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### STUDIES ON THE SMALL INTESTINE IN FOLATE DEFICIENCY

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#### A thesis

submitted to the University of London for the degree of Doctor of Philosophy in the Faculty of Medicine

bу

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#### ABSTRACT

1. An experimental model for folate deficiency was established in the rat, which resulted in a decrease of folate in liver, red cells, serum and intestinal mucosa. Development of folate deficiency was associated with diarrhoea, morphological changes in the intestine, and a reduction in total mucosal DNA, RNA, and protein.

2. A technique was established to isolate epithelial cells from all levels of the mucosal villus. The cells from both control and folate deficient animals were viable for a minimum of 40 minutes, as assessed by oxygen consumption, carbon dioxide production, intracellular  $K^+/Na^+$  ratios, and dye exclusion.

3. Radioautography showed a delay in cell migration time in folate deficiency and RNA/DNA and protein/DNA ratios were greater, especially at the villus-tips. In villus-tip fractions, sucrase activity per cell was sufficiently increased that total gut sucrase was not decreased despite mucosal atrophy. However  $(Na^+-K^+)$ -ATPase activity per villus-tip cell was not increased and total mucosal  $(Na^+-K^+)$ -ATPase was lower. The relationship of these findings to absorptive function are discussed.

4. A new <u>in vitro</u> model was designed to measure the rates of DNA synthesis and relative contributions of <u>de novo</u> and salvage pathways to synthesis in crypt cells. In normal mucosa the <u>de novo</u> pathway was the most important for thymidylate synthesis; its contribution was reduced to a third in folate deficiency. The lowered rate of DNA synthesis in folate deficiency was increased by the provision of thymidine, suggesting that nutrient provision for the salvage pathway can facilitate replication of epithelial cells in folate deficiency. 5. The role of folate in determining the composition and replication of intestinal epithelium is discussed.

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SECTION I

GENERAL INTRODUCTION

### SECTION 1 - 1 NUTRITION AND THE GUT

The association of malnutrition and intestinal disease has been recognised for many years. Malnutrition may occur as a result of failure to absorb certain essential nutrients; this malabsorption is frequently the result of damage to or malfunctioning of the gut. It has been recognised more recently that the nutritional state of the host, and more specifically the intestinal mucosa, may impair cellular metabolism of the epithelial cells lining the gut sufficiently to impair absorption of nutrients. It is the latter aspect that is the subject of this thesis.

The effect of malnutrition on the gut received interest when physicians reported severe diarrhoea occuring in prisoners of war on semi-starvation rations (Lipscomb, 1945). Other reports of diarrhoea in patients with severe megaloblastic anaemia in India also suggested an important role for nutritional status in the development of diarrhoea (Baker, 1957). The onset of severe malabsorption states with the development of pregnancy where there is an increased requirement for folate, and the marked improvement in the intestinal lesion following vitamin therapy. (O'Brien & England, 1971) gave further support for this. In more recent times the nutritional problems of folate deficiency in the alcoholic have received attention. Several careful studies demonstrated that marked mucosal abnormalities exist (Bianchi <u>et al</u>. 1970; Hermos <u>et al</u>. 1972; Rubin <u>et al</u>. 1972).

The problem in many of the human studies remains that it is rare to have isolated nutritional deficiences. Most reported series on folate deficiency for example, occur in the presence of other pathological processes damaging the mucosa, whether infectious (eg. tropical malabsorption; Klipstein, 1972), dietary allergens (eg. gluten enteropathy; Shiner & Doniach, 1960), or toxins (eg. ethanol; Halsted <u>et al</u>. 1973). Nevertheless, a few studies of isolated folate deficiency present evidence that the intestinal morphology is altered (Winawer <u>et al</u>. 1965; Bianchi <u>et al</u>. 1970; Davidson & Townley, 1977). There have also been studies made on the intestinal mucosa in experimental folate deficiency (Klipstein <u>et al</u>. 1973b; Howard <u>et al</u>. 1974) or after anti-folate drugs (Trier, 1962a; 1962b; Ryback, 1962), but the nature of the cellular changes in the mucosa caused by folate deficiency and its effects on cellular metabolism have received scant attention.

#### SECTION 1-2

#### SMALL INTESTINAL MORPHOLOGY

#### 1-2a: HISTOLOGY

The mucosal surface of the small intestine is composed of villous projections and is covered by a continous sheet of epithelial cells (enterocytes) that rest upon a filamentous basement membrane (Rubin, 1971). It is dynamically one of the most active epithelial surfaces in the body - it has been estimated in man that there is a loss of 20-50 million cells per minute (Croft <u>et al</u>, 1968).

Since the introduction of the jejunal biopsy in 1955, the structure of the small intestinal mucosa has been thoroughly investigated and described. The epithelial membrane is not homogeneous. It is composed of different types of epithelial cells which have different physiological functions. The villi are between 3 and 5 times as long as the crypts which take their origins at their bases and dip into the lamina propria. The ratio of crypts to villi is approximately 3:1 and small clusters of crypts often exist between villi. The villous epithelium is continuous with that lining the crypts; crypts and villi being linked by intervillous ridges that run between the crypt mouths. The villous cells are differentiated with a distinct brush border composed of microvilli providing a large surface area, and associated with digestive enzymes such as disaccharidases and dipeptidases. Their primary function therefore, is the transport and absorption of nutrients. Crypt cells have a less distinct brush border than villous cells, and mitotic figures are detectable in the crypt but never villous epithelium. The crypts are specialised as production zones, where cell division occurs and from them the epithelial cells move out and progress up the villus, where they gradually mature and

acquire enzymes for absorptive function. At the tip of the villus the cells are lost from extrusion zones into the gut lumen where they soon disintegrate and their constituents are reabsorbed.

The epithelial cells themselves exhibit polarity ie. for normal functioning there must be net entry of water and electrolytes and other substances into the cell at the luminal surface and net entry into the plasma at the serosal surface. The absorptive enzymes are specifically located within the villous cells. For example, the brush border enzymes are found associated with the microvillous membranes or within the microvilli (eg. disaccharidases, di- and tripeptidases and alkaline phosphatases), while lysosomes contain acid phosphatases and various esterases (Riecken, 1970; Peters <u>et al</u>. 1975). Lateral and basal cell membranes are associated with (Na<sup>+</sup>-K<sup>+</sup>)- adenosine triphosphatase. (Quigley & Gotterer, 1969). The epithelial sheet is therefore highly organised with a clearly defined route for entry and exit of nutrients.

The core of the villus and the rest of the lamina propria is a connective tissue infiltrated by a small number of lymphocytes and occasional plasma cells, eosinophils and histiocytes (Whitehead, 1973). Intraepithelial lymphocytes are sometimes observed in histological samples but are not seen emerging into the lumen. The villi also contain a central blind-ending lacteal which is surrounded by smooth muscle cells upwards from the muscularis mucosa. Lying beneath the basement membrane of the enterocytes is a capillary plexus.

#### 1 - 2b: CELL TURNOVER

The epithelial sheet is therefore being continuously replaced by regeneration in the crypts, with migration up the villi and loss from the extrusion zones at the villus-tips (Creamer, 1974). The structure of the villi depends upon the epithelial sheet remaining constant (Williams et

al, 1958; Padykula <u>et al</u>, 1961). The proliferation of intestinal epithelial crypt cells is a steady state process with a constant distribution of cells through the mitotic cycle (Quastler & Sherman, 1959; Schultz <u>et al</u>, 1972; Maurer <u>et al</u>, 1972). While a great deal is known about rates of transit from crypts to villus in a variety of nutritional and pathological states, little is known about its control and in particular the control of cell division and differentiation.

Models for the mode of cell proliferation within rat jejunal crypts have been proposed. These were based on the labelling index and the mitotic index which were determined for the different positions of the cells in a crypt column (i.e. between the bottom and neck of the crypt), using a  ${}^{3}$ H -  ${}^{14}$ C thymidine double labelling technique (Cairnie et al 1965a; 1965b; Burholt et al 1976). According to these models the proliferating cells in the lower one-third of the crypt comprise an exponentially growing cell population; every mitosis leads to two proliferative cells. In the adjacent, middle region of the crypt, the probability of producing proliferative daughter cells is decreased with each increasing cell position up the crypt. At the neck of the crypt every mitosis leads to only differentiating cells.

Mammalian cells have a remarkably constant turnover time and the timing of events in the proliferative compartment is also similar, although rodents show rather slower times than man. From the crypt, cells move up the villi where they differentiate and mature. The differentiation of epithelial cells as they progress up the villus has been studied in conditions of normal and experimentally altered cell proliferation and lifespan, (de Both et al. 1974; de Both & Plaisier 1974). Such studies have shown a relationship between cell position along the villus, and enzyme activities, suggesting an effect of cell transit time on functional differentiation. The importance of this relationship will be discussed in more depth in section 2 - 3.

At the villus-tip cells are extruded into the lumen. The time taken for a cell to reach the top from the base of the crypt ie. to replace the whole epithelium, is called the turnover or transit time. The dynamic situation which exists between cell production and cell loss means that the rate of both processes must be constant if the epithelial structure is to be preserved.

The epithelial cells have an estimated turnover time from crypt to villus-tip of between 4 and 6 days in man (Lipkin <u>et al</u>. 1963; Macdonald <u>et al</u>. 1964; Rubini <u>et al</u>. 1964) and 2 - 3 days in mice (Loran & Crocher, 1963) and rats (Williams <u>et al</u>. 1958; Koldovsky <u>et</u> <u>al</u>. 1966; Sunshine <u>et al</u>. 1971). There is a slower migration rate and faster cell turnover in ileum than jejunum (Gleeson <u>et</u> <u>al</u>. 1972a; Batt & Peters, 1976). There are three principle techniques for measuring cell turnover rates:

(i) <u>Mitotic counting</u>: The percentage of cells in mitosis ie. the mitotic index, can be calculated by counting the number of cells in mitosis compared with the total population of cells. From labelling experiments it is possible to calculate the duration of mitosis, and thus the turnover time of the epithelium can be calculated. The technique is laborious, but reproducible data has been published (Leblond & Stevens, 1948).

(ii) <u>Autoradiography:</u> Following its injection, tritium (or <sup>14</sup>C) labelled thymidine is taken up by all cells in the S (synthetic) phase of cell mitosis (Winawer & Lipkin, 1970), and incorporated into DNA. When the cell divides, the daughter cells almost equally share the thymidine, and so the label remains within the nucleus for the life of the cell. Histological sections are thinly coated with either stripping film or nuclear emulsion, and the energy emitted by the tritium labelling will precipitate silver in this film. This shows up as dark particles when the film emulsion is developed. The presence of the label and its location can therefore be demonstrated. By sacrificing animals at different time intervals after injection of the label, the progression of cells can be observed. It is assumed that the irradiation in the nucleus causes no significant damage during the period of observations.

In the small intestine the crypt cells are immediately labelled in large numbers. Over the next few hours these cells enter a series of mitoses, following which labelled cells leave the crypts and ascend the villi so that the level of the leading labelled cell at any time interval shows the distance travelled.

(iii) <u>DNA loss</u>: Loehry <u>et al</u>. (1969) have developed a technique which utilises the rate of cell loss as a measure of turnover because of the rapid disintegration of cells. By washing the intestine at a controlled rate, the amount of DNA collected in the washings gives a measure of the rate of cell exfoliation. The rate of cell exfoliation compares well with the other techniques which measure cell production rates. Clarke (1970) also measured cell exfoliation rates and compared them with the rate of cell production per crypt. Epithelial cells shed from the intestinal villus in starved rats were trapped in mucus which the goblet cells were stimulated to secrete. The number of trapped cells per villus was counted in whole mounts of fixed Feulgen stained specimens.

#### 1 - 2c: FACTORS AFFECTING CELL TURNOVER

The size of the epithelial cell population of the small intestine is determined by the balance between the rate at which cells are produced in the crypts and the rate at which they are shed from the villi.

Autoradiographic studies showed that cell renewal was decreased to about half the normal rate following severe starvation in mice (Brown <u>et al</u>. 1963). Morphological changes and impaired differentiation of the epithelial cells accompanied this change. The rate of migration of the epithelial cells to the villus-tip was also reduced. Rats and monkeys with kwashiorkor have shorter villi and crypts than normal, and the mitotic count

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is low (Deo & Ramalingaswami, 1965; Kern <u>et al</u>. 1966). More commonly, a decrease in villous height is associated with crypts longer than normal and this has been suggested to be due to an increased output of cells to balance the increased loss.

In coeliac disease the mucosa is flattened and an increased mitotic rate is usually observed (Padykula <u>et al</u>. 1961), suggestive of a fast cell turnover rate. Specific nutritional deficiencies are known to occur in tropical malabsorption, in particular folic acid and vitamin  $B_{12}$  and specific deficiencies of these enzymes are known to diminish cell production and in some instances villous height (Klipstein, 1972).

Intraluminal nutrition and conditions play an important role in mucosal morphology (Gleeson <u>et al</u>. 1972a). Abnormal food, pH, bacteria and parasites will also increase cell loss and alter villous height. Overeating induces a high cell output and enlarged villi as does small intestinal resection in the rat (Loran & Althausen, 1960; Gleeson <u>et al</u>. 1972b). Germ free animals have an abnormal small intestine with short crypts. The rate of cell renewal is reduced to about half of that in conventional animals, and animals reared with only one species of organism in their gut show a mucosal morphology half way between the germ free and conventional animal (Lescher <u>et al</u>. 1964). The normal intestinal morphology and turnover pattern is therefore not an inherited characteristic, but an interplay between a basic dynamic process of cell production and cell loss, and the gut flora.

#### 1 - 2d: CELL TURNOVER AND ABSORPTION

In conditions of mucosal atrophy such as in severe coeliac disease or giardiasis, the flattening of the jejunal mucosa may be a casual factor of the associated malabsorption. Another consideration is that an alteration in the rate of cell turnover may cause a change in the number of mature villus tip enterocytes with developed brush borders and associated absorptive enzymes. If a normal epithelial cell takes 3 days to ascend a villus and the cells covering the lower part of the villus are still acquiring a full enzymic load, then an abnormal state in which the cell life lasts only one day, may lead to a situation where few of the adult cells are fully developed. Conversely, an increase in cell life may lead to a 'hypermature' population of cells. The relationship between cell turnover rate and absorptive function will be discussed further in section 2 - 3.

### <u>SECTION 1 - 3</u> FOLIC ACID METABOLISM

Folic acid (pteroylglutamic acid, folacin) is a combination of the pteridine nucleus, p-aminobenzoic acid, and 1-glutamic acid (Stokstad & Koch, 1967; Krumdieck & Baugh, 1969) - see figure 1 - 3a. It is present in practically all natural foods, usually as polyglutamyl conjugates (Butterworth <u>et al</u>. 1963). The richest sources are yeast and liver, containing respectively 1.25mg and 300µg/100g net weight (Paul & Southgate, 1978). Significant quantities are present in nuts and vegetables, especially asparagus, lettuce, spinach and dried beans (Toepfer <u>et al</u>. 1951; Santini <u>et al</u>. 1962; Herbert, 1963). Folates are sensitive to light, aerobic conditions, extremes of pH and heat (Herbert, 1963), all of which result in losses of folate activity. A normal human daily folate intake has been estimated to vary from about 700µg (Butterworth <u>et al</u>. 1963; Chanarin <u>et al</u>. 1968) to 1000 - 1500µg (Jandl & Lear, 1956) per day.

Pteroylpolyglutomates have a large molecular weight and molecular radius. They are strongly electronegative and highly water soluble with all carboxyl groups charged at physiological pH (Rosenberg & Godwin, 1971). However, by their very size they represent a convenient intracellular storage form and as such are the major form of intracellular folate in tissues such as red blood cells. Digestion of the pteroylpolyglutamic conjugates to free or monoglutamic forms is necessary before intestinal transport of the latter can occur. The hydrolytic enzyme folate conjugase (arglutamyl hydrolase), is found concentrated in the mucosa of the small intestine (Butterworth <u>et al</u>. 1969; Rosenberg <u>et al</u>. 1969; Hoffbrand and Peters, 1970; Reisenauer <u>et al</u>. 1977), and also in the human intestinal juice (Klipstein, 1967; Hoffbrand & Peters, 1970). The site of hydrolysis of pteroylpolyglutamates, whether intraluminally, at the brush border, or within the intestinal cell, is undetermined



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(Rosenberg, 1975; Halsted et al. 1976; Jagerstad et al. 1976; Reisenauer et al, 1977). However, recent studies suggest that polyglutamates are transported into the epithelial cell prior to hydrolysis by folate conjugase (Halsted et al. 1976). Folate in the human blood circulates as the free or unconjugated form principally as the reduced form, 5-methyl-tetrahydrofolic acid (Stokstad & Koch, 1967). Methylation of the monoglutamate appears to occur within the intestinal cell prior to release into the portal circulation (Rosenberg et al. 1971; Corrocher et al. 1972). It is then converted back to the polyglutamate form in all mammalian cells (Herbert et al. 1962). Studies using radioactively labelled folic acid have shown that endogenous folic acid is concentrated in the mitochondrial and cell sap fractions, and conjugation probably occurs here. (Brown et al. 1965; Corrocher & Hoffbrand, 1972). Intestinal injury such as that which occurs in tropical malabsorption has been shown to cause malabsorption of the polyglutamate forms of folic acid (Hoffbrand et al. 1969; Corcino et al. 1976).

Folic acid is metabolically inactive as such. It is reduced by an enzyme system, to the coenzymatically active tetrahydrofolic acid, which is important in reactions involving the transfer and utilisation of onecarbon moieties (see figure 1 - 3b). It is necessary in the formation of thymidylate by the methylation of deoxyuridylate, in the <u>de novo</u> synthesis of purine and pyrimidine bases, in the interconversion of glycine to serine the methylation uf homocysteine to form methionine (the principle methyl donor in the body), the synthesis of choline via methyl groups from methionine, and the catabolism of histidine. The enzymatic aspects of folic acid metabolism have been reviewed in detail by Friedkin, 1963; Jaeniche, 1964; Stokstad & Koch, 1967; Chanarin, 1969; Halsted <u>et al.</u> 1974.

Folate therefore has a fundamental role in cell growth and reproduction via the synthesis of components of DNA and RNA metabolism, as well as in amino acid and phospholipid metabolism. The role of folate in DNA



metabolism will be discussed in detail in Section 1 - 4. There is also some evidence for a role for folate in protein metabolism, but this is less clearly defined. Folate deficient rats have low plasma protein levels and aminoaciduria (Harper, 1973), as do severely deficient pregnant women (Jacobs & Fleming, 1970), compared with controls. Folate deficient monkeys have a significantly decreased rate of nucleoprotein synthesis (Halta, 1970; Chang & Kaiser, 1972).

Folate deficiency is one of the most important deficiencies both in the industrialised world and in developing countries. Pregnancy is reported as a major cause (Lowenstein <u>et al</u>. 1966; Jacobs and Fleming, 1970; Harper, 1973), but women taking oral contraceptives (Wood <u>et al</u>. 1972) alcoholics (Bianchi et al.1970; Hermos <u>et al</u>. 1972; Halsted <u>et al</u>. 1973) persons with adult coeliac disease, tropical malabsorption and inflammatory bowel disease (Klipstein 1966; 1972; Corcino <u>et al</u>. 1975), and patients treated with anticonvulsants (Wood <u>et al</u>. 1972; Reynolds, 1973), often suffer from folate deficiency. In these situations there is either a disturbance in folate metabolism or an interference with folate absorption. Patients with a malignant disease commonly have an abnormal folate status. This is probably secondary to anorexia, increased requirements caused by the tumor growth, and use of folate antagonists in the treatment (Magnus, 1967; Poirer, 1973). All these conditions can be exacerbated by an insufficient intake or the inappropiate preparation of foods containing folate.

The role of folate in DNA and protein metabolism means that tissues in a growing phase, or with a high cell turnover rate, such as red blood cells and gut epithelial cells, will be most affected by a deficiency of the vitamin. Megaloblastic anaemia is a common feature of folate deficiency. where the basic abnormality is a reduced capacity to double the nuclear DNA complement which is necessary for mitosis. This leads to maturation arrest and the formation of large macrocytic red blood cells. Abnormalities of jejunal morphology and function have been described in severely folate

deficient alcoholics (Bianchi <u>et al</u>. 1970; Hermos <u>et al</u>. 1972; Halsted <u>et al</u>, 1973), and folate deficiency has been implicated in the pathogenesis of the intestinal lesion of tropical malabsorption (Klipstein, 1972). A more detailed account of the relationships between intestinal and folate metabolism is given in section 1 - 5.

### <u>SECTION 1 - 4</u> THYMIDYLATE METABOLISM

Thymidylate (dTMP) and its triphosphate (dTTP) occupy a unique position in DNA synthesis. Three of the substrates for DNA polymerase (deoxyadenosine triphosphate; deoxyguanosine triphosphate and deoxycytosine triphosphate) are obtained by direct reduction of the corresponding ribonucleotides (Laftha & Vane, 1958; Dunlap et al. 1971), but dTTP is synthesised by a more complex route. Thymidine triphosphate is one of the rate limiting factors in DNA synthesis, and if absent DNA production is totally inhibited (Cannellakis et al. 1959; Weissman et al. 1960; Beltz, 1962). It is produced via the reduction of uridine triphosphate, which is then dephosphorylated to deoxyuridylate (dUMP) (see figure 1-4a). Deoxyuridylate is converted (via thymidylate synthetase) to thymidylate, which is rapidly phosphorylated to thymidine triphosphate (via thymidylate kinase) (Dunlap et al. 1971; Hoffbrand & Waters, 1972; Hauschka, 1973). The thymine nucleotide pools are mostly composed of dTTP, with only small amounts of dTMP, and dTDP (Gentry et al. 1965, Lindberg et al. 1969). The conversion of deoxyuridylate to thymidylate by thymidylate synthetase, depends on folic acid in the form of 5,10-methylene tetrahydrofolic acid (Flaks & Cohen, 1957; Friedkin & Komberg, 1957; Humphreys & Greenberg, 1958; Hauschka, 1973). The latter is reduced to dihydrofolic acid during the reaction (see figure 1 - 4b). Since the level of the folate coenzyme is limited within the cell, the continuous synthesis of thymidylate requires that dihydrofolate be reduced to tetrahydrofolate and the latter be reconverted to 5,10-methylene tetrahydrofolate (Killman, 1964; Metz et al. 1968; Hoffbrand & Waters, 1972; Hauschka, 1973). Chronic folate deficiency has been shown to block thymidylate synthesis by this pathway (referred to as the de novo pathway) as has acute folate deficiency following administration of folate antagonists such as methotrexate (Livingston et al. 1968;



Key:

Reactions in the de novo pathway
 Reactions in the salvage pathway
 Pt.Glu. = Pteroylglutamic acid ie. folic acid.



Figure 1-4b; FOLIC ACID METABOLISM - In the synthesis of thymidylate.

1.0

Metz <u>et al</u>. 1968; Tattersall & Harrap, 1973). Methotrexate is an analogue of folic acid, and therefore blocks the enzyme dihydrofolate reductase thereby decreasing the amount of reduced folic acid available for the conversion of deoxyuridylate to thymidylate. (Pastore & Freidkin, 1962; Wahba & Freidkin, 1962).

Thymidylate can also arise from another pathway called the salvage pathway (see figure 1 - 4a). This involves the phosphorylation of thymidine by thymidine kinase. Thymidine comes from thymine by a phosphorylase reaction. These two steps are independent of folate metabolism.

The relative importance of the salvage pathway against the de novo pathway in thymidylate synthesis has not been confirmed. It has generally been considered however, that thymidylate synthetase is the primary enzyme involved in the formation of thymidylate (Thomas, 1969), and that thymidine kinase acts only as a salvage mechanism for the previously formed thymidine and thymine (Beltz, 1962; Lang et al. 1966; Dunlap et al. 1971). This is on the basis of evidence that shows that thymidylate synthetase is blocked by folate analogues which also inhibit cell growth. In addition various mutants of He La cells and Escherichia coli have been produced that lack thymidine kinase but are still mitotically active (Kit et al. 1963; 1966). It has been suggested that thymidine kinase acts only as a salvage mechanism for those thymidine nucleotides, which when degraded, produce the free base thymine (Beltz, 1962). It has been found that when radioactively labelled thymine is added to a cell culture, little is incorporated in the cellular DNA (Lang <u>et al</u>. 1966). However the presence of either deoxyribose-1-phosphate or a deoxynucleoside such as deoxyuridine, as deoxyribosyl donors, leads to the reversible conversion of thymine to thymidine via a deoxyribosyl transferase or deoxythymidine phosphorylase (see figure 1 - 4c). Thymidine can therefore enter the salvage pathway for thymidylate synthesis using thymidine kinase (Gallo et al. 1967). However, the significance of the salvage pathway cannot

(A)

THE SYNTHESIS OF THYMIDINE FOR THE 'SALVAGE' PATHWAY FROM THYMINE + DEOXYURIDINE (GALLO ET AL., 1967)

THYMINE + DEOXYURIDINE + URACIL

Enzyme: deoxyribosyl transferase

\* \* \*

(B) DEOXYURIDINE + P

THYMINE + DEOXYRIBOSE-1-PHOSPHATE THYMIDINE + P

Enzyme: deoxynucleoside phosphorylase

\* \* \*
be ignored. Conceivably the rate of the <u>de novo</u> pathway in producing thymidylate may be too slow to produce the quantity of thymidylate needed in an efficient mitotic system. If there was sufficient thymidine or thymine present, thymidylate could be produced by the salvage pathway to compensate for the inadequency of the <u>de novo</u> pathway (Thomas, 1969; Badcock, 1975). Similary Tattersall <u>et al</u>. (1974), in a study of the effects of the antifolate methotrexate on 5 mammalian cell lines, demonstrated that some of the cells could be rescued from the methotrexate toxicity if excess thymidine was present in the culture medium. In all the cell lines thymidine administered concurrently with methotrexate, prevented the drop in thymidine triphosphate levels caused by the drug and actually increased thymidine triphosphate pools over control values.

The fact that thymidine kinase must have some significance in thymidylate synthesis is also indicated since this kinase, and in fact all the nucleoside kinases, are subject to feedback inhibition by their end-product nucleoside triphosphates (Hauschka, 1973). Thymidine kinase is usually inhibited by both potential end products - dTTP (Reichard <u>et</u> <u>al.</u> 1960; Bresnick & Karjala, 1964) and deoxyuridine triphosphate (dUTP) (Morris & Fischer, 1960). Thymidylate synthetase on the other hand exhibits no significant activation or feedback inhibition by dTTP or other nucleotides (Maley & Maley, 1970; Conrad & Ruddle, 1972). Preliminary work on the small intestine showed that the salvage pathway operated at normal rates of DNA synthesis in control animals, suggesting that it might contribute to DNA synthesis.

Many studies have described the relationship between increased DNA synthesis, as for instance during development and regeneration, and the enzymes for the synthesis of thymidylate. However, none has compared the relative importance of the <u>de novo</u> with the salvage pathway. Maley & Maley (1960; 1969) have reported in regenerating rat liver, that the synthetase increased concomitantly with the initiation of increased DNA synthesis. Flaks and Cohen (1957), Humphreys and Greenberg (1958),

Beltz (1962) and Myers (1962), reported similar increases in thymidylate synthetase.

Following partial hepatectomy DNA synthesis begins about 18 hours post-operatively, reaches a maximum between 24 and 30 hours post-operatively and then declines abruptly (Fausto & Van Lancher, 1965). Bresnick et al. (1967) and Baugnet-Mahieu et al. (1968) found that this pattern was followed closely by thymidine kinase activity. Similarly Bollum and Potter (1958; 1959), Weissman et al. (1960), Bianchi et al. (1962), Eker (1965) and Weinstock and Dju (1967) found that there were higher levels of thymidine kinase in tissues and cells undergoing rapid cellular proliferation such as in regenerating tissues. Most of these studies have been carried out in vitro and the authors have reported an absence of thymidine kinase in normal tissues, suggesting that the salvage pathway is not normally important for DNA synthesis. However, Smellie (1963) has shown that in vitro thymidine kinase was present 20 - 24 hours after partial hepatectomy while in vivo the enzyme was present in normal liver as well as after partial hepatectomy. This evidence further suggest that the salvage pathway could play a role in thymidylate synthesis in normal tissues even if only a minor one.

Considerable work has therefore shown that the enzyme of both pathways are increased when DNA synthesis and therefore cell turnover rates increase. Some interesting work has been done on the importance of both pathways in neonatal and mature muscle cells of chicks (Weinstock et al. 1970). It is known that the rate of muscle cell turnover is most rapid immediately after birth when growth occurs and then slows down at maturity when growth is complete. The work on the chick muscle revealed that at birth and up to 15 days after birth, there was a dependence on the <u>de</u> <u>novo</u> pathway, ie. when cell turnover was rapid. After 15 days and up to maturity the salvage pathway was shown to be of greater importance ie. folate dependence decreases as the rate of cell turnover decreases.

Similarly in studies on the folate coenzymes of the brains of mice, McLain and Bridges (1970) found that they underwent quantitative and qualitative changes during the first week after birth, when there was rapid growth and DNA synthesis, and that the amount of folate coenzymes decreased with maturation ie. DNA synthesis decreases.

