation: measurements and their meaning. in Dowlig RH, EO Riecken,eds. Intestinal adaptation. FK Schattauer Verlag. Stuttgart-New York.

Clarke RM. (1974b).

Control of intestinal epithelial replacement: Lack of evidence for a tissue-specific blood-borne factor. Cell and Tissue Kinetics. 7:241.

Clarke RM. (1975).

Diet, mucosal architecture and epithelial cell production in the small intestine of specified-pathogen free and conventional rats. Laboratory Animals. 9:201.

Coelho KIR, AS Bryden, C Hall, TH Flewett. (1981).

Animal model. Pathology of rotavirus infection in suckling mice: A study by conventional histology, immunofluorescence, ultrathin sections and scanning electron microscopy. Ultrastructural Pathology. 2:59.

Creamer B. (1964).

Variations in the small intestinal villus shape and mucosal dynamics. British Medical Journal. 2:1371.

Creamer B. (1967).

The turnover of the epithelium of the small intestine. British Medical Bulletin. 23:226.

Croft DN, CA Loehry, JFN Taylor, J Cole. (1968).

DNA and cell loss from normal small intestinal mucosa. A clinical method for assessing cell turnover. Lancet. 2:70.

Crouche CF, GN Woode. (1978).

Serial studies of virus multiplication and intestinal damage in gnotobiotic piglets infected with rotavirus. Journal of Medical Microbiology. 11:325.

Davidson GP, DG Gall, M Petric, DG Butler, JR Hamilton. (1977).

Human rotavirus enteritis induced in conventional piglets.Intestinal structure and transport. The Journal of Clinical Investigation. 60:1402.

Dahlqvist A. (1964)

Method for assay of intestinal disaccharidases. Analytical Biochemistry. 7:18.

Eastwood GL. (1977).

Progress in gastroenterology. Gastrointestinal epithelial renewal. Gastroenterology, 72:962.

Ecknauer R, RM Clarke, H Meyer. (1977).

Acute distal intestinal obstruction in gnotobiotic rats: intestinal morphology and cell renewal. Virchows Archives. (Cell Pathology) 25:151.

Elliot KM. (1976).

Acute diarrhoea in childhood. Ciba Foundation Symposium No.42. Elsevier/North Holland/Excerpta Medica, Amsterdam.

Faulk WP, JN McCormick, JR Goodman. (1970).

Peyer's Patches: morphologic studies. Cellular Immunology. 1:500.

Ferguson A. (1978).

Lymphocytes and cell-mediated immunity in the small intestine. In: Wetherall D, ed. Advanced Medicine 14, Tunbridge Wells; Pitman Medical Publishing Co Ltd. 278

Ferguson A, F Allan, DM Al-Thamery. (1979).

Functional maturity of villus enterocytes in states of accelerated cell turnover without villus atrophy. in Cell Proliferation in the Gastrointestinal Tract. ed Appleton, Sunter and Watson. Pitman Press GB.

Ferguson A, AMcI Mowat. (1980).

Immunological mechanisms in the small intestine. In:Wright R, ed. Recent Advances in Gastrointestinal Pathology. Eastboure:WB Saunders, 1980:93.

Ferguson A, D Murray. (1971).

Quantitation of intraepithelial lymphocytes in human jejunum. Gut. 12:988.

Ferguson A, G Paul, DR Snodgrass. (1981).

Lactose tolerance in lambs with rotavirus diarrhoea. Gut. 22:114.

Ferguson A, A Sutherland, TT MacDonald, F Allan. (1977).

Technique for microdissection and measurement in biopsies of human small intestine. Journal of Clinical Pathology. 30:1068.

Flewett TH, AS Bryden, HA Davies. (1975).

Virus diarrhoea in foals and other animals. Veterinary Record. 96:477.

Flewett TH, AS Bryden, HA Davies, CA Morris. (1975)

Epidemic viral enteritis in a long stay children's ward. Lancet. 1:4.

Flewett TH, GN Woode. (1978).

The Rotaviruses - Brief review. Archives of Virology. 57:1.

Frederick GT, EH Bohl. (1976).

Local and systemic cell mediated immunity against transmissible gastroenteritis: An intestinal viral infection of swine. Journal of Immunology. 116:1000.

Fulton RW, CA Johnson, NJ Pearson, GN Woode. (1981).

Isolation of rotavirus from a newborn dog with diarrhoea. American Journal of Veterinary Research. 42.

Gall DG, D Chapman, M Kelly, JR Hamilton. (1977)

Sodium transport in jejunal crypt cells. Gastroenterology. 72:452.

Gouet Ph, M Contrepois, HC Dubourguier, Y Riou, R Scherrer, J Laporte, JF Vautherot, J Cohen, R L'Haridon.

The experimental production of diarrhoea in colostrum deprived Axenic and Gnotoxenic calves with enteropathogenic Escherichia coli, Rotavirus, Coronavirus and a combined infection of rotavirus and E. coli. Ann Rech Vet. 9:433.

Haelterman EO. (1972).

On the pathogenesis of Transmissible Gastroenteritis of Swine. Journal of the American Veterinary Medical Association. 160:531

Hall GA, JC Bridger, RL Chandler, GN Woode. (1976).

Gnotobiotic piglets experimentally infected with neonatal calf diarrhoea reovirus-like agent (rotavirus). Veterinary Pathology. 13:197.

Hamilton JR, DG Gall. (1982).

Pathophysiological and Clinical features of viral enteritis. in Virus Infections of the Gastrointestinal Tract. Tyrrell DAJ and Kapikian AZ, eds.page 227.

Hamilton JR, DG Gall, DG Butler, PJ Middleton. (1976).

Viral gastroenteritis - recent progress, remaining problems. Ciba foundation Symposium. 42:209.

Hanson WR, JW Osborne, JG Sharpe. (1977).

Compensation by the residual intestine after intestinal resection in the rat. 2. Influence of post operative time interval. Gastroenterology. 72:701.

Hanson WR, RPC Rijke, W Ewijk, JW Osborne. (1977).

The effect of intestinal resection on Thiry-Vella fistulae of jejunal and ileal origin: evidence for a systemic control mechanisn of cell renewal. Cell and Tissue Kinetics. 10:543.

