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THE ROLE OF SURFACE PROPERTIES IN THE TRANSPORT OF FOLATES ACROSS THE INTESTINE

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SUMMARY

The importance of the intestinal epithelial cell glycocalyx in the transport of folates in the intestine has been demonstrated. It was proposed that the glycocalyx acts as a retaining layer for the acid microclimate on the jejunal mucosal surface.

Histological investigation of human jejunal biopsies from healthy volunteers and patients with gastrointestinal disease showed an abnormality in the glycocalyx of subjects with conditions associated with folate deficiency. The abnormal appearance, which presented the glycocalyx as knobbed or broken up, may result from abnormal synthesis of the structural components of the glycocalyx, thereby resulting in an incomplete structure similar to that observed on immature cells. The absorptive cells in these conditions may therefore be underdeveloped for their normal function.

The transport of folic acid into everted sacs of rat jejunum was investigated using radiolabelled folic acid. A correlation was observed between the total folic acid transfer and the acidity of the microclimate. An association of fluid transfer in the movement of folic acid from the tissue into the serosal compartment was also demonstrated. The administration of compounds and the application of treatments to rats had varied effects on folic acid transfer which could be explained largely in terms of the existence of an acid microclimate. Conditions have been discussed which in humans, could induce folate deficiency.

The immediate uptake of folates was investigated using isolated rat jejunal epithelial cells, obtained by exposing tissue for different lengths of time to hyaluronidase. There was a marked association between the histological appearance of the glycocalyx and the uptake characteristics of folic acid.

The present investigation has provided definitive evidence to support an important role of surface properties in the transport of folates across the intestine. The mechanism of absorption of folic acid and 5-methyltetrahydrofolic acid involves passive diffusion following conversion to the neutral species within the acid microclimate.

KEY WORDS: FOLATES, GLYCOCALYX, INTESTINAL TRANSPORT, MICROCLIMATE.

To my

FATHER and MOTHER

Jack and Maida Swanston

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§ Magnification ×1280

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Chapter 1.

General introduction.

Folates, a group of compounds including folic acid, are essential for the correct functioning of specific metabolic pathways in the synthesis of DNA. Since man cannot synthesise folates, an adequate dietary intake coupled with the successful absorption of these vitamins is of paramount importance for the maintenance of health.

Folic acid is the most well known folate, the term 'folic' originating from the Latin word 'folium' meaning leaf. This name was provided by Mitchell, Snell and Williams (1944) for the substance isolated from spinach with the characteristics of folic acid. These characteristics were not specific for folic acid but common to folates in general; their ability to act as nutritional factors in the growth of bacteria (Snell & Peterson, 1940; Mitchell et al., 1944) and in the treatment of macrocytic anaemia (Wills, 1931; Wills, Clutterbuck & Evans, 1937). The growth-promoting effect of folates on various bacteria is used extensively as the basis of a microbiological assay, as certain bacteria exhibit different specificity in their response to folates. The term pteroylglutamic acid is used synonomously with folic acid and was applied to this compound by those who elucidated its structure which is shown in figure 1,1 (Angier, Boothe, Hutchings, Mowat, Semb, Stokstad, SubbaRow, Waller, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels & Smith, 1945, 1946).

Dietary folate is predominantly in the form of polyglutamates which have one or more glutamic acid residues linked to folic acid through the γ-carboxyl group. The polyglutamates are hydrolysed to monoglutamates

by intestinal conjugase enzymes prior to absorption (Halsted, Baugh & Butterworth, 1975). The monoglutamate, folic acid, is commonly used in folate investigative studies because it is readily synthesised and quite stable when stored under suitable conditions. In general, it is the tetrahydrofolate derivatives of folic acid which participate in metabolism. The enzyme responsible for the reduction of folate derivatives to tetrahydrofolate is dihydrofolate reductase. Only a small amount of this enzyme is present in the intestine with the highest content in the liver (Braganca & Kenkare, 1964). 5-Methyltetrahydrofolic acid (figure 1,2) is the major folate compound to be detected in the liver (Bird, McGlohon & Vaitkus, 1965). This compound is unstable to work with as it readily undergoes oxidation (Donaldson & Keresztesy, 1962) unless reducing agents are present. The metabolic role of folates has been discussed and reviewed in a comprehensive monograph by Blakley (1969) which encompasses the entire field of folic acid and its related compounds.

Deficiency of dietary folate occurs only rarely in the developed countries (Weir, 1974). However, inadequate dietary folate intake may be present in the elderly (Batata, Spray, Bolton, Higgins & Wolner, 1967) or in chronic alcoholics (Klipstein & Lindenbaum, 1965); these susceptible groups, by the nature of their disability, often do not consume a nutritionally balanced diet. This problem is exacerbated as the deficient folate condition itself reduces the ability of the intestine to absorb folic acid (Elsborg, 1976; Baker, Jaslow & Frank, 1978).

With continued folate deficiency, there arises a haematological disorder known as megaloblastic anaemia. As folate derivatives are involved in the biosynthesis of purine and pyrimidine nucleotides, folate deficiency limits the synthesis of these DNA precursors and hence the synthesis of specific amino acids is reduced (Blakley, 1969). Although deficiency of certain amino acids might well affect the growth of the cell more critically by limiting the synthesis of essential enzymes, than by limiting synthesis of basic proteins involved in nucleoprotein complexes. In this case, the metabolic lesions are in both nucleic acid and in the synthesis of specific proteins rather than in nucleoprotein synthesis.

Inadequate dietary intake is not the only aetiological factor giving rise to a folate deficient state. There are many instances where malabsorption of folates has been associated with diseases or the administration of drugs (tables 3,1 and 3,2). One of these drugs, methotrexate, itself a folate derivative, acts as an antimetabolite by inhibiting dihydrofolate reductase (Olinger, Bertino & Binder, 1973). There is however, much controversy concerning the mechanism by which diseases and drugs result in a malabsorptive condition leading to folate deficiency.

The absorption of folic acid occurs principally in the proximal jejunum (Smith, Matty & Blair, 1970) and the cell which is responsible for the absorption is the columnar, absorptive or border cell. The columnar cell constitutes 94% of the jejunal epithelial cells (Cheng & Leblond, 1974); several other epithelial cell types are present in the intestine (Specht, 1977) and new types are

continually being demonstrated, particularly those responsible for endocrine secretions (Pearse, Polak & Bloom, 1977). Throughout this text, the term epithelial cell is used synonomously with the small intestinal epithelial columnar cell. The capillary network which removes the absorbed folic acid from the intestine, lies directly beneath the epithelial cell layer (Clementi & Palade, 1969; Casley-Smith, 1971).

Details of the gross intestinal structure may be obtained from numerous monographs. However, in recent years the importance of the brush border region as a digestive-absorptive surface has become more evident (Crane, 1975, 1977). The plasma membrane of the microvilli of the brush border, has the classical 'unit' membrane structure (Robertson, 1959). Attached to the inner leaflet of the membrane is the terminal web. This layer is composed of microfilaments and is believed to be at least partially responsible for active movements of the plasma membrane (Wessels, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn & Yamanda, 1971). Extending from the microvilli into the lumen, are numerous filaments which constitute a region known as the glycocalyx (Bennett, 1963). The glycocalyx is being increasingly investigated as it is an integral part of the cell membrane which has been largely neglected until electron microscopic and autoradiographic techniques confirmed that this region was not merely a smearing of mucous on the intestinal mucosal surface (Mukherjee & Wynn Williams, 1967; Leblond & Bennett, 1974). The realization that the 'unit' membrane

is intimately associated with its two peripheral layers has prompted authors to introduce descriptive terms to account for this fact: names such as 'greater membrane' (Revel & Ito, 1967) or 'cell boundary' (Orci, Ravazzola, Amherdt & Malaisse-Lagae, 1974) have been used to describe the triple layer, glycocalyx-unit membrane-terminal web, and it is widely acknowledged that this triple association may be important in functions of the plasma membrane. Further investigation is required to clarify the function of the glycocalyx, either as an individual structure or as a component of the plasma membrane.

The normal luminal pH range in the jejunum is 5.8 to 6.7 (Benn & Cooke, 1971; MacKenzie & Russell, 1976). However, the existence of a region of greater acidity than the lumen has been demonstrated to exist on the mucosal surface (Lucas, Schneider, Haberich & Blair, 1975). It was proposed by Blair and Matty (1974) that the acidity of this microclimate could be altered by a disruption of the glycocalyx and that the absorption of folates was intimately dependent on this acid layer. Although there is now much circumstantial evidence and speculation to support this view, the function of the jejunal glycocalyx and the crucial role of the acid microclimate in the absorption of folates remains to be established.

The pH is an important factor when considering folate absorption as these compounds undergo ionisation dependent on the acidity of the medium. In aqueous solution at a pH of between 6.0 and 7.0 folic acid and 5-methyltetrahydrofolic acid are present entirely as the charged dianion whereas in a more acidic solution, the

percentage fractions of the neutral species increase (Blair & Matty, 1974). Figure 1,3 illustrates the relative concentrations of neutral and ionised species of folic acid at acidic pH values. The characteristics of folates at different pH values are therefore of considerable importance when suggesting a mechanism of folate transport dependent on an acid microclimate.

There is an increasing trend in scientific research to be interdisciplinary in approach. For the development of unity in a study, consideration of possible interrelationships between the main aspect of investigation and other areas is of particular value. As pharmacological research progresses an increasing number of drugs are synthesized, such as those in hormone replacement therapy which have been given in recent years to women with menopausal disturbances. Full investigation must be made with novel drugs such as these, as for example, an association between endocrinological and gastrointestinal disfunction is possible. Other drugs, such as those given to epileptics (Schafer & Nutall, 1975), or oral contraceptives (Streiff, 1970) have been implicated in malabsorption conditions despite their main target tissue being distinct from the intestine. Associations between diseases, such as coeliac disease with malignancy (Holmes, Stokes, Sorahan, Prior, Waterhouse & Cooke, 1976) or with diabetes mellitus (Koivisto, Kuitunen, Tiilikainen & Akerblom, 1977; Walsh, Cooper, Wright, Malins & Cooke, 1978a) require thorough consideration from many angles.

The present text is comprised of detailed investigations designed to enable a more complete

understanding of the folate transport mechanism.

An appropriate commencing point for these investigations concerns the initial structure of the intestinal wall which faces the prospective folate for transport.

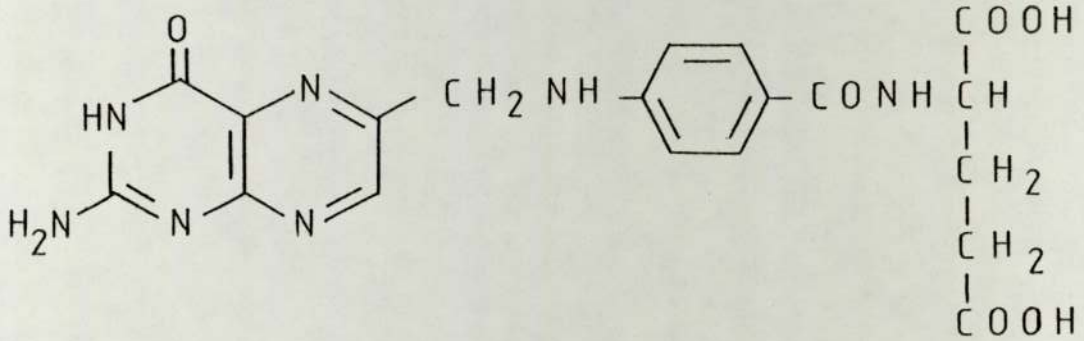


Figure 1,1. The chemical structure of folic acid.

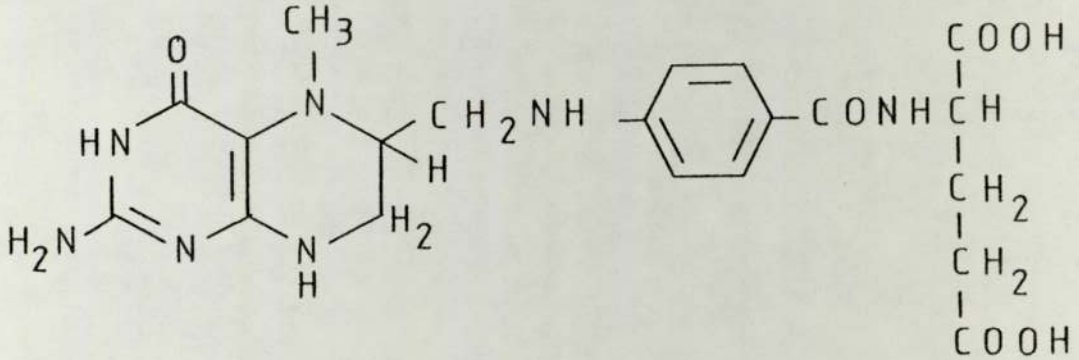


Figure 1,2. The chemical structure of 5-methyltetrahydrofolic acid.

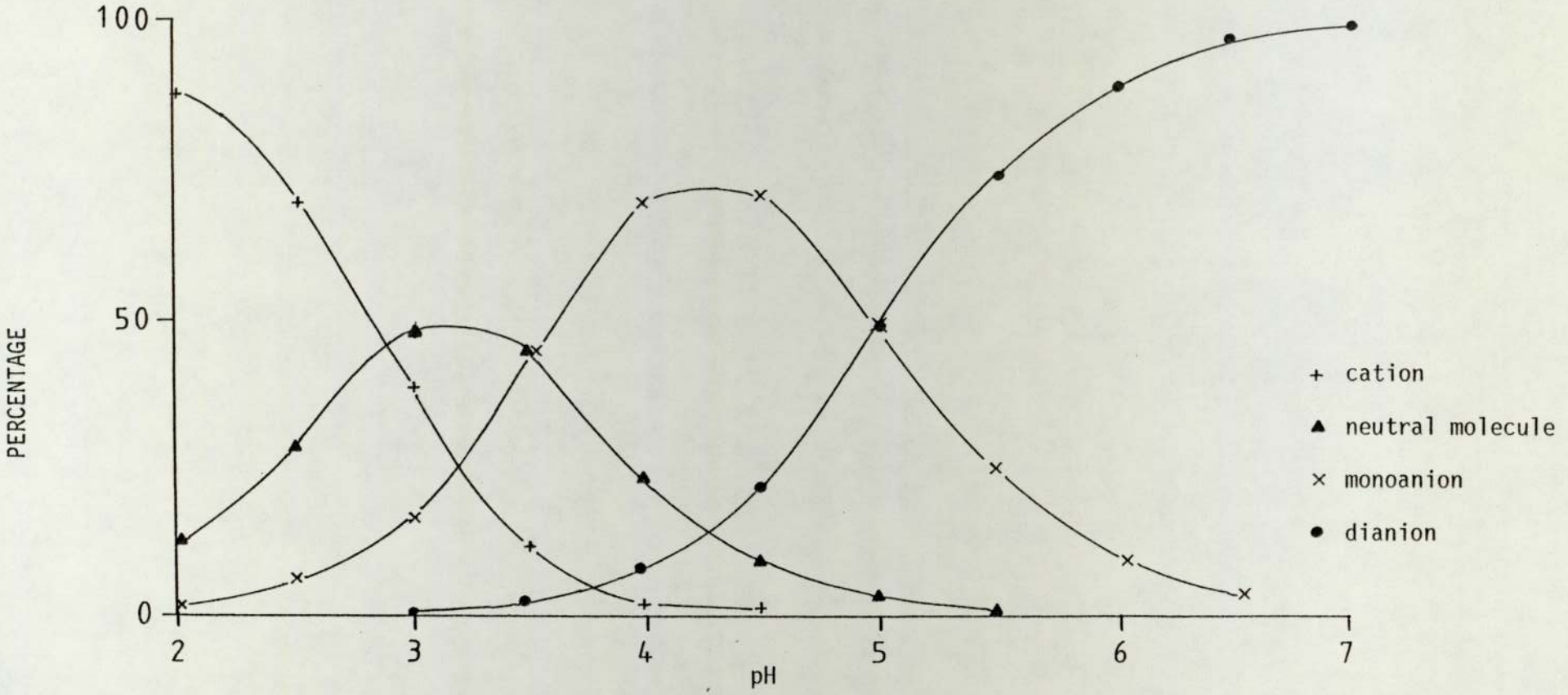


Figure 1,3. Relative concentrations of neutral and ionised species of folic acid at acidic pH values. (Lucas, 1977).

Chapter 2.

The epithelial cell glycocalyx.

Section 1. INTRODUCTION.

The initial research into extraneous cell coats was pioneered by Chambers (1940). His investigation was concerned with the three coats of the sea urchin egg. The animal whose cell surface has been most thoroughly investigated is the amoeba (Revel & Ito, 1967). The extraneous coat of this protozoan, similar to the coats of the sea urchin egg (Monné & Slauterbach, 1950), gave a positive reaction to the periodic acid-Schiff histochemical technique (Pappas, 1954). Other similar periodic acid-Schiff staining layers were observed on the apical surfaces of the epithelial cells in the intestine (Leblond, 1950), epididymis and kidney (Burgos, 1960), pancreatic acinar cells (Fawcett, 1962) and the bladder (Choi, 1963). In a study of a large variety of rat tissue including epithelial cells, nerve cells and cells of mesenchymal origin such as lymphocytes and fibrocytes, Rambourg, Neutra & Leblond (1966) observed that a glycocalyx was present in all these cells.

The term glycocalyx was proposed by Bennett (1963). He considered this apt because the glycocalyx always consisted of a polysaccharide structure, and the Greek word glycocalyx means 'sweet husk'. Despite this, other terms have still been used including antennulae microvillares (Yamanda, 1955), and enteric surface coat (Ito, 1965). Ito (1974) lists further terms, hirsutulous, hispiditious, hairy coats, fuzz and fuzzy coat.

The histochemical techniques for the demonstration of the glycocalyx are discussed fully by Martínez-Palomino (1970), Rambourg (1971) and Parsons & Subjeck (1972).

Winzler (1970) in a comprehensive review on carbohydrates in the cell surface concludes that the glycocalyx is composed of glycoproteins with a polypeptide chain and pendant carbohydrate side chains. Figure 2,1 shows the basic glycoprotein structure. The protein chain may consist of, for example, some 300 amino acid units which can be any of the 20 or so naturally occurring L-α-amino acids (Kennedy, 1973). This protein chain is essentially the backbone of the glycoprotein, and the oligosaccharide chains are covalently bound to specific amino acid residues in the polypeptide chain namely asparagine, serine, threonine or hydroxylysine.

The oligosaccharide chains (10-15 residues long) are often branched. The inner residues are usually N-acetylglucosamine and N-acetylgalactosamine, combined with mannose. Galactose and fucose may also be present but the terminal monosaccharide unit is often a negatively charged residue N-acetylneuraminic acid, a sialic acid. Sialic acid is a characteristic residue of glycoproteins at the cell surface (Pasternak, 1977). Linked with galactose or N-acetylgalactosamine, it is the carboxyl group of sialic acid which is responsible for a significant portion of the net negative surface charge of animal cells (Cooke, 1976).

Viewed by scanning electron microscopy, the glycocalyx appears to form a continuous layer over the microvilli (Toner & Carr, 1969; Marsh, Brown & Swift, 1970). This feature often results in an obstructed view of the microvilli (Balcerzak, Lane & Bullard, 1970; Toner, Carr & Wyburn, 1971).

Transmission electron microscopy has revealed that the glycocalyx is filamentous in nature. These observations

were made in the gallbladder (Yamanda, 1955), bladder (Peachey & Rasmussen, 1961), pancreatic acinar cells (Sjöstrand & Elfin, 1962), gastric mucosa (Ito & Winchester, 1963), small intestine (Ito, 1964), blood capillaries (Luft, 1965), fibrocytes, erythrocytes, kidney and endocrine cells of the pituitary and thyroid (Rambourg & Leblond, 1967), and the β cells of the islets of Langerhans in the pancreas (Orci, Ravazzola, Amherdt & Malaisse-Lagae, 1974). The fine structure of the glycocalyx varies with the different electron microscopy preparative techniques (Trier, 1968; Swift & Mukherjee, 1976; Bernstein, Griffin, Jackson & Lynch, 1976). In the intestine, most commonly it appears as a branching network of fine filaments extending into the lumen from the outer layer of the trilaminar plasma membrane (Ito, 1969). Occasionally microfilaments continue from the microvillous core across the plasma membrane and into the glycocalyx (Mukherjee & Wynn Williams, 1967). Attempts at removing the glycocalyx with various mucolytic and proteolytic agents including EDTA (ethylenediaminetetraacetate), N-acetyl cysteine, chymotrypsin, hyaluronidase, diastase, neuraminidase, lysosyme, trypsin and papain have all failed (Ito, 1965; Revel & Ito, 1967). The glycocalyx was not removed or altered with either <u>in vivo</u> or <u>in vitro</u> incubation with these substances, and it is now generally accepted that the glycocalyx is an integral part of the plasma membrane. Mukherjee and Wynn Williams (1967) presented transmission electron micrographs of mouse intestine to show how the thickness of the glycocalyx varies considerably among adjacent cells. This observation, confirmed by Rao, Mukherjee and Wynn Williams (1972), provides further evidence to suggest that the glycocalyx is not a uniform

smearing of the cells by mucus.

The dimensions of the glycocalyx on human intestinal epithelial cells were measured from transmission electron micrographs by Rifaat, Iseri & Gottlieb (1965). On the tips of the microvilli the fibrils reached a length of 1.5μ, between the microvilli the fibrils were less dense and shorter at 0.5μ. They also reported that on the crypt epithelium, the fibrils constituting the glycocalyx were only about 0.5μ in length, becoming more dense and longer as the cells move up the side of the crypt; this change may be a function of cellular maturation paralleling the development of microvilli and is discussed in more detail later in this section.

Autoradiographic studies have shown that the intestinal glycocalyx is a dynamic surface component requiring an intact, viable cell for its maintenance and synthesis. The incorporation of radioactive sulphate and glucose into the glycocalyx indicated to Ito & Revel (1964) that the synthesis of this layer occurred in the cell. Further autoradiographic studies revealed that the radioactive species were initially accumulated in the Golgi region prior to accumulation at the cell surface (Ito & Revel, 1966; Bennett, 1970). The most detailed autoradiographic work has been performed by Leblond & Bennett (1974) using rat small intestine. They observed that the polypeptide moieties of the cell coat glycoproteins are formed in the rough endoplasmic reticulum with mannose, positioned near the base of the oligosaccharide side chains, being added at this site. Sugars nearer to the end of the side chains, such as galactose and fucose (Bennett, Leblond & Haddad, 1974) are incorporated into the glycoproteins as they pass

through the Golgi apparatus. The completed glycoproteins are then transported to the cell surface, probably in vesicles (Forstner, 1969) which then open at the surface by exocytosis so as to donate their contents to the plasma membrane.

The function of the intestinal epithelial cell glycocalyx has been attributed to many of the processes which in the past have been associated with the cell surface. The most popular theory is that the glycocalyx is the site at which the initial phase of digestion and absorption occurs (Fawcett, 1965; Ugolev, 1965; Dobbins, 1969; Greenberger, 1969). The appearance of the intestinal glycocalyx may vary according to species (Dougherty, 1967) or with the method of tissue preparation. In negatively stained preparations of the hamster intestine the glycocalyx is replaced by rows of 50-60 Å globular units (Overton, Eicholz & Crane, 1965; Johnson, 1967). Johnson (1969) used a cysteine-activated papain treatment and claimed to release these globular units from the plasma membrane. The isolated particles were found to contain maltase and invertase. Similar work by Eicholz (1968) resulted in the release of particles containing maltase, sucrase and isomaltase. According to Ugolev (1965) pancreatic enzymes are selectively adsorbed on the intestinal mucosa resulting in 'membrane or contact digestion'.

In addition to enzymes being bound to the intestinal glycocalyx, other substances such as ferritin may also bind there (Mukherjee, 1972). Mukherjee reported that surface charges vary considerably between villus cells and at pH 4 ferritin is positively charged thus binding to the negatively charged surface membrane. He also suggested that by

distributing glycoproteins on the membrane surface, the cell may be able to regulate the intake of ferritin.

The surface membrane glycoproteins may perform the function of cell protection (Martin & Louisot, 1976). Latta and Johnson (1976) have reported that the glycocalyx in the kidney, acts as a filtration barrier to plasma albumin and it is suggested by Hamilton & McMichael (1968) to be a diffusion barrier for mono and disaccharides in the intestine. Thus the glycocalyx may act as an area of selection for the uptake of substances into the cell (Bennett, 1964; Nakamura, Yoshizaki, Yasuhara, Kimura, Muranishi & Sezaki, 1976). Rambourg (1971) has discussed the literature which suggests that the glycocalyx is involved in cellular immunity and adhesion.

An additional function which may be attributed to the intestinal glycocalyx is that it provides a retaining layer for the hydrogen ions of the acid microclimate (Blair & Matty, 1974). The theory of an acid microclimate at the surface of the intestinal cell was put forward by Hogben, Tocco, Brodie and Schanker (1959) to explain the major features of intestinal absorption of weak acids and bases consistent with the theory of non-ionic diffusion (Schanker, Tocco, Brodie & Hogben, 1959). The role of the acid microclimate in intestinal absorption has been reviewed by Blair & Matty (1974) and a more detailed discussion of the glycocalyx and microclimate may be found in section 4 of this chapter.

Observed alterations to the intestinal cell surface membrane during the normal cell cycle are becoming increasingly numerous. As the glycocalyx is closely related to the microvillous membrane it is not surprising that

changes to the microvilli result in changes to the glycocalyx. Comparisons of villus and crypt cells have shown that crypt cells have only a few, short microvilli (Rubin, 1971; Dognen, Visser, Daems & Galjaard, 1976). Crypt cells exhibit a slow rate of glycoprotein synthesis when compared with villus cells (Weiser, 1976). Not only is the glycoprotein synthesis slower but the resulting crypt cell surface glycoproteins are incomplete in their structure as demonstrated by increased glycosyltransferase activities (Isselbacher, 1974; Weiser, 1976). Many of the observations on crypt cells, e.g. reduced number of microvilli, increase in glycosyl transferase activity, increase in agglutinability and incomplete glycoprotein synthesis have also been observed in fetal and tumour cells (O'Neill & Follett, 1970; Parsons & Subjeck, 1972; Isselbacher, 1974; Weiser, 1976).

As the crypt cells divide actively, it is not unreasonable to find that there are associated cell surface alterations. The cell cycle was initially divided into phases by Howard and Pelc (1953). The cell cycle is commonly expressed in a diagrammatic form (figure 2,2). The major components of the plasma membrane:glycoproteins, gangliosides and glycolipids, are inserted throughout interphase; the amount of these present in G2 being approximately double the amount in G1 (Graham, Sumner, Curtis & Pasternak, 1973). Other cell investigations, made using synchronised cells, have shown with the aid of scanning electron microscopy that during interphase a folding of the plasma membrane occurs resulting in an increase in surface area, the formation of microvilli (Knutton, Sumner & Pasternak, 1975) and an increased thickness of glycocalyx by late G2 (Rosenfeld,

Paintraud, Choquet & Venuat, 1975).

In crypt cells, the Golgi apparatus is poorly developed, becoming more prominent during cell migration along the upper part of the crypt and onto the villus (Dognen et al., 1976). Hence as the Golgi apparatus is involved in the synthesis of the glycocalyx (Ito & Revel, 1966), it may be assumed that the lower regions of the crypt will have a poorly developed glycocalyx (Dognen et al., 1976) which becomes more prominent with cell maturation (Rifaat et al., 1965).

The surface morphological differences between normal and transformed cells in vitro have been reviewed by Pasternak (1977). In addition to other alterations, it is reported that the glycocalyx is apparently thicker on transformed cells although the mechanism of this alteration is unclear. Pasternak hypothesized that the glycocalyx may not span as much of the surface as normal cells and if represented in diagrammatic form (figure 2,3), this would explain an increased thickness whilst not accompanied by an increase in total carbohydrate containing molecules. The glycocalyx is also thicker in 'transitional' mucosa taken from an area a short distance away from a colonic carcinoma (Dawson & Filipe, 1976).

Alterations and damage to the intestinal microvilli and glycocalyx have been observed using electron microscopy in several disease states including coeliac disease (Biempica, Toccalino & O'Donnell, 1968; Falchuk, Gebhard, Sessoms & Strober, 1974), ulcerative colitis (Ceralli, Familiari, Marinozzi & Muccioli-Casadei, 1976), cows milk intolerance (Iancu & Elian, 1976) and intestinal micro-organism infestations (Erlandsen & Chase, 1974). It is not

unreasonable to suggest that intestinal architectural abnormalities are at least one of the underlying causes resulting in nutritional deficiencies exhibited in many of the aforementioned disease states. To date, there seems to be no literature available which discusses the light microscopic appearance of the glycocalyx in disease states.

The aim of the work described in this chapter was to observe the glycocalyx in several disease states, especially those associated with folate malabsorption or folate deficiency. Initially a study was made of some of the light microscopic stains which are positive for the glycocalyx. Light microscopy is a relatively fast technique which provides some information on the chemical composition of the observed feature. Using the most suitable staining method, the glycocalyx was observed on the mucosa of peroral biopsies obtained from human subjects, to see if there were any alterations to the glycocalyx.

Section 2. MATERIALS AND METHODS.

HISTOLOGICAL PROCEDURE.

i) Fixation.

The tissue was obtained from the clinician immediately after extraction from the body (the method of extraction is described later in this section). The importance of the speed of the exercise was understood by the clinician hence no time was lost in immersing the tissue in fixative at room temperature. The aim of fixation is to preserve the cells and tissue in a condition identical to that existing during life. Clearly this aim can never be completely fulfilled but fixation does prevent autolysis, bacterial decomposition and loss of diffusible substances from the tissue. Fixation also fortifies the tissue against the deleterious effects of the various stages in the preparation of sections.

The fixative used in this study was buffered 10% formalin (see appendix). This is one of the most widely used histological fixatives which imparts a firm consistency to tissue and allows a wide variety of staining methods to be applied. Buffered fixative was used so that throughout the histological process the tissue would not be subject to any wide pH range alterations.

ii) Dehydration.

The tissues were dehydrated using graded alcohols (70%, 90% and absolute). Several changes of alcohol were made and the whole dehydration process took at least 5 hours. The aim of dehydration is to remove all water from the tissue thus allowing the embedding medium to penetrate the tissue completely.

iii) Infiltration and embedding.

The glycocalyx may be observed on thin sections by light microscopy as a thin line on the luminal surface of the brush border. It was necessary to cut sections as thin as possible thus preventing the glycocalyx from appearing to merge with the brush border as was the case with thick sections. To cut thin sections, it was essential that a hard embedding medium was used and for this purpose the tissues were embedded in synthetic resin. Polymerizing resins as embedding media for tissues also have the advantage over paraffin in that they produce much less distortion and therefore preserve the tissue much more faithfully (Bennett, Wyrick, Lee & McNeil, 1976). The method used here with glycol methacrylate was that of Sims (1974).

Following dehydration, each tissue specimen was placed into infiltrating solution containing the appropriate concentrations (see appendix) of 2-hydroxyethyl methacrylate (TAAB Laboratories, Reading), 2 butoxyethanol and benzoyl peroxide (BDH, Poole). The infiltration process took at least 6 hours involving 3 changes of solution.

After infiltration, 150 µl of the promoter solution, consisting of polyethylene glycol 400 (E.Gurr, London) and N.N. dimethylaniline (Hopkin and Williams Ltd., Chadwell Heath) was mixed with 8 ml of the infiltrating solution. This final solution was well mixed and poured into a suitable sized foil container which had already been positioned partially immersed in a trough of water. The tissue was then placed into the solution and correctly orientated with forceps. A label with the appropriate details of the biopsy was inserted into the container.

Polymerization usually occurred within 30-40 minutes

of mixing and the block was left overnight to dry off any tackiness left on the surface. The following day, the container was peeled away from the block, and the block trimmed to shape with a fine toothed hacksaw. A border of plastic of approximately 3 mm was left around the tissue with a longer 'tongue' of plastic at the leading edge. This 'tongue' was to facilitate the cutting and handling of the tissue sections.

iv) Cutting.

A base sledge microtome (Type 1400, Leitz, West Germany) was used with a wedge shaped knife (MSE, London). The knife was sharpened regularly on an automatic knife sharpener.

Following the trimming of the embedded biopsy, it was attached to a wooden block using ester wax and then firmly secured to the chuck of the microtome. After initial rough cutting to expose the tissue surface, a fresh portion of knife was selected and the cutting thickness altered to approximately 0.5μ. The sections were transferred to a water bath at room temperature with the aid of the 'tongue' of plastic and forceps. In order to mount the section, a slide, previously cleaned with absolute alcohol, was breathed upon to produce a fine film of moisture on its surface, and then held in the water in a near vertical position and drawn towards the section. When the section and slide touched, the slide was pulled out of the water vertically and gently heated over a bunsen flame to dry. When cool, the slide was appropriately labelled using a diamond writing pencil.

Although the accuracy of thickness at this level cannot be guaranteed, it was assumed the sections were not less than 0.5μ and not greater than 1.0μ thick.

v) Staining.

Most conventional histological stains can be used on glycolmethacrylate sections in a manner similar to their application to paraffin sections (Bennett et al., 1976). It was not necessary to remove the embedding medium from the sections prior to staining (Cole & Sykes, 1974) and thus staining could be commenced immediately after the sections had been mounted.

Before a comparative study of the glycocalyx on various tissues was carried out, a brief résumé was made of the stains suitable, to find which was the best stain to use. The following stains were carried out on tissue obtained from a patient (E.W.) at the time of a jejunoileostomy operation; the tissue had a normal histological appearance. The complete staining schedules are described in the appendix and the stains were obtained from R. A. Lamb, London.

Periodic acid-Schiff.

The most important staining technique for carbohydrates is the periodic acid-Schiff (PAS) reaction. Initially used by McManus (1946) for the staining of mucins, the PAS method has been further developed into a histochemical technique for polysaccharides. The reaction acts by breaking 1,2 glycol groups (vicinal hydroxyl groups) into dialdehydes which are then visualised by combination with Schiff's reagent. The method used here was a modification of that by McManus (1946).

Alcian blue.

The Alcian blue staining procedure is used to stain simple acid and sulphated mucopolysaccharides. Two methods were used; 1% Alcian blue in solutions buffered between

pH 1 and 5 (modification of Steedman, 1950) and 1% Alcian blue in 30% acetic acid (Revel, 1974).

Hale's colloidal iron.

This technique depends upon the interaction of colloidal iron with acidic groups at low pH. The iron forms a chelate with the acid groups of acid mucopolysaccharides, especially the carboxyl groups of sialic acid (Parsons & Subjeck, 1972). Two methods were used in this investigation, one according to Hale (1946) and the other, a modified method by Mowry (1963).

Toluidine blue.

The toluidine blue method also demonstrates the presence of acid mucopolysaccharides. A modification of the method described by Drury and Wallington (1967) was used with 1% toluidine blue buffered between pH 1 and 5.

Uranyl nitrate.

This is another method used for acid mucopolysaccharides and was used as described by Hughesdon (1949).

Schmorl's thionin.

Schmorl's thionin was the last of the series of stains used which reveals the presence of acid mucopolysaccharides. The method followed was that described by Drury and Wallington (1967).

After staining, all the sections were dehydrated in alcohol, cleared in xylene and mounted with DPX resin mountant.

Photomicrographs were made using an Orthomat Leitz (Leitz, West Germany) photomicroscope with Agfa DIN 18 50L film (36 exposures per film) and the pictures were processed at the Agfa laboratories. All sections were photographed under oil emersion and at a magnification of 1280 times unless otherwise stated.

CLINICAL CONDITIONS AND BIOPSY PROCEDURE.

The human subjects from whom tissue was obtained, were either patients at the Gastrointestinal Unit, General Hospital, Birmingham, or normal volunteers. Biopsies were obtained from 6 patients with folate deficiency of unknown aetiology and no other disorder, 4 with untreated and 5 with treated coeliac disease, and 4 patients with Crohn's disease. There were 9 subjects in the control group which included student volunteers, patients with normal histology and no demonstrable gastrointestinal disorder and also patients in hospital for a jejunoileostomy operation for massive obesity. Jejunoileostomy or intestinal bypass is an operation to induce weight reduction (Payne, DeWind, Schwalbe & Kern, 1973; Baddeley, 1976).

Peroral jejunal biopsies were taken from all subjects apart from the 2 jejunoileostomy patients from whom a small piece of jejunum was obtained during the operation. The peroral jejunal biopsies were taken using a suction biopsy capsule as described by Roy Choudhury, Nicholson and Cooke (1964). The patients were fasted overnight, and prior to the operation given 'Maxolon' to facilitate the movement of the capsule to the biopsy site. Lignocaine was sprayed on the throat prior to the biopsy insertion. If the patients

showed signs of stress before the biopsy procedure, they were given 10 mg of diazepam (Valium). The passage of the biopsy capsule was monitered by fluoroscopy. Each biopsy was taken from the same site, just past the duodenojejunal junction. A detailed account of the technique of suction biopsy is given in Perera, Weinstein and Rubin (1975). The size of the biopsy specimens varied from 2-5 mm and went to the depth of the muscularis muscosa.

Section 3. RESULTS.

HISTOLOGICAL STAINS FOR THE GLYCOCALYX.

The efficiency of the staining technique varies with all the conditions employed including the type and length of fixation but especially with the embedding medium. There is no objective method by which one may compare the results; the conclusion being purely subjective. Comparisons of the following stains were made on tissue sections from a jejunoileostomy patient (E.W.). The histology of this tissue shows a normal appearance.

Periodic acid-Schiff.

Of the stains which were tried out, PAS gave the best results by far (plate 1). The glycocalyx appeared as a thin, heavily staining red line on the luminal surface of the brush border. The brush border stained a paler pink and was several times thicker than the glycocalyx. The nuclei were shown clearly by the counterstain (celestine blue haemalum sequence). The break in the continuity of the glycocalyx at the villus tip is the epithelial cell extrusion zone.

Alcian blue.

When 1% Alcian blue in solutions buffered between pH 1 and 5 was used, the reaction of the glycocalyx was strongest at pH 5. At lower levels the staining was less specific. At pH 5 (plate 2) both the glycocalyx and terminal web stained a turquoise colour although their appearance was rather fuzzy and indistinct.

A modification of the above technique, involved using 1% Alcian blue in 30% Acetic acid but the results to this were poor. The glycocalyx was barely visible as a dark line on the epithelial luminal surface.

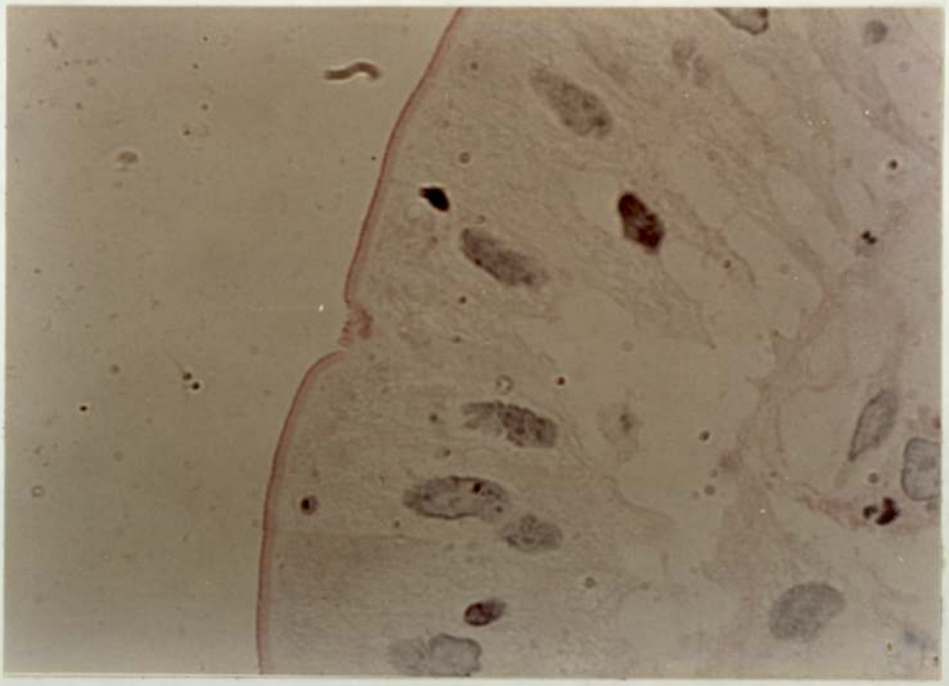


Plate 1. Periodic acid-Schiff.

Plate 2. Alcian blue.

Hale's colloidal iron.

The glycocalyx was visible as a blue-green line on the luminal edge of the villus (plate 3). The terminal web did not appear to have reacted with the stain.

Mowry's modification (1963) of the colloidal iron technique resulted in the glycocalyx appearing very pale and virtually indistinguishable from the brush border or terminal web.

Toluidine blue.

In a similar fashion to the results obtained with buffered Alcian blue solutions, the glycocalyx appeared to be clearest at pH 5. At lower pH values, the glycocalyx was not readily visible. At pH 5 (plate 4) the glycocalyx and terminal web were seen as two distinct white lines against the blue background of the stained plastic.

Uranyl nitrate.

The glycocalyx and terminal web were observed as rather indistinct white lines (plate 5). But a turquoise fuzzed area was apparent on the luminal edge of the glycocalyx. There is a goblet cell in the centre of the picture, this has stained a pale turquoise.

Schmorl's thionin.

This staining technique was not of any use as the whole tissue section was hardly visible.

It was obvious from the staining techniques listed above, that the PAS method was most suitable to use as the routine stain for the histological studies on the glycocalyx of the biopsies obtained from the patients with the various clinical disorders.

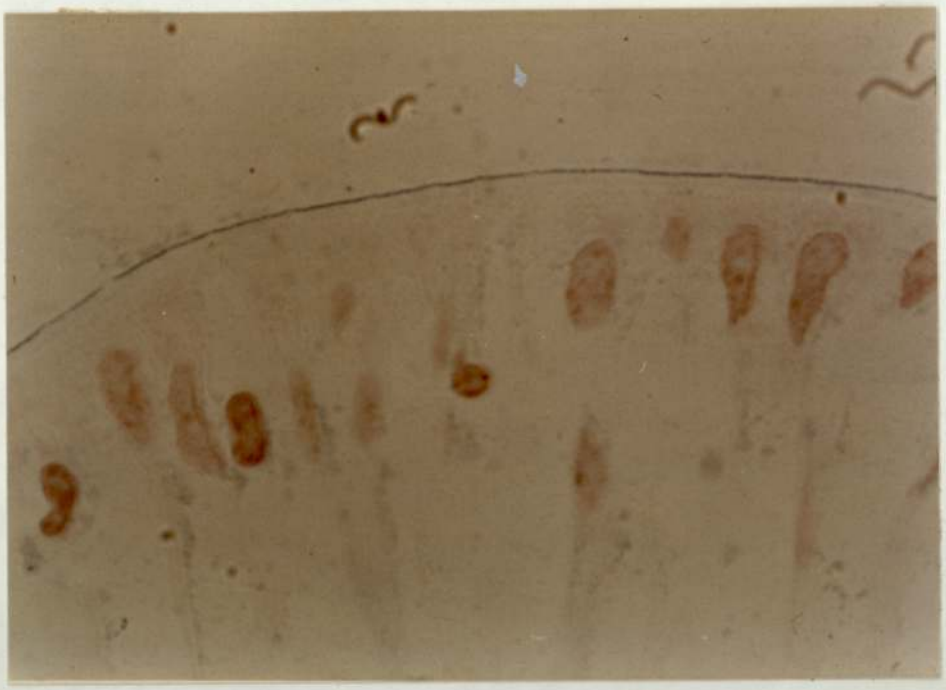


Plate 3. Hale's colloidal iron.

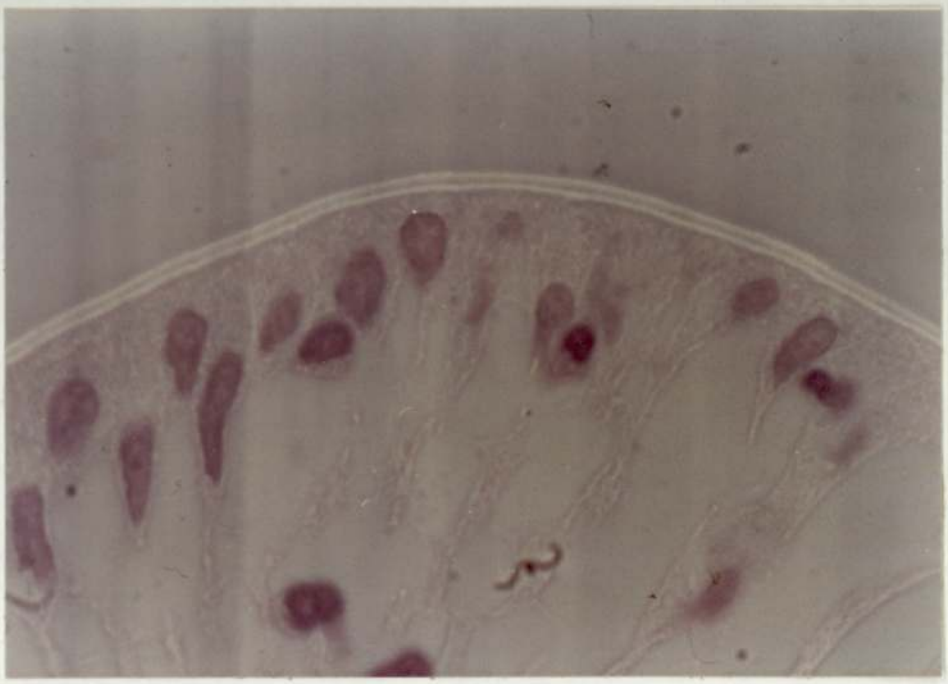


Plate 4. Toluidine blue.

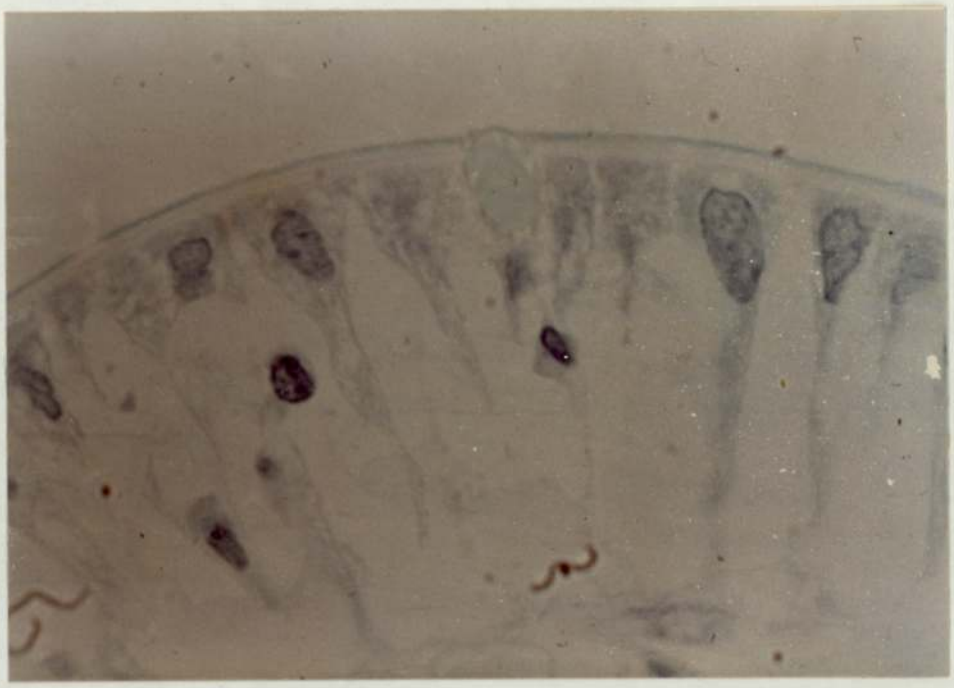


Plate 5. Uranyl nitrate.

THE GLYCOCALYX IN VARIOUS CLINICAL CONDITIONS.

The grading for several of the biopsies was made by the hospital pathologist according to the system of Roy Choudhury, Cooke, Tan, Banwell and Smits (1966). Briefly these grades of abnormality are:

Grade 1 - slight inflammatory cell inflammation of the lamina propria, normal columnar epithelium.

Grade 2 - broader and shorter villi, slight to severe cellular inflammation. Epithelial cells becoming cuboidal in shape with some lymphocytic infiltration.

Grade 3 - no normal villi, the surface is essentially flat being interrupted only by crypt openings. Epithelium abnormal and severe lamina propria infiltration.

Serum folate values were obtained by microbiological assay with Lactobacillus casei (Herbert, 1966), and are expressed as ng folate per ml serum. A value of 5 ng/ml is considered as being fairly normal and <3 ng/ml very low. The patients in the folate deficient group had folate deficiency of unknown aetiology. Patients in other groups may also have shown folate deficiency but there was also an associated disorder, e.g. coeliac disease.

The clinical details which were made available are included in the results. The subjective comments on the glycocalyx were always made from observations of mature epithelial cells close to the villus extrusion zones.

Normal group.

E.W. (plate 6)

This was a 28 year old jejunoileostomy patient who had normal intestinal histology.

The glycocalyx appeared as a continuous red line on the luminal surface of the brush border. The small breaks in continuity correspond to the lateral borders of the epithelial cells beneath the glycocalyx.

N.O. (plate 7)

This tissue was obtained from a female jejunoileostomy patient aged 23. The histological appearance was normal and the glycocalyx appeared continuous.

Normal group.

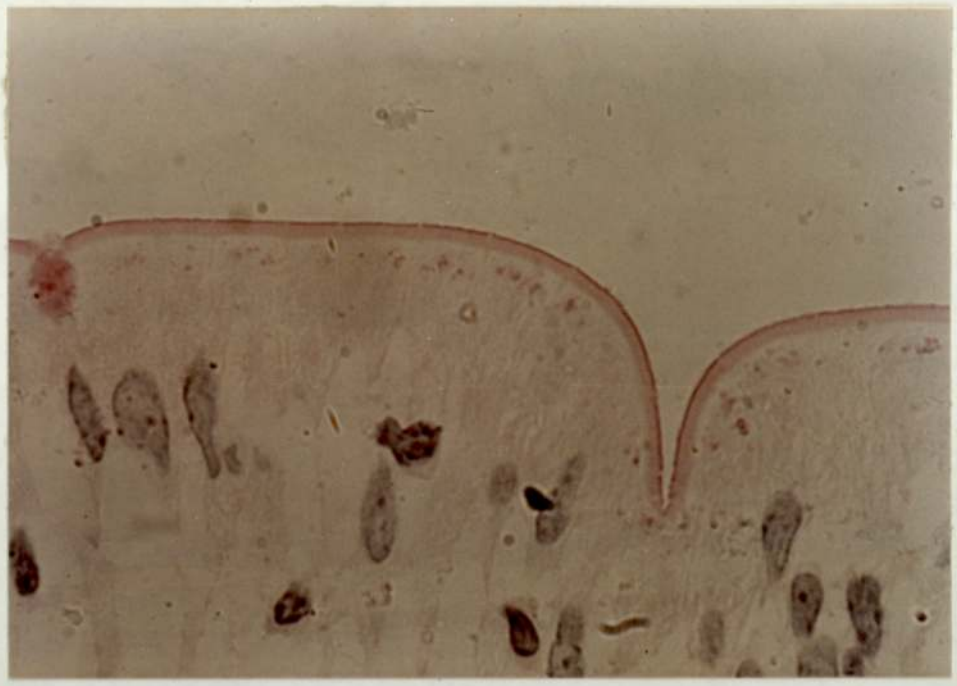


Plate 6.

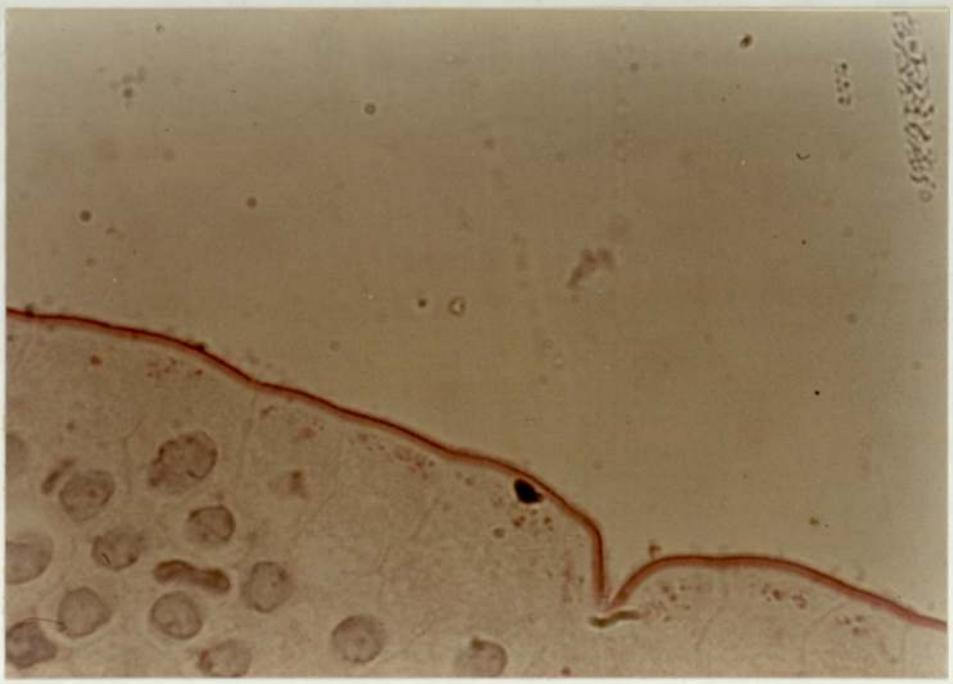


Plate 7.

R.B. (plate 8)

This biopsy was obtained from a 23 year old male student who volunteered for the biopsy procedure. The histology was reported as normal and the serum folate value was 5.8 ng/ml. The glycocalyx appeared continuous.

U.H. (plate 9)

This tissue was obtained from a 23 year old male student who volunteered to donate a biopsy specimen. The histology was normal and his folate level was 6.4 ng/ml. The glycocalyx was continuous.

Normal group contd.

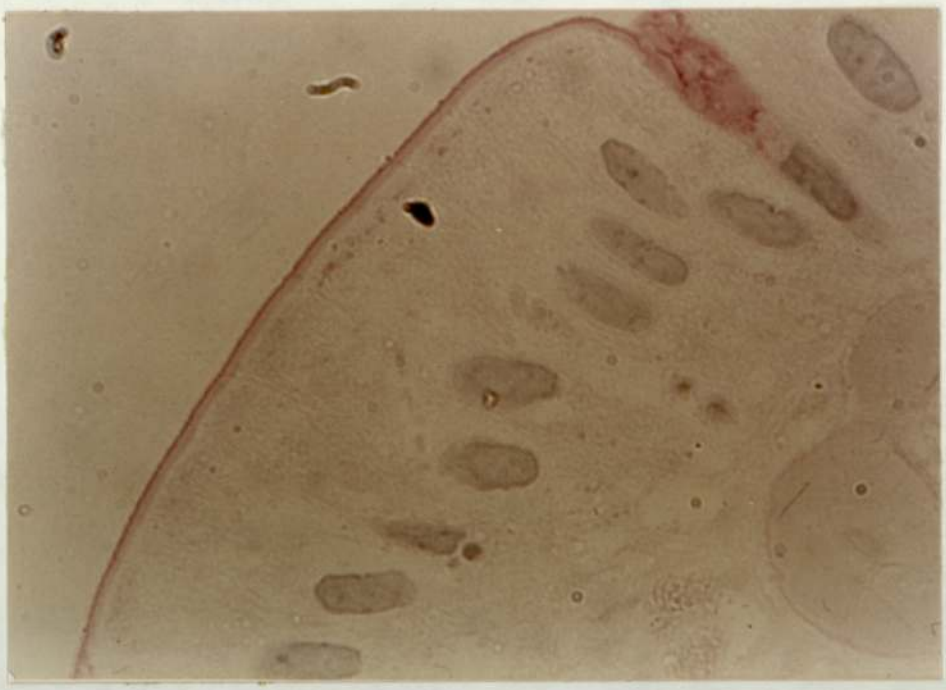


Plate 8.

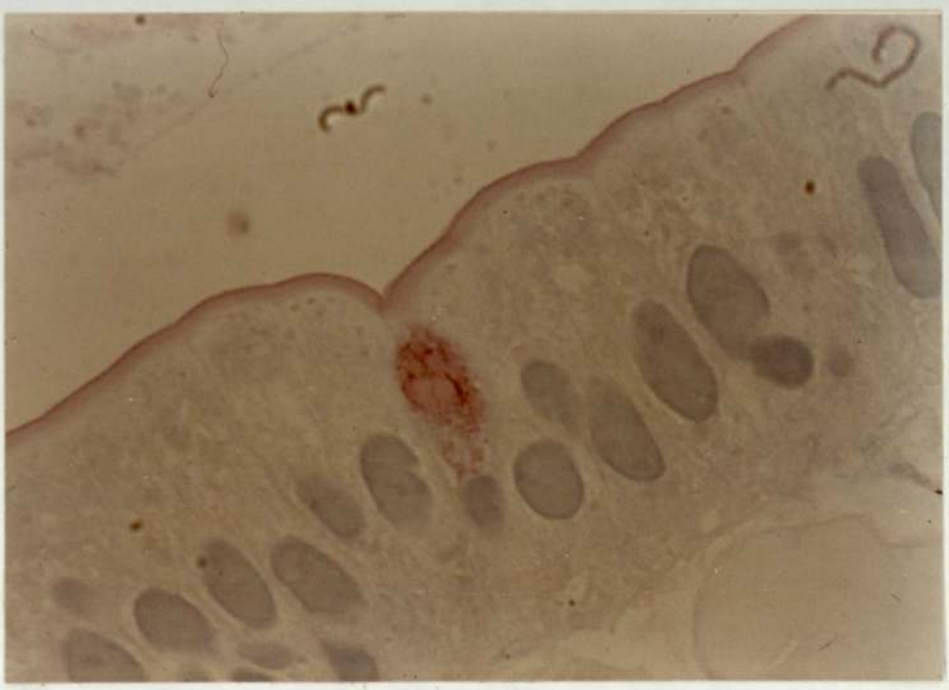


Plate 9.

I.N. (plate 10)

This was a biopsy obtained from a 21 year old female student volunteer. The histology was reported as normal and the serum folate value was 5 ng/ml. The glycocalyx appeared continuous.

O.C. (plate 11)

A biopsy was obtained from this 45 year old male patient because he had reported having abdominal pains. But the pathologist reported normal histology and no treatment was required. The serum folate value was 4.8 ng/ml. There was rather a fuzzed appearance to the glycocalyx although it may be seen as being continuous in places. There was slight lymphocytic infiltration of the epithelium and therefore this tissue may not be completely normal.

Normal group contd.