In adults the mucosa of the small intestine is the most rapidly regenerating tissue in the body (LeBlond & Walker, 1956). The epithelial cells have an estimated turnover time from crypt to villus tip of between 2 and 6 days in man (Lipkin et al. 1963; Macdonald et al. 1964; Rubini et al. 1964) and 2 to 3 days in rats (Sunshine et al. 1971). Cell turnover is directly related to DNA turnover rate (Davidson et al. 1951), and it would seem possible therefore that the enzymes for pyrimidine and purine nucleotide synthesis would also be directly related to cell turnover. Sunshine et al. (1971) found a correlation between increased activities of some of the enzymes necessary for pyrimidine biosynthesis such as aspartate transcarbamylase, dihydrooratase, thymidine kinase and uridine kinase and the increased rate of intestinal cellular proliferation which occurs at weaning. In view of the increases in DNA synthesis and related increases in several enzymes necessary for pyrimidine and purine biosynthesis, one could anticipate that the enzymes for thymidylate synthesis would also increase. This is also supported by the increases in thymidylate synthetase and thymidine kinase found in other rapidly growing or regenerating tissues described previously. Of more interest, however, is the change in utilisation of one pathway for thymidylate synthesis, relative to the other, during changes in DNA synthesis. In view of the work by Weinstock et al. (1970) on chick muscle which has shown that the de novo pathway, requiring folate, is highly active immediately after birth and then decreases in importance relative to the salvage pathway as the muscle matures and DNA synthesis slows down, one could postulate that a few days after birth when cell turnover is very slow in the intestine (Koldovsky et al. 1966), the salvage pathway would

be able to supply sufficient thymidylate for DNA synthesis. There is therefore little requirement for folate for the <u>de novo</u> pathway. However, at weaning when DNA synthesis is expected to suddenly increase, in order for the thymidylate requirements to be met the dependence on the <u>de novo</u> pathway increases to a greater extent than the dependence on salvage pathway. So too therefore does the requirement for folate in the cells increase. Since the intestine of the adult rat maintains a higher cell turnover rate than in the suckling rat, dependence on the <u>de novo</u> pathway could be expected to continue, but always with some synthesis of thymidylate via the salvage pathway.

It seemed relevant therefore to measure the relative use of pathways for the synthesis of thymidylate in the small intestinal mucosa in controls and also in folate deficiency. As will be discussed, the isolation of populations of cells from different parts of the villus which have different degrees of maturity and differentiation provides a suitable model for observing differences in the pathways with respect to cell age. The effects of folate deficiency on the pathways, particularly in crypt cells, can also be studied. The role of folate in thymidylate synthesis by the <u>de novo</u> route, could suggest that in a deficiency state the synthesis of thymidylate in crypt cells is impaired. This would be analogous to the bone marrow cells in megaloblastic anaemia due to folate deficiency, where <u>de novo</u> synthesis is blocked (Hoffbrand & Waters, 1972; Wickramsinge & Saunders, 1976; Hooton & Hoffbrand, 1977).

Most studies of DNA synthesis in mammalian cells use thymidine as radioactive label. To be able to incorporate labelled thymidine, the cells have to use salvage pathway enzymes to synthesise thymidylate. Thymidine is not taken up by the <u>de novo</u> pathway. Deoxyuridine is a precursor for the <u>de novo</u> pathway. The uptake of labelled thymidine after preincubation with deoxyuridine expressed as a percentage of the uptake without preincubation with deoxyuridine, is called the deoxyuridine

suppressed value. It provides an index of the relative use of one pathway for thymidylate synthesis against the other ie. de novo versus salvage (see figure 1 - 4a). It has been shown that bone marrow cells from patients with vitamin  $B_{12}$  or folate deficiency show a subnormal suppression of labelled thymidine uptake after preincubation with deoxyuridine (Killman, 1964; Metz et al. 1968). Addition of 10 microgram of pteroylglutamic acid per millilitre of marrow culture, partially corrects the abnormal deoxyuridine suppressed value shown by all folate deficient bone marrows (Wickramsinghe & Saunders, 1975). Previous work from this department, using an adaptation of the deoxyuridine suppression test suitable for incubated rat small intestinal rings or human intestinal biopsies (Badcock, 1975; Tomkins & McNurlan, 1977), indicated similar trends in folate deficiency. A problem with interpretation of these results was that the tissues contained many cells of differing biological age and thus differing dependence on <u>de novo</u> and salvage pathways. It was hoped that the use of populations of isolated gut epithelial cells from each level of the crypt and villus would overcome this problem.

While the deoxyuridine suppression method provides some information on the relative use of the two pathways for thymidylate synthesis, it is still not possible to quantitate the percentage contribution of one pathway versus the other with respect to thymidylate, since the incorporation of labelled thymidine from the salvage pathway is measured in the end product DNA and not the thymidylate pool. There are also problems in interpretation of results with reference to true rates of DNA synthesis. There was greater incorporation of radioactive thymidine into DNA by bone marrow cells from patients with megaloblastic anaemia due to folate deficiency or vitamin  $B_{12}$  deficiency, compared with corresponding cells treated with these vitamins <u>in vivo</u> or <u>in vitro</u> (Hooton & Hoffbrand, 1977). It was unclear whether the higher incorporation really represented increased rates of DNA synthesis, or was merely due to a higher specific radioactivity of the thymidine triphosphate pool. These problems are also

relevant in gut enterocytes in folate deficiency, particularly in crypt cells. It was proposed therefore to develop a new model for measuring the relative utilisation of <u>de novo</u> and salvage pathways for the synthesis of thymidylate, by measuring the direct product ie. thymidylate. The model could be extended to give an accurate measurement of the rate of DNA synthesis. The importance of the salvage pathway in folate deficiency with respect to changes in the rate of DNA synthesis when the supply of thymidine is increased, could then be examined.

A detailed discussion of the deoxyuridine suppression test and the new model for measuring rates of DNA synthesis are discussed in direct relation to the methods and experimental design in Section 2 - 4 and Appendix 3.

#### SECTION 1 - 5

### FOLATE DEFICIENCY & THE INTESTINAL MUCOSA

Primary nutritional folate deficiency is an uncommon condition in humans, because folate deficiency is often associated with tropical malabsorption (Klipstein, 1966; 1972), coeliac disease (Shiner & Doniach, 1960; Klipstein, 1966) or alcoholism (Halsted et al. 1973), which are all conditions affecting the intestinal mucosa in their own right. Nevertheless a few studies of primary folate deficiency (Winawer <u>et al.</u> 1965; Bianchi <u>et al</u>. 1970; Davidson & Townley, 1977) have shown morphological damage of the jejunal mucosa as assessed by intestinal biopsy. The report of Davidson and Townley (1977), described four infants fed goat's milk for periods of six months or more, who developed folate deficiency with anaemia, lethargy and diarrhoea, in association with megalocytic epithelial cells in the gut mucosa, and suppression of brush border disaccharidases. All these findings were reversible by folic acid therapy.

Early studies demonstrated the sensitivity of the gut mucosa to folate antagonists, in which morphological changes in jejunal biopsies from patients receiving a single large dose of methotrexate included reversible inhibition of mitoses in the crypts (Trier, 1962b), and in which the crypts of mice injected with aminopterin demonstrated mitotic arrest and the appearance of cells with large ruclei (Ryback, 1962).

By assessing the activity of the folate dependent enzymes necessary for the synthesis of DNA and cell replication, Tomkins and McNurlan (1977) demonstrated a functional mucosal folate deficiency in five patients with tropical malabsorption. Only two of these patients had megaloblastic anaemia, the remainder had normal bone marrow morphology and red blood cell folate concentrations; suggesting that mucosal malnutrition occurs in the absence of systemic folate deficiency. The jejunal epithelium has a very high folate requirement because of the demand for DNA synthesis by the crypt cells. They probably rely to a considerable degree on intraluminal supply and any disturbance in intraluminal metabolism could affect mucosal nutrient status. If mucosal folate status is determined more by intestinal than systemic events, then it is likely that secondary folate deficiency is responsible for exacerbating specific intestinal abnormalities. It may be that in tropical malabsorption for example, there is a primary insult, possibly the result of a virus or bacterial enterotoxin. The effect of bacterial metabolism, possibly including the large quantities of ethanol present within the luminal fluid of such cases, may cause an antifolate effect on intestinal epithelium (Klipstein <u>et</u> al. 1973b; Tomkins <u>et al</u>. 1976).

Small intestinal biopsies are usually normal in binge drinking alcoholics, but reports of gut morphology in severely folate deficient alcoholic patients with megaloblastic anaemia, described decreased mitotic counts with villous shortening and enlargement of crypt cell nuclei (Hermos <u>et al.</u> 1972). All the changes were corrected by treatment with folic acid. Similar histological changes are found in patients with tropical malabsorption, although these are not always reversed by folic acid therapy (Shehy <u>et al.</u> 1962; Wheby <u>et al.</u> 1968).

Further support for a role for folate in the regulation of the intestinal mucosa, comes from studies on the wives of British servicemen in Singapore and Bantu women in South Africa, where there is a precipitation of mucosal atrophy and malabsorption during the folate deficiency of pregnancy (O'Brien & England, 1971). Additionally jejunal biopsy specimens from patients with tropical malabsorption, showed a marked change in the megaloblastic appearance of nuclei of crypt cells two days after commencement of folic acid therapy, with an accompanying improvement in villous morphology.

Using the appropiate dietary regime, folate depletion of the intestinal mucosa of rats can be achieved, and these animals develop

diarrhoea and abnormalities of intestinal morphology (Klipstein <u>et al.</u> 1973b; Tomkins <u>et al.</u> 1976). Goetsch and Klipstein (1977) using an <u>in vivo</u> perfusion technique demonstrated normal absorption of xylose, glucose and L-leucine, but a net secretion of water and sodium with persistent diarrhoea in folate deficient rats compared with controls despite morphological changes in the jejunal mucosa. Treatment with folic acid caused net jejunal absorption of water and sodium. Similarly there was no impairment of absorption of glucose, xylose and glycine in African subjects with severe megaloblastic anaemia of primary nutritional origin, despite the presence of morphological abnormalities (Cook, 1976).

Thus despite morphological changes of the jejunal mucosa in folate deficiency, there appears to be relatively normal absorption of most nutrients. By contrast there was marked impairment of sodium absorption and water transport. Some of the experiments to be described here were designed to study this disparity.

### SECTION 1 - 6

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### 'IN VITRO' STUDIES OF INTESTINAL METABOLISM

1 - 6a: REASONS FOR ISOLATING GUT EPITHELIAL CELLS

Numerous methods for studying intestinal metabolism and function have been described, both <u>in vivo</u> e.g. perfused intestine (Ohnell, 1939; Cook, 1976; Goetsch & Klipstein, 1977), but predominantly <u>in vitro</u> e.g. everted sacs (Wilson & Wiseman, 1954; Crane & Wilson, 1958), intestinal rings and segments (Crane & Mandelstam, 1960; Badcock, 1975), isolated mucosal sheets and scrapings (Kay & Entenman, 1959; Crane & Mandelstam, 1960), mucosal homogenates (Dawson & Isselbacher, 1960; Taylor, 1963), isolated subcellular particles (Clark & Hubscher, 1961, Carnie & Porteous 1962; Sherratt & Hubscher, 1963; Porteous & Clark, 1965) and isolated epithelial cell preparations (e.g. ferris, 1966; Stern, 1966; Evans, 1969; Kimmich, 1970; Reiser & Christiansen, 1971; Weiser, 1973; Raul <u>et al</u>. 1977).

The intact small intestine comprises several types of tissue layer, which can be broadly defined as an outer serosal layer of muscles and blood vessels, and an inner mucosal layer which is lined with epithelial cells. Many of these detailed studies on intestinal metabolism and function in recent years have emphasised the need to study the mucosal tissue and in particular the epithelial cells, intact from the underlying tissue. It would then be possible to avoid the assumption that measurements made on intestinal segments or mucos<sup>-1</sup> scrapings, completely represent the activity of the mucosal epithelium, which in fact may only comprise a small and variable porportion of the starting material. This also became apparent from previous studies by the author, in which rings of small intestine were used for assessing the incorporation of tritiated thymidine into DNA (Badcock, 1975). The results were variable and inconclusive since the incubated tissue contained both serosal and mucosal tissue together with lymphocytic and possibly bacterial infiltration.

It is therefore important in studies of mucosal metabolism and

function to distinguish between the varying cell populations found on the villus. Dahlqvist & Nordstrom (1966), Moog & Grey (1967), Imondi et al (1969), have all attempted to remove cells from known heights on the villi, using cryostatic techniques. Whilst achieving this segregation of cell types, these techniques do not give rise to individual cells which are viable for incubation studies. There are now several reports in the literature (e.g. Kimmich, 1970; Evans et al. 1971), which have detailed methods for isolating gut epithelial cells which remain viable for an adequate period of time, but in most cases these only give rise to one heterogeneous population of cells from both crypt and villus or villus alone. Harrison & Webster (1969) have successfully separated two populations of cells ie. villus and crypt, but they did not confirm their viability, although Iemhoff et al. (1970) attempted to follow up these studies but with inconclusive results. Weiser (1973) sequentially separated epithelial cells from different levels of the villus and crypt, but despite using them for incubation studies, he gave no account of a detailed assessment of viability.

### 1 - 6b: ISOLATION TECHNIQUES

Procedures for isolating whole mucosa and isolated epithelial cells have been developed over the last 10 - 15 years, but they are many and varied. They were, however, all considered when deciding on a method suited to the purposes of this study. Isolation methods for intestinal cells and mucosal sheets can generally be subdivided into mechanical and biochemical methods, although in some procedures these methods have been combined.

(i) <u>Mechanical methods</u>: These include the histological planing method of Dahlqvist & Nordström (1966), Moog & Grey (1967) and Imondi <u>et al.</u> (1969). Dahlqvist and Nordström used a cryostatic technique, whereby sections of frozen mucosal tissue were removed successively down the villus. This allowed them to evaluate the enzyme patterns from cells of various levels of crypts and villi. A similar technique, using an 'intestinal planing

apparatus' to obtain a number of villous and crypt fractions, was developed by Imondi <u>et al</u> (1969). Such methods require experience in histological techniques and they do not produce isolated cells free from the lamina propria and villous core tissue, although Imondi and co-workers (1969) attempted to release individual cells from the villous sections using the enzyme hyaluronidase (see biochemical methods).

Isolation of intestinal mucosa can also be achieved by scraping everted intestine with, for example a microscope slide, or spatula. (Dawson & Isselbacher, 1960; Hubscher <u>et al</u>. 1965). The scrapings obtained resulted in high yields of mucosal tissue which did not necessarily contain all the epithelial cells, since very hard, thorough scraping was needed to completely remove all the crypt cells. The preparations were also contaminated with lymphocytes, red blood cells, connective tissue and mucus. The mucus inhibits the disaggregation of the scrapings into individual cells. Filtering the scrapings through fine nylon cloth or similar material can reduce the mucous aggregated factors, but may also be selective in excluding some epithelial cells thereby reducing recovery.

Sjöstrand (1968) used an 'automatic intestinal epithelium peeler' whereby pressure was applied to the surface of an everted intestine on a rotating rod, the pressure point being gradually displaced from the top to the bottom of the rod. The tissue harvested was dependent on the pressure applied. A gentle pressure produced only sheets of epithelial cells from the villi, a greater pressure could harvest whole villi or even crypts and submucosa. Hulsmann (1974) used the same principles applying the pressure by hand, but produced variable types of tissue fractions. The fractions were again contaminated with factors such as lymphocytes, red blood cells etc. Prolonged stirring was needed to break up the obtained sheets of mucosa into isolated cells and subsequent metabolic studies (lemhoff <u>et al.</u> 1970) indicated that such cells were heavily damaged. Other workers (Harrison & Webster, 1969) have used a vibration

technique, coupled with a citrate dissociating agent (see biochemical methods) in the collection medium in order to obtain epithelial cell preparations. Like most of the mechanical techniques this method produced sheets of cells rather than isolated cells, but in addition the damage to the membranes of the tissues and cells by the high frequency vibration to which they are exposed renders them unsuitable for incubation studies (Iemhoff et al. 1970).

(ii) Biochemical methods: More has been accomplished in the isolation of individual epithelial cells by applying biochemical (or chemical) techniques. These include incubation of the intestine with enzymes such as trypsin and pancreatin (Harrer <u>et al</u> 1964; Stern & Reilly, 1965), lysozyme (Huang, 1965), hyaluronidase (Perris, 1966; Kimmich, 1970; Rask & Peterson, 1976), collagenase (O'Doherty & Kuksis, 1975) and papain (Padron <u>et al.</u> 1973). The principle behind the technique is that these enzymes attack the connective tissue of the intestinal mucosa and release the epithelial cells. Some of the enzymes such as trypsin are non-specific proteolytic enzymes, and so a choice of enzyme more specific to collagen e.g. hyaluronidase and collagenase would seem more appropiate.

Other methods are based on the principle of calcium dissociation of tissues (Dawson & Isselbacher, 1960; Stern, 1966; Evans, 1969; Evans <u>et</u> al. 1971; Weiser, 1973; Raul <u>et al</u>. 1977). Citrate or ethylenediamine tetracetic acid (EDTA) chelate with calcium ions which have a binding role in connective tissue. Thus, when intestinal tissue is incubated with EDTA or citrate, individual epithelial cells are released. Reiser & Christiansen (1971), Feters & Shio (1976) and Kremski <u>et al</u> (1977) have used a combination of the principles of citrate and enzyme dissociation. Detailed studies, in particular by Evans (1969) and Kimmich (1970) have shown that cells isolated by these methods remain viable for suitable time periods to allow incubation studies to be carried out.

### 1 - 6c: ASSESSMENT OF VIABILITY

With any method of tissue disaggregation there is the possibility of cell damage, so it is essential to demonstrate that isolated cells to be used for metabolic studies are functionally as well as structurally intact. This is particularly important in these present studies where folate may affect several aspects of small intestinal metabolism and function in both crypt and villous zones of the mucosa. Many workers have made little or no attempt to do this. This was particularly true of the early workers where vibration techniques were used. Iemhoff et al. (1970) did attempt to evaluate the methods of Sjöstrand (1968) and Harrison & Webster (1969) for yield and integrity, but this was based largely on morphological appearance, which is inconclusive in terms of the functional capacity of a cell and its membrane. Cells obtained from the Harrison and Webster technique displayed glycolytic activity of the same order as intestinal mucosa in situ, but a more comprehensive study considering the collective results of several morphological and metabolic studies is required to be conclusive. It would seem likely that the degree of handling and exposure in some instances to high frequency vibration is likely to cause damage to membranes.

In each of the early attempts at preparing cell suspensions from rat intestinal mucosa by biochemical methods (Harrer <u>et al.</u> 1964; Stern & Reilly, 1965; Huang, 1965; Stern, 1966), the cells once isolated rapidly lost their metabolic activity as assessed by the rate of oxygen consumption. Perris (1966) reported a constant rate of respiratory activity for a period of up to 40 minutes with cessation after that time. Kimmich (1970), using hyaluronidase to isolate cells from the chicken intestine, demonstrated a linear production of carbon dioxide and lactate from glucose for more than 2 hours, and morphological appearance as assessed by light microscopy was normal. In addition concentration gradients of both sugars and amino acids could be generated and the formation of such gradients could be prevented by inhibitors of energy metabolism. Evans

et al. (1971) carried out a detailed assessment of viability in cells isolated from guinea pig small intestine by using citrate. The cells retained lower sodium ion concentrations and higher potassium ion concentrations than those of the suspending medium, lost only small amounts of their soluble enzyme, and had a respiratory activity characteristic of intact cells over a period of up to 2 hours. Evans (1969) also compared the viability of cells isolated by enzyme and citrate techniques. She concluded that cells isolated by citrate dissociation techniques, retained properties of intact cells for longer periods and with more consistency than those isolated by enzyme techniques. This was especially so for membrane integrity, and she suggested that this was due to the non-specificity of some of the general proteolytic enzymes used, such as trypsin and pancreatin, which can digest cell membrane proteins as well as connective tissue proteins. Enzymes specific to connective tissue such as hyaluronidase and collagenase are therefore preferable in enzyme isolation techniques.

Weiser (1973) using a citrate dissociation technique was able to isolate epithelial cells sequentially from different levels of the villus as a villous-crypt gradient. He used them to investigate the composition and synthesis of membrane glycoproteins as a reflection of the developmental state of the cell. However, despite using the cell preparations in incubation studies he has published no detailed assessment of their viability and functional integrity.

From the literature it was concluded that the most viable preparations of isolated epithelial cells could be obtained by incubating the gut with enzymes such as hyaluronidase and collagenase or citrate buffers (or a combination of both) in order to disaggregate the cells from their connective tissue. Detailed studies by Evans (1969) showed that the citrate dissociation method was preferable, in the guinea pig at least, to enzyme dissociation. The method of Weiser (1973) which sequentially separated cells from the villus and crypt, also used a citrate dissociation

technique. It was decided therefore to develop a technique similar to that used by Weiser to isolate cells from the different regions of the gut mucosa, and characterise the cell populations obtained from both control and folate deficiert animals with respect to enzyme and functional markers. A thorough evaluation of the morphology and functional integrity of these cells would then be necessary to establish their suitability for incubation studies.

## SECTION 1 - 7

## PROJECT AIMS

The epithelium of the jejunal mucosa has a high requirement for folate because of the demand for DNA synthesis by the crypt cells. It would seem likely therefore that folate plays an important role in the regulation of function and composition of the intestine, particularly with reference to clinical conditions of secondary folate deficiency, where mucosal atrophy and malabsorption have been reported.

It was decided therefore to establish an experimental model for folate deficiency and to develop a method for isolating the different populations of cells from the villus. It would then be possible to study the different aspects of gut metabolism and function specific to each region of the villus, and to relate the composition and kinetics of cell turnover with functional changes as cells mature, with particular reference to the influence of folate on these changes. An assessment of changes in pathways for, and actual rates of, synthesis of DNA in crypt cells in folate deficiency, would also be made to help clarify the reasons for, and the mechanisms of, changes in small intestinal morphology in folate deficiency. SECTION 2

EXPERIMENTAL WORK

## MATERIALS

In the following experimental sections, most of the chemicals used were of Analar grade, and were purchased from British Drug Houses (Poole, Dorset) or Sigma Chemicals Ltd. (Poole, Dorset). All radioactive isotopes were purchased from Radiochemicals Ltd. (Amersham, Buckinghamshire). Where an alternative source or note of purity was required, this has been indicated in the text, as have notes on the equipment used.

## Animals.

In the following experimental sections, the animals used were bred from Lister hooded dams (200 g) obtained from Olac 1976 (Bicester, Oxfordshire), which were mated with Lister hooded males (250-300 g) from the C.N.M.Unit animal house.

In the following series of experiments, all the animals used were sacrificed by decapitation.

## PRESENTATION OF RESULTS

Unless otherwise stated, all results in the tables are given as mean values ± standard errors. Significance levels were assessed by Student's 't' test. 'p' is the probability that any difference is due to chance alone. 'p' values of 0.05 or less were considered to be significant.

#### SECTION 2 - 1

### EXPERIMENTAL FOLATE DEFICIENCY

## 2 - 1a: INTRODUCTION

Folic acid is found in most foodstuffs and can be synthesised by normal intestinal bacteria in man and mammals. It is not easy therefore, to produce experimental folate deficiency in man or rats. The synthesis of folate by gut bacteria is of considerable importance in rats for example, but is of dubious physiological significance in man. Wills et al. (1935; 1937) produced a megaloblastic anaemia by feeding rhesus monkeys, for 3 - 9 months, a diet based on that of poor classes of Indians in Bombay. This consisted of polished rice, margarine, salt, white bread, cod-liver oil, tomato and carrot. Similarly folate deficiency was readily produced in baboons by dietary deprivation alone (Siddons, 1974). These studies suggest that the intestinal flora does not play a major part in folate nutrition in primates. Herbert (1962) succeeded in producing folate deficiency in a healthy male physician on a diet consisting primarily of foods thrice boiled in large quantities of water to extract the water soluble vitamins, which were replaced except folate, by supplementation in pure form. Megaloblastic anaemia associated with folate deficiency developed after a period of 19 weeks. Chanarin et al. (1969) have described a folate deficient diet which they fed to 6 week old rats. As a result there was a rapid loss of folate from the liver, together with a slower fall in serum and red cell folates. Williams et al. (1975) used a similar diet fed to weanling rats for up to 3 months and showed reductions in serum folate levels. Chronic folate deficiency was described by Kodicek and Carpenter (1950). From these studies it was apparent that the animals needed to be housed individually in wire bottom cages in order to prevent coprophagy (eating faeces). The bacteria of the intestine in rats, can produce vitamins, including folic acid, which if not absorbed immediately can be recycled and absorbed after coprophagy. The addition of an antibiotic to the diet was required to decrease the number of vitamin

producing bacteria in the first instance. Using a diet modified from Kodicek and Carpenter (1950) and fed to 6 week old rats, we were unable in a preliminary investigation, to produce folate deficiency, despite individual caging in wire bottom cages. Klipstein et al (1973b) described a method whereby a folate deficient diet was fed to pregnant rats one week prior to giving birth, in order to deplete their own and therefore the stores of their pups. The mothers remained on the folate deficient diet throughout lactation and the pups were weaned on to the diet at 3 weeks of age. At 4 weeks of age Klipstein et al. (1973b) reported a decline in liver and intestinal folate levels and the development of diarrhoea in rats fed the deficient diet. There were no further significant changes after 6 more weeks on the regime. Most of the deficient animals had an increase in crypt height and size of the epithelial cell nuclei and a decrease in the mitotic index. However, intestinal function as measured by xylose and fat absorption remained normal, although the watery diarrhoea was associated with impaired sodium and water transport (Goetsch & Klipstein, 1977).

It was decided therefore to continue using the diet modified from Kodicek and Carpenter, and to adopt the method of Klipstein <u>et al</u>. in order to deplete new born rats of folate. Further precautions of sterility and hygiene were also introduced to eliminate any bacterial contamination which could synthesise and therefore supply folate.

#### 2 - 1b: METHODS

(i) Animal model: Seven days before delivery of their litters, pregnant, hooded rats were started on an experimental diet, the composition of which is described in Figure 2 - la. One group received a diet fed <u>ad</u> <u>libitum</u>, which was deficient in folate and contained 1% of the antibiotic sulfasuccidine, while a second group received a diet fed <u>ad libitum</u>, which was supplemented with 3mg/kg of folic acid. Third and fourth groups received diets supplemented with 3mg/kg of folic acid and containing

# FIGURE 2-1a: COMPOSITION OF BASIC ANIMAL DIET (folate free diet).

Vitamin free casein	18%
Sucrose	73%
Ground nut oil	3%
ISalt mixture	5%
Fat soluble vitamins	0.1%
Cystine	0.15%
Choline	0.2%
Water soluble vitamins (excluding folic acid)	<0.1%

<sup>1</sup>Payne & Stewart, 1972.

<sup>2</sup>Based on the requirements for rats (Coates <u>et al.</u>1969).

# FIGURE 2-15: ANIMAL GROUPS.

GROUP	1	Basic diet +	3mg/Kg	folic	acid -	ad	libutum.
GROUP	2	Basic diet +	3mg/Kg	folic	acid +	1%	sulfasuccidine - ad libitur
GROUP	3	Basic diet.					
GROUP	4	Basic diet +	3mg/Kg	folic	acid +	1%	sulfasuccidine - pair
		fed to GROUP	3.				

# Specific precautions:

- 1. Daily cage change.
- 2. Individual caging in wire bottom cages.
- 3. Daily replacement of sterile drinking water.

1% of sulfasuccidine, fed either <u>ad libitum</u> or pair fed to the deficient group, (see Figures 2 -la and 2 - lb). The antibiotic was necessary to reduce the number of gut bacteria which normally produce and supply folate to the rat. The possibility of remaining bacteria reentering the gut after coprophagy was reduced by frequent cage changing, and the bacterial contamination of the drinking water was reduced by using distilled boiled water, administered in sterilised bottles. These were also changed daily.

The mothers were placed on the dietary regime one week prior to giving birth in order to deplete their folate stores and hence the stores of their pups. Each litter was restricted to eight pups, which were weaned at 3 weeks of age on to the same experimental diet as their mothers and subjected to similar precautions, including individual wire bottom cages and sterile drinking water, both of which were changed daily. Three weeks after weaning, serum, red cell, liver and mucosal folates were measured by a microbiological technique. Villous height, crypt depth and cell turnover rates in the small intestine were also measured. Growth rates and food intakes were recorded daily in all groups of rats.

(ii) Folate assay: Microbiological assay methods are usually employed for assaying folate in biological materials (Stokstad & Koch, 1967). New techniques using radioactive precursors have now been developed, but have been applied principally to human serum folate assays, and are expensive. A reproducible assay using <u>Lactobacillus casei</u> was therefore developed. Microbiological assays are in general not highly reproducible because they rely on the growth properties of an organism and are subject to biological variation and to contamination with other organisms if sterilisation procedures are not employed. The development of an assay using a strain of <u>Lactobacillus casei</u> which was resistant to chloramphenicol (chloromycetin), greatly improved the reproducibility of the conventional assay described by Herbert (1961) and required less rigid sterilisation

procedures, although these were usually retained for an additional safety margin. The new method was based on that described by Davis <u>et al.</u> (1970) and Millbank <u>et al.</u> (1970). Despite the improvement in reproducibility a 10 - 15% variation still existed between samples measured on different assay runs. Subsequently, batches of samples from each experiment were run together with the same assay ie. control and folate deficient animal samples, were compared under the same conditions on the same day. All samples were measured in triplicate.

<u>Assay procedure:</u> All dilutions, and buffers, culture media and sample preparations were carried out using double deionised, sterile water (using Elgastat deioniser and autoclaving at 151b/square inch for 15 minutes). Where possible all equipment was cleaned in acid, rinsed in double deionised, sterile water and sterilised prior to use in the assay.