Harding JD, AB Cairnie. (1975).

Changes in intestinal cell kinetics in the small intestine of lactating mice. Cell and Tissue Kinetics. 8:135.

Hart R, JM Mackay, CR McVittie. (1971).

A technique for the derivation of lambs by hysterectomy. British Veterinary Journal. 127:419.

Holmes IH, SM Rodger, RD Schnagl, BJ Ruck, ID Gust, RF Bishop, GL Barnes. (1976).

Is lactase the receptor and uncoating enzyme for infantile enteritis (rota) viruses? Lancet 1:1387

Hopper AF, PM Rose, RW Wannamacher. (1972).

Cell population changes in the intestinal mucosa of protein depleted or starved rats. 2. Changes in cellular migration. Journal of Cell Biology.53:225.

Jacobs LR, J Polak, S Bloom, RH Dowling. (1975).

Does enteroglucagon play a trophic role in intestinal adaptation? Clinical Science and Molecular Medicine. 48:14P

Johnson LR, PD Guthrie. (1974).

Mucosal DNA synthesis, a short term index of the trophic action of gastrin. Gastroenterology. 67:453.

Kelly M, DG Butler, JR Hamilton. (1972).

Transmissible gastroenteritis in piglets: A model of infantile viral diarrhoea. The Journal of Pediatrics. 180:925.

Kenworthy R, WD Allen. (1966).

Influence of diet and bacteria on small intestinal morphology with special reference to early weaning and Escherichia coli. Studies with gernfree and gnotobiotic pigs. Journal of Comparative Pathology. 76:291.

Kerzner B, MH Kelly, DG Gall, DG Butler, JR Hamilton. (1977).

Transmissible gastroenteritis: sodium transport and the intestinal epithelium during the course of viral enteritis. Gastroenterology. 72:457.

Koldovsky O, J Sunshine, N Kretchmer. (1966).

Cellular migration of intestinal epithelia in suckling and weaned rats. Nature. 212:1389.

Leblond CP, H Cheng. (1976).

Identification of stem cells in the small intestine of the mouse. in Cairnie AB, PK Lala, DG Osmond eds Stem cells of Renewing populations. New York. Academic Press. P 7.

Leblond CP, B Messier. (1958).

Renewal of chief cells and goblet cells in the small intestine as shown by radioautography after injection of thymidine-H3 into mice. Anatomical Record. 132:247.

Leblond CP, BE Walker. (1956).

Renewal of cell populations. Physiological Review. 36:255.

Lecce JG, MW King, WB Dorsey. (1978).

Rearing regime producing piglet diarrhoea (rotavirus) and its relevence to acute infantile diarrhoea. Science. 199:776.

Lecce JG, MW King, R Mock. (1976).

Reovirus-like agent associated with fatal diarrhoea in neonatal pigs. Infection and Immunity. 14:816.

Lecce JG, G Matrone, DO Morgan. (1961).

The effect of diet on the maturation of the neonatal piglets serum protein profile and resistance to disease. Annals of the New York Academy of Science. 94:250.

Lesher S, J Bauman. (1969).

Cell kinetic studies of the intestinal epithelium; maintainance of the intestinal epithelium in normal and irradiated animals. In Human Tumour Cell Kinetics; National Cancer Research Monogram. 30:185.

Light JS, HL Hodes. (1943).

Studies on epidemic diarrhoea in the newborn: isolation of filterable agent causing diarrhoea in calves. American Journal of Public Health. 33:1451.

Loehry CA, B Creamer. (1969a).

Three dimensional structure of the human small intestinal mucosa in health and disease. Gut. 10:6

Loehry Ca, B Creamer. (1969b)

Three dimensional structure of the rat small intestinal mucosa related to mucosal dynamics. Gut. 10:112.

Madeley CR, BP Cosgrove. (1975).

28nm particles in faeces in infantile gastroenteritis. Lancet. 2:451.

Malherbe HH, M Strickland-Cholmley. (1967).

Simian Virus SA.11 and related O agent. Archiv.fur die gesamte Virusforschung. 22:235.

Marsh MN, JS Trier. (1974).

Morphology and cell proliferation of subepithelial fibroblasts in adult mouse jejunum. 1. Structural features. Gastroenterology. 67:622.

Mebus CA (1978).

Pathogenesis of coronavirus infection in calves. Journal of the American Veterinary Medical Association. 173:631.

Mebus CA, LE Newman. (1977).

Scanning electron, Light and immunofluorecent microscopy of intestine of gnotobiotic calf infected with Reovirus-like agent. American Journal of veterinary Research. 38:553

Mebus CA, EL Stair, MB Rhodes, MJ Twiehaus (1973).
Neonatal calf diarrhoeas - propagation, attenuation and characteristics of a coronavirus-like agent. American Journal of Veterinary Research. 34:145.

Mebus CA, EL Stair, NR Underdahl, MJ Twiehaus. (1971).
Pathology of neonatal calf diarrhoea induced by a Reo-like virus. Veterinary Pathology. 8:490.

Mebus CA, NR Underdahl, MB Rhodes, MJ Twiehaus. (1969).
Calf diarrhoea (scours) reproduced with a virus from a field outbreak. Bulletin of the Nebraska Agricultural Experimental station. Research Bulletin. 233:1

Mebus CA, RG White, EP Bass, MJ Twiehaus. (1973).
Immunity to neonatal calf diarrhoea virus. Journal of the American Veterinary Medical Association. 163:880.

Mebus CA, RG Wyatt, AZ Kapikian (1977).
Intestinal lesions induced in gnotobiotic calves by the virus of human infantile Gastroenteritis. Veterinary Pathology. 14:273.

Middleton PJ, M Petric, MT Szymanski. (1975).
Propagation of infantile gastroenteritis virus (orbi-group) in conventional and gernfree piglets. Infection and Immunity. 12:1276.

Middleton PJ, MT Szymanski, M Petric. (1977).
Viruses associated with acute gastroenteritis in young children. American Journal of Diseases of Childhood. 131:733.

Moon HW. (1971).

Epithelial cell migration in the alimentary mucosa of the suckling pig. Proceedings of the Society for Experimental Biology and Medicine. 137:151.