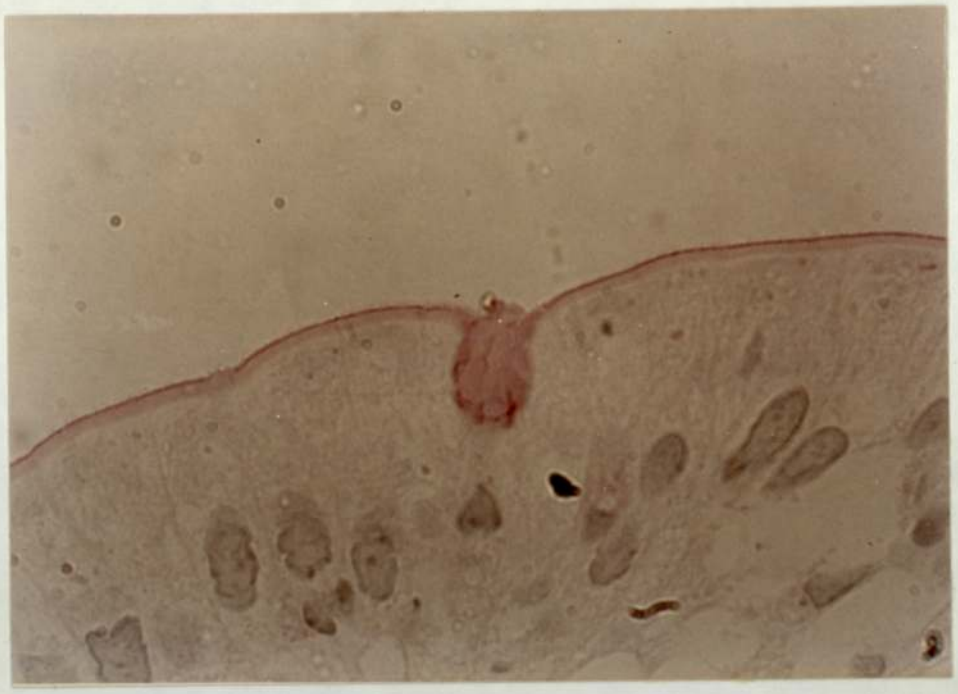


Plate 10.

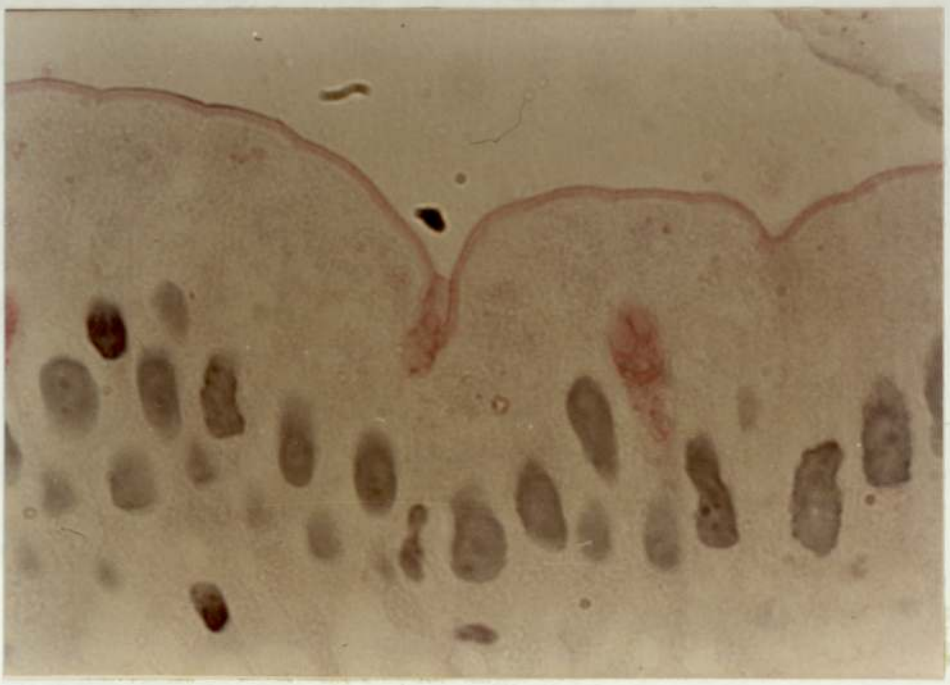


Plate 11.

A.S. (plate 12)

This female patient, aged 49, had reported having diarrhoea but the pathologist's report was of normal histology. The serum folate value was 6.2 ng/ml and the glycocalyx appeared continuous.

B.H. (plate 13)

This 46 year old male patient had reported with diarrhoea but the pathologist observed a normal histological appearance. The folate value was 5.8 ng/ml and the glycocalyx was continuous.

Normal group contd.

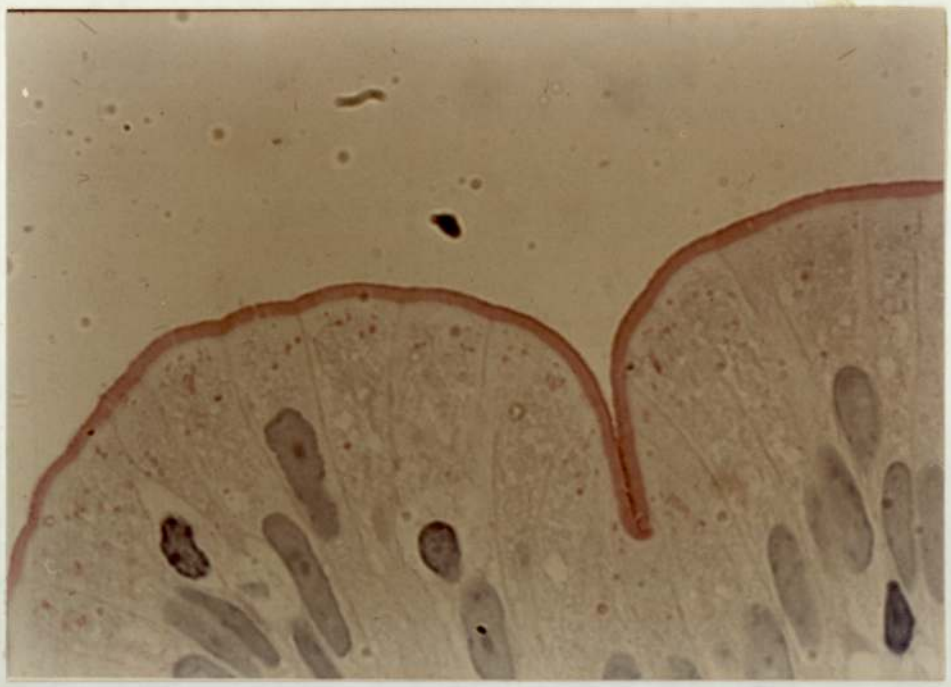


Plate 12.

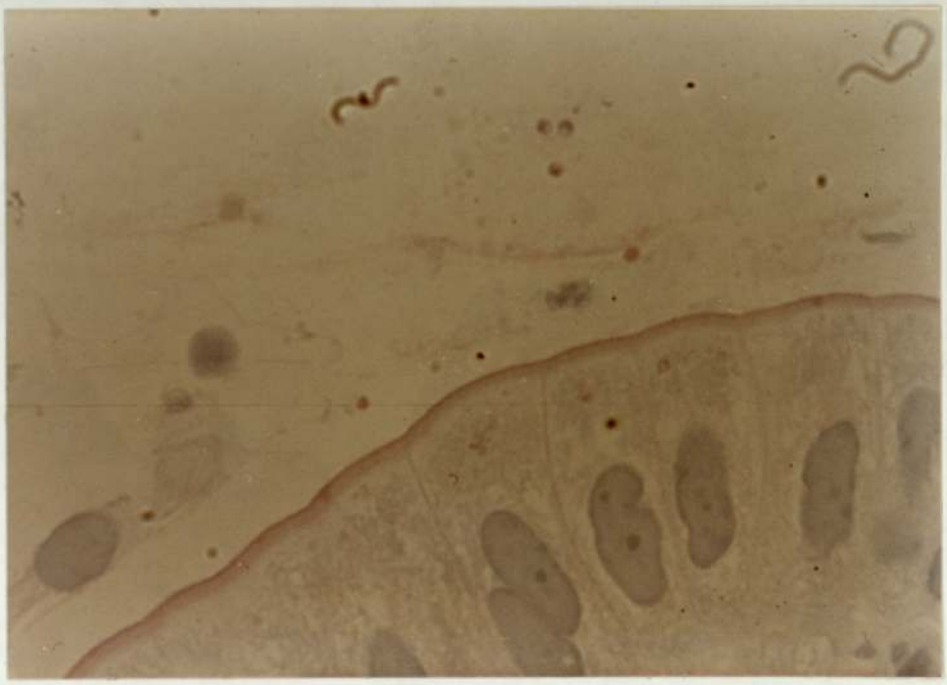


Plate 13.

O.H. (plate 14)

This 38 year old male patient had reported having diarrhoea. The histology was normal and the folate was 5.0 ng/ml. Overall, the glycocalyx appeared continuous.

Folate deficient group.

A.F. (plate 15)

This 51 year old female patient had normal histology but a folate value of 2.0 ng/ml. The majority of the glycocalyx appeared continuous although there were some patches where it appeared to be slightly disrupted.

Normal group contd.

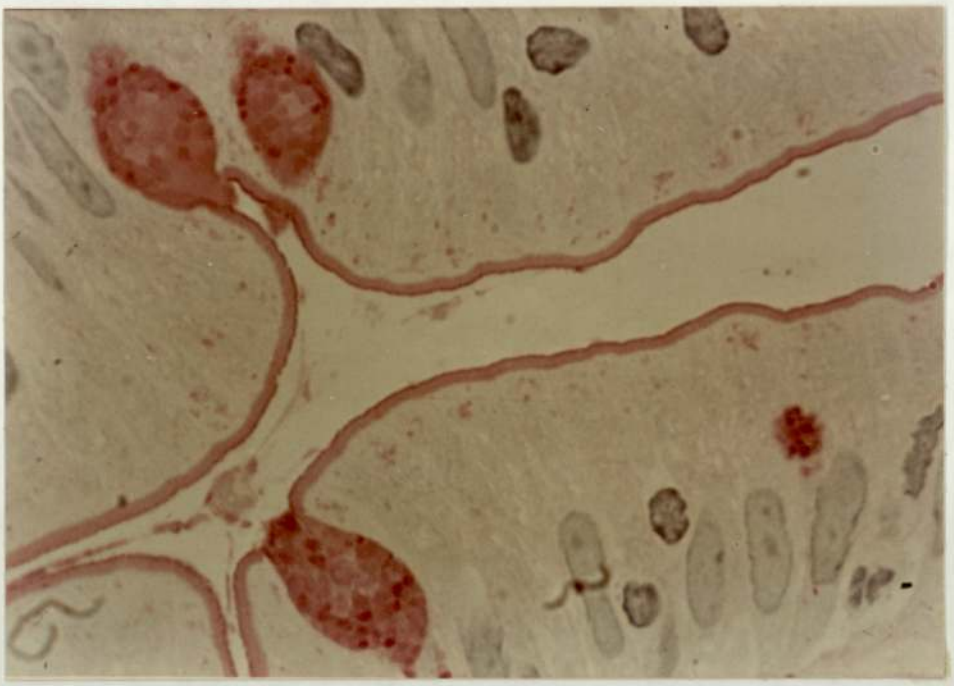


Plate 14.

Folate deficient group.

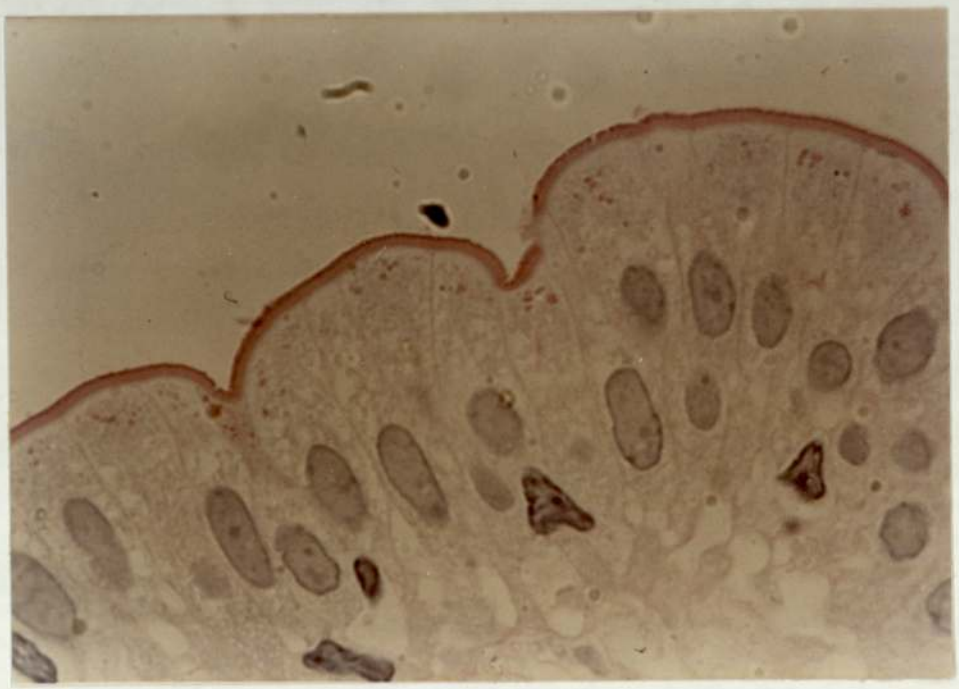


Plate 15.

T.S. (plate 16)

This 24 year old female patient had a report of grade 1 histology. The folate value was 2.6 ng/ml. The appearance of the glycocalyx was variable although the majority of epithelial cells seemed to have a glycocalyx which was patchy with rather a knobby appearance.

D.E. (plate 17)

This biopsy was obtained from a male patient, aged 25 with grade 1 histology and a folate level of 1.4 ng/ml. The glycocalyx over the whole of this biopsy had a knobbed appearance and did not appear to be a continuous line.

Folate deficient group contd.

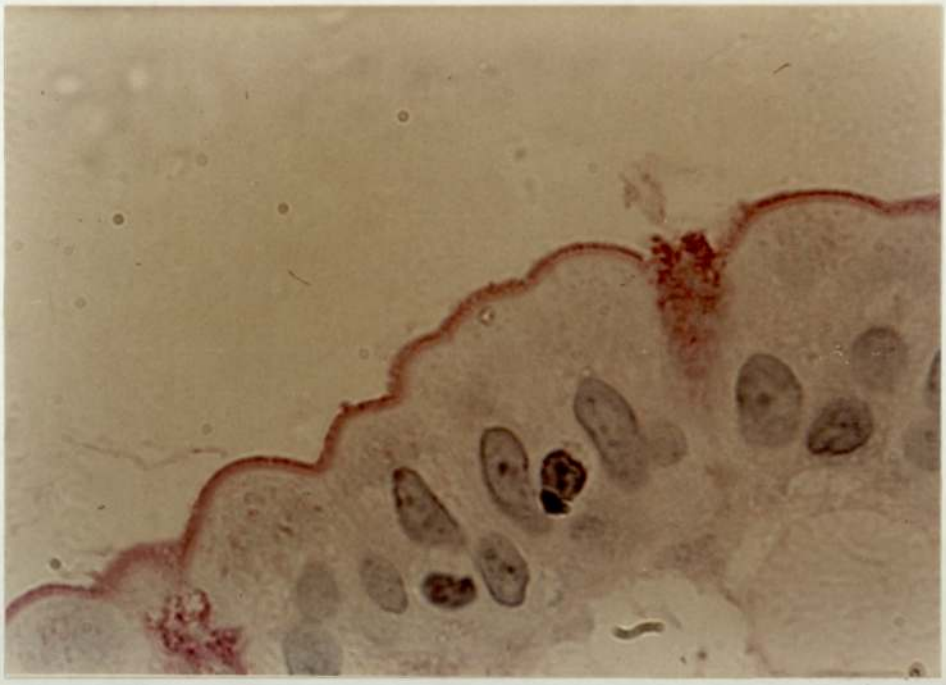


Plate 16.

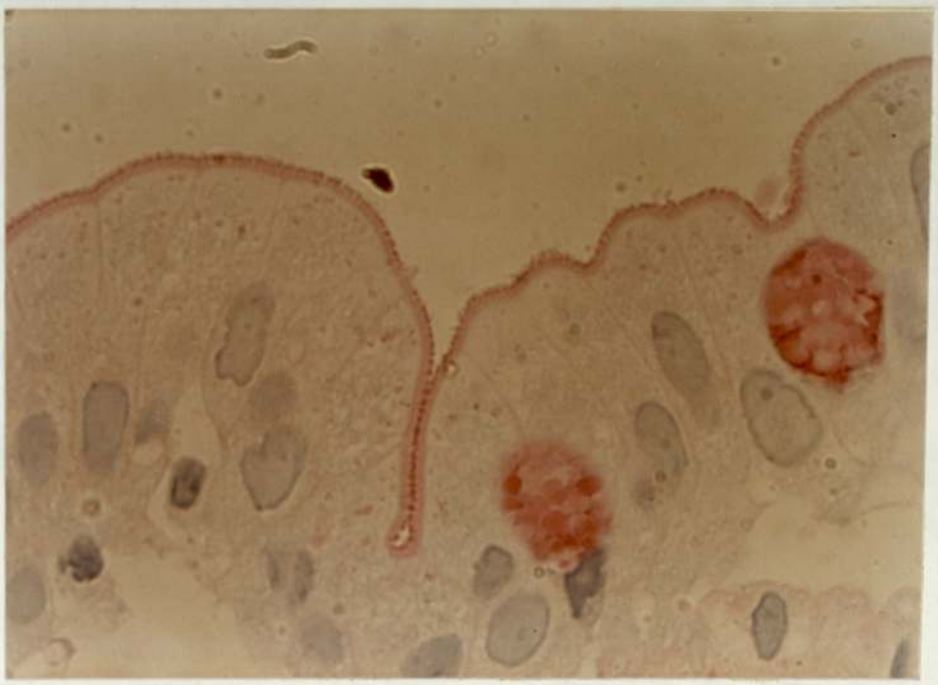


Plate 17.

A.P. (plate 18)

This was a 40 year old male patient with normal histology and a folate value of 2.0 ng/ml. There was rather a hazy appearance to the brush border and glycocalyx although a continuous line could be seen to represent the glycocalyx over several of the epithelial cells. Over other cells, the glycocalyx appeared either absent or discontinuous.

U.A. (plate 19)

This biopsy was obtained from a female patient, aged 42 who had previously had a colonic carcinoma. Her folate value was 0.8 ng/ml. The continuity of the glycocalyx was variable; in many areas it appeared to be knobbed.

Folate deficient group contd.

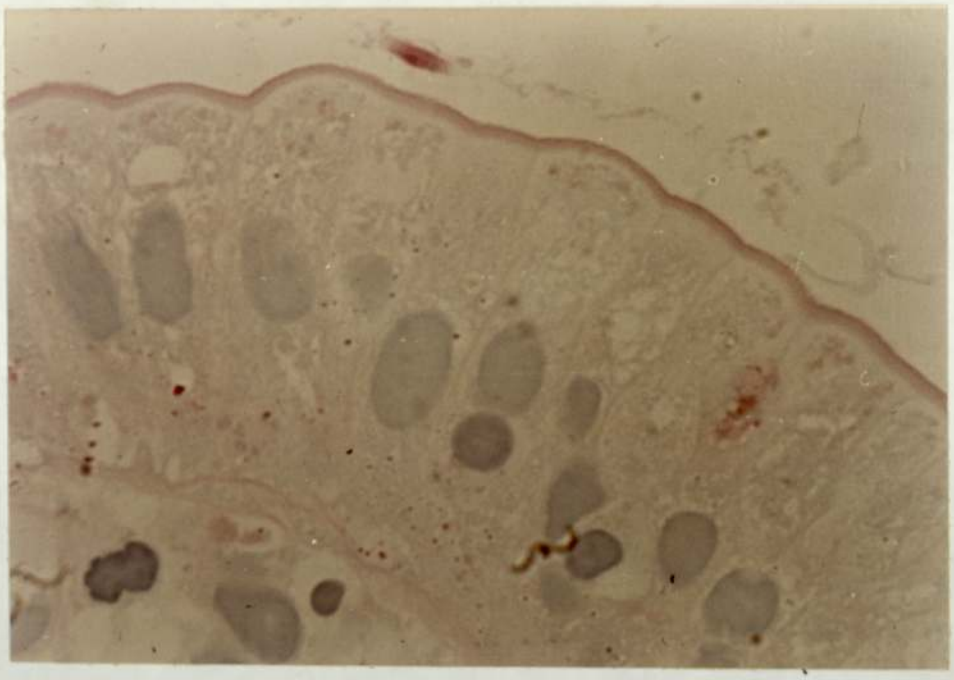


Plate 18.

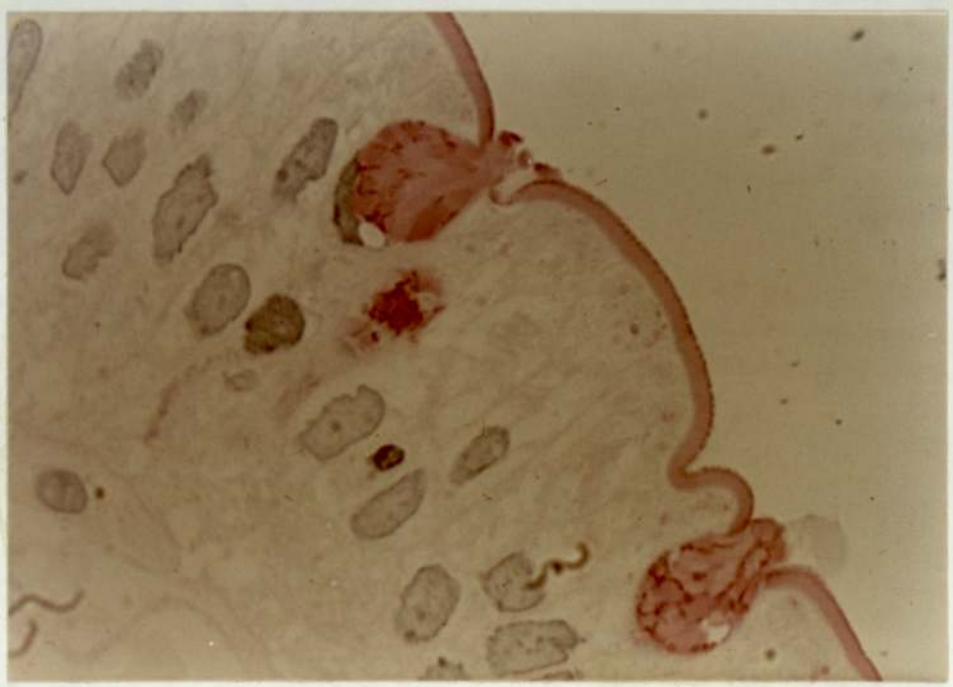


Plate 19.

H.S. (plate 20)

This was a 36 year old female epileptic patient who had shown persistent folate deficiency. She was receiving Phenytoin for her epilepsy. The biopsy was grade 1 histology and the folate value was 1.0 ng/ml. Although the glycocalyx initially appeared continuous, on close examination it was distinctly patchy.

Untreated coeliac disease.

A.B. (plate 21)

This female patient, aged 39 had grade 3 histology and a folate value of 2.2 ng/ml. The glycocalyx appeared to be broken.

Folate deficient group contd.

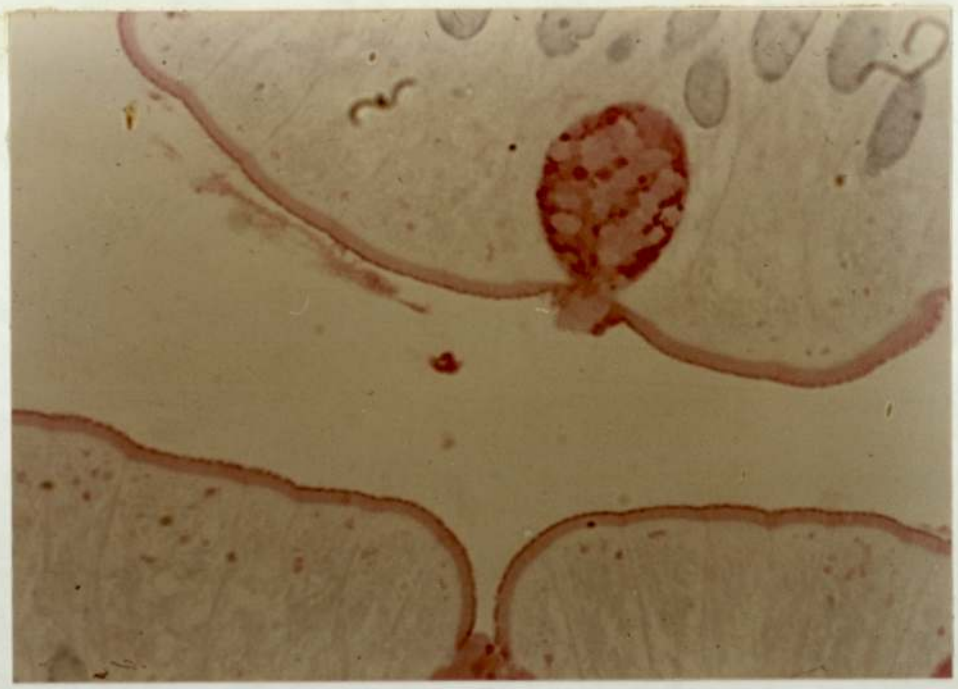


Plate 20.

Untreated coeliac disease.

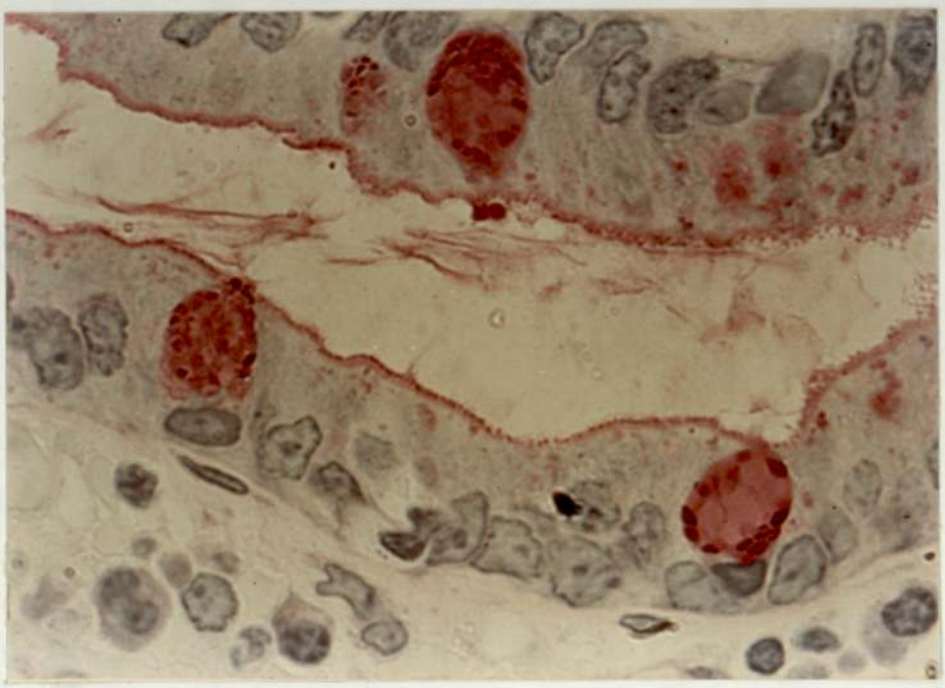


Plate 21.

A.M. (plate 22)

This 16 year old female patient with a folate level of 2.0 ng/ml, was reported to have a histological grading of 2 and 3. The glycocalyx has a knobby appearance and the fuzzy areas result from a tangential view of the surface of the epithelium.

H.P. (plate 23)

This was a female patient, aged 35 with grade 2 and 3 histology and a folate value of 1.5 ng/ml. The glycocalyx had a hazy appearance, it may have been absent in some areas although a continuous line could also be detected.

Untreated coeliac disease contd.

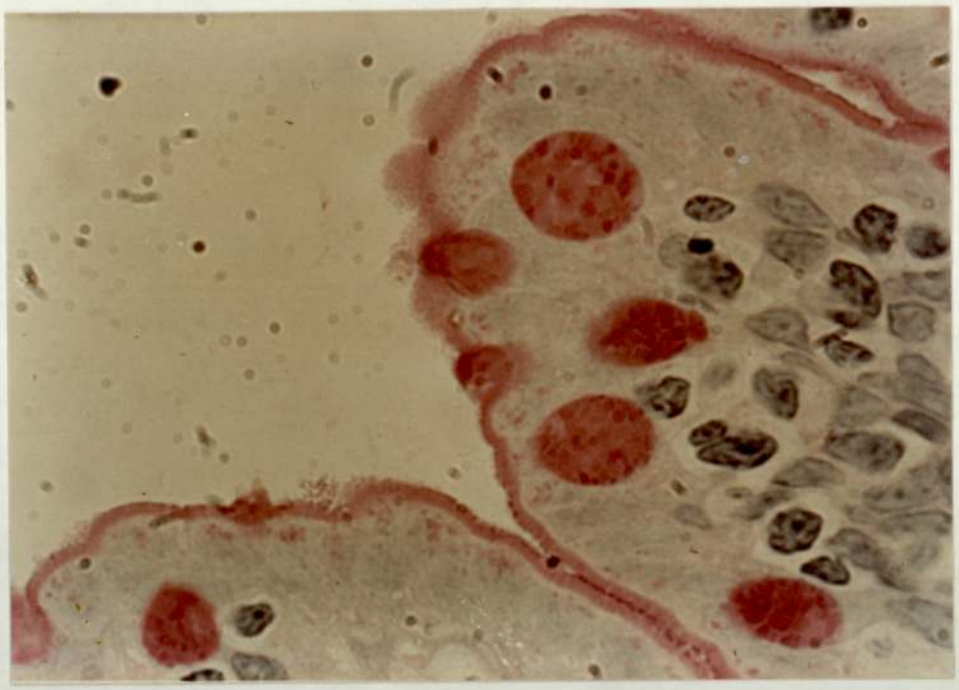


Plate 22.

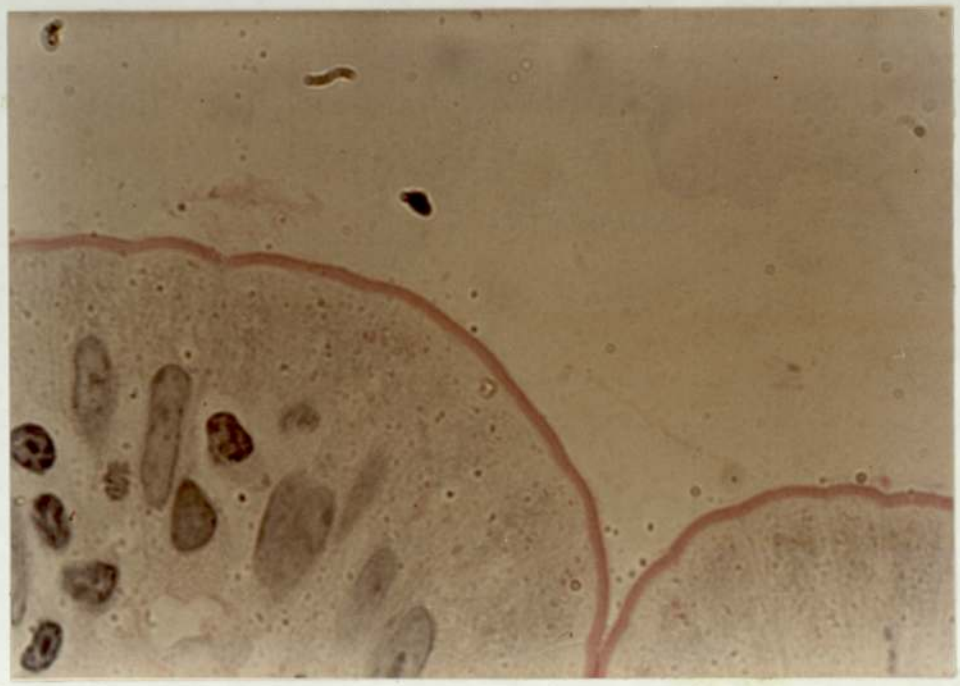


Plate 23.

U.M. (plate 24)

This biopsy was from a 66 year old male patient who also had dermatitis herpetiformis. The histology was grade 2 and 3 and the folate was 1.2 ng/ml. The glycocalyx appeared to be broken up over the majority of the biopsy.

Treated coeliac disease.

O.H. (plate 25)

This 48 year old male patient had a histology report of grade 1. He had been on a gluten-free diet for 10 years and his folate level was 2.4 ng/ml. The glycocalyx over the majority of the biopsy looked continuous but there were a few areas where it was patchy.

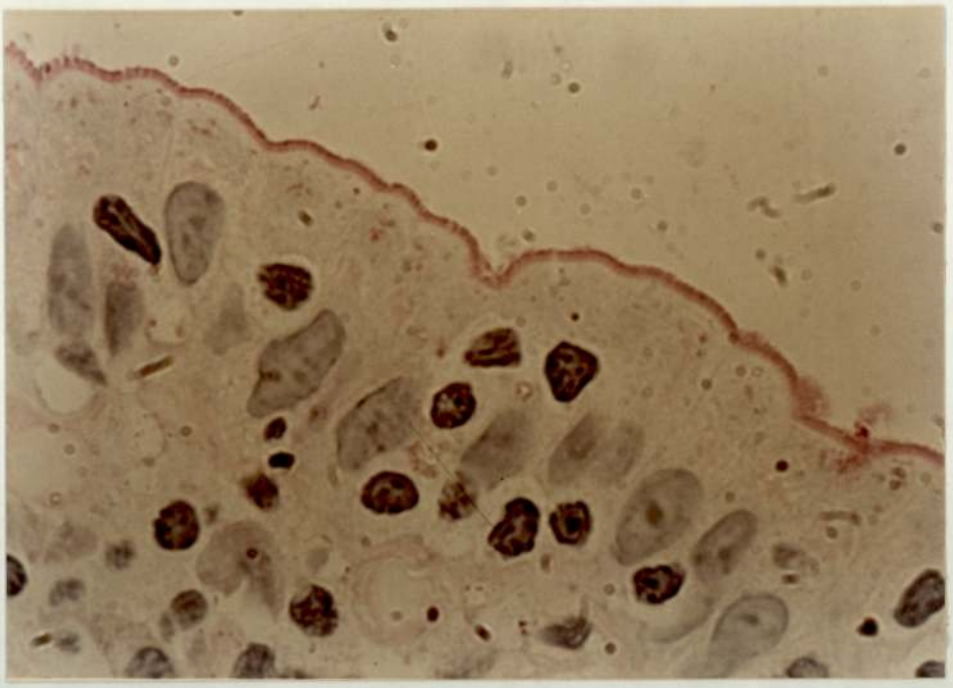


Plate 24.

Treated coeliac disease.

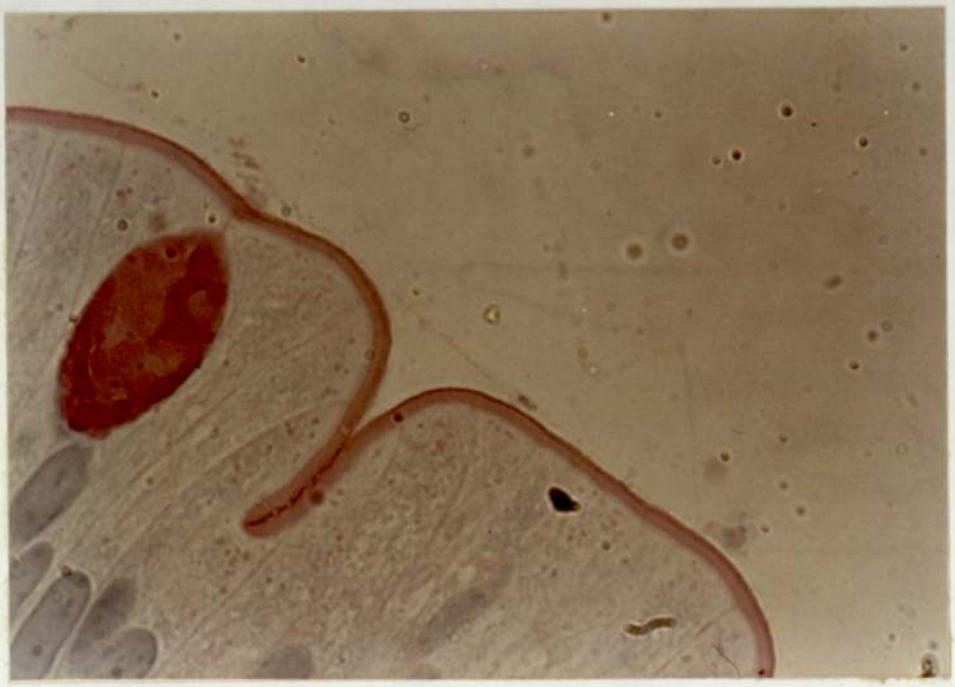


Plate 25.

A.C. (plate 26)

This 59 year old female patient had been on a gluten-free diet for 17 years. She was receiving folate supplementation and her folate value was >32 ng/ml. There was rather a hazy appearance to the glycocalyx although it was possible to detect some knobs of glycocalyx on the brush border.

A.D. (plate 27)

This female patient, aged 49, had been on a gluten-free diet for 2 years and her folate level was 3.6 ng/ml. The glycocalyx appeared fairly continuous although in some areas, it was rather pale.

Treated coeliac disease contd.

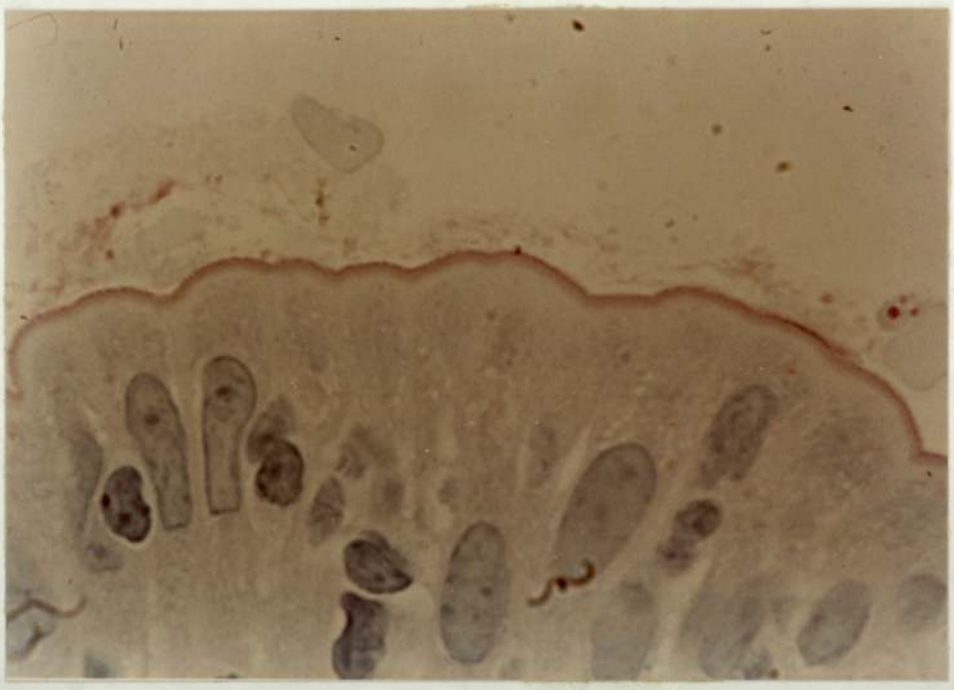


Plate 26.

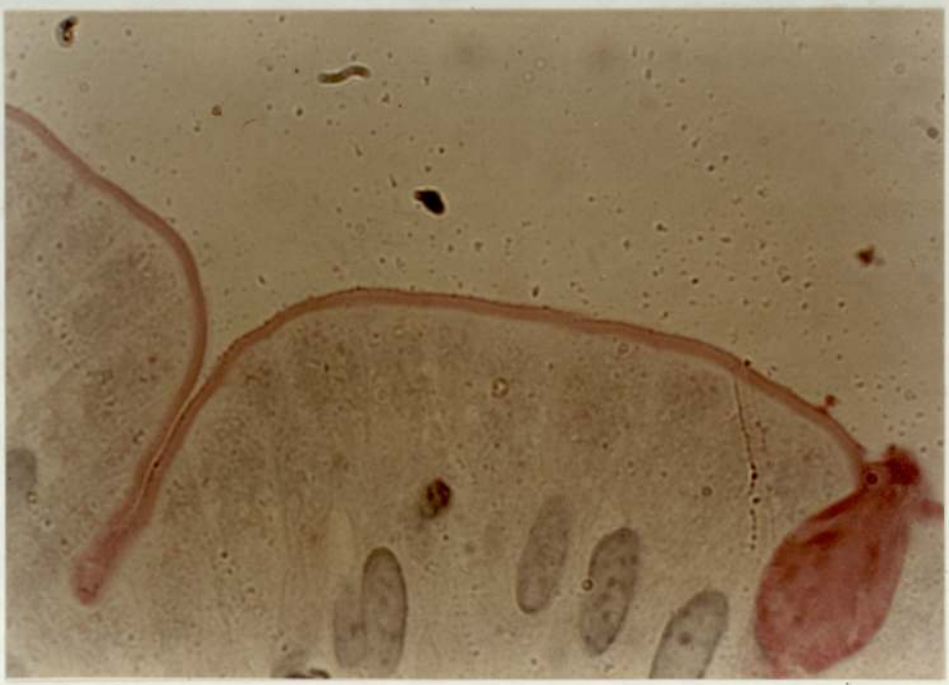


Plate 27.

O.W. (plate 28)

This 75 year old male patient had been on a gluten-free diet for 3 years. The serum folate value was 4.0 ng/ml. The majority of the glycocalyx was continuous although over a few cells it appeared patchy.

A.N. (plate 29)

This male patient, aged 49, had been on a gluten-free diet for 4 years. His folate value was 3.0 ng/ml. The glycocalyx was mostly continuous although some areas were patchy.

Treated coeliac disease contd.

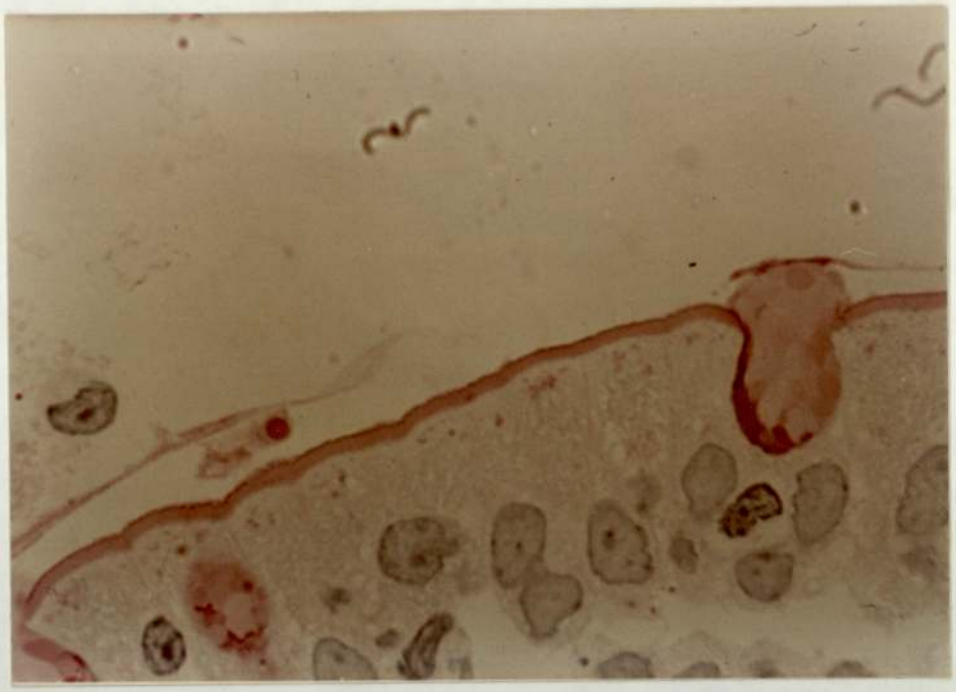


Plate 28.

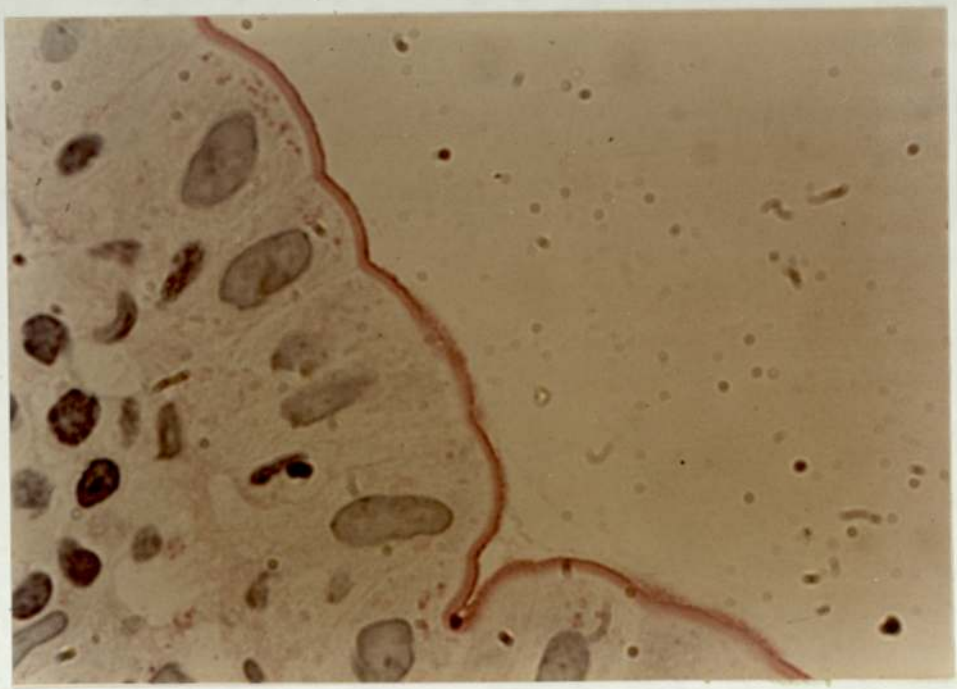


Plate 29.

Crohn's disease.

G.H. (plate 30)

This 61 year old female patient had a grade 1 histology and was on folate supplementation. Her serum folate value was >32 ng/ml. The glycocalyx had a rather hazy appearance and seemed to be disrupted.

T.S. (plate 31)

This female patient, aged 34, had a folate level of 0.2 ng/ml. The glycocalyx was very pale and thin with a knobbed appearance in some areas.

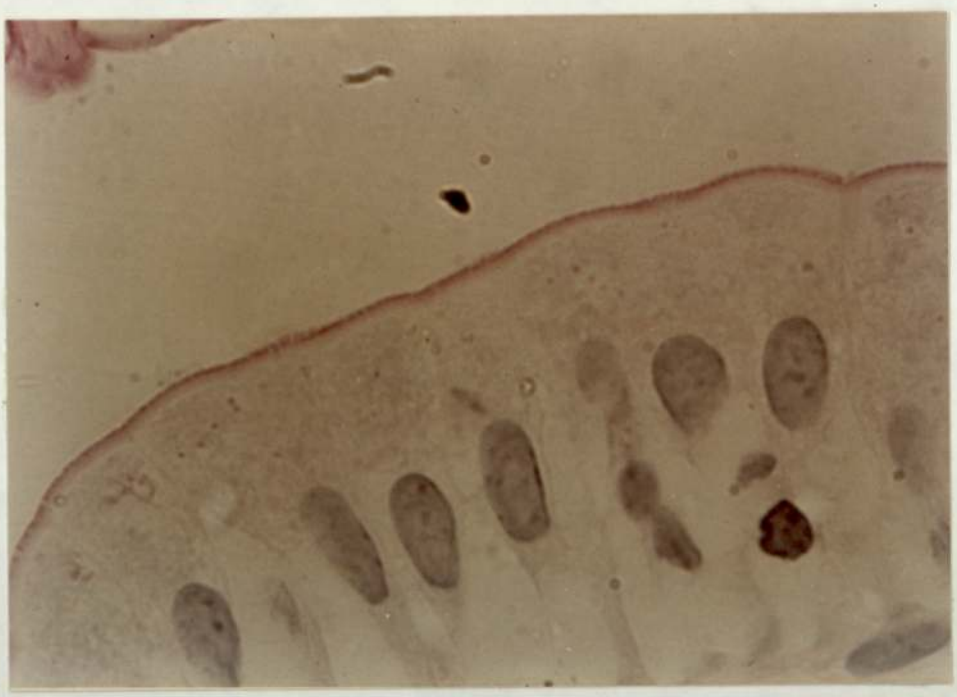


Plate 30.

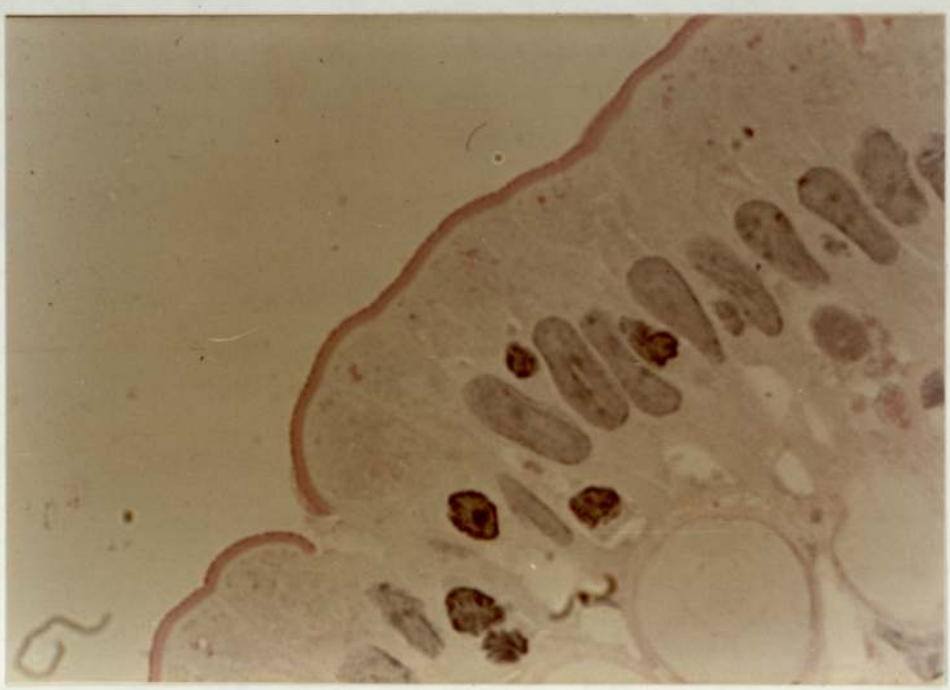


Plate 31.

L.C. (plate 32)

This biopsy was obtained from a 45 year old female patient who had a serum folate value of 1.0 ng/ml. The glycocalyx was very pale and hazy, appearing almost absent in places.

E.P. (plate 33)

This was a female patient, aged 37, who had a folate value of 2.0 ng/ml. The glycocalyx appeared very pale and almost absent.

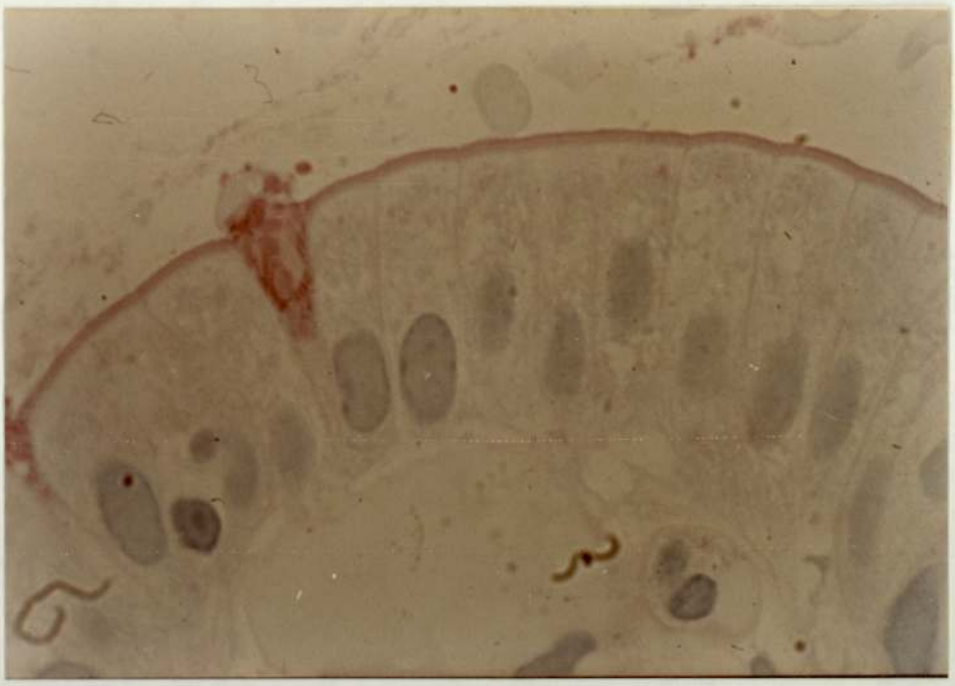


Plate 32.

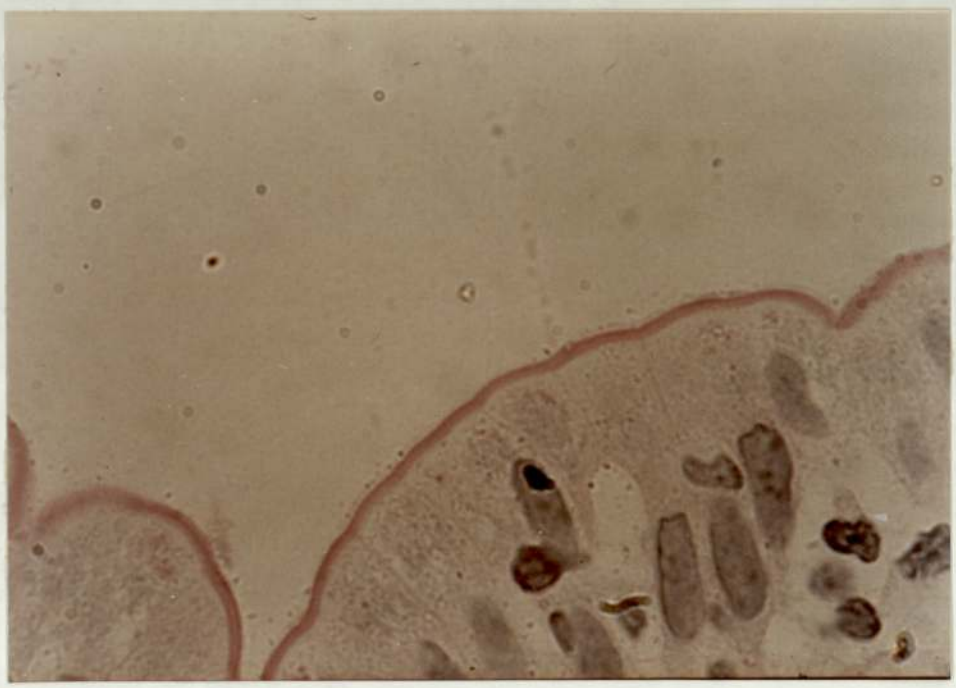


Plate 33.

A common feature of all the tissues examined, including the control group, was that in the crypt region, the glycocalyx was always broken up and not continuous (plate 34).

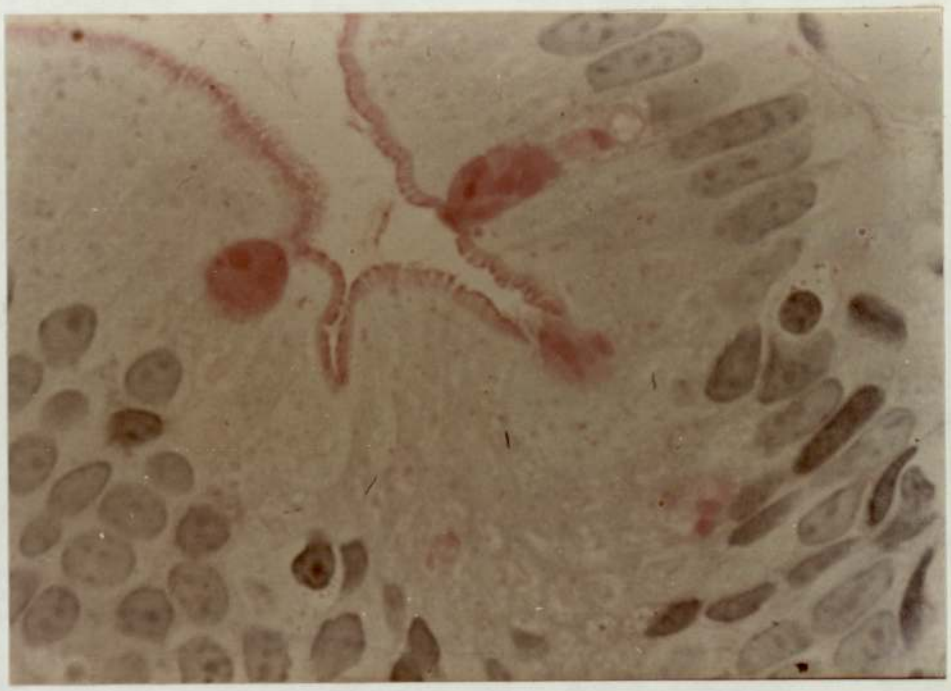


Plate 34.

Section 4. DISCUSSION.

HISTOLOGICAL STAINS FOR THE GLYCOCALYX.

The main advantage of using a plastic embedding medium is that thin sections can be cut (0.5-1.0μ) when compared with the limit of paraffin embedded tissues (3-5μ). Using thin plastic sections, optical definition is improved and tissue distortion is reduced (Germain, 1974; Bennett et al., 1976). But despite these advantages, it should not be assumed that a staining procedure which is reliable on paraffin embedded sections will be the same with plastic embedded tissues (Cole & Sykes, 1974).

The staining procedures used here to demonstrate the glycocalyx were applied in a manner identical to that which would be used for paraffin sectioning. But there was limited success with these stains and the periodic acid-Schiff (PAS) technique proved to be the most suitable for further work. Authors have reported difficulties with staining plastic sections (Lumsden & Aparicio, 1970) and several authors state that modifications to the normal staining procedures may be necessary to obtain successful results (Aparicio & Marsden, 1969; Cole & Sykes, 1974; Sims, 1974; Bennett et al., 1976).

The glycocalyx reacted positively with the PAS reaction. Carbohydrates which are positive to PAS include polysaccharides, mucopolysaccharides, mucoproteins, glycoproteins and glycolipids (Drury & Wallington, 1967). The PAS reaction has commonly been used to demonstrate surface coats and glycocalyces as was discussed in section 1 of this chapter.