Samples of serum, whole blood and liver were taken for measurement of folate content immediately after sacrifice. Intestinal epithelial cells were isolated (Section 2-2 ) and all fractions were pooled for analysis of folate. Samples of serum were stored at  $-20^{\circ}$ C. Samples of whole blood (0.3ml) were mixed with 2.7ml of freshly prepared ascorbate buffer (1.36% KH<sub>2</sub>PO<sub>4</sub>, 1.42% Na<sub>2</sub>HPO<sub>4</sub>, 1% ascorbic acid, pH7.4) and stored at  $-20^{\circ}$ C. Packed cell volume (haematocrit) was measured in fresh whole blood. Liver (50mg) was homogenised in 1ml of the ascorbate buffer, and 2ml of mucosal cell suspension was homogenised in 2ml of ascorbate buffer. Both liver and mucosal homogenates were frozen and thawed twice very rapidly to kill any contaminating bacteria and then incubated for 5 hours at  $37^{\circ}$ C to release folic acid analogues from protein bound complexes. The samples were then filtered through Whatman No. 1 filter papers, and the filtrates stored at  $-20^{\circ}$ C. Mucosal cell protein concentration was determined by the technique described in Section 2 - 2.

A culture of <u>Lactobacillus casei var Rhammous</u> which was resistant to chloramphenicol, was maintained in a Lactobacillus broth containing

lml of 300mg% chloramphenicol solution. Subcultures were taken fortnightly to maintain the activity of the culture.

Folic acid Casei medium (Difco Ltd.) was prepared (4.7%) using double deionird, sterile water and autoclaved at 151b/sq. in. for 10 minutes. An overnight culture was prepared ready for the assay the next day by culturing lml of Lactobacillus casei culture in 8ml of folic acid Casei medium containing lml of 100mg% chloramphenicol solution at 37°C for 12 hours. On the day of the assay the overnight culture was washed 3 times with 10 ml of folic acid Casei medium containing 10ml of 100mg% chloramphenicol per litre and 25mg% ascorbic acid (assay medium). The organism was finally suspended in approximately 1 litre of this assay medium to give an opacity of 1.2 Absorbance at 650mu (innoculated assay media). Triplicate samples (500µl) of serum, whole blood, mucosal and liver filtrates and standard folic acid solutions  $(0 \rightarrow 15 \text{ ng/ml})$  were mixed with 5 mls of the innoculated assay medium in sterile test tubes and incubated at 37°C for 18 hours. Following incubation all tubes were vigorously shaken and the absorbance (ie. opacity which corresponds to the density of growth of the organism) measured at 650m; on a Gilford SP240. A blank was set up using uninoculated assay medium to assess the contamination of the assay with other microbiological growth. Dilutions of samples to be analysed were adjusted to fall within the range of the standard curve.

Serum folate (ng/ml), red cell folate (ng/ml), liver folate ( $\mu$ g/g wet weight) and mucosal folate ( $\mu$ g/g protein) levels were calculated.

(<u>iii)Histology</u>: Samples (0.5cm) of everted jejunum were fixed by immersion in Carnoy's fixative (10% glacial acetic acid, 60% absolute alcohol, 30% chloroform), for one hour, followed by two hours dehydration period in absolute alcohol. Samples were then stored in Cedarwood Oil, prior to histological section (4µm) and differential staining with haematoxylin and eosin blue. Villous height and crypt depth were measured in control

and folate deficient animals using an occular micrometer. Measurements were made on three groups of ten villi and crypts per slide and on four sections per animal.

(iv) Cell turnover rates: Cell turnover rates were measured autoradiographically (see Appendix 1). Half of the animals from each group were injected with <sup>3</sup>H thymidine (100µCi/100g body weight), 4 hours prior to sacrifice, the other half 22 hours prior to sacrifice. Samples of distal jejunum were fixed and unstained histological sections (4µm) were prepared on 3" x 1" slides. Autoradiographic techniques were established according to the method of Kopriwa & Leblond (1962). The histological sections were dewaxed and stored under distilled water for no more than 1 hour prior to coating with nuclear emulsion. All subsequent operations were carried out in a darkroom kept at 20°C with an electric fan and equipped with three Ilford S safety lamps (No. 902). Nuclear emulsion (K5 Ilford Ltd. K5 has a mean crystal diameter of  $0.2\mu$  and is sensitive to all charged particles) was heated at 50°C in a clean beaker, with very gently stirring until dissolved. It was then filtered very carefully through fine muslin into another clean beaker in order to remove air bubbles, and then kept at 50°C until required. Occasional gentle stirring prevented local overheating. The slides were carefully dried with tissues and dipped vertically in the emulsion so that the section was covered, and then carefully removed after 2 seconds, keeping the slide vertical. The emulsion was drained off the slide and the back of the slide wiped clean, leaving a fine even film of emulsion coating the section. Thick layers of emulsion would affect the final degree of resolution of the section under the light microscope. The slides were left to dry vertically and then placed horizontally in slide boxes with the section side uppermost, containing 30g of silica gel per 30 slides. The boxes were carefully wrapped in 3 layers of opaque, light proof black paper. The boxes could then be removed from the darkroom and stored at 4°C. After 6 weeks the slides

were developed. Extreme care had to be taken to keep all the developing reagents at  $16^{\circ}$ C or less, or the nuclear emulsion 'dissolved' off the slides. The slides were developed horizontally, section side uppermost, in small plastic slide boxes with large holes cut in the sides to allow entry of solutions. They were first soaked in distilled water for ten minutes and then developed in ID19 solution (0.1% metol, 0.44% hydroquinone, 3.6%sodium sulphite, 2.4% sodium carbonate and 0.2%potassium bromide) with continuous agitation for 16-18 minutes depending on the temperature (Ilford Handbook). The development was stopped by dipping the slides in an acid bath (1% acetic acid) for 10 minutes. The slides were then left under cold running tap water for 15 minutes and dehydrated by successive immersion in 96\% alcohol and 2 x 100\% alcohol. The slides were then removed from the darkroom and stained differentially with haematoxylin and eosin and then permanently mounted.

When examined under the light microscope, cells which contained radioactivity were covered by black dots of precipitated silver. Villous height, crypt depth and the height of the black dots were measured using an occular micrometer. Epithelial cell turnover time and migration rate were then calculated. Cell turnover time is the time for a cell to move from the base of the crypt to the tip of the villus assuming a constant migration rate (hours); migration rate is the distance travelled by a cell in unit time (µm/hour).

(v) Blood data: Samples of heparinised blood were analysed for red cell counts and white cell counts, using the Coulter counter in University College Hospital Pathology Department. Measurements of haematocrit value and haemoglobin concentration (Wootton, 1964) were made in the laboratory.

### 2 - 1c: RESULTS

(i) <u>Growth rates</u>: The growth rates for each group of animals are given in Figure 2 - 1c. Average daily food intakes for each of 3 weeks are given in Table 2 - 1a. Control animals with no antibiotic grew at a comparable rate to other animals of the same strain in the department fed on a standard pelleted diet. Control animals on the diet containing antibiotic grew less well and had mild diarrhoea for about the first 10 days, but this subsequently cleared. Animals fed the deficient diet grew poorly in comparison with both control groups. The mean final weights at 6 weeks of age were  $59.2g \pm 0.45g$  in deficients,  $81.9 \pm 2.06g$  in controls and  $72.4 \pm 2.84g$  in controls with antibiotic. Hence, at 24 days folate deficient rats weighed significantly less than all controls (p<0.001). The fourth group which were pair fed to the deficient animals with control diet containing antibiotic, grew as well as the control group fed <u>ad</u> <u>libitum</u>. Food intakes were not significantly decreased in the deficient group.

(ii) Folate Status: Table 2 - 1b summarises the mean values and standard errors of each animal group for red cell, serum, liver and mucosal folates. Figure 2 - 1d, demonstrates the distribution of folate levels within each group which is probably in part due to the variability of a microbiological assay. However, the folate status of the group fed the folate deficient diet was significantly poorer than all control groups, particularly with respect to serum (10.0  $\pm$  3.8 ng/ml v 62.5  $\pm$  9.8ng/ml in control + antibiotic, p<0.001) and mucosal (14.6  $\pm$  2.4 µg/g protein 39.8  $\pm$  4.4 µg/g protein in control + antibiotic, p<0.001). Red cell folate (196  $\pm$  31.0 ng/ml in folate deficients v 600  $\pm$  85.0 ng/ml in controls + antibiotic, p<0.01) and liver folate levels (6.5  $\pm$  2.5µg/g wet wt. in folate deficients v 17.9  $\pm$  2.3 µg/g wet wt. in controls + antibiotic, p<0.01) were also reduced. The folate levels in each of the tissues of control groups were similar to the values recorded by Klipstein <u>et al.</u>(1973b).



## FIGURE 2 - 1c

Growth rates of weanling rats (42 days old) fed control, control + antibiotic, folate deficient or control + antibiotic (pair fed) diets. Mean values for groups of 8 animals (21- 41 days). At 42 days of age mean values ± standard errors are given - rats fed the folate deficient diet weighed less than all the control animals(p<0.001)

# TABLE 2 - la

Average daily food intakes (g) of control, control + antibiotic, pair fed and folate deficient animals for 3 weeks post weaning.

	Week 1	Week 2	Week 3
Control	4.1±1.1	7.1±0.7	8.3±0.9
Control + Antibiotic	3.8±0.9	7.6±0.6	8.0±0.7
Deficient	3.9±1.2	7.9±0.7	7.8±0.7
Pair fed	3.9±1.2	7.9±0.7	7.8±0.7

Means ± Standard Errors from groups of 8 animals.

## TABLE 2 - 1b

<u>Tissue folate levels of control, control + antibiotic.</u> pair fed. and folate deficient animals (42 days old).

Tissue	Control	Control + Antibiotic	Deficient	Pair fed
Red Cell folate (ng/ml)	684.4±116.0	600 ±85.0	196 ±31.0*	618.1±66.2
Serum folate (ng/ml)	88.2± 12.2	62.5± 9.8	10.0± 3.8***	76.9± 7.6
Liver folate (µg/g wet wt)	17.0± 1.3	17.9± 2.3	6.5± 2.5	18.3± 5.0
Mucosal folate (μg/g protein)	49.6± 5.3	39.8± 4.4	14.6± 2.4***	41.3± 5.2

Mean values ± standard errors from groups of 8 animals.

\* p < 0.05 compared with pair fed group, p < 0.01 compared with other groups.

\*\* p < 0.01 compared with all control groups.</pre>

\*\*\* p < 0.001 compared with all control groups.



## FIGURE 2 - 1d

Folate status of rats (aged 42days) fed control or folate deficient diets. Tissue folates were measured by a modified <u>Lactobacillus casei</u> assay. Mean values ± standard errors from groups of 8 animals.

continued over page.....



FIGURE 2 - 1d (continued from previous page)

(iii) Mucosal Histology: Figure 2 - le demonstrates the villous heights and crypt depths of each animal group. The villous height of sections from folate deficient animals was reduced compared with the control groups (410  $\pm$  30µm in folate deficients v 480  $\pm$  23µm in controls + antibiotic), but this difference was not significant. Crypt depth was much greater in deficients than all controls (220  $\pm$  10µm in folate deficients v 150  $\pm$  10µm in controls + antibiotic, p<0.01). There was no difference in villous height or crypt depth between the control groups. Figure 2 - lf illustrates the morphological appearance of sections from the jejunum of control and folate deficient animals.

Cell turnover rates: Figure 2 - 1g demonstrates the rate of cell (iv) migration up the villus, and the time it takes for a cell to migrate from the base of the crypt to the villus-tip ie. cell turnover time. In folate deficient animals there was a slowing of the cell migration rate from 10.0  $\pm$  0.95µm per hour in controls + antibiotic, to 6.4  $\pm$  0.83µm per hour, (p<0.01). Consequently, because the total villous and crypt height were not altered in folate deficiency the cell turnover time was increased from  $63.0 \pm 6.28$  hours in controls + antibiotic, to  $98.4 \pm 7.9$  hours, (p<0.01). There was a problem in calculation and interpretation of migration rates and therefore cell turnover rates in folate deficient animals because the leading line of labelled cells (ie. those at 22 hours post injection of labelled thymidine) were still in the crypts. It is not known if the migration rate changes at the crypt-villus junction, and it is therefore more accurate to compare groups of animals with one time point of cells on the villus, with another time point where cells are still in the crypt. The fact that the cells were still in the crypt at 22 hours - even when allowance was made for the increased crypt depth in folate deficiency - was still indicative of a slower cell migration and hence increased cell turnover rate. (In fact

68





\* p<0.01 compared with all control groups.</pre>

FIGURE 2 - le

Villous height ( $\mu$ m) and crypt depth ( $\mu$ m) of the jejunal mucosa of control, control + antibiotic, folate deficient and pair fed rats.

## FIGURE 2 - 1f

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Light microscopic appearance of the jejunal mucosa from control, control + antibiotic, folate deficient and pair fed control rats.

Photomicrograph A: control intestinal mucosa.

- B: control + antibiotic intestinal mucosa.
- C: folate deficient intestinal mucosa (3 photographs)
  - D: pair fed intestinal mucosa.

The villi of the folate deficient mucosa appear swollen but there is no significant difference in overall villous height between all control groups and the folate deficient group. Crypt depth is greater in the folate deficient mucosa (p<0.01 compared with all control groups).








# FIGURE 2 - 1g

<u>Cell migration rate (µm/hour) and cell turnover time (hours) in control,</u> <u>control + antibiotic, folate deficient and pair fed control rats.</u> Mean values ± standard errors from groups of 8 animals.

# p<0.01 compared with all control groups.</pre>

a subsequent study at 36 hours post injection of labelled thymidine in folate deficient animals, showed a slight but not significant decrease in the calculated turnover rate (Section 2 3)).

It must be remembered that the method assumes a linear progression of cells up the villus. Since the rat villus has a tapering villus-tip, and if no cell loss occurs until the villus-tip is reached, the rate of migration must increase as the villus-tip is approached, since the cells are migrating over a basement membrane whose surface area is steadily diminishing. This means that transit times calculated by extrapolation from measurement of migration between two points will be greater than in reality. However, the difference is likely to be serious only where the taper of the villus is marked (Clarke, 1974).

Using female albino Wistar rats (150-200g) Gleeson et al. (1972a) noted migration rates of 11.4±4.6µm/hour and 8.8±1.2µm/hour in jejunum and ileum respectively, and cell turnover times of 41.1±4.1 hours and 35.9±4.9 hours in jejunum and ileum respectively. The control groups in this study had similar migration rates but slower cell turnover rates in the jejunum due to a comparatively higher jejunal villous height (500±41µm) than that reported by Gleeson et al. (1972a) (393±24µm). Batt & Peters (1976) reported jejunal villous heights and crypt depths in male Wistar rats (200-300g) of 510µm and 190µm respectively, but migration rates of 17µm/hour and cell turnover times of 35 hours. Other reports have estimated cell turnover time in the rat to be 2-3 days (Williams et al. 1958; Koldovsky et al. 1966; Sunshine et al. 1971). The rate of cell turnover (63.0±6.28 hours) in control rats (80-85g) reported here, therefore falls within this range. Differences in villous height and cell turnover times could be a reflection of species differences, differences in diet or possibly an artifact of the fixation procedure. The age of the animals studied may also be relevant since the intestinal mucosa of young rats has a slower rate of cellular proliferation and migration as compared to that of adult animals (Koldovsky et al. 1966).

## FIGURE 2 - 1h

Autoradiographs of mucosal sections prepared 4 and 22 hours after administration (intraperitoneal) of  ${}^{3}$ H labelled thymidine (100 $\mu$ Ci/100g body weight), from the jejunum of control and folate deficient rats.

Photomicrograph A: control - 4 hours post <sup>3</sup>H injection.
" B: control - 22 hours post <sup>3</sup>H injection.
" C: folate deficient - 22 hours post <sup>3</sup>H injection
(2 photographs).

Dark spots of precipitated silver over cell nuclei correspond to labelled cells ie. those cells in, or from, the proliferative crypt zone of the mucosa. At 22 hours labelled cells are found on the lower villus in controls, whereas in folate deficient mucosa only the crypts are labelled.



A (X 190)



<u>B</u> (X 190)



<u>c</u> (X 190)



<u>c</u> (X 190)

## TABLE 2 - 1c

<u>Blood composition data</u>: Haemaglobin concentration (mg%); packed cell volume (%); white blood cell count  $(x10^9/1)$ ; red blood cell count  $(x10^{12}/1)$  of rats fed control, control + antibiotic, folate deficient, and control + antibiotic (pair fed) diets.

ANIMAL GROUP	Hb mg%	PCV %	WBC (x10 <sup>9</sup> /1)	RBC (x10 <sup>12</sup> /1)
CONTROL	12.18±0.57	33.89±3.5	33.72±2.52	3.76±0.85
CONTROL + ANTIBIOTIC	11.85±1.14	37.25±5.0	÷	
FOLATE DEFICIENT	15.76±1.83	27.50±5.0	37.86±6.18	3.31±0.20
PAIR FED	13.01±1.08	34.71±4.6	-	

Mean values ± standard errors from 8 animals.

No significant differences between deficient and control values for each parameter.

(v) <u>Blood Composition Data</u>: Table 2 - 1c shows the data on blood composition compiled from analysis in the laboratory or the hospital pathology department. Haemaglobin concentration was slightly increased and packed cell volume (PCV) slightly decreased in the deficient group, but these differences were not significant. White blood cell count and red cell counts were the same in control and deficient groups.

#### 2 - 1d: DISCUSSION

Klipstein <u>et al</u>. (1973b) reported changes in folate status of rats born to mothers fed a diet deficient in folate, accompanied by diarrhoea and caecal enlargement, but with no abnormalities of total mucosal height or villous structure. In the present study further measurements have been made on animals used in a similar model for producing experimental folate deficiency.

Growth rates of animals fed the deficient diet were slower compared with the controls. This was not due to the addition of antibiotic to the diet because the control group with added antibiotic also had a faster growth rate, although not as fast as controls without added antibiotic. The control group with antibiotic had a watery diarrhoea over the first ten days post weaning with an initial lag in growth. The diarrhoea subsequently cleared and growth accelerated at a rate parallel to ordinary controls. The deficient group had diarrhoea continuously for the 3 week post weaning period, and a careful watch on cage cleanliness had to be made throughout the day. The diarrhoea associated with the addition of sulfasuccidine to the diet, which was also accompanied by an increase in caecal size, was probably related to associated changes in the small intestinal flora (Loesche 1969; Klipstein & Lipton 1970). The diarrhoea which persisted after 10 days in the folate deficient rats was probably due to the malabsorption of one or more nutrients. Food intakes in each group were closely similar and the group pair fed to the deficients grew as well as the controls with antibiotic suggesting that the deficient

rats had an impairment in absorption, which could also be contributing to their depressed rate of growth. Goetsch and Klipstein (1977) found normal absorption of monosaccharides, fat and amino acids, but impaired transport of sodium and water, and it could be this which contributes to the diarrhoea of folate deficiency (see Section 2-3).

A decrease in the level of folate in red cells and serum, was accompanied by reductions in liver and mucosal folate levels in rats fed the deficient diet. The addition of antibiotic to the diet, also decreased the folate concentrations in red cell, serum and mucosa, but not significantly in comparison with the decreases in the deficient diet group. By decreasing the number of folate producing bacteria in the gut with the addition of antibiotic to the diet, the supply of available folate to the rat was limited to the diet alone. Coupled with the initial period of diarrhoea, folate supply to this group of controls was therefore slightly reduced. Serum leveis in particular reflect acute changes in folate status, but tissue folates, particularly liver, demonstrate the state of body stores of folate. The liver folate concentration was not altered in control rats fed antibiotic and their overall folate status was therefore not considered to be significantly altered. The rats fed a deficient diet as well as the antibiotic had a minimal supply of folate, and this was reflected in tissue folates such as liver and red cell folates. Serum folate levels were especially reduced, reflecting the lack of supply of folate immediately from the diet. Folate status of the mucosal enterocytes was also considerably reduced in parallel with the other tissues. Feeding the folate deficient diet did not produce anaemia in the rats.

Intestinal structure was normal in control rats with or without added antibiotic, but the folate deficient group had morphological changes with respect to an increase in crypt depth. Villous height was decreased (although this was not significant) such that total mucosal height was not altered. In addition, the villous structure, and the degree of inflammatory cell infiltration within the lamina propria were not altered in the

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deficient group. However, the enterocytes appeared larger which Klipstein et al. (1973b) attributed to being analagous to megaloblastic changes. Cell migration rate was slower in the deficient rats, yet villous height remained normal. Villous height is determined by the dynamic process which exists between cell production and cell loss. If the rate of one process is altered the rate of the other must be altered in a direct relationship (Creamer 1973). In the deficient animals the increase in crypt depth and megaloblastic appearance of the enterocytes suggests some impairment in the production of new cells. If cell migration rate were to be maintained at the same rate as in the control animals, villus height would be expected to be decreased. That villous height in folate deficiency is not altered, suggests that the rate of cell loss is reduced, possibly reflected by the slowing of cell migration. This decrease in the cell migration rate and maintenance of total villous height, means that cell turnover time is increased. Since the normal course of events is that cells gradually differentiate and mature as they increase in age and migrate up the villus (Creamer 1973; Weiser 1973), the folate deficient cells will have the opportunity to mature for a longer period ie. become 'hypermature' and this may be reflected in terms of increased acquisition of enzyme activities. The importance of this concept and its implications in absorption in folate deficiency are discussed in Section 2 - 3.

From this series of measurements it was concluded that feeding a diet deficient in folic acid and containing an antibiotic such as sulfasuccidine, to rats born to mothers fed on the same diet one week prior to giving birth, resulted in a depletion of folate levels in serum, red cell and liver. These changes in folate status were also reflected in the mucosal enterocyte levels. The addition of antibiotic to the diet contributed to these changes, but addition of folate to this diet allowed tissue stores, particularly those of the liver, to remain normal. All the tissues studied reflected any change in folate status, but since serum

levels probably reflect more acute changes in folate status it was decided in subsequent studies to use red cell and mucosal folate levels as routine indices of folate status. Haematological indices gave no useful index of folate status as has been found in previous studies on acute folate deficiency (Klipstein et al. 1973b; Asfour, 1975). Growth rates gave a useful index of possible folate deficiency prior to sacrifice of the rats, and were monitored routinely together with food intakes and observations of the persistence or not of diarrhoea.

The fact that food intake was not contributing to the changes in the growth rate of folate deficient animals was demonstrated by the normal growth of the pair fed group compared with <u>ad libitum</u> controls on the same diet. The pair fed group was therefore omitted from subsequent studies. The addition of antibiotic to the diet produced minor changes in folate status and growth rate, but there were no changes in mucosal morphology and cell turnover rates. It was decided to retain this group along with a control group containing no antibiotic for further comparison with the folate deficient group in subsequent experiments.

#### SECTION 2 - 2

#### **ISOLATED CELL PROCEDURE:**

## CHARACTERISATION OF CELLS & ASSESSMENT OF VIABILITY

## 2 - 2a: INTRODUCTION

(i) Isolation procedure: An account of the numerous methods available for isolating gut epithelial cells was given in Section 1 - 6. It was concluded that citrate or enzyme dissociation methods produce the most consistently viable isolated cell preparations. Studies by Weiser (1973) employed such a technique for sequentially separating nine fractions of cells from different regions of the crypt and villus. Cells from villous and crypt regions have different functions and probably different composition in terms of DNA/RNA/protein contents. It was important therefore to choose a method which would result in reproducibly sequential fractions of cells and so the method described by Weiser was adapted for this study. The cell populations were characterised with respect to enzyme and functional markers. A thorough evaluation of the morphology and functional integrity of these cells was then carried out to establish their suitability for incubation studies. Whether folate deficiency, because of its effect on gut morphology and function, would effect the cell preparations was not known and so the effects of folate deficiency on the separation and viability of the cells was also assessed.

(ii) Characterisation: The cell populations can be characterised according to their enzyme patterns - for example, cells isolated from the crypt where new epithelial cells are synthesised, will have high activities of enzymes associated with DNA synthesis, such as thymidine kinase, but low activities of enzymes associated with absorptive function such as disaccharidases. On the other hand cells isolated from the villus-tip will have high activities of the disaccharidases and low activities of thymidine



Diagram of a single villus of the small intestinal mucosa,



kinase (See Figure 2 - 2).

Another means of characterising the cell types is the incorporation of tritiated thymidine ( ${}^{3}$ HTDR) into DNA <u>in vivo</u>. Intraperitoneal administration of  ${}^{3}$ HTDR, 22 hours prior to removal of the gut, has been shown by autoradiographical techniques to be incorporated into the crypts and lower villus of the mucosal villi. Cell populations sequentially isolated 22 hours following administration of  ${}^{3}$ HTDR should therefore only have detectable counts in the DNA of crypt and lower villous fractions. Radioactivity detected in other fractions could indicate the degree of contamination of villous fractions by crypt cells and villus base cells.

(iii) Viability: In order to study the integrated metabolism and function of epithelial cells a preparation was needed that contained large numbers of epithelial cells which were undamaged by the isolation procedure and uncontaminated with connective tissue cells or mucus. The integrity of isolated cells cannot be assessed merely by the measurement of metabolic activity. Similarly studies of morphology give no indication of the functional capacity of the cells. A comprehensive assessment of integrity can only be made by considering collectively the results of morphological and metabolic studies.

There are many parameters which can be used to evaluate the viability of isolated cell systems. These parameters can be broadly defined in 3 groups:

- (a) membrane integrity and morphological appearance
- (b) respiratory and metabolic function of the cell
- (c) functional capacity

Membrane integrity has frequently been assessed by the ability of the cells to exclude colour dyes eg. Trypan blue and nigrosine. Intact cell membranes do not allow the large dye molecules to enter the cells, whereas damaged cells become stained by the dye molecules. Intact cells also retain

their normal intracellular concentrations of protein, enzymes and ions such as sodium and potassium. Cells which begin to leak protein and enzymes, and do not retain the normal low intracellular  $Na^+$  concentrations and high  $K^+$  concentration compared with the surrounding medium, are no longer viable. Examination of morphological appearance by light or phase contrast microscopy can be useful to complement these studies on membrane integrity.

Respiratory activity can be assessed by measuring oxygen consumption and end products of respiratory pathways, such as lactate, pyruvate and carbon dioxide production. The ATP content of cells will also remain stable if the cells are metabolising normally.

With respect to the nature of the <u>in vitro</u> investigations to be carried out on the isolated cells, some assessment of the normality of their function had to be made, and where possible, compared with <u>in vivo</u> results on function. In absorption and transport studies of sugars and amino acids for example, it is important that isolated intestinal cells can generate concentration gradients of these molecules. Investigations into protein and DNA synthesis should only be made on cells which can linearly incorporate labelled precursors such as leucine and thymidine respectively.

For the development of the isolated cell technique in this study, investigations were made on the morphological appearance, the ability to exclude Trypan blue dye and to maintain concentration gradients of sodium and potassium ions, and the rates of carbon dioxide production and oxygen consumption. Studies were also made on the ability of the cells to incorporate tritiated thymidine into DNA in relation to subsequent studies on DNA metabolism and will be discussed in the relevant section (ie. Section 2-4).

2 - 2b: METHODS

Fractions containing individual epithelial cells were isolated from 3 groups of animals (control, control and antibiotic, folate deficient - 8 animals per group). Growth rates and serum, liver, red cell and mucosal folate status were assessed as described in Section 2 - 1.

(i) Isolation procedure: Details of the buffers used and schematic design of the method for isolating epithelial cells are given in Figures 2 - 2a & 2 - 2b. All buffers were pregassed, (95%02, 5%002) for 15 minutes prior to use. The upper 3/5ths of the small intestine (excluding duodenum) was excised and rinsed with ice cold normal saline, containing 0.015% dithiothreitol (buffer A), and its length measured (vertically using a standard 5g weight). The distal end was occluded and the lumen filled with buffer B (0.06ml/cm intestine ie. without distention of the gut wall). After occlusion of the proximal end, the gut was incubated at 37°C in a phosphate buffered saline bath at 37°C, with continuous gassing. After 15 minutes buffer B was discarded together with excess mucus and digestive material. The gut was refilled with successive washes of buffer C (containing 0.056% ethylenediamine tetra-acetic acid, 0.01% dithiothreitol), and incubated with continuous gassing for the times listed in Figure 2 - 2b. Citrate dissociates Calcium ions (Ca<sup>++</sup>) from connective tissue, releasing individual cells. Dithiothreitol solubilises mucus and helps prevent cell aggregation. For washes 1 - 6, 0.06ml/cm intestine of buffer C was used, and for washes 7 - 9, 0.1ml/cm intestine, of buffer C was used. The luminal contents containing epithelial cells were collected in plastic tubes, on ice, after each incubation. The cells were gently centrifuged (500 x g for 1 minute) and washed twice with ice cold oxygenated phosphate buffered saline. The final cell pellet was weighed and resuspended in ice cold pregassed Krebs Henseleitt buffer (Krebs & Henseleitt, 1932), (approximately 10 x wet weight of pellet-

### FIGURE 2 - 2a

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Buffers for epithelial cell isolation procedure.

- A 0.90% NaCl pH 7.3 0.015% Dithiothreitol (DTT)
- B 0.79% Na Citrate In phosphate buffered saline (Ca<sup>++</sup>, Mg<sup>++</sup> free) pH 7.3
- C 0.056% EDTA In phosphate buffered saline (Ca<sup>++</sup>, Mg<sup>++</sup> free) pH 7.3 0.01% DTT

## Notes

All buffers are free of  $Ca^{++}$  and  $Mg^{++}$  ions. Citrate dissociates  $Ca^{++}$  and  $Mg^{++}$  ions which are associated with connective tissue, thus releasing individual cells. DTT solubolises mucus and helps prevent cell clumping.

Phosphate buffered saline (  $Ca^{++}$  and  $Mg^{++}$  free):

0.02% KC1 0.02% KH<sub>2</sub>PO<sub>4</sub> 0.80% NaC1 0.115% Na<sub>2</sub>HPO<sub>4</sub>

## FIGURE 2 - 2b

Method for isolating rat, small intestinal epithelial cells, to give nine sequential fractions representing cells from the villous tip to the crypt base.