Moon HW, DD Joel. (1975).

Epithelial cell migration in the small intestine of sheep and calves. American Journal of Veterinary Research. 36:187.

Moon HW, LJ Kemeny, GL Lambert, SL Stark, GD Booth. (1975).

Age dependent resistance to transmissible gastroenteritis of swine. 3. Effects of epithelial cell kinetics on coronavirus production and atrophy of intestinal villi. Veterinary Pathology. 12:434.

Moon HW, EM Kohler, SC Whipp. (1973).

Vacuolation: A function of cell age in porcine ileal absorptive cells. Laboratory Investigation. 28:23.

Morin M, LG Morehouse, RF Solorzano, LD Olson (1973).

Transmissible gastroenteritis of feeder swine: Clinical, Immunofluorescence and Histopathological observations. Canadian Journal of Comparative Medicine. 37:239.

Mouwen JMVM. (1971).

White scours in piglets. 2. Scanning electron microscopy of the mucosa of the small intestine. Veterinary Pathology. 8:401.

Mowat AMcI, A Ferguson. (1982).

Intraepithelial lymphocyte count and crypt hyperplasia measure the mucosal component of the graft-versus-host reaction in mouse small intestine. Gastroenterology 83:417.

McAdaragh JP, ME Bergeland, RC Meyer, MW Johnshoy, IJ Stotz, DA Benfield, R Hammer. (1980).
Pathogenesis of rotaviral enteritis in gnotobiotic pigs. A microscopic study. American Journal of Veterinary Research. 41:1572.

McClung HJ, DG Butler, B Kernzer, DG Gall, JR Hamilton. (1976).
Transmissible gastroenteritis: mucosal ion transport and acute viral enteritis. Gastroenterology. 70:1091.

McDermott FT, B Roundnew.(1976).
The crypt cell population kinetics after 40 percent small bowel resection. Gastroenterology. 70:707.

McDonald TT, A Ferguson. (1977).
Hypersensitivity reactions in the small intestine. 2. Effects of allograft rejection and of graft- versus-host disease on epithelial cell kinetics. Cell and Tissue Kinetics. 10:301.

McNulty MS. (1978).
Review article. Rotaviruses. Journal of General Virology. 40:1.

McNulty MS, GM Allan, GR Pearson, JB McFerran, WI Curran, RM McCracken. (1976).
Reovirus-like agent (rotavirus) from lambs. Infection and Immunity. 14:1332.

McNulty MS, GM Allan, JC Stuart. (1978).
Rotavirus infection in avian species. Veterinary Record. 103:319.

McNulty MS, GR Pearson, JB McFerran, DS Collins, GM Allan. (1976).
A reovirus-like agent (rotavirus) associated with diarrhoea in neonatal pigs. Veterinary Microbiology. 1:55.

Nordstrom C, A Dahlqvist. (1973).

Quantitative distribution of some enzymes along the villi and crypts of human small intestine. Scandinavian Journal of Gastroenterology. 8:407.

Owen RL.(1977).

Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: An ultrastructural study. Gastroenterology. 72:440.

Owen RL, AL Jones. (1974).

Epithelial cell specialisation within human Peyer's Patches: An ultrastructural study of intestinal lymphoid follicles. Gastroenterology. 66:189.

Owen RL, P Nemanic. (1978).

Antigen processing structures of the mammalian intestinal tract: An SEM study of lymphoepithelial organs. Scanning Electron Microscopy. 2: 367.

Parrott DVM. (1976).

The gut as a lymphoid organ. in Clinics in Gastroenterology. Immunology of G.I. and Liver Disease. R Wright ed. Saunders. Philadelphia. 5:211.

Parker FG, EN Barnes, GI Kaye. (1974).

The pericryptal fibroblast sheath. 4. Replication, migration and differentiation of the subepithelial fibroblasts of the crypts and villus of rabbit jejunum. Gastroenterology. 67:607.

Pearson GR,MS McNulty. (1977).

Pathological changes in the small intestine of neonatal pigs infected with a pig reovirus-like agent (rotavirus). Journal of Comparative Pathology. 87:363.

Pearson GR, MS McNulty. (1979).

Ultrastructural changes in small intestinal epithelium of neonatal pigs infected with pig rotavirus. Archives of Virology. 59:127.

Pearson GR, McNulty MS, EF Logan. (1978).

Pathological changes in the small intestine of neonatal calves naturally infected with reo-like virus (rotavirus). Veterinary Record. 102:454.

Pensaert M, EO Haelterman, EJ Hinsman. (1970).

Transmissible gastroenteritis of swine: Virus-intestinal cell interactions. 2. Electron microscopy of the epithelium in isolated jejunal loops. Archiv fur die gesante Virusforschung. 31:335.

Phillips RW, LD Lewis. (1973).

Viral induced changes in intestinal transport and resultant body fluid alteration in neonatal calves. Ann Rech Vet. 4:87.

Piazza M, G Pane, L Picciotto. (1967).

Effect on the infectivity of various viruses by the intestinal factor of normal mice which inactivates murine hepatitis virus. Nature. (London). 213:293.

Pink IJ, DN Croft, B Creamer. (1970).

Cell loss from small intestinal mucosa: a morphological study. Gut. 11:217.

Porter P. (1973).

Intestinal defence in the young pig. A review of the secretory antibody system and their possible role in oral immunization. Veterinary Record. 92:658.

Potten CS, TD Allen. (1977).

Ultrastructure of cell loss in intestinal mucosa. Journal of Ultrastructural Research. 60:272.

Quastler H, FG Sherman. (1959).

Cell population kinetics in the intestinal epithelium of the mouse. Experimental Cell Research. 17:420.

Reed DE, CA Daley, HJ Shane. (1976).

Reovirus-like agent associated with neonatal diarrhoea in pronghorn antelope. Journal of Wild Diseases. 12:488.

Rey J, J Schmitz, F Rey, J Jos. (1971).

Cellular differentiation and enzymatic defects. Lancet 2:218.

Rijke RPC, JM van Dongen. (1980).

Control mechanisms of crypt cell production in the small intestinal epithelium. Acta Histochemistry. supplement, 21:81

Rijke RPC, WR Hanson, H Plaisier. (1977).