Ito (1965) also used methacrylate sections to demonstrate the glycocalyx in the cat with different staining procedures, although the method of methacrylate polymerization was different to that used here (Sims, 1974). In agreement with Ito, a positive reaction was also obtained with the glycocalyx and Alcian blue and Hale's colloidal iron although these results, especially with Alcian blue, were not as crisp as those with PAS. Positive reactions of the glycocalyx with Alcian blue and Hale's colloidal iron imply the presence of mucopolysaccharides.

The indistinct appearance of the glycocalyx with Alcian blue might be an artefact. If one cuts a section with a knife that is not absolutely sharp then in some instances, especially at the edge of the tissue, the knife causes the tissue to 'drag' resulting in a structural artefact (Drury & Wallington, 1967). This could account for the rather fuzzed appearance on the luminal side of the glycocalyx.

Alternatively this appearance may be the result of a staining artefact whereby the chemical reaction of the dye is affected by the tissue and plastic lying in such close proximity (Ayres, 1977).

Toluidine blue and uranyl nitrate are metachromatic stains in which the acid mucopolysaccharides combine with the dyes to produce a colour which is different from the colour of the original dye. The glycocalyx ideally should have coloured red with toluidine blue and uranyl nitrate but in fact reversed metachromasia has occurred whereby the metachromatic reaction has not taken place correctly. The glycocalyx appears as a distinct white line, especially clear with the toluidine blue. There is a turquoise

coloured fuzzy appearance with the uranyl nitrate on the luminal side of the glycocalyx although it seems more likely that this is a staining artefact rather than a cutting artefact for the glycocalyx is white in colour. The reversed metachromasia and other artefacts imply that these methods as such, are not suitable for the methacrylate sections embedded according to Sims (1974).

The results obtained from the other staining procedures used here were either unsuccessful (Schmorl's thionin) or very poor (1% Alcian blue in 30% Acetic acid and Mowry's colloidal iron). These techniques were not initially developed for plastic sections but for either paraffin or frozen cryostat sections. In addition to the above-mentioned artefacts which may result in unusual staining, the sections may just be too thin to bind the dye properly (Barton, 1959) or the presence or absence of impurities in the dye or salts in the tap water may affect the characteristics of the stains (Lillie, 1969).

THE GLYCOCALYX IN VARIOUS CLINICAL CONDITIONS.

In the control group of normal subjects, the glycocalyx appeared as a continuous line on the surface of the brush border. Occasionally, as with patient E.W., gaps could be observed in the glycocalyx which corresponded to the lateral borders of the epithelial cells. A similar light microscopic observation was made in the mouse intestine by Rao *et al*. (1972).

There was no difference in the appearance of the glycocalyx in tissue from the jejunoileostomy patients and the biopsy subjects. It is possible to get a haemorrhagic

suction artefact with peroral biopsies (Perera et al., 1975) but this was not observed here. Thus it was concluded that there were no adverse effects from the biopsy procedure as a result of comparison with tissue obtained at laparotomy.

In all the tissues observed, the glycocalyx appeared broken in the crypts. There was not a smooth continuous line around the crypts but patchy PAS stained areas. This is consistent with the work of Rifaat et al. (1965) and Dognen et al. (1976) on the electron microscopic appearance of the crypt cell glycocalyx and also the observation that on immature epithelial cells, the glycocalyx or cell surface glycoproteins are incomplete in their structure (Weiser, 1976).

Varying degrees of glycocalyx disruption were observed in all the tissues other than the control group. Glycocalyx disruption on the mature epithelial cells predominantly took the form of a knobbed or budded appearance, rather similar to the phenomenon observed in the crypts. Occasionally the glycocalyx had a rather hazy, indistinct appearance. Using transmission electron microscopy, the glycocalyx appeared discontinuous in ulcerative colitis (Ceralli et al., 1976) and diminished or nearly absent in coeliac disease (Biempica et al., 1968; Falchuk et al., 1974) although one author has reported no apparent structural change to the glycocalyx in untreated coeliac disease (Shiner, 1967). The uncommon hazy appearance of the glycocalyx may have been artefactual, with the sections being too thin to bind the dye properly.

Estimations of serum folate values were obtained for all the biopsy subjects. All the folate levels, except those in the control group, were considered to be low although

folate deficiency is one of the common aspects of coeliac disease (Cooke, 1968). Two patients (A.C. and G.H.) were receiving folate supplementation for their deficiency hence their serum folate levels were temporarily very high. It is also not unusual for patients (e.g. H.S.) on anti-convulsant therapy, e.g. Phenytoin, to have low serum folate levels (Hoffbrand & Necheles, 1968).

The degree of disruption of the glycocalyx was not proportional to the amount of folate deficiency. For example, D.E. with a folate level of 1.4 ng/ml had a glycocalyx which was very knobby compared with U.A. whose level was 0.8 ng/ml and yet the disruption was not as severe. Also, the patients with very high folate levels as a result of clinical dosing with folate for their deficiency (A.C. and G.H.) did not have a continuous glycocalyx, thus the disruption did not correct itself with high doses of folic acid.

The two essential criteria for the diagnosis of coeliac disease are the characteristic flat biopsy and the unequivocal response to a gluten-free diet (Weinstein, Shimoda, Brow & Rubin, 1970). The characteristic flat lesion is present in the duodenum and upper jejunum (Creamer, 1974), but there are differing degrees of intestinal involvement and a flat biopsy might not necessarily be obtained from a coeliac disease patient if only one specimen was examined (Cooke, 1968). Thus although the glycocalyx of H.P. could be seen as continuous in areas, another biopsy from her may have shown a much worse situation. The association of dermatitis herpetiformis and coeliac disease (patient U.M.) is not uncommon (Marks & Shuster, 1970).

The knobbed appearance of the glycocalyx viewed

on the coeliac tissue, may correspond to the irregular clumps of glycocalyx observed on coeliac biopsies by scanning electron microscopy (Marsh et al., 1970).

The group of treated coeliac disease patients, that is those who had been on a gluten-free diet for a suitable length of time to produce marked improvement in their condition, showed low folate levels and altered glycocalyces when compared to the normals. Histological improvement, as the result of commencement of a gluten-free diet, may be observed in a few months (Anderson, 1960) but only the exceptional patient will return to histologic normality after a long term diet (Bolt, Parrish, French & Pollard, 1964). From the appearance of the glycocalyx in these subjects, none was indeed similar to the normal state. But the rate of improvement of the intestinal lesion is dependent on the degree of adherence to the diet or the amount of gluten present in the reduced diet (Dissanayake, Truelove & Whitehead, 1974).

Crohn's disease is an inflammatory condition of the terminal ileum and associated folic acid deficiency is not uncommon (Creamer & Lockhart-Mummery, 1974). There is increasing evidence to suggest that Crohn's disease is a diffuse lesion of the gastrointestinal tract (Tootla, Lucas, Bernacki & Tabor, 1976; Nugent, Richmond & Park, 1977) with particular effects on the epithelium and the microvilli being the site of abnormality resulting in reduced levels of brush border enzymes (Dunne, Allan & Cooke, 1976). The glycocalyx appeared altered, although not severely disrupted, in the patients with Crohn's disease. This would agree with the other work which suggests that the disease is more diffuse than at first thought.

The disruption of the glycocalyx could feasibly be the result of incomplete oligosaccharide synthesis which would cause an incomplete surface glycoprotein layer (Swanston, Blair, Matty, Cooper & Cooke, 1977). Especially if there were 1:2 glycol groups absent then the PAS reaction would appear patchy. Degradation, loss or rearrangement of glycoproteins at the cell surface of the erythrocyte membrane, change the glycocalyx structure thus deteriorating the conditions for its staining with toluidine blue (Makovitzky & Geyer, 1977). A hypothesis of structural change in the cell surface membrane glycoproteins produced by incomplete oligosaccharides was also proposed by Weiser and Douglas (1976) to explain the toxicity of gluten in coeliac disease. The gluten would bind to altered exposed incomplete cell surface membrane glycoproteins and act as a toxic lectin which would initiate cell toxicity. Douglas (1976) substantiated this hypothesis by isolating a toxic fraction of gluten which bound to coeliac mucosa and not to normal mucosa.

There is a similarity of the appearance of the diseased glycocalyx to that on the immature crypt cells. The glycoproteins are also incomplete on the crypt cell surfaces (Isselbacher, 1974; Weiser, 1976). In coeliac disease, there is an increased cell loss from the epithelium and the cell cycle time is halved resulting in crypt elongation. Although there is a reduction in surface area, the increased cell cycle time is a compensatory reaction whereby the rate of loss from the surface epithelium is minimised (Watson & Wright, 1974). The accelerated epithelial cell replacement provides the surface with cells which are functionally immature (Weinstein, 1974). These

cells have an enhanced ability to synthesize DNA (Jones & Peters, 1977) and they accumulate on the mucosa, failing to differentiate (Cooper & Lipkin, 1973). A similar failure of repression of DNA synthesis also occurs in cancer conditions (Lipkin & Deschner, 1976). One might hypothesise therefore, that the cells which are functionally immature on the diseased mucosal surface will have the characteristics similar to crypt cells previously described. Namely, surface alterations during the cell cycle and increased glycocalyx thickness by late G2. Thus the patchy or knobbed appearance may be explained by the fact that the epithelial cells are still dividing and the glycocalyx is incomplete as in crypt cells.

Abnormal mucosal villous architecture and absorptive abnormalities may occur in folate deficient states when the deficiency occurs as a primary event without underlying disease (Davidson & Townley, 1977). Rats which have been kept on a folate-free diet exhibit significant lengthening of the jejunal crypts (Klipstein, Lipton & Schenk, 1973). An increase in crypt size was also observed in untreated coeliac mucosa (Wright, Watson, Morley, Appleton & Marks, 1973). Folate is required for DNA synthesis and the reduced transport of low concentrations of thiamine in folate deficient rats (Howard, Wagner & Schenker, 1974) does suggest decreased mitosis which is the opposite in coeliac disease. Even so, the structural abnormalities in folate deficient states appear to result from altered cell renewal whatever the stimulus for alteration.

It is suggested therefore that there may be altered surface glycoproteins in folate deficiency as well as in coeliac disease. These alterations to the glycoproteins

constituting the glycocalyx may worsen the situation for folate absorption by disrupting the retaining layer for the acid microclimate which is thought to be necessary for folate absorption.

The acid microclimate pH has been measured in human jejunal biopsy samples using microelectrodes (Lucas, Blair, Cooper & Cooke, 1976). For the control tissue, a value of pH 5.9 was obtained but the measurements for untreated coeliac and Crohn's tissue were significantly less acidic at pH 7.0 and pH 6.4 respectively. Recent work has shown that the microclimate value for coeliacs on a gluten-free diet was pH 6.1 which although not significantly different from the control group, it is a value less acidic (Lucas, 1978).

It has been reported here that the glycocalyx appears abnormal in treated and untreated coeliac disease, Crohn's disease and folate deficient states. Altered microclimate values have been reported in all these conditions (Lucas, 1977), with the exception of folate deficient states of unknown aetiology, for which the corresponding microclimate values remain to be established. Hence it is suggested that the maintenance of a complete glycocalyx is necessary for the effective functioning of the microclimate.

Section 5. SUMMARY.

1) On methacrylate sections, the glycocalyx was best observed by light microscopy following the periodic acid-Schiff staining technique.

2) In normal human jejunal mucosa, the glycocalyx appeared as a distinct continuous staining border on the luminal surface of the microvilli.

3) The glycocalyx appeared disrupted in certain disease states; especially untreated coeliac disease but also treated coeliac disease, Crohn's disease and folate deficient states.

4) The disruption of the glycocalyx probably occurred as the result of incomplete oligosaccharide synthesis in the epithelial cell surface glycoproteins.

5) Disruption of the glycocalyx was associated with alterations of the acid microclimate. The glycocalyx may therefore be important in the absorption processes in the intestine.

Figure 2,1. General representation of glycoprotein structure. (From Kennedy, 1973).

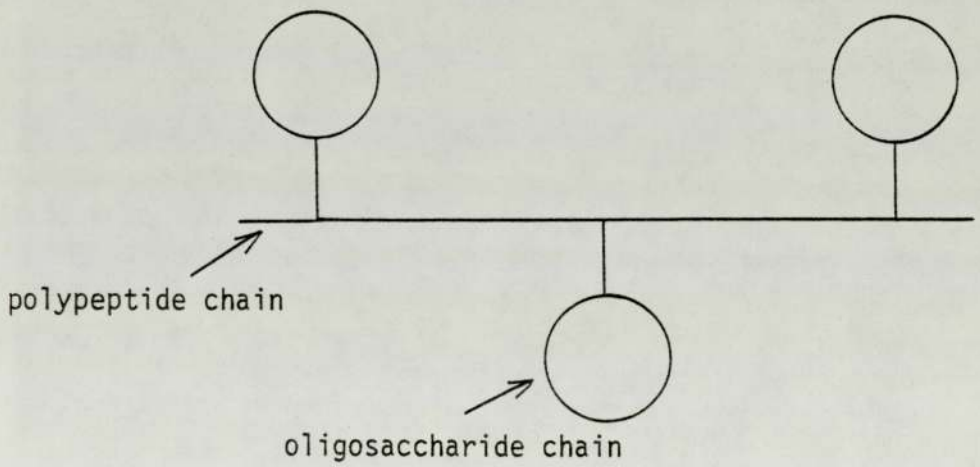


Figure 2,2. Phases of the cell cycle.

M = mitosis

G1 = interval between mitosis and DNA synthesis

S = DNA synthesis

G2 = interval between DNA synthesis and mitosis

Go = prolonged interphase, fertile cells not proliferating

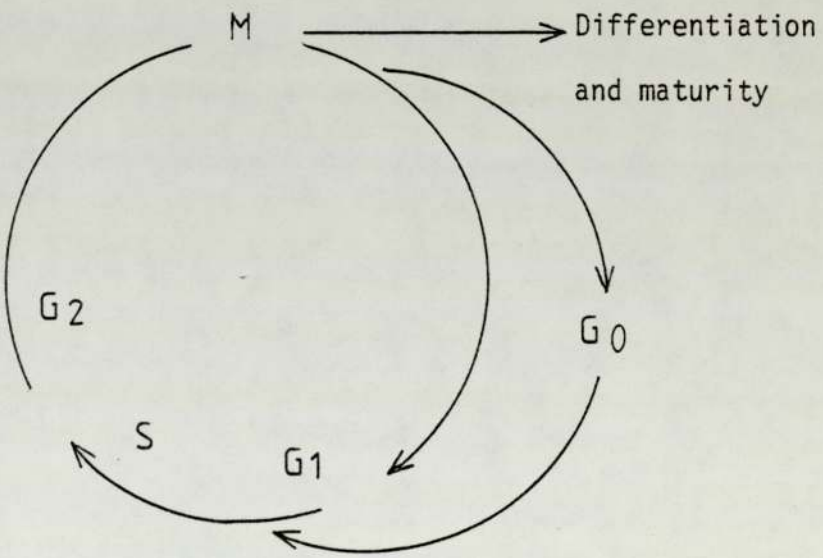
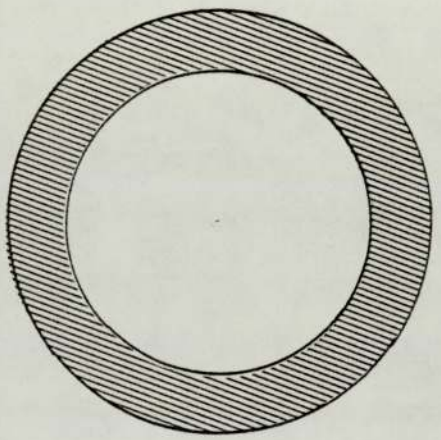
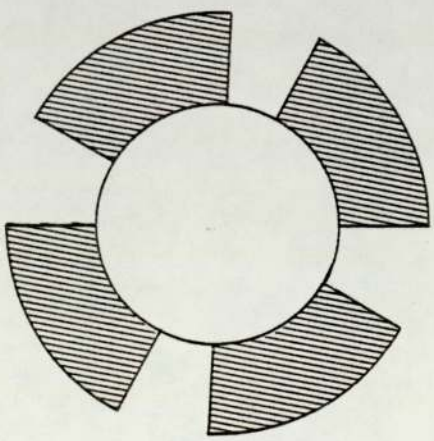


Figure 2,3. Diagrammatic representation of the possible disposition of the glycocalyx on a normal cell (a) and a transformed cell (b). (From Pasternak, 1977).

(a)



(b)



Chapter 3.

Absorption of folic acid.

Section 1. INTRODUCTION.

The major proportion of dietary folate is ingested in the form of polyglutamates, these being abundant in both plant and animal tissues (Blakley, 1969). Most of the current concepts of folate absorption however, have been derived from studies utilizing synthetic pteroylmonoglutamic (folic) acid. Folic acid is employed for clinical and research purposes because it is readily synthesised, inexpensive and easily absorbed. It is converted in the body into metabolically active folates.

The ingested polyglutamates are broken down in a stepwise fashion to monoglutamates by hydrolytic conjugase enzymes, first described by Bird, Robbins, Vanderbelt and Pfiffner,(1946). The location of polyglutamate hydrolysis by conjugase enzymes within the intestine is still undecided. However, it has been demonstrated that the digestion of the heptaglutamate requires its contact with the intestinal mucosa prior to absorption, and hence the site of hydrolysis is not intraluminal (Halsted, Baugh & Butterworth, 1975). Although one might expect the enzymes to be associated with the brush border similar to several other hydrolytic enzymes, it was observed that in tropical sprue, the activity of the brush border enzymes lactase, sucrase and maltase decreased, whilst the folate conjugase activity increased (Halsted, Reisenauer & Corcino, 1975). Thus it was postulated that the location of the folate conjugase was intracellular, although no firm conclusion has yet been reached.

Monoglutamyl folate derived from polyglutamate

shares a common pathway of absorption with the monoglutamate alone (Santiago-Borrero, Santini, Perez-Santiago & Maldonado, 1973; Wagonfeld, Dudzinsky & Rosenberg, 1975; Rosenberg, 1976) but the rate of absorption appears to be inversely proportional to the chain length (Baugh, Krumdieck, Baker & Butterworth, 1971).

Following digestion or hydrolysis of conjugated folate, the next event in absorption involves the transport of the folate monoglutamate, either present as such in foods, or released from conjugates by intestinal enzymes. The site of folic acid absorption is principally the proximal jejunum (Smith, Matty & Blair, 1970; Izak, Galevski, Grossowicz, Jablonska & Rachmilewitz, 1972), although its naturally occurring derivative 5-methyl-tetrahydrofolic acid, is absorbed equally well in the jejunum as in the proximal ileum (Strum, Nixon, Bertino & Binder, 1971). Transport of folic acid may proceed without metabolic alterations (Smith et al., 1970); neither reduction nor substitution being obligatory for transport (Rosenberg, 1976).

No other topic in the field of folate absorption has produced more conflicting observations than has inquiry into the mechanism of folate uptake by the intestinal epithelial cell. The controversy exists as to whether the transport process is active or passive. An active transport system is energy dependent and may be demonstrated where the transport occurs against a concentration gradient. If there is a structure-specific mechanism, at high substrate concentrations there is a saturation of transport rate. The mechanism of passive

transport is simple diffusion a process which is energy independent.

The transport system of folates requires the presence of the pteridine ring, the para-aminobenzoyl moiety and at least one glutamate in order to proceed. These are necessary structural requirements as neither pteroic acid, nor tetrahydrobiopterin, a natural pteridine, are taken up by the intestine to any significant extent (Blair, Ratanasthien & Leeming, 1974a).

The transport of folic acid in the jejunum, after much controversy (see Rosenberg, 1976), appears to be that of a saturable system with a saturation value of approximately 5.1×10^{-6}M (Blair, Johnson & Matty, 1974). The transport of 5-methyltetrahydrofolic acid is not saturable at the concentrations which have been investigated so far (Strum *et al.*, 1971; Blair, Matty & Razzaque, 1975). Folic acid has been shown to accumulate within the intestinal tissue (Selhub, Brin & Grossowicz, 1973; Blair *et al.*, 1974), and Smith (1973) suggested that in addition to the rate limiting process into the tissue, there was a separate passive transport system into the serosal solution.

The importance of pH in the absorption of folic acid has been demonstrated both *in vivo* (Benn, Swan, Cooke, Blair, Matty & Smith, 1971; Elsborg, 1974; MacKenzie & Russell, 1976) and *in vitro* (Smith *et al.*, 1970; Elsborg, 1974; Blair, Johnson & Matty, 1976). The existence of an acid microclimate next to the jejunal wall was proposed by Hogben *et al.* (1959) and has been

confirmed by Lucas, Schneider, Haberich and Blair (1975). Blair and Matty (1974) suggested that the acid microclimate might influence folate transport in the small intestine. It was hypothesised that the existence of an acid microclimate would enhance the transfer of the folic acid molecule across the jejunal mucosa. Within the normal luminal jejunal pH range of 5.8 to 6.7 (Benn & Cooke, 1971; MacKenzie & Russell, 1976), almost no folic acid would be available for diffusion as the neutral species. However, if a microclimate were present at pH5 or below, enough of the neutral form of the folate molecule would be present to diffuse across the intestinal membrane consistent with a diffusional system of the non-ionic species (Schanker et al., 1958).

Alternatively, folic acid may be absorbed actively at specific receptor sites (McIntyre, Dow, McIntyre & Harding, 1975). The receptor site theory is supplied by evidence of saturability (Burgen & Goldberg, 1962) and hexose energy requirement (Gerson, Cohen, Brown, Lindenbaum, Hepner & Janowitz, 1974). However, this evidence may also be explained by the hypothesis of Blair and Matty (1974) involving a passive diffusion mechanism; saturability of absorption would be explained by the low folic acid solubility in the acid microclimate, thus as the solubility in water drops with decreasing pH the microclimate would be saturated before the bulk medium. Hence initially the kinetic pattern for transport of folic acid via the microclimate would be linearly related to concentration followed by saturation. The hexose energy requirement may be necessary to maintain the

microclimate. Indeed, some aspect of the intestinal absorption of folate is dependent upon energy because the presence of metabolic inhibitors and the absence of glucose and oxygen result in a decrease of tissue accumulation and serosal transfer of folic acid by rat everted sacs (Smith et al., 1970; Blair et al., 1976).

Several theories have been proposed to explain the mechanism of the acidification process, these include: intracellular lactic acid production followed by differential migration of the proton mucosally and the lactate anion serosally (Wilson, 1954), and from studies of acidification in turtle bladders, hydration of metabolic carbon dioxide to carbonic acid (Schilb & Brodsky, 1966), and carbonic acid shuttling across the secreting membrane, with the bicarbonate ion being transferred back leaving behind a hydrogen ion in the compartment that is being acidified (Schilb & Brodsky, 1972). Blair, Lucas and Matty (1975) have put forward evidence, based mainly on studies involving metabolic inhibitors, that the acidification in the jejunum results from the hydrolysis of ATP. Anaerobiosis, dinitrophenol and phloridzin all resulted in decreased acidification. Thus glucose metabolism was essential to the acidification process. Acidification was also depressed by aminophylline which inhibits phosphodiesterase activity, leading to the accumulation of cAMP and a reduction in ATP turnover. A detailed discussion of this proposed model for acidification has been given by Blair et al. (1975). Lucas (1976) also suggests that there is a potassium-hydrogen ion exchange at the mucosal membrane and that the hydroxyl ions formed

from the ATP hydrolysis enter into the cell, where they combine with an intracellular metabolic source of carbon dioxide, this process being balanced by movement of chloride ions out of the cell.

The transport of 5-methyltetrahydrofolate is also consistent with the Blair and Matty (1974) hypothesis of folic acid transport via an acid microclimate. At a pH of 6 to 7, this compound is present in its ionized form. In an acid microclimate, 5-methyltetrahydrofolate would be present in its neutral form of a zwitterion having one positive and one negative charge. At a pH similar to that of the acid microclimate, 5-methyltetrahydrofolate does not become saturated until a concentration of 3.2×10^{-2}M has been reached (Robb, 1975). This concentration is far in excess of the physiological range and would explain why saturation of 5-methyltetrahydrofolate has not been observed.

Folic acid is the principal species to appear in the blood stream following its transport across the intestine although a certain amount of methylation may occur within the intestine. Using a method of umbilical vein catheterization, Whitehead and Cooper (1967) demonstrated that folic acid was absorbed unaltered from the human intestinal lumen and appeared as the intact molecule in the portal vein. Consistent with this observation, no evidence was found to suggest the reduction or methylation of plasma folate during the first two hours following the oral administration of folic acid to leukemia patients (Butterworth, Baugh & Krumdieck, 1969). However, Chanarin and Perry (1969) demonstrated

that during normal absorption, an oral dose of folic acid was followed by its appearance in the bloodstream substantially as the monoglutamate but with a small amount methylated. Smith et al. (1970), using everted sacs of rat intestine incubated in the presence of tritiated folic acid also found that the predominant form transported was unaltered but there was a minor radioactive component present which was not identified.

Persistent malabsorption of folates and dietary deficiency of folates will give rise to a condition known as megaloblastic anaemia. The developing cells, particularly erythrocytes but also cervical, vaginal and gastrointestinal epithelia, are affected by a basic biochemical defect of inhibition of DNA synthesis, while RNA synthesis is unaffected (Stebbins & Bertino, 1976). This is directly due to the necessity of folates in the synthesis of DNA.

Folate malabsorption has been associated with a number of disease states and ingested drugs (tables 3,1 & 3,2). The mechanisms of folate malabsorption are complex and there may be more than one factor which contributes to the abnormality. Several of the diseases cause structural damage to the intestine, e.g. coeliac disease, tropical sprue, dermatitis herpetiformis, and thus may directly affect the functional integrity of the intestine.

Although the amount of intestinal conjugase has been reported to increase in both untreated coeliac disease (Jägerstad, Lindstrand, Nordén, Westesson & Lindberg, 1974) and in tropical sprue (Halsted et al., 1975), the actual rate of hydrolysis appears to be decreased only in coeliac disease (Halsted, Reisenauer,

Romero, Cantor & Ruebner, 1977). The defective mechanism resulting in the malabsorption of folates in coeliac disease is therefore not due to a deficiency of the hydrolysing enzymes. It has been suggested that there is disruption of an affinity specific transport system for the reduced folates (Weir, 1974) although Blair and Matty (1974) hypothesised that folate malabsorption may result from a decreased acidity of the microclimate at the mucosal surface. This latter suggestion has been confirmed in biopsies from untreated coeliac disease and Crohn's disease patients (Lucas et al., 1976).

Alterations to villous structure, especially villous atrophy, may be observed in diabetes mellitus (Cabarrou, Laguens, Caíno, Schaposnik, Zamboni, Laguens, Costa Gil, Etcheverria, Doria, Caíno & Cendagorta, 1975) and there is evidence to suggest a relationship between abnormalities of glucose homeostasis and coeliac disease (Thain, Hamilton & Ehrlich, 1974; Walsh, Cooper, Wright, Malins & Cooke, 1977; Cooper, Holmes & Cooke, 1978). Although the precise nature of this association remains to be established and is the subject of much recent investigation (Besterman, Bloom, Sarson, Blackburn, Stewart, Guerin, Modigliani & Mallinson, 1978; Walsh, Cooper, Holmes, Wright, Cooke, Besterman & Bloom, 1978), it is conceivable that alterations to the villous architecture of these patients represents one of the many complications of diabetes mellitus. Folate malabsorption has been associated with diabetic enteropathy (Klipstein, 1966) although it has not been considered to be of significant importance in diabetes (Davis, Calder & Curnow, 1976).

In pregnancy, there are increased demands on the maternal folate stores by the growing foetus. This has resulted in several reports of associated folate deficiency and also of folate malabsorption (Chanarin, MacGibbon, O'Sullivan & Mollin, 1959; Giles, 1966; Pinto, Santos, Almeida & Cantuaria, 1973; Yusufji, Mathan & Baker, 1973). Despite these numerous reports, tracing the absorption of tritiated folic acid, produced no evidence of altered folate absorption during pregnancy (Iyengar & Babu, 1975). This area remains controversial but it is quite usual for pregnant women to receive supplementary folate in their diet.

There is a group of drugs which are known as antimetabolites and interfere with folate metabolism. Methotrexate, the commonly used antifolate, may have more than one role as an inhibitor of folic acid transport. It is a powerful inhibitor of dihydrofolate reductase (Olinger, Bertino & Binder, 1973) and as a consequence DNA synthesis is impaired. If there is a specific folic acid carrier system, the methotrexate might compete for this (Nahas, Nixon & Bertino, 1972). Alternatively the toxic effects on the intestine by methotrexate (Robinson, Antonioli & Vannotti, 1966) and aminopterin (Vitale, Zamcheck, DiGiorgio & Hegsted, 1954) might be expected to lead to reduced transport.

Low serum folate levels are found in the majority of patients receiving long term anti-convulsant therapy (Schafer & Nuttall, 1975). Proposed explanations for the low levels include inhibition of gastrointestinal conjugase (Hoffbrand & Necheles, 1968), altered folic

acid metabolism (Klipstein, 1964) and altered luminal pH (Benn et al., 1971).

Megaloblastic anaemia is a common complication of chronic alcoholism (Herbert, Zalusky & Davidson, 1963) although the mechanism of alcohol induced megaloblastosis is complex, as alcohol produces various toxic effects on cellular metabolism (Lundquist, 1975; Gitlow, Dziedzic, Dziedzic & Wong, 1976). Ethanol is reported to decrease serum folate levels (Eichner & Hillman, 1971) and impair folate absorption (Halsted, Robles & Mezey, 1973) in alcoholics administered folic acid. In vitro, ethanol inhibits the activity of tetrahydrofolate formylase (Bertino, Ward, Sartorelli & Silber, 1965) but has no effect on serum conjugase (Eichner, Loewenstein & Cox, 1976). Abnormalities of the intestinal mucosa have been described in severely folate deficient alcoholics (Hermos, Adams, Lui, Sullivan & Trier, 1972), however it was not possible to distinguish the respective roles of folate deficiency versus other factors in the pathogenesis of the intestinal abnormalities, as folate deficiency itself is associated with intestinal structure alterations (Davidson & Townley, 1977). Goetsch and Klipstein (1977) observed that folate deficiency did not alter the transport of xylose, glucose and L-leucine in rats, and suggested that when transport abnormalities of these solutes occurred in folate deficient alcoholics (Halsted et al., 1973), it was not due to the folate deficiency but to the pathogenic effects of the ethanol. Thus the mechanism of folate malabsorption in alcoholism remains unclear, but the poor nutrition common to alcoholics (Halsted, Robles & Mezey, 1971) is no doubt a substantial factor

contributing to the folate deficiency observed in alcoholism.

Another group of drugs which may lower the serum folate levels are the oral contraceptives (Shojania, Hornady & Barnes, 1968). The red blood cell folate values may also be decreased (Martinez & Roe, 1977). The mechanism of these alterations, as with other drugs, is unclear. The serum folate levels may be reduced in spite of adequate dietary folate intake (Pietarinen, Leichter & Pratt, 1977). But it has been reported that there is decreased absorption of folate polyglutamate yet normal monoglutamate absorption in subjects taking oral contraceptives, while control subjects had equivalent absorption of both compounds (Streiff, 1970). However, when similar studies were performed in women presaturated with folate, other investigators found no consistent malabsorption of polyglutamates in subjects taking oral contraceptives (Shojania & Hornady, 1973).

Streiff and Greene (1970) reported that oral contraceptive hormones caused <u>in vitro</u> inhibition of isolated folate conjugase, but Stephens and colleagues (1972) were unable to confirm this effect using human or guinea pig jejunal preparations. Shojania (1975) observed that women on oral contraceptives showed increased urinary folate excretion when compared with controls and noted that this may explain, at least in part, the low serum folate and red blood cell folate values in many of these women.

The aim of the work described in this chapter was to compare the effects of various drugs and conditions on the absorption of folic acid. Recent measurements of the rat jejunal microclimate, performed in this laboratory, have enabled more detailed analyses of alterations in folic acid uptake, following various treatments, into rat intestine. Hence where such joint studies have been undertaken, mention is made of the trend, if any, in changes of the microclimate values.

In this investigation of folic acid absorption, certain drug treatments and conditions have been included, which, although there are no reports of associated folate malabsorption, might feasibly induce such a state. The experimental conditions of substituting sucrose for glucose and depleting sodium from the incubation medium, were included to examine in greater depth the mechanism of folic acid absorption and the necessity of such compounds in the normal absorptive process.

The classic everted sac technique has been used throughout as it is a well established method of investigating intestinal absorption and in view of the number of studies involved, provided numerous results for their comparison.

In each study, a histological examination was made on the proximal jejunum to observe if there had been any alterations to the glycocalyx initiated by the drugs or the conditions.

Section 2. MATERIALS AND METHODS.

ANIMALS.

Albino rats (Rattus norvegicus albinus) of the Wistar strain were used throughout at a weight of 150 - 250 g which corresponds approximately to 7 weeks of age. Male rats were used at all times apart from the experiments which required the use of female, e.g. ovariectomy. The animals were purchased from Bantin and Kingman Ltd., Aldbrough and accommodated in The University of Aston animal house prior to experimentation. Groups of 6 animals were housed per cage (50 x 30 x 15 cm), and the bedding of wood shavings changed every third day. The room was air conditioned at 25 $\pm$ 2°C with a regular lighting schedule of 10 hours light (09.00 to 19.00 hours) and 14 hours darkness. The required number of animals were removed from the animal house 24 hours prior to experimentation. This was to prevent additional stress which would have been caused by moving the animals to the site of the experiment on the actual day.

Water and a standard 3/8" pellet diet (Heygate's Breeding Diet, L.A. Pilsbury, Birmingham) were supplied ad libitum. Tap water was given to all animals apart from those in the sodium depleted experiment where the tap water was replaced with double distilled deionised water 48 hours before death. The animals were not fasted at any stage. Apart from fasting giving rise to a stress condition, a 24 hour fast results in alterations to the intestinal epithelial cell cycle in rats and mice; cell proliferation is inhibited (Orr & Benet, 1975) and the G1 phase is lengthened (Hagemann & Stragand, 1977).

A 48 hour fast results in significant changes to the carbohydrate moiety of the intestinal brush border in guinea pigs (Bouhours, André, Guignard & Lambert, 1973). Such alterations include increased levels of fucose, sialic acid and sulphate particularly in the proximal intestine.

The animals were sacrificed by a sharp and immediate cervical fracture and the proximal intestine removed as carefully and quickly as possible. There is evidence to suggest that etherization, the most common method of sacrifice, has adverse effects on the physiological viability of subsequently isolated tissue (Johnson, 1976) apart from the unpleasant induction phase, irritant action on mucous membranes (Croft, 1966) and other pharmacological actions (Price, 1975). However, there is some controversy in this area (Levine, McNary, Kornguth & Le Blanc, 1970).

CHEMICALS.

All reagents used were of analytical grade and solutions were prepared with distilled water. The inorganic saline constituents, glucose and sucrose were obtained from Fisons Ltd., Loughborough, L-leucine, 5,5-diphenylhydantoin (sodium salt), cortisone, 17α-ethynyl-1,3,5-estratrien-3,17β-diol, and 1,3,5(10)-estratrien-3,17β-diol were supplied by Sigma Chemical Co., London. Folic acid was obtained from Koch Light Ltd., Colnbrook, and the streptozotocin (U-9889, Lot no. 10518-GGS-37A,) from Upjohn Co.,

Kalamazoo, Mich., U.S.A. The methotrexate was a gift from Lederle Laboratories Ltd., London.

^{14}C absorber 'P' scintillation cocktail and emulsifier mix No. 1 were obtained from Fisons Ltd., and other associated chemicals, 2,5-diphenyl-oxazole (PPO) and scintillation grade toluene were from Koch Light Ltd.

The radioisotopically labelled compounds were supplied by The Radiochemical Centre, Amersham and special precautions in accordance with the code of practice recommended by The Department of Employment and Productivity (1968) and the University of Aston (1969) were observed when working with the radioactive compounds.

The labelled compounds used, were L-leucine-^{14}C(U) (10mCi/mmol); [2-^{14}C] folic acid, potassium salt (58.2 mCi/mmol); and n-[1-^{14}C] hexadecane, benzene solution (50 mCi/mmol). The radiochemical purity of the leucine and folic acid was confirmed by column chromatography (see appendix).

Contaminated glassware was rinsed in tap water and immersed for 48 hours in a solution of Decon 90 (Decon Laboratories Ltd., Brighton) prior to further rinses in tap water and distilled water and then it was dried in an oven at approximately 60°C.

THE EVERTED SAC TECHNIQUE.

The everted intestinal sac technique was introduced by Wilson and Wiseman (1954). The method is described in detail by Wiseman (1961) and is only briefly outlined here.

The animals were killed by a sharp blow on the

neck. The abdomen was opened with a mid-line incision and the intestines moved to one side of the body cavity. The duodenojejunal flexure was located, and the intestine cut between the first and second jejunal arteries. The proximal jejunum was excised, this being considered as the first fifth of the intestine excluding the duodenum, and immediately placed in a trough with oxygenated saline (154 mM NaCl) at 0-4°C.

It was not found necessary to flush out the intestine as little food was ever present in the proximal jejunum and such an action would slow down the experimental procedure. It was also not necessary to tie the intestine to the everting rod prior to eversion as long as 3 cm of everted jejunum from the initially invaginated end of intestine was discarded to avoid the use of damaged intestine. The everting rod was made of surgical quality stainless steel (1.5 mm diameter, 40 cm length) the ends of which were rounded and smooth.

Four sacs of approximately 2.5 cm were made from the intestine of each rat, and the sacs were filled with 0.2 ml of buffer as the initial serosal solution. The buffer used in the everted sac study was Kreb's Ringer phosphate (Umbreit, Burris & Stauffer, 1951) and unless otherwise stated, contained glucose (20 mM). The manipulations of the sacs were performed on filter paper saturated with cold saline. A torsion balance (0-1g range, White Electrical Instrument Co., England) was used to weigh the sacs empty and full both before and after incubation.

The sacs were incubated in 25 ml polypropylene Erlenmeyer flasks (Xlon Products Ltd., London) containing

10 ml of incubation medium at 37°C, and gassed continuously with 100% oxygen. The flasks were maintained at 37°C in a Griffin 100 series (Gallenkamp, Birmingham) shaking incubator running at 100 oscillations per minute (amplitude 3 cm).

One sac was removed at the end of each of the incubation times: 15, 30, 45 and 60 minutes. The final serosal fluid was collected in a test tube and a 50 µl aliquot placed in 10 ml of scintillation cocktail (500 ml Toluene, 250 ml emulsifier and 2.5 g PPO). The vials were counted in a liquid scintillation spectrometer (NE 8305, Nuclear Enterprises, Edinburgh).

The tissue, with the ligatures removed, was then weighed on a pre-weighed boat and both placed in an oven at 90°C to dry overnight. The boats with dry tissue, were then re-weighed and burnt in a biological material oxidiser (Beckman Ltd., Glenrothes, Scotland) and the resulting carbon dioxide was collected in 15 ml of ^{14}C absorber 'P' scintillation cocktail. The oxygen flow rate was kept steady during burning at 300 ml per minute and the apparatus checked frequently with ^{14}C hexadecane although standards of ^{14}C folic acid were counted with each experiment.

CALCULATION OF RESULTS.

The weight of the ligatures was estimated by determining the average values of wet and dry sutures of standard length. These were subtracted from the measurements made on the torsion balance and the following data were obtained: weights of the sac empty and full,

both prior to and after incubation.

From the measurement of the empty sac after incubation on pre-weighed oxidiser boats, the wet weight of the sac and the dry weight of the sac after incubation were obtained.

A computer programme was written to calculate firstly, (figure 3,1) the following results from the original data: initial and final serosal fluid per sac, gut (tissue), serosal and total fluid uptake per sac or mg dry weight, initial wet weight and final dry weight of sac.

The second part of the programme (figure 3,2) took the mean of the background counts for each of the 3 runs on the liquid scintillation spectrometer, and subtracted this figure from the corresponding counts of the standards and experimental aliquots. The mean values of both the standard and experimental data were then calculated.

The final section of the programme (figure 3,3) brought the data from the previous two parts together and included calculations for the uptake of folic acid, expressed as pmoles per mg dry weight into either the serosal or tissue compartment.

The programme was run on a desk computer (Programma 101, British Olivetti Ltd., London). Statistical calculations including standard error of the mean, least squares test and correlation coefficient were also calculated using this computer. Different groups of results were compared using the student's t-test and a statistically significant difference was accepted for

probability levels of $p = < 0.05$.

ABSORPTION STUDIES.

i) Folic acid controls.

In this series, male and female rats were used but no drugs were given to the animals. The everted sac technique was described earlier in this section. The 10 ml of incubation medium contained Kreb's phosphate buffer with 20 mM glucose and folic acid at a concentration of 10^{-6}M. The activity of the radioactive species per 10 ml buffer was 0.1 µCi (specific activity of 2-^{14}C folic acid, 58.2 mCi/mmol). The 0.2 ml initial serosal solution contained phosphate buffer with glucose (20 mM) and no folic acid.

Initially the sacs were made in a latin square order (Snedecor & Cochran, 1967) to establish whether there was any adverse effect caused by the delay in producing, or the order of production of the sacs on their absorptive capacity of folic acid. Subsequently, the order of sac production was constant in that sac 1 (mouth end) was made first and used for the 15 minute incubation and sac 4 (anal end) was made fourth and used for the 60 minute incubation.

In the female control group, prior to the experiment, a vaginal smear was taken from each rat to determine at which stage of the oestrus cycle the rat was undergoing. Young adult female rats have a regular 4 day oestrus cycle (Long & Evans, 1922), each day of which is associated with a characteristic vaginal smear:

pro-oestrus - mostly nucleated epithelial cells,

oestrus - cornified epithelial cells only,

metoestrus - few cornified cells, many leucocytes,

dioestrus - predominantly leucocytes with a few nucleated epithelial cells.

The vaginal smear technique of Zarrow, Yochim and McCarthy (1964) was employed.

ii) Leucine controls.

The everted sacs from male rats were prepared as previously described and incubated in 10 ml Kreb's phosphate buffer with 20 mM glucose and leucine at a concentration of 10^{-6}M. There was 0.1μCi of ^{14}C leucine (specific activity of 10 mCi/mmol) in the incubating medium and the initial serosal solution contained only phosphate buffer with glucose (20 mM).

iii) Replacement of glucose by sucrose.

Male rats were used to make everted sacs which were incubated in phosphate buffer with folic acid (10^{-6}M, 0.1 μCi) in which the glucose had been replaced with sucrose (20 mM).

iv) Sodium depletion of the buffer.

48 hours prior to sacrifice, the rats in this experiment were given double distilled deionised water to drink rather than tap water.

Once each proximal jejunum had been excised, it was placed into ice cold sodium depleted saline solution containing 30 mM sodium ion with osmolarity compensated for by mannitol (normal sodium ion

concentration was 154 mM).

The sacs were incubated in low sodium phosphate buffer (30 mM NaCl) with mannitol (248 mM), glucose (20 mM) and folic acid (10^{-6}M, 0.1μCi) present. The initial serosal solution consisted of the sodium depleted phosphate buffer with mannitol and glucose but no folic acid.

(v) Methotrexate.

A series of experiments were performed with methotrexate. Initially, sacs were incubated in buffer with folic acid (10^{-6}M, 0.μCi), glucose (20 mM) and methotrexate (10^{-6}M).

Intraperitoneal injections of methotrexate were also administered to rats at either 24 or 48 hours before sacrifice. The two doses of methotrexate employed were 10 mg/Kg dissolved in saline, and 40 mg/Kg in suspension in arachis oil. The uptake of folic acid was studied with these doses as was the uptake of leucine at the high dose only.

A single oral dose (10 mg/Kg in water) was also given 24 hours before sacrifice to a group of rats, and the uptake of folic acid was subsequently examined.

(vi) Phenytoin (5,5-diphenylhydantoin).

In the first of the studies with phenytoin, everted sacs were incubated in phosphate buffer, glucose (20 mM), folic acid (10^{-6}M, 0.1 μCi) and also phenytoin (10^{-6}M).

A group of rats was also given the equivalent human dose of phenytoin (10 mg/Kg body wt/day) intra-muscularily in arachis oil for 2 weeks prior to

their sacrifice. The uptake of folic acid into sacs from these animals was then observed.

vii) Alcohol.

An acute experiment was performed whereby everted sacs were incubated in buffer, glucose, folic acid and 3% (v/v) alcohol.

A second experiment involved chronic exposure of the rats to 20% (v/v) alcohol in their drinking water for 3 weeks. The fluid intake was recorded during the three weeks of exposure to alcohol. The 6 animals in this study were kept in the same cage and the amount of solution consumed from the one water bottle was observed. The total body weight and the liver weight of each animal was also noted.

viii) Diabetogenic agents.

Streptozotocin is a specific β cytotoxic agent (Rakieten, Rakieten & Nadkarni, 1963) which rapidly brings about a diabetic state as a result of one injection. The streptozotocin was dissolved in 0.1M citrate buffer at pH 4.5 and immediately injected intraperitoneally at a dose of 70 mg/Kg body weight. The rats were used in a study of folic acid uptake into everted sacs 3 weeks after the treatment.

Cortisone is an insulin antagonist (Fajans & Conn, 1956). A dose of 40 mg/Kg body weight was administered daily to a group of rats for 3 weeks. The vehicle was arachis oil and the injections were intraperitoneal. The everted sacs from these animals were used to observe folic acid uptake.

One of the diagnostic procedures for diabetes mellitus is the glucose tolerance test. This test provides a profile of the glycaemic alterations in response to a specific glucose challenge and is used to estimate the rate of glucose entry into and/or removal from the blood (Duffy, Phillips & Pellegrin, 1973). Intraperitoneal glucose tolerance tests were performed 48 hours before sacrifice on both groups of diabetic animals and on a group of normal controls. Blood samples were collected from the rats immediately before, and exactly 30, 60 and 120 minutes after an intraperitoneal injection of glucose (2g/Kg body weight) in a 40% (w/v) solution at 37°C. The samples were obtained from the rats by tail-tip amputation (Grice, 1964). The rat was introduced into a cylindrical plastic restrainer with the tail protruding. The extreme tip of the tail was removed with a sharp scalpel blade and blood was 'milked' drop-wise from the wound by applying gentle pressure with a downwards motion of the hand over the tail. The samples were collected into small plastic tubes that had been prewashed with a solution of saline heparin (80 U/ml). The samples were centrifuged and the plasma separated. Two 20 µl aliquots of plasma were obtained, one was used for the assay of glucose and the other for the assay of insulin. The samples were stored at -20°C until the time of analysis.

The plasma glucose was determined by the glucose oxidase method using a Beckman analyser (Beckman Instruments Ltd., Glenrothes, Scotland). 10 µl of plasma

was inserted in the analyser where, in a solution containing the enzyme glucose oxidase the following reaction took place:

$$\text{Glucose} + O_2 \xrightarrow[H_2O]{\text{glucose oxidase}} \text{Glucuronic acid} + H_2O_2$$

An oxygen electrode measured the disappearance of oxygen and this was translated into the equivalent glucose concentration. The analyser was standardised using the glucose reagent supplied by Beckman.

Plasma insulin was determined by radioimmunoassay using rat insulin as standard. Free and antibody bound hormone moieties were separated by modification of the charcoal technique using horse serum and dextran coated charcoal (Albano, Ekins, Maritz & Turner, 1972). The radioactivity associated with the charcoal pellet was counted in a well-type crystal scintillation counter (ICN Tracer Lab Gamma Set, 500). A fully automated and computerised radioimmunoassay data processing system (Flatt & Thornburn, 1978) was employed for the analysis of the results. The assay had a detection limit of 1 pg/ml and interassay coefficient of variation was in the region of 2 to 5% over the observed concentration range.

ix) Pregnancy.

A group of female rats were made pregnant. For two days each pair of females was left with one adult male, and on the second day the male was exchanged with

another in case the first was sterile. The gestation period in the rat is 21 to 23 days (Porter, 1966) and the rats were killed 18 days after the introduction to the first male. An uptake study of folic acid was made with the everted sacs from these animals.

x) Oral contraceptive hormone.

One of the oral contraceptives used by women is the synthetic hormone ethynyl oestradiol (17α-ethynyl-1,3,5-estratrien-3,17β-diol). The equivalent dose was given to a group of female rats (2 μg/Kg body wt/ day) dissolved in 0.1 ml of arachis oil. The subcutaneous route of injection was employed since this reduces the rate of entry of the hormone into the bloodstream. The rats were injected daily with ethynyl oestradiol for 3 weeks after which they were used in a study of folic acid uptake.

xi) Ovariectomy.

The surgical procedures for the ovariectomy operations were carried out with the maximum of asepsis possible. The operations were performed in an operating theatre using a temperature controlled (37°C) stainless steel operating table and a standard Boyle's apparatus (British Oxygen Co. Ltd., London) fitted with a miniature face mask. Anaesthesia was induced by inhalation of a gaseous mixture of 80% nitrous oxide and 20% oxygen containing 3.5% (v/v) halothane (Fluothane, ICI Ltd., Macclesfield). Anaesthesia was maintained with 1 to 1.5% (v/v) halothane in the same gaseous mixture. The

instruments were sterilized in a mixture of 5% hibitane (ICI Ltd.) and 70% methyl alcohol 2:5 v/v, and rinsed in a solution of sterilized saline (Steriflex, Vestric Ltd., Brierley Hill) before contact with the animal tissues. The operation site was washed with hibitane-methyl alcohol and sterilized saline before and after surgery. To minimise post-operative infection the animals were placed in clean cages bedded with absorbent paper.

The bilateral ovariectomy was performed according to D'Amour, Blood and Belden (1969). The animal was anaesthetized and the operation sites were shaved and washed. The abdomen was opened by a small (1 cm) vertical incision in the mid-lateral region of the abdomen. By exploration of the exposed abdominal cavity, the ovary could be identified as a bright red globular mass embedded in much fat. The fallopian tube and associated fat and blood vessels were doubly ligated (size 0000 polyamide suture, Armour Pharmaceutical Co. Ltd., Eastbourne), and the tissue was transected between the two ligatures. The ovary was removed and the remaining tissue returned into the abdomen. The body wall was closed with standard surgical clips.

Four weeks after the operation, the rats were sacrificed and the proximal jejunum of each animal was used to observe the transport of folic acid.

xii) Hormone replacement therapy.

Oestradiol(1,3,5(10)-estratriene-3,17β-diol), is one of the hormones given to menopausal women receiving replacement therapy. The equivalent dose to women was

given to a group of ovariectomised rats (10 μg/Kg body weight/day). The vehicle was arachis oil and the subcutaneous route was chosen to delay the entry of the hormone into the blood stream.

HISTOLOGICAL STUDIES.

From each of the above experiments, the proximal jejunum was set aside from one animal for a histological evaluation of the glycocalyx. The method used was as described in chapter 2 section 2 and the periodic acid-Schiff staining procedure was employed.

Section 3. RESULTS.

ABSORPTION STUDIES.

The calculation of the results was described in the previous section of this chapter. The transport of folic acid has been expressed as:tissue uptake, that which has entered into the tissue and is there at a specific time; serosal uptake, folic acid which has entered into the serosal compartment and has therefore passed through the tissue; and total uptake which is the total folic acid taken up from the mucosal (bathing) solution, i.e. the summation of the tissue and serosal uptakes.

Values for the rate of uptake, and uptake at time zero (intercept on the ordinate) were obtained from theoretical estimations derived from the best fit line as estimated by the least squares test.

i) Folic acid controls.

There was no significant difference in the absorptive capacities of the four sacs prepared from each proximal jejunum. Accordingly there was no necessity to use a latin square régime in subsequent experiments.

Figure 3,4a illustrates the time course of the transport of folic acid into everted sacs of jejunum. Each point represents the mean and standard error of the number of animals indicated. The regression coefficients of the total, tissue and serosal uptakes were all significant. Backwards extrapolation of the uptake on the ordinate showed an apparently immediate total and tissue uptake, but a delayed entry of folic acid into the serosal compartment

until 12 minutes after the start of incubation. The rate of uptake of folic acid into the serosa was slower than into the tissue (0.06 and 0.18 p. moles/mg dry weight/min respectively).

The initial mucosal folic acid concentration in all the experiments was 10^{-6}M. The final concentration in the tissue after 60 minutes was 2.11 x 10^{-6}M and in the serosal compartment, 0.69 x 10^{-6}M. Thus, there had been a relative accumulation of folic acid in the tissue but not in the serosal compartment.

Figure 3,4b illustrates the uptake of fluid into everted sacs. The total and serosal uptakes were linear ($P = < 0.01$), but the tissue uptake was not significantly linear ($P = > 0.05$). Hence over 60 minutes, fluid was continually entering the serosal compartment but in the tissue, the maximal amount of fluid was taken up after 30 minutes of incubation.

With a mucosal bathing concentration of folic acid at 10^{-6}M, the highest concentration of folic acid available for transport by solvent drag with the fluid was 1 pmole/µl. By assuming this, the minimum folic acid transported by mechanisms other than with the fluid may be calculated and are shown in table 3,3. Further evidence that folic acid was not transported entirely by solvent drag, was provided by the differences in immediate uptake and rate of uptake over 60 minutes of both folic acid and fluid. The difference in immediate uptake was particularly obvious with the serosal transfer of folic acid and fluid, where the folic acid was not observed in the serosal compartment until 12 minutes after the start

of incubation and yet the increase in fluid was immediate, as shown in figure 3,4. The rate of uptake of folic acid over 60 minutes was always more rapid than that of fluid uptake; this may also be seen from figure 3,4.

The female rats proved to be in different stages of the oestrus cycle as determined by vaginal smears. Of the group of 5, 2 were in prooestrus, 2 in dioestrus and 1 in metoestrus. The rats also exhibited a wide variation in their capacity to transport folic acid as shown by the standard errors of the mean results (figure 3,5a and table 3,4). Further details concerning the uptake of folic acid are shown in table 3,5.

The female total uptake of folic acid was increased after 60 minutes incubation when compared with those results obtained for the males. The main component of this increase was a marked serosal uptake of folic acid; tissue uptake was slightly depressed. The rates of total and tissue folic acid uptake were unaltered. Female serosal uptake was slightly faster but not as linear as with the males although the actual time of appearance of folic acid in the serosal compartment was approximately 13 minutes with both sexes.

The serosal fluid uptake and folic acid uptake by mechanisms other than solvent drag were greater than in the males, although this did not achieve statistical significance. Hence the significant increase in serosal folic acid uptake may have been due to a combination of these 2 factors.

ii) Leucine controls.

The time dependent uptake of leucine, and the associated regression coefficients are shown in figure 3,6a. Uptake into both compartments was linear with time; serosal transfer not being observed until 6 minutes after the start of incubation. The initial mucosal bathing concentration of leucine was 10^{-6}M. After 60 minutes, the serosal concentration was 4.24 x 10^{-6}M indicating that an active process had been functioning to transfer the leucine across the tissue and into the serosal compartment.

The fluid uptake (figure 3,6b) was not as linear as the leucine uptake, and reached a maximum rate in the tissue after 30 minutes but serosal fluid transfer was still linear after 60 minutes incubation.

iii) Glucose replacement by sucrose.

Substituting 20 mM sucrose in the buffer instead of glucose resulted in a significant depression in tissue folic acid uptake (table 3,6). There was no other significant difference in folic acid uptake although the serosal uptake was slightly raised. This was reflected in the final concentration of folic acid in the serosal compartment (0.82 x 10^{-6}M) being higher than with the control group (0.69 x 10^{-6}M). Although the total (1.47 x 10^{-6}M) and tissue (1.97 x 10^{-6}M) concentrations were lower than the control group (1.54 x 10^{-6}M and 2.11 x 10^{-6}M respectively).

Neither fluid transfer or folic acid transfer by mechanisms other than solvent drag were significantly

altered by the replacement of glucose with sucrose, although both showed an overall depression.

iv) Sodium depletion.

The uptake of folic acid was significantly depressed when sodium was depleted from the incubating medium. Figure 3,7 shows the uptake of folic acid into everted sacs presented in the form of a histogram. The P values demonstrate the predominantly significant decrease in uptake compared with the control group.

The effect of sodium depletion on the uptake of fluid by everted sacs is presented in table 3,7. Total fluid uptake was reduced although this was entirely accounted for by reduced serosal fluid transfer as the tissue uptake was unaltered. Hence the depression in tissue folic acid uptake cannot be accounted for by reduced tissue fluid uptake. This is substantiated by comparison of the folic acid transported by mechanisms other than solvent drag where tissue folic acid uptake was significantly reduced compared with the control group. However, assuming that solvent drag does occur, the serosal folic acid uptake was unaffected by sodium depletion, implying that the observed depression in figure 3,7 of serosal folic acid transfer was due to the decreased serosal fluid transfer.

Thus it appears that in everted sacs incubated in sodium depleted buffer, tissue folic acid uptake was to some extent, independent of fluid uptake, whereas serosal folic acid uptake was dependent on fluid uptake.

v) Methotrexate.

Incubating everted sacs in the presence of 10^{-6}M methotrexate resulted in no significant alteration to the serosal or total uptakes of folic acid or fluid. Although the final concentrations of folic acid were depressed in these compartments (1.46 x 10^{-6}M and 0.68 x 10^{-6}M respectively) compared with the control values (1.54 x 10^{-6}M and 0.69 x 10^{-6}M).

Tissue folic acid transfer was significantly depressed after 45 minutes of incubation ($P = <0.05$); this was reflected in reduced concentration of folic acid after 60 minutes incubation (2.01 x 10^{-6}M against a control value of 2.11 x 10^{-6}M). Although the importance of fluid transfer in this depression could not be accounted for since neither tissue fluid uptake nor folic acid transfer associated with mechanisms other than solvent drag were significantly altered.

When an intraperitoneal low dose (10 mg/Kg) of methotrexate was given 24 hours before sacrifice, a marked increase was observed in the tissue uptake of folic acid, particularly in the earlier stages of incubation (table 3,8). The immediate tissue uptake of folic acid, derived from backwards extrapolation of the slope on the ordinate, was also greater than the control value (5.95 and 3.89 p. moles/mg respectively). It might appear that the increased folic acid transfer resulted from the increased fluid transfer, i.e. solvent drag; although this may be partly true, other transport mechanisms must have been affected by the methotrexate because assuming

solvent drag was occurring, the minimum folic acid transported was still greater with the methotrexate treatment after 30 minutes ($P = <0.01$).

The serosal transfer of folic acid and fluid was also slightly increased by the methotrexate treatment although this was not significantly altered from the control results. The overall effect of the intraperitoneal methotrexate on the total folic acid transfer was to increase the amount transported, with most of this effect resulting from increased tissue uptake with some degree of the associated increase in fluid uptake.

Similar to the 10 mg/Kg dose of methotrexate given 24 hours prior to sacrifice, a dose given 48 hours before death resulted in an increase in total folic acid transport (after 60 minutes, $P = <0.01$). However, whereas the 24 hour dose increased total uptake as a result of enhanced tissue uptake, the main component of the overall increase with the 48 hour dose was the increased serosal uptake. Neither tissue folic acid nor fluid uptakes were significantly different from the control group although the values were higher than the control results.