- Note: All buffers gassed prior to (15 mins) and during the procedure. Cells collected in plastic tubes or siliconised glassware.
- 1. Intestine excised, rinsed, ice cold Buffer A.
- 2. Distal end occluded, lumen filled with phosphate Buffer B.
- 3. Incubated in phosphate buffered saline bath, 37°C for 15 mins.
- Gut emptied and steps 2 and 3 repeated using citrate Buffer C and incubated for the following times to give 9 sequential cell fractions.

Fraction		Time (mins)	Volume Buffer ( ml/cm intestine
Villus•	- 1	5	0.06
Tip	2	4	0.06
Cells	3	4	0.06
Mid-	4	5	0.06
Villus	5	5	0.06
Cells	6	6	0.06
Crypt Cells	7	7	0.10
	8	10	0.10
	9	15	0.10

 Cells collected in plastic tubes and washed with phosphate buffered saline and suspended in ice cold Krebs Henseleitt Buffer with a plastic pipette. Kept on ice until required. equivalent to approximately 200µg DNA/ml), using a plastic pipette, and stored on ice until required. Cells could be stored on ice for up to 4 - 5 hours before use without impairment to viability.

(ii) Sucrase assay: Aliquots of each cell fraction were homogenised (Kontes glass homogeniser-clearance 200µ), and centrifuged for 20 minutes at 1000 x g,  $4^{\circ}$ C. The supernatant was assayed for sucrase activity by a method based on that of Dahlqvist (1968). Enzyme activity remained constant for at least 6 months after storage at  $-20^{\circ}$ C. All samples were assayed in duplicate. Enzyme solution (100µ1) was incubated with 100µl substrate buffer containing 1.92% sucrose in 0.1M sodium maleate buffer pH 6.0, at 37°C, with continuous agitation. After one hour samples were boiled for 2 minutes to inactivate the enzyme. For each sample a control was set up by boiling the enzyme and substrate buffer prior to incubation. The glucose produced was assayed using a standard kit of glucose oxidase reagent. (GOD-Perid method - Boehringer Mannheim Ltd.). This reagent contains a small amount of sucrase which was inactivated by heating to 60°C for 15 minutes, without affecting the activity of the glucose oxidase. The glucose oxidase reagent (5ml) was added to standards of glucose (20, 40, 60, 80,  $100\mu g$  glucose/200µl) and the incubated enzyme and substrate buffer mixture, and the colour reaction allowed to develop over 40 minutes at room temperature. Absorbance at 590 mp was measured in a Gilford 240 spectrophotometer.

Protein content was determined by the Lowry technique described on Page 95.

The concentration of enzyme solution was adjusted so that glucose production was linear for the period of incubation and concentration of substrate used.

Sucrase activity was defined as µg glucose produced per µg enzyme protein per hour.

(iii) Thymidine kinase assay: Aliquots (2ml) of each cell fraction were homogenised (Kontes glass homogeniser-clearance 200µ) and centrifuged for 25 minutes at 15,000 x g, 4°C. The supernatant was assayed for thymidine kinase based on a number of published methods, but with particular reference to those of Breitman (1963), Ives, Morse & Potter (1963), Klemperer & Haynes (1968). Conditions and techniques for the assay procedure were adapted to give optimal activities in gut cells. Thymidine kinase activity is measured by its ability to convert thymidine to thymidylate (dTMP). The enzyme was stable for up to 6 months when stored at -20°C. All samples were assayed in duplicate. Enzyme solution (250µ1) was incubated with an equal volume of substrate buffer containing 10mM ATP. 5mM MgCl2, 10mM phosphoglyceric acid, 0.1M Tris-HCl buffer pH 7.4 and 4.68 x  $10^{-3}$  µmoles <sup>14</sup>C-thymidine (58mCi/m mole) at 37<sup>o</sup>C with continuous agitation. After 20 minutes the samples were boiled for 2 minutes to inactivate the enzyme. Precipitated protein was removed by centrifugation at 1000 x g, for 5 minutes. Controls for each sample were set up by boiling the enzyme and buffer prior to incubation.

Sample or control supernatants  $(100\mu1)$  were pipetted on to discs of DEAE cellulose paper (4cm diameter, Whatman Diethylaminoethyl Cellulose Papers DE20), which were immediately placed on a scintered glass filter funnel, and washed under gentle vaccum pressure (150mm Hg) with the following solutions:

- (a) 10ml ammonium formate (0.001N pH4.5)
- (b) 10ml distilled water
- (c) 10 ml ammonium formate (0.001N pH4.5)
- (d) 10ml distilled water
- (e) 10ml ethanol

All the free thymidine was removed, leaving  $^{14}C-dTMP$  bound to the DEAE cellulose disc. The extent of removal of free thymidine was assessed from blank samples where enzyme solution was replaced with Krebs Henseleitt (250µl) or from the control samples. That  $^{14}C-dTMP$  was bound to the DEAE

cellulose paper was checked using a standard  $^{14}$ C-dTMP solution (4.68 x  $10^{-3}$  ]moles, 58mCi/m mole).

The discs were air dried overnight, placed in scintillation vials containing 18ml of scintillation cocktail (0.4% 2, 5-diphenyloxazole (PPO) in toluene/triton X 100 (2:1 v/v) and counted in a Packard 2420 or Nuclear Chicago Delta 300 liquid scintillation counter to an efficiency of 3%.

The counting efficiency was reduced by the binding of the labelled dTMP to the paper discs. The reduction in efficiency was determined using a  $^{14}$ C-dTMP standard solution (4.68 x  $10^{-3}$ µmoles, 0.1µCi. Specific activity, 58mCi/m mole) counted both with and without binding to a DEAE cellulose disc. All results were adjusted to account for this reduction in counting efficiency as well as for the efficiency of the wash procedure (ie. less the control or blank value).

The protein content was determined by the Lowry technique described on Page 95.

The concentration of enzyme was adjusted so that the production of thymidylate was linear for the time of incubation and concentration of substrate used. Thymidine kinase activity was defined as p moles dTMP produced per  $\mu$ g enzyme protein per hour.

(iv) <sup>3</sup>H thymidine incorporation in vivo: Half of each group of animals (ie. 4 animals per group) were injected intraperitoneally with <sup>3</sup>Hthymidine 22 hours prior to separation of enterocytes. The DNA content of the cell suspensions was extracted and quantitatively assessed using a diphenylamine method based on that described by Croft & Lubran (1965). An aliquot (0.5ml) of DNA hydrolysate was added to 6ml of scintillation cocktail (0.4% PPO in toluene/triton X 100, 2:1 v/v) and the radioactivity counted in a Packard 2420 to a 3% level of efficiency. DNA extraction: DNA was extracted from homogenised samples of the cell fractions, after several washings with 2% PCA (to remove any free



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intracellular thymidine) using hot 10% PCA. The method is summarised in Figure 2 - 2c.

Diphenylamine Reaction: Standard Solutions  $(8 \rightarrow 40 \mu g \text{ hydrolysed DNA/}$ 500µl) and sample hydrolysates (500µl) were mixed with 500µl of freshly prepared diphenylamine reagent (2% diphenylamine, 49ml glacial acetic acid, lml conc. H<sub>2</sub>S0<sub>4</sub>,0.25ml aqueous 2% acetaldehyde). The reaction was allowed to develop at 30<sup>o</sup>C for 18-24 hours, when the absorbance at 567mm, was measured in a Gilford SP240 spectrophotometer. This method was modified from that described by Croft and Lubran (1965).

Uptake of labelled thymidine was expressed as disintegrations per minute per mg DNA (DPM/mg DNA).

(v) Protein assay: Protein contents of the cell fractions and enzyme preparations were assessed by the Lowry method (Lowry et al. 1951).

All samples were set up in duplicate. Aliquots of cell suspension were homogenised by hand (Kontes glass homogeniser-clearance  $200\mu$ ), and  $100\mu$ l samples were added to  $900\mu$ l of 8% PCA in order to precipitate the protein. The samples were centrifuged for 15 minutes at  $1000 \times g$ ,  $4^{\circ}C$ , and the supernatant discarded. The precipitate was resuspended in  $450\mu$ l distilled water and  $50\mu$ l 3N NaOH was added. The suspension was incubated at  $37^{\circ}C$ , for up to one hour to dissolve the precipitate, which was then diluted to a final volume of 5ml with 0.1N NaOH.

Aliquots  $(500\mu1)$  of standard solutions of bovine serum albumin in O.1N NaOH  $(50\mu g + 1000\mu g)$  and samples were mixed rapidly with a copper reagent (1ml of a solution containing 0.5% CuSO<sub>4</sub>  $.5H_20$  and 1% Na or K tartrate, mixed with 50ml of 2% Na<sub>2</sub>CO<sub>3</sub> solution). The reaction mixture was left at room temperature for 10 minutes when 250µl Folin and Ciocalteau reagent was added. The colour reaction was left to develop at room temperature for 30 minutes and the absorbance at 700mµ was measured using a Gilford SP-240 spectrophotometer.

This method has been automated in this department using a Technicon Autoanalyser proportionating pump, spectrophotometer and pen recorder. When large numbers of samples were being assayed, this method was used. Samples and standards assayed by hand or by autoanalyser gave comparable results.

(vi) DNA assay: The DNA content of the cell fractions was assayed by a fluorometric technique using ethidium bromide, adapted from that of Boer, (1975). This technique requires the DNA to be present intact and unhydrolysed ie. with an unbroken helix, and is particularly suited to isolated cell preparations which can readily be homogenised to release intact RNA and DNA. In this technique ethidium bromide (2, 7, diamino-10ethyl-9-phenyl phenanthridinium bromide)intercalates with the helical, double stranded DNA, and fluoresces. The measureable fluorescence is then proportional to the DNA present. Samples of cell fractions were homogenised (Kontes glass homogeniser-clearance 200µ) and diluted 1 in 10 with phosphate buffered saline (0.01% CaCl<sub>2</sub>, 0.02% KCl, 0.02% KH<sub>2</sub>PO<sub>4</sub>, 0.01% MgCl<sub>2</sub>.6H<sub>2</sub>O, 0.8% NaCl, 0.115% Na<sub>2</sub> HPO<sub>4</sub> pH 7.5). Triplicate aliquots (100µ1) of sample and standard solutions (containing 10-100ng DNA) were mixed on ice with 50µl pronase (B grade, nuclease free, Calbiochem Ltd) and 50µl RNA-ase (71 K unitz/mg, protease free) to give a final concentration of 200µg/ml and 150µg/ml respectively, and then incubated at room temperature for 30 minutes. Blanks were set up using the phosphate buffered saline alone and incubated with pronase and RNA-ase. The enzymes were then inactivated by dilution with 750µl phosphate buffered saline. Ethidium bromide (50µg/ml) was added (50ul) to two of the triplicate samples or standards. The third sample or standard was used for assessing background fluorescence. Fluorescence was measured at 583mp in a Locarte fluorimeter using filters -blue and orange LF7.

\* 1 K unitz will produce a  $\triangle$ A260 of 0.001/min/ml at pH 5.0 at 25°C using RNA as substrate.

(vii) Oxygen consumption: Cell suspensions were mixed in a siliconised 50ml flask with an equal volume of Krebs Henseleitt incubation medium (to contain at final concentration- 10mg/ml fatty acid free boyine serum albumin (FAFBSA), 250 mg% glucose pH 7.2). A mixed population of cells (ie. from villus-tip, mid-villus and crypt cell fractions) was used. The cells were incubated at  $37^{\circ}$ C with continuous gassing (95% 0<sub>2</sub>, 5% CO<sub>2</sub>) and intermittant, gentle shaking. The depth of cell suspension never exceeded 0.75cm in the bottom of the flask to allow for easy diffusion of gases. Aliquots (1m1) were taken at 0 minutes and thereafter at 10 minute intervals for 1 hour. The rate of oxygen consumption was measured in the oxygen electrode system described below. DNA content of the cells was measured by the fluorometric assay described on Page 96. Oxygen electrode - (refer to Figure 2 - 2d): An oxygen electrode was obtained from Rank Brothers Ltd., which consisted of an air tight incubation chamber surrounded by a water jacket. The electrode was incorporated into the base of the incubation chamber with a platinum electrode (cathode) and a silver/silver chloride electrode (anode). The principle of operation was that first described by Clark et al. (1953). Oxygen diffuses through a thin 0.0005" teflon membrane and is reduced at a platinum surface immediately in contact with the membrane.

 $0_2 + 2E + 2H^+ + H_2 0_2$ .  $H_2 0_2 + 2E + 2H^+ + 2H_2 0$ This allows a flow of current which is proportional to the amount of oxygen present. The current is recorded via a polarising unit, and a change in current recorded represents a change in oxygen concentration. Viable cells which consume oxygen will decrease the oxygen concentration of the suspending medium, and hence the current. The incubation mixture of cells and medium is constantly stirred to prevent a local build up of reduced oxygen in the electrode area.

The amount of oxygen  $(\mu 1)$  in a saturated buffer (incubation medium) was determined (see below). Zero oxygen content can be obtained by adding a small amount of sodium dithionite to the buffer. The rate of



- A air tight stopper. B - water jacket at 37°C. C - cell suspension.
- D plastic coated magnet. R flat-bed recorder.
- E anode  $(Ag/Ag/C1_2)$ .
- F cathode (Pt).
  - G magnetic stirrer.
    - P potentiometer.

# FIGURE 2 - 2d: Oxygen electrode system.

For assessing rates of oxygen consumption of isolated epithelial cells incubated at 37°C, in Krebs Henseleitt medium (containing 10mg/ml FAFBSA: 250mg% glucose).

oxygen consumption in microlitres per minute can then be calculated from the slope of the recorder trace.

<u>Calibration of electrode and recorder trace</u>: At  $37^{\circ}C$ , 0.0235ml of oxygen is dissolved in lml of water, when the atmospheric pressure is 760mm Hg. The partial pressure of oxygen in air is 20.9% of 760mm Hg and therefore contains 20.9% of 0.0235 ml of oxygen (Henry's Law) (Handbook of Chemistry and Physics; Evans, 1969) ie. 4.9µl oxygen per ml of water. The system can therefore be readily calibrated by comparing the height of the recorder trace, with the volume (µl) of oxygen dissolved in known amounts of water or incubation medium.

The rate of oxygen consumption  $(QO_2)$  is expressed as  $\mu$ l oxygen taken up per mg cell DNA per hour.

(viii)Carbon Dioxide production - (refer to Figure 2 - 2e): Measurement of carbon dioxide production from labelled substrate (ie. glucose) was adapted from the method used by Kimmich (1970) on isolated chicken epithelial cells. A mixed population of cells was used for the study ie. from villus-tip, mid-villus and crypt fractions. Cell suspension (lml) was added to 1ml of pregassed incubation medium (Krebs Henseleitt buffer containing 20mg/ml FAFBSA) with 500mg% glucose and 0.1µCi of the corresponding (<sup>14</sup>C)-substrate, (D-6-<sup>14</sup>C glucose; specific activity 59.6mCi/ m mole) in a siliconised 25ml Marie flask. A small glass vial (0.3ml) was placed on the centre well, the flask top closed with a serum stopper, and the flask incubated at 37<sup>0</sup>C with gentle shaking (60 cycles/minute). Flasks were set up in duplicate for incubation periods of 0, 15, 30, 45 and 60 minutes. A blank was also set up whereby the cell suspension was replaced by lml of Krebs Henseleitt buffer (pH 7.2) in order to assess the  $^{14}CO_{2}$ released from glucose by the addition of acid alone. After the appropriate incubation period, 250µl  $2NH_2SO_4$  was injected into the flask to stop metabolic activity. The flasks were removed from the incubation bath, 0.2ml of hydroxide of Hyamine was injected into the glass vials without



- A water bath at  $37^{\circ}C$ .
- B serum stopper.
- C glass vial containing 0.2ml hyamine hydroxide.
- D cell suspension in incubation medium.
- E siliconised Marie flask with central well.

FIGURE 2 - 2e: Carbon dioxide production - incubation system. For measuring rates of carbon dioxide production in isolated epithelial cells incubated in Krebs Henseleitt buffer(containing 10mg/ml FAFBSA; 0.1 $\mu$ Ci D-6-<sup>14</sup>C glucose 250mg%), at 37<sup>o</sup>C. Carbon dioxide released at the end of the incubation period by the addition of 2N H<sub>2</sub>SO<sub>4</sub>, was trapped in the hyamine hydroxide solution after 1 hour incubation at room temperature. removing the stoppers, and they were shaken for a further 60 minutes at room temperature to allow time for complete diffusion of liberated carbon dioxide into the hyamine. That all the carbon dioxide was trapped in one hour was assessed. Addition of 2N  $H_2SO_4$  to 2 ml of incubation medium containing 0.01 µCi, <sup>14</sup>C-bicarbonate (specific activity 59.1mCi/m mole), released <sup>14</sup>CO<sub>2</sub>,which was all quantitatively trapped in 0.2ml of hyamine hydroxide after one hour. The hyamine hydroxide was not added prior to the incubation because it solidifies at 37°C and absorption of <sup>14</sup>CO<sub>2</sub> becomes variable. At the end of the hour the Hyamine vial was dropped directly into scintillation cocktail (0.4% PPO, in toluene/triton X 100 2:1 v/v) and counted for radioactivity in a Packard 2420 liquid scintillation counter.

DNA content of the cell suspension was determined by the fluorometric assay described on Page 96.

Carbon dioxide production was expressed as disintegrations per minute (DPM) in  $^{14}\mathrm{CO}_2$  from  $^{14}\mathrm{C}\text{-glucose}$  per mg DNA.

(ix) Potassium/Sodium intracellular ion ratios: Cellular concentrations of potassium ions  $(K^+)$  and sodium ions  $(Na^+)$  were determined by flame photometry. The method of tissue preparation was adapted from that described by Medzihradsky & Metcalf (1975) in red cells. A mixed population of cells was used for the study ie. from villus-tip, mid-villus and crypt fractions. Cell suspension (5ml) was added to 5ml of incubation medium (Krebs Henseleitt buffer pH 7.2, 20mg/ml FAFBSA, 500µg% glucose) in a siliconised flask and incubated at  $37^{\circ}C$  with continuous gassing  $(95\%0_2, 5\%0_2)$  with occasional gentle agitation. Cell suspensions (1ml) were centrifuged in siliconised glass test tubes at 1000 X g for 2 minutes. After removing the supernatants the pellets were resuspended in isotonic MgCl<sub>2</sub>-Tris buffer (110mM MgCl<sub>2</sub>, 2mM Tris) pH 7.2, and centrifuged again. This removed the extracellular Na<sup>+</sup> and K<sup>+</sup> and did not damage the cells, which when resuspended in incubation medium (Krebs Henseleitt buffer

pH 7.2, containing 10mg/m1 FAFBSA, and  $250 \mu g\%$  glucose) retained their normal respiratory characteristics and morphological appearance. After washing, the pellets were covered with  $500\mu$ 1 concentrated  $HNO_3$ , the tubes were sealed with foil, and then boiled for 1 hour in order to dissolve the cellular material. The digest obtained was diluted with double deionised water and the concentrations of Na<sup>+</sup> and K<sup>+</sup> were measured using an EEL (Harlow, Essex) flame photometer and by comparison with standards for both ions (KC1 0.1-1.6m equivalents/1, NaC1 0.01-0.08 m equivalents/1). DNA content of the cell suspensions was determined by the fluorometric assay described on Page 96.

(x) Dye exclusion: A mixed population of cells was used ie. from villus-tip, mid-villus and crypts. Cell suspension (5ml) was added to 5ml of incubation medium (Krebs Henseleitt buffer pH 7.2, 20mg ml FAFBSA, 500 $\mu$ g% glucose) in a siliconised flask and incubated at 37°C with continuous gassing (95%0<sub>2</sub>, 5%C0<sub>2</sub>) with occasional gentle agitation. At time 0 minutes and at ten minute intervals thereafter for 1 hour, 200 $\mu$ l aliquots were mixed with 200 $\mu$ l of 0.4% trypan blue, and subjected to microscopic and cytographic analysis. The percentage of cells which excluded dye, was assessed.

(<u>xi</u>) <u>Morphological assessment</u>: Cells of all fractions were examined by light microscopy and under phase contrast using a Vickers 2000 light microscope. Morphological appearance and evidence of lymphocytic contamination were assessed.

#### 2 - 2c: RESULTS

The growth rates, folate status, mucosal morphology and cell turnover rates of the animal groups used for this study have been described in Section 2 - 1.

(i) Isolation procedure: The method as described here, produced nine fractions of cells of sequential washings, over a period of one hour, in both control and deficient groups of animals. Total cell yields, in controls, controls with antibiotic and folate deficients contained  $7.89 \pm 0.59$ mg,  $7.21 \pm 0.89$ mg and  $3.42 \pm 0.35$ mg DNA respectively. The length of the intestine was not affected by folate deficiency. The percentage of total cells isolated in each fraction (mg DNA per fraction/total mg DNA x 100) was similar in both controls and folate deficient groups as shown in table 2 - 2a. There are a number of crypts per villus which could account for the greater proportion of cells in the crypt fractions than one could expect from observations on villous height and crypt depth. Similarly measurements of total villous height do not give estimates of cell number up the villus because of changes in cell size and shape as they migrate from the crypt up the villus.

Suspensions of individual cells uncontaminated by mucus were found in the final preparations. The cells tended to reaggregate if left standing on ice or room temperature, but were easily redispersed with a plastic pipette to give a single cell suspension.

A series of preliminary studies showed that the conditions described were optimal in removing all the enterocytes from the mucosa, as assessed by histological examination of the remaining tissue. Lowering the citrate concentration, and temperature of the buffers, reduced the yield of cells. Increasing the citrate concentration did not release more cells. Increasing the temperature of the incubation resulted in loss of cell viability as did a change of pH. The percentage of cells per fraction was variable, and the cells were not easily dispersed and washed when dithiothreitol was omitted from the isolation buffers.

# TABLE 2 - 2a

The percentage of intestinal epithelial cells per fraction (mg DNA per fraction/mg total DNA x 100), isolated from control, control + antibiotic and folate deficient rats.

AN IMAL GROUPS	1 - 3	CELL FRACTIONS 4 - 6	7 - 9
Control	21.9±1.9	40.2±2.8	37.9±2.3
Control + antibiotic	22.3±1.1	40.6±2.7	37.1±2.0
Folate deficient	19.9±2.3	39.8±1.9	40.3±2.2

Means ± standard errors from groups of 8 animals.

All the cells were removed at the end of the ninth wash, since a tenth wash failed to release more cells, ie. there was no detectable DNA or protein in this fraction. Histological examination of the intestine at the end of the isolation procedure showed intact villous cores and submucosal tissue, but an absence of epithelial cells even in the crypts after the ninth wash as illustrated in Figure 2 - 2f. Failure to distend the intestine for the last three washes resulted in a decreased yield of crypt cells.

Examination of the cell suspensions after they had been washed in phosphate buffered saline, by light and phase contrast microscopy demonstrated that there was a minimal contamination of the fractions by lymphocytes, in both control and deficient animals.

(ii) Validation of method: The activities of sucrase and thymidine kinase per cell fraction in control and deficient animals are shown in Tables 2 - 2b and 2 - 2c and Figures 2 - 2g and 2 - 2h. In all groups of animals sucrase activity was greatest in fractions 1 - 3 with a gradual decline in activity thereafter, to the lowest level in fraction 9. Thymidine kinase activity was lowest in fraction 1 in all groups, and increased steadily to a maximum in fraction 9. Activities of thymidine kinase in folate deficient cells were significantly higher in all fractions compared with both control groups, but there was still a gradation of activity from crypt to villus tip. The importance of these higher activies will be discussed further in Section 2 - 4.

Twenty two hours after injection of labelled thymidine in both control groups, there was an initial sharp increase in radioactivity per mg DNA at fraction 6, and this was maintained in fractions 7, 8 and 9 (Table 2 - 2d, Figure 2 - 2j). Fractions 1 - 5 contained lower concentrations of radioactivity in DNA, but incorporation was significantly higher than that expected from autoradiographical analysis. Autoradiography showed cells containing radioactivity to be located in the crypts and the base of

# FIGURE ? - 2f

Histological appearance of the jejunal muucosa after Wash 9 of the isolation procedure, demonstrating intact villous cores with removal of the majority of the epithelial cells.



(X 540)
## TABLE 2 - 2b

Sucrase activities (µg glucose/µg enzyme protein/hour) of epithelial cell fractions isolated from control, control + antibiotic and folate deficient rats.

	% CELLS I SOLATED	CONTROL	CONTROL + ANTIBIOTIC	FOLATE DEFICIENT
villus-tip	5	0.89±0.061	0.95±0.071	1.06±0.083
	10	0.83±0.053	0.91±0.069	0.97±0.070
	20	0.68±0.049	0.64±0.056	0.67±0.060
	30	0.55±0.050	0.53±0.033	0.66±0.045
	40	0.50±0.033	0.50±0.040	0.54±0.040
	50	0.47±0.040	0.44±0.029	0.49±0.039
	60	0.42±0.028	0.43±0.030	0.48±0.030
	70	0.40±0.025	0.44±0.032	0.47±0.035
	80	0.31±0.010	0.40±0.027	0.47±0.030
	90	0.33±0.011	0.31±0.025	0.44±0.029
crypt base	100	0.23±0.012	0.29±0.018	0.37±0.029

Mean values ± standard errors from groups of 8 animals.



## FIGURE 2 - 2g

Validation of cell isolation procedure: Sucrase activities (ug glucose/ ug enzyme protein/hour) of epithelial cell fractions isolated from control, control + antibiotic and folate deficient rats. Mean values from groups of 8 animals.

## TABLE 2 - 2c

Thymidine kinase activities (p moles dTMP/µg enzyme protein/hour) of epithelial cell fractions isolated from control, control + antibiotic and folate deficient rats.

% CE ISOLA	LLS TED	CONTROL	CONTROL + ANTIBIOTIC	FOLATE DEFICIENT
villus- 5	5	126± 13.3	450± 36.3	659± 72.1
tip 1 10	)	144± 15.6	510± 39.9	1044± 98.3
20	)	258± 30.1	832± 72.8	982±100.9
30	)	298± 22.3	1151± 96.7	1719±191.8
40	5	341± 33.9	1259±111.2	2364±260.7
50	0	381± 45.1	1369±139.1	2517±200.3
60	0	479± 39.3	1575±150.4	3711±393.1
70	0	1059± 98.6	2085±189.5	4390±440.5
8	0	1256± 99.1	2158±209.3	5342±500.4
9	0	1302±169.3	2177±201.6	5833±309.7
crypt base 10	0	1624±222.1	2456±231.7	6631±590.9

Mean values  $\pm$  standard errors from groups of 8 animals.

## TABLE 2 - 2c

Thymidine kinase activities (p moles dTMP/ug enzyme protein/hour) of epithelial cell fractions isolated from control, control + antibiotic and folate deficient rats.

% CELLS ISOLATED	CONTROL	CONTROL + ANTIBIOTIC	FOLATE DEFICIENT	
villus- 5	126± 13.3	450± 36.3	659± 72.1	
tip   10	144± 15.6	510± 39.9	1044± 98.3	
20	258± 30.1	832± 72.8	982±100.9	
30	298± 22.3	1151± 96.7	1719±191.8	
40	341± 33.9	1259±111.2	2364±260.7	
50	381± 45.1	1369±139.1	2517±200.3	
60	479± 39.3	1575±150.4	3711±393.1	
70	1059± 98.6	2085±189.5	4390±440.5	
80	1256± 99.1	2158±209.3	5342±500.4	
90	1302±169.3	2177±201.6	5833±309.7	
crypt base 100	1624±222.1	2456±231.7	6631±590.9	

Mean values  $\pm$  standard errors from groups of 8 animals.



FIGURE 2 - 2h: Validation of cell isolation procedure: Thymidine kinase activities (p moles  $dTMP/\mu g$  enzyme protein/hour) of epithelial cell fractions isolated from control, control + antibiotic and folate deficient rats. Mean values from groups of 8 animals.

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## TABLE 2 - 2d

The incorporation of tritiated thymidine into the DNA of epithelial cell fractions (DPM/mg DNA) from control, control + antibiotic and folate deficient rats, following the in vivo administration (intraperitoneal) of 100uCi/100g body weight <sup>3</sup>H TDR, 22hours prior to isolation of the cells.

% CELLS ISOLATED	CONTROL	CONTROL + ANTIBIOTIC	FOLATE DEFICIENT	
	2350+110	2160+ 99	200±12	
tip 1 10	2470±170	2050±103	280±11	
20	2666±230	2150±141	198±21	
30	4133±305	2375±169	240±15	
40	3666±300	2275±201	323±24	
50	3466±319	2550±231	573±37	
60	8333±632	3850±359	715±68	
70	9966±301	6250±302	780±52	
80	8033±490	6475±401	923±90	
90	8700±367	6350±298	1038±83	
crypt base 100	9367±498	6700±306	1085±80	

Mean values ± standard errors from groups of 4 animals.

## FIGURE 2 - 2j

The incorporation of tritiated thymidine into the DNA of epithelial cell fractions (DPM/mg DNA) from control, control + antibiotic and folate deficient rats, following the in vivo administration (intraperitoneal) of  $100\mu$ Ci/100g body weight <sup>3</sup>H TDR, 22 hours prior to isolation of the cells. Mean values from groups of 4 animals.



the villus, with no detectable radioactivity in the villus-tip and midvillus cells.

In folate deficient cells, the incorporation of labelled thymidine per mg DNA was significantly decreased in all cell fractions, and while there was a smaller gradient increasing from villus-tip to crypt fractions, compared with controls, the difference between fraction 1 and fraction 9 and fraction 6 and fraction 9 were significantly different, (p<0.01). Autoradiography showed cells containing radioactivity to be located at the base of the crypts only, in folate deficient rats.

Figure 2 - 2k demonstrates the stages of the wash procedure for isolating cells. They show a sequential removal of cells from villus tip in fraction 1, down to villus base by fraction 6, and total removal of crypt cells by fraction 9.