The effect of transposition to jejunum on epithelial cell kinetics in an ileal segment. Cell and Tissue Kinetics. 10:399.

Rijke RPC, WR Hanson, H Plaisier, JW Osborne. (1976).

The effect of ischemic villus cell damage on crypt cell proliferation in the small intestine: evidence for a feedback control mechanism. Gastroenterology. 71:786.

Rijke RPC, H Plaisier, AT Hoogeveen, LF Lamerton, H Galjaard. (1975). The effect of continuous irradiation on cell proliferation and maturation in the small intestinal epithelium. Cell and Tissue Kinetics. 8:441.

Rijke RPC, H Plaisier, H de Ruiter, H Galjaard. (1977). Influence of experimental bypass on cellular kinetics and maturation of small intestinal epithelium in the rat. Gastroenterology. 72:896.

Saif LJ, KW Theil, EF Bohl. (1978). Morphogenesis of porcine rotavirus in porcine kidney cell cultures and intestinal epithelial cells. Journal of General Virology. 39:205.

Scott TM, CR Madeley, BP Cosgrove, JP Stanfield. (1979). Stool viruses in babies in Glasgow. 3. Community studies. Journal of Hygiene. (Cambridge) 83:469.

Shepherd RW, DG Butler, E Cutz, GD Gall, JR Hamilton. (1979). The mucosal lesion in viral enteritis. Extent and dynamics of the epithelial response to viral invasion in Transmissible Gastroenteritis of piglets. Gastroenterology. 76:770.

Shepherd RW, DG Gall, DG Butler, JR Hamilton. (1979). Determinants of diarrhoea in viral enteritis.The role of ion transport and epithelial changes in the ileum in Transmissible gastroenteritis in piglets. Gastroenterology. 76:20.

Smith MW, LG Jarvis. (1980). Use of differential interference contrast microscopy to determine cell renewal times in mouse intestine. Journal of Microscopy. 118:153.

Smith MW, LG Jarvis, IS King. (1980).

Cell proliferation in Follicle-Associated epithelium of mouse Peyer's patch. American Journal of Anatomy. 159:157.

Smith MW, MA Peacock. (1980).

'M' cell distribution in Follicle-Associated epithelium of mouse Peyer's patch. American Journal of Anatomy. 159:167.

Smith MW, MA Peacock. (1981).

Lymphocyte induced formation of antigen transporting 'M' cells from fully differentiated mouse enterocytes. in Mechanisms of Intestinal Adaptation. Robinson JWL, RH Dowling, EO Riecken, eds. Falk Symposium. 30. p573.

Snodgrass DR, KW Angus, EW Gray. (1977).

Rotavirus infection in lambs: Pathogenesis and Pathology. Archives of Virology. 55:263.

Snodgrass DR, KW Angus, EW Gray.(1979).

A rotavirus from kittens. Veterinary Record. 104:222.

Snodgrass DR, JA Herring, EW Gray. (1976).

Experimental rotavirus infection in lambs. Journal of Comparative Pathology. 86:637.

Snodgrass DR, CR Madeley, PW Wells, KW Angus. (1977).

Human rotavirus in lambs. Infection and passive immunity. Infection and Immunity. 16:268.

Snodgrass DR, W Smith, EW Gray, JA Herring. (1976).

A rotavirus in lambs with diarrhoea. Research in Veterinary Science. 20:113.

Snodgrass DR, PW Wells. (1976).

Rotavirus infection in lambs. Studies on passive protection. Archives of Virology. 52:201.

Snodgrass DR, PW Wells. (1978).

The influence of colostrum on neonatal rotaviral infections. Ann Rech Vet. 9:335.

Sobhon P. (1971).

The light and electron microscopic studies of Peyer's patches in non germfree adult mice. Journal of Morphology. 135:457.

Sprinz H. (1971).

Factors influencing intestinal cell renewal. A statement of principles. Cancer. 28:71.

Tallet S, RN MacKenzie, P Middleton, B Kerzner, R Hamilton. (1977).

Clinical laboratory and epidemiological features of a viral gastroenteritis in infants and children. Paediatrics. 60:217.

Tannock IF. (1967).

A comparison of the relative efficiencies of various metaphase arrest agents. Experimental Cell Research. 47:345.

Thake DC. (1968).

Jejunal epithelium in Transmissible Gastroenteritis of Swine. An electron microscopic and histochemical study. American Journal of Pathology. 53:149.

Thake DC, HW Moon, G Lambert. (1973).

Epithelial cell dynamics in Transmissible Gastroenteritis of neonatal pigs. Veterinary Pathology. 10:330.

Theil KW, EH Bohl, AG Agnes. (1977).

Cell culture propagation of porcine rotavirus (reovirus-like agent). American Journal of Veterinary Research. 38:1765.

Theil W, EH Bohl, RF Cross, EM Kohler, AG Agnes. (1978).
Pathogenesis of porcine rotaviral infection in experimentally inoculated gnotobiotic pigs. American Journal Of Veterinary Research. 39:213.

Thornhill TS, G Wyatt, AR Kalika, R Dolin, M Chanock, AZ Kapikian. (1977).
Detection by immune electron microscopy of 26-27nm virus particles associated with two family outbreaks of gastroenteritis. Journal of Infectious Diseases. 135:20.

Torres-Medina A, NR Underdahl. (1980).
Scanning electron microscopy of intestine of gnotobiotic piglets infected with porcine rotavirus. Canadian Journal of Comparative Medicine. 44:403.

Trier JS, TH Browning. (1970).
Epithelial cell renewal in cultured duodenal biopsies in coeliac sprue. New England Journal of Medicine. 283:1245.

Tutton PJM. (1973).
Control of epithelial cell proliferation in the small intestinal crypt. Cell and Tissue Kinetics. 6:211.

Tzipori S, M Walker. (1978).
Isolation of rotavirus from foals with diarrhoea. Australian Journal of Experimental Biology and Medical Science. 56:453.

Tzipori S, IW Caple, K Butler. (1976).
Isolation of a rotavirus from deer. Veterinary Record. 99:398.