Table 3,9 shows the results for the serosal folic acid and fluid uptakes. There was an increase in folic acid transport at all the times investigated, this was also accompanied by a small increase in fluid transfer. Thus, the increase in folic acid uptake cannot be accounted for by increased solvent drag but another mechanism of folic acid transfer must have been affected

by the methotrexate. This is substantiated by a significant increase in the minimum amount of folic acid transferred corrected for fluid movement after 60 minutes ($P = < 0.01$).

A large dose of methotrexate (40 mg/Kg) administered by the intraperitoneal route 48 hours before death caused a dramatic effect on the uptake of folic acid (figure 3,8a) reducing the rate of tissue uptake to virtually zero at 0.005 p.moles/mg dry wt/min. After 60 minutes the folic acid in the tissue amounted to only 4% of that in the control tissue (table 3,10).

The amount of folic acid entering the serosal compartment was not significantly altered (table 3,10) although the time of entry was retarded to 16 minutes (figure 3,8a) from the control time of 12 minutes (figure 3,4a). The overall effect of the high dose of methotrexate on the total transfer was a significant reduction in folic acid uptake.

In accordance with the reduced tissue folic acid uptake, tissue fluid uptake was also depressed, as shown in table 3,10. Although the tissue fluid was only reduced by 22% whereas the tissue folic acid was depressed by 96%. Maximal tissue fluid was taken up by 15 minutes (figure 3,8b), to a lower value than with the controls (1.41 and 2.03 mg/dry wt respectively) and this remained virtually unaltered throughout the rest of the incubation period.

Whereas the high dose of methotrexate was without effect on the serosal folic acid transfer, the fluid uptake into the serosal compartment was markedly increased

(table 3,10). With the serosal fluid increased, and the tissue fluid decreased, the overall total effect was not different from the control total fluid transfer.

It is important to observe any effects which the drug may have on the animal, indeed with the 40 mg/Kg intraperitoneal dose of methotrexate, the stomach and intestines appeared markedly distended and filled with fluid although the general appearance and behaviour of the animals was not affected. Similar abnormalities, especially fragility of the intestines, were also observed with a much lower dose (10 mg/Kg) given by the oral route.

With the oral 10 mg/Kg dose of methotrexate given 24 hours prior to death, the total transfer of folic acid was decreased and the total fluid transfer was increased (table 3,11). The effect on the total folic acid transfer was caused by a considerable depression in tissue uptake which was not accompanied by a depression in fluid transfer. The rate of folic acid uptake was also reduced (0.01 p. moles/mg dry wt/min) as shown in figure 3,9a.

Serosal folic acid transfer was increased along with an increase in serosal fluid transfer (table 3,11), the latter of which gave rise to an increase in total fluid transfer. Appearance of folic acid in the serosal compartment at 10 minutes (figure 3,9a) was slightly more rapid than the 12 minutes observed in the control group and the rate of uptake, 0.16 pmoles/mg dry wt/min, was also more rapid than the control rate of 0.06 p. moles/ mg dry wt/min. But the ratio of serosal folic acid to

serosal fluid after 60 minutes was 1.24×10^{-6}M which is similar to the control value of 1.25×10^{-6}M. Thus, in spite of increased serosal rate of uptake and amount of uptake, a similarity still remained between the methotrexate treated rats and control rats, such that the ratio of serosal folic acid to fluid was unaltered; whereas the tissue ratios were markedly altered (MTX = 0.29×10^{-6}M, control = 2.44×10^{-6}M) with an overall effect of depression of folic acid uptake.

To summarize the rather complex effects of methotrexate on the intestinal transport of folic acid, figure 3,10 shows the significant alterations from the control values of the folic acid and fluid uptakes. The data given is for a 60 minute period of incubation.

The most pronounced effects of methotrexate were on the tissue uptake of folic acid with the oral dose (10 mg/Kg, 24h) and the intraperitoneal high dose (40 mg/Kg,48h), where in both cases the uptake was markedly depressed without an equal depression of fluid uptake. Incubation of intestine with 10^{-6}M methotrexate also caused tissue depression of folic acid uptake with an insignificant depression of tissue fluid uptake. Methotrexate did not in any instance cause an increase in tissue folic acid uptake.

Serosal quantities of folic acid were increased with the oral dose which paralleled the increased fluid transfer in this case. The intraperitoneal low dose (10 mg/Kg, 48h) also showed an increased serosal folic acid uptake but with an insignificant increase of fluid

transfer. However, there was no associated serosal folic acid transfer with the raised fluid values for the intraperitoneal high dose (40 mg/Kg, 48h). In no instance did methotrexate decrease serosal folic acid transfer.

The total transfer of folic acid was depressed with the oral dose (10 mg/Kg, 24h) and the intraperitoneal high dose (40 mg/Kg, 48h) as a result of depression of tissue folic acid uptake, and the total transfer of folic acid was increased with the intraperitoneal low dose (10 mg/Kg, 48h) as a result of the increased serosal folic acid uptake.

A single intraperitoneal high dose of methotrexate (40 mg/Kg) given 48 hours prior to sacrifice did not alter the uptake of leucine (table 3,12) from the control leucine results described earlier in this section. The final serosal concentration of leucine with the methotrexate treated animal tissue, was $3.21 \pm 0.84 \times 10^{-6}$M. This was not significantly different from the control value of $4.24 \pm 0.26 \times 10^{-6}$M where in both cases the initial mucosal concentration of leucine was 10^{-6}M.

The rate of uptake of leucine into the tissue was no longer linear following the methotrexate (table 3,13). The tissue also took up a lesser amount of fluid after 60 minutes compared with the control leucine experiment ($P = < 0.01$); this observation was also made with a similar dose of methotrexate and folic acid uptake as shown in figure 3,10. Although the serosal fluid was also increased with the leucine and methotrexate,

this was not a significant difference from the leucine control group.

Following correction for solvent drag into the tissue, leucine uptake was unaltered from the controls ($P = > 0.05$). It would therefore seem that the reduced rate of entry of leucine into the tissue might have been as a result of the depressed tissue fluid uptake caused by this dose of methotrexate.

vi) Phenytoin.

Incubating everted sacs in buffer with 10^{-6}M phenytoin resulted in an increase in total folic acid uptake which was caused by the marked increase in serosal uptake (table 3,14). Serosal fluid was also increased above the control value and this was responsible for the raised values of folic acid transfer because when the results were corrected for solvent drag, there was no difference in folic acid transfer from the control group.

Intra-muscular injections of phenytoin for a period of 2 weeks prior to death, did not significantly alter the uptake of folic acid or fluid into everted sacs although the total folic acid transfer was reduced by 12% and the total fluid transfer by 21%.

vii) Alcohol.

Acute exposure of everted sacs to 3% alcohol in the incubation medium did not result in a significant change of folic acid transport despite the mean values

of uptake being lower than normal. Although the serosal fluid was also unaffected by the alcohol treatment, the tissue fluid was markedly increased in uptake. Table 3,15 shows the tissue folic acid values and the corresponding increased fluid values.

The group of rats which had undergone chronic exposure to 20% alcohol in the drinking water for 3 weeks, appeared at the end of the period to have reduced physical activity with a staggered gait and rough, dry fur. The mean body weight of these animals was 219g with the control group being 250g. At the time of death, the livers were removed and weighed. The appearance of the alcohol exposed liver was paler in colour, smaller and weighed 40% less than normal. The decrease in the weight of the liver contributed to 13% of the overall body weight loss. During the period of exposure to alcohol, fluid intake was reduced from the control value of 33g per day to 18g per day. This level of consumption was maintained throughout the course of study and the rats were therefore drinking 4.5g alcohol per day corresponding to approximately 21g/Kg body weight.

The exposure of rats to chronic alcohol ingestion had a marked effect on increasing the uptake of folic acid into everted sacs (table 3,16). Fluid uptake was also increased (table 3,16) but solvent drag was not solely responsible for the increased folic acid uptake since when the tissue results were corrected for this, a significant difference remained, implying that

an additional mechanism had also increased the folic acid transfer ($P = <0.02$). Solvent drag could, to some extent, explain the increased serosal folic acid transfer as here the corrected difference was not significant from the controls ($P = >0.05$).

viii) Diabetogenic agents.

As illustrated in table 3,17, the animals which received streptozotocin and cortisone treatment exhibited various alterations with respect to body weight and both fluid and food intakes. The greatest effect was observed with the streptozotocin treated animals whose body weight was markedly reduced whilst the fluid and food intake increased. The fed plasma glucose concentration of the streptozotocin treated group was approximately four times greater than the normal value whilst the fed plasma insulin concentration was almost zero. The insulin level of the cortisone treated rats was greatly increased whereas the plasma glucose level was not reduced despite the magnitude of the prevailing insulin concentration.

The presence of a diabetic state in both groups of animals was further substantiated by a glucose tolerance test (figure 3,11). Following a glucose stimulus, the concentration of glucose in the plasma of the streptozotocin diabetic animals was raised to a high level indicating the lack of insulin availability. The high level of plasma glucose decreased gradually; most likely as a result of increased excretion in the urine, particularly as the rats showed obvious polyuria.

The failure of glucose to evoke an insulin secretory response in these animals confirmed the total lack of pancreatic β cell function.

In the cortisone treated animals, the plasma insulin concentration was greatly increased following a glucose challenge whereas the glucose level was not reduced to normal. Thus although the β cells were functionally intact and hyperactive, there was a state of insulin resistance in which the normal hypoglycaemic action of insulin was greatly reduced.

It is noteworthy that at the time of the everted sac study, in situ the stomach and intestine of the streptozotocin treated animals appeared markedly distended and filled with fluid.

Streptozotocin and cortisone both caused a depression in the total transfer of folic acid into everted sacs, although the greatest depression was observed with cortisone, these results were not significant. The effects of these drugs on tissue transfer and serosal transfer of folic acid and fluid are shown in table 3,18. The depression of folic acid uptake into the tissue by cortisone was significant. Although the tissue fluid was not significantly depressed by cortisone, the folic acid transported by mechanisms other than solvent drag was significantly decreased. The tissue uptakes of folic acid&fluid were also reduced with the streptozotocin treatment, but the data were not significant.

Both treatments caused a decrease in serosal folic acid and fluid transfer. With the streptozotocin

treated animals, the folic acid transported following correction for solvent drag, was very low at 0.05 pmoles, implying that nearly all of the folic acid transported into the serosal compartment must have been associated with solvent drag.

ix - xii) Female rat studies.

The results of the total uptake of folic acid and fluid into everted sacs from female rats after various treatments are shown in table 3,19. The 4 treatments of ethynyl oestradiol (oral contraceptive hormone), oestradiol (replacement therapy hormone), pregnancy and ovariectomy reduced the amount of folic acid entering into everted sacs although none to any significant extent. The effects of the treatments on the fluid uptake was variable, most reducing fluid uptake, except oestradiol which slightly increased fluid uptake. Correcting the total uptake results for solvent drag, showed the ovariectomy treatment to have a much lower uptake than the controls and ethynyl oestradiol a slightly enhanced uptake. The standard errors of the control group were larger than those of other groups and this may have prevented any significance arising.

The tissue and serosal folic acid uptakes, which constitute the total uptake, are shown in figures 3,12 and 3,13. Of the tissue uptake, the folic acid transfer was depressed, except with the ethynyl oestradiol treatment which was slightly increased. The ovariectomised animals in particular,

exhibited a marked depression in folic acid uptake. Fluid uptake into the tissue was mostly increased, especially during pregnancy, however the ethynyl oestradiol treated group again showed exception with a slight depression of fluid uptake. Transfer of folic acid by mechanisms other than solvent drag was generally depressed, particularly with the ovariectomy treatment although a slight increase was shown with the ethynyl oestradiol treatment.

Of the serosal uptakes (figure 3,13), all treatments without exeption decreased the values of folic acid and fluid transfer. Large standard deviations prevented any significant differences from becoming apparent; this was especially obvious with the folic acid transported by mechanisms other than solvent drag where the ovariectomy treatment was considerably reduced but not significantly different from the control group.

Indeed, the ovariectomised animals exhibited the greatest differences from the control group with the administration of oestradiol as replacement therapy, shifting the results of the ovariectomised group towards normal. Although significant differences were not apparent in this study it should be remembered that the treatments employed were strictly physiological and that a slight depression in uptake of folic acid over a long period of time might result in a state of folate deficiency. Ovariectomy was employed to produce a condition similar to the human menopausal state and the low doses of hormone were corrected to suit the

experimental animal employed.

HISTOLOGICAL STUDIES.

The appearance of the glycocalyx in normal rat jejunum (plate 35) was similar to that in the human, as previously described in chapter 2. However, in the rat, this continuous glycoprotein layer attached to the brush border did not appear quite as distinct, or as thick, as it did in the human biopsies. After 60 minutes incubation, the glycocalyx was still clear on the everted jejunum (plate 36). No obvious tissue disruption was associated with the time of incubation although a larger amount of oedematus tissue and a slight alteration in the shape of the nuclei was noted.

The many treatments, described earlier in this chapter, did not produce any gross alterations in the appearance of the glycocalyx; certainly there was no disruption similar to that observed with some of the diseased biopsies reported in chapter 2. However, minor subjective observations were made on the histological sections and a brief account of some of these is given here, particularly of those treatments which affected folic acid transport.

Depletion of sodium in the incubation medium resulted in a tissue section which showed a brush border which did not quite have a normal appearance (plate 37). The brush border appeared to be thin and the glycocalyx was indistinct.

Methotrexate at an intraperitoneal dose of 40 mg/Kg, 48 hours prior to death, had the greatest

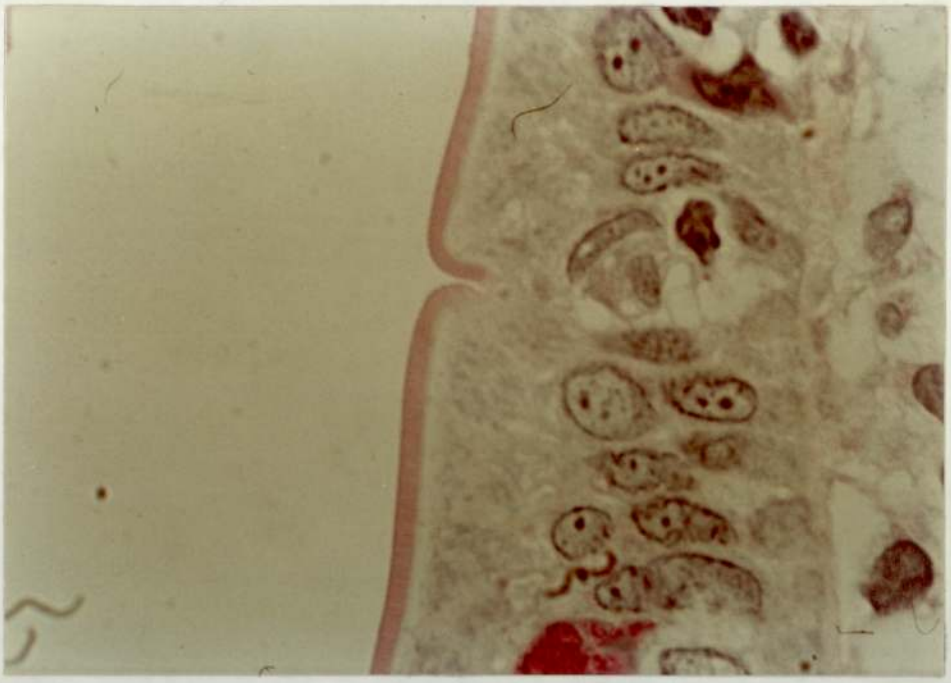


Plate 35. Rat jejunum.

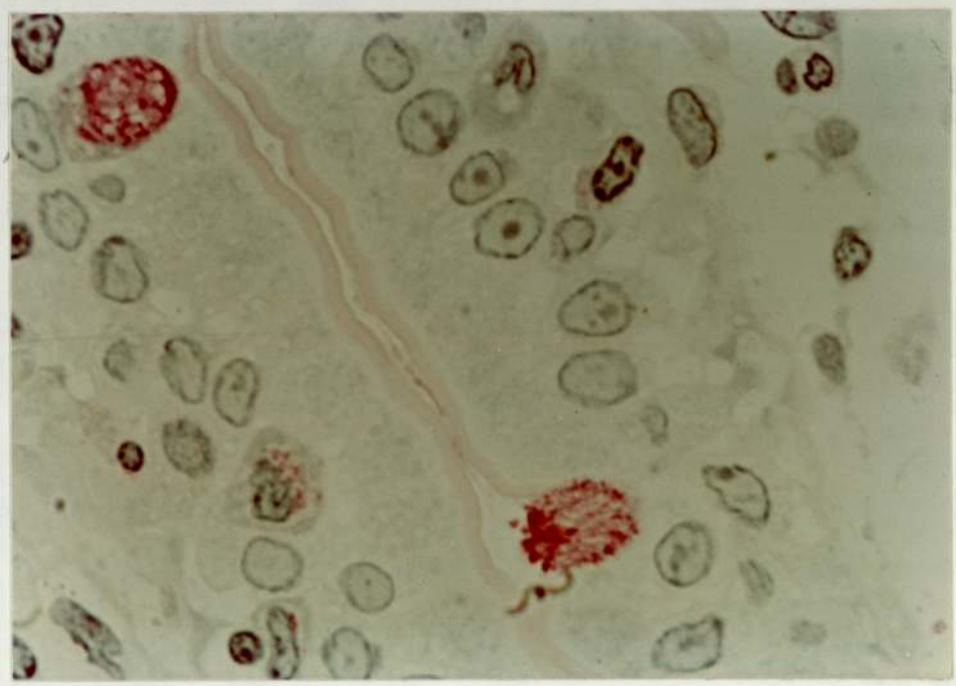


Plate 36. Everted rat jejunum after 60 minutes incubation in Kreb's phosphate buffer.

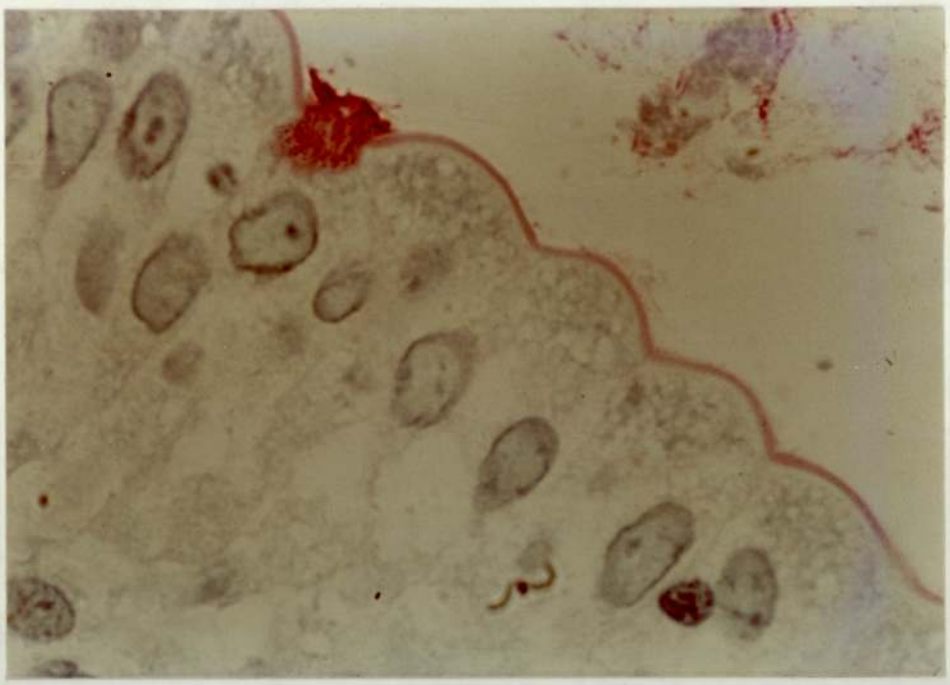


Plate 37. Everted rat jejunum, depletion of sodium from the incubation medium.

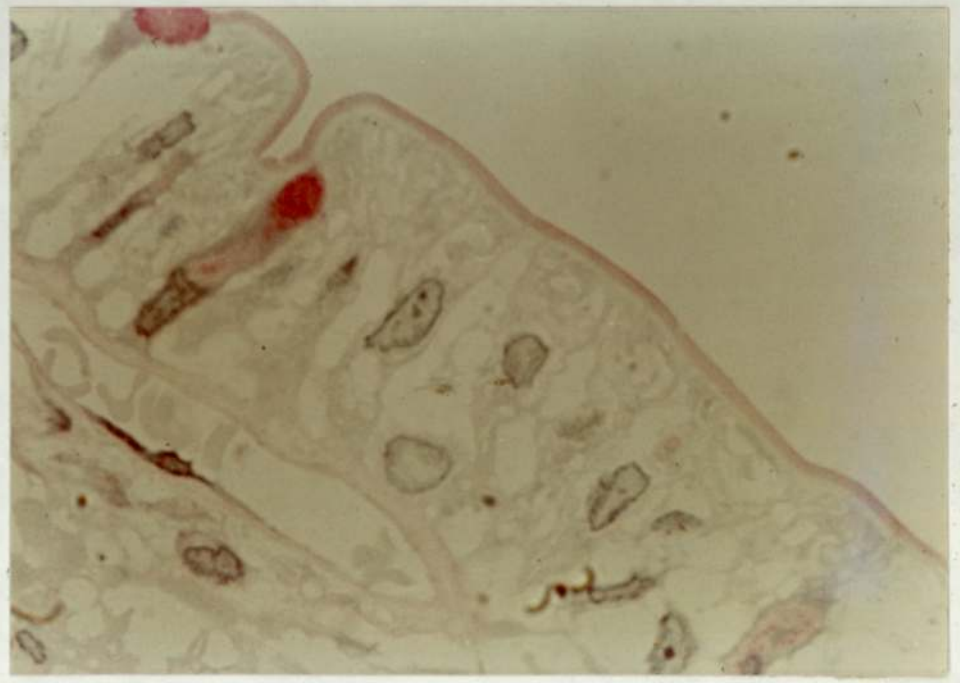


Plate 38. Everted rat jejunum, intraperitoneal methotrexate (40 mg/Kg, 48h).

effect of all the treatments on folic acid transfer, reducing the total uptake by 74% after 60 minutes incubation. Although the gross appearance of the villi was stunted, the glycocalyx was still visible (plate 38) but was paler and less distinct than in normals. The plate shows the tissue prior to incubation; the epithelial cells, apart from the glycocalyx, appear to be changed, especially the shape and position of the nuclei and the organisation of the columnar cells.

An oral dose of methotrexate (10 mg/Kg) resulted in a tissue appearance (plate 39), which was pale compared with the control tissue. Prior to incubation the villi were stunted and there was a notable lack of cell types present in the lamina propria e.g. plasma cells and lymphocytes. The glycocalyx was not readily visible and the brush border was thin.

Although without having the gross effect of folic acid transport of the above mentioned methotrexate treatments, incubating tissue for 60 minutes in methotrexate at a concentration of 10^{-6}M (plate 40), resulted in a brush border appearance which was more intensely stained than that of the control material. The glycocalyx was occasionally visible.

The tissue sections resulting from the incubation of an everted sac in the presence of 10^{-6}M phenytoin for 60 minutes (plate 41), showed markedly enlarged interepithelial cell spaces compared with the control tissue. This could be associated with the

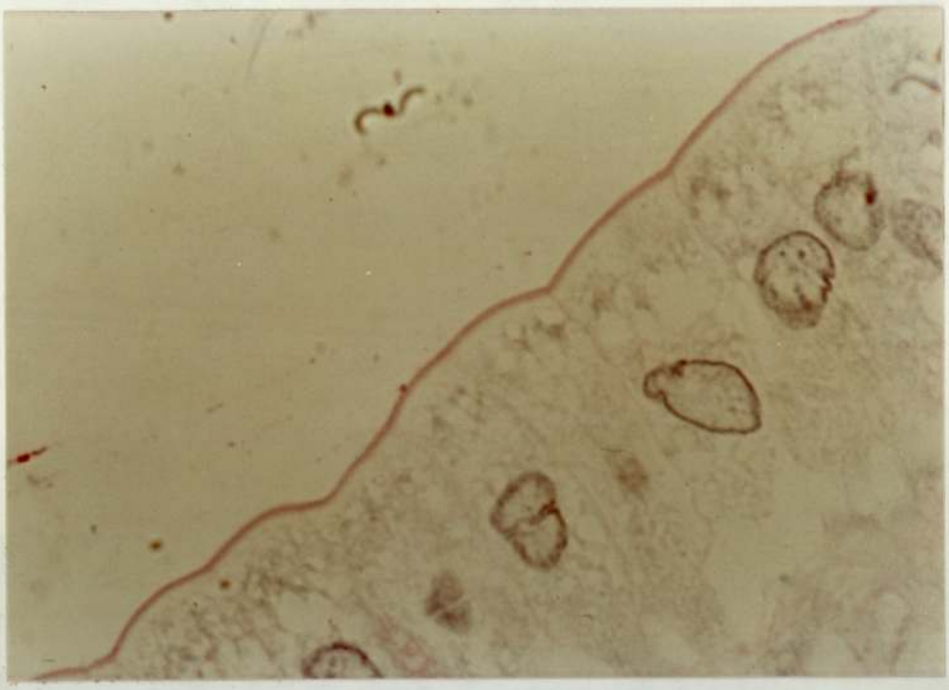


Plate 39. Everted rat jejunum, oral methotrexate (10 mg/Kg, 24h).

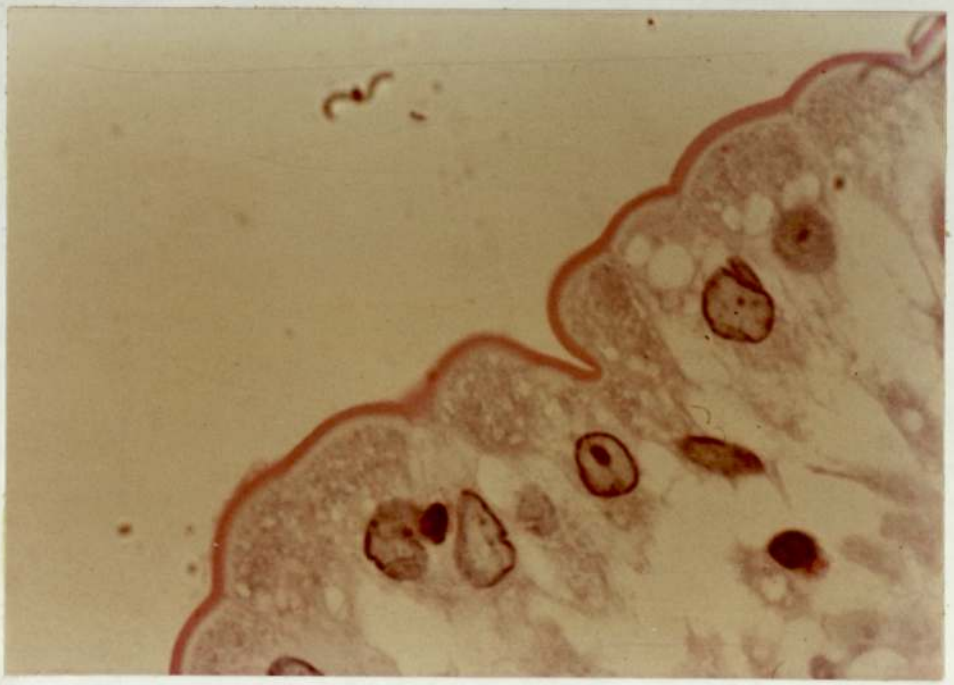


Plate 40. Everted rat jejunum, 10^{-6}M methotrexate in the incubation medium.

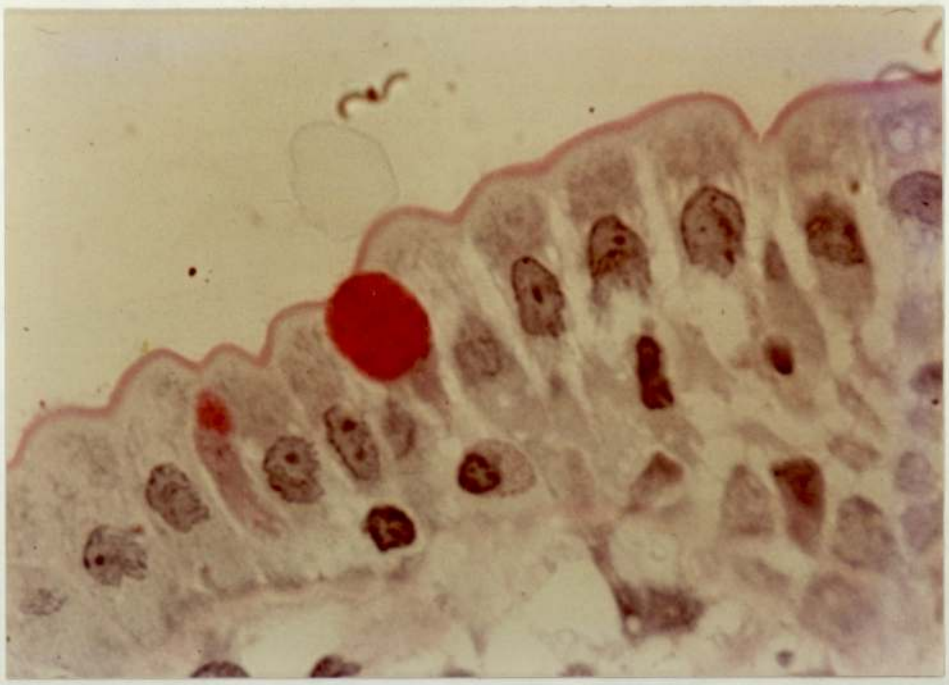


Plate 41. Everted rat jejunum, 10^{-6}M phenytoin in the incubation medium.

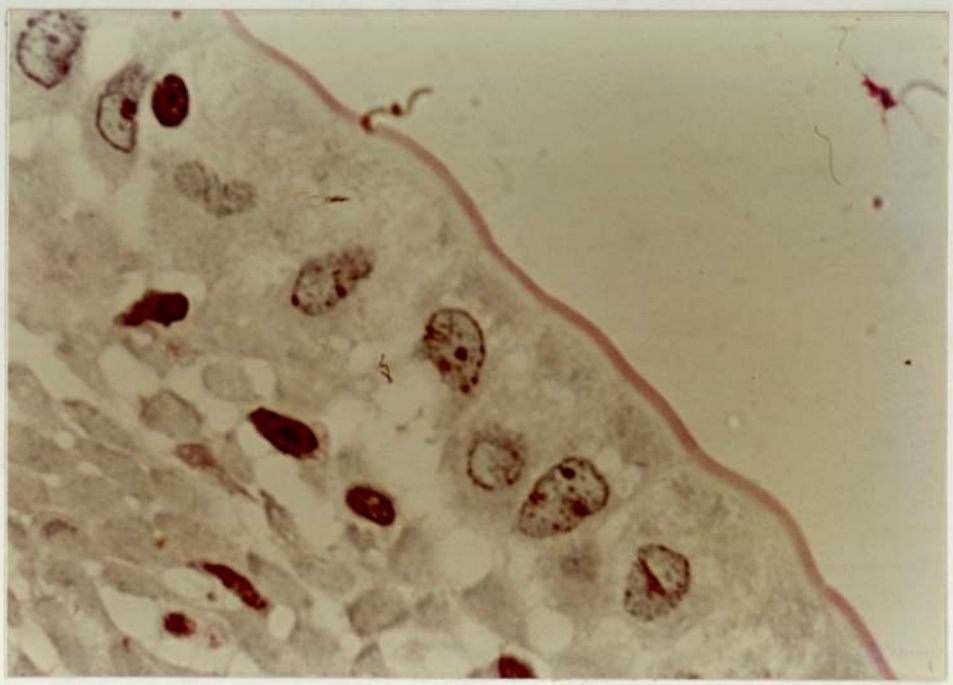


Plate 42. Everted rat jejunum, chronic exposure to 20% alcohol.

highly significant increase in fluid transfer into the serosal compartment which was observed with this treatment.

Exposure of rats to chronic alcohol ingestion (plate 42), provided tissue sections which did not appear very different from control tissue, although the distinction of staining between the brush border and glycocalyx was not as clear as with the control material, Acute exposure of tissue to alcohol (plate 43) resulted in a pale appearance of the glycocalyx with the nuclei slightly disrupted.

Streptozotocin treatment (plate 44) resulted in the villi appearing longer than those of the normal rat. The glycocalyx was pale but was still present on the brush border. Cortisone treatment (plate 45) produced the appearance of a thick brush border which stained intensely and on the luminal surface of this, the glycocalyx was continuous.

The female treatments provided no alterations to histological appearance of the intestine. The untreated female was no different to the male, and the tissue from the ovariectomised animals (plate 46), which showed the greatest effect of the female treatments on folic acid transfer, still supported a glycocalyx and intensely stained brush border.

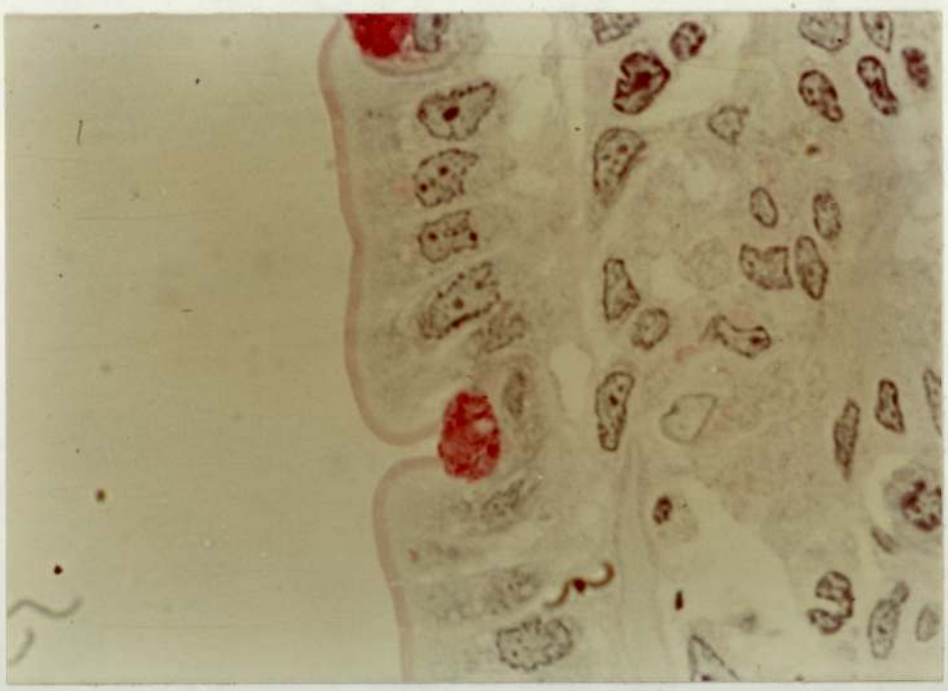


Plate 43. Everted rat jejunum, acute exposure to 3% alcohol in the incubation medium.

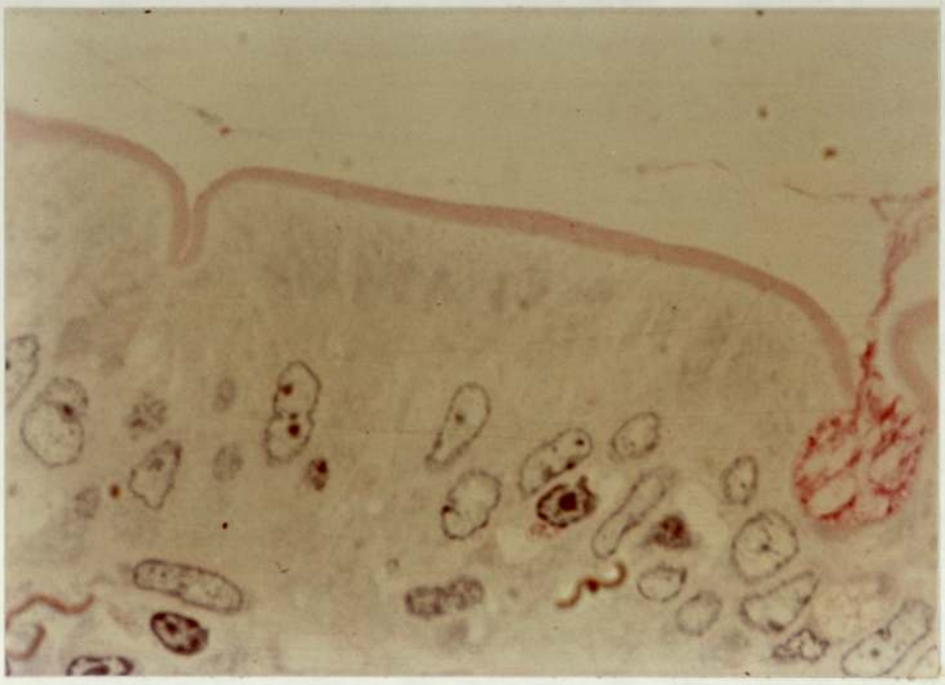


Plate 44. Everted rat jejunum, streptozotocin diabetes.

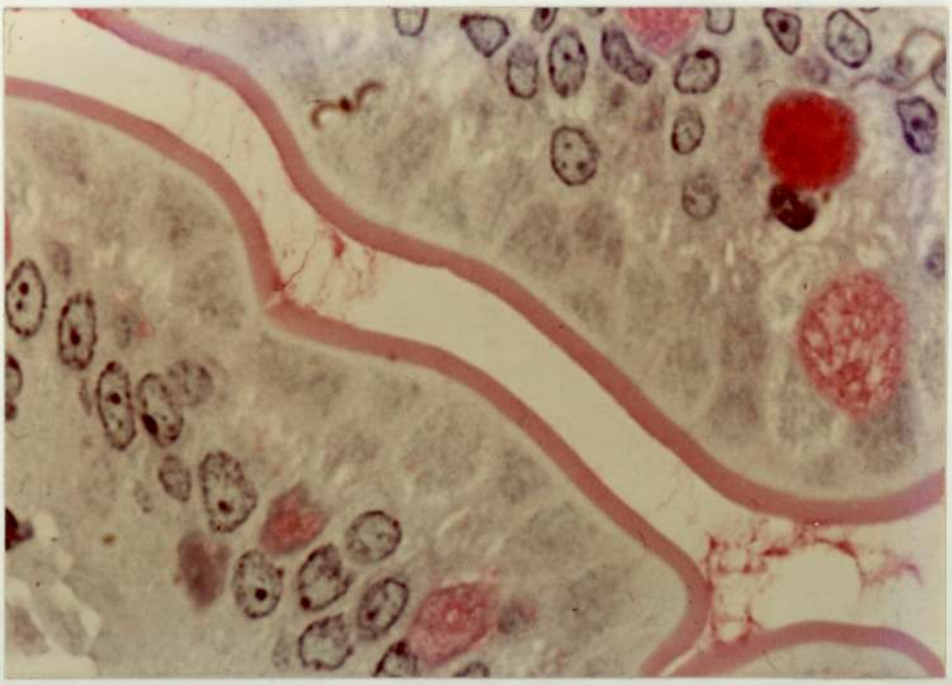


Plate 45. Everted rat jejunum, cortisone diabetes.

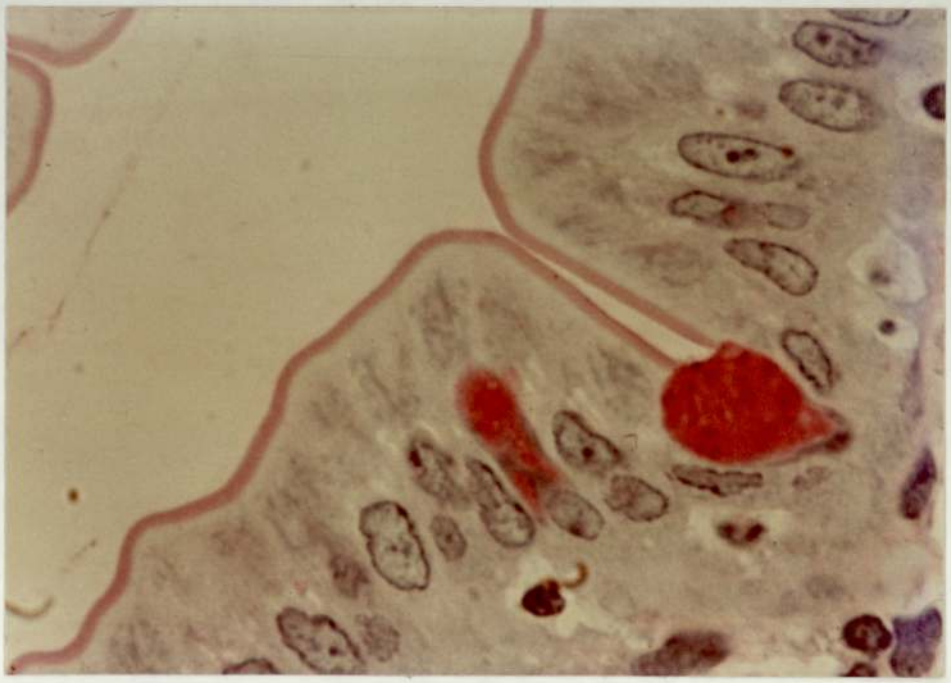


Plate 46. Everted rat jejunum, ovariectomy.

Section 4. DISCUSSION.

In vitro intestinal preparations have been used for many years although their use has particularly flourished since the 1950's. Leading up to this period was the work by Fisher and Parsons (1949) which described one of the basic requirements of a viable tissue preparation: adequate mucosal oxygenation. The everted sac technique was exploited by Wilson and Wiseman (1954) and the primary virtue of this technique is undoubtedly its simplicity. Many other methods exist for the investigation of intestinal absorption; the methodology, interpretation and history of these and the everted sac technique have been reviewed (Wilson, 1962; Levin, 1967; Parsons, 1968; Smyth, 1974).

A criticism of *in vitro* techniques is that they may bear little relationship to the normal *in vivo* absorption process, particularly as the intestine is devoid of its normal blood supply. Hence the products of transfer from the mucosal solution (that which would be the intestinal lumen *in vivo*) appear in the serosal fluid instead of the blood. With the everted sac technique, the small volume of serosal fluid may then be directly examined for the presence of the substance under investigation.

The size of the sacs used in the original Wilson and Wiseman experiments were 2-3 cm in length and in the present study, sacs of approximately 2.5 cm were employed. The importance of the degree of

distention has been pointed out by Wilson (1956), and in the work presented here, sufficient fluid was inserted into the sac so as to distend it and separate the villi. It was found that with the proximal jejunum, 0.2 ml fluid per sac was most suitable and obviously allowed for the additional uptake of fluid during incubation. A bubble of oxygen was not introduced into the sac as this has been considered unnecessary (Wilson, 1962).

When glucose is omitted from the mucosal solution, the intestine noticeably deteriorates during the period of incubation (Turner, 1959, Bhanthumnavin, Wright & Halsted, 1974) and the importance of glucose for fluid transfer, particularly in the jejunum, has been observed (Barry, Matthews & Smyth, 1961). Adequate oxygenation of the everted sac preparation has been shown to be important in the absorption of glucose and fluid; with anaerobic conditions, no active transport occurs, and fluid is lost from the everted sac (Wilson & Wiseman, 1954). In the present study, the mucosal solution was gassed gently with 100% oxygen. A mixture of oxygen/carbon dioxide was not used as a certain amount of the carbon dioxide would dissolve to make the bathing solution more acid over the period of incubation.

It is imperative with a study involving the use of an _in vitro_ technique, that evidence is presented to establish the viability of the tissue. The ability of the intestine to transport fluid was the initial indicator of viability in this study. The mucosal

fluid transfer in the control experiments was 0.04 mg/mg dry wt/min, this is consistent with the original data obtained by Wilson and Wiseman (1954). The serosal fluid uptake rose constantly with time to a value of 2.44 mg/mg dry wt after 60 minutes whereas the tissue uptake reached a saturation value of 2.40 mg/mg dry wt after about 30 minutes. A similar observation was also made by Blair *et al.*(1975a). It is likely that the tissue has a maximum capacity for the volume of fluid which it can retain.

The second indicator of viability in this study was the ability of the everted sacs to concentrate leucine within the serosal fluid, as leucine is transported by an active mechanism (Wiseman, 1974). The everted sacs had an ability to transport leucine at a rate of 1.55 p.moles/mg dry wt/min. The ratio of the final serosal concentration to the initial mucosal concentration was 4; this is in agreement with the results obtained at a similar concentration by Larsen and colleagues (1964).

Uptake of folic acid into everted sacs was linear with time. There was a rapid tissue uptake which might imply an immediate binding of folic acid onto specific binding sites in the brush border (Leslie & Rowe, 1973); the aspect of rapid uptake of folic acid onto the epithelial cells is considered in greater detail in chapter 4. Not surprisingly, the rapid uptake was not observed in the serosal compartment as the folic acid had initially to traverse the tissue to reach the serosa, a process which took approximately

12 minutes.

The rate of folic acid uptake into the tissue was 0.18 pmoles/mg dry wt/min and the rate into the serosa was 0.06 pmoles/mg dry wt/min. Folic acid was accumulated in the tissue to a concentration of 2.11×10^{-6}M after 60 minutes. Therefore, it appears that there were at least three components involved in the mechanism of folic acid transport; an initial rapid binding stage, an accumulation process within the tissue, and a slower transfer of folic acid from the tissue into the serosal compartment. The observation that some form of accumulation occurred within the intestine is consistent with previous reports (Selhub et al., 1973; Smith, 1973; Blair et al., 1974).

Although one may assume at least the above components to be involved in folic acid transport, the importance of pH in the absorption of folic acid cannot be overlooked. The acid microclimate has a maximum value in the rat jejunum and becomes more alkaline in the ileum (Lucas & Blair, 1978); this is coexistent with the rate of folic acid transport being greater in the jejunum than in more distal regions of the small intestine (Smith et al., 1970). Blair and Matty (1974) proposed that the acid microclimate might influence folate transport in the small intestine, as was discussed in section 1 of this chapter.

In vitro measurements of the acid microclimate of rat proximal jejunum have shown the pH to be 5.7, significantly more acid than the buffer pH of 7.08(Lei,

Lucas & Blair, 1977). In the absence of glucose, the acid microclimate became markedly more alkaline (Lucas & Blair, 1978), clearly demonstrating that glucose was necessary for the maintenance of acid surface pH values. Indeed the model for acidification of the microclimate proposed by Blair et al.(1975), involving the hydrolysis of ATP at the mucosal surface, has shown the necessity for glucose. In the study presented here, when the glucose in the incubation medium was replaced with sucrose, there was a marked reduction in the tissue uptake of folic acid. This depression could not be ascribed to reduced fluid transfer or solvent drag but parallels the observation of reduced acidity of the microclimate following the omission of glucose from the buffer (Lucas & Blair, 1978). However, the sucrose would be hydrolyzed at the surface of the epithelial cells by the enzyme sucrase, to glucose and fructose which are liberated in equal amounts and are readily absorbed when derived from sucrose in vitro (Davidson & Leese, 1977). Thus there would have been a small amount of glucose present, but this cannot have been sufficient for maintenance of the acid microclimate, and consequently, folic acid uptake into the tissue was reduced.

There is close agreement with several of the other studies reported here and alterations of the pH of the microclimate (Lucas, Swanston, Lei, Mangkornthong & Blair, 1978). Details of the precise nature of this correlation are presented in chapter 5. Depletion of

sodium from the buffer resulted in an overall depression in folic acid transfer. The reduced tissue folic acid uptake was not associated with reduced fluid uptake; there was a significant depression in the tissue folic acid uptake by mechanisms other than solvent drag. The pH of the acid microclimate has been shown to be significantly less acidic when tissue was incubated in sodium depleted buffer (Lei, et al., 1977). Since depletion of sodium from the incubation medium decreases glucose transfer in vitro (Faust, 1962), the reduced acidity of the microclimate probably reflects a decrease in availability of glucose, which as has been described above, affects the pH of the microclimate. Thus, tissue uptake of folic acid was reduced directly as a result of decreased acidity of the microclimate.

The serosal uptake of folic acid in sodium depleted buffer, was directly associated with the serosal fluid uptake and a depression was observed in both cases. Fluid movement is therefore important in the serosal transfer of folic acid, a process which is slower than the initial uptake of folic acid into tissue, and does not lead to accumulation within the serosal compartment. The dependence of serosal folic acid transfer on fluid uptake into the serosal compartment is however in contrast to the fluid independent uptake of folic acid into the tissue.

Water transport is closely linked to sodium and total solute transport (Curran & Schwartz, 1960), and fluid absorption may be regarded as a passive consequence of net solute movement (Curran, 1968).

The basic mechanism of water absorption involves the extrusion of sodium from the epithelial cells into the intercellular spaces, giving rise to a concentration gradient; water movement across the cell from the lumen is consequently driven by the increased concentration of sodium. Edmonds and Pilcher (1972) observed a clear relationship between the rates of sodium absorption and water absorption in the human colon *in vivo*. Decreased sodium concentration in a dialysis bag resulted in decreased water absorption. However, another hypothesis has been proposed which attributes water absorption to glucose, which creates a gradient for the flow of water through the tight junction of the epithelial cells; water then carries sodium by solvent drag (Fordtran, Rector & Carter, 1968). In either model, it is clear that sodium is intimately involved in fluid absorption. The relationship of sodium and fluid absorption have been extensively reviewed by Sidorov (1976) and Binder (1977). In the present study, depletion of sodium from the buffer caused a depression in fluid transfer, and no doubt affected other metabolic processes of the intestine such as reduced oxygen consumption as demonstrated by Levin and Syme (1975).

Methotrexate (4-amino-4deoxy-10-methyl-pteroylglutamic acid) is a folate analogue which is used therapeutically as a folic acid antagonist (Holland, 1958). The most widely acknowledged action of methotrexate is to act as an inhibitor of folic acid metabolism by irreversibly binding with the enzyme

dihydrofolate reductase (Osborn, Freeman & Huennekens, 1968), which is necessary for the reduction of folic acid to the metabolically active tetrahydrofolate form. However, a recently established action of methotrexate concerns the alteration to the pH of the acid microclimate (Lei et al., 1977). Correlation of the acidity of the jejunal microclimate and folic acid uptake following methotrexate treatment has recently been made (Lucas et al., 1978) and includes two of the conditions discussed here. When tissue was incubated in buffer supplemented with 10^{-6}M methotrexate, there was a significant depression in tissue folic acid uptake; measurement of the microclimate under similar conditions revealed a small reduction in its acidity. The total uptake of folic acid was also reduced and the reduced acidity of the microclimate could explain the depressed folic acid transfer. Other authors have also reported reduced tissue folic acid uptake without significant total depression under similar in vitro conditions (Selhub et al., 1973).

In contrast, when the oral dose of methotrexate (10 mg/Kg, 24h) was given to a group of animals, the total and tissue folic acid uptakes were both markedly depressed whereas there was an increase in serosal uptake. In a complementary study of microclimate measurements with this oral dose, the acid microclimate had become significantly more alkaline. Thus the folic acid would not be able to enter into the tissue via the microclimate. However, the level of folic acid was significantly increased in the serosal compartment, an observation

also made by Olinger and colleagues (Olinger et al., 1973). The transport of folic acid into the serosa may have been by solvent drag, not through the cell, but between the epithelial cells via the terminal bar, into the intercellular space with free passage to the serosal compartment. Indeed, such a mechanism might be facilitated by the lack of glycocalyx at the lateral borders of epithelial cells (Rao et al., 1972), particularly as the glycocalyx may be the retaining layer for the acid microclimate (Blair & Matty, 1974). Alternatively, folic acid may have entered the tissue, and if there are specific folate binding sites within the cell, these may have been saturated by the methotrexate and thus the folic acid would pass through by diffusion into the serosal compartment. Indeed, the tissue might have been damaged morphologically so that it was no longer viable and therefore folic acid would not be taken up by the preparation. However, a histological examination of the tissue showed no evidence of severe damage although the glycocalyx was not readily visible and this may have been associated with the altered microclimate values. Even when some morphological damage was produced, using doses of methotrexate greater than that used here, active absorption of amino acids still persisted (Robinson et al., 1966).

A severe reduction in folic acid transfer was observed with a high dose of methotrexate (40 mg/Kg) administered by the intraperitoneal route 48 hours before death. Although concomitant microclimate

measurements were not performed in this experiment, the tissue was shown to actively transport leucine and was therefore still viable. The histological appearance of the tissue showed mild cellular abnormalities and stunted villi but no severe morphological damage. The effect of this 40 mg/Kg dose on folic acid transfer was as severe as a much lower 10 mg/Kg oral dose. Obviously the effect of the single oral dose was predominantly due to direct contact with the intestinal mucosal surface, although a single dose does not produce the severe mucosal damage which may be caused by daily administration of an oral low (0.6 mg/Kg) dose (Jolly & Fletcher, 1977). Contact with the mucosal surface would allow the methotrexate to directly affect the microclimate. However, the question is raised as to how an intraperitoneal dose might affect the microclimate, particularly if altered microclimate values are presented as the cause for the depressed folate transport. Intraperitoneal methotrexate would enter the blood circulation, via the blood supply to the tissues in the intraperitoneal cavity. In the liver, methotrexate would enter the bile, where it is known to concentrate (Strum & Liem, 1976), and from there it would pass into the intestinal lumen. Folic acid is also known to be removed from the blood by the liver and secreted in the bile at a higher concentration than that in the serum (Baker, Kumar & Swaminathan, 1965). The flow rate of bile in the rat is virtually constant over 24 hours (Bailey, Flatt, Atkins & Matty, 1976) and following a dose of

methotrexate 48 hours before death, some of the methotrexate would be secreted in the bile and thereby make contact with the intestinal mucosal surface. By this mechanism, a dose of intraperitoneal methotrexate may have affected the acid microclimate and thus have reduced folic acid transfer.

Another hypothesis for the action of methotrexate, particularly if the intestinal mucosal surface was directly affected, might involve the saturation of binding sites present in the brush border (Leslie & Rowe, 1972). With both the oral dose (10 mg/Kg, 24h) and intraperitoneal high dose (40 mg/Kg, 48h) of methotrexate, the immediate uptake of folic acid was markedly reduced and the rate of entry into the tissue retarded compared with the controls. This might suggest the existence of preoccupied binding sites which would, in the absence of methotrexate, become occupied by folic acid at the initiation of the incubation period.

The time course of methotrexate action appears to be complex, although this may be expected as methotrexate is an inhibitor of DNA synthesis (Altmann, 1974) affecting cells particularly in the S phase of the cell cycle (Weinstein, 1977). The effect of subcutaneous methotrexate on the rat intestine has been studied during the first 36 hours following treatment (Altmann, 1974). The crypts diminished in size although the crypt cells still migrated onto the villi. Since methotrexate inhibits mitosis the villus cell population would consist increasingly of immature cells,

and as discussed in chapter 2, these cells would have incomplete glycocalyces. If the glycocalyx is the retaining layer for the acid microclimate, an incomplete glycocalyx would result in abnormal microclimate pH values. Indeed, in the histological sections of methotrexate treated rat intestine, the glycocalyx did appear paler than normal and not so readily distinguished although it was not markedly disrupted.

Following a low dose (10 mg/Kg) of methotrexate, given by the intraperitoneal route 24 hours prior to death, tissue folic acid was significantly increased with no effect on serosal uptake. Whereas 48 hours after the same dose, there was a marked increase in serosal folic acid uptake with no effect on tissue uptake. These changes in folic acid uptake could not be attributed to increased solvent drag, but one may attempt to explain the mechanism of methotrexate action in terms of binding sites within the cell. After 24 hours, methotrexate may have been bound to intracellular sites, and the exposure of the tissue to folic acid would result in its uptake via the acid microclimate, which at this low dose might have been unaffected. Once inside the cell, the folic acid would cause an efflux of methotrexate from the binding sites and consequently a greater quantity of folic acid would enter the cell. Since methotrexate has been reported to stimulate folic acid efflux from pre-loaded jejunal sacs (Blair et al., 1976) then due to the similar chemical structure of the compounds, the reverse might be feasible. However, even if the immediate increase

in tissue folic acid was due to a stimulated efflux, the effect was not irreversible, since after 60 minutes of incubation the increase was no longer significant.

Following the intraperitoneal low dose (10 mg/Kg) of methotrexate administered 48 hours prior to death, folic acid entered the serosal compartment but did not accumulate in the tissue. The folic acid may still have diffused into the epithelial cells via the microclimate, but did not displace the methotrexate from binding sites as with the previous methotrexate dose (10 mg/Kg, 24h), since in this instance the methotrexate had had an additional period of time to accumulate inside the cells. Hence, there may have been a greater quantity of methotrexate within the cells after 48 hours. The concentration of folic acid may not have been sufficient to cause a net efflux of the bound methotrexate from the tissue and thus the folic acid progressed into the serosal compartment.

The histological sections of the methotrexate treated tissue showed the glycocalyx was not disrupted but its appearance was paler than the control tissue. Thus the anomalies of folic acid transfer cannot be explained in terms of an abnormal glycocalyx. However, those associated microclimate values which were available, showed a high degree of involvement of the acid microclimate in the varied results of folic acid absorption following methotrexate treatment. Obviously the effects of methotrexate on folic acid absorption are complex; important factors are the magnitude of dose, route of administration and time of treatment prior to death.

Nevertheless, the functional state of the microclimate and the processes necessary for its unimpaired function are also prominent features which may succumb to the methotrexate treatment.

Phenytoin, at a concentration of 10^{-6}M in the buffer, caused an increase in the total uptake of folic acid; this appeared to result from solvent drag associated with the large increase in fluid transfer into the serosal compartment. The histological appearance of this tissue showed marked enlargement of the intercellular epithelial spaces. Similar enlargement of intercellular spaces has been observed in gall bladder epithelium both in vivo and in vitro (Kaye, Wheeler, Whitlock & Lane, 1966; Tormey & Diamond, 1967). Diamond and Bossert (1967) suggested that the ultrastructural geometry of the intercellular spaces was important for solvent drag. Indeed, when the uptake was corrected for solvent drag, there was no difference in the folic acid transfer of everted sacs incubated in the presence and absence of phenytoin. Microclimate values were available for the present study, and these showed that there was a slight increase in acidity corresponding to the increased folic acid uptake. Although the change in pH was slight, the direction of altered pH from control was such that more folic acid would be transported into the cell. Phenytoin imparts an alkaline pH in solution, but the concentration in the buffer was sufficiently low not to appreciably change the pH. At higher concentrations, the alkaline solution would depress the uptake of folic

acid as has been demonstrated by Benn et al. (1971). Phenytoin is not generally associated with an increase in folic acid transfer, but with a malabsorption condition which has been attributed to the rise in jejunal pH produced by phenytoin under clinical conditions (Elsborg, 1974b). Indeed, when phenytoin was administered to rats by intramuscular injection, a slight depression in folic acid uptake was observed.

The role of anti-convulsants in the production of a folate deficient state in epileptics remains controversial (Rosenberg, 1972; Norris & Pratt, 1974; Nutrition reviews, 1974). The depressed serum folate levels may only occur in patients who have been receiving phenytoin therapy for a number of years (Klipstein, 1964), although other authors have observed subnormal serum folate levels after the onset of treatment (Dahlke & Mertens-Roesler, 1967). It has also been reported that in persons with normal folate levels, phenytoin was unable to depress the intestinal absorption of folic acid (Perry & Chanarin, 1972), but folate absorption may also be normal whilst serum folate levels are reduced in patients receiving therapy (Shafer & Nuttall, 1975).