(iii) Viability studies: Figure 2 - 21 shows the rate of oxygen consumption per mg DNA per hour (ie.  $QO_2$ ) in a mixed population of cells in controls and folate deficient animals, incubated at  $37^{\circ}$ C in Krebs Henseleitt medium (containing 10mg/m1, fatty acid free B.S.A., 250mg% glucose, pH 7.2). There was a lag of 4 - 5 minutes before the cells consumed oxygen at a maximum and constant rate. The rate of consumption remained constant,( $QO_2 \equiv$ 0.53 ± 0.04, 0.47 ± 0.03, 0.43 ± 0.03µ1/hr/mg DNA, in controls, controls + antibiotic and folate deficients respectively for a period of 40 minutes, and then steadily declined in all groups. The constant  $QO_2$  value was significantly lower for the folate deficient cells compared with the controls (p<0.05) suggesting a slight impairment of metabolic activity in the folate deficient gut.

Figure 2 - 2m shows the rate of carbon dioxide production  $(DPM \ ^{14}CO_2/hour/mg\ DNA)$  in a mixed population of cells in controls and folate deficient groups in the Krebs Henseleitt incubation medium. From 10 minutes to 40 minutes the rate of production remained constant in all groups (40.8 ± 2.1, 38.5 ± 1.9, 33.8 ± 3.8 DPM/hour/mg\ DNA in controls,

#### FIGURE 2 - 2k

Histological appearance of the jejunal mucosa after release of epithelial cells at various stages of the wash procedure(from control animals).

Photomicrograph A: After fraction 1 has been collected.

8	В:	"	fraction 2			
0	C:	ŧ	fraction 3		н	0
n	D:	"	fraction 4	"	"	8
	E:	u	fraction 5		"	81
	F:	11	fraction 6		*	61
u	G:	**	fraction 7	n		
п	Н:		fraction 8	0	0	н
н	Ι:		fraction 9		н	16

Between washes 1 & 6 cell fractions are collected progressively from down the villus. After fraction 6 has been collected, only intact crypts and a few villous base cells remain. After fraction 8 has been collected, cells are left in the base of the crypts. Following wash 9 only villous cores remain.















Viability studies - respiratory metabolism - on mixed populations of cells (ie. villus-tip, mid-villus + crypt cells) isolated from the small intestine of control, control + antibiotic and folate deficient rats. Cells were incubated in Krebs Henseleitt medium, at 37°C.

controls + antibiotic and folate deficients respectively). The rate decreased slightly at 50 minutes and again by 60 minutes in all groups. The rate of carbon dioxide production was lower in folate deficient cells, again indicating a possible effect of folate deficiency on cellular respiratory metabolism.

Figure 2 - 2n shows the intracellular ratios of potassium ions  $(K^+)$  and sodium ions  $(Na^+)$  in a mixed population of cells in controls and folate deficient groups incubated at  $37^{\circ}$ C, in the Krebs Henseleitt incubation medium. The ratios remained constant from 0 to 40 minutes in all groups since the individual concentrations of K<sup>+</sup> and Na<sup>+</sup> remained constant and began to decline with a leakage of sodium into and/or potassium out of the cell at 50 minutes. This was shown by increases in intracellular Na<sup>+</sup> and decreases in intracellular K<sup>+</sup> concentrations. By adding ouabain (0.2mg/ml), which inhibits the energy dependent sodium pump mechanisms (Medzihradsky & Marks, 1975), to the suspension before incubation, the gradients could not be maintained.

Figure 2 - 2p shows the results of the dye exclusion test in a mixed population of cells in controls and folate deficient groups incubated at  $37^{\circ}$ C in the Krebs Henseleitt incubation medium. The percentage of cells that excluded the dye trypan blue molecules remained constant over the 60 minute incubation period.

Figure 2 - 2q shows the cells as seen under phase-constrast microscopy of control and folate deficient isolated villous cells after 30 minutes incubation at  $37^{\circ}$ C in the Krebs Henseleitt incubation medium. The cells from all groups of animals were morphologically normal, having a cuboidal shape and a distinct brush border on what could then be defined as the luminal side of the cells. A slight rounding of the cell shape began at about 50 minutes and the cells were completely rounded after 90 minutes.

The results shown in all studies of viability are for a mixed population of isolated cells taken from fractions 1 - 9. Preliminary investigations showed that the cells from fractions 1 - 3, 4 - 6 and 7 - 9



FIGURE 2 - 2n: Intracellular potassium/sodium ratios.

#### FIGURES 2 -2n & 2 -2p

Viability studies - membrane integrity - on mixed populations of cells (ie. villus-tip + mid-villus + crypt cells) isolated from the small intestine of control, control + antibiotic and folate deficient rats. Cells were incubated in Krebs Henseleitt buffer(containing lomg/ml FAFBSA & 250mg% glucose -at final concentrations; pH 7.2) at 37°C.

## FIGURE 2 - 29

The appearance of epithelial cells released from the villi of control and folate deficient rat small intestine - phase contrast microscopy. Photomicrographs A & B: control villous cell (fraction 3).

Photomicrographs C & D: folate deficient villous cell (fraction 4).





gave comparable results for viability as assessed by oxygen consumption and  $K^+/Na^+$  ratios. In control animals between 10 and 40 minutes oxygen consumption under standard incubation conditions was 0.56 ± 0.05, 0.53 ± 0.05 and 0.49 ± 0.07µl oxygen/hour/mg DNA in fractions 7 - 9, 4 - 6 and 1 - 3 respectively, and intracellular  $K^+/Na^+$  ratios were 0.43 ± 0.02, 0.43 ± 0.03, 0.45 ± 0.04 in fractions 7 - 9, 4 - 6 and 1 - 3 respectively. The logistics of handling large numbers of fractions did not allow viability to be assessed on them all, but this preliminary work justified the use of a mixed population of cells for such studies.

The cells in this viability study were suspended and incubated in a Krebs Henseleitt medium containing lOmg/ml, fatty acid free B.S.A. and 250mg% glucose. The albumin stabilises membranes of cells in suspension and glucose is included as a substrate. Preliminary investigations using predominantly oxygen consumption measurements, showed this to be a suitable incubation medium. Lowering the concentration of fatty acid free B.S.A. from 10mg/ml to 5mg/ml lowered the rate of oxygen consumption which rapidly diminished after 20 minutes. Increasing the concentration to 15 or 20mg/ml did not alter the rate of oxygen consumption, nor did it increase the period of constant consumption to exceed 40 minutes. Similar observations were made when the concentration of glucose was altered. In some of the subsequent experiments (Section 2 - 4), a commercially available medium TC199 (the composition of which is given in Appendix 2) was used for incubating the cells. The rate of oxygen consumption was the same for cells incubated in TC199 as for the Krebs Henseleitt medium containing 10mg/ml, fatty acid free B.S.A. and 250mg% glucose, and intracellular K<sup>+</sup>/Na<sup>+</sup> ratios were normal. However, the rate of oxygen consumption and maintenance of K<sup>+</sup> and Na<sup>+</sup> ratios were constant for a period of at least 60 minutes in TC199 medium.

Failure to gas the cells continuously throughout the incubation period resulted in a decrease in the rate of oxygen consumption. Cells could be kept on ice in their initial suspending medium (Krebs Henseleitt

buffer only, pH 7.2), with no gassing for up to 5 hours. After this period if they were then suspended in the usual incubation medium and incubated at  $37^{\circ}$ C, the rate of oxygen consumption was maximal and constant for up to 40 minutes as in the cells incubated immediately after isolation. Boiled cells did not utilise any oxygen. Homogenised suspensions of cells maintained a rate of oxygen consumption of 0.35µl oxygen/hour/mg DNA (compared with 0.53µl oxygen/hour/mg DNA in intact cells) for only 20 minutes. The rate of consumption then declined rapidly. This emphasises that only intact cells consume oxygen at an optimal rate, but that cells with broken membranes can also respire (eg. in remaining intact mitochondria) at a reasonable and constant rate for a significant length of time, such that tests of membrane integrity should always accompany metabolic studies.

#### 2 - 2d: DISCUSSION

In order to study aspects of the metabolism and function of the epithelial cell in vitro, a preparation in which a large proportion of epithelial cells were undamaged by the isolation procedure used was required. In order to distinguish between the varying cell populations found on the villus, the citrate dissociation method described was used to produce nine cell fractions representing populations ranging from villus tip to crypt base cells. The cell isolation procedure used was based on that first described by Weiser (1973) for separating fractions of enterocytes. Minor modifications were made with respect to time of incubation of the gut for separating the different fractions, and it was found to be necessary to gas all buffers and tissues throughout the isolation period in order to maintain the subsequent viability of the cells in an incubation system. The cell fractions obtained in both control and folate deficient animals were characterised according to their enzyme patterns as suggested by Weiser (1973). Weiser expressed his data for percentage of cells in each fraction per milligram protein, but as protein concentration of the epithelial cell changes with position of the villus (see Section 2 - 3)

it was decided that cell number would be measured by the amount of DNA per fraction. The discussion of results applies to all animal groups, which all conformed to the same patterns of enzyme activities. Twenty percent of the total population of cells isolated were in the first 3 fractions. These cells had high activities of sucrase and low activities of thymidine kinase. Villus tip cells are normally associated with high activities of digestive and absorptive enzymes (Miller & Crane, 1961; Nordstrom et al. 1967; Weiser, 1973) and the high activities of sucrase found in the first 3 isolated fractions suggest that they were predominantly villus-tip cells. The next 40% of total cells isolated had intermediary activities of thymidine kinase and sucrase, with a slow gradation of activities upward and downward respectively and corresponded to mid-villus and villous base cells. After 62% of the cells had been isolated there was a sharp increase in thymidine kinase activity, a high incorporation of  ${}^{3}$ H thymidine into DNA and a continued fall in sucrase activity. These cells corresponded to crypt cells (see Figure 2 - 2r). Subsequent experiments were therefore simplified by grouping fractions. Fractions 1 - 3 corresponded to villus-tip cells. Fractions 4 - 6 corresponded to mid-villus cells. Fractions 7 - 9 corresponded to crypt fractions. This breakdown of fractions was supported by the histological sequencing of the isolation procedure.

The integrity of isolated cells cannot be assessed merely by metabolic activity, or by morphological appearance alone. It can only be made by considering collectively the results of morphological and metabolic studies. There were no differences in the length of time in which the cells maintained a maximum degree of viability, between both control groups and the deficient group, or villus-tip, mid-villus and crypt cell fractions. Slightly longer viability was obtained in cells from both controls and folate deficient animals if TC199 (folate and thymine free, with added fatty acid free bovine serum albumin - 10 mg/ml) was used rather than the Krebs Henseleitt based medium. Medium TC199 contains all the essential nutrients and substrates for maintaining cells in culture (details of the composition of TC199 are

FIGURE 2 - 2r

Characterisation of cell populations.



Sucrase activities ( $\mu g$  glucose/ $\mu g$  enzyme protein/hour), and thymidine kinase activities (p moles dTMP/ $\mu g$  enzyme protein/hour) of villus-tip, mid-villus and crypt cell fractions from the small intestine of control rats. Mean values from groups of 8 animals.

given in Appendix 2). These two media used, were selected to give the most optimal rates of oxygen consumption and carbon dioxide production without affecting membrane integrity. The studies reported in this section used the Krebs Henseleitt solution as the incubation medium.

Cells had a constant rate of respiratory activity with respect to oxygen consumption and carbon dioxide production from 5 to 40 minutes. The initial lag phase corresponded to the cells warming from  $0 \rightarrow 37^{\circ}C$ , at which temperature the cells function optimally under the conditions of this experimental procedure. Previous workers using isolated cells where rates of oxygen consumption  $(Q0_2)$  or  $^{14}C0_2$  production were measured, have either used a different species of animal or expressed their results per mg cell protein. Kimmich's (1970) data on  $^{14}CO_2$  production, whose method was adapted in this study, was poorly expressed in terms of counts per minute, ie. without allowing for the percentage efficiency of counting, and making a direct comparison difficult. Evans et al. (1971) recorded a QO2 of about 9µl oxygen per mg cell protein per hour in guinea pig small intestinal cells isolated by a citrate dissociation method. By extrapolating our values where the Protein/ DNA ratio of a mixed population of control cells corresponded to  $15.1 \pm 1.1$ , the maximum QO2 of 0.56µ1/mg DNA/hour corresponds to 8.46µ1 oxygen/ mg cell protein/hour. Stern and Reilly (1965), in cells isolated from rat small intestine using a trypsin-pancreatin digest method, recorded a QO2 of 5.0ul oxygen/hour/mg cell protein, when they were incubated in a 0.05M potassium phosphate buffer containing 0.2M sucrose. Perris (1966) reported  $QO_{p}$  values of 11 ± 1 and 9 ± 0.5µl oxygen/hour/cell protein for a period of 40 minutes in everted sacs of rat jejunum and ileum respectively. Paterson & Zbarsky (1958) recorded initial values of QO2 of 6 - Bul/hour/mg cell protein in mucosal strips. The rates recorded in this study appeared to fall in the range of previously recorded in vitro levels of oxygen consumption in normal cells. Rates of oxygen consumption and carbon dioxide production in folate deficient cells were lower than in control cells, suggesting alterations in

respiratory metabolism in the deficient mucosa. This may be important with respect to active transport of nutrients and will be discussed further in Section 2 - 3.

An intact and functioning cell will normally maintain low concentrations of Na ions and high concentrations of K ions compared with the extracellular environment. This is achieved by an energy requiring sodium pump mechanism. The ability of cells to maintain normal intracellular  $K^+/Na^+$  concentrations is therefore a useful index of cell membrane integrity. Intracellular concentrations of  $K^+$  and  $Na^+$  were similar to those reported by Evans <u>et al</u>. (1971) in guinea pig small intestinal cells and ratios of  $K^+/Na^+$  were therefore comparable.

Between the parameters chosen for study in this project, there was a close relationship with respect to length of time that the cells could be considered viable - with one exception - ie. the dye exclusion test. This test has been used by a great many workers as the only test of viability of their tissues or cell suspensions. Even before including it in this study it was felt that such a test was too subjective and open to errors in quantitative assessments. Some cells aggregate under the microscope making counting and distinction between cells containing dye, and those not, difficult. It is also easy to select portions of the slide containing no dyed cells or alternatively containing a high proportion of dyed cells. Every attempt was made in this study to systematically count a large proportion of the cells visible by scanning the whole amount of cell suspension on the slides. However, the operation was time consuming and the results still failed to reflect the apparent depreciation of cell viability after 40 minutes, as detected by other more quantitative tests. Medzihradsky and Marks (1975) made similar comments with respect to assessing the viability of isolated blood cell preparations.

When observed by phase contrast microscopy, the cells appeared of normal cuboidal shape and brush borders were visible in villous cells.

The cells maintained a normal morphological appearance for up to 50 minutes when a slight rounding off in shape was observed. By comparison, indices of respiratory function and also  $K^+/Na^+$  ratios indicated that cell viability was not maintained after 40 minutes, and were therefore the more sensitive means of assessing cell viability.

When incubated in a Krebs Henseleitt incubation medium, the cells could be maintained on ice for up to four hours before being used in incubation studies. When incubated at 37°C the cells demonstrated rates of oxygen consumption and carbon dioxide production considered to be normal for small intestinal epithelial cells and maintained a normal intracellular ratio of K<sup>+</sup>/Na<sup>+</sup> for periods of up to 40 minutes. After this period cell function was diminished. This could have corresponded to either a diminution of functional integrity in every cell of the population or cell death and total loss of function in a proportion of the population. In either situation it would be invalid to use the cells for periods of more than 40 minutes in incubation studies. The intended studies on DNA metabolism required an incubation period of 30 - 40 minutes (allowing for the initial 5 minute lag period) and the cell preparations were therefore considered suitable for use in these studies. Further studies were required to ensure that the cells maintained a normal functional integrity with respect to the functional processes under direct study ie. with respect to DNA metabolism. Studies were therefore made into the rate of in vitro incorporation of labelled thymidine into the DNA of incubated cells, particularly crypt cells. The results of these studies demonstrated the functional integrity of the isolated crypt cells. The methods and results of this study are detailed with respect to the subsequent studies on DNA metabolism in Section 2 - 4.

It was concluded from this study therefore, that the method described for isolating epithelial cell fractions gave populations of cells corresponding to villus-tip, mid-villus and crypt cell populations, in

both control and folate deficient animals. Since these populations could be characterised according to their enzyme characteristics, sucrase and thymidine kinase activities were therefore measured routinely after all cell isolation procedures.

The epithelial cells from both folate deficient and control animals were metabolically and functionally viable, and therefore suitable for incubation studies of up to 40 minutes duration. Routine assessments of oxygen consumption and intracellular  $K^+/Na^+$  concentrations were made on all cells used for incubation studies.

# SECTION 2 - 3 CHANGES IN MUCOSAL COMPOSITION AND ABSORPTIVE ENZYME ACTIVITIES IN FOLATE DEFICIENCY

#### 2 - 3a: INTRODUCTION

(i) <u>Mucosal composition</u>: The migration of cells from the undifferentiated crypt cell region to the villus tip is associated with structural and biochemical changes that equip the cells for their mature functional activities, which include transport. The nature of the biochemical changes in terms of composition ie. DNA, RNA and protein content have not been fully described. Values for total mucosal content of DNA, RNA and protein in rats have been reported (Gleeson <u>et al</u>. 1972b; Batt & Peters, 1976; Tomkins <u>et al</u>. 1976), but the composition of the varying cell populations down the villus have not been described. It would be expected that newly synthesised, cuboidal cells in the crypts would be small, having a high DNA content compared with protein. As they migrate up the villus and mature, the enterocytes become larger, columnar and acquire digestive enzymes. Protein content compared with DNA would therefore be much higher than in crypt cells.

The biochemical composition of cells could have importance in delineating changes in the functional capacity eg. absorption, of the intestine which occur in certain disease and deficiency states. Folate deficiency is a common finding in tropical malabsorption syndromes, alcoholism and coeliac disease. In tropical malabsorption the improvement in symptoms and tests of absorption on folic acid therapy alone suggest folate deficiency may contribute to the mucosal lesion, but the precise mechanisms of malabsorption in folate deficiency are unclear.

Cook (1976) has studied absorption, using intestinal perfusion techniques in patients with severe megaloblastic anaemia of primary nutritional origin in Africa. There was no impairment of absorption of glucose, xylose or glycine, when compared with African control subjects. He was unable to measure sodium and water transport because the perfusion

system used was not suitable. Despite the results obtained, there were discernable morphological abnormalities of the jejunal mucosa. Goetsch & Klipstein (1977), using an in <u>vivo</u> marker perfusion technique, evaluated the intestinal transport of water, sodium, xylose, glucose and L-leucine in control and folate deficient rats. Their results indicated that folate deficiency of the intestinal mucosa altered the transport of water and electrolytes, but not that of such solutes as xylose, glucose and L-leucine.

It was proposed therefore to study the activities of two enzymes associated with absorption ie. sucrase and  $(Na^+-K^+)$ -ATPase, in both folate deficient and control rats and relate their activities to the observed differences in absorption of disaccharides, water and sodium in folate deficiency, as seen in the study of Goetsch & Klipstein (1977). Changes in enzyme activity could be related to biochemical composition and cell turnover rates as has been postulated in a number of studies on both disaccharidase activities and  $(Na^+-K^+)$ -ATPase activities in disease and experimental conditions as will be discussed.

(ii) Disaccharide absorption: Intestinal disaccharidases are associated with the region of the brush border of mature, non-dividing differentiated cells of the villus, and little activity is found in the proliferative cells of the crypts (Miller and Crane, 1961; Nordstrom <u>et al</u>. 1967). Disaccharidase activity in man is maximal in the jejunum and proximal ileum. The principle disaccharidases are sucrase, maltase and lactase and they convert disaccharides into monosaccharides. The resultant monosaccharides are taken up by carriers and are transported across the lipid membrane accompanied by a dependence on sodium, which is also attached to the carrier. The energy for the transport system and its apparent one way direction is due to the sodium gradient, which is itself dependent on the sodium pump (Section 2 - 3a(iii)), which efficiently removes sodium from the interior of the epithelial cell. The monosaccharides leave the

lateral wall of the epithelial cell and enter the portal venous circulation.

Malabsorption of disaccharides resulting from a hereditary deficiency of these enzymes has been noted in man (Auricchio <u>et al.</u> 1963; Bayless & Rosenweig, 1966). Exogenous factors such as infection, parasitic infestations, hormones, diet and drugs are also capable of having a deleterious effect on intestinal disaccharidases (Herbst <u>et al</u>. 1969). The specific biological mechanisms by which these exogenous factors produce a decrease in enzymatic activity have not been elucidated.

Sucrase and lactase have been correlated with cell position (cell age) under a variety of cell renewal states (Doell et al. 1965). Dramatic changes in enzyme activities could therefore be expected in association with alterations of the normal cell lifespan. A lengthening of the enterocyte lifespan with a decrease in cell proliferation occurs after prolonged fasting (Goldsmith, 1973). Conversely, a shortening of the enterocyte lifespan in association with an increased proliferative activity, occurs in rats treated with colchicine (Herbst et al. 1970), which has a dose dependent effect of decreasing sucrase and lactase activities. A marked reduction in cell transit time was characteristically associated with the enzyme defects, thus inferring that the cells are not able to mature adequately and acquire sufficient enzymes for normal absorptive function. It could be therefore, that when cell turnover time is increased, cells can acquire more of the enzymes and therefore increase their digestive and absorptive capacities. Disaccharidases can be readily induced to levels exceeding normal by feeding high concentrations of the appropriate disaccharide (Deren et al. 1967; Rosenweig & Herman, 1969). Newly developed immunohistochemical methods applied to enzyme localisation along the crypt-villus axis of adult rat intestine have suggested that inactive proteins are present in crypt cells, at least for sucrase, peptidases and alkaline phosphatase (Raul et al. 1977). In addition, Silverblatt et al. (1974) suggested that sucrase protein is synthesised in the crypts and is firmly

bound to cell surfaces as an inactive precursor of the villous enzyme. Whether sucrase is in fact present in an inactive form, which can be 'activated' under certain conditions, or rather is inducible under these conditions, is still in some doubt.

(iii) Sodium and water transport: The volume of water absorbed is comparatively small compared with the total fluid load presented to the small intestine. Salivary, gastric, biliary, pancreatic, and mucosal secretions total about 7 litres, while the dietary fluid intake from food and drinks provide another 1 - 2 litres daily. Water absorption is passive and accompanies net movement of solute out of the lumen in isotonic proportions (Curran, 1968; Burland and Samuel, 1972).

Sodium absorption is an active process, differing in certain aspects in the jejunum and ileum. Sodium absorption in the ileum can occur independently of other solutes, and can take place against a large concentration gradient. Net transport rather than equilibration of ions is a feature of the ileum. Sodium absorption in the jejunum is always linked with other actively transported solutes, in particular, monosaccharides, amino acids and bicarbonate. Monosaccharides and amino acids are transported on a molar basis with sodium, such that one molecule of non-electrolyte accompanies one atom of sodium (Crane, 1965).

The driving force for sodium transport comes from the sodium pump situated at the lateral or basal surface of the cell. This actively removes sodium from the cell maintaining the low intracellular sodium ion concentrations (Na<sup>+</sup>) and high potassium ion (K<sup>+</sup>) concentrations compared with the extracellular fluid.

The enterocytes are different to other cells eg. red blood cells or liver cells, because they exhibit polarity ie. for normal functioning, there must be net entry of water and electrolytes and other substances into the cell at the luminal surface and net entry into the plasma at the serosal surface. The hydrolysis of ATP under the catalytic action of an adenosine triphosphatase enzyme, provides the energy for many processes within cells (Katz and Epstein, 1968). The enzyme ( $Na^+-K^+$ ) stimulated adenosine triphosphatase ( ( $Na^+-K^+$ )-ATPase ) has been closely linked to sodium transport in many tissues, including intestinal tissue (Katz & Epstein, 1968; Charney <u>et al</u>. 1974). Approximately 85% of the measureable ( $Na^+-K^+$ )-ATPase activity in rat and rabbit small intestinal mucosa is located in a plasma membrane fraction devoid of brush border, nuclei and mitochondria ie. in the lateral or serosal membrane (Quigley & Gotterer, 1969; Parkinson <u>et al</u>. 1972; Fujita <u>et al</u>. 1972). The enzyme is thought to straddle the membrane and for each molecule of ATP hydrolysed, 3 sodium ions are extruded from the cell, and 2 potassium ions are driven into the cell, both against concentration gradients. This pump mechanism is therefore electrogenic in that current is generated (Rowland, 1978). The mechanism whereby these uphill movements are achieved is unknown.

The locus for the interaction of sodium and actively transported monosaccharides is responsible, for example, for generating (a) a sugar gradient and increases in Na<sup>+</sup> influx (located in the brush border) and (b) the Na<sup>+</sup> pump responsible for maintaining the low intracellular Na<sup>+</sup> concentration and Na<sup>+</sup> absorption (located in the lateral membrane) (Gall <u>et al.</u> 1974). The evidence for this separation is that actively transported sugars have a stimulatory effect on Na<sup>+</sup> absorption only when present in the mucosal solution (Schultz & Zalusky, 1963) and that ouabain inhibition of (Na<sup>+</sup>-K<sup>+</sup>)-ATPase occurs only at the serosal side of the epithelial cell (Schultz & Zalusky, 1964).

Adenosine triphosphatase activity is greater in the villus than in the crypt cells (Charney <u>et al.</u> 1974; Gall <u>et al.</u> 1976) and this may signify important differences in sodium transport between villus tip and crypt cells. The  $(Na^+-K^+)$ -ATPase activity in villus tip or crypt cells will reflect the level of ATPase linked Na<sup>+</sup> transport and associated water transport at this site, and the steep gradient of activity between villus tip and crypt cells may be important when there is a proportional change

in the number of, or biochemical composition of, these cell populations. Such a change might be related to the alteration in transport that accompanies compensatory small intestinal hypertrophy following resection (Dowling & Booth, 1967; Gleeson <u>et al.</u> 1972a) and the many diarrhocal disorders characterised by shortened villi and crypt cell hyperplasia (Hendrix & Bayless, 1970). Total reduction in cell numbers could also affect ( $Na^+-K^+$ )-ATPase activity and therefore sodium and water transport.

Hamilton et al. (1976) have described the effects of transmissable qastroenteritus (TGE) virus, and emphasised the importance of electrolyte transport, particularly sodium transport, as a major determinant of the diarrhoea caused by the virus. The mucosal lesion in TGE is variable. Light microscopy of sections made during in vitro studies (Hamilton et al. 1976) showed mild but significant shortening of the villi and an increase in crypt depth. Stool volumes were increased and faecal electrolyte concentrations were significantly higher. There was no steatorrhea and despite a decrease in disaccharidase activity in the jejunal mucosa, there was no excess of sugar in the stool. In the infected segment of the small intestine there was net movement of water and electrolyte into the gut lumen; Na<sup>+</sup> flux from the lumen to the extracellular fluid was decreased, and Na<sup>+</sup> flux from the extracellular fluid to the lumen was increased. Total mucosal (Na<sup>+</sup>-K<sup>+</sup>)-ATPase activity was diminished in TGE when isolated villous enterocytes were studied (Hamilton et al. 1976). Pensaert et al. (1970), using experimental conditions similar to those of Hamilton et al., showed that within 10 hours of its administration TGE virus invaded the intestinal epithelium and there replicated predominantly in the enterocytes on the villi. When these cells were shed, their replacements migrated from the crypts much faster than normal; by 40 hours, when Na<sup>+</sup> transport was most deranged, probably few infected cells were left in the (Hamilton et al. 1976). Hamilton suggests from data measured intestine in isolated enterocytes, that at this stage of acute diarrhoea, enterocytes on the villi have retained some qualities of less differentiated crypt cells.

They are rich in thymidine kinase - normally confined to crypt cells - but have little sucrase activity, which normally becomes functional as cells migrate to the villi (Weiser, 1973; Kerzner <u>et al.</u> 1975). Thymidine kinase activity increases as Na<sup>+</sup> transport deteriorates during the course of the disease. The transport properties of crypt cells are not known. Nevertheless, direct invasion of the cell by virus may not be the only, or even the main factor disturbing ion transport in viral diarrhoea. Reparative events in the mucosa after infection, and changes in migration and differentiation of the epithelial cells are suggested therefore as possible factors contributing to the secretory condition.

Gastrointestinal fluid loss in human and experimental cholera, arises throughout the small intestine, occurring by way of an epithelium which remains histologically, as well as functionally, intact. The permeability to macromolecules is not increased and active absorptive processes for non-electrolytes are not impaired (Field et al. 1972). The effector of this intestinal secretion is a Vibrio cholerae exotoxin (cholera toxin). It has been postulated that adenylate cyclase is the mediator in choleragen induced secretion (Field et al. 1972), but despite this it has also been shown that methylprednisilone may prevent and reverse the secretory effects of the choleragen by increasing mucosal  $(Na^+-K^+)$ -ATPase activity (Charney & Donowitz, 1976). Charney et al. (1975) and Rachmilewitz et al. (1978) have shown that  $(Na^+-K^+)$ -ATPase activities are increased following methylprednisilone administration in normal rats, with an associated increase in water transport. There were no alterations in intestinal histology and villous and crypt heights after the corticosteroid treatment.

Diarrhoea is a frequent side-effect of colchicine therapy, a drug used in the treatment of gout. It depresses intestinal disaccharidase activities in man (Herbst <u>et al</u>. 1970), guinea pig (Cohen & Macnamara, 1970) and rats (Herbst <u>et al</u>. 1970). In the study of Herbst <u>et al</u>.(1970) histological examination showed an increase in crypt depth, but no

decrease in the length of the villi in rat mucosa. Mitotic arrest in metaphase was observed in the crypts. Cell migration rate was increased. Rachmilewitz <u>et al</u>. (1978) tested the hypothesis that colchicine and vinblastine inhibit water transport from the rat small intestine by inhibiting  $(Na^{4}-K^{+})$ -ATPase activities, using an <u>in vitro</u> intestinal infusion system. Changes in  $(Na^{+}-K^{+})$ -ATPase activities were mirrored by changes in water secretion, both two and four hours after colchicine or vinblastine administration.