Walker-Smith J. (1978).
Review Article. Rotavirus gastroenteritis. Archives of Disease in Childhood. 53:355.

Walker-Smith JA. (1979).

Diseases of the Small Intestine in Childhood. Tunbridge Wells. Pitman Medical Publishing Company.

Welch AB, MJ Twiehaus. (1973).

Cell culture studies of a neonatal calf diarrhoea virus. Canadian Journal of Comparative Medicine. 37:287.

Williamson RCN. (1978).

Medical Progress. Intestinal Adaptation. 2. Mechanisms of control. New England Journal of Medicine. 298:1444.

Wilsnack RE, JH Blackwell, JC Parker. (1969).

Identification of an agent of epizootic diarrhoea of infant mice by immunofluorescent and complement fixation tests.American Journal of Veterinary Research. 30:1195.

Wimber DR, LF Lamerton. (1963).

Cell population studies on the intestine of continuously irradiated rats. Radiation Research. 18:137.

Woode GN, JC Bridger. (1978).

Isolation of small viruses resembling astroviruses and caliciviruses from acute enteritis of calves. Journal of Medical Microbiology. 11:441.

Woode GN, JC Bridger, GA Hall, MJ Dennis. (1974).

The isolation of a rotavirus-like agent associated with diarrhoea in colostrum deprived calves in Great Britain. Research in Veterinary Science. 16:102.

Woode GN, J Bridger, GA Hall, JM Jones, G Jackson. (1976).

The isolation of reovirus-like agents (rotavirus) from acute gastroenteritis of piglets. Journal of Medical Microbiology. 9:203.

Woode GN, CF Crouche. (1978).

Naturally occurring and experimentally induced rotaviral infections of domestic and laboratory animals. Journal of the American Veterinary Medical Association. 173:522.

Wright NA. (1978).

The cell population kinetics of repopulating cells in the intestine. in Stem Cells and Tissue Homeostasis. Lord BI, CS Potten, RJ Cole. eds. Cambridge: University Press. p335.

Wright NA, HS Al Dewachi, DR Appleton, AJ Watson. (1975).

Cell population kinetics in the rat jejunal crypt. Cell and Tissue Kinetics. 8:361.

Wright NA, DR Appleton. (1980).

The metaphase arrest technique. A critical review. Cell and Tissue Kinetics. 13:643.

Wright NA, DR Appleton, J Marks, AJ Watson.(1979).

Cytokinetic studies of crypts in convoluted human small intestinal mucosa. Journal of Clinical Pathology. 32:462.

Wright NA, AR Morley, DR Appleton. (1972).

Variation in the duration of mitosis in the crypts of Lieberkuhn of the rat; a cytokinetic study using vincristine. Cell and Tissue Kinetics. 5:351.

Wright N, A Watson, A Morley, D Appleton, J Marks, A Douglas. (1973).

The cell cycle time in the flat (avillous) mucosa of human small intestine. Gut. 14:603.

Wright NA, AJ Watson, AR Morley, DR Appleton, J Marks, S Young. (1979). Cell kinetics in convoluted human mucosae. Journal of Clinical Pathology. 32:462.

Zajiceck G. (1977). The intestinal proliferon. Journal of Theoretical Biology. 67:515.

Zucoloto S, N Wright, M Bramble, C Record. (1979). Assessment of villus and crypt population sizes in human small intestinal biopsies. Gut 20:A921.

Small Intestinal Morphology and Epithelial Cell Kinetics in Lamb Rotavirus Infections

D. R. SNODGRASS, ANNE FERGUSON, FRANCES ALLAN, K. W. ANGUS, and B. MITCHELL
Moredun Research Institute, Edinburgh, and Gastro-Intestinal Unit, University of Edinburgh, and Western General Hospital, Edinburgh, Scotland

Morphologic changes in the small intestine of rotavirus-infected gnotobiotic lambs were investigated by measurement of villi and crypts in histologic sections of jejunum, midgut, and posterior ileum. In midgut, villus atrophy developed within 12 hr of infection and was apparent until 72 hr after infection. Crypt hypertrophy was evident from 42 hr after infection until the end of the observation period (6 days after infection). Changes in posterior ileum were similar in extent, but jejunal changes were much less marked. The relatively mild effect in the jejunum is in accord with reports from other species, and provides a basis for questioning the assumption that human rotavirus affects mainly the foregut.

Studies of epithelial cell kinetics were made on midgut using a microdissection and metaphase accumulation technique on sequential samples from anesthetized lambs. An increase in the cell production rate per crypt per hour from the overall control level of 5.8 was detected by 48 hr after infection. The maximum level of 21.2 was reached 8 days after infection, and this had returned to near normal by 15 days after infection. This large and sustained increase in crypt cell production probably underlies other previously described functional abnormalities.

Rotaviruses have been implicated as important causes of diarrhea in children and the young of several animal species.[1] Studies based on viral immunofluorescence, histology, and electron microscopy in calves, piglets, and lambs, have led to the concepts (a) that rotaviruses infect mature epithelial cells in the small intestine, and to a lesser extent in the caecum and colon; (b) that infected cells are sloughed, leading to partial villus atrophy; and (c) that the atrophic villi are rapidly reclad with relatively undifferentiated crypt cells, which mature over a few days and lead to healing of the lesion.[2–6] Reduced disaccharidase and increased thymidine kinase levels have been observed in rotavirus-infected small intestine,[7,8] which supports the contention that immature enterocytes are present on the villi during rotavirus infection.

The basic assumption underlying this hypothesis, i.e., that there is substantially accelerated production of immature enterocytes from the crypts, has not been tested. In this paper, the authors have performed experiments on lambs infected with lamb rotavirus[5,9]; these experiments investigated morphologic changes in small intestine, and kinetic studies on crypt cell production with a metaphase accumulation technique, from initial infection through to apparent recovery.

Materials and Methods

Animals

Twenty-two gnotobiotic lambs were delivered by hysterectomy and maintained in plastic isolators. Fourteen lambs were infected when 2–4 days old with 2–3 ml of a bacteria-free 20% fecal filtrate containing lamb rotavirus from the second to fifth gnotobiotic lamb passage.[5,9] Eight lambs were kept as uninfected controls.