The glycocalyx appeared relatively indistinguishable from the brush border following either incubation of tissue with phenytoin or the intramuscular injection of the drug into rats, however, no inference could be drawn from this observation. Further work is required to elucidate the mechanism of action of phenytoin

induced folate deficiency; from the present study it is evident that the effect is ambiguous.

Acute exposure of rat intestine to alcohol caused a slight depression in folic acid transport and a considerable reduction in tissue fluid uptake. Following acute exposure of either rat or hamster jejunum to alcohol, oxygen consumption and the transport of glucose, amino acids and water were reduced (Chang, Lewis & Glazko, 1967; Baraona, Pirola & Lieber, 1974; Dinda, Beck, Beck & McElligott, 1975; Dinda & Beck, 1977). These authors propose that active transport processes are inhibited by alcohol, and as the net transport of water depends on the active transport of solutes (Curran, 1965), water uptake is therefore depressed following acute exposure to alcohol. Microclimate values for the acute alcohol treated tissue (Lucas et al., 1978) show a decreased acidity and this correlates with the depressed folic acid uptake observed in the present study.

Rats exposed to chronic ingestion of alcohol did not show the same gain in weight as the control rats throughout the period of treatment; this observation is consistent with the data of Wang, Marvin, Abel and Pierson (1976). The alcohol treated rats also consumed less fluid than the control group, this would no doubt result in dehydration, particularly when combined with the diuretic effect of alcohol (Zeballos, Basulto, Munoz & Salinas-Zeballos, 1976). This dehydration would contribute to reduced body weight; weight loss of the liver was responsible for 13% of the total weight reduction. The liver is particularly affected by

alcohol (Lieber, 1975) although at autopsy of alcoholic patients, it has been observed that the heart is affected to a greater extent, with numerous other organs showing lesions (Corrigan, 1976).

Inadequate diet appears to be of importance in chronic alcoholics in causing a folate deficient state (Halsted et al., 1971; Mezey, 1975) although the absorption of vitamin B12 has been impaired following chronic alcohol administration in the absence of nutritional deficiency (Lindenbaum & Lieber, 1975). The chronic exposure of rats to alcohol resulted in an increased uptake of folic acid and fluid into everted sacs. As the tissue would be dehydrated following a period of reduced fluid consumption, it is not unexpected that fluid uptake was enhanced. The tissue, once excised from the rats, was incubated in alcohol-free buffer and therefore the alcohol previously located at the intestinal surface would be washed away, thereby alleviating the inhibitory effect on fluid uptake. Chang and colleagues (1967) have reported a reversal of the inhibitory effects of alcohol on phenylalanine transport following a 10 minute wash in alcohol-free buffer. 30 minutes following washing, there was an increased uptake of phenylalanine above normal; this was described as being an overshoot. Increased folic acid absorption in the present study was not entirely attributable to solvent drag in spite of increased fluid uptake. Thus, there may have been an overshoot of the folic acid absorption following depressed uptake during the period of chronic

exposure to alcohol. Alternatively the microclimate may have been more acid during incubation. Indeed, complimentary microclimate measurements (Lucas et al., 1978) have shown that the microclimate was slightly more acid than normal and hence may have contributed to the increased folic acid uptake.

Morphological damage to intestinal tissue caused by alcohol has been reported (Baraona et al., 1974; Dinda et al., 1975), but in the present study no marked injury was observed with either the acute or chronic alcohol conditions. It has been reported that severe chronic alcohol ingestion results in a population of immature epithelial cells (Baraona et al., 1974); as discussed in chapter 2, immature cells would have an incomplete glycocalyx, and the pH of the microclimate might be altered resulting in folate malabsorption. This could be the mechanism of alcohol induced folate deficiency observed in alcoholics.

The two drugs, methotrexate and phenytoin, are administered for therapeutic purposes, and alcohol is readily consumed by humans; diabetes mellitus, a disease characterised by a relative or absolute deficiency of insulin, occurs in both humans and animals (Meier, 1960; Hunt, Lindsey & Walkley, 1976) and has been associated with intestinal morphological abnormalities (Cabarrou et al., 1975) which could precipitate a condition of malabsorption. Diabetes has been found in association with coeliac disease (Thain et al., 1974) which itself is readily associated with malabsorption. Indeed, diabetes occurs more

often in coeliac disease than would be expected by chance (Walsh et al., 1977; Cooper et al., 1978).

Streptozotocin exerts a direct cytotoxic action on the β cells of the pancreatic islets of Langerhans and is occasionally used to treat insulinomas. It is also commonly employed to produce an animal model for the experimental study of diabetes, where only a single injection is required to produce permanent diabetes (Brosky & Logothetopoulos, 1969). Cortisone is a steroid hormone frequently used in pharmaceutical preparations which can, under certain conditions, produce a diabetic state as a result of its insulin antagonistic actions (Fajans & Conn, 1954; Perley & Kipnis, 1966).

There is increasing interest in the effect of hormones on the gastrointestinal tract (Levin, 1969), particularly those produced by the intestine, which is rapidly becoming considered as an important endocrine organ in its own right (Buchanan, 1976; Bloom, 1977; Johnson, 1977). The role of gastrointestinal hormones in the aetiology of diabetes has still to be elucidated although there appears to be a close relationship (Buchanan, 1975). In the normal state, gastrointestinal hormones including glucagon, secretin, pentagastrin, cholecystokinin, gastric inhibitory polypeptide and vasoactive intestinal polypeptide have been implicated in the absorption of fluid and electrolytes (Binder, 1977).

The absolute and relative actions of

streptozotocin and cortisone on the β cells of the pancreatic islets and glucose homeostasis, were confirmed in the present study. Streptozotocin treatment resulted in a complete lack of glucose induced insulin secretory activity and severe hyperglycaemia; cortisone treatment resulted in the production of hyperinsulinaemia and moderate hyperglycaemia, indicative of a state of insulin resistance which would eventually lead to β cell exhaustion. Such complimentary assays confirmed that a diabetic state had been induced in the rats.

The fluid consumption was increased following both the streptozotocin and cortisone treatments, but particularly with the streptozotocin, in response to the very high concentration of glucose in the blood. Increased fluid intake and polyuria are characteristic signs of severe diabetes in human patients.

The rats in both groups exhibited hyperphagia; this may have been partly in response to the deficient action of insulin stimulating a hunger response, but particularly in the streptozotocin treated animals, the villi were seen to be longer in the histological sections, thus providing a larger area for absorption. It has been shown that the lengthening of villi following streptozotocin treatment is a result of enhanced cell proliferation (Lorenz-Meyer, Thiel, Menge, Gottesbüren & Riecken, 1977). Enteroglucagon secreting tumours have also been shown to increase villus length (Gleeson, Bloom, Polak, Henry & Dowling, 1971) and one of the postulated physiological rôles of gastrin is

the control of growth in the mucosa of the small intestine (Grossman, 1976; Bloom, 1977). As epithelial cell growth would be affected in these instances, it is feasible to assume that the glycocalyx would also be affected, as it is produced by the cell. Increased activities of several brush border enzymes have also been observed in streptozotocin diabetic rats (Lorenz-Meyer et al., 1977; Olsen & Kormso, 1977) and in human diabetics (Caspary, Winckler, Lankisch & Creutzfeldt, 1975). Body weight was reduced in the diabetic rats despite the increased food intake although this is common in experimental models of diabetes (Anderson, 1974; Nakabou, Okita, Takamo & Hagihara, 1974).

Malabsorption of folates is not generally associated with diabetes although this has occasionally been reported (Doig & Girdwood, 1960; Davis et al., 1976). In the present study, folic acid uptake was depressed in both the conditions although not significantly with the streptozotocin treatment. The cortisone treatment resulted in a depression of tissue folic acid uptake which was due to a mechanism other than solvent drag as the fluid uptake was not significantly affected with either of the diabetogenic agents. The histological sections of the cortisone treated rat jejunum showed a considerable intensity in staining of the brush border with a relatively clear glycocalyx. This intensity may have been associated with the depressed folic acid uptake; however, it would be more likely that the intense

staining implied a thicker brush border and glycocalyx, and hence a larger area for retention of the acid microclimate. The effect of cortisone on folic acid uptake may have resulted from direct contact with the drug, or by the increased amount of insulin, particularly as this is secreted directly into the lumen via the pancreatic and bile ducts (Bailey et al., 1976). As has been mentioned above, numberous hormones are affected in diabetes (Buchanan, 1975), several of which are intimately associated with the gastrointestinal tract; whether any of these were involved in the inhibition of folic acid uptake cannot be ascertained from the present study. At least the intestinal morphology was not severely disrupted with either treatment, hence this could not have been the cause of the reduced uptake. Thus the depression could indeed have been the result of altered hormone action.

Recent interest has been focused on the investigation of the hormone levels of patients with treated and untreated coeliac disease (Besterman et al., 1978; Walsh et al., 1978). In these studies, the untreated coeliac patients, probably folate deficient by nature of their disease, exhibited abnormalities of glucose homeostasis and diminished insulin secretory responses following the ingestion of either an oral glucose load or a standard test breakfast, indicative of a diabetic or near diabetic state. The untreated patients also displayed paradoxical increases of pancreatic glucagon, together with grossly elevated enteroglucagon levels and markedly diminished gastric

inhibitory polypeptide responses. Treated coeliac patients on gluten-free diets showed slightly improved insulin secretory responses, suppressed pancreatic glucagon concentrations and marginally increased enteroglucagon levels but the gastric inhibitory polypeptide responses remained similar to those observed in untreated patients. Such evidence that treated coeliacs are not normal is in accordance with the finding presented in chapter 2 that the glycocalyx of treated coeliacs was still altered in appearance although to a lesser degree than that of the untreated patients. The folate levels of the treated coeliac patients, whose glycocalyces were not yet normal, also showed low serum folate values. Hence the interrelationship of altered hormone levels, the presence of a disease state, appearance of the glycocalyx and depressed serum folate levels is an intricate problem.

To investigate further the possible involvement of hormones in the uptake of folic acid, various studies were performed on female rats. Female rats differ from males in that they have a regular 4-day oestrus cycle (Long & Evans, 1922) during which the levels of ovarian hormones fluctuate. The circulating levels of oestrogens (Shaikh, 1971; Neill & Smith, 1974) and progestins (Feder, Resko & Goy, 1968; Sutter-Dub, Leclercq, Felix, Jacquot & Sutter, 1973) rise rapidly during pro-oestrus. During oestrus the circulating level of sex hormones declines, and with the exception of a secondary peak of progestins at mid-metoestrus, the levels during the other stages of the cycle are minimal.

The results of folic acid uptake into everted sacs from female rats showed a much wider variation than those made from male rats. This large standard error could possibly be as a result of the rats being in various stages of the oestrus cycle, as this was determined prior to the experiment. The uptake of folic acid and fluid, particularly into the serosal compartment, was greater in the female rats than the males. Association of increased water and sodium transport has been observed with increased levels of ovarian hormones (Hinsull & Crocker, 1970).

As fluid transport was increased in the female rats compared with the males, this resulted in an increased uptake of folic acid by solvent drag although this could not entirely account for the observed results. It is therefore suggested that the fluctuating levels of ovarian hormones in the females may have been responsible for the increased folic acid transfer.

Oral contraceptives and pregnancy have been implicated in the malabsorption of folic acid as discussed in section 1 of this chapter. Oral contraceptives act by simulating pregnancy as a result of inhibition of ovulation; they consist mainly of oestrogens, either synthetic or natural. The endocrine aspects of pregnancy in the rat appear to be generally similar to those of human pregnancy (Heap, 1972) and in both there is a rise in the circulating level of oestrogens (Yoshinaga, Hawkins & Stocker, 1969; Shaikh, 1971). Hence the notion that ovarian hormones, particularly oestrogens, are involved in folic acid

absorption is not new although the subject is controversial.

Ethynyl oestradiol (contraceptive hormone) and pregnancy both reduced the uptake of folic acid into everted sacs although the results were not significantly different from the controls. Fluid uptake was slightly reduced with the exception of the pregnant tissue fluid uptake which was slightly increased. Pregnant women may also exhibit increased tissue fluid uptake in the form of fluid retention. Normal absorption of folates in pregnant women and oral contraceptive users has been reported by several authors (Shojania & Hornady, 1973; Iyengar & Babu, 1975) whereas others have reported folate malabsorption (Strieff, 1970; Yusufji et al., 1973). However the present study showed that there was no significant depression in folic acid transport with oral contraceptives or in pregnancy.

Oestrogens are also given in pharmaceutical preparations to women receiving replacement therapy for menopausal disturbances. Ovariectomy in the rat produces a situation similar to that of the menopause; the ovariectomised rat is equivalent to the woman whose ovaries have ceased to function. In the ovariectomised rat, as in the menopause, oestrogen levels decline (Labhsetwar, 1972; Saez, Morera, Dozord & Bertrand, 1972).

The natural oestrogen, oestradiol was given as the replacement therapy hormone to ovariectomised rats. Folic acid uptake was depressed, although not

significantly, in both the replacement therapy treated rats and ovariectomised rats. The depression was greatest following ovariectomy, and replacement therapy moved the depression towards normal.

Overall, folic acid uptake was mildly depressed by the various treatments on female rats although it is noteworthy that adding excess oestrogen to the rat, particularly the potent synthetic oral contraceptive hormone ethynyl oestradiol, did increase the folic acid transported by mechanisms other than solvent drag, whereas pregnancy, a condition with elevated oestrogen levels, did not appear to increase this transport mechanism. However ovariectomy, which would reduce the oestrogen levels in the rat, resulted in the greatest depression in folic acid transport, and this was corrected by replacing the deficiency of oestrogen with a replacement therapy hormone. The intestinal morphology after all the treatments was normal, this included the ovariectomised rats in which the glycocalyx was still apparent despite this condition having the greatest depression on tissue folic acid uptake.

Hormonal systems are complex; ovarian hormones and contraceptive steroids are noted to have numerous metabolic effects (Salhanick, Kipnis & Vande Wiele, 1969), and it is apparent from the present study that although oestrogen itself does not directly affect folic acid transport to any great extent, minor depressions were observed and as such, alterations to the level of this hormone for a longer duration of time together

with the intricate interaction of many associated hormones might result in greater abnormalities in the absorption of folic acid.

Section 5. SUMMARY.

1) The absorption of folic acid in the rat jejunum *in vitro* showed an immediate rapid uptake on to the tissue followed therein by a slower accumulation of folic acid. Appearance of folic acid in the serosal compartment was delayed following commencement of incubation and no concentration of folic acid was observed, hence there is not an active mechanism specifically for folic acid transport.

Some parallelism was seen with fluid movement and thus solvent drag was responsible for a component of folic acid absorption particularly transfer from the tissue into the serosal compartment. However, solvent drag did not account for all the folic acid movement into the intestinal preparation and uptake into the tissue from the mucosal bathing medium and appeared to be largely fluid independent.

2) Abnormal folic acid absorption occurred following certain drug treatments, the ommision of glucose and depletion of sodium from the incubation medium. There was a close involvement in the trend of alteration of the pH microclimate values and the results of folic acid transport following the conditions. Thus, although folic acid followed a system of passive diffusion an active

mechanism was involved, in the form of maintenance of an acid microclimate.

3) The effect of conditions, namely diabetes mellitus, pregnancy, the menopause, non-fertility with an oral contraceptive and replacement of oestrogen lost in ovariectomy with hormone replacement therapy together with various treatments including alcohol, methotrexate, phenytoin and cortisone on folic acid absorption were studied in the rat. In most instances mild distrubances of both folic acid and fluid uptake were observed usually resulting in an overall depression in the absorption of folic acid. Although folate malabsorption is not fully established in diabetes mellitus, pregnancy, the menopause or during hormone replacement therapy, the present study suggests that these conditions, together with the administration of cortisone, might eventually lead to a state of folate deficiency.

4) The glycocalyx did not appear as distinct in the rat as it did in the human. Disruption of the glycocalyx, similar to that observed in human disease states, was not observed. There did not appear to be an association of the glycocalyx with malabsorption of folic acid and any of the treatments described here.

5) A degree of hormonal involvement was proposed in the absorption of folic acid; particularly as the importance of gastrointestinal tract hormones is rapidly becoming more evident.

Figure 3,1. Computer programme for the processing of everted sac data. Part 1. Weights and fluid measurements.

Input data:

W1 = weight of empty sac + 1 dry suture

W2 = weight of full sac + 1 dry suture + 1 wet suture

W3 = weight of full sac after incubation + 2 wet sutures

W4 = weight of empty sac after incubation + 2 wet sutures

W5 = weight of oxidiser boat

W6 = weight of boat + wet sac

W7 = weight of boat + dry sac

Output data:

W8 = weight of initial serosal fluid (W2-W1-82)

W9 = weight of final serosal fluid (W3-W4)

W10 = initial wet weight (W1-28)

W12 = tissue fluid uptake per sac (W6-W5)×1000-W10

W13 = serosal fluid uptake per sac (W9-W8)

W11 = total fluid uptake per sac (W12+W13)

W14 = dry weight (W7-W5)×1000

W15 = total fluid uptake per mg dry wt (W11÷W14)

W16 = tissue fluid uptake per mg dry wt (W12÷W14)

W17 = serosal fluid uptake per mg dry wt (W13÷W14)

Where 82 = weight of 1 wet suture in mg

28 = weight of 1 dry suture in mg

Figure 3,1 contd.

The information stored was as follows:

		AV
Set up machine and		b*
clear registers		B*
		c*
		d*
		D*
		e*
Feed in data	Enter W1	MS
		b↑
	Enter W2	MS
		M↓
		b-
Calculate & store	W8	F-
		b↕
	W10	f-
		c↕
	Enter W3	MS
		M↓
	Enter W4	MS
Calculate & store	W9	M-
		B↕
	Enter W5	MS
		d↑
	Enter W6	MS
		M↓
Calculate & store		d-
		EX
	W12	c-
		C↕

Figure 3,1 contd.

	Enter W7	MS
Calculate & store		M↓
		d-
	W14	EX
		e↕
		B↓
	W13	b-
		d↕
		d↓
	W11	C+
		D↕
Print	W8	b◇
	W9	B◇
	W10	c◇
	W12	C◇
	W13	d◇
	W11	D◇
		MW
Jump over F		MS
		MS
		AW
Print	W14	e◇
		D↓
		e÷
Calculate & print	W15	a◇
		C↓
		e÷
Calculate & print	W16	A◇
		MY
Jump over f		MS

Figure 3,1 contd.

		MS
		MS
		AY
		d↓
		e÷
		M↕
Calculate & print	W17	M◇
Space		/◇
Back to start		MV
	Constant	82F↑
	Constant	28f↑
	Constant	1000E↑

Figure 3,2. Computer programme for the processing of everted sac data. Part 2. Radioactivity in standards and samples of tissue and serosal fluid.

a) Data from liquid scintillation spectrometer: serosal fluid.

	AV	
Space	/◊	
Enter blank 1	MS	
	M↓	
Enter blank 2	MS	
	M+	
Calculate & store	F÷	
Mean blank	B↕	
	BW	
Enter sample in counts	MS	
	M↓	
or	CY	
Enter sample in seconds	FY	
	D↓	
	M÷	
	BY ↵	
	B-	
	A◊	
Print counts per 600 secs (with background subtracted)	CW	Repeat for each of the two serosal fluid samples from each sac and also for the experimental standard.

Repeat from AV to CW with data from the second run on the liquid scintillation spectrometer.

Figure 3,2 contd.

With data from the third run on the liquid scintillation spectrometer, the mean values of the serosal samples and experimental samples are calculated:

	AV
	AW
Enter blank 1	MS
	M↓
Enter blank 2	MS
	M↓
Enter sample count	CV
or time	FZ
	D↓
	M÷
	BV
	B-
	CZ
Enter previous data from runs	AY
1-3 with background subtracted	M↓
	BZ
	C+
Data stored from runs 1-3,	C↕
with background subtracted	E↓
	F+
	E↕
	CW
	AZ
	E↓
	F÷
	C↓
	C÷

Figure 3,2 contd.

Number of pieces of information entered	M◊
Mean count	A◊
Clear for new batch	C✱
	E✱
	CW
Constant	2F↑
Constant	6000000D↑

After repeating this procedure, the five mean count values (A◊) were obtained. These represent the radioactive content of the serosal fluid from the four everted sacs and the experimental standard of each experiment.

b) Data from liquid scintillation spectrometer: tissue samples.
The above procedure was repeated, using the data obtained for tissue samples and the appropriate standards burned in the biological oxidiser. Five mean values (A◊) were obtained for each experiment: the counts for each of the four everted sacs and the standard counts.

Figure 3,3. Computer programme for the processing of everted sac data. Part 3. Tissue and serosal uptake of folic acid.

a) Serosal uptake of folic acid:

	AV	
Enter standard count per 10 min	MS	
	M↓	
	F÷	
Print efficiency	a◇	
	fX	
	B↕	
Print cpm per picomole	B◇	
	BV	Repeat for each sample from the four incubation times.
Enter sample cpm	MS	
	M↓	
Enter W9	MS	
	MX	
Enter W14	MS	
	M÷	
	B÷	
	E÷	
Print uptake (pmol/mg dry wt)	A◇	
	CV	

b) Tissue uptake of folic acid:

	AW
Enter standard count per 10 min	MS
	M↓
	e÷
	B↕
Print cpm per picomole	B◇

Figure 3,3 contd.

	BW	
Enter sample cpm	MS	
	M↓	
	DX	Repeat for each sample from
Enter W14	MS	the four incubation times.
	M÷	
	B÷	
Print uptake (pmol/mg dry wt)	A◇	
	CW	

Constant	111F↑
Constant	1.29222f↑
Constant	8.616E↑
Constant	171.8e↑
Constant	5.8033D↑

Figure 3,4. Total (•), tissue (×) and serosal (+) transfer of folic acid (a) and fluid (b) in everted sacs from a control group of male rats (m ± sem, n = 12). Significant regression coefficients: § = P = < 0.05, §§ = P = < 0.01.

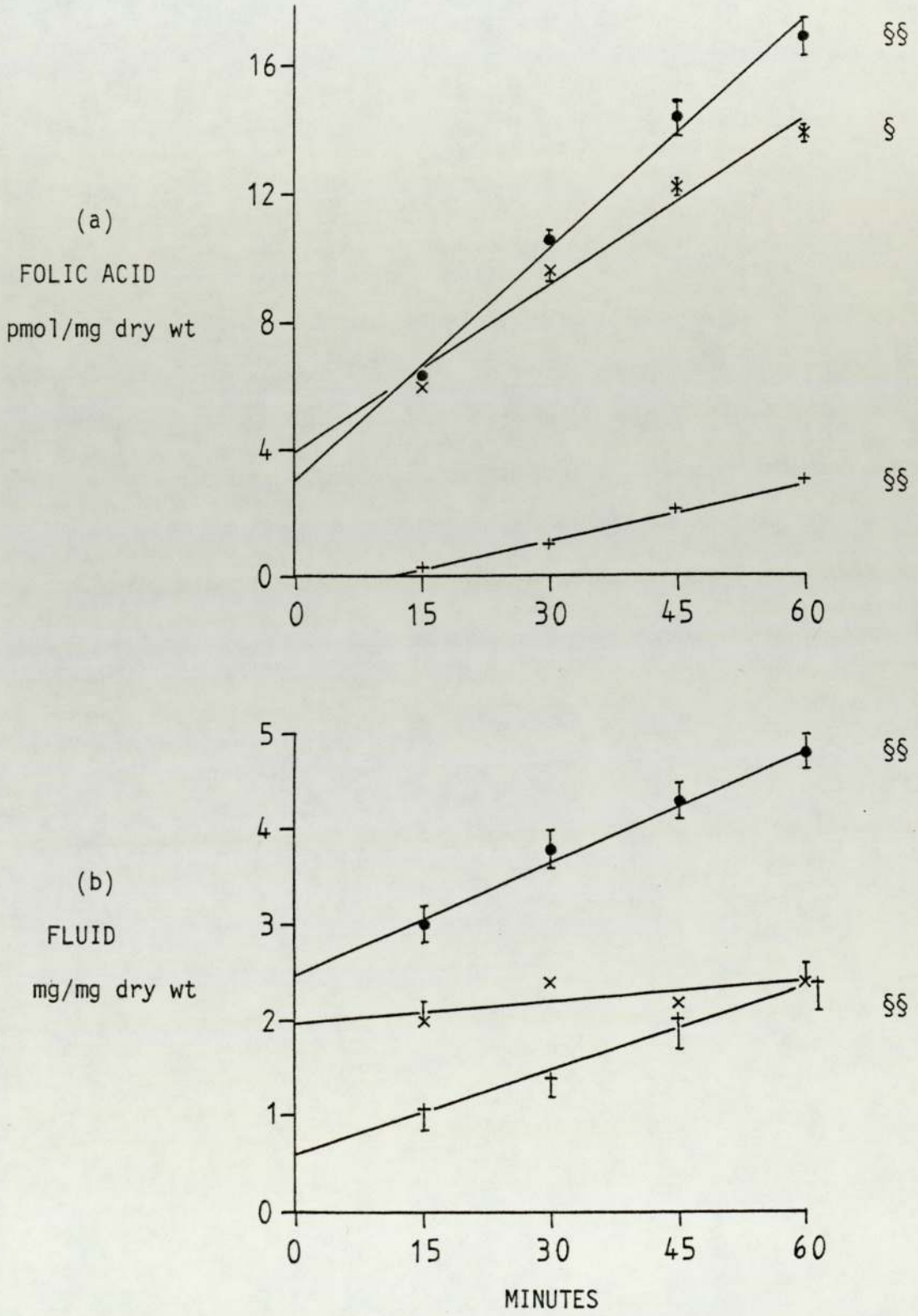


Figure 3,5. Total (•), tissue (×) and serosal (+) transfer of folic acid (a) and fluid (b) in everted sacs from a control group of female rats (m ± sem, n = 5). Significant regression coefficients: § = P = < 0.05, §§ = P = < 0.01.

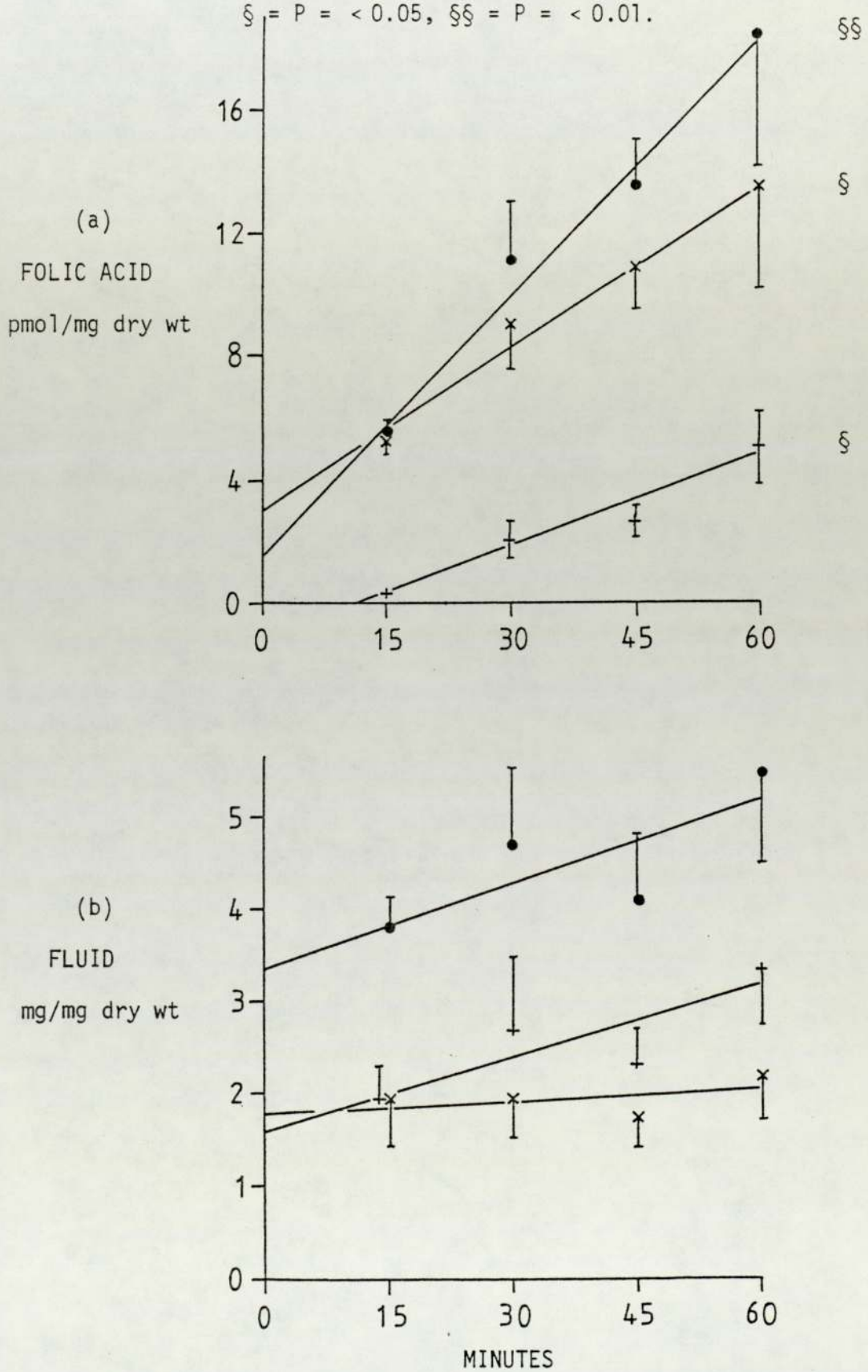


Figure 3,6. Total (•), tissue (×) and serosal (+) transfer of leucine (a) and fluid (b) in everted sacs from a control group of male rats (m ± sem, n = 5). Significant regression coefficients: § = P =<0.05, §§ = P =<0.01.

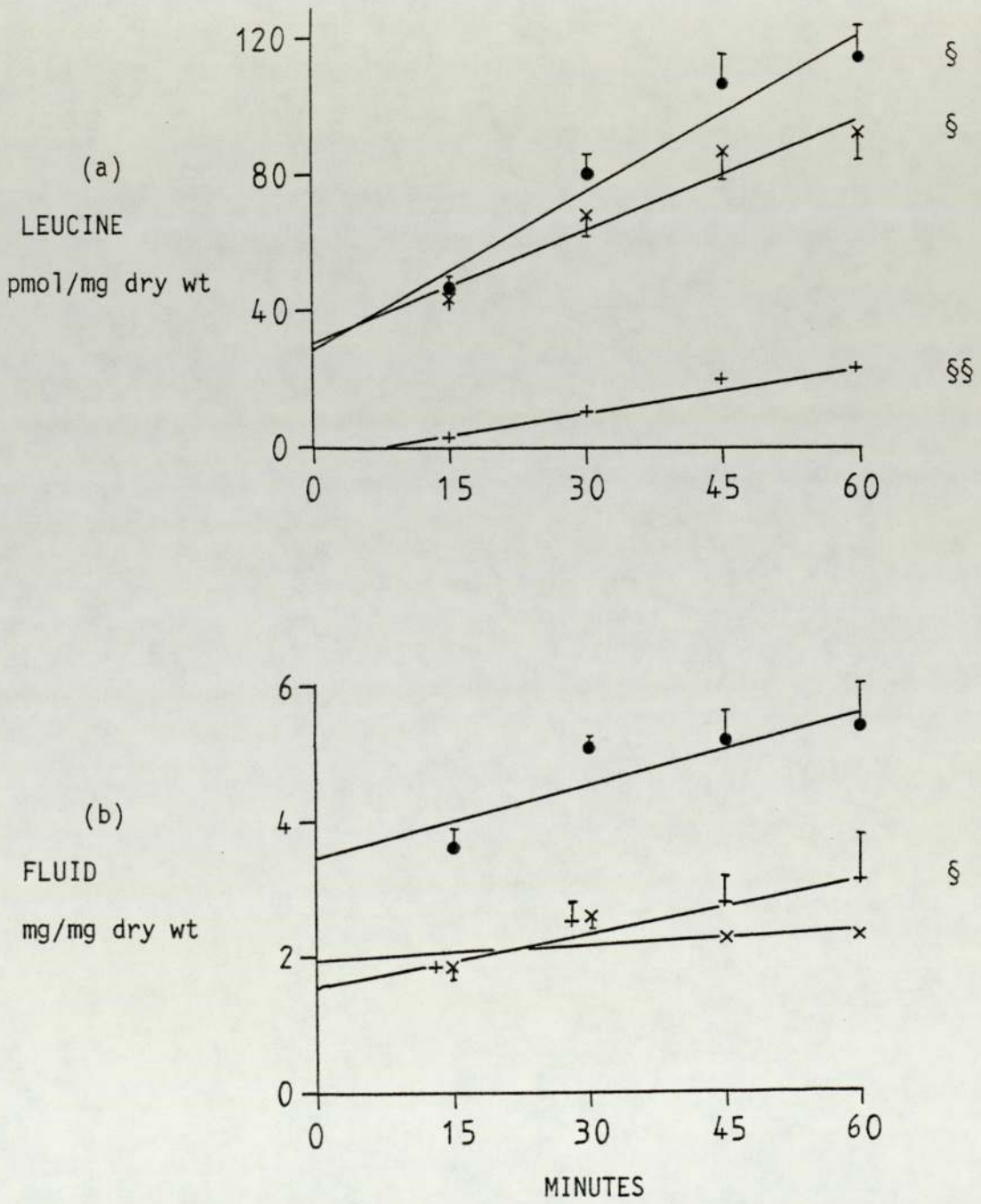


Figure 3,7. Total, tissue and serosal transfer of folic acid in everted sacs incubated in sodium depleted buffer and in control buffer (m ± sem). ▭ sodium depleted n = 6
▨ control n = 12

Values of P compared with control: § = P = <0.05
§§ = P = <0.02
§§§ = P = <0.01

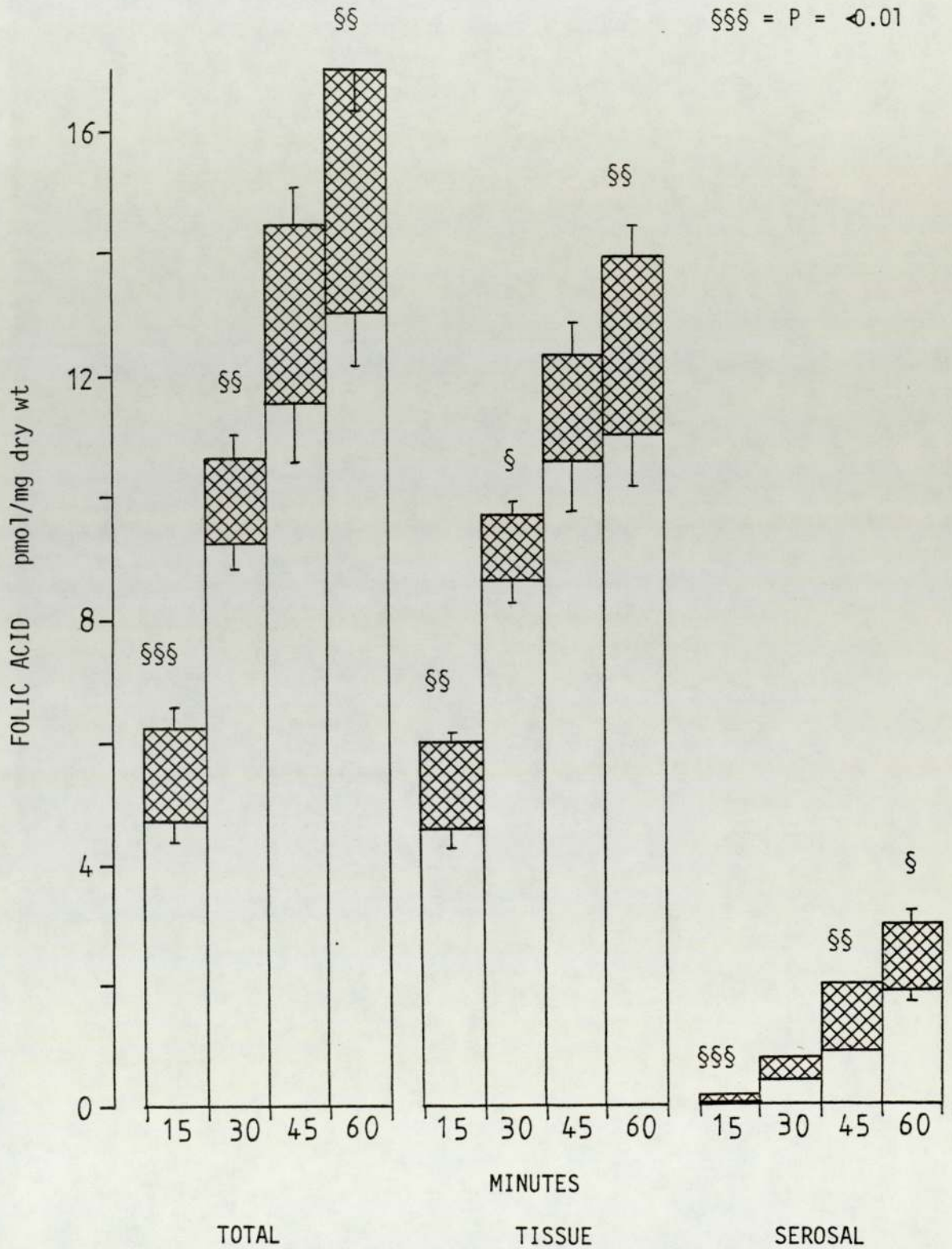


Figure 3,8. Total (•), tissue (×) and serosal (+) transfer of folic acid (a) and fluid (b) in everted sacs from rats pretreated with methotrexate (ip, 40mg/Kg, 48h). m ± sem, n = 6. Significant regression coefficient: § = P = <0.05.

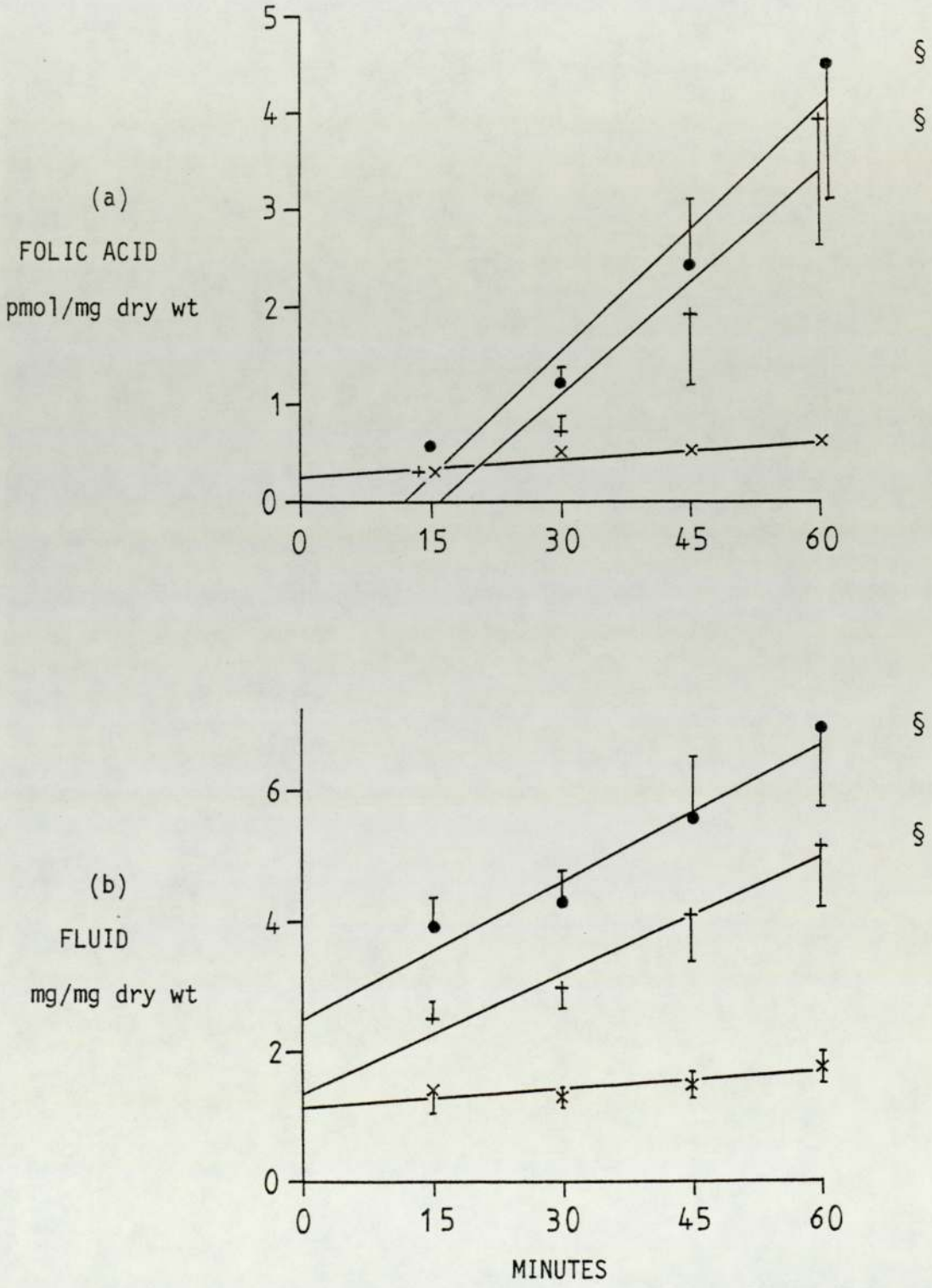


Figure 3,9. Total (•), tissue (×) and serosal (+) transfer of folic acid (a) and fluid (b) in everted sacs from rats pretreated with methotrexate (oral, 10mg/Kg, 24h). m ± sem, n = 6. Significant regression coefficients: § = P = < 0.05, §§ = P = < 0.01.

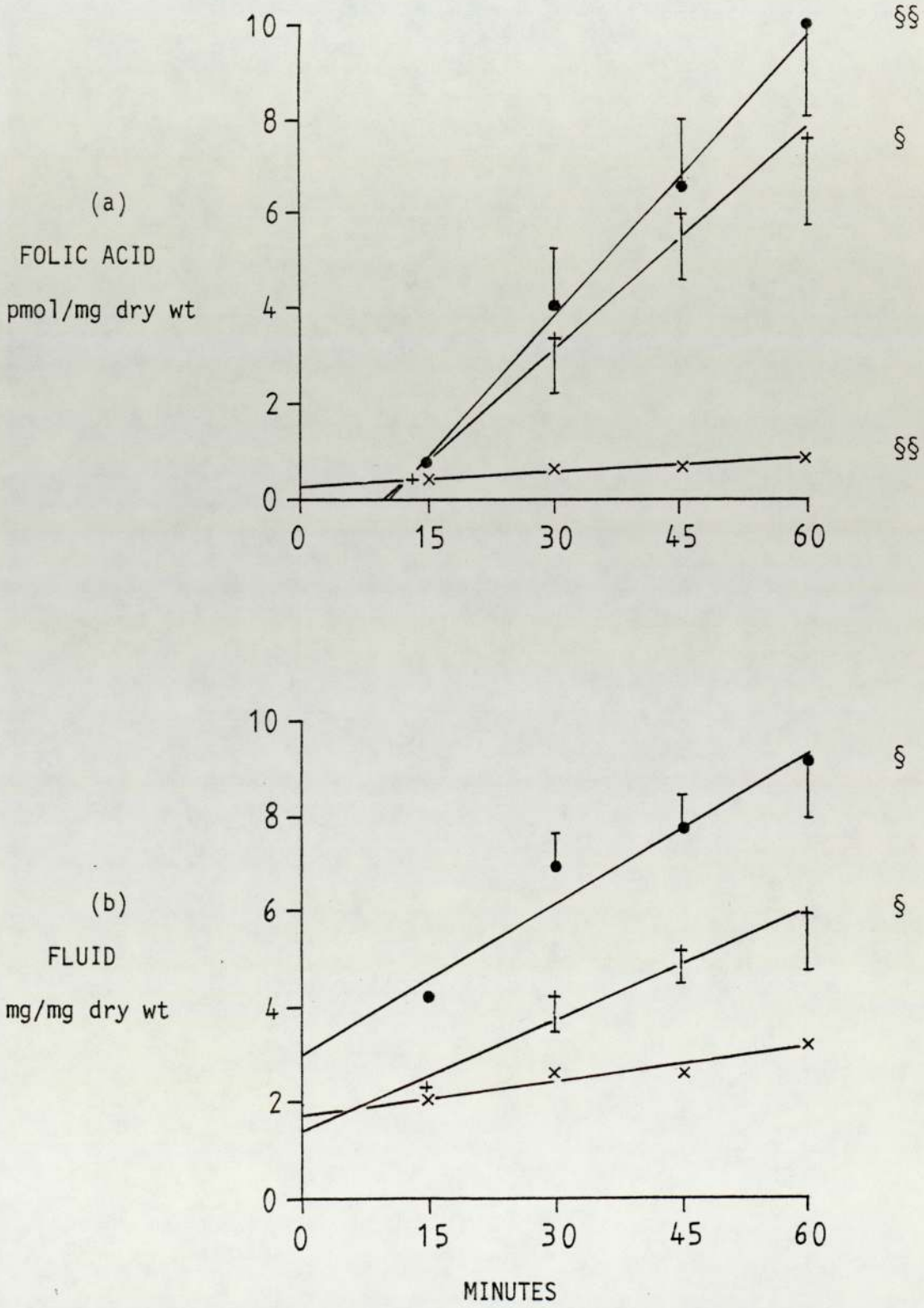


Figure 3,10. Significant alterations with methotrexate treatment when compared with control values.

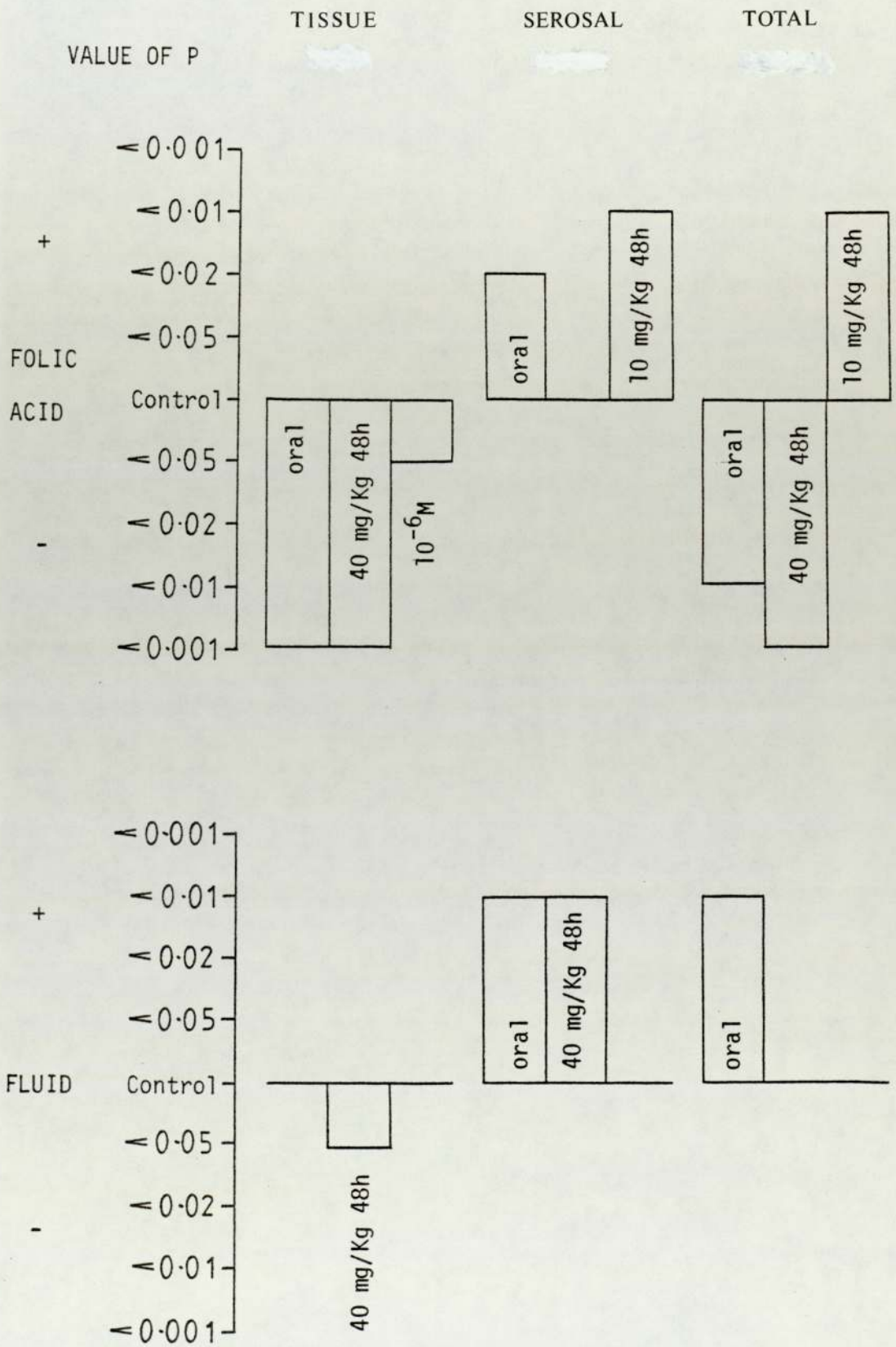


Figure 3,11. Plasma glucose and plasma insulin levels in streptozotocin diabetic, cortisone treated and control rats following an intraperitoneal glucose load (2g/Kg). m ± sem. • streptozotocin n = 4
o cortisone n = 4
× control n = 4

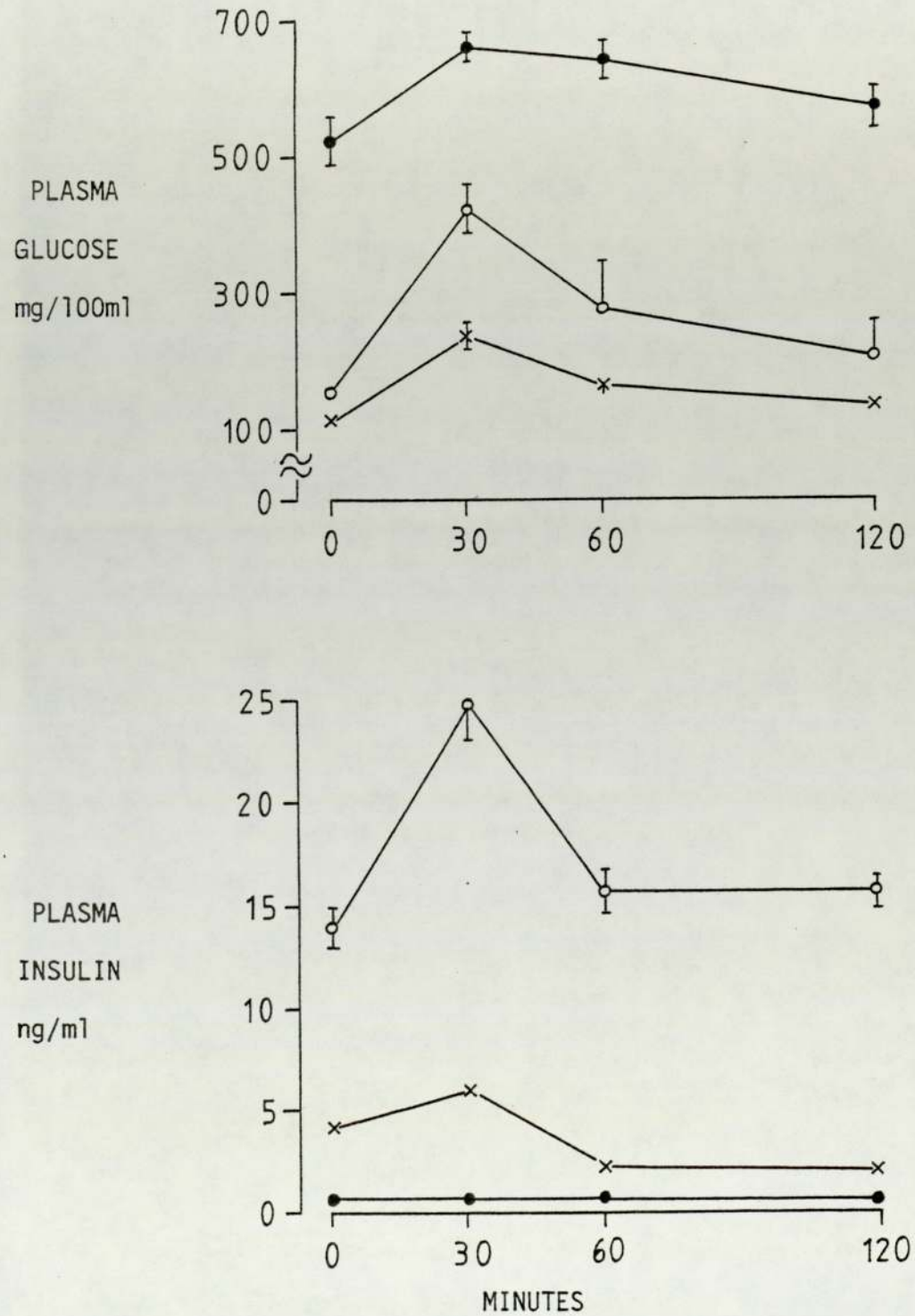
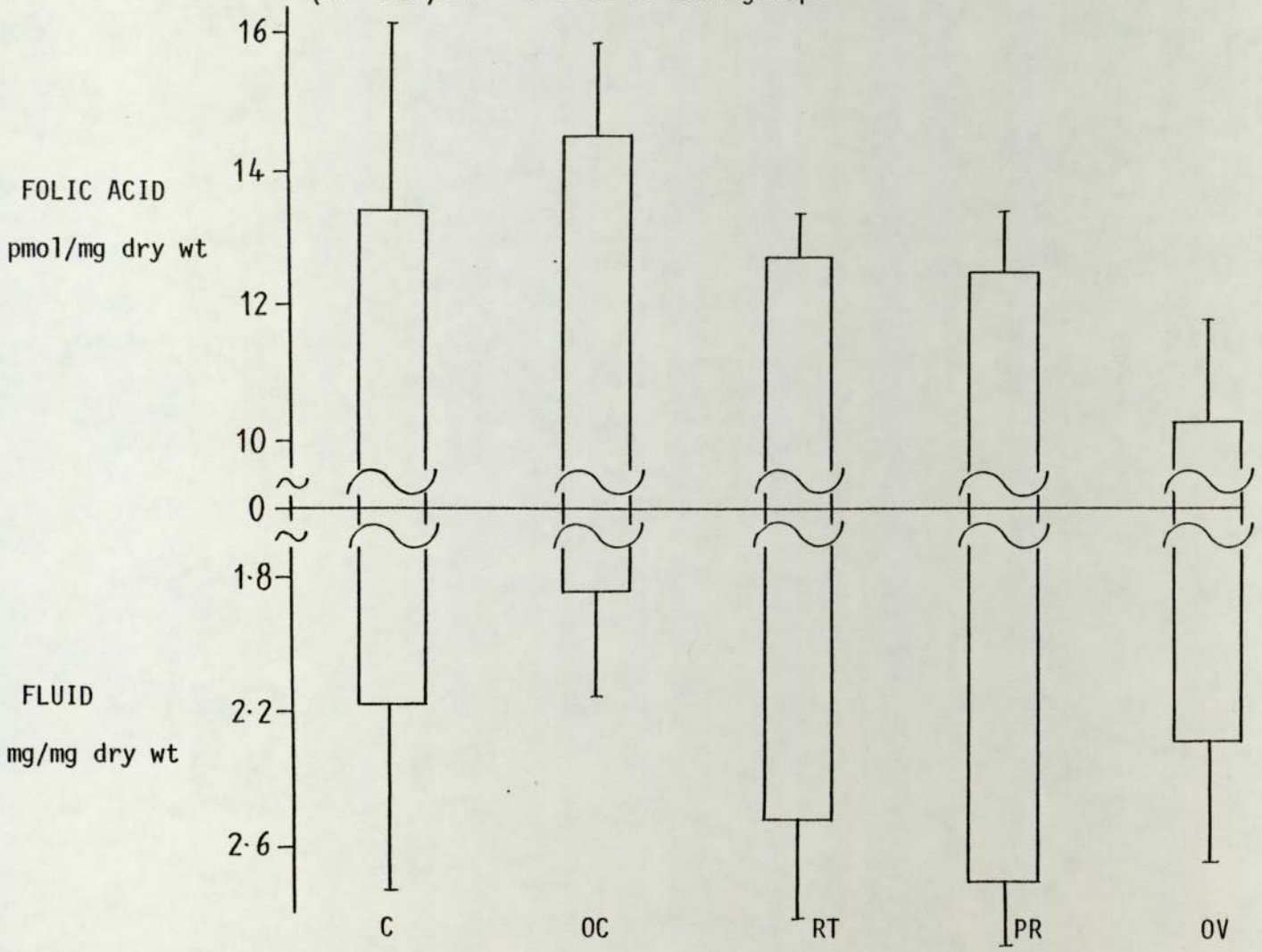
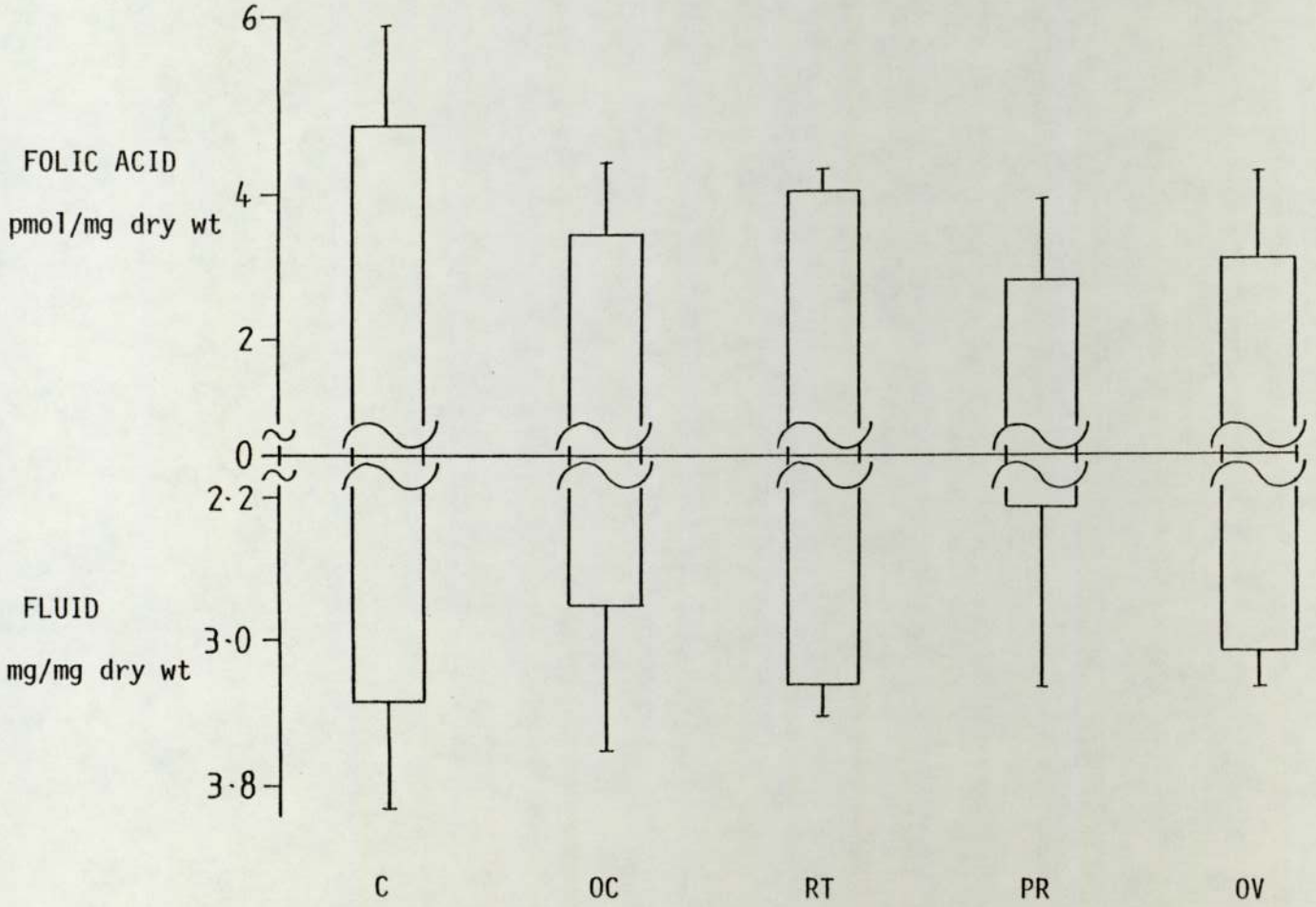


Figure 3,12. Tissue transfer of folic acid in everted sacs from female rats subjected to various conditions. (m ± sem). n = 5 rats in each group.