Human erythrocyte membrane  $(Na^+-K^+)$ -ATPase has been studied in several disease states. Kaplay <u>et al</u>. (1978) demonstrated elevated  $(Na^+-K^+)$ -ATPase activities in the erythrocyte membrane of children suffering from kwashiorkor, but not in marasmic children.  $(Na^+-K^+)$ -ATPase activity in the erythrocyte membrane is reported to be higher in hereditory spherecytosis (Parker & Welt, 1972). On the other hand, reduced activity is found in uraemic disorders, malarial infection and thyrotoxicosis (Parker & Welt, 1972; Cole & Waddell, 1972). The mechanism of these changes appears to be different in each clinical disorder.

Electrolytes play an important role in the maintenance of structure and function of a living cell. Membrane bound enzymes such as  $(Na^+-K^+)$ -ATPase are sensitive indices of altered cellular environment, and thus their study could be useful in the understanding of the biochemical basis of the pathogenesis of a number of disease states. In particular, the role of  $(Na^+-K^+)$ -ATPase in gut epithelial cells, not only in maintaining electrolyte gradients, but in absorption processes, could be important in evaluating the pathogenesis of diarrhoea conditions.

(iv) Experimental plan: The study described in Section 2 - 2 which outlined the method for isolating epithelial cells, included measurements of sucrase activity, primarily to validate the cell populations obtained. It was noted at the time that the total activity of the folate deficient mucosa was the same as that of the control levels of activity. It was
shown in Section 2 - 1 that cell turnover rates were decreased in deficient animals, and from previous work on whole mucosa (Tomkins <u>et al.</u> 1976), it was apparent that there were decreased levels of total mucosal DNA in deficient animals. The increases in sucrase activities found in this study were therefore surprising, and suggested that there was some compensatory mechanism for increasing disaccharide absorption despite the apparent decrease in cell number. The improvement of the mucosal lesion following folic acid therapy in certain tropical malabsorption syndromes, suggests that folate deficiency may produce a mucosal lesion in man. However, the precise mechanisms of malabsorption in folate deficiency are unclear. The role and location of enzymes such as the disaccharidases and (Na<sup>+</sup>-K<sup>+</sup>)-ATPase in experimental and disease situations associated with diarrhoea and malabsorption, have frequently been discussed in relation to mucosal histology and cell turnover rate.

It was proposed therefore to measure sucrase and  $(Na^+-K^+)$ -ATPase activities in the villus-tip, mid-villus and crypt populations of isolated mucosal enterocytes, and relate these changes to the composition (DNA, RNA and protein contents) of these cells in folate deficient and control animals. These changes could then be related to the observed changes of Goetsch and Klipstein (1977) in the absorption of nutrients in folate deficient rats.

#### 2 - 3b: METHODS

Three groups of 16 rats each (control, control + antibiotic, and folate deficient) were bred as described in Section 2 - 1. Growth rates, mucosal and red cell folates of each group were comparable with those described in Section 2 - 1. Epithelial cell fractions were isolated from each animal and the fractions characterised according to the method described in Section 2 - 2. Fractions 1 - 3, 4 - 6 and 7 - 9 were pooled into villus-tip, mid-villus and crypt fractions respectively. Measurements

of RNA, DNA and protein concentration were made on all fractions from all animals. In the isolated enterocytes from each group (16 animals per group) sucrase activity was measured.  $(Na^+-K^+)$ -ATPase activity was measured in enterocytes from half of the animals (8 animals per group). Cell turnover rates were measured in all animals:

(i) Protein: Protein concentration was measured by the Lowry technique described in Section 2 - 2.

(ii) DNA: DNA concentration was measured by the fluorometric technique described in Section 2 - 2.

(iii) RNA: RNA concentration was measured by a fluorometric assay based on that described for DNA (Section 2 - 2).

Aliquots (100µ1) of cell fractions were treated exactly as for DNA samples with the exception that the RNA-ase (150µg/ml final concentration) was replaced by 50µl buffered saline. The measurable fluorescence after addition of ethidium bromide was therefore from total nucleic acid content. RNA content was calculated by subtraction of DNA content from total nucleic acid content.

Replacement of RNA-ase by  $50\mu$ l DNA-ase ( $150\mu$ g/ml final concentration) (3185<sup>\*</sup>K unitz/mg solid) gave the same result.

(iv) Cell turnover & migration rates: Cell turnover and cell migration rates were measured by the autoradiographic technique described in Section 2 - 1. Eight animals from each group were injected with labelled thymidine 4 hours prior to sacrifice, four animals 22 hours and the remaining four animals 36 hours prior to sacrifice.

\* lkunitz will produce a  $\Delta A 260$  of 0.001 per min. per ml, at pH 5.0, at 25°C, using DNA as substrate.

(v) Sucrase activity: Sucrase activity was measured by the technique described in Section 2 - 2. Activity was defined as the glucose ( $\mu$ g) liberated by the enzyme in one hour per mg of enzyme protein.

(vi)  $(Na^+-K^+)$ -ATPase activity: The method used was based on that described by Quigley and Gotterer (1969) and Charney et al (1974).

Aliquots of cell suspension (2ml) were homogenised (Konteshomogeniser clearance 200µ), with 2 ml buffer containing 130mM NaCl, 10mM Na<sub>2</sub> EDTA, 60mM imidazole, & 4.8mM Na-deoxycholate. The homogenates were centrifuged at 700 x g for 10 minutes, at  $4^{\circ}$ C. The supernatant was centrifuged for a further 10 minutes at 10,000 x g, at  $4^{\circ}$ C. The pellets obtained were resuspended in 1ml buffer containing 130mM NaCl, 5mM Na<sub>2</sub> EDTA, 30mM imidazole and then assayed for (Na<sup>+</sup>-K<sup>+</sup>)-ATPase activity

Aliquots of enzyme preparation (200µ1) were mixed in duplicate with 800µl substrate buffer containing (final concentration in lml reaction mixture) 5mM ATP, 5mM Mg<sup>2+</sup>, 100mM Na<sup>+</sup>, 20mM K<sup>+</sup>, 10 mM imidazole - HCl buffer pH 7.2. Aliquots of enzyme (200µ1) were also mixed with the same medium but containing 120mM Na<sup>+</sup> and no K<sup>+</sup>. The reaction mixtures were incubated at 37<sup>0</sup>C in a shaking water bath. After ten minutes 1ml 10% PCA was added to inactivate and precipitate the enzyme. The supernatant obtained after centrifugation at 200 x g for 10 minutes was assayed for inorganic phosphorous ( $P_i$ ) by the method described by Fiske and SubbaRow (1925). The supernatant was diluted 1 in 5 with 5% PCA and 500µl aliquots were mixed in duplicate with reagent containing 1% ammonium molybdate - $(NH_4)_6MO_7O_24.4H_2O$ , 5% FeSO<sub>4</sub>.7H<sub>2</sub>O in 1N H<sub>2</sub>SO<sub>4</sub>. After 30 minutes the P<sub>1</sub> content was assessed spectrophotometrically at 660mp using standards containing 30 - 200n moles inorganic phosphorous. Blanks containing no enzyme were run with each incubation, and controls were set up for each enzyme preparation. Control reaction mixtures were inactivated with 10% PCA prior to the incubation period.  $(Na^+-K^+)$ -ATPase activity was

defined as the difference between inorganic phosphate released in the presence and absence of potassium in the incubation mixture.  $(Mg^{2+})$ -ATPase activity was defined as the amount of enzyme released in the absence of potassium from the incubation mixture. Addition of 0.091% ouabain to the standard substrate buffer gave the same results as omission of K<sup>+</sup> from the buffer. Activities were expressed as µmoles inorganic phosphate liberated per mg enzyme protein per hour.

#### 2 - 3c: RESULTS

Table 2 - 3a shows the red cell folate, mucosal folate and final weights of each of the animal groups. The pattern of results was similar to those of the original model described in Section 2 - 1. The cell fractions isolated had comparable gradients of sucrase activity and thymidine kinase activity to those described in Section 2 - 2, (Table 2 - 3b). Cell turnover rates were also similar to those previously described in Section 2 - 1, (Table 2 - 3c) showing a reduction in migration rate and an increase in cell turnover time in deficient animals compared with controls (52.8 ± 6.12 v 91.6 ± 8.1 hours respectively, p<0.01). Villous height was slightly, but not significantly, decreased in folate deficient animals, and crypt depth was increased (200  $\pm$  15 v 140  $\pm$  10µm in controls, p<0.01) (Table 2 - 3c). A third group was included in this study who were not sacrificed until 36 hours after injection with labelled thymidine instead of 22 hours. This allowed time for labelled cells in folate deficient mucosa to migrate onto and up the villus. At 22 hours labelled cells were still in the crypts, and since it is not known whether migration rate changes at the crypt-villus junction this could lead to an error in calculating cell turnover rates. In fact cell migration rate in folate deficient animals, when calculated from the 36 hours time point was slightly higher (7.0  $\pm$  0.61 $\mu$ m/hr) than that calculated from the 22 hour time point (6.4  $\pm$  0.52µm/hr) giving cell turnover times of 87.1  $\pm$  7.2 hours and

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### TABLE 2 - 3a

Validation of animal model: Red cell folate levels (ng/ml), mucosal folate levels (µg/g protein) and final weights at 42 days (g) of rats from control, control + antibiotic and folate deficient groups.

	Red Cell Folate	Mucosal Folate	Final Weight
CONTROL	696.3±101.5	45.3±6.1	85.6±3.01
CONTROL + ANTIBIOTIC	651.2± 98.9	50.6±4.9	80.0±2.56
FOLATE DEFICIENT	219 ± 42.3 <sup>*</sup>	16.0±3.1*	61.6±1.69 <sup>*</sup>

Mean values ± standard errors for groups of 16 animals.

\* p < 0.001 compared with both control groups.

#### TABLE 2 - 3b

Validation of cell isolation procedure: Thymidine kinase activities (p moles dTMP/µg enzyme protein/hour) and sucrase activities (µg glucose/ µg enzyme protein/hour) of isolated epithelial cell fractions from villustip, mid-villus and crypt regions of the small intestine of control, control + antibiotic and folate deficient rats.

ANIMAL GROUP & SUCRASE ACTIVITY THYMIDINE KINASE ACTIVITY CELL FRACTION

CONTROL	villus-tip	2.28±0.14	398± 25
	mid-villus	1.47±0.12	1601± 240
	crypt	0.96±0.08	5034± 282
CONTROL +	villus-tip	2.34±0.10	810± 63
ANTIBIOTIC	mid-villus	1.44±0.12	2400± 312
	crypt	1.08±0.08	8876±1242
FOLATE	villus-tip	2.46±0.19	1342± 49
DEFICIENT	mid-villus	1.62±0.11	7400± 991
	crypt	1.32±0.10	10373±1245

Mean values ± standard errors for groups of 16 animals.

## TABLE 2 - 3c

Villous height (µm), crypt depth (µm), cell migration rate (µm/hour) and cell turnover time (hours) of the jejunal mucosa of control, control + antibiotic and folate deficient rats.

	VILLOUS HEIGHT	CRYPT DEPTH	MIGRATION RATE	CELL TURNOVER TIME
	μΜ	μm	µm∕hr	hrs
CONTROL	460±49	140±10	10.0±1.3	59.8±5.49
CONTROL + ANTIBIOTIC	455±52	140±13	11.3±1.0	52.8±6.12
FOLATE DEFICIENT	410±31	200±15 <sup>**</sup>	6.7±0.7*	91.6±8.1**

Mean values ± standard errors from groups of 8 animals.

\* p < 0.05 compared with both control groups.

**\*\*** p < 0.01 compared with both control groups.

95.31  $\pm$  8.3 hours respectively. These differences were not significant and the results from 22 hour and 36 hour time points were therefore grouped.

With respect to the proportion of cells per fraction, there were fewer cells contributing to the villus-tip fractions (14% v 22% previously) and an increase in the proportion of cells in the crypt fractions (46% v 38% previously). This may have been attributable to the slightly decreased villous heights in this experiment compared with the previous experiment, although there were no apparent differences in crypt depth. The increase in crypt depth in folate deficient animals was again reflected by the increased proportion of cells from the crypt fraction compared with controls.

Tables 2 - 3d, 2 - 3e and Figure 2 - 3a give data on the composition of the isolated cell fractions with respect to protein, DNA and RNA contents. Total mucosal DNA, RNA and protein were significantly reduced in folate deficient animals to 41.3%, 67.1% and 71.2% of control animals respectively. The control group with added antibiotic had reduced total mucosal protein, DNA and RNA contents compared with ordinary controls, but these differences were not significant. Concentrations of DNA were lower in all levels of the villus in deficient animals, compared with both control groups. Protein and RNA concentrations were also decreased in each fraction, but not to the same extent as DNA.

Ratios of protein/DNA and protein/RNA were higher at all levels in folate deficient animals than the groups of control animals, especially in the villus tips  $(34.54 \pm 2.61 \text{ in folate deficients v } 19.26 \pm 1.91 \text{ in}$ controls with antibiotic, p<0.001 and 14.37 ± 0.79 in folate deficients v 8.93 ± 0.69 in controls with antibiotic p<0.001, respectively) (Table 2 - 3f). In both control groups, the ratios of protein/DNA and protein/RNA increased from crypt to villous fractions. Ratios of RNA/DNA were significantly greater in the crypts and mid-villus regions of the folate deficient mucosa.

## TABLE 2 - 3d

Total protein, DNA and RNA contents (mg) in isolated cell preparations from control, control + antibiotic and folate deficient rats.

	PROTEIN	DNA	RNA
	mg	mg	mg
CONTROL	131.30±11.90	8.69±0.62	11.27±1.53
CONTROL + ANTIBIOTIC	112.34±13.21 <sup>ns</sup>	7.20±1.02 <sup>ns</sup>	10.34±1.43 <sup>ns</sup>
FOLATE DEFICIENT	93.52±11.78 <sup>*</sup>	3.59±0.43**	7.56±0.95 <sup>*</sup>

\* p<0.05 compared with control group.

\*\* p<0.01 compared with both control groups.

ns not significantly different from control group.

Mean values ± standard errors for groups of 16 animals.

# TABLE 2 - 3e

<u>Composition of cell fractions</u>: Protein, DNA and RNA concentrations (mg/cell fraction) in isolated epithelial cell fractions from villustip, mid-villus and crypt regions of the small intestine of control, control + antibiotic and folate deficient rats.

ANIMAL GROUP & CELL FRACTION		PROTEIN	DNA	RNA
		mg	mg	mg
CONTROL	villus-tip	24.01±3.47	1.22±0.17	2.49±0.41
	mid-villus	54.13±3.26	3.43±0.09	4.56±0.62
	crypt	53.16±5.17	4.04±0.36	4,22±0.50
CONTROL + ANTIBIOTIC	villus-tip	19.85±3.18	1.03±0.35	2.22±0.09
	mid-villus	39.35±6.06	2.73±0.20	3.76±0.63
	crypt	53.15±7.97	3.45±0.47	4.36±0.71
FOLATE DEFICIENT	villus-tip	19.69±2.11	0.57±0.07**	1.37±0.10 <sup>*</sup>
	mid-villus	34.68±6.51	1.06±0.09***	2.25±0.36*
	crypt	39.15±3.16 <sup>*</sup>	1.96±0.27***	3.94±0.49

\* p<0.05 compared with both control groups.

\*\* p<0.01 compared with both control groups.

\*\*\* p<0.001 compared with both control groups.

Mean values  $\pm$  standard errors for groups of 16 animals.



FIGURE 2 - 3a: Composition of cell fractions: Protein, DNA and RNA concentrations (mg/cell fraction) of villus-tip, mid-villus and crypt epithelial cell fractions isolated from the small intestine of control, control + antibiotic and folate deficient rats. Mean values ± standard errors from groups of 16 animals.

#### TABLE 2 - 3f

<u>Composition of cell fractions</u>: Protein/DNA, Protein/RNA and RNA/DNA ratios in isolated epithelial cell fractions from villus-tip, midvillus and crypt regions of the small intestine of control, control + antibiotic and folate deficient rats.

ANIMAL GROU	JP & ION	PROTEIN DNA	PROTEIN RNA	RNA DNA
CONTROL	villus-tip	19.68±2.04	9.64±0.91	2.04±0.24
	mid-villus	15.78±2.10	11.87±1.06	1.33±0.16
	crypt	13.16±1.44	12.59±1.03	1.26±0.13
CONTROL + ANTIBIOTIC	villus-tip	19.26±1.91	8.93±0.69	2.15±0.26
	mid-villus	14.47±1.01	10.47±0.96	1.38±0.13
	crypt	15.41±1.49	12.19±1.23	1.26±0.13
FOLATE	villus-tip	34.54±2.61***	14.37±0.79***	2.40±0.21
DEFICIENT	mid-villus	32.72±2.36***	15.41±1.31**	2.12±0.16**
	crypt	19.97±1.83 <sup>*</sup>	9.94±1.02	2.01±0.12**

\* p<0.05 compared with both control groups.

\*\*\* p<0.001 compared with both control groups.

Mean values ± standard errors for groups of 16 animals

# TABLE 2 - 3g

Sucrase activity ( $\mu g$  glucose/mg enzyme protein/hour) and ( $Na^+-K^+$ )-ATPase activities ( $\mu$ moles  $P_i/mg$  enzyme protein/hour) of isolated epithelial cell fractions from villus-tip, mid-villus and crypt regions of the small intestine of control, control + antibiotic and folate deficient rats. Enzyme activities are expressed as activity per cell, ie. per mg DNA, or as specific activities, ie. per mg protein.

ANIMAL GROUP & CELL FRACTION		SUCRASE ACTIVITY		(Na <sup>+</sup> -K <sup>+</sup> )-ATPase ACTIVITY <sup>††</sup>	
		per mg DNA	per mg Protein	per mg DNA	per mg Protein
CONTROL	villus-tip	1860± 82	94.9±6.7	66.66±13.11	3.21±0.34
	mid-villus	429± 33	27.2±1.3	18.28± 1.41	1.19±0.08
cr	crypt	238± 13	18.1±1.3	8.22± 0.56	0.66±0.04
CONTROL + ANTIBIOTIC	villus-tip	2000±125	103.8±6.2	73.53± 7.12	3.76±0.29
	mid-villus	529± 39	36.6±2.8	20.55± 2.34	1.47±0.11
	crypt	313± 21	20.3±1.1	6.90± 0.56	0.56±0.06
FOLATE DEFICIENT	villus-tip	4316±216***	124.9±7.1*	58.00± 5.22	1.59±0.12***
	mid-villus	1529± 76***	46.7±2.7**	25.25± 1.51	0.89±0.06**
	crypt	673± 29***	33.7±1.9**	8.89± 0.54	0.50±0.02***

+ mean values ± standard errors from groups of 16 animals.

tt mean values ± standard errors from groups of 8 animals.

\* p < 0.05 compared with both control groups.</pre>

\*\* p < 0.01 compared with both control groups.</pre>

\*\*\* p < 0.001 compared with both control groups.</pre>

FIGURE 2 - 3b: (Page 156) -- legend

Sucrase activity (ug glucose/mg enzyme protein/hour) and Protein/DNA ratios of isolated epithelial cell fractions from villus-tip, mid-villus and crypt regions of the small intestine of control, control + antibiotic and folate deficient rats. Enzyme activities are expressed per mg DNA and per mg protein.

FIGURE 2 - 3c: (Page 157) -- legend

 $(Na^+-K^+)$ -ATPase activity (µmoles  $P_i/mg$  enzyme protein/hour) and Protein/ DNA ratios of isolated epithelial cell fractions from villus-tip, mid-villus and crypt regions of the small intestine of rats. Enzyme activities are ezpressed per mg DNA and per mg protein.





Table 2 - 3g and Figures 2 - 3b, 2 - 3c show the activities of sucrase and  $(Na^+-K^+)$ -ATPase expressed per cell and per milligram protein in each animal group. Sucrase activity was higher in villus tip than in crypt cells in both control and deficient animals. When expressed as enzyme activity per mg DNA, the concentration of enzyme per cell was much greater at all levels of the villi of the deficient mucosa, compared with controls. It has been shown that the cells of the deficient mucosa contain more protein per mg DNA. It seemed possible therefore that the greater concentration of sucrase per cell merely reflects this increased concentration of protein per mg DNA. However, when sucrase was expressed as specific activity, ie. sucrase activity per mg protein, there were significantly higher levels of activity at all levels of the villi.

There was also a gradation of  $(Na^+-K^+)$ -ATPase activity in both control and deficient animals, activities being higher in the villus tip than the crypt cells. When expressed as enzyme activity per mg DNA, the concentration of enzyme per cellwas the same at all levels in both control and deficient animals. There was no change in  $(Mg^{2+})$ -ATPase activities in any group. Since the cells of the deficient mucosa contain more protein per mg DNA, the specific activity of  $(Na^+-K^+)$ -ATPase was lower in all levels of the villi compared with controls.

### 2 - 3d: DISCUSSION

The proliferating cells of the small intestine are separated from the non-proliferating cells which are involved in absorptive and secretory functions. As cells move from a proliferative state to a mature state, they undergo changes in size, shape and function, such as the change from cuboidal to columnar shape, the development of microvilli and the development of absorptive and secretory functions, as well as the loss of ability to undergo cell division. It would seem likely that changes in function and structure would be accompanied by changes in composition of the

different cell types on the villus.

Few workers have described the composition of the epithelium of the small intestine with respect to protein, DNA and RNA content. Gleeson et al. (1972b) described the composition of scraped mucosa in control and by-passed jejunum and ileum. Similarly Tomkins et al. (1976) described the protein, DNA and RNA contents of control and folate deficient mucosa, while Batt & Peters (1976) described the composition of control mucosa and effects of prednisolone on it. Each group of workers used rats. It is difficult to make direct comparisons between the previously reported composition data on the jejunum, with this present study because of the differences in methodology. From the work of Gleeson et al. (1972b) using mucosal scrapings, which probably represent the villous population, it can be calculated that Protein/DNA, RNA/DNA and Protein/RNA ratios were 8.63, 0.64 and 11.04 respectively. Batt & Peters (1976) using isolated enterocytes consisting predominantly of villous cells, reported Protein/DNA, RNA/DNA ratios of 12.8 and 0.4 respectively. Tomkins et al. (1976) using mucosal scrapings reported Protein/DNA, RNA/DNA and Protein/ RNA ratios of 13.3, 0.95 and 14.0 respectively, in control animals. In this present study, looking at villus alone, or an average of all the cells isolated, the values for Protein/DNA, RNA/DNA and Protein/RNA were similar to the pattern of values cited, although RNA/DNA and Protein/DNA were slightly higher. In folate deficiency Tomkins et al. (1976) did not find any differences in Protein/DNA ratios - in fact ratios were slightly decreased in folate deficiency suggesting a smaller cell size, and there were only small changes in Protein/RNA and RNA/DNA.

Webster & Harrison (1969) using a vibration technique to isolate cells from villus and crypt found no change in cell size as they migrate from the base of the villus to the villus tip, but the concentration of DNA relative to the protein content was double in the crypts compared with the villus. The model for separating sequential fractions of cells down

the villus described here, provides a useful means of comparing the composition of cells as they mature. Crypt cells had a high activity of DNA per mg of protein. As cells migrate up the villus they acquire enzymes associated with absorptive function and they increase in size ie. Protein/DNA ratios increase. The concentration of RNA in cells increased in parallel with the increases in protein concentration. This pattern of compositional development was altered in folate deficiency where cell turnover time was increased. The total concentration of DNA in the jejunum was markedly decreased, which emphasises the importance of folate in pathways for DNA synthesis. Protein and RNA concentrations were also lower in folate deficient animals but not in proportion to the decrease in ENA. Since villous height was not significantly decreased in folate deficiency, the decrease in DNA must reflect an atrophy of the gut with respect to the actual number of crypts and villi, rather than a significant decrease in the number of cells on each villus. The folate deficient cells were also considerably larger compared with the control cells. This was observed in histological sections and was also shown by the high Protein/DNA ratios at all levels of the villus in folate deficient animals. The fact that the cells were larger and yet the villus height not altered, suggests that there may in fact be fewer cells per villus as well as a decrease in the number of villi in folate deficiency.

The slow cell turnover rate of the cells could be an important factor in determining the increase in cell size in folate deficiency. The cells spend longer on the villus in folate deficient animals (Section 2 - 1) and therefore have more time to synthesise protein and to acquire enzymes, thus giving rise to populations of large 'hypermature cells'. The large appearance of the cells, particularly in crypts, are characteristic of the megaloblastic type of cell observed in the bone marrow of folate deficient subjects, and also in crypts of patients with secondary folate deficiency in tropical sprue or chronic alcoholism (Swanson & Thomassen, 1965; Hermos <u>et al</u>. 1972). The high ratios of RNA/DNA and Protein/DNA

which were demonstrated in this study were also found in studies on megaloblastic anaemia in humans. Thorrell (1947) showed that with normal maturation of the erythroblast there was a decline and finally the disappearance of RNA. In contrast, levels of RNA persisted in the megaloblast. Further, Glazer <u>et al.</u> (1954) demonstrated high ratios of RNA/DNA in megaloblastic bone marrows, and this was confirmed by a higher than normal rate of tritiated uridine incorporation into the RNA of megaloblasts together with a lower rate of DNA synthesis (Yoshida <u>et al</u>. 1968). There was also a high rate of protein synthesis as evidenced by a high uptake of tritiated leucine.

The work of Goetsch & Klipstein (1977) indicated that experimental folate deficiency of the intestinal mucosa alters the transport of water and electrolytes, but not that of such solutes as xylose, glucose and L-leucine. An association between enterocyte life span and development of absorptive and digestive enzymes has been suggested (Doell et al. 1965; de Both & Plaiser, 1974; de Both et al. 1974). In colchicine treated rats for example, shortening of the enterocyte lifespan in association with an increase in cell transit time has a close drug dependent effect of decreasing sucrase and lactase activities (Herbst et al. 1970). Similarly Prosper et al. (1968) demonstrated decreased total activities of sucrase, lactase and maltase in association with an increased rate of mucosal cell turnover in rats fed a protein free diet. In experimental folate deficiency it has been shown that there is an increase in the enterocyte lifespan, and it could be postulated that this allows cells to acquire greater concentrations of sucrase. As a result, despite an overall decrease in the number of cells in the folate deficient mucosa, total sucrase activity is not decreased, and absorption of disaccharides is likely to be normal.

Sucrase is an inducible enzyme. It can be induced for example, by increasing the sucrose content of the diet (Deren <u>et al</u>. 1967; Rosenweig

& Herman, 1969). Silverblatt et al. (1974) have suggested that inactive precursors for sucrase enzyme are present in crypt cells, and are firmly bound to cell surfaces prior to activation. The enzyme becomes active as the brush borders of the enterocytes develop suggesting that the enterocytes have a reserve capacity of 'inactive' sucrase, which can be activated to give sucrase levels over and above normal requirements. Since the diet fed to both controls and folate deficient animals had a very high sucrose content (72%), the reserve capacity would have to have been very large indeed, in order for activity to increase 3 fold in folate deficiency. Mucosal, RNA & protein concentrations were increased in the folate deficient animals, indicating an increase in protein synthesis, which was possibly associated with increases in the synthesis of enzymes. It would seem more likely therefore, that the increased sucrase activities observed in folate deficiency were due to an induction of sucrase in response to the decreased total number of enterocytes, rather than activation of an already present inactive precursor protein. This is further supported by data from James et al. (1971) who measured the turnover of disaccharidases and brush border proteins in rat intestine. They demonstrated a continuous turnover and incorporation of protein into the brush border as cells migrated up the villi. This suggested that the synthesis or catabolism of proteins can be modified in the mature villous cells, and therefore that the control of disaccharidase synthesis was not totally dependent on cell formation in the crypt, but was instead located at the villous level.

The activities of  $(Na^+-K^+)$ -ATPase do not follow the same pattern as sucrase activities in folate deficiency.  $(Na^+-K^+)$ -ATPase activity per cell was the same in controls as in the folate deficient mucosa, but because of the decreased number of cells, total gut activity was decreased. This decrease in total activity was probably responsible for the watery diarrhoea associated with folate deficiency, since a number of disease

states eg. TGE virus (Hamilton <u>et al</u>. 1976) or experimental conditions eg. colchicine induced diarrhoea (Rachmilewitz <u>et al</u>. 1978), have demonstrated a decrease in  $(Na^+-K^+)$ -ATPase activity, in association with the onset of diarrhoea. It is interesting to note that changes in  $(Na^+-K^+)$ -ATPase activity are not necessarily associated with histological changes. In cholera for example the mucosa remained histologically and functionally intact with respect to the permeability of macromolecules and the active processes for non-electrolytes (Field <u>et al</u>. 1972), yet there was a development of net secretion of water and electrolytes from the gut, with an associated decrease in  $(Na^+-K^+)$ -ATPase activity. Conversely administration of prednisolone ( Charney <u>et al</u>. 1975; Rachmilewitz <u>et al</u>. 1978), even in normal rats, was associated with an increase in water transport, again with no alteration in intestinal histology and villous and crypt height.

Following the administration of colchicine or vinblastine (Rachmilewitz et al. 1978) there was an increase in cell migration rate with a decrease in  $(Na^+-K^+)$ -ATPase activity, which might suggest a similar relationship between cell turnover rate and enzyme activity, as that observed with brush border enzymes. Similarly in TGE virus, reduced  $(Na^+-K^+)$ -ATPase activities are associated with a decrease in cell migration rate. However, the reverse does not appear to be true since in folate deficiency where cell migration was decreased there was no increase in  $(Na^+-K^+)$ -ATPase activities over normal to compensate for the reduced number of cells.