Experiment 1: Histologic Observations

One lamb was anesthetized with pentobarbitone sodium at each of the following hours after infection: 12, 18, 27, 42, 48, 72, 96, and 144 hr. Control lambs were anesthetized at 72, 96, 96, 144, and 144 hr of age, respectively. Segments of small intestine were collected from jejunum, midgut, and posterior ileum of anesthetized lambs into formol-saline as described[5] or into corrosive formalin.

Received August 7, 1978. Accepted October 11, 1978.

Address requests for reprints to: Dr. D. R. Snodgrass, Animal Diseases Research Association, Moredun Institute, 408 Gilmerton Road, Edinburgh, EH17 7JH, Scotland.

The authors thank Mr. J. Menzies for skilled assistance and Mr. M. McLauchlan for help with the statistical analysis.

0016-5085/79/030477-05$02.00

Table 1. Histologic Measurements of Villi and Crypts (Experiment 1)

Time of sample	Villi			Crypts		
	Jejunum	Midgut	Posterior ileum	Jejunum	Midgut	Posterior ileum
hr postinfection		(*μm, mean ± SE*)			(*μm, mean ± SE*)	
Controls	600 ± 15	540 ± 17	614 ± 20	110 ± 5	100 ± 3	94 ± 2
12	527 ± 16	335 ± 21[a]	427 ± 19[a]	116 ± 7	94 ± 3	101 ± 4
18	552 ± 22	213 ± 5[a]	NS	116 ± 7	105 ± 3	NS
27	489 ± 24[b]	279 ± 10[a]	231 ± 8[a]	154 ± 4[a]	106 ± 7	111 ± 5[b]
42	541 ± 16	334 ± 23[a]	192 ± 9[a]	134 ± 6	124 ± 4[a]	102 ± 6
48	579 ± 33	307 ± 13[a]	278 ± 8[a]	153 ± 7[a]	125 ± 5[a]	143 ± 7[a]
72	606 ± 20	475 ± 20	321 ± 27[a]	223 ± 8[a]	134 ± 6[a]	116 ± 6[a]
96	531 ± 15[c]	681 ± 18[b]	346 ± 20[a]	152 ± 7[a]	151 ± 4[a]	104 ± 8
144	699 ± 33[b]	432 ± 8[c]	285 ± 13[a]	153 ± 6[a]	136 ± 6[a]	100 ± 5

Significance of deviation from control values. NS = no sample.
[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$.

Hemalum and eosin stained 5 μm sections were examined, and villus heights and crypt depths measured by ocular micrometer on 10 properly orientated villi and crypts at each site of small intestine.

Experiment 2: Microdissection and Epithelial Cell Kinetics

At each of 1, 2, 4, 8, 11, and 15 days postinfection an infected lamb and an age-matched uninfected control lamb were inoculated with vincristine sulfate (Oncovin, Eli Lilly & Co. Ltd) at 1 mg/kg by slow i.v. injection to block cells entering mitosis in metaphase. The animals were then anesthetized by inhalation of halothane and nitrous oxide and maintained anesthetized for 2 hr. At three time intervals over the 2-hr period, portions of midgut were ligated, and samples were placed in Clarke's fixative (75% absolute alcohol—25% acetic acid). After the last sampling, the lambs were killed.

The midgut tissues were stained by the Feulgen reaction. A line of villi with their associated crypts was cut from the edge of each specimen, and the lengths of 10 villi and 10 crypts were measured.[10,11] The crypts were separated from the lamina propria by pressure, and the number of cells in metaphase was then counted in each of 10 crypts per specimen.[11] The number of crypts per villus was calculated from counts of villus and crypt numbers on small areas of mucosa.[11]

The crypt cell production rate (CCPR—expressed as cells produced per crypt per hour) was calculated from a regression line of the accumulated numbers of metaphases on time after Oncovin inoculation. The rate of cell loss per villus per hour was calculated by multiplying the figure for CCPR by the mean number of crypts per villus for that section.

Results

Clinical and Virological Response

All infected lambs, except one killed 12 hr postinfection, developed watery diarrhea within 24 hr of inoculation, and most of them also showed apathy and decreased appetite. Control lambs continued to pass firm brown feces throughout the period of clinical reaction of the infected lambs.

No rotavirus was detected in the feces of lambs before infection or in the control lambs. Rotavirus was detected in feces of all diarrheic lambs, either by immunofluorescence on cell culture[12] or by enzyme-linked immunosorbent assay.[13]

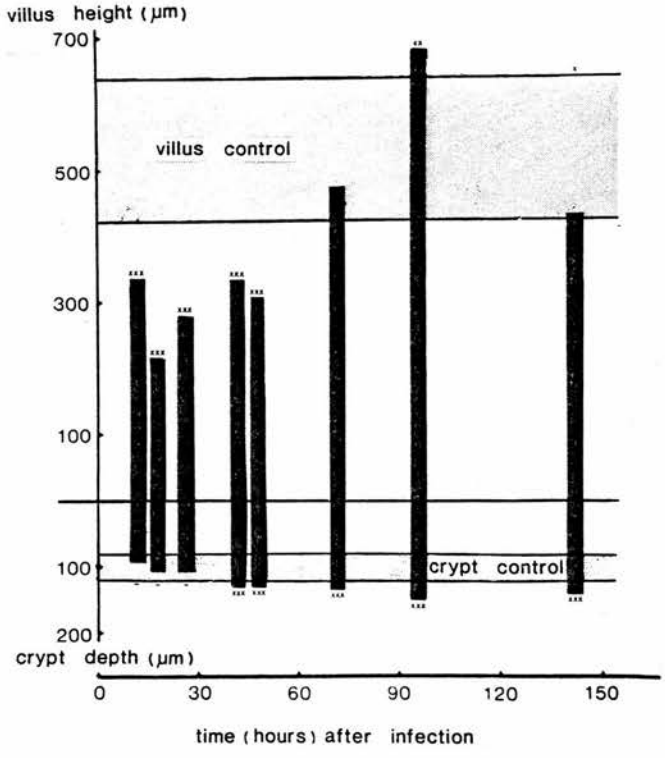

Figure 1. Villus height and crypt depth in midgut site (experiment 1). The crypt and villus controls are represented by a *band* for mean ± SD. The significances of the deviations from these control values are shown: —, not significant; X, $P < 0.05$; XX, $P < 0.01$; and XXX, $P < 0.001$.