C control
OC oral contraceptive (ethynyl oestradiol)
RT replacement therapy (oestradiol)
PR pregnant
OV ovariectomised

Figure 3,13. Serosal transfer of folic acid in everted sacs from female rats subjected to various conditions. ($m \pm sem$). n = 5 rats in each group.



C control
OC oral contraceptive (ethynyl oestradiol)
RT replacement therapy (oestradiol)
PR pregnant
OV ovariectomised

Table 3.1. Diseases associated with folate malabsorption.

Coeliac disease	Gerson et al., (1974)
Tropical sprue	Corcino, Coll & Klipstein (1975)
Crohn's disease	Hoffbrand, Stewart, Booth & Mollin (1968)
Ulcerative colitis	Franklin & Rosenberg (1973)
Dermatitis herpetiformis	Hoffbrand, Douglas, Fry & Stewart (1970)
Diabetic enteropathy	Klipstein (1966)
Small intestine lymphoma	Klipstein (1966)
Amyloid	Klipstein (1966)
Whipple's disease	Klipstein (1966)
Systemic bacterial infections	Cook, Morgan & Hoffbrand (1974)
Cardiac failure	Hyde & Loehry (1968)
Small intestine resection	Booth (1961)
Gastrectomy	Elsborg (1974a)

Table 3.2. Drugs associated with folate malabsorption.

Methotrexate	Hepner (1969)
Anticonvulsants	Schafer & Nuttall (1975)
Ethanol	Halsted et al., (1973)
Oral contraceptives	Shojania et al., (1968)
Sulfasalazine	Franklin & Rosenberg (1973)
Antituberculosis drugs	Klipstein, Berlinger & Reed (1967)
Paramino salicylate	Longstreth, Newcomer & Westbrook (1972)
Metformin	Stebbins, Scott & Herbert (1973)

Table 3,3. Folic acid:controls. Total, tissue and serosal uptakes of folic acid and fluid, and rates of uptake following the incubation of everted sacs for 60 minutes in 10^{-6}M folic acid. Minimum folic acid represents folic acid transported by mechanisms other than solvent drag. Where appropriate the results are given as mean $\pm$ standard error of the mean. (n = 12 rats).

	TOTAL	TISSUE	SEROSAL
FOLIC ACID (pmol/mg dry wt at 60 min)	17.04 $\pm$ 0.76	13.91 $\pm$ 0.55	3.05 $\pm$ 0.26
FOLIC ACID (pmol/mg dry wt/min)	0.24	0.18	0.06
FLUID (mg/mg dry wt at 60 min)	4.85 $\pm$ 0.29	2.45 $\pm$ 0.15	2.45 $\pm$ 0.26
FLUID (mg/mg dry wt/min)	0.03	0.01	0.03
MINIMUM FOLIC ACID (pmol/mg dry wt at 60 min)	12.19 $\pm$ 0.66	11.46 $\pm$ 0.65	0.61 $\pm$ 0.23

Table 3,4. Folic acid: male and female controls. Differences in the transport of folic acid, fluid and folic acid corrected for solvent drag. Results are given as mean ± standard error.

	MALES (n = 12 rats)	FEMALES (n = 5 rats)	t	P
MAXIMUM FOLIC ACID (pmol/mg dry wt at 60 min)				
TOTAL	17.04 ± 0.76	18.49 ± 4.07	0.53	> 0.05
TISSUE	13.91 ± 0.55	13.45 ± 2.91	0.24	> 0.05
SEROSAL	3.05 ± 0.26	5.04 ± 1.32	2.25	< 0.05
FLUID (mg/mg dry wt at 60 min)				
TOTAL	4.85 ± 0.29	5.56 ± 1.15	0.87	> 0.05
TISSUE	2.44 ± 0.15	2.19 ± 0.56	0.63	> 0.05
SEROSAL	2.45 ± 0.26	3.38 ± 0.67	1.61	> 0.05
MINIMUM FOLIC ACID (mg/mg dry wt at 60 min)				
TOTAL	12.19 ± 0.66	12.93 ± 2.95	0.35	> 0.05
TISSUE	11.46 ± 0.65	11.26 ± 2.36	0.09	> 0.05
SEROSAL	0.61 ± 0.23	1.67 ± 0.74	1.84	> 0.05

Table 3,5. Folic acid: male and female controls. Immediate uptake, and rate of uptake of folic acid with regression coefficient (r). (Male n = 12 rats, female n = 5 rats).

	IMMEDIATE UPTAKE (pmol/mg dry wt)	RATE OF UPTAKE (pmol/mg dry wt/min)	r	P
TOTAL				
MALE	3.06	0.24	0.99	< 0.01
FEMALE	1.77	0.28	0.99	< 0.01
TISSUE				
MALE	3.89	0.18	0.98	< 0.05
FEMALE	3.04	0.18	0.98	< 0.05
SEROSAL				
MALE	- 0.79	0.06	0.99	< 0.01
FEMALE	- 1.27	0.10	0.98	< 0.05

Table 3,6. Glucose replacement by sucrose. Differences in the transport of folic acid, fluid and folic acid corrected for solvent drag between controls and tissue incubated in medium with glucose replaced by 20 mM sucrose. Results are given as the mean ± standard error after 60 minutes incubation: folic acid is expressed as pmol/mg dry wt and fluid as mg/mg dry wt.

	CONTROL (n = 12 rats)	SUCROSE (n = 6 rats)	t	P
TISSUE				
MAXIMUM FOLIC ACID	13.91 ± 0.55	11.72 ± 0.64	2.47	< 0.05
FLUID	2.44 ± 0.15	2.23 ± 0.16	0.92	> 0.05
MINIMUM FOLIC ACID	11.46 ± 0.65	9.50 ± 0.54	1.98	> 0.05
SEROSAL				
MAXIMUM FOLIC ACID	3.05 ± 0.26	3.74 ± 0.23	1.81	> 0.05
FLUID	2.44 ± 0.26	2.44 ± 0.21	0.01	> 0.05
MINIMUM FOLIC ACID	0.61 ± 0.23	1.30 ± 0.17	2.07	> 0.05
TOTAL				
MAXIMUM FOLIC ACID	17.04 ± 0.76	15.46 ± 0.85	1.33	> 0.05
FLUID	4.85 ± 0.29	4.67 ± 0.24	0.43	> 0.05
MINIMUM FOLIC ACID	12.19 ± 0.66	10.80 ± 0.64	1.42	> 0.05

Table 3.7. Sodium depletion. Total, tissue and serosal transport of fluid and folic acid corrected for solvent drag after 60 minutes incubation of everted sacs in sodium depleted and control buffers. Results are given as mean ± standard error. (Sodium depleted n = 6 rats, control n = 12 rats).

	TOTAL	TISSUE	SEROSAL
FLUID			
(mg/mg dry wt)			
SODIUM DEPLETED	3.86 ± 0.20	2.19 ± 0.11	1.66 ± 0.14
CONTROL	4.85 ± 0.29	2.44 ± 0.15	2.45 ± 0.26
t	2.45	1.12	2.21
P	< 0.05	> 0.05	< 0.05
MINIMUM FOLIC ACID			
(pmol/mg dry wt)			
SODIUM DEPLETED	9.17 ± 0.92	8.87 ± 0.91	0.30 ± 0.23
CONTROL	12.19 ± 0.66	11.43 ± 0.65	0.61 ± 0.23
t	2.88	2.30	0.88
P	< 0.02	< 0.05	> 0.05

Table 3,8. Methotrexate, 10 mg/Kg 24 hr ip. Differences in tissue uptake of folic acid and fluid between everted sacs from methotrexate pre-treated and control rats. Results are given as mean ± standard error.

	CONTROL (n = 12 rats)	METHOTREXATE 10 mg/Kg 24hr ip (n = 6 rats)	t	P
FOLIC ACID (pmol/mg dry wt)				
15 min	6.01 ± 0.26	7.63 ± 0.19	4.20	< 0.001
30 min	9.67 ± 0.34	12.24 ± 0.45	4.58	< 0.001
45 min	12.29 ± 0.54	14.31 ± 0.42	2.52	< 0.05
60 min	13.91 ± 0.55	15.59 ± 0.71	1.85	< 0.05
FLUID (mg/mg dry wt)				
15 min	2.03 ± 0.18	2.34 ± 0.13	1.22	> 0.05
30 min	2.40 ± 0.11	3.29 ± 0.36	3.74	< 0.01
45 min	2.27 ± 0.11	2.96 ± 0.17	3.63	< 0.01
60 min	2.44 ± 0.15	3.19 ± 0.43	1.98	> 0.05

Table 3.9. Methotrexate, 10 mg/Kg 48hr ip. Differences in serosal uptake of folic acid and fluid between everted sacs from methotrexate pretreated and control rats. Results are given as mean ± standard error.

	CONTROL (n = 12 rats)	METHOTREXATE 10 mg/Kg 48hr ip (n = 6 rats)	t	P
FOLIC ACID (pmol/mg dry wt)				
15 min	0.26 ± 0.05	0.62 ± 0.11	3.46	< 0.01
30 min	0.96 ± 0.14	2.44 ± 0.59	3.64	< 0.01
45 min	2.16 ± 0.28	3.47 ± 0.39	2.67	< 0.02
60 min	3.05 ± 0.26	5.55 ± 0.69	3.40	< 0.01
FLUID (mg/mg dry wt)				
15 min	1.11 ± 0.16	1.53 ± 0.33	1.31	> 0.05
30 min	1.43 ± 0.17	1.99 ± 0.52	2.12	> 0.05
45 min	2.02 ± 0.33	2.79 ± 0.56	1.27	> 0.05
60 min	2.45 ± 0.26	3.07 ± 0.88	0.83	> 0.05

Table 3.10. Methotrexate 40 mg/Kg 48 hr ip. Total, tissue and serosal uptake of folic acid and fluid. Figures in parentheses indicate value of t when compared with control, followed by value of P, and if significantly different, an arrow indicates whether the alteration is an increase or decrease in uptake compared with control. Values of folic acid are expressed as pmol/mg dry wt with standard error of the mean and values of fluid are expressed as mg/mg dry wt with standard error of the mean. (Methotrexate n = 6 rats, control n = 12 rats).

Table 3.10. Methotrexate 40 mg/Kg 48 hr ip.

(Legend previous page).

	TOTAL	TISSUE	SEROSAL
FOLIC ACID			
15 min	0.65 ± 0.12 (12.35) < 0.001↓	0.33 ± 0.04 (14.92) < 0.001↓	0.29 ± 0.09 (0.26) > 0.05
30 min	1.21 ± 0.21 (21.87) < 0.001↓	0.50 ± 0.05 (28.07) < 0.001↓	0.71 ± 0.17 (1.22) > 0.05
45 min	2.39 ± 0.75 (10.32) < 0.001↓	0.52 ± 0.07 (15.42) < 0.001↓	1.88 ± 0.72 (0.18) > 0.05
60 min	4.50 ± 1.40 (7.48) < 0.001↓	0.61 ± 0.07 (16.13) < 0.001↓	3.90 ± 1.34 (0.53) > 0.05
FLUID			
15 min	3.92 ± 0.48 (1.77) > 0.05	1.41 ± 0.35 (1.78) > 0.05	2.51 ± 0.27 (4.80) < 0.001↑
30 min	4.33 ± 0.49 (1.10) > 0.05	1.33 ± 0.17 (5.47) < 0.001↓	3.00 ± 0.35 (4.55) < 0.001↑
45 min	5.60 ± 0.98 (1.57) > 0.05	1.50 ± 0.24 (3.39) < 0.01↓	4.10 ± 0.77 (2.92) < 0.02↑
60 min	7.00 ± 1.24 (2.14) > 0.05	1.79 ± 0.33 (2.13) < 0.05↓	5.21 ± 0.95 (3.48) < 0.01↑

Table 3.11. Methotrexate 10 mg/Kg 24hr oral. Total, tissue and serosal uptake of folic acid and fluid. Figures in parentheses indicate value of t when compared with control, followed by value of P, and if significantly different, an arrow indicates whether the alteration is an increase or decrease in uptake compared with control. Values of folic acid are expressed as pmol/mg dry wt with standard error of the mean and values of fluid are expressed as mg/mg dry wt with standard error of the mean. (Methotrexate n = 6 rats, control n = 12 rats).

Table 3.11. Methotrexate 10 mg/Kg 24 hr oral.
(Legend previous page)

	TOTAL	TISSUE	SEROSAL
FOLIC ACID			
15 min	0.80 ± 0.22 (11.49) < 0.001↓	0.40 ± 0.04 (14.76) < 0.001↓	0.41 ± 0.18 (0.59) > 0.05
30 min	4.11 ± 1.28 (5.71) < 0.001↓	0.63 ± 0.07 (27.49) < 0.001↓	3.48 ± 1.28 (2.21) < 0.05↑
45 min	6.73 ± 1.46 (4.78) < 0.001↓	0.78 ± 0.08 (15.05) < 0.001↓	5.95 ± 1.45 (3.06) < 0.02↑
60 min	10.04 ± 2.07 (3.25) < 0.01↓	0.94 ± 0.12 (15.64) < 0.001↓	7.43 ± 1.81 (3.03) < 0.02↑
FLUID			
15 min	4.19 ± 0.38 (2.58) < 0.05↑	2.03 ± 0.13 (0.01) > 0.05	2.28 ± 0.38 (3.35) < 0.01↑
30 min	6.99 ± 0.77 (5.07) < 0.001↑	2.73 ± 0.24 (1.41) > 0.05	4.27 ± 0.67 (5.29) < 0.001↑
45 min	7.87 ± 0.66 (5.47) < 0.001↑	2.66 ± 0.24 (1.70) > 0.05	5.22 ± 0.51 (5.49) < 0.001↑
60 min	9.24 ± 1.24 (4.35) < 0.001↑	3.24 ± 0.23 (2.12) > 0.05	5.99 ± 1.11 (3.92) < 0.01↑

Table 3.12. Uptake of leucine (pmol/mg dry wt) after 60 minutes incubation of everted sacs from control rats, and rats treated 48 hrs before death with 40 mg/Kg intraperitoneal methotrexate. Results are given as mean ± standard error. (Leucine control n = 5 rats, methotrexate n = 5 rats).

	CONTROL LEUCINE	LEUCINE + METHOTREXATE	t	P
TOTAL	115.54 ± 11.23	128.85 ± 24.81	0.53	> 0.05
TISSUE	92.03 ± 8.31	103.40 ± 19.14	0.59	> 0.05
SEROSAL	23.51 ± 4.09	24.96 ± 5.09	0.22	> 0.05

Table 3.13. Rate of uptake of leucine, with regression coefficients (r), for everted sacs from control rats and rats treated 48 hrs before death with 40 mg/Kg intraperitoneal methotrexate. Rate is expressed as pmol/mg dry wt/min. (Leucine control n = 5 rats, methotrexate n = 5 rats).

	CONTROL LEUCINE			LEUCINE + METHOTREXATE		
	RATE	r	P	RATE	r	P
TOTAL	1.56	0.97	< 0.05	1.50	0.82	> 0.05
TISSUE	1.10	0.96	< 0.05	0.77	0.77	> 0.05
SEROSAL	0.46	0.99	< 0.01	0.44	0.98	< 0.05

Table 3.14. Phenytoin 10^{-6}M. Total, tissue and serosal uptake of folic acid, fluid and folic acid corrected for solvent drag after 60 minutes incubation of everted sacs in 10^{-6}M phenytoin (n = 6 rats).

	TOTAL	TISSUE	SEROSAL
MAXIMUM FOLIC ACID (pmol/mg dry wt)	20.58*	15.68	4.90**
FLUID (mg/mg dry wt)	6.31**	2.50	3.81**
MINIMUM FOLIC ACID (pmol/mg dry wt)	14.24	13.15	1.05

* = P = <0.05 and ** = P = <0.01 increased transfer compared with control values.

Table 3.15. Acute exposure to 3% alcohol. Differences in tissue uptake of folic acid and fluid, between everted sacs incubated in either control buffer or buffer supplemented with 3% alcohol. Results are given as mean ± standard error.

	CONTROL	ACUTE ALCOHOL	t	P
FOLIC ACID	(n = 12 rats)	(n = 6 rats)		
(pmol/mg dry wt)				
15 min	6.01 ± 0.26	5.55 ± 0.29	1.10	> 0.05
30 min	9.67 ± 0.34	9.37 ± 0.52	0.51	> 0.05
45 min	12.29 ± 0.54	11.62 ± 0.72	0.74	> 0.05
60 min	13.91 ± 0.55	11.72 ± 0.94	2.11	> 0.05
FLUID				
(mg/mg dry wt)				
15 min	2.03 ± 0.18	1.11 ± 0.25	3.10	< 0.01
30 min	2.40 ± 0.11	1.51 ± 0.13	4.98	< 0.001
45 min	2.27 ± 0.11	1.61 ± 0.13	3.75	< 0.01
60 min	2.44 ± 0.15	1.79 ± 0.19	2.63	< 0.02

Table 3.16. Chronic exposure to 20% alcohol in drinking water. Total, tissue and serosal uptake of folic acid and fluid. Figures in parentheses indicate value of t when compared with control, followed by value of P, and if significantly different, an arrow indicates whether the alteration is an increase or decrease in uptake compared with control. Values of folic acid are expressed as pmol/mg dry wt with standard error of the mean and values of fluid are expressed as mg/mg dry wt with standard error of the mean.
(Chronic alcohol n = 6 rats, control n = 12 rats).

Table 3.16. Chronic exposure to 20% alcohol in drinking water. (Legend previous page).

	TOTAL	TISSUE	SEROSAL
FOLIC ACID			
15 min	8.99 ± 0.95 (2.77) < 0.02↑	8.32 ± 0.87 (2.58) < 0.05↑	0.70 ± 0.24 (1.72) > 0.05
30 min	16.59 ± 1.47 (4.44) < 0.001↑	14.00 ± 1.08 (4.36) < 0.001↑	2.59 ± 0.47 (3.47) < 0.01↑
45 min	20.44 ± 2.37 (2.79) < 0.02↑	16.25 ± 1.51 (2.71) < 0.02↑	4.19 ± 0.91 (2.55) < 0.05↑
60 min	23.47 ± 1.87 (3.09) < 0.02↑	17.97 ± 1.24 (3.08) < 0.01↑	4.96 ± 0.71 (2.31) < 0.05↑
FLUID			
15 min	4.47 ± 0.40 (3.01) < 0.01↑	2.32 ± 0.15 (1.13) > 0.05	2.15 ± 0.46 (2.64) < 0.02↑
30 min	6.91 ± 0.74 (5.09) < 0.001↑	2.61 ± 0.06 (1.30) > 0.05	4.30 ± 0.76 (4.82) < 0.001↑
45 min	7.59 ± 0.62 (5.21) < 0.001↑	2.69 ± 0.17 (2.18) < 0.05↑	4.90 ± 0.70 (4.25) < 0.001↑
60 min	7.85 ± 0.80 (4.22) < 0.001↑	3.11 ± 0.15 (2.85) < 0.02↑	4.74 ± 0.74 (3.49) < 0.01↑

Table 3.17. Diabetogenic agents. Effects of streptozotocin and cortisone treatments of rats on various parameters including plasma glucose and insulin levels. Results are expressed as mean ± standard error.

	CONTROL (n = 4 rats)	STREPTOZOTOCIN (n = 4 rats)	CORTISONE (n = 4 rats)
BODY WEIGHT (g)	286 ± 10	260 ± 21	280 ± 22
FLUID INTAKE (g/24h)	33 ± 5	60 ± 11	39 ± 8
FOOD INTAKE (g/24h)	29 ± 3	38 ± 5	31 ± 6
PLASMA GLUCOSE (mg/100ml)	125 ± 0.67	529 ± 58.17	166 ± 14.18
PLASMA INSULIN (ng/ml)	4.20 ± 0.98	0.62 ± 0.31	13.47 ± 1.32

Table 3.18. Diabetogenic agents. Tissue and serosal transport of folic acid, fluid and folic acid corrected for solvent drag, following 60 minutes incubation of everted sacs from diabetic rats. Folic acid is expressed as pmol/mg dry wt and fluid as mg/mg dry wt. Results are given as mean ± standard error.

	CONTROL (n = 4 rats)	STREPTOZOTOCIN (n = 4 rats)	CORTISONE (n = 4 rats)
TISSUE			
MAXIMUM FOLIC ACID	13.91 ± 0.55	13.60 ± 4.24	9.62 ± 2.10 *
FLUID	2.44 ± 0.15	2.36 ± 0.61	2.13 ± 0.40
MINIMUM FOLIC ACID	11.43 ± 0.65	11.23 ± 4.00	7.50 ± 1.70 *
SEROSAL			
MAXIMUM FOLIC ACID	3.05 ± 0.26	2.36 ± 0.61	2.99 ± 1.73
FLUID	2.45 ± 0.26	2.30 ± 0.65	2.29 ± 1.47
MINIMUM FOLIC ACID	0.61 ± 0.23	0.05 ± 0.31	0.55 ± 0.23

* = P = <0.05 compared with control value.

Table 3.19. Female rat studies. Total transport of folic acid, fluid, and folic acid corrected for solvent drag, following 60 minutes incubation of everted sacs from female rats subjected to various conditions. Results are given as mean ± standard error. (n = 5 rats per group).

	MAXIMUM FOLIC ACID (pmol/mg dry wt)	FLUID (mg/mg dry wt)	MINIMUM FOLIC ACID (pmol/mg dry wt)
FEMALE CONTROL	18.49 ± 4.07	5.56 ± 1.15	12.92 ± 2.95
ETHYNYL OESTRADIOL	18.00 ± 1.32	4.69 ± 0.80	13.31 ± 1.96
OESTRADIOL	16.77 ± 0.84	5.81 ± 0.44	10.95 ± 1.18
PREGNANCY	15.49 ± 1.73	4.99 ± 0.85	10.51 ± 1.07
OVARIECTOMY	13.61 ± 1.76	5.38 ± 0.54	8.22 ± 1.5

Chapter 4.

Isolated cells and folate uptake.

Section 1. INTRODUCTION.

A logical progression from the everted sac preparation was the development of techniques for the isolation of intestinal epithelial cells. *In vitro* preparations employing whole tissue have the disadvantage in transport studies that the accumulation of metabolic substrates is a composite of numerous processes with the multiple cell types. Diffusional entry and diffusion following active transfer can cause ambiguity in interpreting the nature of the transport process. Isolated intact epithelial cells represent a sequential step in the breakdown from *in vivo* preparations to progressively smaller components of the mucosal cell that are more intimately involved in transport processes across the brush border membrane. Such preparations essentially represent a homologous cell population in which transport rates and metabolic processes are uninfluenced by other components of the intestinal wall. There are only 2 compartments within the isolated cell preparation, the cell and the medium, whereas in whole tissue there are the mucosal, submucosal, muscular and serosal layers; these may act as physical barriers to diffusion or as storage space in the accumulation of compounds.

In 1890, the dissociation of tissue cells in calcium-free media was reported by Ringer. However, the first method used for the removal of intestinal epithelial cells was digestion with trypsin (Harrer, Stern & Reilly, 1964). Other methods were subsequently developed for cell isolation, including mechanical

techniques (Harrison & Webster, 1969; Levine & Weintraub, 1970), chemical disruption (Stern & Jensen, 1966; Søgnen, 1967) and enzymatic digestion using lysozyme (Huang, 1965), hyaluronidase (Perris, 1966; Kimmich, 1970), papain (Padron, Gallagher & Kent, 1973) and collagenase (O'Doherty & Kuksis, 1975).

With any method of tissue disaggregation, there is a possibility of cell damage. However, numerous authors have reported that isolated intestinal epithelial cells are structurally intact, possess the morphological characteristics of epithelial cells prior to isolation and retain their metabolic transport capabilities (Stern, 1966; Kimmich, 1970; Marsh, Peters & Brown, 1971; Wilson & Treanor, 1975; Kimmich, Carter-Su & Randles, 1977). Studies with epithelial cells isolated from rat intestine have been utilized widely to investigate metabolic and transport processes (Iemhoff, Van den Berg, De Pijper & Hülsmann, 1970; Reiser & Christiansen, 1971; Yousef & Kuksis, 1972; Sayeed & Baue, 1973; Gall, Butler, Tepperman & Hamilton, 1974).

The metabolic activity of isolated cells, as assessed by their respiratory activity, has generally been shown to decline rapidly following many of the isolation techniques. One of the longer surviving preparations was reported by Perris (1966) and involved the use of hyaluronidase; oxygen uptake was linear for up to 40 minutes. However, a more elaborate isolation technique also based on the use of hyaluronidase was described by Kimmich (1970) and yielded a harvest of cells which produced carbon dioxide and lactate from glucose at a

constant rate for a time period of greater than 2 hours.

It is probably unreasonable to assume that isolated epithelial cells are identical in behaviour to those cells in the intact tissue. By virtue of their situation they will be different. One important factor is the loss of orientation; the lateral and serosal surfaces as well as the mucosal surface become exposed to the incubation medium. However, isolated cells offer advantages in their ease of reproducibility of sampling and homogeneity of cell population.

Recently, vesicles have been employed for the investigation of transport processes. These may either be formed from lipids and as such are called liposomes, or alternatively isolated membrane vesicles may be prepared from the isolated brush border and basolateral plasma membrane fractions of intestinal epithelial cells. The preparation and use of liposomes as models of biological membranes has been discussed by Bangham, Hill & Miller (1974). The role of liposomes as a model system for the study of lipid bilayers has also developed into the medicinal field where therapeutic substances may be trapped between the lipid bilayers and administered to patients (Colley & Ryman, 1976). Isolated membrane vesicles have only been used to analyze epithelial transport (Hopfer, 1977). Such vesicles are devoid of intracellular metabolism and hence the complexity of epithelial transport is reduced by investigating only the membrane. Nevertheless one must approach with caution experimental models such as liposomes or isolated membrane vesicles which are so far removed from the in vivo situation, particularly if the observations derived from such experiments are to be

applied to the intact tissue.

In the present investigation, isolated rat jejunal epithelial cells, prepared by a modified method of that described by Kimmich (1970), were used to study folate uptake. The method of isolation employed the use of hyaluronidase and Millipore filters. Kimmich demonstrated that isolated small intestinal epithelial cells from the chick had the ability to actively transport amino acids and sugars, and that following treatment with dinitrophenol, ouabain and oligomycin, these specific transport processes ceased. Consonant with other studies utilizing this technique, Kimmich concluded that enzymatic digestion using hyaluronidase, produced a high yield of viable cells with good morphological, metabolic and transport properties. Several other authors have successfully employed hyaluronidase in cell isolation (Perris, 1966; Leslie & Rowe, 1972; Bihler & Cybulsky, 1973; Barrett, 1975; Blair et al., 1975a; Wilson & Treanor, 1975; Blair et al., 1976). The use of Millipore filters is also advantageous since it both facilitates the rapid collection of cell samples and allows the cell pellet to be extensively washed of contaminating fluid. One of the range of commercially available Millipore filters becomes translucent when placed in toluene-based scintillation cocktail and thus direct counting of the radioactivity bound to the cell pellet is possible.

A model of folic acid transfer was proposed in the preceeding chapter, based on the observation that following the conversion of folic acid to its neutral form within the acid microclimate, there was a rapid

uptake of folic acid into the tissue. This was followed by accumulation therein which appeared to be largely fluid independent. 12 minutes following the commencement of incubation, folic acid appeared slowly in the serosal compartment with a close association of fluid transfer. With the aid of isolated cells, the rapid uptake component could be investigated commencing 60 seconds after the start of incubation and at similar short time intervals thereafter.

It would seem quite feasible that the use of the enzyme hyaluronidase, which specifically catalyzes the hydrolysis of hyaluronic acid, itself abundant in the cell coats of vertibrates (Lehninger, 1975), might disrupt or remove the glycocalyx from isolated cells. Leslie and Rowe (1972) reported that following hyaluronidase treatment, no glycoproteins were demonstrated on the brush border membrane. If this was the case, then hyaluronidase treatment should remove the glycocalyx from the epithelial cells and hence the pH of the acid microclimate might be altered. Thus one of the objectives of the present study was to investigate the process of folate uptake in the absence of the glycocalyx. As discussed in the previous chapter, the transport of folic acid is a saturable process, whereas that of the related species, 5-methyltetrahydrofolic acid is unsaturable at physiological concentrations. However, for successful absorption of both compounds, there should be an acid microclimate at the surface of the epithelial cell (Blair & Matty, 1974) and thus a loss or depletion of the glycocalyx, the postulated retaining layer for the acid microclimate,

would result in the depression of folate transport.

The aim of the present study was to use a viable preparation of isolated rat jejunal epithelial cells to investigate the uptake process of folic acid and 5-methyltetrahydrofolic acid within the first 6 minutes of incubation. The histological appearance of the isolated cells was also examined, especially to determine whether the glycocalyx was altered in any way following the enzymatic isolation of the cells.

Section 2. MATERIALS AND METHODS.

ANIMALS.

Details of the care and maintenance of the rats used for the preparation of isolated jejunal epithelial cells were given in chapter 3 section 2. Food and water were allowed ad libitum until the time of death by cervical fracture.

CHEMICALS.

Details of the chemicals and preparation of solutions involved in this study are similar to those given in chapter 3 section 2. In addition, hyaluronidase (type 1), bovine serum albumin (fraction v) and 5-[^{14}C] methyltetrahydrofolic acid, barium salt (>53 mCi/mmol) were also used and were supplied by Sigma Chemical Co., London and The Radiochemical Centre, Amersham. The purity of the radiochemicals was confirmed by column chromatography (see appendix).

ISOLATED EPITHELIAL CELLS.

Following the death of the rat by an acute cervical fracture, the abdomen was opened and the jejunum excised. The tissue was placed in a plastic dish with saline (154 mM NaCl) at 0-4^{o}C. The proximal jejunum was everted as previously described, and 12 cm was cut into approximately 4 cm lengths. Each piece was cut longitudinally to form a flat sheet of tissue. The same procedure was followed using a second rat, and the sheets

of tissue from both rats were gently blotted on saline-damp filter paper and transferred to a 50 ml polypropylene Erlenmeyer flask (Xlon Products Ltd., London) containing 20 ml of the isolation media at 37°C. This medium, described by Kimmich (1970), was a Tris-chloride buffer (20mM, pH 7.4) containing sodium chloride (120 mM), dipotassium hydrogen phosphate (3 mM), magnesium chloride (1 mM), calcium chloride (1 mM), bovine serum albumin (1 mg/ml) and hyaluronidase (1 mg/ml).

The tissue was incubated at 37°C for a time period of either 30 or 60 minutes in the presence of hyaluronidase, using a Griffin 100 series shaking incubator running at 100 oscillations per minute. Gentle gassing with a mixture of carbon dioxide and oxygen (95%:5%) was achieved via a soft silicone rubber tube; such tubing was chosen to prevent structural damage to the tissue. Plastic apparatus was used throughout for the same reason, particularly since Gall et al. (1974) observed lysis of isolated intestinal epithelial cells when glassware was used rather than plastic apparatus.

At the end of the incubation period, the contents of the flask were transferred to a plastic dish where the epithelial cells still adherent to the underlying mucosa were gently removed. This was accomplished by using a specifically designed polythene utensil with a blunt tip of approximately 5 mm width. The cells thus harvested were transferred to ice cold 15 ml plastic centrifuge tubes and centrifuged at 650 rpm for 1 minute. The supernatant fluid was discarded and the cell pellet was gently resuspended in the incubation medium (0 - 4°C),

namely the isolation medium minus the hyaluronidase. This washing procedure was repeated twice, and following transfer to a single tube, the cells were finally spun down to form a loose pellet.

The isolated cells were then added to a 25 ml polypropylene Erlenmeyer flask with 10 ml of the Kimmich incubation medium containing the appropriate concentration of substance under investigation. This solution had previously been gassed with a mixture of carbon dioxide and oxygen (95%:5%), and was at a temperature of 37°C. A second flask was also incubated to provide blank results; to this an equivalent volume of incubation medium minus cells was added. As time was limited, only two samples of 100 µl were withdrawn as blanks from this flask: at the initiation of cell incubation and at the end of the 6 minute incubation period. The flasks were shaken in a Griffin 100 series shaking incubator running at 100 oscillations per minute (amplitude 3 cm).

Cell samples were withdrawn from the cell incubation flask at minute intervals for up to 6 minutes. These samples were inserted into plastic Millipore 'swinnex' filter holders each containing a single dry MF-Millipore filter (25 mm, 0.8 µ pore size)obtained from Millipore (UK) Ltd., London. The filter and cell pellet, or filter and blank sample, were then washed with 10 ml ice cold 154 mM sodium chloride drawn through the filter by negative pressure provided by a vacuum pump. The entire unit comprising 12 such filters is shown in plate 47, and was specifically designed to facilitate rapid and precise manipulations. The syringes were filled with ice

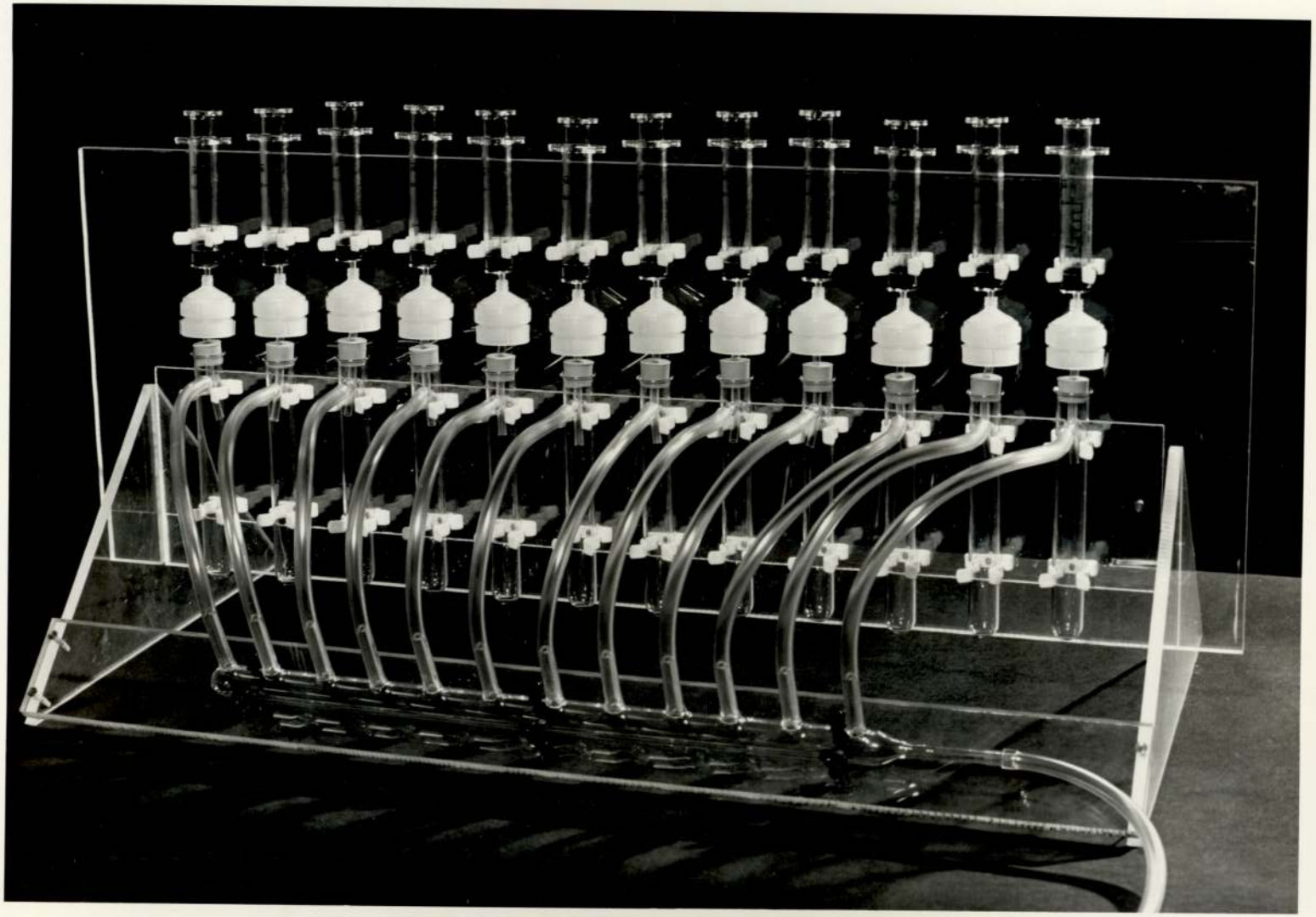


Plate 47. The unit designed to hold 12 Millipore filters.

cold saline immediately prior to cell incubation and following the introduction of each sample in the 'swinnex' holder, the washing procedure could be implemented within seconds. Contaminating fluid was collected together with the waste saline in a side arm test tube beneath the filter.

After each sample had been obtained, the filters were oven-dried and placed into standard size liquid scintillation vials. Each filter was positioned at the bottom of the vial and the filter orientation was always with the cell pellet facing upwards. 10 ml of scintillation cocktail (500 ml toluene and 2.5g PPO) was added to each vial causing the moistened filter to become transparent. This occurred since the filters had the same refractive index as the scintillation cocktail. The radioactive content of the vials was counted for 3 runs of 10 minutes each in a liquid scintillation spectrometer (NE 8305, Nuclear Enterprises, Edinburgh).

CALCULATION OF RESULTS.

Aliquots were taken from the blank flask containing the incubation medium minus cells, to provide a mean value for the standard count. These aliquots were placed directly onto Millipore filters in scintillation vials, oven-dried and 10 ml of scintillation cocktail was added to each vial which was then placed into a liquid scintillation spectrometer and counted as described above.

Additional aliquots were also removed at the end of the experiment from both the blank and cell incubation flasks. These aliquots were placed on preweighed

planchets and dried overnight in an oven at 90°C. The mean weight of the blank aliquots was subtracted from the mean cell weight to provide the mg dry weight of cells per aliquot.

Counts obtained for the filters onto which had been placed individual blank samples, at both the initiation of cell incubation and after 6 minutes as described above, were only minimal but were still subtracted from the experimental counts. Assays of background radiation were also made routinely with each experiment and the mean value was subtracted from the data obtained from the liquid scintillation spectrometer. It was found unnecessary to correct for quenching as this was similar in all the instances; each vial contained a single Millipore filter. Using the experimental standard data for each experiment, the uptake of the compound under investigation could be calculated as p.moles uptake per mg cellular dry weight.

Statistical calculations including standard error of the mean, student's t-test, least squares regression and correlation coefficient were performed with the aid of a desk computer (Programma 101, British Olivetti Ltd., London). Probability levels of $p = < 0.05$ were accepted as being statistically significant.

UPTAKE STUDIES.

i) Leucine.

Estimations of isolated cell viability were made by examining the capability of jejunal epithelial cells to transport L-leucine. Isolated cells were obtained from

tissue incubated for either 30 or 60 minutes in the presence of hyaluronidase (1 mg/ml). The cells were subsequently incubated for up to 6 minutes in 10 ml test medium containing 2.25 x 10^{-3}M L-leucine and 0.2 μCi ^{14}C L-leucine (specific activity of 10mCi/m.mol).

ii) Folic acid.

Folic acid was added to the incubation medium to cover a range of concentrations from 0.5 x 10^{-6}M to 2.3 x 10^{-5}M. Unlabelled folic acid was used with ^{14}C folic acid (specific activity of 10 mCi/m.mol) to achieve the concentrations required. The amount of radioactivity per 10 ml incubation medium was 1.2 μCi for the higher concentrations of folic acid and slightly less for the lower concentrations. Folic acid uptake over 6 minutes was investigated at all concentrations with cells which had been obtained as a result of 30 minutes incubation with hyaluronidase (1 mg/ml). In addition, the uptake of 10^{-6}M folic acid was examined with cells which had been isolated by 60 minutes incubation with hyaluronidase (1 mg/ml).

iii) 5-methyltetrahydrofolic acid.

Isolated cells were obtained following incubation for 30 minutes with hyaluronidase (1 mg/ml). The cells were subsequently incubated for up to 6 minutes in the presence of 5-[^{14}C] methyltetrahydrofolic acid (specific activity of >53 mCi/mmol) over a concentration range from 0.5 x 10^{-6}M to 2.5 x 10^{-5}M. On opening the manufacturer's vial of 5-methyltetrahydrofolic acid, the unstable contents

were immediately mixed with 2% sodium ascorbate to prevent oxidation, and used rapidly thereafter.

HISTOLOGICAL STUDIES.

In addition to leucine uptake, cell viability was also estimated by determining the fraction of the cell population capable of extruding 0.2% trypan blue (Girardi, Michael & Henle, 1956).

A histological examination was made on proximal jejunum following the isolation of epithelial cells. The histological method used was as described in chapter 2 section 2 and the periodic acid-Schiff staining procedure was employed. The photomicrographs of such tissue were taken at a lower magnification (x 320) than that shown on previous plates (x 1280).

Isolated epithelial cells were examined histologically: following 6 minutes incubation in the presence and absence of radioactive (^{14}C) folic acid and after either 30 minutes or 60 minutes incubation with hyaluronidase (1 mg/ml). For examination, a smear of cells was made on a clean microscope slide which was allowed to air dry before being placed in a solution of 95% alcohol overnight. The cell smear was subsequently rinsed with water and stained by the periodic acid-Schiff method. Photomicrographs of the isolated cells were taken at a magnification of x 1280.

Section 3. RESULTS.

UPTAKE STUDIES.

i) Leucine.

The uptake of leucine (2.25×10^{-3}M) over 6 minutes by isolated rat jejunal epithelial cells prepared by tissue incubation for 30 minutes in medium containing hyaluronidase (1 mg/ml), is shown in figure 4,1. The uptake was significantly linear corresponding to a rate of 0.62 n.moles/mg dry wt/min, and the estimated final concentration of leucine within the cells was 1.01×10^{-3}M. Backwards extrapolation of the line of best fit did not pass through zero but through the ordinate at 1.6 n.moles per mg dry weight.

Figure 4,2 shows the uptake of leucine by isolated cells which were obtained following tissue incubation for an extended period of 60 minutes in the presence of hyaluronidase (1 mg/ml). At the end of the incubation period with leucine, the estimated concentration in the cells was 3.73×10^{-3}M. The uptake was linear with time and the regression coefficient was significant ($P = < 0.001$). Backwards extrapolation of the line of best fit passed essentially through the origin and the rate of uptake was calculated as 3.49 n.moles/mg dry wt/min.

Table 4,1 presents a comparison of results for leucine uptake by cells isolated either as a result of 30 minutes or 60 minutes incubation with hyaluronidase. Leucine was taken up to a greater extent by cells which were isolated after 60 minutes incubation with hyaluronidase;

this was reflected by an approximate 5-fold increase in the rate of uptake. After 6 minutes of incubation with leucine, the cells isolated after 30 minutes hyaluronidase contact only accumulated 27% leucine compared with the other group of cells. Another marked contrast between the two groups of cells was the difference in the intercept of the ordinate caused by backwards extrapolation of the line of best fit; cells incubated in hyaluronidase for 30 minutes showed a raised intercept on the ordinate compared to cells incubated for 60 minutes.

ii) Folic acid.

The uptake of folic acid over a concentration range of 0.5×10^{-6}M to 2.3×10^{-5}M was investigated during the first 6 minutes of incubation with isolated cells. These cells had been isolated as a result of 30 minutes contact with hyaluronidase and the results are illustrated in figure 4,3. The details of the regression coefficients, rate of uptake and intercept are given in table 4,2. Uptake was most linear at the 10^{-6}M and 10^{-5}M concentrations and the rate of uptake increased with increasing concentration. Backwards extrapolation of the slope at all concentrations, resulted in a positive intercept on the ordinate. The amount of folic acid associated with the cells increased as a function of time (table 4,3) and the standard errors of the mean values, which were of some magnitude, also increased with both time and concentration (table 4,3).

Figure 4,4 illustrates the rate of uptake of folic acid plotted as a function of concentration. The

regression coefficient of the slope was 1.00; this pattern of uptake is not consistent with that for a structurally specific transport system, which would saturate with increasing concentration. The concentrations investigated were within the experimental range for folic acid used by other authors (Smith et al., 1970; Halsted & Mezey, 1972; Leslie & Rowe, 1972; Blair et al., 1974). An attempt to apply these results to Michaelis-Menten kinetics by forming a Lineweaver-Burk plot from the reciprocals of substrate concentration and reaction velocity, gave a poor fit to a linear curve ($P = >0.05$). However, this was not surprising in view of the degree of linear fit in figure 4,4 obtained without plotting the reciprocals. The estimated final concentration of folic acid associated with the cells was always lower than the concentration of the folic acid in the incubation medium (table 4,4). Thus, in view of the poor fit to a Lineweaver-Burk plot and the concentration in the cells, the uptake of folic acid by isolated cells in the first 6 minutes of incubation would appear to conform to a passive mechanism.

The above-mentioned positive intercept on the ordinate at all concentrations (figure 4,3) could be artefactual, reflecting incomplete removal of adherent folic acid during the post-incubation washing; alternatively it might be the result of rapid binding of folic acid onto the cells prior to the first minute of incubation. With the aim of investigating this phenomenon further, the magnitude of the intercept values or rapid component was plotted against concentration, and the results are shown in figure 4,5. The resultant curve was linear ($P = <0.01$)

indicative that the immediate uptake process was not a structurally specific mechanism, however, neither was the slope 1.00 which would have suggested that the observation of an intercept on the ordinate was purely artefactual. Thus, there is support for either a passive uptake process occurring prior to the first minute of incubation, as well as for an artefact due to incomplete washing of substrate from the cells. Figure 4,6 illustrates the relationship between the intercept values and the rate of folic acid uptake. Although not unexpected in view of previous correlations, a linear relationship was obtained ($P = < 0.01$).

Figure 4,7 illustrates the distinct difference in uptake at 10^{-6}M folic acid, by cells isolated as the result of either 30 minutes or 60 minutes of tissue incubation with hyaluronidase. The rate of folic acid uptake was depressed by the 60 minute hyaluronidase incubated cells to 0.18 p. moles/mg dry wt/min and the relationship was no longer significantly linear ($P = > 0.05$), compared to the 30 minute incubated cells which had a rate of 0.39 p. moles/mg dry wt/min and showed a linear relationship ($P = < 0.001$). 60 minutes incubation in medium containing hyaluronidase caused a depression of the intercept on the ordinate; such an observation was also made when the uptake of leucine was investigated with cells previously incubated in medium containing hyaluronidase for an extended period of 60 minutes. The folic acid uptake by the 60 minute incubated cells was significantly lower than that for the 30 minute incubated cells (table 4,5); the estimated final concentration of folic acid associated with the cells being 3.05×10^{-7}M and 8.83×10^{-7}M respectively.

iii) 5-Methyltetrahydrofolic acid.

The isolated cell uptake of 5-methyltetrahydrofolic acid during the first 6 minutes of incubation, over a range of concentrations from 0.5×10^{-6}M to 2.5×10^{-5}M is illustrated in figure 4,8, with the corresponding data with standard errors of the mean values shown in table 4,6. The cells were isolated by tissue incubation for 30 minutes in the presence of hyaluronidase. There was a degree of variation in the uptake and although the 5-methyltetrahydrofolic acid taken up by the cells at each concentration was essentially linear over 6 minutes, the statistical fit to a linear curve was poor as reflected by the regression coefficients (table 4,7). Table 4,7 also illustrates the proportionately increased rate of uptake and intercept values with concentration. The estimated final concentration of 5-methyltetrahydrofolic acid within the cells did not reach the respective concentration of substrate in the incubation medium (table 4,8). Such an observation would implicate a passive transport mechanism, or alternatively that 6 minutes was not a sufficient time period for the uptake against a concentration gradient to have occurred, particularly as saturation of uptake with time was not obvious.

Figure 4,9 illustrates the relationship between the 5-methyltetrahydrofolic acid rate of uptake values from table 4,5 as a function of concentration. The rate of uptake increased with concentration in a linear fashion ($P = <0.01$). A poor fit ($P = >0.05$) to a linear curve was obtained with a Lineweaver-Burk plot of this data.

Thus, the immediate uptake of 5-methyltetrahydrofolic acid would appear to be that of a non-saturable mechanism which does not have a structurally specific transport system.

Similar to the results of cell uptake of folic acid described above, a positive intercept was obtained on the ordinate when the uptake of 5-methyltetrahydrofolic acid was plotted against time (figure 4,8). The magnitude of this intercept value showed a linear relationship ($P = <0.01$) with concentration (figure 4,10); this could imply that the rapid uptake of 5-methyltetrahydrofolic acid on to cells prior to the first minute of incubation was an artefactual process, or alternatively a non-saturable uptake mechanism, but certainly not due to a specific transport mechanism. Figure 4,11 illustrates the significant linear relationship ($P = <0.01$) between the intercept values and the rate of uptake; this would be expected in view of the linear relationship between rate and concentration.

HISTOLOGICAL STUDIES.

Estimations of cell viability by the trypan blue exclusion method were performed routinely at intervals throughout the series of experiments described above. Cells were counted using a haemocytometer, but in view of the difficulty in quantitating cell numbers accurately, particularly as groups of cells may clump together, only approximate estimations of viability could be obtained. Overall, trypan blue stained less than 20% of the total cell population indicating approximately 80% viability.

Plate 48 shows the appearance of rat jejunal tissue following the isolation of epithelial cells by hyaluronidase. It may be observed that only lamina propria remained and the complete layer of mature epithelial cells was stripped off at the basement membrane; a strip of cells may be seen to be detached from the villus. Under higher magnification, the isolated epithelial cells appeared to have an intact glycocalyx. Immature epithelial cells in the crypts of Lieberkühn were not removed by the isolation technique.

Isolated epithelial cells appeared normal under histological examination (plate 49). The majority were observed as single cells although a large number could be seen as continuous strips of 2 to 5 cells, unbroken at their lateral membranes. There was a degree of cell clumping in the samples, and an amount of mucus was generally associated with such cells. No detrimental effect was observed following cell incubation in the presence of radioactive folic acid at a quantity and specific activity of that used in the experiments. However, there was a difference in the appearance of cells which had been incubated for 30 minutes and for 60 minutes prior to cell isolation. The cells which had been incubated in hyaluronidase for 30 minutes (plate 49) had an apparently continuous or normal glycocalyx whereas those previously incubated for 60 minutes (plate 50) had a glycocalyx which appeared altered, either paler than normal or absent.

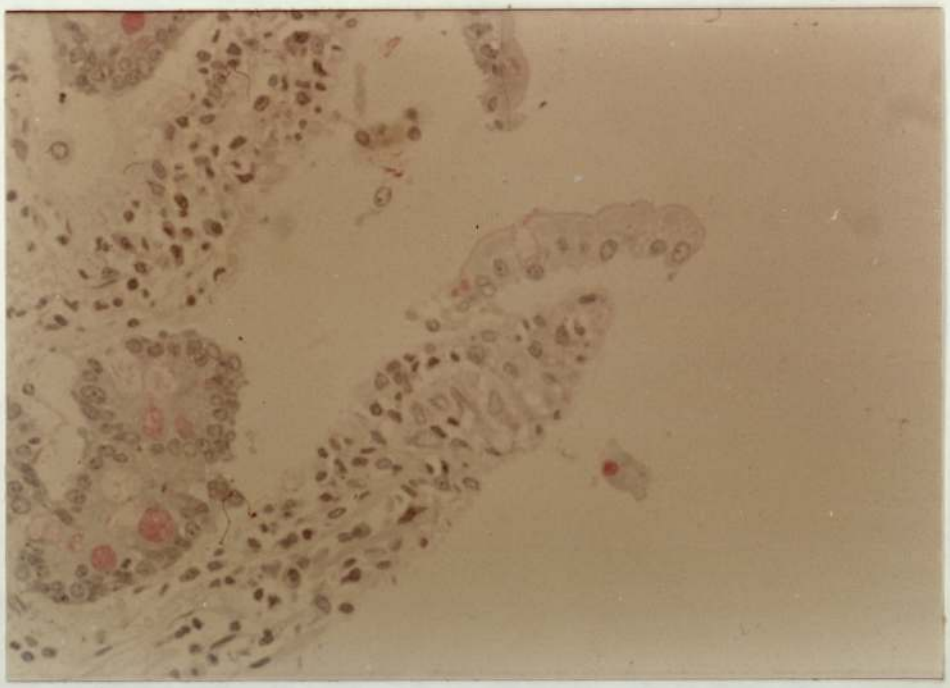


Plate 48. Rat jejunum following epithelial cell isolation.

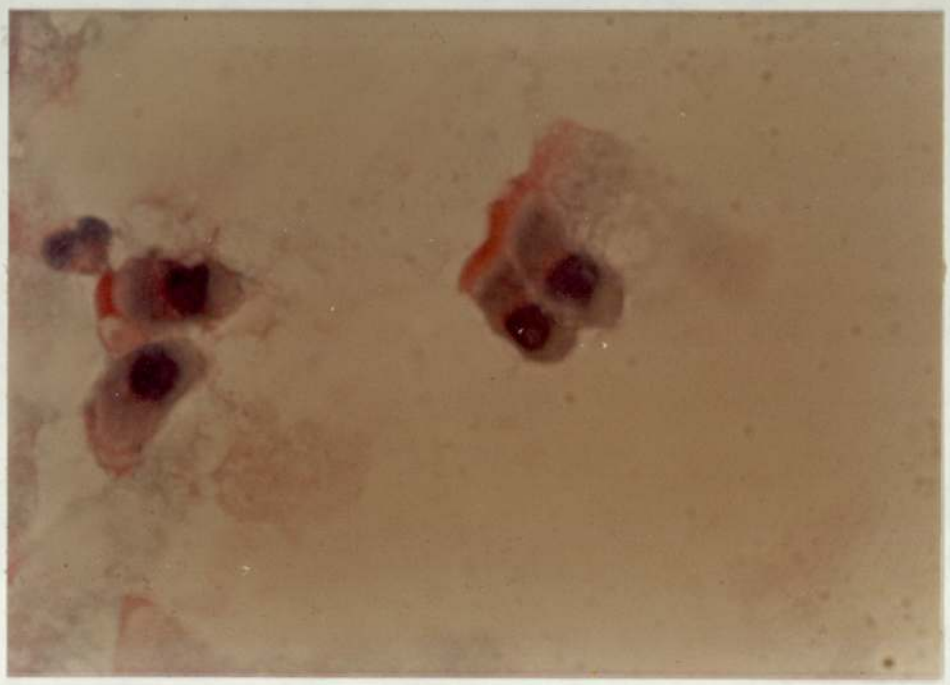


Plate 49. Isolated epithelial cells, 30 minutes hyaluronidase incubation.

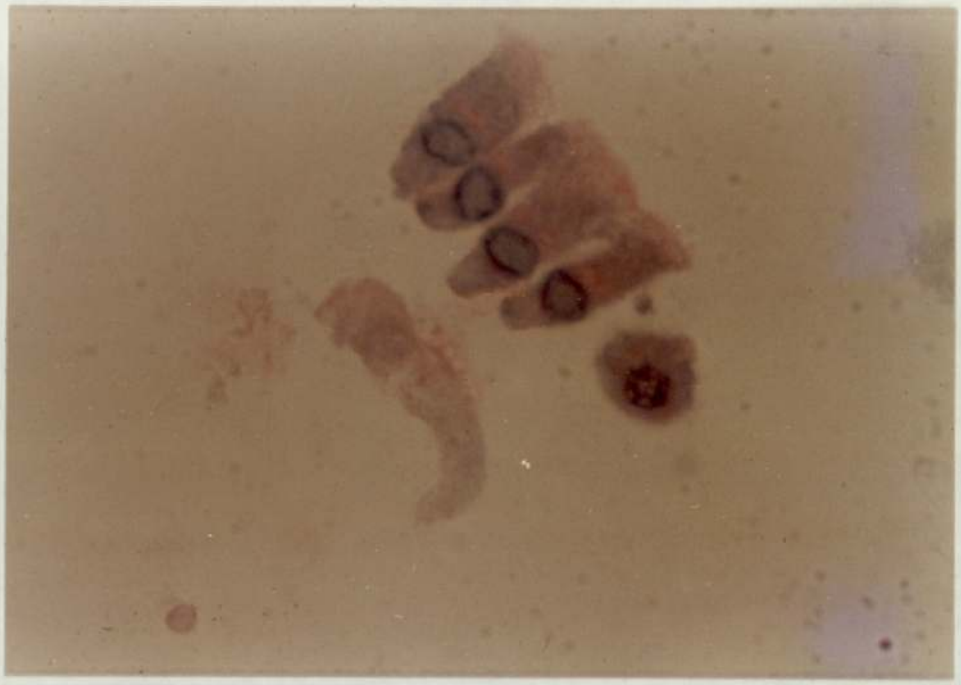


Plate 50. Isolated epithelial cells, 60 minutes hyaluronidase incubation.

Section 4. DISCUSSION

An isolated cell technique using hyaluronidase, based on the method described by Kimmich (1970), produced a high yield of morphologically intact, viable rat jejunal epithelial cells suitable for use in transport studies. Initial viability assessment using trypan blue gave an approximate estimation of 80% viability. However, this simple dye exclusion method and widely applied criterion for investigation of cell viability has been criticised (Tennant, 1964; Medzihradsky & Marks, 1975). A more suitable indicator of cell viability was the capacity of the cells to transport L-leucine; this was an indication of a functional active transport mechanism and therefore of a viable preparation.