That  $(Na^+-K^+)$ -ATPase activity can be increased by the administration of hormones such as prednisolone (Rachmilewitz <u>et al</u> 1978), and aldosterone (Schmidt <u>et al</u>. 1975) suggests that activation of this enzyme is controlled independently of cell turnover and cell age. That the rapid activation of  $(Na^+-K^+)$ -ATPase induced by aldosterone was completely prevented by actinomycin-D and cycloheximide, suggests that  $(Na^+-K^+)$ -ATPase activation requires an intact protein synthetic process. Increases of enzyme activity

are therefore probably associated with increased synthesis of the enzyme rather than activation of inactive precursors. However, contradictory to this theory is the fact that colchicine in the dose range which decreases  $(Na^+-K^+)$ -ATPase activity has been shown to have no effect on RNA or protein synthesis (Rossignol <u>et al</u>. 1972). Similarly in this study the activity of  $(Na^+-K^+)$ -ATPase in folate deficiency did not alter in conjunction with the greater concentrations of protein per cell. This suggests that  $(Na^+-K^+)$ -ATPase activity is in fact independent of an intact protein synthesising system, or that there is some other functional abnormality related to the enzyme activity in folate deficiency.

It is a characteristic of the cell membrane transport systems of most animal cells to require ATP as the energy substrate. The chief substrate for the  $(Na^+-K^+)$ -ATPase is ATP, since with other nucleoside triphosphates, such as inosine, guanosine and uridine triphosphate as substrates, phosphatase activity is either very small or absent (Hoffman, 1960; Rendi & Uhr, 1964). The mechanism by which the energy of ATP is ultimately converted to the movement of ions is unknown. It has been suggested that folic acid may regulate intestinal glycolysis, since several glycolytic enzymes are induced in man by large (15mg) daily doses of folic acid (Rosenweig <u>et al</u>. 1968). In the viability studies on the isolated cells, rates of oxygen consumption and carbon dioxide production were shown to be slightly lower in folate deficient cells, suggesting some impairment of respiratory metabolism in these cells. It could be therefore that in folate deficiency there is a limitation of ATP production from the respiratory pathways, on the activities of  $(Na^+-K^+)$ -ATPase.

The fact still remains however, that  $(Na^+-K^+)$ -ATPase activity is essentially normal per folate deficient epithelial cell. If folic acid was in some way affecting protein synthesis, energy metabolism or even hormone status, it could be expected that  $(Na^+-K^+)$ -ATPase levels would be decreased per cell.  $(Na^+-K^+)$ -ATPase activities seem in fact to be

unpredictable in many disease situations, not only those affecting the gut eg. reduced activity found in uraemic disorders, malarial infection and thyrotoxicosis (Parker & Welt, 1972; Cole & Waddell 1976), and yet elevated activities in children suffering from kwashiorkor (Kaplay <u>et al</u>. 1978). It is therefore apparent that a more detailed study is required into the fundamental mechanisms of  $(Na^+-K^+)$ -ATPase synthesis and/or activation, and its relationship with normal cell metabolism and maturity, before the effects of hormones and disease or deficiency states can be fully elucidated.

From this study it would seem that folic acid itself does not affect the normal synthesis of  $(Na^+-K^+)$ -ATPase in enterocytes, since activities remain normal per cell. Total gut activity is decreased due to a reduction in the number of cells, and the enterocytes are unable to respond in the same way as they do for brush border enzymes such as sucrase, and increase in activity. One possible explanation for this failure to increase activity, may be related to the fact that  $(Na^+-K^+)$ -ATPase enzyme is closely associated with the baso-lateral membrane of the cells, and despite an increase in cell size in folate deficiency, there is not a proportional increase in cell membrane surface area to allow more  $(Na^+-K^+)$ -ATPase to be functioning upon it.

In conclusion therefore, in folate deficiency, it is apparent that epithelial cells acquire sucrase and  $(Na^+-K^+)$ -ATPase activity as they migrate up the villus. In folate deficiency, it is suggested that the delay in cell migration rate allows cells to remain on the villus for a longer time, and to acquire greater concentrations of sucrase. This is sufficient to prevent the malabsorption of disaccharides that would be expected due to the decrease in total number of villous cells in folate deficiency.  $(Na^+-K^+)$ -ATPase activity is concentrated in the lateral membrane of the cell. Despite an increase in cell size reflected by an increase in Protein/DNA ratio in folate deficiency, cell surface area

does not increase proportionately, and thus the capacity of the cell for transporting sodium and water will not increase. A reduction in cell number will therefore decrease the transport efficiency of sodium and water. This may then be a causal factor in the diarrhoea of mucosal folate deficiency.

# SECTION 2 - 4 THYMIDYLATE AND DNA SYNTHESIS IN FOLATE DEFICIENCY

#### 2 - 4a: INTRODUCTION

Having attributed many of the compositional and enzyme changes to differences in cell number and cell turnover, it was decided to study changes in DNA metabolism in the crypt cells, with particular respect to thymidylate synthesis. A model has been proposed for (a) quantitating the relative utilisation of the two pathways for thymidylate synthesis and (b) for directly measuring rates of DNA synthesis.

(i) Methods for measuring DNA synthesis and pathways associated with DNA synthesis: The role of folate in DNA metabolism, particularly with respect to thymidylate synthesis, has been discussed in detail in the General Introduction (Section 1 - 4). The principle pathways for thymidylate synthesis ie. de novo and salvage are summarised again in Figure 1 - 4a. Folate is required for the de novo synthesis of thymidylate from deoxyuridylate and deoxyuridine. Thymidylate can also be formed from thymidine via the salvage pathway, which is measured by incubating tissues with labelled thymidine and estimating the amount incorporated into DNA. Preincubation of the tissue with deoxyuridine will, in the presence of folate, give a reduced uptake of labelled thymidine by the salvage pathway, because of the enzymic conversion of deoxyuridine supplying the thymidylate pool. Deficiency of folate, or the presence of a folate antagonist in the tissue will block the de novo synthesis, and uptake of labelled thymidine will not then be limited (Metz et al. 1968; Hoffbrand & Waters, 1972; Wickramasinghe & Saunders, 1975). This method therefore enables one to study the two pathways for thymidylate synthesis being actively utilised relative to one another, rather than merely estimating the potential enzyme activity of each separately as has often been done. Killman, (1964)



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..... Reactions in the salvage pathway

Pt.Glu. ≡ Pteroylglutamic acid ie. folic acid.

and Metz et al. (1968) developed an incubation technique using bone marrow cells for assessing the effect of folate and vitamin B12 deficiency on thymidylate synthesis, using the deoxyuridine suppressed value. These and subsequent studies have shown that bone marrow cells from patients with vitamin  $B_{12}$  or folate deficiency, show a subnormal suppression of labelled thymidine uptake after preincubation with deoxyuridine (Killman, 1964; Metz et al. 1968; Wickramasinghe & Saunders, 1976). Addition of 10µg of folic acid per ml of bone marrow culture, partially corrected the abnormal deoxyuridine suppressed value. (Wickramasinghe & Saunders, 1976). The incubation technique was adapted to assess the relative activity of the <u>de novo</u> and salvage pathways for thymidylate synthesis, in human jejunal biopsies and rat intestinal rings in controls and in folate deficiency (Badcock, 1975; Tomkins and McNurlan, 1977). However, there were problems in the interpretation of these data, because of the variation in tissue types and cell age. The intestinal rings for example, consisted of muscle, connective, serosal and mucosal tissue, while the jejunal biopsies comprised of mucosal tissue containing cells of varying maturity. It was hoped that these problems could be overcome by using populations of cells isolated from different regions of the villus and crypt. A series of incubations was therefore carried out using crypt cell fractions isolated from control and folate deficient groups of rats. The results obtained showed no apparent differences in suppression of uptake of tritiated thymidine into DNA, by deoxyuridine preincubation. However, it was found that the uptake of tritiated thymidine whether with or without preincubation with deoxyuridine, was greater in folate deficient cells, than control cells. There was a wide range of variation in the results, and problems of binding of labelled thymidine to the albumin in the incubation medium were encountered (a resume of the methodology and results of these experiments is given in Appendix 3).

It has often been suggested that the measurement of <u>in vitro</u> incorporation of thymidine into DNA alone is an unreliable measurement

for the quantitative determination of DNA synthesis (Lindberg et al. 1969; Hauschka, 1973; Hooton & Hoffbrand, 1977). Changes in the rate of uptake and in the size of the thymidine pool may result in changes in the incorporation rate of thymidine into DNA, which are unrelated to the rate of DNA synthesis. This could lead to erroneous results when comparing DNA synthesis in the different cells of an organism, cells of different species or when following DNA synthesis in one cell over a period of time. Discrepancies between the rate of incorporation of labelled thymidine into DNA and the rate of DNA synthesis, have been reported in mammalian cells (Smets, 1969; Hooton & Hoffbrand, 1977). There was a greater incorporation of labelled thymidine into DNA (ie. higher specific activity) in the jejunal crypt cells used in the deoxyuridine suppression test in folate deficiency compared with controls. It was unclear whether the higher specific activity was due to a true increase in DNA synthesis or to an increase in the specific radioactivity of the thymidine nucleoside phosphate pool, as a result of the higher level of thymidine kinase. In folate deficient animals, the higher in vitro incorporation of tritiated thymidine into DNA compared with in vivo incorporation (described in Section 2 - 2) was striking. In the light of the evidence shown so far that in folate deficiency there are fewer epithelial cells ie. less DNA in the small intestine, and slower rates of turnover of these cells, the in vivo measurements were likely to be a more accurate reflection of rates of DNA synthesis. There are a number of assumptions underlying the in vivo use of radioactive thymidine as a cell label, and these have been well summarised by Wickramasinghe (1975), together with the problems of making such assumptions in certain tissues eg. lymphoid cells. Briefly the assumptions are (a) that all cells actively synthesising DNA will incorporate labelled thymidine into their DNA, (b) that DNA is metabolically stable, (c) that there is no reutilisation of DNA, and (d) that incorporation of radioactive thymidine into DNA does not cause any disturbance in cell proliferation. Despite difficulties in making some of these assumptions,

in vivo trace labelling of DNA can still be a useful indicator of DNA synthesis.

Since the discrepancy between results of incorporation of DNA in vivo and in vitro were likely to be due to the in vitro technique, and since the deoxyuridine suppression test is an <u>in vitro</u> technique and therefore subject to the same limitations of measuring labelled thymidine incorporation into DNA, a new <u>in vitro</u> model for measuring rates of DNA synthesis, together with the relative utilisation of the two pathways for thymidylate synthesis, was established to overcome some of these limitations.

(ii) Proposed model: The enzyme activities for the salvage pathway are high in folate deficiency suggesting a greater utilisation of this pathway. This could also be predicted if the postulate, that folate deciciency restricts thymidylate synthesis by the <u>de novo</u> pathway, is correct). The specific activity of the intracellular thymidylate pool may therefore be higher and hence the specific activity of DNA is also higher. It is therefore the specific activity of the thymidylate pool which affects the specific activity of the DNA, and not that of added thymidine. Measurement of the specific activity of the direct end-product of <u>de novo</u> and salvage pathways ie. thymidylate following incubation with labelled thymidine, will give a more appropriate indication of the relative utilisation of the 2 pathways, since such measurements are independent of the limitations of specific activity of DNA being influenced by the specific activity of thymidylate.

The principles of the model are based on those first proposed for nucleotide pools by Quastler, (1963), and are best summarised in the following diagram (Figure 2 - 4a).

# FIGURE 2 - 4a

Model for thymidine nucleotide pools: for measuring the relative utilisation of the pathways for thymidylate synthesis and fractional rates of DNA synthesis.



The specific activity (S) of a substance containing a labelled atom, is the amount of radioactivity per unit of substance.

ie.	DPM µmole/DNA	•	S <sub>DNA</sub>
	DPM µmole/dTTP		Sdttp
	DPM µmole/TDR		STDR

The specific activity of the intracellular thymidine pool (TDR<sub>i</sub>) is assumed to be equal to the known concentration of exogenous thymidine (TDR<sub>e</sub>) in the incubation medium. The specific activity of DNA after a suitable incubation period, can be measured following extraction of acid insoluble material from the cells. The specific activity of the soluble nucleotide pool of thymidylate, after the same period of incubation, can also be measured by chromatographic techniques.

1 mole of DNA was calculated as the mean of the molecular weights of its four major component deoxyribonucleoside triphosphates, ie. dCTP, dGTP, dATP and dTTP (after weighting for the proportion of each base found in rat tissue DNA - Biochemists' Handbook, 1961).

The utilisation of the <u>de novo</u>  $(V_{dn})$  relative to the utilisation of the salvage pathway  $(V_s)$  can be calculated as the proportion of radioactivity in the dTTP pool relative to the radioactivity in the thymidine pool.

$$\frac{S_{dTMP}}{S_{TDR}} = \frac{V_s + V_{dn}}{V_s}$$

By knowing the specific activity of the product i.e DNA, and the specific activity of its precursor ie. dTMP (or dTTP), at a given time, the fractional rate of DNA synthesis can also be calculated:

The absolute rate of synthesis (V) is the rate at which DNA is being synthesised ie. the amount of dTTP incorporated into DNA per unit time (t) ie.  $\mu$ moles/minute.

V can be calculated as the rate of transfer of radio- activity to DNA (DPM\_{DNA})

ie  $V \times S_{TTP} = \frac{DPM_{DNA}}{t}$ 

The fractional rate of synthesis  $(k_s)$  is the fraction or percentage of DNA that is being synthesised per unit time

Since V can be calculated, the fractional rate of synthesis can be expressed as:

$$\frac{V}{\mu moles DNA} \times S_{TTP} = \frac{DPM_{DNA}}{\mu mole DNA} \times \frac{1}{t}$$
  
ie.  $k_s \times S_{TTP} = \frac{S_{DNA}}{t}$  or  
 $k_s = \frac{S_{DNA}}{S_{TTP}} \times \frac{1}{t}$ 

#### Assumptions:

There are a number of assumptions made when applying this model for measuring <u>in vitro</u> rates of DNA synthesis:

1. That  $\text{TDR}_{e}$  and  $\text{TDR}_{i}$  are equal. The intracellular pool of thymidine is very small and is unlikely to affect the specific activity of the added labelled thymidine. Similarly the time of incubation (40 minutes) and the large volume of incubation medium compared to cell mass, would probably limit any decrease in specific activity of  $\text{TDR}_{e}$  caused by DNA breakdown. Uptake of  $\text{TDR}_{e}$  by cells is usually very rapid and is by simple diffusion (Gentry <u>et</u> <u>al</u>. 1965; Cleaver, 1967; Hauschka, 1973). It is also assumed that the dilution of  $\text{TDR}_{i}$  from thymine is minimal and that there is no synthesis of thymidine from deoxyuridylate (dUMP). There is no evidence to suggest that this latter reaction is important. The predominant supply of thymine or thymidine to the incubated cells is therefore the known amount of labelled thymidine added to the incubation medium ie.  $\text{TDR}_{e}$ .

2. That the thymidylate pool maintains a constant specific activity over the period of the incubation (following an initial equilibration period), and that the incorporation of label into DNA is linear over this time period. This assumption has been shown to be valid by measuring the specific activity of dTMP and the specific activity of DNA at varying time points (0+40 minutes). Preliminary investigation:

Following a five minute preincubation period at  $37^{\circ}$ C, crypt cells (1 ml) (prepared, characterised and assessed for viability by the methods described in Section 2 - 2) were incubated at  $37^{\circ}$ C in 1 ml of TC199 medium (folate and thymine free, with added fatty acid free bovine serum albumin-FAFBSA 20mg/ml), containing a tracer dose of tritiated thymidine (0.81µM, 0.013µCi/ml) for time intervals of up to 40 minutes. Cells were continuously gassed with 95%0<sub>2</sub>, 5%CO<sub>2</sub>. At the end of the incubation times, the cells were washed 3 times in



ice cold Krebs Henseleitt buffer (pH 7.2) and the specific activities of the dTTP pool and DNA were measured by the methods described in Section 2 - 4b (Page181) and Section 2 - 2b (Page 93) respectively.

The results from a preliminary study of 3 control animals (see Figure 2 - 4b) indicated that incorporation of label into the DNA of crypt cells was linear over the 40 minute time period studied following an initial 5 minute lag period ie. the rate of DNA synthesis was therefore assumed to be constant between 5 and 40 minutes. The specific activity of the dTTP pool increased from 0 - 4 minutes and then remained constant for the remaining time period (Figure 2 - 4b). Due to the fact that there was an initial lag phase, the gradient of the rate of incorporation after 5 minutes was greater than if the rate increased linearly from time 0 (Figure 2 - 4c).




This could mean that the rate of DNA synthesis calculated could be faster than the actual rate. However, by using a long incubation time period of 40 minutes prior to measuring incorporation, the effect of the lag phase would become less significant.

3. A third assumption is that the added thymidine does not affect the rate of DNA synthesis. It was decided to look at the effect of increasing thymidine concentration on the rate of DNA synthesis in both control and folate deficient animals. It was postulated that in normal cells the concentration of thymidine has no effect on the rate of DNA synthesis, probably because the salvage pathway is not normally the major pathway for thymidylate synthesis. In folate deficiency however, if there is a slower rate of DNA synthesis compared with controls, it might be that by the addition of thymidine it is possible to increase this rate. This would be such that thymidine 'rescues' the cells and allows DNA synthesis to proceed at a more normal rate since thymidylate is no longer limited by the block in de novo synthesis caused by folate deficiency.

4. Thymidylate (dTMP) is rapidly phosphorylated to thymidine triphosphate (dTTP) via thymidine diphosphate. The rapidly equilibrating mono-, di- and triphosphates of a nucleoside, are generally considered to be in the same pool, and will have the same specific activities following incubation with tritiated thymidine. Since dTTP is the major thymidine nucleoside phosphate in cells (Gentry <u>et al</u>. 1965; Lindberg <u>et al</u>. 1969), it was appropriate to extract and measure the specific activity of this pool from the epithelial cells.

#### 2 - 4b: METHODS

(i) Three groups (ie. control, control + antibiotic and folate deficient) containing 24 rats each, were bred as described in Section 2 - 1. Growth rates, red cell and mucosal folate status were assessed in all animals as described in Section 2 - 1. Fractions containing indiviual epithelial cells were isolated and characterised from 16 animals in each group, according to the method described in Section 2 - 2. Cell fractions 1 - 3, 4 - 6 and 7 - 9 were pooled into villus-tip, mid-villus and crypt fractions respectively, and corresponding fractions from 2 animals in the same group were also pooled. Viability of the crypt cell fractions when incubated at 37°C in TC199 (thymine and folate free), was assessed by measurement of oxygen consumption, and intracellular ratios of  $K^+/Na^+$  (Section 2 - 2). Additional assessment of functional viability with respect to DNA metabolism in vitro was made by measuring the linearity of incorporation of labelled thymidine into DNA as described above. The crypt cell fractions were then used for incubation studies to assess the rates of utilisation of the de novo and salvage pathways as described below. Measurements of DNA and protein concentration were made on all fractions from all animals by the methods described in Section 2 - 2. Cell turnover rates were measured in the remaining 8 animals from each group, following intraperitoneal administration of tritiated thymidine at 4 or 36 hours, by the method outlined in Section Section 2 - 1.

(ii) Incubation studies: Aliquots of crypt cell suspension (6ml) were mixed in a siliconised 50ml Erlenmeyer flask, with 5.7ml of the pregassed incubation medium TC199 (Prepared by Wellcome Reagents Limited to contain all the essential nutrients for cell culture except thymine and folic acid acid. Compositional details are given in Appendix 2. Fatty acid free bovine serum albumin (20 mg/ml) was added to the TC199 medium on the day of the assay). The cells were then incubated at  $37^{\circ}$ C for 5 minutes, after which

300µl of tritiated thymidine of varying concentrations(0.035+16.2µM; 0.156µCi) was added. The flasks were then incubated for a further 40 minutes with continuous gassing (95%02, 5%002) and intermittent agitation. At the end of the incubation the flasks were removed onto ice and the cells transferred to siliconised test tubes. The cells were centrifuged (200 x g for 1 minute) to remove the incubation medium, and then resuspended and washed three times in ice cold phosphate buffered saline (5ml) to remove the remaining incubation medium, and therefore any thymidine bound to the albumin. The cells were next homogenised in 4ml of a mixture of methanol, chloroform and water (12 parts:5 parts:3 parts) and centrifuged at 1000 x g for 10 minutes. The supernatant was retained and the precipitate rehomogenised and centrifuged in a further 4ml of methanol, chloroform and water. The second supernatant obtained was pooled with the first supernatant and 2ml of chloroform, followed by 3ml of water were added to the pooled supernatants. The mixture was centrifuged at 1000 x g for 20 minutes in order to separate the aqueous phase from the chloroform and methanol phase. The top aqueous layer contained the soluble nucleotide pools, and this layer was removed and stored at -20°C prior to separation of thymidylate by thin layer chromatographic techniques as outlined below. This method of pool extraction was based on that described for crude extracts from plants for analysis of amino acid content by chromatographic techniques (Bieleski & Turner, 1966). It was chosen in preference to the usual acid extraction procedure because the nucleotides were present in an aqueous medium which could be vacuum dried, resuspended in a small volume of water (50µl) and used directly for nucleotide analysis. The frequently used perchlorate or trichloacetate extraction techniques require neutralisation and desalting steps prior to chromatographic analysis.

The precipitated material which remained after extraction of the nucleotide pools and which contained the DNA was homogenised in lml of 2% PCA and stored at  $-20^{\circ}$ C prior to extraction and analysis of DNA content and specific activity. The incorporation of tritiated thymidine into DNA

ic. specific activity of DNA, was measured by the method described in Section 2 - 2b, (ie. acid hydrolysis followed by diphenylamine analysis of DNA content, and scintillation counting of the hydrolysate).

(iii) Analysis of thymidine nucleoside phosphate pools: The resolution of a complex mixture of ribonucleotides and deoxyribonucleotides can be achieved using separation methods involving paper chromatography, electrophoresis, thin layer chromatography or ion exchange chromatography. The appropriate use of each of these methods has been well summarised by Hauschka (1973). Thin layer chromatographic techniques are convenient requiring a minimum of apparatus, and give excellent resolution with extremely small samples, and they have been widely adopted for pool studies. Neuhard et al. (1965) and Randerath and Randerath (1967) have made valuable contributions to the development of thin layer chromatographic (TLC) techniques. Randerath and Randerath (1967) have summmarised the main advantages of TLC methods, as being sharpness of resolution, great sensitivity, simplicity and speed. Two dimensional anion exchange TLC allows the resolution of complex mixtures of nucleotides that are difficult to separate on paper or on a single column. Layers of P.E.I. cellulose (cellulose impregnated with polyethylene imine as the anion exchanger), are commonly used for the analysis of nucleic acid components ie. nucleotides, nucleosides, nucleobases and sugar phosphates.

The method of Neuhard <u>et al</u>, 1965 (Procedure 1 - using P.E.I. cellulose plates), was adopted (with minor modifications) for use in these experiments. <u>TLC Method</u>: Samples (50µ1) of aqueous cell extract or standard solution (50µ1) containing 15nmoles of nucleotide, were spotted on to ready prepared P.E.I. cellulose plates (Merck Limited 20cm x 20cm. Cellulose layer was 0.1mm thick). The position of the applied spot in relation to the direction of the eluting runs, is shown in Figure 2 - 4d. The plates were first washed in Direction B with anhydrous methanol (50 minutes) to remove unwanted salts from the origin. The plates were then developed in Chromatogram of a mixture of ribonucleotide and deoxyribonucleotide standards (15n moles of each compound) applied at origin X.



Chromatogram developed in direction A:

i) 1.0M LiCl saturated with borate (pH 7.0 with  $NH_3$ ), to 12cm from X.

11) 2.0N HCOOH/1.6M LiCl (1:1, v/v) to 12cm from X.

Chromatogram developed in direction B:-stepwise elution. with:

i) 0.5M  $(NH_4)_2SO_4$  to 4cm from X.

ii) 0.7M  $(NH_4)_2SO_4$  to 15cm from X.

\* - \* solvent front direction A.

\*\* - \*\* solvent front direction B.

Direction A to 12 cm from the origin with a borate system: 1.0M LiCl was saturated with boric acid at room temperature and brought to pH 7 by the additon of ammonia (specific gravity 0.9). The plates were then dried in a stream of air and electrolytes were removed by treatment in a bath of anhydrous methanol for ten minutes. The plates were again dried and chromatographed in the same direction with 2.0N HCOOH 1.6M LiCl (1:1 v/v) up to 12cm from the origin. The plates were dried in a stream of warm air and then neutralised by laying flat in a bath of Tris-methanol (120mg% Tris in anhydrous methanol) for 5 minutes. The plates were again dried and treated for 10 minutes with anhydrous methanol. After drying, development in the second direction was carried out by stepwise elution with 0.5M  $(NH_4)_2SO_4$  up to 4cm from the origin and 0.7M  $(NH_4)_2SO_4$  up to 15cm from the origin. After drying the plates were again treated for 10 minutes in methanol and dried at room temperature. All the development reagents were used at room temperature and were made up using Aristar grade materials. The whole procedure took 33+4 hours.

Separated nucleotide compounds were detected by examination under a U.V. lamp. The prominence of each spot was enhanced by the fluorescing indicators present in the P.E.I. cellulose. Using mixtures of standards, and standards alone, the position of each ribonucleotide and dexoyribonucleotide on the developed chromatogram was ascertained. The solvent containing borate, separates deoxyribonucleotides from ribonucleotides, and the rate of migration decreased in the order of deoxyribonucleoside diphosphates > ribonucleoside diphosphates > deoxyribonucleoside triphosphates > ribonucleoside triphosphates, and in a base sequence of uridine/ thymidine compound > cytidine > adenine > guanine. Ribonucleoside and deoxyribonucleoside monophosphates run closely associated with the solvent front. The formic acid system clearly separates di- and triphosphates the rate of migration of diphosphates being greater than that for triphosphates. Ammonium sulphate in the second direction separates chiefly

according to the base moiety. The migration rate for uridine/thymidine compounds was greater than cytidine > adenine > guanine compounds, with the diphosphates preceding the corresponding triphosphates. The final position of each nucleoside phosphate is shown in Figure 2 - 4d. These positions correspond closely with those of Neuhard et al (1965) and Randerath and Randerath (1967). It was not possible to separate dTTP and deoxyuridine triphosphate (dUTP) by this method, but since dUTP is not a commonly found nucleoside phosphate, the contamination of the dTTP pool from cell extracts was expected to be minimal.

<u>Elution of nucleotides:</u> Standard spots, and the spots from the cell extract chromatograms which corresponded to the dTTP standard, were eluted from the plate on to a paper wick (Whatman No. 1 filter paper) using 0.7M MgCl<sub>2</sub>; 2MTris HCl pH 7.4. (100:1 v/v) (Randerath & Randerath, 1967). The paper wick was dried and cut up finely and the dTTP was eluted from it in  $500\mu$ l of the MgCl<sub>2</sub>/Tris eluent in a test tube for 2 hours. The paper was removed by centrifugation (1000 x g for 5 minutes). The dTTP was characterised by its absorption spectrum (measured in a Gilford SP240 spectrophotometer) and quantitated by comparison with standard solutions of dTTP. A blank sample was eluted from a spot on the plate adjacent to the dTTP spot.

That the spot isolated from the cell extract was dTTP was confirmed by its absorption spectrum (max.  $267m\mu$ ). In addition, by adding a standard solution of dTTP to the cell extract, it was possible to detect that the migration characteristics were the same as when dTTP standards were run alone or with a known mixture of standard nucleoside phosphates.

Following spectrophotometric analysis, an aliquot  $(50\mu1)$  of the dTTP solution was added to 10ml of scintillation fluid (0.4% PPO in toluene/ triton X 100; 2:1 v/v) and the radioactivity counted in a Nuclear Chicago Delta 300 - liquid scintillation counter, to a 3% level of efficiency. The specific activity of the dTTP pool could then be calculated ie. DPM per umole dTTP.

#### 2 - 4c: RESULTS

At 42 days, rats from control, control + antibiotic and folate deficient groups weighed 83.6  $\pm$  3.9g, 78.4  $\pm$  5.0g and 60.9  $\pm$  3.2g respectively. The growth rates and daily food intakes were similar to those found in the previous experiments. Table 2 - 4a summarises the data for villous heights, crypt depth, cell migration rates and cell turnover times for each of the three groups of animals, demonstrating similar trends in folate deficiency as found in previous data ie. crypt depth was increased (190  $\pm$  17µm in folate deficiency v 130  $\pm$  12µm in controls + antibiotic) as was cell turnover time  $(89.2 \pm 9.4 \text{ hours in folate deficiency})$ v 58.3  $\pm$  5.7 hours in controls + antibiotic). For a period of 8 months there was no supply of Folic acid Casei medium in the United Kingdom, which meant that samples for mucosal and red cell folate analysis were kept frozen for a prolonged period of time. When finally analysed the results were considerably more variable than usual. However, there was still a significant difference between controls and folate deficient groups, and so the group fed the folate deficient diet were considered to have a poor folate status compared with both control groups. This was supported by the poor growth rates and the presence of diarrhoea in this animal group.

Table 2 - 4b gives the results for sucrase and thymidine kinase activities in the 3 fractions of isolated enterocytes, which were characterised as cells from villus-tip, mid-villus and crypts. The crypt cells from all groups had high activities of thymidine kinase, (5910  $\pm$  370pmolesdTMP/µg enzyme protein/hour, in controls) compared with the villus-tip cells (410  $\pm$  40pmolesdTMP/µg enzyme protein/hour, in controls) and low activities of sucrase (0.89  $\pm$  0.06µg glucose/µg enzyme protein/hour, in controls) compared with villus-tip cells (2.36  $\pm$  0.19µg glucose/µg enzyme protein/hour in controls).