Histology of Small Intestine

The villus heights and crypt depths at the three sampling sites in the five control lambs of 3–6 days of age (experiment 1) did not vary significantly with age. Normal measurements for villi and crypts at each site were therefore obtained by pooling observations for all five lambs. Measurements from individual infected lambs were compared with these normal values (Table 1).

The most marked changes were observed in midgut and posterior ileum. Significant villus atrophy occurred within 12 hr of inoculation and lasted for 2 days in midgut and throughout the experiment in posterior ileum. There was a temporary increase in villus height 4 days postinfection in midgut. By 1–2 days postinfection, the crypts showed a significant increase in length; this lasted throughout the experiment in midgut. In jejunum, a less consistent and less severe pattern developed, but villus atrophy and crypt hypertrophy were again evident.

The changes in midgut are illustrated graphically in Figure 1. As the abnormalities were more consistent and marked in midgut than in jejunum, and as it was sometimes difficult to select a posterior ileum sample free of Peyer's patches, it was decided to confine the microdissection and epithelial cell kinetic experiments (experiment 2) to the midgut site in the first instance.

Microdissection Measurements

The values obtained by measurement of villi and crypts in these specimens (experiment 2) were in proportion to but substantially greater than measurements in conventionally prepared sections from lambs in experiment 1, which is in accord with previous observations.[10] Villus atrophy was apparent in the lambs killed 1 and 2 days postinfection, while crypt hypertrophy developed by day 2 post infection and was still present at the end of the experiment.

Epithelial Cell Kinetics

All 12 lambs showed increasing accumulations of crypt cells in metaphase throughout the period of sampling. The regression lines of the numbers of accumulated metaphases with time are drawn in Figure 2.

The CCPR was calculated from the slope of the regression line. The CCPR for the control lambs did not vary significantly with age, and the production rate calculated from the combined regression line for all the controls was 5.8 cells per crypt per hour. The CCPR increased significantly by 2 days after rotavirus infection, reached a maximum of 21.2 cells/hr on the 8th day, and had returned to near normal values by the 15th day (Figure 3).

The number of crypts supplying cells to each villus varied between 2.1 and 4.2. The calculated cell loss per villus per hour increased roughly in proportion to the CCPR, to a maximum of 59.3 cells on the 8th day postinfection compared with a control rate of 15.5 cells/hr.

Discussion

The morphologic changes observed in the rotavirus-infected lambs confirmed and extended the authors' previous subjective descriptions.[5] Significant villus atrophy was apparent within 12 hr of infection and, in the distal site, was still present at 6 days postinfection. Crypt hypertrophy was first detected between 27 and 42 hr postinfection in all sites, and, in jejunum and midgut, was still present at 6 days postinfection. The measurements obtained and the sequence of events are in general agreement with those reported for pig rotavirus infections.[4,6]

These measurements of villus height indicated that jejunum was less consistently and less severely damaged in lamb rotavirus infections than the other more distal sites. This observation corroborates the less severe jejunal changes found independently by histology, immunofluorescence, and electron microscopy.[5] The anterior small intestine has also been observed to be least damaged in pig rotavirus infections[4,6] and in calf rotavirus infections in piglets.[3] The consistent occurrence of vomiting in infected children[14,15] indicates damage to the proximal small

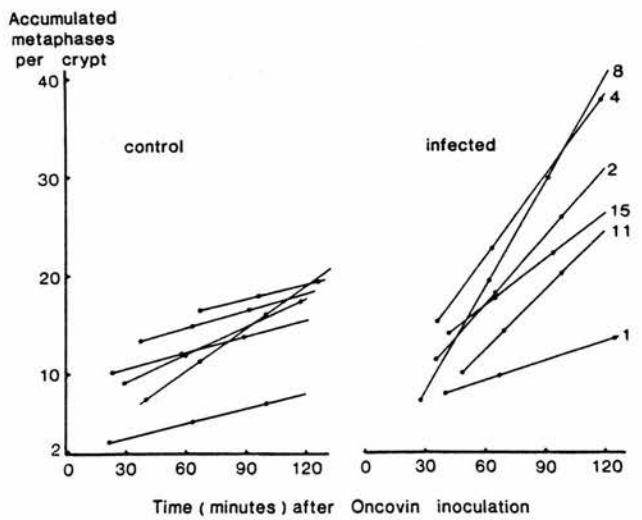

Figure 2. Accumulation of blocked metaphases in control and rotavirus-infected lambs. The regression *lines* are drawn for each lamb, and the *points* on each line represent the times when the lamb was sampled. The *numbers* beside the lines for the infected lambs indicate the day after rotavirus infection on which the lamb was killed.

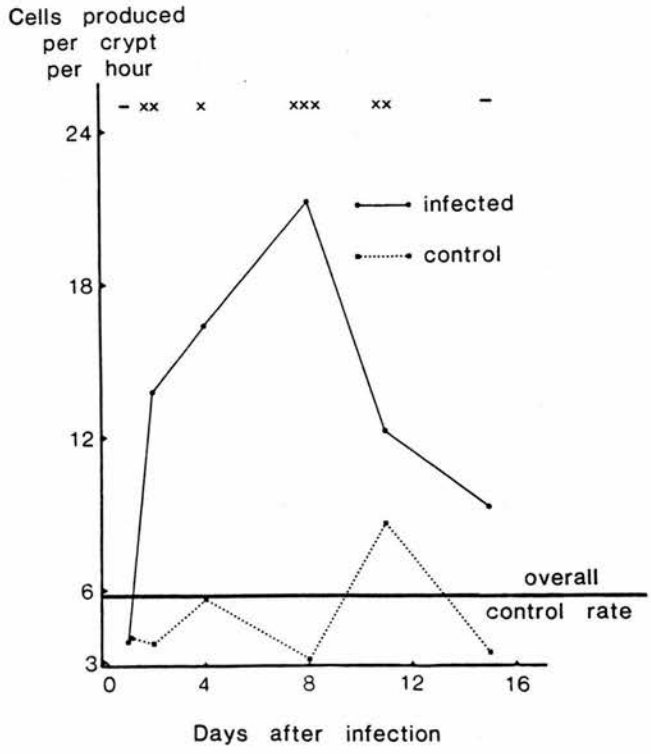

Figure 3. Crypt cell production rates (CCPR) compared for infected and control lambs. The data were converted to logarithms to minimize the positive association between means and variances. The regression lines for data from each infected lamb were compared with the regression line for all the control data after the differences between sheep had been removed. The levels of significance for the infected lamb CCPRs are shown: —, not significant; X, $P < 0.05$; XX, $P < 0.01$; and XXX, $P < 0.001$.