Linear transport of leucine was observed over 6 minutues incubation. Cells isolated as a result of incubation with hyaluranidase for 30 minutes had not obtained an estimated internal concentration of leucine greater than that in the incubation medium although maximal leucine uptake had not yet been achieved with time. The quantity of leucine taken up by the cells was comparable to that obtained under similar conditions by other authors (Reiser & Christiansen, 1971; Leslie & Rowe, 1972). Cells isolated as a result of 60 minutes incubation with hyaluronidase showed an uptake of leucine which was markedly greater than the 30 minute incubated cells, and also achieved a concentration within the cells which was greater than that in the incubation medium. An explanation of this increased uptake of leucine

following 60 minutes incubation with hyaluronidase may be postulated from a study of the morphological appearance of the cells examined histologically.

Tissue treatment with the enzyme hyaluronidase, provided isolated cells which had a normal morphological appearance. However, there was a difference in the appearance of the glycocalyx on cells incubated with hyaluronidase for 30 minutes compared to those incubated for 60 minutes. With the latter group of cells the glycocalyx appeared less obvious, if not completely absent; reiterating the discussion from the preceeding chapters, if the glycocalyx is disrupted, the microclimate pH might feasibly be altered such that it would become less acid, having lost its intact retaining area for the hydrogen ions which give rise to an acid microclimate on the epithelial cell surface. The additional 30 minutes of exposure of the cells to hyaluronidase would appear to have disrupted the glycocalyx such that the luminal side of the epithelial cells would present an abnormally less acid region to any prospective substrates for transport. The transport of leucine in isolated cells has been reported to increase with increasing pH of the incubation medium (Reiser & Christiansen, 1971). It would seem feasible therefore that the link between the observed altered appearance to the glycocalyx and hypothesised altered microclimate pH values was valid. A move towards neutrality in the pH of the incubation medium or acid microclimate, would increase the percentage of the zwitterionic form of leucine present and one could postulate

that leucine would be transferred as the neutral species according to the theory of non ionic diffusion (Schanker et al., 1959). However, there is little doubt concerning the existence of an active transport mechanism for leucine uptake utilizing a sodium dependent carrier system (Curran, Schultz, Chez & Fuisz, 1967; Reiser & Christiansen, 1971; Wiseman, 1974) which varies in efficiency with pH (Thompson, Levin & Jackson, 1970; Reiser & Christiansen, 1971).

It is relevant in a study utilizing isolated cells, to consider the problems related to loss of polarity and modified function of isolated intestinal epithelial cells. In the isolated cell population, both brush border and basolateral cell boundaries are exposed, and solute fluxes at all surfaces can contribute to observed transport rates. This problem has concerned several authors and there is evidence to suggest that there is a monosaccharide transport system situated on the basolateral border of the epithelial cell; this system however, is sodium independent and functions as a facilitated diffusion process which only occurs down a chemical gradient (Bihler & Cybulsky, 1973; Kimmich & Randles, 1975). A similar sodium independent neutral amino acid transport system has been established on the basolateral membrane of the epithelial cell (Hopfer, Sigrist-Nelson, Ammann & Murer, 1976). This would also function as a facilitated diffusion process. However, Kimmich (1975) considers that solute accumulation by isolated cells represents primarily brush border transport capability; approximately 75% active valine transport may be inhibited by 3 - O-methylglucose in a manner completely

abolished by phloridzin which is a potent inhibitor of epithelial cell sugar transport at the mucosal boundary. Such an observation may indicate that a high proportion of amino acid entry occurs at the mucosal pole of the cell. Thus the loss of polarity in isolated cell preparations is a factor of legitimate concern, but if solute transport occurs primarily via the brush border then the concern need not be so great as to daunt the use of isolated cell preparations.

According to the theory of folic acid transport via an acid microclimate, presented in the preceeding chapters, loss of cell polarity should not greatly influence folic acid uptake since the acid microclimate is hypothesised to exist at the mucosal pole of the epithelial cell within the glycocalyx. The basolateral membrane is without a structurally suitable retaining layer for a microclimate. However, the unstirred layer which is proposed to exist within the intestinal lumen, is without a retaining structure. This layer is a region of unstirred water (Westergaard & Dietschy, 1972) whose resistance to solute molecules has been claimed to give marked under-estimations of permeability coefficients for passive transport processes, and significant over-estimation of apparent Km values for active transport processes (Wilson & Dietschy, 1974; Wilson & Treanor, 1975). The unstirred water layer is not considered to involve the glycocalyx (Westergaard & Dietschy, 1972) and has a thickness in jejunum <u>in vitro</u>, dependent on the rate of mixing of the bulk solution (Wilson & Dietschy, 1972), of 110-330μ (Westergaard & Dietschy, 1974; Wilson & Dietschy, 1974) and in jejunum

in vivo of at least 530μ (Winne 1976). The thickness of the jejunal acid microclimate is estimated to be less than 20μ (Lucas et al., 1975) and is therefore of a much smaller dimension than the unstirred water layer. Appropriate correction calculations have been provided for the existence of an unstirred water layer (Winne, 1977, 1977a) and should it become the custom to involve such complex calculations in kinetic analysis no doubt an amount of the kinetic data on various solutes would be considered vastly inaccurate. Nevertheless, Wilson and Treanor (1975) have reported that isolated epithelial cell preparations are ideal in providing the conditions in which the unstirred water layer artefacts are minimized, and in their study of bile acid uptake, the apparent permeability coefficients were considered to be true. It was with this feature in mind that the appropriate correction calculations were not performed on the data in the present study.

One of the aims of the present investigation was to examine the cellular uptake of folates within the first few minutes of incubation. One feature which was constant with both folic acid and 5-methyltetrahydrofolic acid was the intercept on the ordinate when the line of best fit was extrapolated backwards. It was discussed in the preceeding section that this observation was not due to a structurally specific uptake mechanism since plotting the intercept values as a function of concentration gave no evidence of saturation. There was support for either a passive mechanism or an artefactual process. Error in determination of uptake rates may be caused by bathing solution adherent on to cells and carried over into the counting vials (Sallee, Wilson & Dietschy, 1972). No

radioactivity was retained on the filters following the 10 ml wash in saline, as this was checked with each experiment. However, how much of the cell-associated radioactivity was adherent cell fluid could not be determined. There is no suitable method for quantifying the volume of adherent extracellular fluid associated with a cell pellet after Millipore filtration, as extracellular markers such as polyethylene glycol and inulin bind to the filters and therefore give incorrect values for trapped extracellular fluid (Kimmich, 1975). In spite of this, Wilson and Treanor (1975) estimated the amount of adherent extracellular fluid on rat isolated intestinal epithelial cells to be approximately 50 µl per 100 mg cell protein; this volume was achieved within the first 0.5 minutes of cell incubation.

A saturable binding of folic acid onto isolated rat jejunal epithelial cells has been reported (Leslie & Rowe,1972) using a similar method as that in the present study. Isolation of the brush border region of such cells by Leslie and Rowe, demonstrated a protein which had a high affinity for folic acid. Maximum binding of folic acid had occurred by the time they removed the cell samples for the initial analysis (approximately 2 minutes) and following this, no further accumulation of folic acid was observed. Binding of folic acid to a binding protein could therefore have occurred in the present study, although not apparently saturable at the concentrations investigated; Leslie and Rowe obtained a binding constant of 3.98×10^{-5}M which was beyond the concentration range of folic acid investigated in the present study. Another factor is that the

investigations of Leslie and Rowe were performed on chick intestinal cells and there could be a species difference in folic acid transport although this would seem unlikely. Hence it cannot be determined whether the observation of a positive intercept on the ordinate represents an immediate binding of folic acid onto tissue or merely an experimental artefact; however, at least a minimal amount of the phenomenon must be considered to be an artefact. A similar argument must apply to the immediate uptake of 5-methyltetrahydrofolic acid prior to the first minute of incubation, although it would be more likely for 5-methyltetrahydrofolic acid to bind to a protein and exhibit saturation kinetics as it occurs naturally in man whereas folic acid, a compound not occurring naturally, would be unlikely to have a specific binding protein.

The present investigation differs further from the studies of Leslie and Rowe (1972) in that they observed no further uptake of folic acid following its initial rapid binding. Blair *et al*. (1976) however, did obtain an uptake of folic acid using the same method and produced results in agreement with those in the present study. The reported failure of Leslie and Rowe's isolated cells to accumulate folic acid may have been due to the loss of the glycocalyx and hence microclimate, as they failed to detect any glycoproteins within the brush border membrane proteins. In the present study, the glycocalyx remained present following 30 minutes of tissue incubation with hyaluronidase. In a transmission electron microscopic investigation of chick cells obtained following 30 minutes incubation with

hyaluronidase, Barrett (1975) presented photomicrographs which also clearly showed the presence of a glycocalyx. It would therefore appear that the Leslie and Rowe preparation, in spite of appearing largely identical, was at variance with other authors. One possible explanation could be differences in the purity, and activity of the hyaluronidase batches employed.

Further discrepancy is to be found in the results of folic acid uptake by isolated cells obtained using methods other than hyaluronidase disaggregation. Both Hepner and Herbert (1969) and Momtazi and Herbert (1973), unlike Leslie and Rowe (1972), did observe cellular uptake of folic acid. However the first Herbert report described apparent saturation kinetics whereas the latter publication described a passive mechanism. The controversy which has existed over the mechanism of folic acid transport was mentioned in the previous chapter, although here it is additionally apparent with isolated cells. Nevertheless, few folate investigations have been performed with this preparation. The Hepner and Herbert (1969) work is only presented in abstract form and therefore details are not given. However, they reported a significant uptake of folic acid, by guinea pig small intestinal mucosal cells over a lengthy period of 5 hours, which decreased in percentage uptake with increasing concentration; in their later work (Momtazi & Herbert, 1973), using rat small intestinal epithelial cells isolated by vibration, they demonstrated linear uptake with concentration over 60 minutes with unchanged percentage uptake with increasing concentration. The Momtazi and Herbert method of

expressing results as uptake in pg per 10^6 cells complicates discussion of their work, indeed the range of concentrations at which these experiments were performed is unclear (0.28 to 25 ng per flask or per approximately 10^6 cells). By recalculating the data provided by Momtazi and Herbert, it would appear that the concentration of folic acid in their experiments was approximately 5×10^{-8}M. It is therefore not surprising that they did not observe saturation kinetics at this low concentration and indeed more surprising that Hepner and Herbert did observe saturation kinetics at an apparently lower concentration (assuming the experimental conditions were similar). In those folic acid transport investigations using preparations other than isolated cells (see Rosenberg, 1976), the bias is towards a saturable system of folic acid transport with a saturation value around 5.1×10^{-6}M (Blair *et al.*, 1974).

In the present study, folic acid transport was investigated over a time period restricted to the first 6 minutes of cell incubation, using folic acid over a range of concentrations from 0.5×10^{-6}M to 2.3×10^{-5}M. The first component of the transport process was observed as an immediate uptake, or intercept on the ordinate; this feature has been discussed above. The following component consisted of folic acid uptake which was linear with increasing concentration. This would initially appear inconsistent with the saturation of uptake which occurs with longer periods of tissue incubation either *in vitro* or *in vivo* (Smith *et al.*, 1970; Blair *et al.*, 1974; Halsted, Bhanthumnavin & Wright, 1974). It was

demonstrated in the preceeding chapter that it takes approximately 12 minutes after the commencement of incubation for folic acid to appear in the serosal compartment of everted sacs; this requires passage through the epithelial cells, submucosal and muscular layers. However, as the present study was carried out at a minimum of 60 seconds it has been assumed that in such a short time period, the folic acid would not have been transported completely through the epithelial cells. Indeed, in those studies reporting tissue accumulation of folic acid (Selhub et al., 1973; Smith, 1973; Blair et al., 1974) one can not be specific as to whether the accumulation was within the epithelial cells or deeper tissue layers. A component of the folic acid transport process which undoubtedly occurs within the first 6 minutes of incubation, is the transfer of folic acid as the neutral species, by diffusion into the cell, according to the theory of the acid microclimate and folate absorption (Blair & Matty, 1974). Therefore, the passive transport of folic acid into isolated epithelial cells described above, could be exactly according to this mechanism of diffusion as the neutral species.

Isolated cells, by the nature of their situation, having lost the structural support provided in the intact tissue, may assume alterations in their appearance including wider spaced or 'fanned out' microvilli (Dybing, Nafstad & Søgnen, 1969). Such an alteration could be associated with a decreased acidity of the microclimate as hydrogen ions would be lost more readily from their retaining area, despite the apparent continued

presence of the glycocalyx. The saturability of absorption of folic acid observed by many authors has been explained by Blair and Matty (1974) by the solubility of folic acid within the acid microclimate. In an acid microclimate of approximately 3, folic acid would reach saturation solubility at a concentration of approximately 2.3×10^{-6}M (Biamonte & Schneller, 1951). Indeed, the observed saturation value of 5.1×10^{-6}M (Blair et al., 1974) would indicate an actual microclimate pH around 3.5 (Blair & Matty, 1974). The present study may be interpreted to indicate that even in the presence of a normal glycocalyx, the microclimate was slightly less acid resulting in the demonstration of non-saturable kinetics. A seemingly less attractive alternative is that the saturation invariably observed, results from the use of whole tissue preparations.

5-Methyltetrahydrofolic acid transport by isolated cells during the first 6 minutes of incubation also showed linear uptake following the initial rapid uptake component, either artefactual or binding as discussed above. The literature available on this folate is not so abundant as that on folic acid; however, the reports to date have not demonstrated saturation of uptake at the concentrations investigated (Strum et al., 1971; Blair et al., 1975a). Only a single study exists of 5-methyltetrahydrofolic acid uptake by isolated cells (Blair et al., 1975a); these authors observed a small, but insignificant, uptake at pH7 between third and sixth minutes of incubation, and at pH5 there was a significant increase in uptake. Blair et al.(1975a) also observed a similar rapid uptake component to that demonstrated in the present investigation. The

unsaturable uptake described above, was investigated as being the component of transport occurring immediately after the rapid uptake component or artefact. The transport of 5-methyltetrahydrofolic acid conforms to the theory implicating an acid microclimate in folate absorption (Blair & Matty, 1974; Blair et al., 1975a) in which 5-methyltetrahydrofolic acid is absorbed passively as the neutral zwitterion. The importance of the jejunal acid microclimate in the transport of 5-methyltetrahydrofolic acid has recently been established (Kesavan & Noronha, 1978). These authors confirmed the dependence of 5-methyltetrahydrofolic acid uptake on an acid microclimate. Saturation of this folate within the acid microclimate would not be attained at physiological concentrations since at a pH of approximately 3, the saturation solubility is 3.2×10^{-2}M (Robb, 1975). Thus the passive component of uptake observed with both folic acid and 5-methyltetrahydrofolic acid immediately after the initial rapid component, could be the transfer of these folates as their neutral species through the microclimate. Indeed, the cells were observed by light microscopy to have retained their glycocalyces, and thus would have a suitable environment for retention of an acid microclimate.

The appearance of the glycocalyx on cells incubated with hyaluronidase for 30 minutes was different to those incubated for 60 minutes. There was also a marked difference between these two groups of cells in their ability to transport leucine and folic acid, the uptake of leucine being increased whilst the uptake of folic acid was decreased. Explanation of the effect on leucine uptake

has already been provided above, based on the morphological observation that the glycocalyx was either absent or disrupted on the cells incubated for the longer period of time. Hence the acid microclimate was less acid and favoured an increase in the transport of leucine. In accordance with this, folic acid transport was decreased as in a microclimate of lower acidity than normal, folic acid transport is reduced (Lucas et al., 1978). However, the mode of action of hyaluronidase must also be considered in order to provide an interpretation of its more pronounced effect on the glycocalyx with time. The isolated cells were dissociated from the rest of the tissue at the basement membrane, although the glycocalyx appeared unaffected in the 30 minute incubated cells. Two theories may be postulated to explain this bias in effect: firstly, the presence of an electrical charge, or the low pH of the acid microclimate, may have been unsuitable to the electrical charge of the hyaluronidase, thus inhibiting enzyme action on the luminal membrane. Alternatively, the stereochemical configuration of the hyaluronic acid in the glycocalyx may have been inconvenient for attack by hyaluronidase. Thus the effect of hyaluronidase, to catalyze the hydrolysis of the $\beta[1 - 4]$ linkages of hyaluronic acid, may have occurred preferentially in the basement membrane which resulted in the isolation of the epithelial cells. Increasing the cell contact time with hyaluronidase resulted in further morphological alterations. Enzyme activity increases with time and it may have taken the additional 30 minutes for the glycocalyx to become disrupted by the hyaluronidase.

Indeed, hyaluronidase may not have been the only enzyme exposed to the cells, as commercial enzyme preparations, particularly hyaluronidase, contain impurities and especially proteolytic enzymes (Kohnert, 1978). The presence of other enzymes would further disrupt the glycocalyx. Tissue permeability increases with exposure to hyaluronidase (Day, 1952) and as hyaluronidase is likely to affect all the membranes of the cell, the cell contents, including lysosomal enzymes would eventually be released, finally resulting in total cell disruption. However, this terminal stage had not been attained as the cells were demonstrated to be both structurally intact and viable after 60 minutes contact with hyaluronidase.

Before departing from the discussion of the effect of hyaluronidase on cells which were incubated in contact with the enzyme for 60 minutes, a further point must be raised. Although in these cells, uptake of leucine was increased and uptake of folic acid decreased, a common observation to both these experiments was a lowering of the intercept on the ordinate when the line of best fit was extrapolated backwards. This feature could challenge the possibility that the immediate component was an artefact, as increased leucine uptake would be expected to be associated with an increased value of the artefact. However, the same statistical observation with folic acid could imply the loss of a binding site, particularly as one has been postulated (Leslie & Rowe, 1972), and would be situated in the glycocalyx region which was disrupted by longer incubation of the cells. Only speculation can be made about the immediate component of uptake prior to the first

minute of incubation but the results in this chapter have implied a subsequent diffusional transport phase of the two folates, folic acid and 5-methyltetrahydrofolic acid; this transport occurring in the acid microclimate of the epithelial cells.

Section 5. SUMMARY

1) A hyaluronidase disaggregation method was used to prepare isolated rat intestinal epithelial cells which were shown to be viable as assessed by their ability to uptake leucine and exclude trypan blue. Normal morphological appearance of these mature epithelial cells was demonstrated by light microscopy using the periodic acid-Schiff staining technique.

2) The cellular uptake of the folates, folic acid and 5-methyltetrahydrofolic acid was studied during the first 6 minutes of incubation. There was shown to be an immediate component of uptake onto the isolated cells, which occurred prior to the first 60 seconds of incubation. A discussion has been given to the possibility that this represents either an artefact or binding onto a membrane site. However, the non-saturability of this process adds more weight towards the contention that it represents an artefactual observation.

3) Following the initial uptake, a second non-saturable process was observed to be the subsequent phase in the transport of the folates. This occurred between the first and sixth minutes of incubation, and was considered to be concomitant with the theory of passive diffusion of folic acid and 5-methyltetrahydrofolic acid into the cell, following conversion to their neutral species in the acid microclimate within the glycocalyx. The non-saturability observed with folic acid may be explained in terms of the minor

alterations in structure which occur to isolated cells by the nature of their detachment from intact tissue.

4) The glycocalyx was shown histologically to be less obvious, or absent, from cells which had been isolated as a result of 60 minutes incubation with hyaluronidase as opposed to 30 minutes. These cells were shown to have remained viable after the prolonged period of exposure to hyaluronidase. The removal of the glycocalyx was associated with a depression in the cellular uptake of folic acid. An explanation of this effect on folic acid was given as an inability of the folic acid to be converted into its neutral form; the acidity of the microclimate being less acid since its retaining layer, the glycocalyx, appeared structurally abnormal.

Figure 4,1. Uptake of leucine (2.25×10^{-3} M) by isolated cells, harvested following 30 minutes incubation in medium containing 1mg/ml hyaluronidase. (m ± sem, n = 6 pairs of rats).

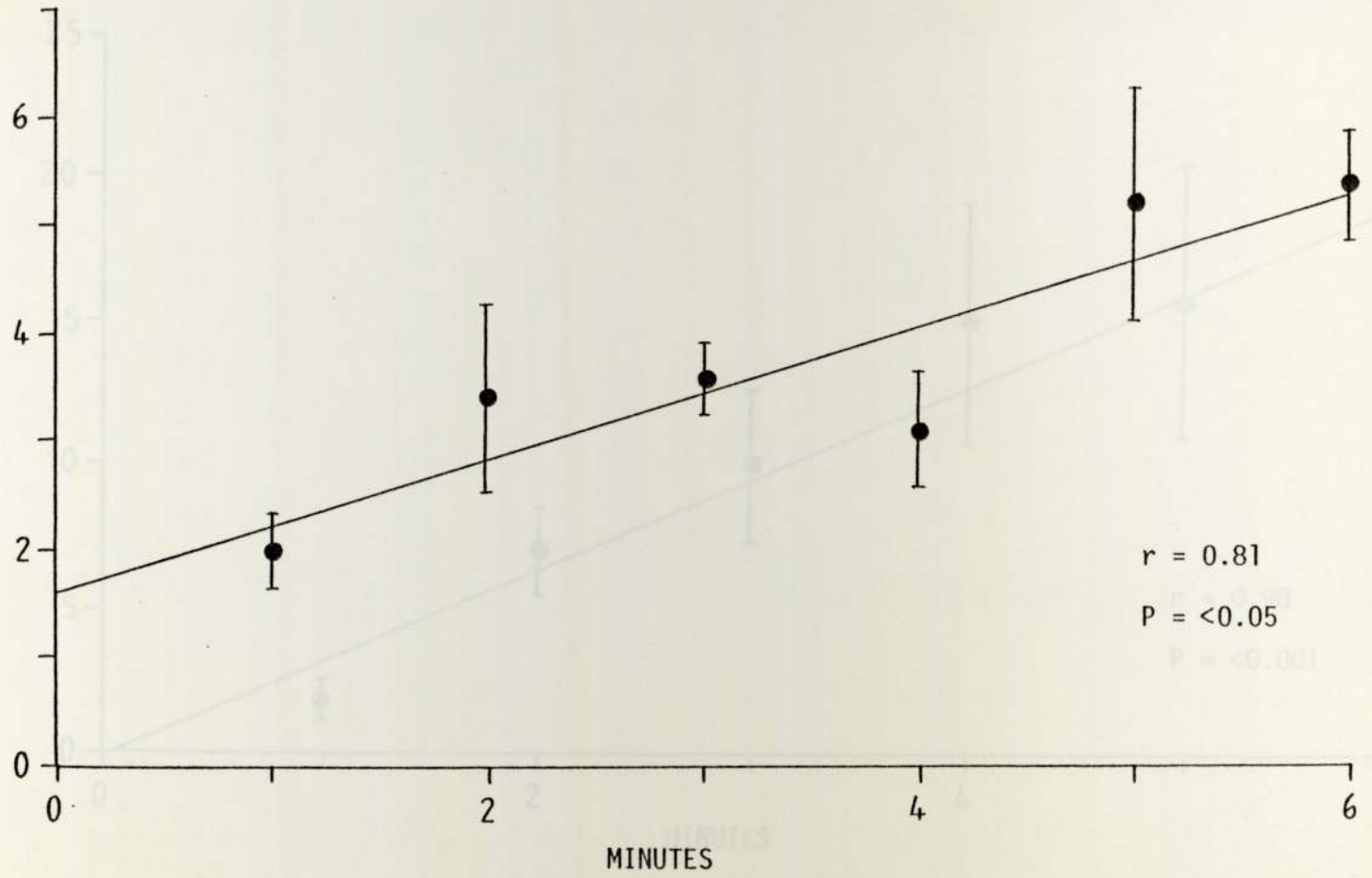


Figure 4,2. Uptake of leucine (2.25×10^{-3}M) by isolated cells, harvested following 60 minutes incubation in medium containing 1mg/ml hyaluronidase. (m ± sem, n = 6 pairs of rats).

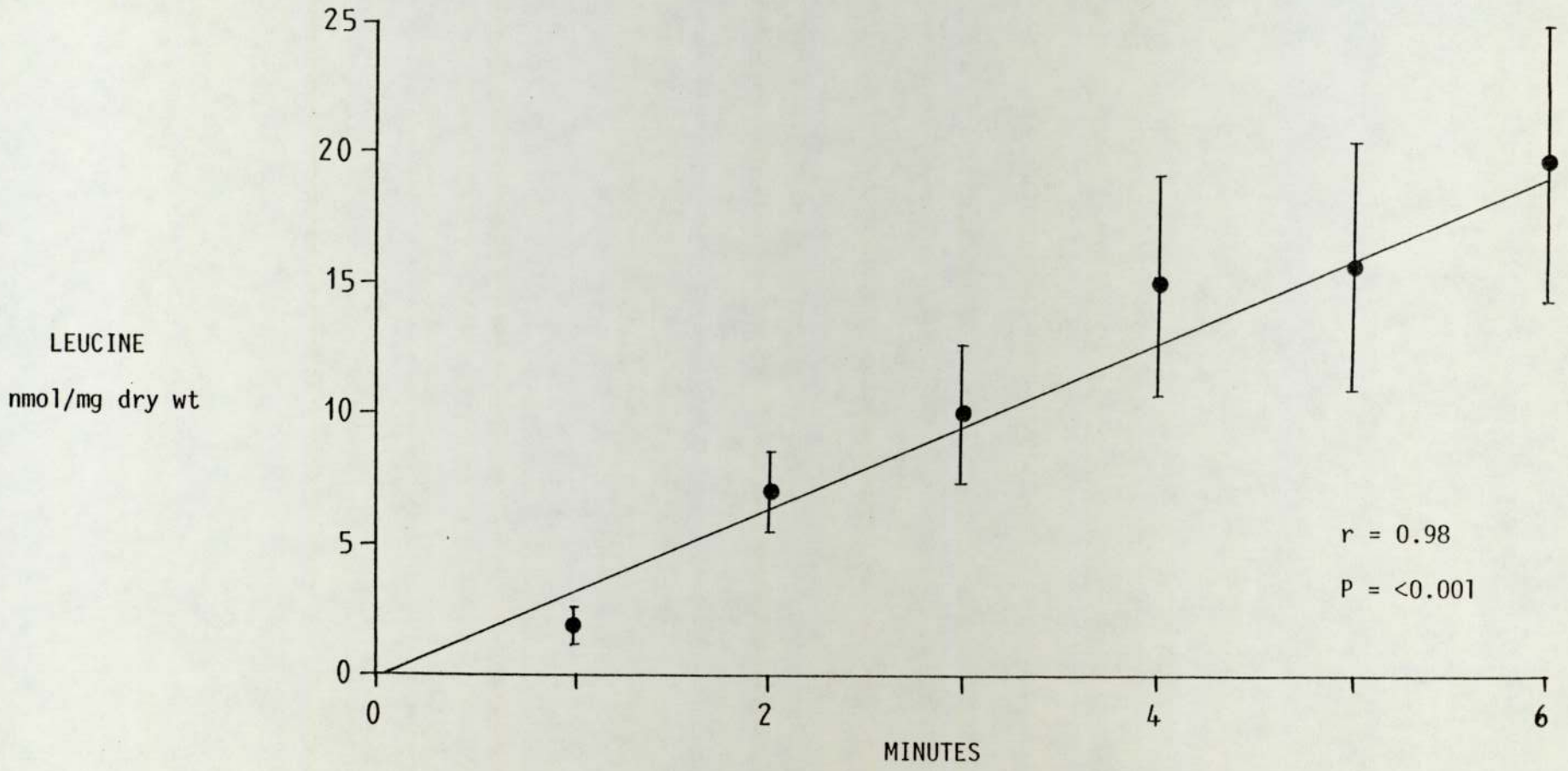


Figure 4,3. Uptake of folic acid by isolated cells incubated in different concentrations of folic acid. Cells were isolated by 30 minutes incubation with hyaluronidase. Regression coefficients are shown in table 4,2.

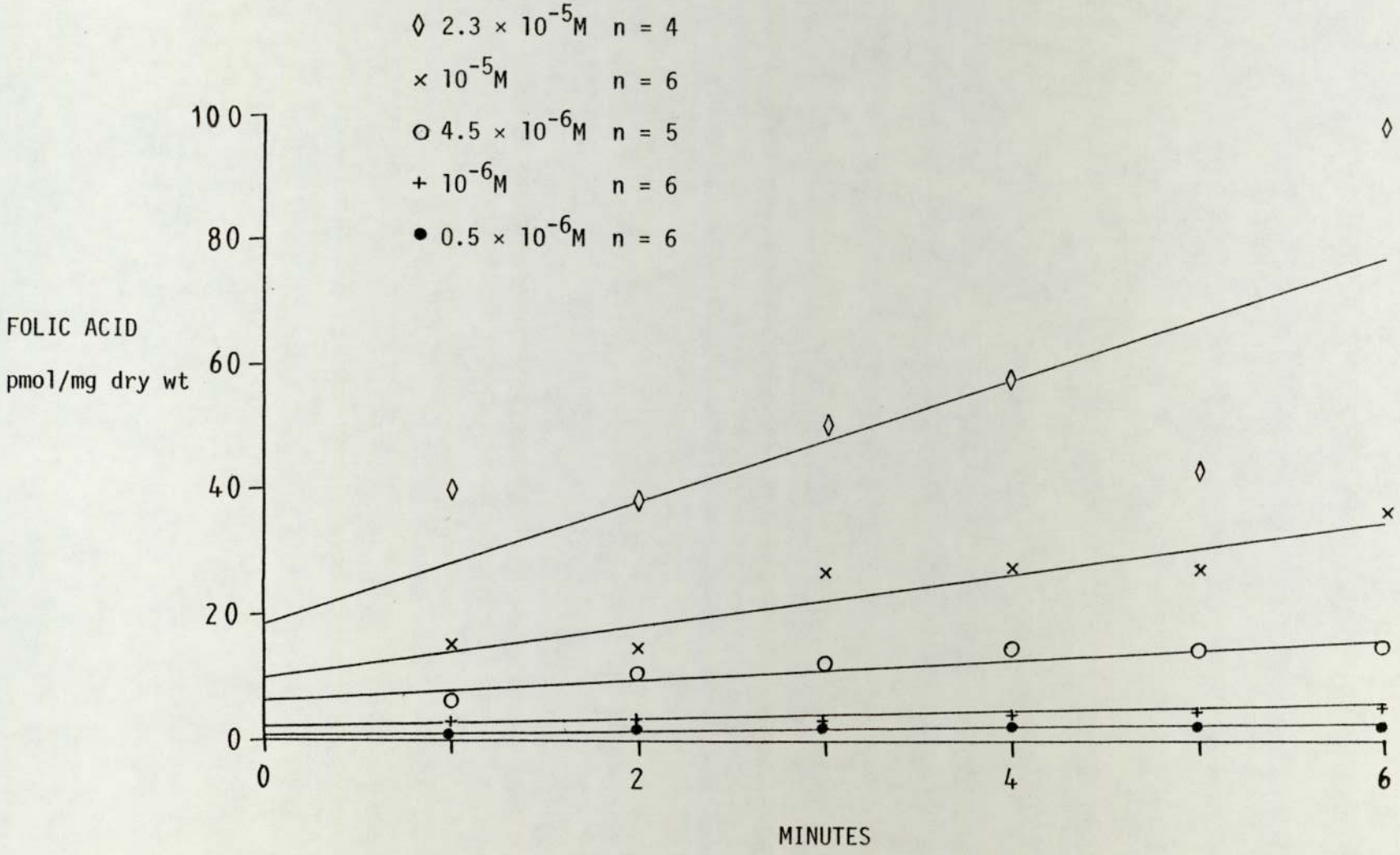


Figure 4,4. Relationship between rate of uptake of folic acid (10^{-6}M) by isolated cells harvested following 30 minutes incubation with hyaluronidase, and folic acid concentration. (n = 5 pairs of rats).

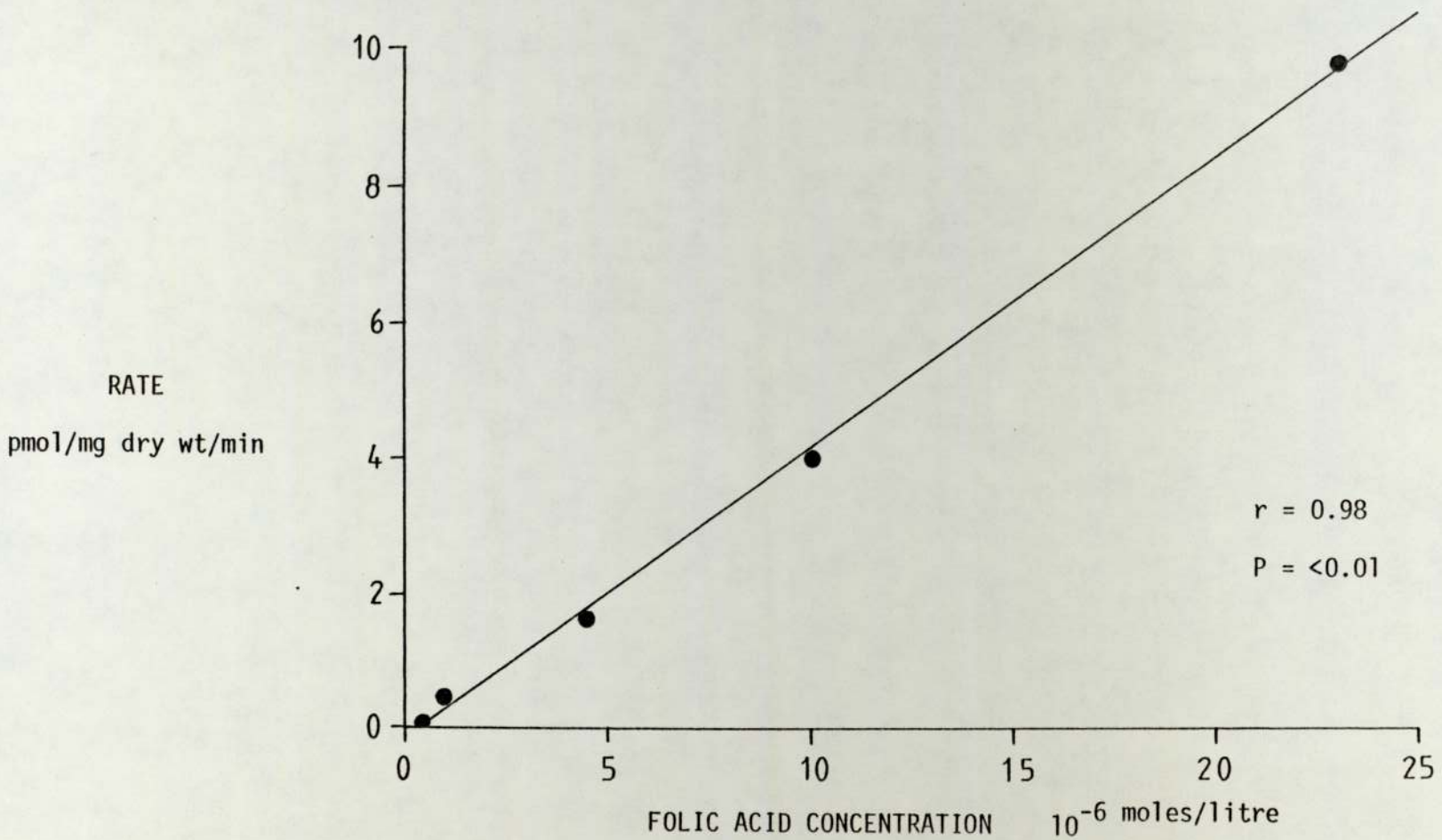


Figure 4,5. Relationship between the immediate uptake of folic acid (derived from the intercept values on the ordinate of figure 4,3) and folic acid concentration.

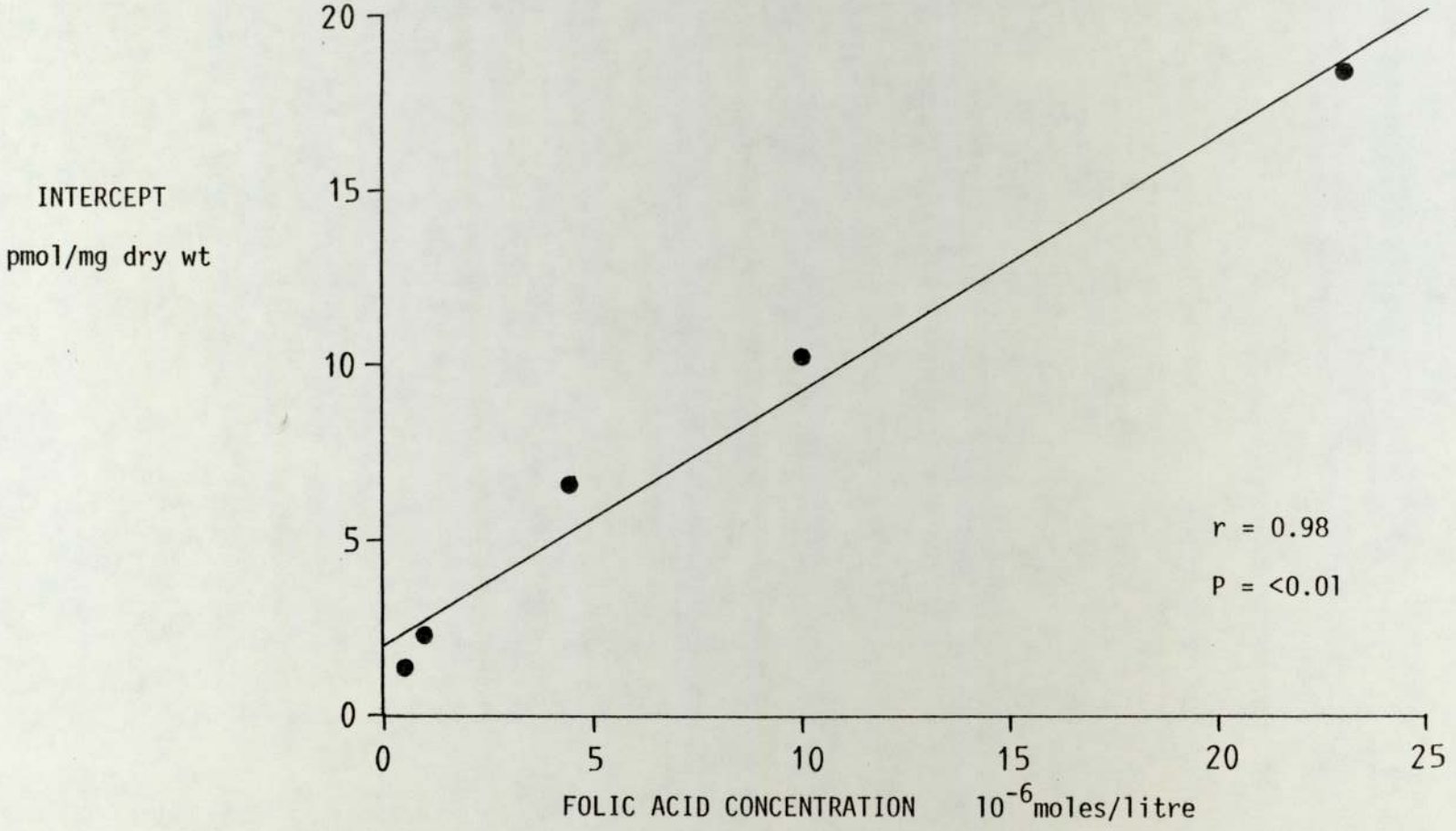


Figure 4,6. Relationship between the immediate uptake of folic acid (derived from the intercept values on the ordinate of figure 4,3) and rate of folic acid uptake.

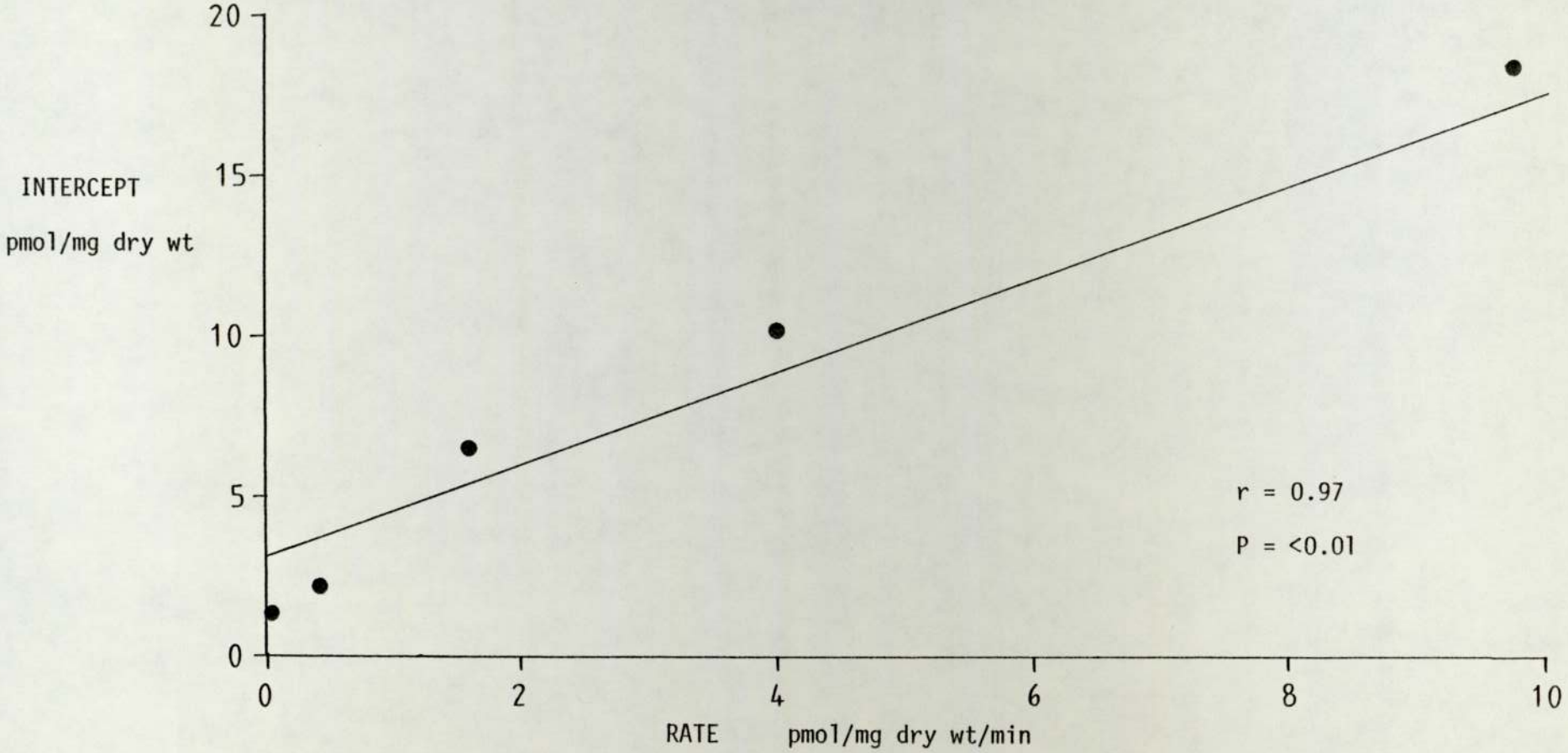


Figure 4,7. Uptake of folic acid (10^{-6}M) by isolated cells harvested following either 30 minutes or 60 minutes incubation with hyaluronidase. 30 mins n = 6 pairs of rats, 60 mins n = 5 pairs of rats.

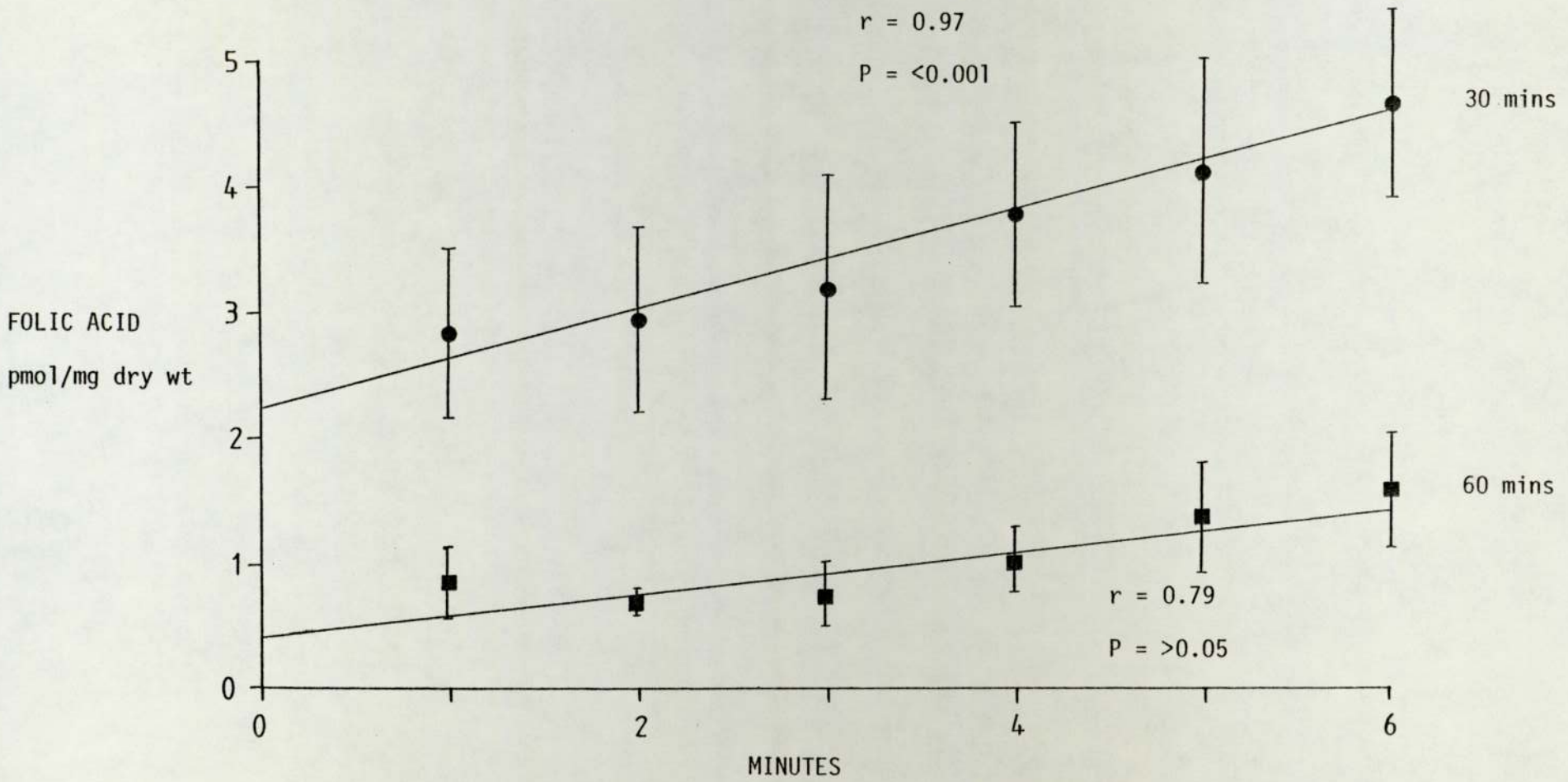


Figure 4,8. Uptake of 5-methyltetrahydrofolic acid by isolated cells incubated in different concentrations of 5-methyltetrahydrofolic acid. Cells were isolated by 30 minutes incubation with hyaluronidase. Regression coefficients are shown in table 4,7.

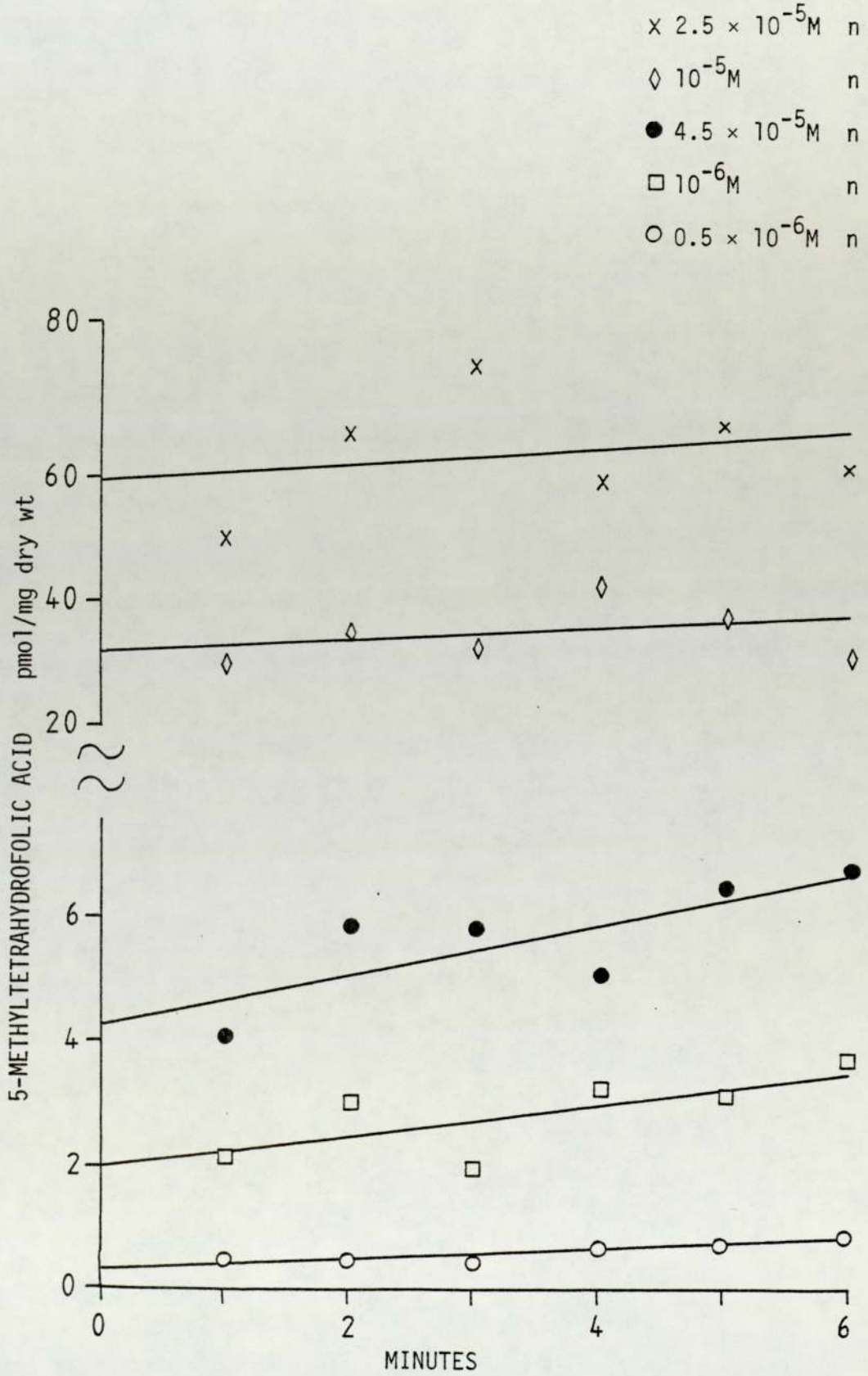


Figure 4,9. Relationship between rate of uptake of 5-methyltetrahydrofolic acid (10^{-6}M) by isolated cells harvested following 30 minutes incubation with hyaluronidase, and 5-methyltetrahydrofolic acid concentration. (n = 6 pairs of rats).

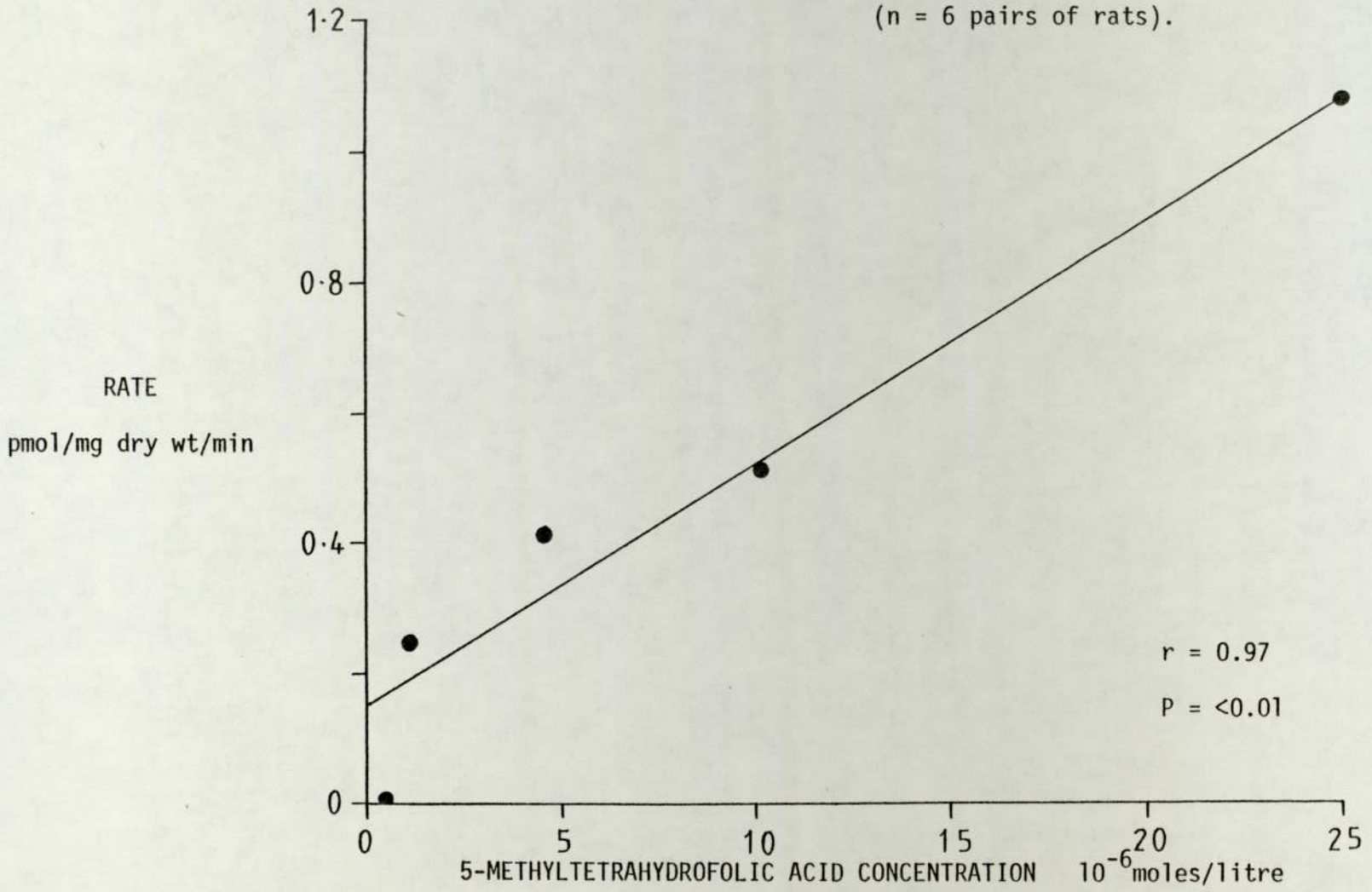


Figure 4,10. Relationship between the immediate uptake of 5-methyltetrahydrofolic acid (derived from the intercept values on the ordinate of figure 4,8) and 5-methyltetrahydrofolic acid concentration.

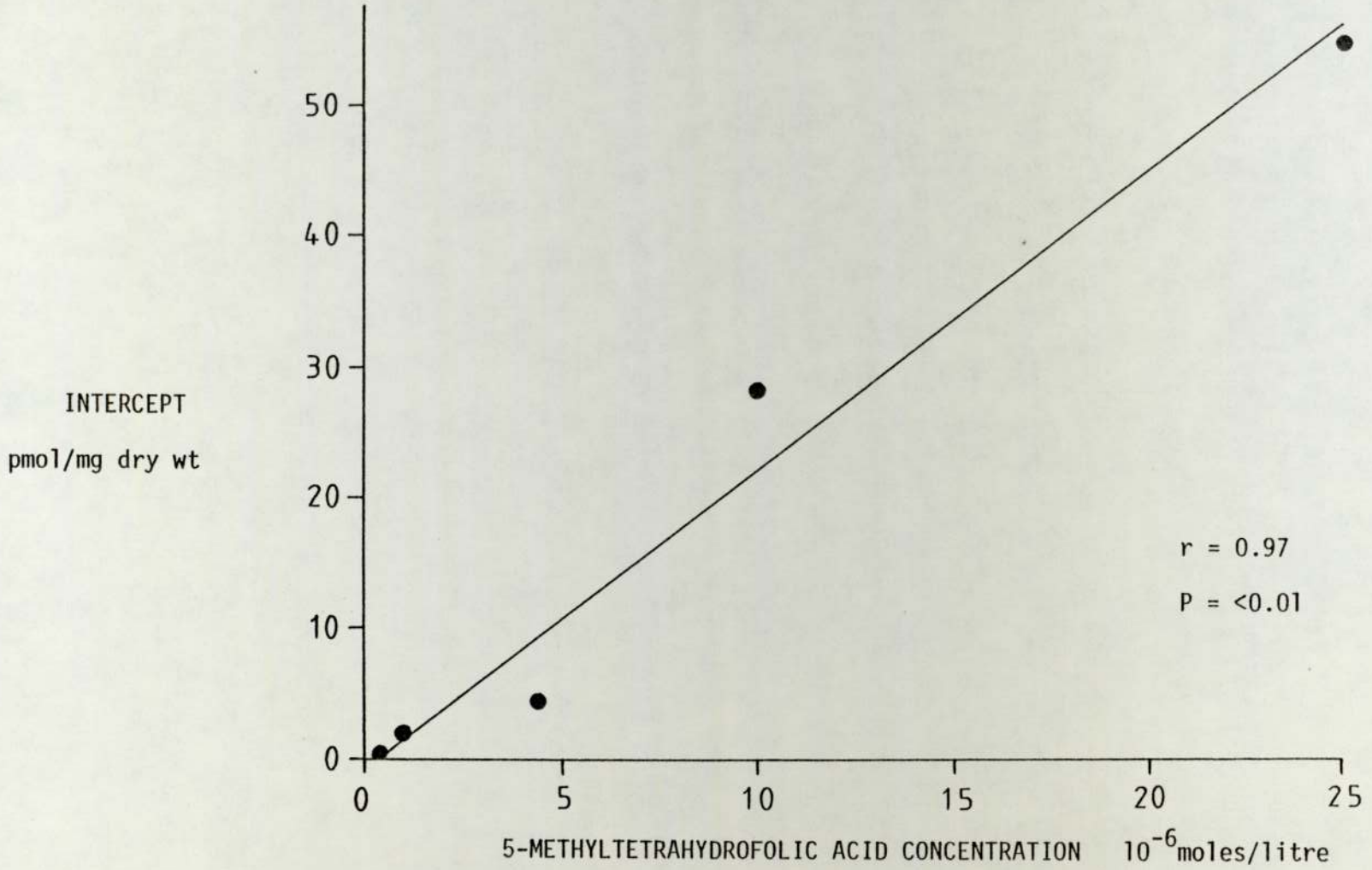


Figure 4,11. Relationship between the immediate uptake of 5-methyltetrahydrofolic acid (derived from the intercept values on the ordinate of figure 4,8) and rate of 5-methyltetrahydrofolic acid uptake.