Figure 2 - 4e demonstrates the results of oxygen consumption and intracellular  $K^+/Na^+$  ratio studies, made on the crypt cells under incubation conditions. The rate of oxygen consumption was similar to that reported

## TABLE 2 - 4a

Villous height (um), crypt depth (um), cell migration rate (um/hour) and cell turnover time (hours) of jejunal mucosa of control, control + antibiotic and folate deficient rats.

	VILLOUS HEIGHT	CRYPT DEPTH	MIGRATION RATE	CELL TURNOVER TIME
	рш	μπ	µm/hr	hrs
CONTROL	450±40	130±11	9.65±1.2	60.1±5.4
CONTROL + ANTIBIOTIC	445±40	130±12	9.86±0.9	58.3±5.7
FOLATE DEFICIENT	<b>390±30</b>	190±17 <sup>**</sup>	6.5 ±0.5 <sup>*</sup>	89.2±9.4**

Mean values ± standard errors from groups of 8 animals.

\* p < 0.05 compared with both control groups.

\*\* p < 0.01 compared with both control groups.

## TABLE 2 - 4b

Validation of cell isolation procedure: Sucrase activities (µg glucose/ µg enzyme protein/hour), and thymidine kinase activities (p moles dTMP/ µg enzyme protein/hour) of villus-tip, mid-villus and crypt cell fractions isolated from the small intestine of control, control + antibiotic and folate deficient rats.

ANIMAL GRO CELL FRACT	DUP & TION	SUCRASE ACTIVITY	THYMIDINE KINASE ACTIVITY
CONTROL	villus-tip	2.36±0.19	410± 40
	mid-villus	1.51±0.20	1830±150
	crypt	0.89±0.06	5910±370
CONTROL +	villus-tip	2.26±0.13	870± 70
ANTIDIUTIC	, mid-villus	1.39±0.12	2000±140
	crypt	0.97±0.09	7020±580
FOLATE	villus-tip	2.51±0.19	1500±800
DEFICIENT	mid-villus	1.43±0.09	8210±900
	crypt	1.36±0.13	11000±1000

Mean values ± standard errors from groups of 16 animals.



# FIGURE 2 - 4e

<u>Viability studies on crypt cells isolated from the small intestine of</u> <u>control, control + antibiotic and folate deficient rats.</u> Cells were used for incubation studies at  $37^{\circ}$ C, using TC199 (folic acid & thymine free) as incubation medium. Mean values from groups of 16 animals. Rates of oxygen consumption were measured using an oxygen electrode system;  $K^+/Na^+$  ratios were measured by flame photometry after digestion of the cell-

ular material in hot acid.

in Section 2 - 2, but was maintained at a constant rate for up to 60 minutes compared with 40 minutes. This was attributed to a change in the incubation medium from Krebs Henseleitt (containing lOmg/ml FAFBSA and 250mg%glucose) to TC199 (containing lOmg/ml FAFBSA; free of thymine and folic acid).

Table 2 - 4c gives the results of the composition of the cell fractions with respect to protein and DNA concentrations and Protein/DNA ratios, for each of the animal groups. In the folate deficient group DNA concentrations were lower at all levels of the villus, particularly in the crypts (p<0.001) when compared with both control groups. (Crypt fractions contained 1.87  $\pm$  0.15, 4.19  $\pm$  0.42 and 4.0  $\pm$  0.32mg DNA/cell fraction in folate deficients, controls and controls + antibiotic respectively.) Protein concentrations were also slightly decreased in the folate deficient animals, but not as much as DNA concentrations. Protein/DNA ratios were therefore greater in folate deficient cells, at all levels of the villus. These results were similar to those from the experiments discussed in Section 2 - 3. There was a similar distribution of cells between fractions to that reported in Section ? - 2 (ie. 12.7%, 13.8%, 16.4% in villus-tip fractions, 39.2%, 35.6%, 34.3% in mid-villus fractions, 48.0%, 50.6%, 49.3% in crypt fractions, in controls, controls + antibiotic and folate deficients respectively).

(ii) Utilisation of <u>de novo</u> and salvage pathways: Table 2 - 4d gives the percentage utilisation of the salvage pathway for thymidylate synthesis at each concentration of added thymidine, in both control groups and the folate deficient group. In both control groups the salvage pathway accounted for 20 - 41% of thymidylate synthesis when the rate of DNA synthesis was constant ( $0.81\mu$ M+16.2 $\mu$ M TDR). In folate deficiency increasing the exogenous concentration of thymidine increased the relative utilisation of the salvage pathway from 60+97% ( $0.81\mu$ M+16.2 $\mu$ M TDR).

## TABLE 2 - 4c

<u>Composition of cell fractions</u>: Protein and DNA concentrations (mg/cell fraction) and protein/DNA ratios in isolated epithelial cell fractions from villus-tip, mid-villus and crypt regions of the small intestine of control, control + antibiotic and folate deficient rats.

ANIMAL GROUP & CELL FRACTION		PROTEIN	DNA	PROTEIN DNA
		mg	mg	
CONTROL	villus-tip	27.42±3.01	1.11±0.09	24.70±3.42
	mid-villus	<b>49.31</b> ±3.90	3.42±0.31	14.42±1.31
	crypt	58.62±5.62	4.19±0.42	13.99±1.08
CONTROL + ANTIBIOTIC	villus-tip	21.03±1.91	1.09±0.03	19.29±6.3
	mid-villus	42.62±4.01	3.81±0.19	15.17±1.3
	crypt	55.09±4.69	4.00±0.32	13.77±1.5
FOLATE DEFICIENT	villus-tip	19.72±1.72	0.62±0.06 <sup>+</sup>	31.81±2.9
	mid-villus	36.39±3.29 <sup>*</sup>	1.30±0.09 <sup>+</sup>	27.99±3.7 <sup>†</sup>
	crypt	41.00±4.00**	1.87±0.15 <sup>+</sup>	21.93±2.7 <sup>***</sup>

\* p<0.05 compared with control group.

**\*\*** p<0.05 compared with both control groups.

\*\*\* p<0.01 compared with both control groups.

+ p<0.001 compared with both control groups.

Mean values ± standard errors for groups of 16 animals.

# TABLE 2 - 4d

Percentage utilisation of the salvage pathway for thymidylate synthesis in crypt cells isolated from control, control + antibiotic and folate deficient rats. Cells were incubated at  $37^{\circ}$ C for 40 minutes in TC199 medium (folic acid and thymine free; containing 10mg/ml FAFBSA) with varying concentrations of added thymidine (0.013µCi/ml; 0.035 + 16.2µM).

EXOGENOUS THYMIDINE	CONTROL	CONTROL + ANTIBIOTIC	FOLATE DEFICIENT
μМ	%	%	%
0.035	15± 2	19± 2	66±18
0.081	20± 2	14± 5	75±13
0.162	42±14	14± 3	67±11
0.324	23± 3	27± 4	67±11
0.810	26± 5	20± 3	60±14
1.620	39± 6	26± 8	101± 9
3.240	28± 6	41±11	91± 8
6.480	28± 4	29± 8	81± 3
16.200	37±12	37±12	97±11

Mean values ± standard errors from 6 incubations.

(iii) Rates of DNA synthesis: The fractional rates of DNA synthesis (% per day), calculated for each concentration of thymidine added to the incubation medium, are given in Table 2 - 4e and Figure 2 - 4f. The rate of DNA synthesis in both control groups plateaued at a concentration of 0.81µM thymidine. At this concentration of thymidine, the precursor pool becomes saturated with label after 4 minutes, and the specific activity of thymidine triphosphate remains constant for the remainder of the incubation period (see Figure 2 - 4b). Increasing the exogenous supply of thymidine over 0.81µM, had no effect on the thymidine triphosphate pool, which incorporated thymidine at the same rate, and maintained a constant rate of DNA synthesis at 43% per day. In control cells which were incubated with thymidine concentrations between  $0.035 \mu M$  &  $0.81 \mu M$ , the rate of DNA synthesis was not constant. This is because at low concentrations of exogenous thymidine, there is a longer equilibration time between the exogenous thymidine and dTTP pool - exceeding the time of incubation. In other words there is a longer lag period before the specific activity of the dTTP pool becomes constant and the rate of incorporation of label into DNA becomes linear. In folate deficiency, the rate of DNA synthesis did not plateau until a much higher concentration of exogenous thymidine was supplied. This may be due to several reasons. Firstly, it may be that in folate deficiency it takes longer for the thymidylate (precursor) pool to equilibrate with the exogenous thymidine pool, so that incorporation of label into DNA has not become linear even at concentrations of  $0.81 \mu M$  TDR, - when the rate of DNA synthesis was constant in controls. However, this was thought to be unlikely because appproximately 76% of thymidylate is synthesised via the salvage pathway in folate deficiency, compared with 25% in controls. Further, the fact that the specific activity of the dTTP is higher in folate deficiency, suggests a smaller pool size. A smaller pool size was reported following acute folate deprivation caused by methotrexate in vitro in PHA stimulated lymphocytes (Hoffbrand et al. 1974). It would therefore be expected that, in folate deficient cells, equilibration of the dTTP pool with

## TABLE 2 - 4c

Fractional rates of DNA synthesis in crypt cells isolated from control, control + antibiotic and folate deficient rats. Cells were incubated at  $37^{\circ}C$  for 40 minutes in TC199 medium (folic acid and thymine free; containing l0mg/ml FAFBSA) with varying concentrations of added labelled thymidine (0.013µCi/ml; 0.035 + 16.2µM).

EXOGENOUS THYMIDINE	CONTROL	CONTROL + ANTIBIOTIC	FOLATE DEFICIENT
μМ	% /day	% /day	% /day
0.035	10.2±1.3	9.9±0.3	5.5±0.7
0.081	10.0±1.0	12.1±0.6	6.3±0.7
0.162	13.7±1.6	17.9±1.2	11.1±1.7
0.324	27.6±1.2	31.4±2.6	19.7±2.7
0.810	39.9±1.3	39.5±2.2	30.1±2.6
1.620	42.3±1.4	39.0±1.9	45.2±5.5
3.240	44.1±2.2	38.6±2.7	58,8±3,1
6.480	43.4±2.8	40.5±2.5	66.7±2.6
16.200	44.9±3.7	39.9±2.6	64.5±2.3

Mean values ± standard errors from 6 incubations.

Fractional rate of DNA synthesis(% per day)  $\equiv \frac{S_{DNA}}{S_{dTTP} \times time(days)} \times 100$ 



exogenous thymidine would be at a faster rate than in control cells, if anything. The rate of DNA synthesis in folate deficient cells at the concentration of thymidine required for thymidine triphosphate pool saturation in control cells (ie.  $0.81\mu$ M), was 30% per day (p<0.001 compared with controls), but this rate increased to 65.5% per day if sufficient thymidine was supplied to the cells (ie. >6.8µM TDR).

#### 2 - 4d: DISCUSSION

The significance of the salvage pathway in the synthesis of thymidylate has been the subject of frequent discussion, as was described in the General Introduction (Section 1 - 4). The results of this present study suggest that the salvage pathway has a role in jejunal crypt cells from control animals in that, an average 26% of the thymidylate synthesised is via this pathway. Previous work using incubated gut rings and the deoxyuridine suppression test (Badcock, 1975) suggested that 25% of the thymidylate synthesised was from the salvage pathway. The results from the deoxyuridine suppression test in isolated crypt cells (see Appendix 3) similarly suggested a utilisation of 21%. Weinstock et al. (1970) demonstrated changes in the relative utilisation of the 2 pathways with changes in maturity and rates of DNA synthesis. Mature cells were more dependent on the salvage pathway than immature cells. Immature cells therefore had a high requirement for folate for the de novo synthesis of thymidylate. It would seem that the immature cells of the gut epithelium are also highly dependent on the presence of folate for normal DNA synthesis. This is emphasised by the fact that approximately 75% of the thymidylate synthesised in control cells was via the <u>de novo</u> pathway, and also by the slower rates of DNA synthesis and lower concentrations of DNA in the folate deficient crypt cells. However, it was interesting to note that in folate deficiency, in association with higher activities of thymidine kinase, the relative utilisation of the salvage pathway for thymidylate synthesis was considerably greater than in controls. However, if the supply of thymidine was limiting, then the potential

for maintaining the synthesis of thymidylate and therefore of DNA could not be utilised, and DNA synthesis was impaired. By supplying increasing amounts of thymidine it was possible to increase the rate of DNA synthesis in folate deficient crypt cells to a level which was greater than the normal rate of DNA synthesis in control cells. This was surprising, but suggests that the cells compensate for the decreased DNA concentrations and rapidly synthesise new DNA. The increased DNA synthesis rate was presumably a reflection of increased thymidylate synthesis via the salvage pathway. This was supported by the fact that increasing the concentration of thymidine supplied to the crypt cells in folate deficiency, increased the percentage utilisation of the salvage pathway compared with the de novo pathway the de novo pathway is already restricted in activity to its lowest limits because of the reduced supply of folate. The reduction in thymidine triphosphate levels of 5 cell culture lines following the administration of lethal doses of the antifolate drug methotrexate has been reported (Tattersall et al. 1974). The administration of thymidine at the same time as methotrexate prevented the fall in the thymidine triphosphate pool size. This suggested that thymidine may be able to 'rescue' the cells from methotrexate toxicity and/or folate deficiency, providing there was not an intracellular purine deficiency. Similarly in megaloblastic anaemia, the activity of the enzyme thymidine kinase is greater than normal, and Bock et al (1967), Hoffbrand et al. (1974) and Wickramasinghe (1975), have suggested that the impairment of endogenous thymidylate synthesis resulting from folate deficiency may be partly or wholly offset by the enhanced activity of the salvage pathway. Significant reticulocyte responses and increases in red cell counts were reported by Spies et al. (1946; 1948) and Frommeyer et al. (1946), with daily doses of 5g of thymine or thymidine, given to patients with pernicious anaemia, tropical sprue and megaloblastic anaemia. Killman (1964) infused 5g of thymidine into 5 patients with pernicious anaemia over a few days, and obtained reticulocyte responses in them all.

Whether the elevated rates of DNA synthesis following thymidine administration in folate deficiency can be maintained until normal concentrations of DNA are restored is not known. It may be that sufficient DNA is not synthesised until folate is supplied again. The treatment of folate deficient rats <u>in vivo</u> with increasing concentrations of thymidine alone or thymidine plus folate, followed by an assessment of total gut DNA concentration and synthesis rates may answer some of these questions. The studies of Spies <u>et al</u>. (1946; 1948) and Frommeyer <u>et al</u>. (1946) while demonstrating initial reticulocyte responses in their patients, following thymidine administration, failed to show a restoration of normal red cell counts or normoblastic bone marrow. Similarly, Butterworth and Perez-Santiago (1956) found no evidence of improvement in the megaloblastic anaemia of tropical sprue following thymidine therapy. This suggests that a minimum amount of folate is always required for some thymidylate to be synthesised via the <u>de novo</u> pathway.

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In the folate deficient gut there was a decrease in the total amount of DNA synthesised, presumably because of the block in utilisation of the <u>de novo</u> pathway, and it can be postulated that the salvage pathway enzymes increase in response to this decreased synthesis. The fact that utilisation of the salvage pathway is increased, and in particular that thymidine kinase activities are greater under conditions of increased requirements for DNA such as in regenerating tissues, has frequently been demonstrated (Bollum & Potter, 1958; 1959; Smellie, 1963; Bresnick <u>et al.</u> 1967; Baugnet-Machieu <u>et al</u>. 1968). It would be interesting to study the changes in the two pathways as folate deficiency progresses, to delineate whether the utilisation of the two pathways changes progressively as folate becomes limiting, such that thymidine kinase activity and the subsequent utilisation of the salvage pathway increase in response to a decrease in synthesis of thymidylate, or whether there is an initial decrease in the concentration of DNA, before the salvage pathway activity is increased. Since thymidine kinase is subject to feedback inhibition by thymidine triphosphate (Reichard <u>et al</u>. 1960; Bresnick & Karjala, 1964) the former proposal of progressive changes in the utilisation of the two pathways is more likely. Repletion of the deficient animals with folate would also be expected to show a progressive reversal from the dependence of the crypt cells on the salvage pathway to 'normal' utilisation of the <u>de novo</u> pathway.

In the control group of rats, the calculated fractional synthesis rate was 43% in gut crypt cells, which is indicative of rapidly synthesising tissue. In view of the fast turnover rate of gut epithelial cells this rapid rate of DNA synthesis was not unexpected. The reduced DNA content of the folate deficient enterocytes and the slower cell turnover rates would suggest a slower rate of DNA synthesis, and when thymidine as a precursor is limiting this was indeed the case. These results are contrary to the results obtained from the usual method for measuring DNA synthesis rates in vitro ie. the incorporation of thymidine into DNA directly which ignores the size of the thymidylate pool. In folate deficiency this pool is depleted because of the block in de novo synthesis, and if the salvage pathway activity is increased. then the specific activity of thymidylate can be expected to increase following labelled thymidine administration, and therefore give rise to a higher specific activity of DNA ie. greater incorporation of labelled thymidine, which is totally independent of the rate at which the label is incorporated ie. the rate of DNA synthesis. In this experiment for example, the incorporation of label into DNA after a 40 minute incubation with 0.81µM thymidine was  $102 \pm 13 \times 10^3$  DPM/umole DNA in controls compared with  $176 \pm 19 \times 10^{3}$  DPM/umole DNA in the folate deficient group. However, the specific activities of the thymidylate pools (equivalent to the thymidine triphosphate (dTTP) pools measured) were  $91.5 \pm 3.1 \times 10^5 DPM/\mu mole dTTP and$ 211  $\pm$  14.2 x 10<sup>5</sup> DPM/umole dTTP in controls and folate deficients respectively. The high level of incorporation of tritiated thymidine into DNA in folate deficiency was therefore a reflection of the high specific activity of the

thymidylate pool, and not an increase in the rate of DNA synthesis. This emphasises the limitations of interpreting data on DNA synthesis rates which have been calculated from <u>in vitro</u> incorporation of labelled thymidine.

In summary, it was demonstrated that there was a slower rate of DNA synthesis in the mucosal epithelium of folate deficient animals compared with control animals. This would account for the decreased DNA concentrations found in these deficient animals. Decreased rates of DNA synthesis would decrease the rate of cell production, and if there was no change in the rate of cell extrusion from the villus-tips, and no alterations in villous height, then cell turnover time would be increased.

In folate deficiency, because of a block in the <u>de novo</u> synthesis of thymidylate, the salvage pathway enzyme - thymidine kinase - was increased. The relative utilisation of this pathway for thymidylate synthesis, compared with the <u>de novo</u> pathway, was also increased when compared with controls. The increased rate of DNA synthesis observed following administration of increasing amounts of exogenous thymidine suggest that the salvage pathway may be able to 'rescue' folate deficient cells and allow DNA synthesis to proceed.



#### SUMMARY

1. Mucosal folate deficiency is associated with diarrhoea and abnormalities of water and electrolyte transport, and malabsorption may occur. Previous reports on the intestine in folate deficiency have mostly been in association with other pathological processes damaging the mucosa such as infections or alcohol. However, the few studies of primary nutritional deficiency, have shown morphological abnormalities of the small intestine.

2. The studies reported here describe the establishment of an experimental model of folate deficiency in the rat, and a system for isolating viable epithelial cells from different regions of the villus of the jejunal mucosa. Studies on the isolated cells from control and folate deficient animals, have investigated the nature of the cellular changes in the mucosa, caused by the folate deficiency.

3. The development of folate deficiency was associated with diarrhoea, increases in crypt depth of the jejunal mucosa, and a reduction in total DNA, RNA and protein contents. The cell turnover time of the folate deficient mucosa was shown to be markedly increased, and the higher ratios of RNA/DNA and protein/DNA, found particularly in the villus-tip cells, reflected the longer time spent by the cells on the villus. The 'hypermature' cells had higher activities of disaccharidase enzymes such as sucrase, than normal villus-tip cells. Thus, despite an overall reduction in the total number of cells in the folate deficient intestine, total gut sucrase activity was normal. In contrast, activities of  $(Na^+-K^+)$ -ATPase per cell did not increase above normal in folate deficiency. The mechanisms controlling the activity of these two enzymes were discussed with reference to the nutritional significance of sucrase activity and the significance of abnormalities of fluid and electrolyte transport in the diarrhoea of mucosal folate deficiency.

4. A method for measurement of fractional rates of DNA synthesis was developed. Rates were lower in folate deficient crypt cells. Methods were also developed for the measurement of the relative contribution of <u>de novo</u> and salvage pathways for thymidylate synthesis. In control mucosa the <u>de novo</u> pathway was the most important, but in the folate deficient mucosa, the salvage pathway was predominant. DNA synthesis in folate deficiency was stimulated by the provision of thymidine. The role of nucleotides in promoting epithelial cell replication even in the presence of folate deficiency, were discussed.



#### APPENDIX 1

\* "The word radioautography is preferred to the word autoradiography, since the latter describes the technique as a form of 'radiography'. Radiography is a procedure by which the object under investigation is located between the source of radiation and emulsion. It is commonly held that radiography yields a negative picture of the object. In contrast, the procedure by which the object under investigation is itself the source of the energy influencing the emulsion, yields a positive picture and may be termed 'autography'. When done with radioactive rays, it is rightly called radioactive autography or, for short, radioautography".

\* From Kopriwa & Leblond (1962).

However, autoradiography still seems to be the usual word used in the literature, and has therefore been used throughout this thesis.

COMPOSITION OF TC199 (Wellcome Reagents Ltd. - working strength)

Inorganic Salts	mg/1	Lipid Sources	mg/1
NaC1	6800.0	Cholesterol	0.2
ксі	400.0	Tween	5.0
CaCl	200.0		
MgS04.7H20	200.0	Vitamins	
NaH2PO4.2H20	150.0	Aneurine HCl	0.01
Fe(NO3)3.9H20	0.72	Riboflavin	0.01
55 2		Pyridoxine HCl	0.025
Amino Acids		Pyridoxal HCl	0.025
1-arginine HC1	70.0	Nicotinic acid	0.025
1-histidine HC1	20.0	Nicotinamide	0.025
1-1ysine HCl	70.0	Ca Pantothenate	0.01
dl-phenylalanine	50.0	Inositol	0.05
dl-serine	30.0	p-amino-benzoic acid	0.05
dl-threonine	50.0	Choline chloride	0.5
dl-leucine	60.0	Ascorbic acid	0.05
dl-isoleucine	120.0	d-biotin	0.01
dl-valine	40.0	Folic acid <sup>*</sup>	0.01
dl-glutamic acid	50.0	Menaphthone	0.01
dl-aspartic acid	150.0	Calciferol	0.1
dl-alanine	60.0	Vitamin A acetate	0.1
1-proline	50.0	a-Tocopherol phosphate	0.01
1-hydroxyproline	40.0		
Glycine	10.0		
d1-tryptophane	50.0		
1-tyrosine	40.0		
1-cystine	20.0		
1-cystine HCl	0.1		

Nucleic Acid_Derivatives		Miscellaneous	
	mg/1		
Adenine HCl	5.1	Sodium acetate (hydrated)	
Guanine HCl	0.3	Glucose	
Xanthine	0.3	Phenol red	
Hypoxanthine	0.3	Glutathione	
Thymine *	0.3	l-glutamine	
Uracil	0.3		
Adenylic acid	0.2		
Adenosine triphosphate	5.0		
Deoxyribose	0.5		

\* The medium used in this study was specially ordered omitting the folic acid and thymine constituents.

# Notes

The medium came as a 10x concentrate and was diluted 1:9 with distilled water containing 50 ml of 4.4% sodium bicarbonate per 900 ml and then gassed for 15 minutes with 95%  $0_2$ , 5%  $C0_2$  prior to use.

mg/1 84.0 1000.0 10.0 0.05 100.0

# INCUBATION STUDIES USING THE DEOXYURIDINE SUPPRESSION TEST IN INCUBATED CRYPT CELLS

Three groups (control, control + antibiotic and folate deficient) of 24 rats each, were bred as described in Section 2 - 1, and fractions of epithelial cells were isolated and characterised from 16 rats from each group according to the methods described in Section 2 - 2. Cell fractions 1 - 3, 4 - 6 and 7 - 9 were pooled into villus-tip, mid-villus and crypt fractions respectively. In the deficient group of rats, when compared with both control groups, growth rates were slower (final weights, at 42 days, were 62  $\pm$  3.9g in the deficient group v 86.1  $\pm$  4.2g in controls and 80.9  $\pm$  6.4g in controls + antibiotic, p<0.01), folate status was poor (mucosal folates:  $18.0 \pm 3.1$ ,  $49.1 \pm 7.2$ ,  $48.3 \pm 7.2 \mu g/g$  protein, p<0.001, red cell folates: 200 ± 29.3, 49.1 ± 7.2, 48 ± 7.2ng/ml in folate deficients, controls and controls + antibiotic respectively), and cell turnover rates were slower (assessed in the remaining 8 animals,  $86.5 \pm 7.1$  hrs,  $59.3 \pm 4.6$  hrs,  $58.2 \pm 2.5$ 3.9hrs in folate deficients, controls and controls + antibiotic respectively). These results were similar to those found previously - such as those described in Section 2 - 1. The oxygen consumption and intracellular  $K^+/Na^+$  ratios of crypt cells were compatable with optimally viable cell systems for each animal group, with a similar pattern of results to those described in Section 2 - 2.

Aliquots (1ml) of Krebs Henseleitt buffer containing 20mg/ml FAFBSA and 500mg% glucose, were added to siliconised flasks. Quadruplicate flasks were set up per animal, and deoxyuridine (0.25mM) was added to each of 2 flasks. Aliquots of crypt cell suspension (1ml) were added to the media, and incubated at  $37^{\circ}$ C for 15 minutes with continuous gassing. Tritiated thymidine (0.81µM, 0.026µCi) was added to each flask and the incubation continued for a further 15 minutes. The reaction was then stopped by adding 40µl of 72%

PCA to each flask, and samples were transferred to test-tubes and centrifuged at 1000 x g for 20 minutes. The precipitated cellular material was homogenised in 1ml of 2% PCA and stored at -20°C, prior to extraction of DNA (by the acid hydrolysis method described in Section 2 - 2). The method of stopping the reaction by precipitating the cellular material, also precipitated the albumin in the incubation medium. Labelled thymidine has been shown to bind to albumin (Morley & Kingdom 1972; Goldspink & Goldberg, 1973), and this was demonstrated again in this study. This bound thymidine was released when the DNA content of the precipitate was extracted by hot acid hydrolysis. Blank incubations where the viable crypt cell suspension was replaced with lml of Krebs Henseleitt buffer, or lml of boiled cells, or 1ml of PCA precipitated cells, gave counts of 3378 ± 58 DPM in the acid hydrolysate. This radioactivity could not be associated with DNA synthesis since none could have occured under these conditions. Further experiments demonstrated a non-specific binding of labelled thymidine, which was consistently associated with a protease sensitive substance and which was attributable to the albumin containing medium. Results were therefore calculated to account for this binding factor, and in subsequent incubation studies with labelled thymidine, the cells were washed free of albumin medium, and therefore the bound thymidine, prior to precipitation of cellular material with PCA.

Uptake of labelled thymidine into DNA (after allowance for the amount of albumin bound thymidine) was expressed as DPM per mg DNA. The uptake of labelled thymidine into DNA after preincubation with deoxyuridine expressed as a percentage of the uptake of labelled thymidine without preincubation, was calculated as the deoxyuridine suppressed value. The results for the incorporation of label into DNA with or without preincubation with deoxyuridine are shown in the following table, together with the calculated deoxyuridine suppressed value.

ANIMAL GROUP	NO PREINCUBATION DPM/mg DNA × 10 <sup>-3</sup>	+ PREINCUBATION DPM/mg DNA x 10 <sup>-3</sup>	DEOXYURIDINE SUPPRESSED VALUE %
control	137 ± 12	29 ± 4	21
control + antibiotic	326 ± 29	62 ± 9	19
folate deficient	520 ± 63	119 ± 20	21

#### TABLE Appendix 3a:

Incorporation of  ${}^{3}$ H TDR (0.81µM; 0.013µCi/ml) with or without preincubation with deoxyuridine (0.25mM) into isolated crypt cell DNA from control, control + antibiotic and folate deficient animals. (Preliminary experiments with crypt cells fron control animals, demonstrated that the deoxyuridine suppressed value could not be decreased further, by increasing the concentration of the deoxyuridine added). Mean values ± standard errors from groups of 8 animals.

There was a greater incorporation of labelled thymidine into the DNA of folate deficient crypt cells compared with the control groups. There were also greater activities of thymidine kinase in these cells and it was therefore unclear whether the higher specific activity was a reflection of a true increase in DNA synthesis, or to an increase in the specific activity of the thymidylate pool due to the increased thymidine kinase activities.

There was no apparent change in the relative utilisation of the <u>de novo</u> pathway compared with the salvage pathway in folate deficient crypt cells compared with controls. This was surprising when (a) the greater incorporation of  ${}^{3}$ H TDR into DNA and increased thymidine kinase activities would suggest an increased utilisation of this pathway, and (b) the deficiency of folate would suggest a block in the <u>de novo</u> synthesis of dTMP. Whether these results were a true indication of the relative utilisation of the two pathways or whether they reflected the insensivity of the deoxyuridine suppressed value as an index of folate deficiency in gut cells, was unclear.

The results of this method are discussed more fully with respect to subsequent experiments which have been detailed in Section 2 - 4. These experiments measured the incorporation of labelled thymidine into the

# APPENDIX 4

# ENZYME CLASSIFICATION

Sucrase Thymidine Kinase Thymidylate Synthetase Folate Conjugase (Na<sup>+</sup>-K<sup>+</sup>)-ATPase E.C. 3.2.1.48. E.C. 2.7.1.21. E.C. 2.1.1.b E.C. 3.4.12.10. E.C. 3.6.1.3.



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