intestine, and studies in children are of necessity usually limited to this site.[7,16] Some workers using human rotavirus to infect piglets suggested that infection occurs primarily in the foregut,[8,17] whereas other studies on human rotavirus infections in monkeys[18] and calves[19] have reported involvement mainly of the mid and distal small intestine. Negative biopsy specimen results from children with suspect rotavirus gastroenteritis should be interpreted cautiously until this point is clarified.

By the end of the period of histologic observation (experiment 1), crypt hypertrophy in midgut was associated with normal villus height, and this suggests an increased turnover rate of epithelial cells. The cell kinetic studies of experiment 2 confirmed this, and show a substantial and prolonged increase in CCPR, to a maximum value at 8 days postinfection of approximately four times the normal level. This confirms previous suggestions that accelerated enterocyte migration might be the basic pathogenic mechanism underlying other abnormalities.[5,8]

The significance of this abnormality can only be partly assessed. Specific malfunctioning of glucose-coupled sodium transport,[8] impaired D-xylose absorption,[20] and reduced disaccharidase levels[7,8] have all been recorded in rotavirus infections and can probably be ascribed to increased CCPR, which leads to relatively immature undifferentiated enterocytes. It is likely that other functional abnormalities exist also as a result of the increased CCPR.

Diarrhea due to experimental rotavirus infections lasts for only a few days,[6,9] while the CCPR increased until 8 days postinfection and was abnormally high for nearly 2 wk. Thus, although the maximum CCPR is not responsible for the period of most severe diarrhea, it may cause a longer period of suboptimal gut function. Further work is necessary to determine the effect of rotavirus infections on such parameters as food conversion efficiency and liveweight gain.

This study leaves many questions unanswered, in particular as to what is the stimulus to prolonged increase in CCPR after villus atrophy has healed, and what are its functional effects. It does, however, confirm an important basic pathogenic mechanism of rotavirus infections.

References

1. Flewett TH, Woode GN: The Rotaviruses. Brief review. Arch Virol 57:1–23, 1978
2. Mebus CA, Stair EL, Underdahl NR, et al: Pathology of neonatal calf diarrhoea induced by a reolike-virus. Vet Pathol 8:490–505, 1971
3. Hall GA, Bridger JC, Chandler RL, et al: Gnotobiotic piglets experimentally infected with neonatal calf diarrhoea reovirus-like agent (rotavirus). Vet Pathol 13:197–210, 1976
4. Pearson GR, McNulty MS: Pathological changes in the small intestine of neonatal pigs infected with a pig reovirus-like agent (rotavirus). J Comp Pathol 87:363–375, 1977
5. Snodgrass DR, Angus KW, Gray EW: Rotavirus infection in lambs: pathogenesis and pathology. Arch Virol 55:263–274, 1977
6. Theil KW, Bohl EH, Cross RF, et al: Pathogenesis of porcine rotaviral infection in experimentally inoculated gnotobiotic pigs. Am J Vet Res 39:213–220, 1978
7. Bishop RF, Davidson GP, Holmes IH, et al: Virus particles in epithelial cells of duodenal mucosa from children with acute nonbacterial gastroenteritis. Lancet II:1281–1283, 1973
8. Davidson GP, Gall DG, Petric M, et al: Human rotavirus enteritis induced in conventional piglets. Intestinal structure and transport. J Clin Invest 60:1402–1409, 1977
9. Snodgrass DR, Herring JA, Gray EW: Experimental rotavirus infection in lambs. J Comp Pathol 86:637–642, 1976
10. Ferguson A, Sutherland A, MacDonald TT, et al: Technique for microdissection and measurement in biopsies of human small intestine. J Clin Pathol 30:1068–1073, 1977
11. MacDonald TT, Ferguson A: Small intestinal epithelial cell kinetics and protozoal infection in mice. Gastroenterology 74:496–500, 1978
12. Snodgrass DR, Wells PW: Rotavirus infection in lambs: studies on passive protection. Arch Virol 52:201–205, 1976
13. Ellens DJ, de Leeuw PW: Enzyme-linked immunosorbent as-

say for diagnosis of rotavirus infections in calves. J Clin Microbiol 6:530–532, 1977

14. Shepherd RW, Truslow S, Walker-Smith JA, et al: Infantile gastroenteritis: a clinical study of reovirus-like agent infection. Lancet II:1082–1084, 1975
15. Carr ME, McKendrick DW, Spyridakis T: The clinical features of infantile gastroenteritis due to rotavirus. Scand J Inf Dis 8:241–243, 1976
16. Middleton PJ, Szymanski MT, Abbott GD, et al: Orbivirus acute gastroenteritis of infancy. Lancet I:1241–1244, 1974
17. Middleton PJ, Petric M, Szymanski MT: Propagation of infantile gastroenteritis virus (Orbi-group) in conventional and germfree piglets. Infect Immunol 12:1276–1280, 1975
18. Wyatt RG, Sly DL, London WT, et al: Induction of diarrhoea in colostrum-deprived newborn rhesus monkeys with the human reovirus-like agent of infantile gastroenteritis. Arch Virol 50:17–27, 1976
19. Mebus CA, Wyatt RG, Kapikian AZ: Intestinal lesions induced in gnotobiotic calves by the virus of human infantile gastroenteritis. Vet Pathol 14:273–282, 1977
20. Mavromichalis J, Evans N, McNeish AS, et al: Intestinal damage in rotavirus and adenovirus gastroenteritis assessed by D-xylose malabsorption. Arch Dis Child 52:589–591, 1977