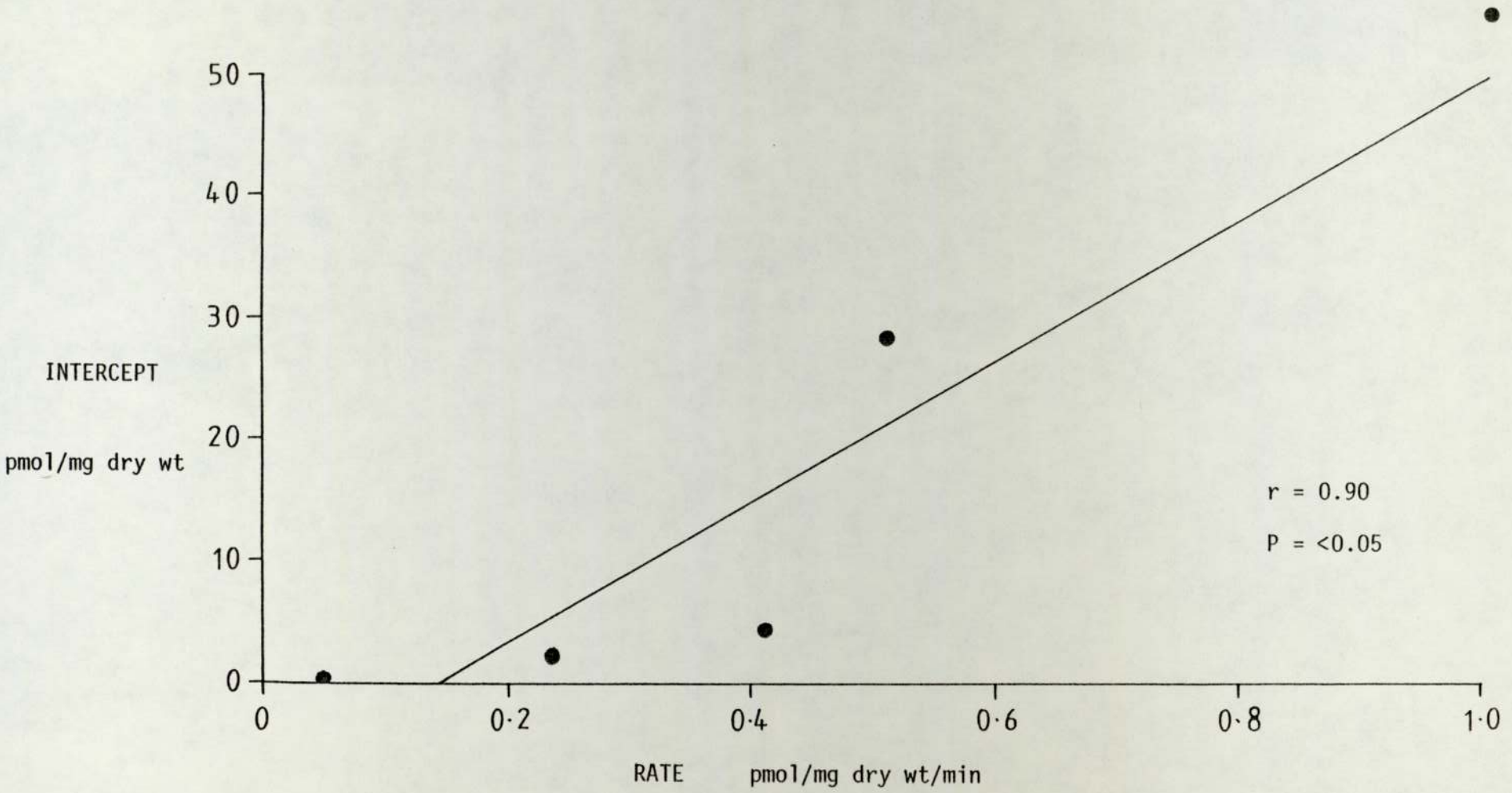


Table 4,1. Uptake of leucine (nmol/mg dry wt) during the first 6 minutes of incubation, by cells isolated as a result of either 30 minutes or 60 minutes tissue incubation with hyaluronidase. Results are given as mean ± standard error. (n = 6 pairs of rats for both conditions).

MINUTES OF INCUBATION WITH LEUCINE	HYALURONIDASE 30 MIN	HYALURONIDASE 60 MIN	t	P
1	1.99 ± 0.39	1.87 ± 0.75	0.13	> 0.05
2	3.41 ± 0.91	7.01 ± 1.56	1.98	> 0.05
3	3.64 ± 0.37	10.17 ± 2.76	2.34	< 0.05
4	3.10 ± 0.47	15.16 ± 4.38	2.73	< 0.05
5	5.24 ± 0.53	16.34 ± 4.96	2.22	< 0.05
6	5.36 ± 0.69	19.69 ± 5.54	2.56	< 0.02

Table 4,2. Folic acid uptake at various concentrations by cells isolated as a result of 30 minutes tissue incubation with hyaluronidase. Details of regression coefficients, rates of uptake and intercepts on the ordinate are given. (n = number of pairs of rats).

	CONCENTRATION OF FOLIC ACID (M)				
	0.5×10^{-6} (n = 6)	10^{-6} (n = 6)	4.5×10^{-6} (n = 5)	10^{-5} (n = 6)	2.3×10^{-5} (n = 4)
REGRESSION COEFFICIENT	0.72	0.97**	0.73	0.85*	0.54
RATE OF UPTAKE (pmol/mg dry wt/min)	0.18	0.39	1.59	3.99	9.75
INTERCEPT (pmol/mg dry wt)	1.07	2.25	6.53	10.20	18.45

Significant regression coefficients: * = P = <0.05, ** = P = <0.001.

Table 4.3. Folic acid uptake at various concentrations by cells isolated as a result of 30 minutes tissue incubation with hyaluronidase. Details of uptake (pmol/mg dry wt) during the first 6 minutes of cell incubation are given. Results are expressed as mean ± standard error. (n = number of pairs of rats).

	CONCENTRATION OF FOLIC ACID (M)				
	0.5×10^{-6} (n = 6)	10^{-6} (n = 6)	4.5×10^{-6} (n = 5)	10^{-5} (n = 6)	2.3×10^{-5} (n = 4)
MINS OF INCUBATION					
1	1.01 ± 0.22	2.81 ± 0.76	5.65 ± 0.44	15.59 ± 5.64	39.67 ± 22.64
2	1.38 ± 0.48	2.94 ± 0.78	10.81 ± 1.63	14.23 ± 2.51	28.83 ± 7.87
3	1.89 ± 0.40	3.27 ± 0.96	12.68 ± 2.76	26.16 ± 8.36	49.86 ± 25.48
4	2.03 ± 0.41	3.79 ± 0.76	14.84 ± 2.57	26.27 ± 8.96	56.87 ± 25.41
5	1.99 ± 0.89	4.24 ± 0.94	14.53 ± 1.49	26.69 ± 7.24	41.01 ± 24.08
6	1.93 ± 0.19	4.67 ± 0.77	14.16 ± 3.13	36.03 ± 16.78	99.22 ± 40.57

Table 4,4. Estimated final concentration of folic acid associated with isolated cells, after 6 minutes incubation in different concentrations of folic acid.

CONCENTRATION IN INCUBATION MEDIUM (M)	0.5×10^{-6}	10^{-6}	4.5×10^{-6}	10^{-5}	2.3×10^{-5}
ESTIMATED FINAL CONCENTRATION (M)	1.76×10^{-7}	8.83×10^{-7}	2.68×10^{-6}	6.82×10^{-6}	1.88×10^{-5}

Table 4,5. Uptake of folic acid (pmol/mg dry wt) during the first 6 minutes of incubation, by cells isolated as a result of either 30 minutes or 60 minutes tissue incubation with hyaluronidase. Results are given as mean ± standard error. (n = number of pairs of rats).

MINUTES OF INCUBATION WITH FOLIC ACID	30 MIN (n = 6)	60 MIN (n = 5)	t	P
1	2.81 ± 0.76	0.84 ± 0.30	2.21	> 0.05
2	2.94 ± 0.78	0.68 ± 0.11	2.57	< 0.05
3	3.27 ± 0.96	0.74 ± 0.27	2.32	< 0.05
4	3.79 ± 0.76	1.04 ± 0.26	3.12	< 0.02
5	4.24 ± 0.94	1.38 ± 0.45	2.55	< 0.05
6	4.67 ± 0.77	1.61 ± 0.46	3.22	< 0.02

Table 4,6. 5-Methyltetrahydrofolic acid uptake at various concentrations by cells isolated as a result of 30 minutes tissue incubation with hyaluronidase. Details of uptake (pmol/mg dry wt) during the first 6 minutes of cell incubation are given. Results are expressed as mean ± standard error. (n = number of pairs of rats).

	CONCENTRATION OF 5-METHYLTETRAHYDROFOLIC ACID (M)				
	0.5×10^{-6} (n = 7)	10^{-6} (n = 6)	4.5×10^{-6} (n = 7)	10^{-5} (n = 4)	2.5×10^{-5} (n = 3)
MINS OF INCUBATION					
1	0.52 ± 0.33	2.18 ± 0.57	4.02 ± 1.82	25.62 ± 21.88	45.42 ± 17.04
2	0.47 ± 0.17	3.04 ± 0.76	5.89 ± 2.60	30.98 ± 19.46	61.95 ± 28.45
3	0.39 ± 0.21	1.89 ± 0.64	5.71 ± 2.31	27.41 ± 10.86	73.24 ± 13.76
4	0.59 ± 0.36	3.22 ± 0.59	5.04 ± 1.18	37.26 ± 16.33	53.96 ± 23.08
5	0.68 ± 0.28	3.05 ± 0.57	6.41 ± 1.81	32.36 ± 7.04	62.92 ± 56.29
6	0.73 ± 0.13	3.62 ± 0.68	6.68 ± 1.30	26.40 ± 14.43	56.22 ± 32.43

Table 4,7. 5-Methyltetrahydrofolic acid uptake at various concentrations by cells isolated as a result of 30 minutes tissue incubation with hyaluronidase. Details of regression coefficients, rates of uptake and intercepts on the ordinate are given. (n = number of pairs of rats).

	CONCENTRATION OF 5-METHYLTETRAHYDROFOLIC ACID (M)				
	0.5×10^{-6} (n = 7)	10^{-6} (n = 6)	4.5×10^{-6} (n = 7)	10^{-5} (n = 4)	2.5×10^{-5} (n = 3)
REGRESSION COEFFICIENT	0.56	0.48	0.61	0.05	0.05
RATE OF UPTAKE (pmol/mg dry wt/min)	0.05	0.24	0.41	0.51	1.08
INTERCEPT (pmol/mg dry wt)	0.39	1.99	4.21	28.22	55.19

Table 4,8. Estimated final concentration of 5-methyltetrahydrofolic acid associated with isolated cells, after 6 minutes incubation in different concentrations of 5-methyltetrahydrofolic acid.

CONCENTRATION IN INCUBATION MEDIUM (M)	0.5×10^{-6}	10^{-6}	4.5×10^{-6}	10^{-5}	2.5×10^{-5}
ESTIMATED FINAL CONCENTRATION (M)	6.98×10^{-8}	6.85×10^{-7}	1.26×10^{-6}	4.99×10^{-6}	1.06×10^{-5}

Chapter 5.

General discussion and conclusions.

The paramount intention of the research described here was to illuminate our knowledge of the mechanism of folate transport, and thus enable further comprehension of the diseases and conditions which display folate malabsorption in man. In confronting the realms of folates, gastro-intestinal transport, and gut ultrastructure, it was apparent that in each of these vast areas there were both controversies and unresolved theories, but as is the nature of scientific research, there is always scope for novel concepts to further assist comprehension. The initial barrier in such a physiological inquiry is the application of the results to the human, when the study has been largely undertaken using the rat. Indeed, the departure from the _in vivo_ state such that the characteristics of barely a few cells are being examined, must undoubtedly lead to the questionable application of the theories to the human who displays malabsorption of folates. However, an awareness of the diversity of species must not be exaggerated as the comparative physiology between rat and man is relatively similar.

The initial approach to examining intestinal folate transport was to study the appearance of the intestine when this mechanism of intestinal function was operating inadequately. The intestinal epithelial cell glycocalyx is the first structure which faces any prospective substance for absorption. Using light microscopy and suitable histochemical technique, the glycocalyx was demonstrated as a thin, heavily stained line on the luminal surface of the brush border with greater clarity

on human biopsy tissue than on rat tissue. This species difference could be directly due to the dimensional aspect. The altered appearance of the glycocalyx was associated with certain diseases linked with folate malabsorption: coeliac disease, Crohn's disease, folate deficiency of unknown aetiology and also coeliac disease patients who had been on a gluten-free diet. Severe glycocalyx disruption took the form of a knobbed appearance which implied that the regular continuity of the structure was broken and could feasibly reflect abnormal biosynthesis of the glycocalyx (Swanston et al., 1977). The knobbed appearance could not be mimicked in rats by the administration of substances implicated in folate malabsorption in man; although mild alterations were observed predominantly as a thin and indistinct glycocalyx compared to the normal rat jejunum. It was possible, using hyaluronidase treatment for a prolonged length of time, to remove or reduce the glycocalyx of isolated jejunal epithelial cells as observed by light microscopy. The initial barrier to the absorption of compounds in the intestine is therefore a dynamic structure which, by alteration in morphology could possibly affect the normal course of absorption.

Malabsorption of folic acid has been reported to be associated with a number of diseases and drugs (tables 3,1 and 3,2). A sufficient quantity of material was not available to investigate folic acid transport in human tissue although authors have successfully isolated cells from coeliac disease biopsies with the intention of examining the epithelial cell characteristics (Hudson,

Anderson & Cooper, 1977). In the present research, drugs known to affect folate absorption, in addition to drugs which could be potential depressants of folate absorption were administered to rats. Controversy over certain drugs and their implicated effects on folate absorption has not been entirely resolved. The important factor that anticonvulsant and antimetabolite drugs are given to patients who have a primary condition which is not reproducible in the rat must not be neglected; indeed, the state of alcoholism was not necessarily induced in the rat simply by forced administration of alcohol. However, the human conditions of pregnancy, menopause and the disease diabetes mellitus were successfully produced in the rat. The novel factor of hormonal involvement in folic acid absorption was evident with cortisone and oestrogen affecting transport although these hormones are naturally implicated in numerous other hormonal interactions in the body.

Streptozotcin-induced diabetes mellitus is the treatment most commonly used to provide an experimental model of human diabetes in the rat. Only the cortisone-induced diabetes mellitus had an effect on tissue uptake of folic acid but this did not significantly alter the overall transport of folic acid. In the human, diabetes mellitus of the type produced by cortisone, is a rare condition which results from an adrenal cortical tumour where glucocorticoids are secreted in massive amounts (Fajans, 1961) and their effects, as with any hormone, may be exceedingly complex. In the present investigation, diabetes mellitus characterised by impaired

β cell function, did not significantly affect folic acid transport in rat jejunum. Recent debate on the association of diabetes mellitus and coeliac disease suggests that the two diseases are linked by some common aetiological factor (Koivisto, Kuitunen, Tiilikainen & Akerblom, 1977), and although the diabetes mellitus is usually diagnosed prior to the coeliac disease (Walsh, Cooper, Wright, Malins & Cooke, 1978a), further investigation is required relating to the interaction of these diseases. The present investigation showed that production of a diabetic state in the rat neither altered the total transfer of folic acid nor produced an altered glycocalyx or flattened mucosa similar to that observed in coeliac disease; typical characteristics of coeliac disease are folate deficiency and a flat mucosa (Cooke, 1968; Weinstein et al., 1970). It could be inferred from the present study that coeliac disease would occur prior to diabetes mellitus as the latter showed no evidence of producing those characteristics typical of coeliac disease. Indeed, in coeliac disease, the levels of secretin, and in particular, gastric inhibitory polypeptide secreted from the jejunum in response to a test breakfast, were markedly reduced (Besterman, Bloom, Sarson, Blackburn, Johnston, Patel, Stewart, Modigliani, Guerin & Mallinson, 1978a); this altered hormonal response could be an important factor as folic acid is absorbed preferentially in the jejunum (Smith et al., 1970). Impaired stimulation of gastric inhibitory polypeptide following normal feeding would reduce the insulin secretory response of the pancreatic β cells, resulting in impaired glucose tolerance and the

induction of a pre-diabetic state. Therefore, the implication is that the coeliac disease occurs prior to the diabetes mellitus and coeliac patients should be monitored carefully for the manifestation of glucose intolerance.

An abnormal appearance of the glycocalyx was observed in patients with coeliac disease who had been treated with a gluten-free diet. This evidence that complete normality was not achieved is supported by the persistently depressed levels of gastric inhibitory polypeptide (Besterman et al., 1978, 1978a) and tendency to reduced acidity of the intestinal acid microclimate observed in treated patients when compared with normal (Lucas, 1978). Indeed, an abnormal glycocalyx was also observed in the jejunum of Crohn's disease biopsies which suggests that this disease is not restricted to the terminal ileum; other authors have observed depressed levels of brush border enzymes in the jejunum (Dunne et al., 1976) and the jejunal acid microclimate has been found to be significantly more alkaline in such patients (Cooper, Lucas, Lei, Blair & Cooke, 1977). Hence Crohn's disease appears to be a diffuse lesion of the gastro-intestinal tract.

It has been suggested in this thesis that the appearance of an abnormal glycocalyx is associated with altered pH of the intestinal acid microclimate. When assessed by light microscopy and histochemical staining, an abnormal glycocalyx must imply a structural modification. Such a change could result from a direct disruptive action on the glycocalyx as is suggested by the effect of

hyaluronidase on isolated cells, or alternatively by a modification of the biosynthesis of the glycocalyx (Swanston et al., 1977). However, deviation from the normal pH of the acid microclimate could be the consequence of more numerous stimuli. Evidence for the existence of the acid microclimate has been provided by pH-microelectrode studies (Lucas et al., 1975; Lucas & Blair, 1978) and the mechanism of acidification depends on the production of hydrogen ions on the intestinal mucosa, a process which is dependent on energy from ATP hydrolysis (Blair et al., 1975) and can be reduced by metabolic and other inhibitors (Blair, Lucas & Matty, 1972; Lucas, 1976). The pH at the intestinal mucosal surface has been measured in both rat and man, but until recently, only speculation had been made concerning its relationship to folate absorption and malabsorption (Lucas, Blair, Cooper & Matty, 1975a; Lucas et al., 1976; Lei et al., 1977). With the proposed association of the glycocalyx to folate malabsorption (Swanston et al., 1977), the logical progression was to correlate folate transport with the acid microclimate.

In the experiments described in chapter 3, where rat jejunal everted sacs were employed for the investigation of folic acid absorption, a number of inter-relationships were demonstrated between the transport of folic acid and other factors. Intestinal fluid transport was shown to be involved in folic acid absorption, particularly from the tissue into the serosal compartment where solvent drag could account for a significant proportion of folic acid transfer. The clear relationship between these two factors is illustrated in figure 5,1, which includes the data from

a total of 19 treatments and conditions. In contrast, tissue fluid uptake was not significantly related to tissue folic acid uptake (figure 5,2). The associations presented in figures 5,1 and 5,2 are from data obtained following 30 minutes incubation of the everted sacs, as maximal fluid uptake was obtained at this time. A correlation was demonstrated between folic acid transfer and the change in pH of the mucosal surface acid layer, although pH values were not available for all the conditions studied; a significant regression coefficient was obtained with the data for which there was a corresponding estimation of surface pH (figure 5,3). The treatments involved in this collaborative study (Blair, Lucas & Swanston, 1978) are listed in table 5,1 together with the values for folic acid transfer and change in pH from the control value of 5.6 (Lucas et al., 1978). Correspondingly, the relationship between folic acid transfer and fluid transfer was not significant; the values for fluid transfer are given in table 5,1. A correlation has also been demonstrated between surface pH and serum folate levels in various clinical states (Lucas, 1977); this data is presented in figure 5,4. The influence of the acid microclimate pH on folate absorption may therefore be reflected in the eventual serum folate values.

Surface pH measurements give overestimated values of the actual microclimate pH. Such measurements are restricted by the relatively large (30μ) size of the pH-microelectrodes (Lucas & Blair, 1978) when the microclimate is estimated to be less than 20μ (Lucas et al.,

1975). More accurate estimations of the acid microclimate pH may be obtained by studying the appropriate physiochemical properties of known compounds. The saturation of folic acid and non-saturability of 5-methyltetrahydrofolic acid have been explained by assuming that the acid microclimate has a pH of approximately 3.5 (Blair & Matty, 1974; Kesavan & Noronha, 1978). Neither tetrahydrobiopterin nor pteroic acid were absorbed to any significant extent in the intestine (Blair et al., 1974a). In an acid microclimate of about 3.5 the availability of the neutral forms of these compounds would be negligible and hence no transport was observed (Blair et al., 1974a); but had the microclimate been less acid they would have been transported. Until pH-microelectrodes are made with even greater precision, unfortunately the true pH value of the acid microclimate will have to remain an estimation.

It is proposed that the glycocalyx acts as a retaining layer for the hydrogen ions which constitute the acid microclimate, and that a morphological abnormality of the glycocalyx is likely to result in an alteration of the pH of the microclimate. In the present study, it was only possible to use subjective assessment in ascertaining the relative appearance of the glycocalyx between various treatments and conditions in rat and man. A marked depression of folic acid uptake was observed using isolated intestinal epithelial cells which had been subjected to a prolonged period of treatment with hyaluronidase. These cells also presented a glycocalyx which appeared pale and

occasionally absent when compared with cells which had been incubated in hyaluronidase for a shorter period of time. It was suggested that with the cells which did not show a clear glycocalyx, the hydrogen ions produced by ATP hydrolysis were no longer retained at the mucosal surface and that the pH of the microclimate was therefore less acid.

The degree of maturity of epithelial cells has been considered to affect the appearance of the glycocalyx. Cells in the crypts of Lieberkühn showed a knobbed glycocalyx and it has been suggested that the functionally immature cells on the surface of coeliac mucosa (Weinstein, 1974) may either still be within the cell cycle, especially as the glycocalyx thickness is hypothesised to change during the cycle, or at least have incomplete surface glycoproteins as determined by their histochemical staining. In the investigations on folic acid transport using everted tissue and isolated cells, the cells most pertinent to transport would be those functionally mature cells situated in the upper regions of the villi. However, it has been proposed that in certain conditions such as following methotrexate or alcohol treatment, the villus cell population would consist of a large number of immature epithelial cells which would not transport folic acid as efficiently as normal. Indeed, not only has the time course of the cell cycle been implicated in the effects of certain treatments on folic acid transport, but also the time course of drug action. The time at which the transport study was performed following the administration of methotrexate has been shown to

remarkably affect results; similar variation with time and methotrexate treatment has been observed by other authors (Altmann, 1974; Jolly & Fletcher, 1977; Jeynes & Altmann, 1978).

Folic acid transport by tissue following methotrexate, phenytoin or alcohol treatment was shown to correlate well with surface pH measurements. The complex interpretation of the results for the methotrexate treatment involved the assumption of tissue binding sites as a supplement to explanation in terms of the acid microclimate. The basic mechanism of folic acid transport was shown to require an adequate supply of glucose and sodium; this was proposed to be necessary for sufficient maintenance of the acid microclimate. A degree of folic acid transport may occur via an intercellular route, although the investigations with phenytoin showed that the intercellular spaces are especially involved with fluid transport, with corresponding dilatation of the intercellular spaces being observed in both this and other studies (Tomasini & Dobbins, 1970).

Folic acid uptake into the tissue is largely intracellular with only a minimal association with tissue fluid uptake. An initial rapid uptake phase occurs as the tissue makes contact with the folic acid, although this phase may be artefactual. Within the glycocalyx, the acidity of the microclimate enables the formation of the neutral species of folic acid which is transported by passive diffusion into the cell. The acid microclimate requires active maintenance and the pH can be altered either by lack of metabolic substrates or disruption of the

glycocalyx. In an acid microclimate with a pH of less than 5, there is an increased amount of the neutral species of folic acid available, with enhanced availability proportional to decreased pH (Blair & Matty, 1974). Although there is increasing discussion that the pH-partition hypothesis or theory of non-ionic diffusion (Schanker et al., 1959) may have been misconceived (Smolen, 1973; Bridges, Houston, Humphrey, Lindup, Parke, Shillingford & Upshall, 1976) with the gut wall being partially permeable to ionic forms of weak acids (Nogami & Matsuzawa, 1961; Naupert & Rommel, 1975), the transport characteristics of folic acid would appear to be restricted entirely to transport as the neutral species (Blair, 1978).

In the present study, the subsequent uptake of folic acid by isolated epithelial cells immediately following the rapid phase, was observed as a non-saturable process, although in the intact tissue this process could feasibly be observed as a saturable process. Authors have suggested that both a saturable and unsaturable transport system may be involved in folate transport (Smith, 1973; Rosenberg, 1976; Horne, Briggs & Wagner, 1978). Within the tissue, there is some accumulation of folic acid; this may constitute a cellular pool of folic acid, or a reaction with dihydrofolate reductase, however, folic acid appears largely unaltered in the blood following transport (Whitehead & Cooper, 1967; Butterworth et al., 1969; Smith et al., 1970). The transport of folic acid from the tissue into the serosal compartment or blood stream is markedly associated with fluid which is

transferred at a constant rate.

The mechanism of 5-methyltetrahydrofolic acid uptake is similar to that of folic acid. 5-Methyltetrahydrofolic acid assumes a neutral form within the acid microclimate, enabling the species to diffuse into the epithelial cell. The transport shows the characteristics of an unsaturable mechanism.

It is proposed that the glycocalyx is a surface property of the intestine which is involved in the transport of folates. Malabsorption of folates may result from abnormalities of the glycocalyx and acid microclimate, these being affected by either direct or indirect factors. The present investigation has researched the properties which immediately concern the transport of compounds in the intestine and naturally will not be restricted to those specific substances examined. Further dimensions have been provided to supplement the expanding knowledge of drugs, conditions and diseases which, by affecting the normal absorptive mechanisms in the intestine, induce a state of folate deficiency.

Figure 5,1. Relationship between serosal folic acid transfer and serosal fluid transfer in everted sacs. Data obtained following 30 minutes incubation of everted sacs. (n = 19 treatments and conditions).

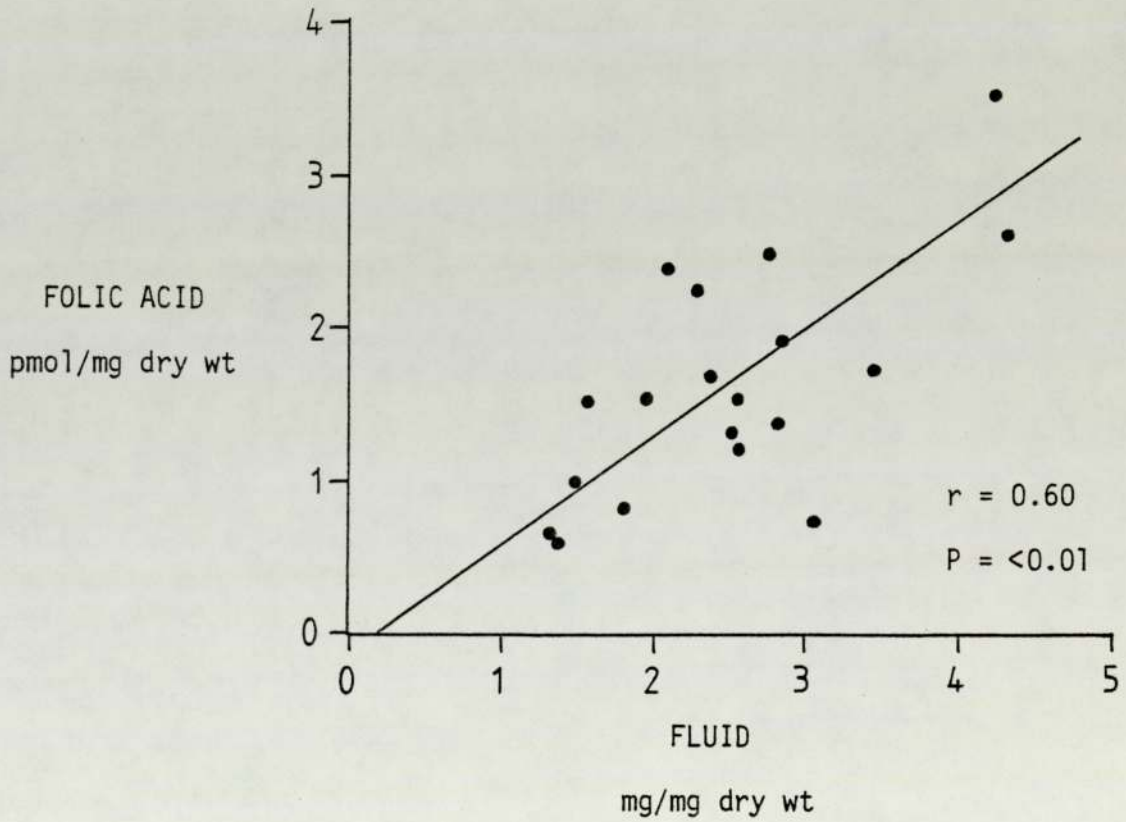


Figure 5,2. Relationship between tissue folic acid transfer and tissue fluid transfer in everted sacs. Data obtained following 30 minutes incubation of everted sacs. (n = 19 treatments and conditions).

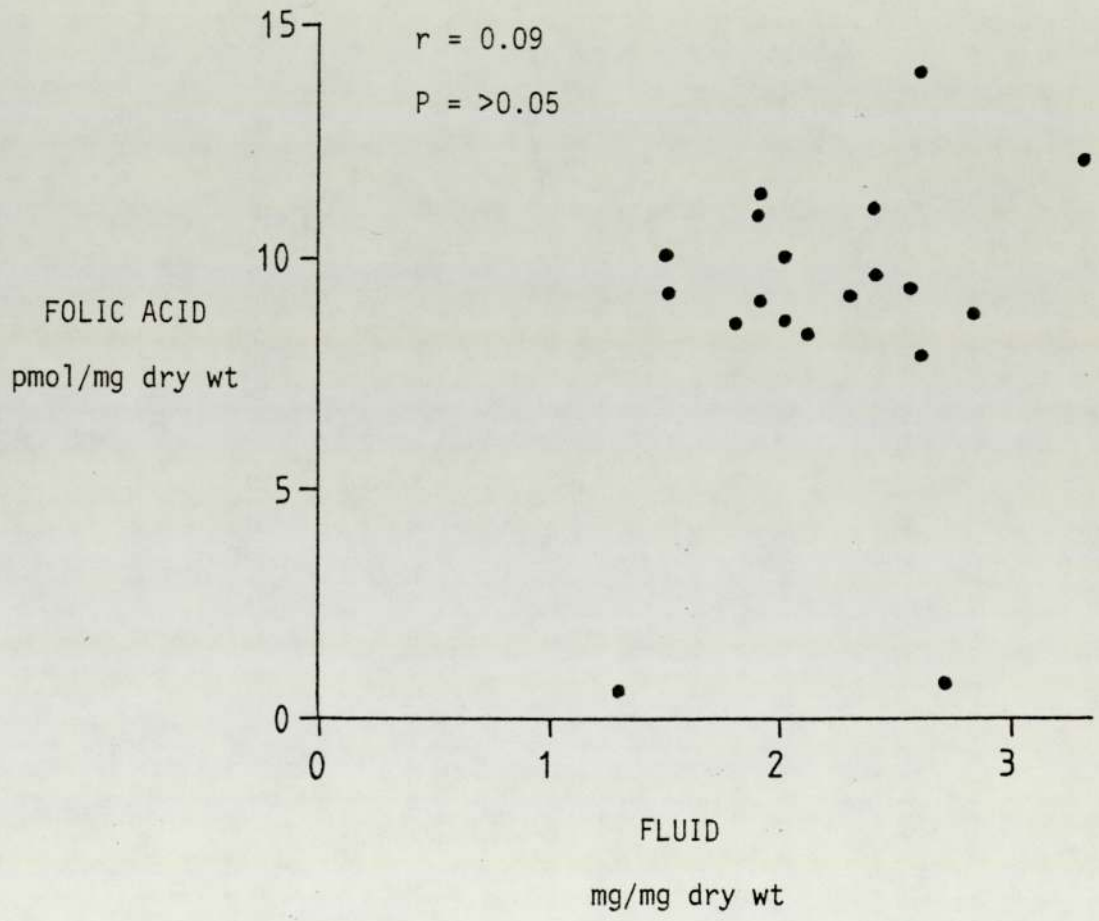


Figure 5,3. Total folic acid transfer as a function of change in surface pH in tissue following the incubation of everted sacs for 60 minutes in 10^{-6}M folic acid. The treatments are listed in table 5,1.

+ △pH increased acidity.

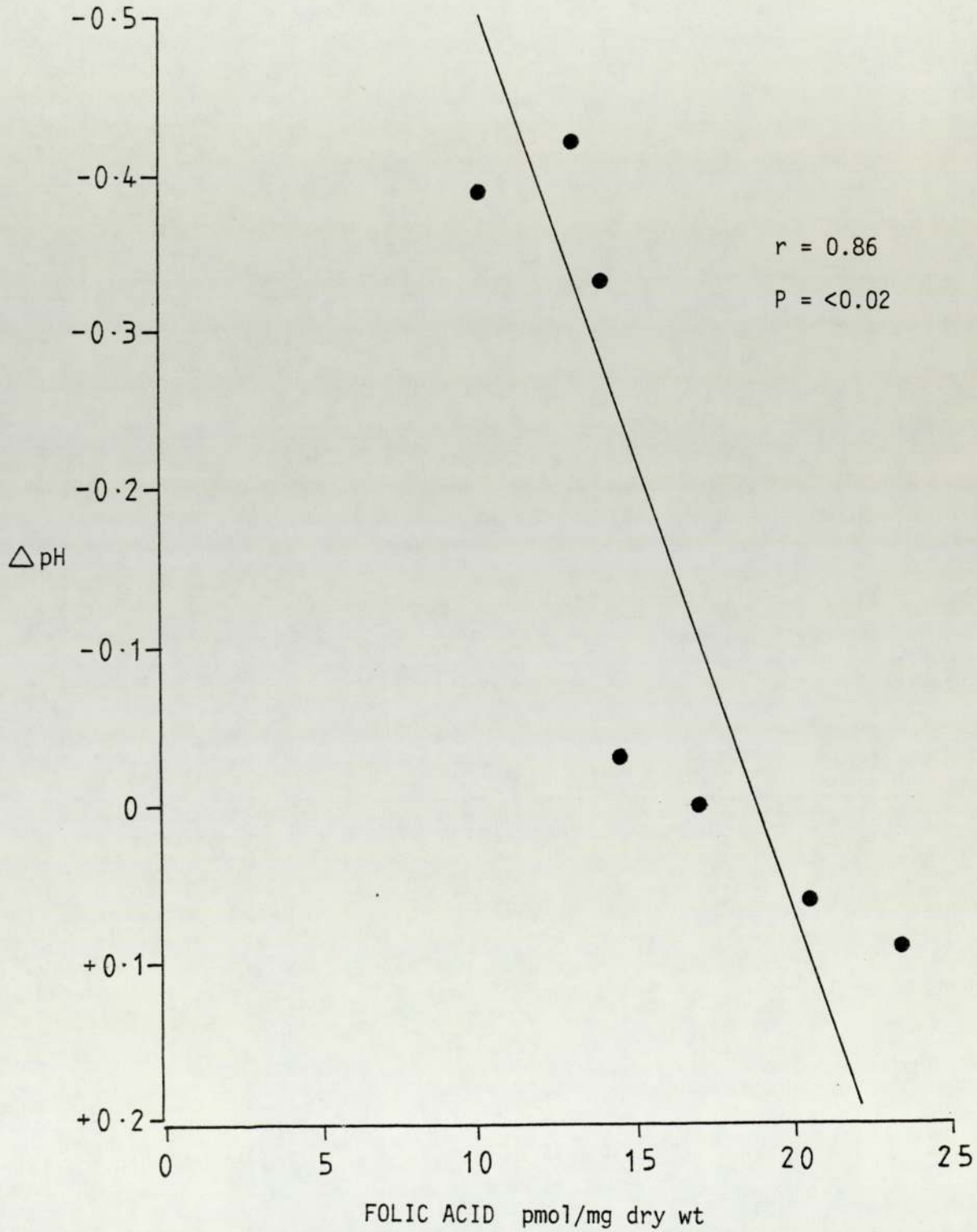


Figure 5,4. The regression of surface pH and serum folate levels in various clinical states (adapted from Lucas, 1977).

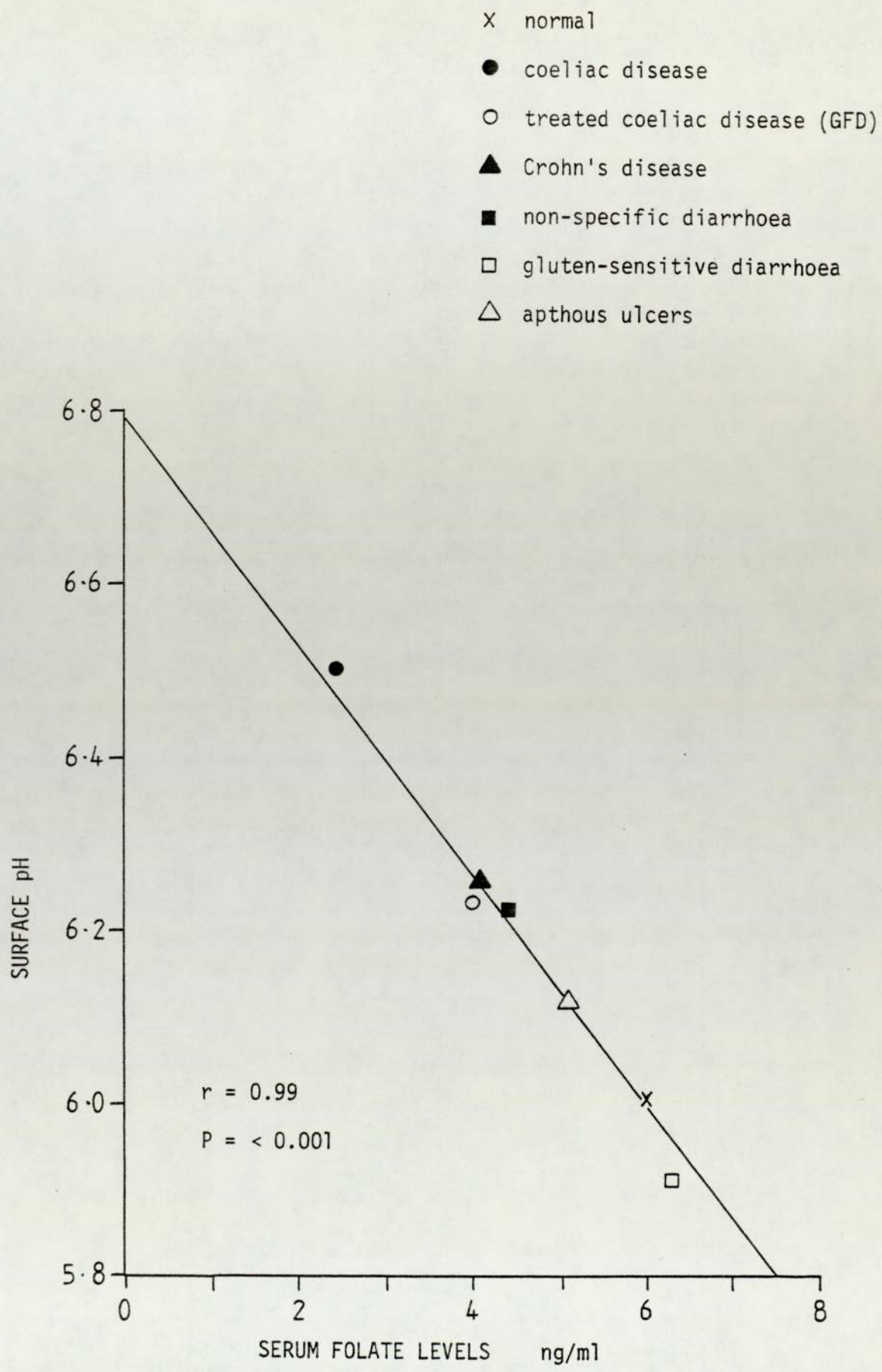


Table 5.1. Alterations in surface pH, total folic acid and fluid transport in rat proximal jejunum after 60 minutes incubation of everted sacs. Results are given as mean ± standard error. Number of rats in parentheses.

TREATMENT	TOTAL FLUID TRANSPORT * (mg/mg dry wt)	TOTAL FOLIC ACID TRANSPORT ** (pmol/mg dry wt)	CHANGE IN SURFACE pH (ΔpH)
Chronic exposure to 20% alcohol	7.9 ± 0.8 (6)	23.5 ± 1.9 (6)	+ 0.09 (8)
Phenytoin, 10^{-6}M	6.3 ± 0.4 (6)	20.6 ± 0.9 (6)	+ 0.06 ± 0.20 (8)
Control	4.9 ± 0.3 (12)	17.0 ± 0.8 (12)	0.0
Methotrexate, 10^{-6}M	4.5 ± 0.8 (6)	14.5 ± 1.4 (6)	- 0.03 ± 0.09 (6)
Acute exposure to 3% alcohol	4.2 ± 0.3 (6)	13.9 ± 1.5 (6)	- 0.33 ± 0.10 (6)
Sodium depletion	3.9 ± 0.2 (6)	13.0 ± 1.0 (6)	- 0.42 ± 0.10 (12)
Methotrexate 10 mg/Kg oral	9.2 ± 1.2 (6)	10.0 ± 2.1 (6)	- 0.39 ± 0.11 (4)

Regression coefficients with change in surface pH: * = P = >0.05, ** = P = <0.02

+ ΔpH increased acidity.

Appendix A.

Histological procedure.

The following schedules were those used in the histological methods.

Buffered 10% formalin (pH 7.0). Drury & Wallington (1967).

Formalin	100 ml
Tap water	900 ml
Sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$)	4 g
Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4)	6.5 g

Infiltrating solution. Sims (1974).

2 Hydroxyethyl methacrylate	80 ml
2 Butoxyethanol	8 ml
Benzoyl peroxide	0.5 g

Promoter solution. Sims (1974).

Polyethylene glycol 400	8 ml
N.N. Dimethylaniline	1 ml

Periodic acid-Schiff. Modified after McManus (1946).

1) Bring sections to water
2) Oxidize for 10 mins in 1% periodic acid
3) Wash in running tap water 3 mins, rinse in distilled water
4) Immerse in Schiff's reagent for 20 mins
5) Wash in running tap water 5 mins
6) Immerse in 5% thionyl chloride 2-3 mins
7) Wash in running tap water 10 mins

8) Counterstain with celestine blue-haemalum sequence:

10 mins in celentine blue

3 mins wash

10 mins in Mayers haemalum

3 min wash

9) Differentiate if necessary in 1% acid alcohol

10) Wash in running water 15 mins

11) Dehydrate in alcohol, clear in xylene and mount.

Alcian blue. Modification of Steedman (1950).

1) Bring sections to water

2) 1% Alcian blue in Walpole buffer (pH range 1-5) Stain in this for 10 - 30 mins

3) Rinse in distilled water and wash in running water 5 mins

4) Counterstain

5) Dehydrate, clear and mount.

Alcian blue. Revel (1974).

1) Bring sections to water

2) Place the sections in a solution of 1% Alcian blue in 30% acetic acid at 60°C for 30 - 60 mins

3) Rinse in 30% acetic acid

4) Counterstain

5) Dehydrate, clear and mount.

Hale's colloidal iron. Hale (1946).

1) Bring sections to water
2) Flood with dialysed iron solution for 10 mins
3) Wash well with several changes of distilled water
4) Immerse in equal parts 2% aqueous potassium ferrocyanide and 2% hydrochloric acid made up in distilled water, leave 15 mins
5) Rinse in distilled water and wash in running tap water
6) Counterstain
7) Wash in water and dehydrate rapidly in alcohol
8) Clear and mount.

Colloidal iron. Modification by Mowry (1963).

1) Bring sections to water
2) Immerse for 2 hours in colloidal iron solution
3) Rinse in 3 changes of 30% acetic acid, 10 mins each
4) Immerse in equal parts 2% aqueous potassium ferrocyanide and 2% hydrochloric acid for 20 mins
5) Wash 5 mins in running tap water and rinse in distilled water
6) Counterstain
7) Wash in water
8) Dip slides quickly into alcohol
9) Clear and mount.

Toluidine blue. Modification of Drury & Wallington(1967).

1) Bring sections to water
2) 1% toluidine blue solution in Walpole buffer (pH range 1-5), stain for 10 mins
3) Rinse in distilled water
4) Buffer rinse of appropriate pH
5) Distilled water rinse
6) Blot with xylene and mount.

Uranyl nitrate. Hughesdon (1949).

1) Bring sections to water
2) Oxidise with 1% aqueous potassium permanganate for 1-5 mins. Rinse in tap water
3) Treat with 5% aqueous oxalic acid until colourless, rinse in distilled water and then tap water
4) Stain with Azur B for 25 mins
5) Rinse in 0.2% aqueous uranyl nitrate for approximately 10 seconds
6) Rinse in tap water, dehydrate in alcohol
7) Clear and mount.

Schmorl's Thionin. Drury & Wallington (1967).

1) Bring sections to water
2) Treat with aqueous mercuric chloride
3) Wash briefly in water
4) Stain in diluted Thionin solution for 15 mins
5) Wash in distilled water
6) Wipe slide with tissue, small drop of hydramount aqueous mount, place on coverslip and seal with ringing medium.

Appendix B.

Radiochemical purity.

Radiochemical purity is of importance in absorption studies involving radiochemicals, since the absorption and metabolism of radioactive impurities may be quite different to that of the labelled folate (Blakley, 1969).

The purity of ^{14}C folic acid, ^{14}C L-leucine and ^{14}C 5-methyltetrahydrofolic acid were checked by column chromatography. A 100 µl aliquot of each compound was applied, on separate occasions to a column (60 x 2 cm) of Sephadex G-15 (particle size 40 - 120µ, Pharmacia Fine Chemicals AB, Uppsala, Sweden) in 0.05 M phosphate buffer at pH 7.0. An antioxidant, dithiothreitol (5 mg %) was added to the buffer. The columns were run at room temperature and eluted with the phosphate buffer.

Small quantities of folic acid, L-leucine, sodium ascorbate and bovine serum albumin were also passed in solution through the column.

The compounds were detected using a LKB Uvicord II instrument and a chart recorder (LKB Instruments, Croydon).

Folic acid (figure i)

The ^{14}C folic acid elution profile showed a peak which corresponded exactly to that obtained with unlabelled folic acid. However, an additional, minor peak was also detectable with the unlabelled compound.

L-leucine (figure ii)

The peak obtained for the ^{14}C L-leucine corresponded exactly to that obtained for the unlabelled L-leucine.

5-Methyltetrahydrofolic acid (figure iii)

An isolated peak was obtained for this radioactive compound. Single peaks were also obtained for the unlabelled substances, sodium ascorbate and bovine serum albumin.

All the radiochemicals investigated displayed a high degree of purity. A minor component was however, detected in the unlabelled folic acid preparation although this appeared to represent only a minor feature.

Figure i . Gel chromatographic profile of folic acid and ^{14}C-labelled folic acid.

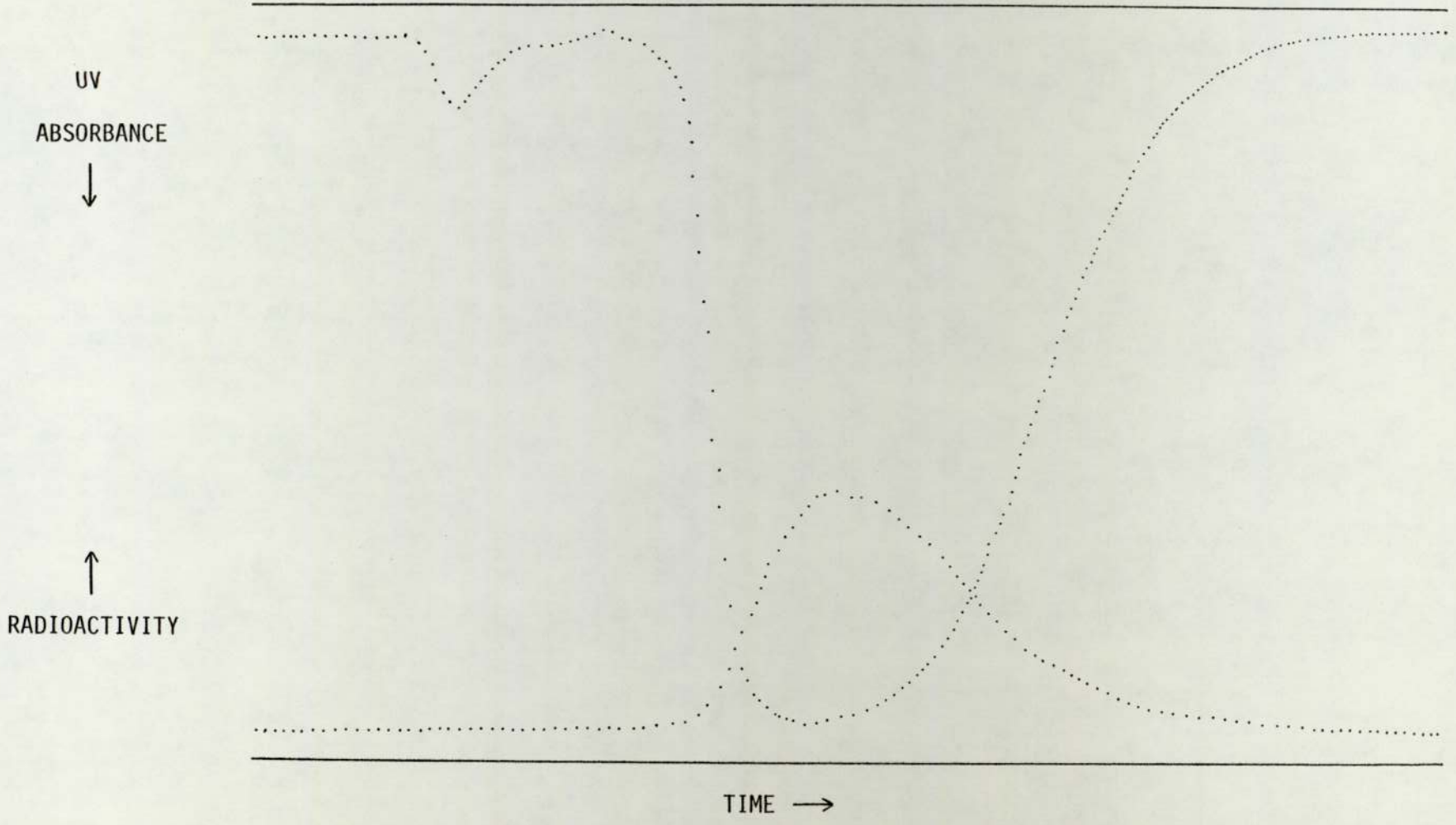


Figure ii. Gel chromatographic profile of leucine and ^{14}C -labelled leucine.

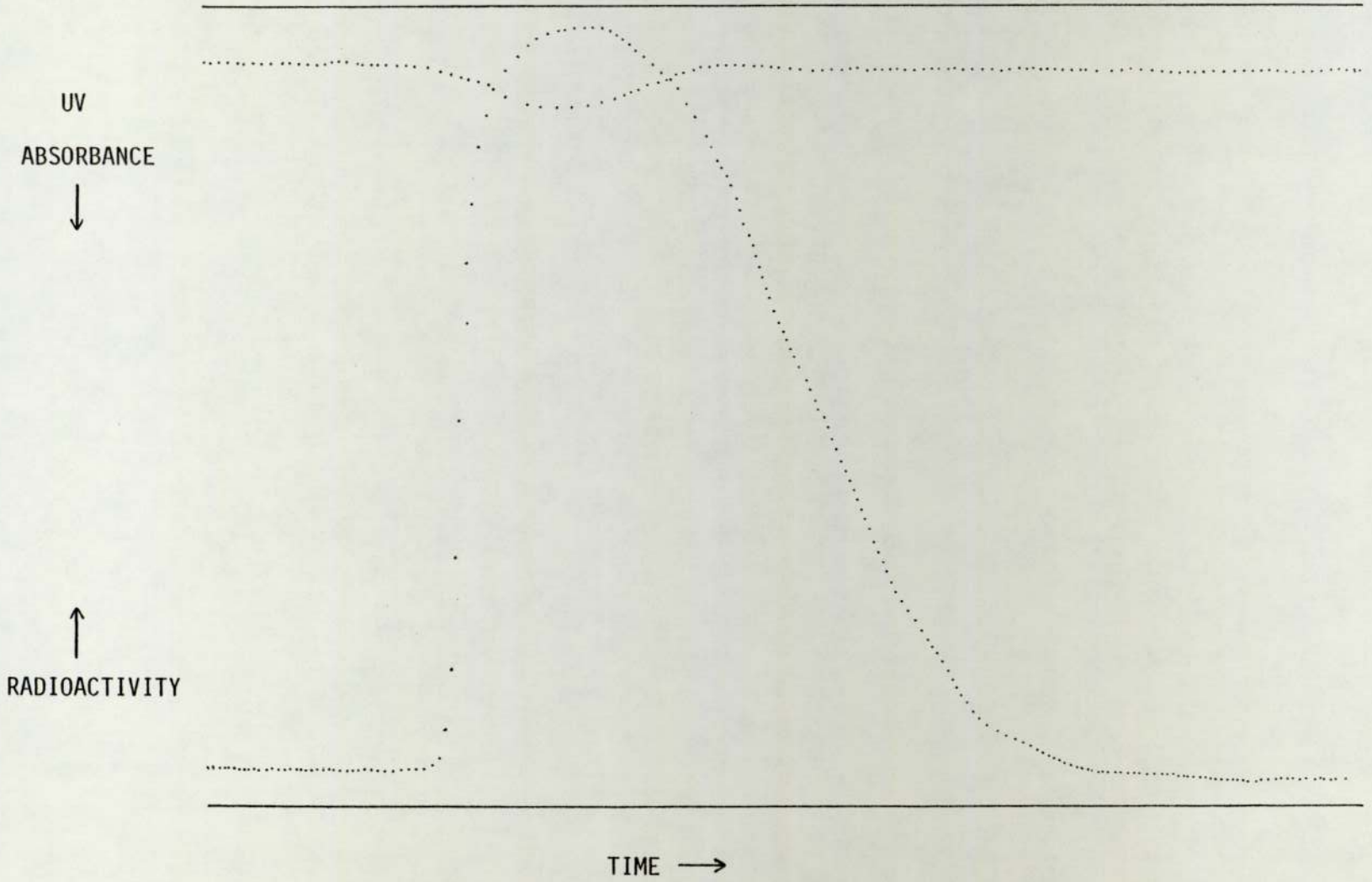
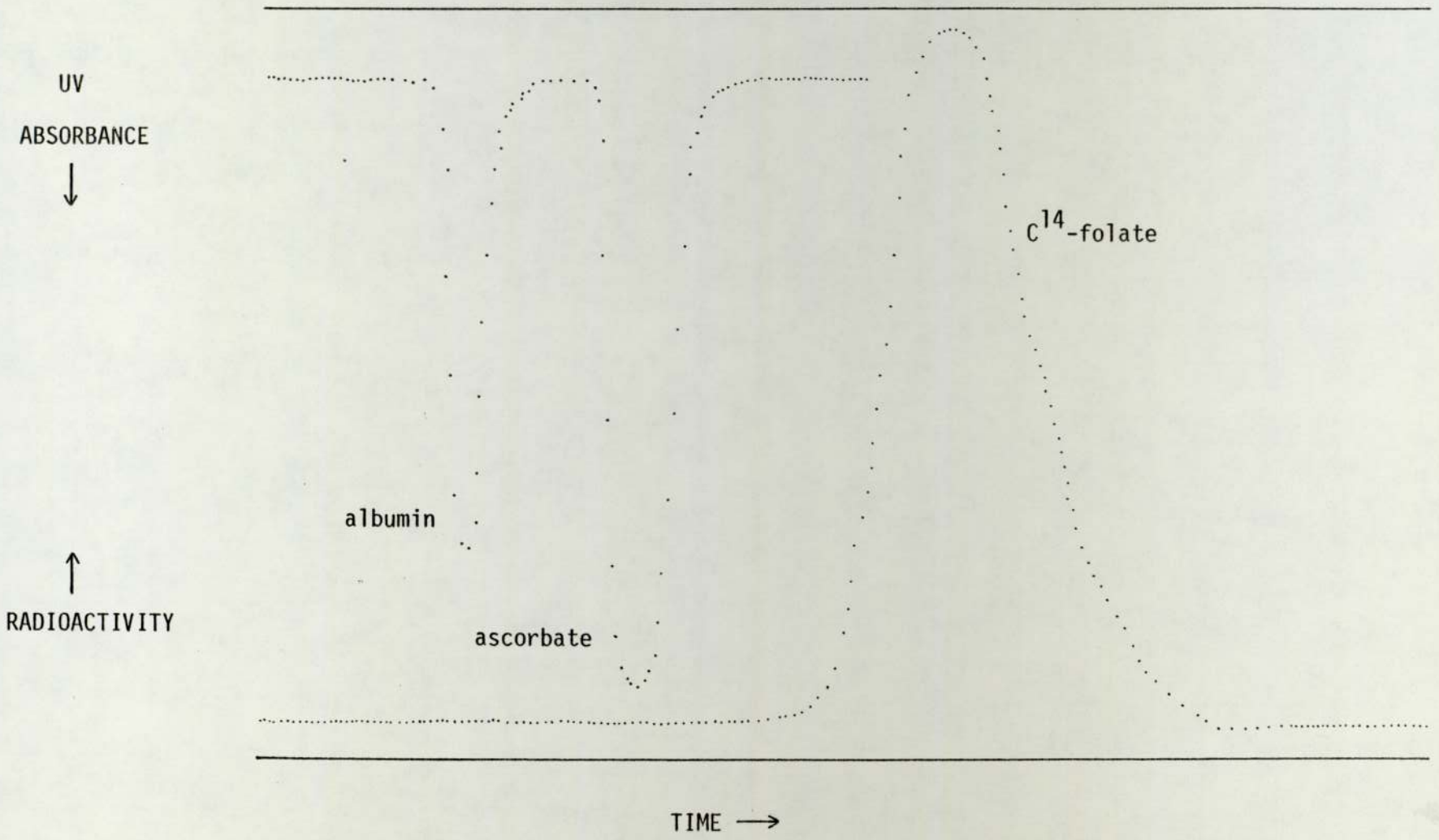


Figure iii. Gel chromatographic profile of ^{14}C -methyltetrahydrofolic acid, sodium ascorbate and bovine serum albumin.



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