THE SURFACE PROPERTIES OF THE MAMMALIAN SMALL INTESTINE ; THEIR ROLE IN THE ABSORPTION OF NUTRIENTS AND THEIR VARIATION IN HEALTH AND DISEASE

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by

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SUMMARY

The intestinal surface microclimate pH was measured in rat and man *in vitro* and rat *in vivo* using a pH-microelectrode. The mechanism of its creation and maintenance, its role in the absorption of nutrients particularly folate, and its variation in normal and diseased human intestinal conditions was investigated.

The maximum acidity of the surface microclimate was measured in the proximal jejunum. The surface acid microclimate is created by the external hydrolysis of adenosine triphosphate (ATP) produced intracellularly through aerobic metabolism, by mucosally located ATPase. The hydrogen ions of the microclimate are retained at the surface by the hydrated glycocalyx. Normal intracellular aerobic metabolism, adequate amount of glucose or other metabolisable substrates that produce enough quantities of ATP and an active mucosally located ATPase in sufficient amount, together with a structurally intact and biosynthetically complete glycocalyx are the necessary requirements for the creation and maintenance of a normal surface acid microclimate.

The absorption of folic acid and 5-meTHF was studied in rats using the everted sac technique. It was demonstrated that folate absorption occurs preferentially in the proximal jejunum but decreases distally. The experimental evidence was inconsistent with the concept of an active specific carrier transport mechanism but supported a model of facilitated passive diffusion of folates after conversion to neutral forms in the surface acid microclimate. An elevation in the surface microclimate pH was found to cause a decrease in folate absorption. 5-meTHF does not undergo metabolic alteration during transport, but a considerable amount of folic acid is metabolised.

A secondary isotopic effect during folate absorption was observed in which the ^{3}H-labelled species was absorbed at a faster rate than the ^{14}C-labelled and unlabelled species. This phenomena was studied and possible explanations discussed.

The surface acid microclimate was also measured in normal human health and diseased conditions of the small intestine using jejunal biopsies. It was found that the surface acid microclimate exists at the human intestinal surface and that an elevation in its pH occurs in coeliac disease, Crohn's disease and in subjects with non-specific diarrhoea with abnormal histology. The increase in the surface pH observed with these clinical states was found to be associated with a decrease in serum folate levels.

The present investigation has provided evidence that the surface acid microclimate is a normal physiological phenomena of a healthy small intestine , and that folate is absorbed by means of a facilitated passive diffusion after conversion into a neutral form in the surface acid microclimate.

KEY WORDS

Intestinal surface microclimate

Glycocalyx

Folate absorption

Intestinal diseases

To my

FATHER and MOTHER

SISTERS and BROTHERS

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CHAPTER ONE

INTRODUCTION

1.1. INTESTINAL MORPHOLOGY :-

The small intestine in common with the rest of the gastrointestinal tract is a hollow tube composed of four separate layers; the mucosa, the submucosa, the muscularis and the serosa. In this section, it is proposed to discuss only the mucosa and its epithelial cells, their brush border and surface coat (glycocalyx). The glycocalyx is an important morphological feature that is thought to play an important role in the digestion and absorption of nutrients (Ugolev, 1965; Greenberger, 1969, Crane, 1966) as well as being responsible for the maintenance of an acid microclimate at the surface of the small intestine (Blair and Matty, 1974).

The mucosa of the small intestine can be conveniently divided into three layers. The innermost of these, the muscularis mucosa, consists of a thin sheet of smooth muscle. The middle layer, the lamina propria, is a continuous subepithelial connective tissue space bounded below by the muscularis mucosa and above by intestinal epithelium. The composition of the lamina propria is heterogenous. It contains a variety of cell types including fibroblasts, macrophages and plasma cells, as well as non cellular connective tissue elements including collagen and reticular fibres, blood and lymph vessels, nerve fibres and strands of smooth muscle. The lamina propria with the epithelium forms numerous finger like villi which project from the surface of the mucosa into the intestinal lumen and so increase the area available for absorption.

Crypts formed at the base of adjacent villi are the site of cell

proliferation and differentiation (Cheng and Leblond 1974). It is only after differentiation and migration that the immature crypt cells acquire their characteristic structure and specialized function (Lipkin, 1973).

The intestinal epithelium consists of a continuous single lining of epithelial cells which cover both the villi and the crypts. The epithelial cells rest on the basement membrane which separates them from the lamina propria. The blood capillary vessels which carry the absorbed nutrients from the intestine into the body cavity lie directly beneath.

94% of the epithelial cells are absorptive cells (Cheng and Leblond, 1974). The small number of goblet cells are responsible for the secretion of mucus.

The intestinal epithelial cells are polarized as their apex differs from their base. The apex, or luminal surface called the brush border is characterized by minute parallel rod-like projections termed microvilli which together with the villi greatly increase the area available for absorption.

The plasma membrane of the microvilli has the classical membrane structure described by Singer and Nicolson (1972) and is composed of lipids, proteins and some carbohydrates. Phosphoglyceride is the major component of the lipid in the membrane, but other forms such as glycolipid and cholesterol are also present (Finean, Coleman and Michell, 1978; Lewis, Gray, Coleman and Michell, 1975). The phos-

pholipid molecules of the membrane are amphiphilic with a hydrocarbon region which is lipophilic and phosphate groups which is hydrophilic. The phospholipid molecules aggregate to form a bilayer with the lipophilic region orientated inwardly forming a non-polar environment, and the polar heads orientated outwardly. The polar groups of the phospholipid are negatively charged and are the major contribution towards the negative-charged character of the surface of the epithelial cells. The protein components of the membrane are asymmetrically distributed. They also have an am phiphilic character with the non-polar region inserted in or spanning the non-polar region of the lipid bilayer and the polar groups orientated towards the outside of the membrane.

Extending from the microvilli into the lumen are numerous branched filaments which constitute a region known as the glycocalyx (Bennett 1963; Ito 1965). This surface layer is also known as antennulae microvillares (Yamada, 1955), enteric coat, hirsutulous, hispiditious, hairy coat, fuzz or fuzzy coat (Ito, 1965; 1974).

The existence of an extraneous cell coat has been shown to exist on a variety of cells and tissues in different animal species. These include, the amoeba, bacteria and sea urchin egg (Ito, 1969; Revel and Ito, 1967), the microvilli of the small intestine of cat, rat, bat and man (Ito, 1965; 1969; Yamada, 1955; Swanston, Blair, Matty, Cooper and Cooke, 1977), kidney (Burgos, 1960), pancreatic acinar cells (Fawcett, 1962) and the toad urinary bladder (Choi, 1963).

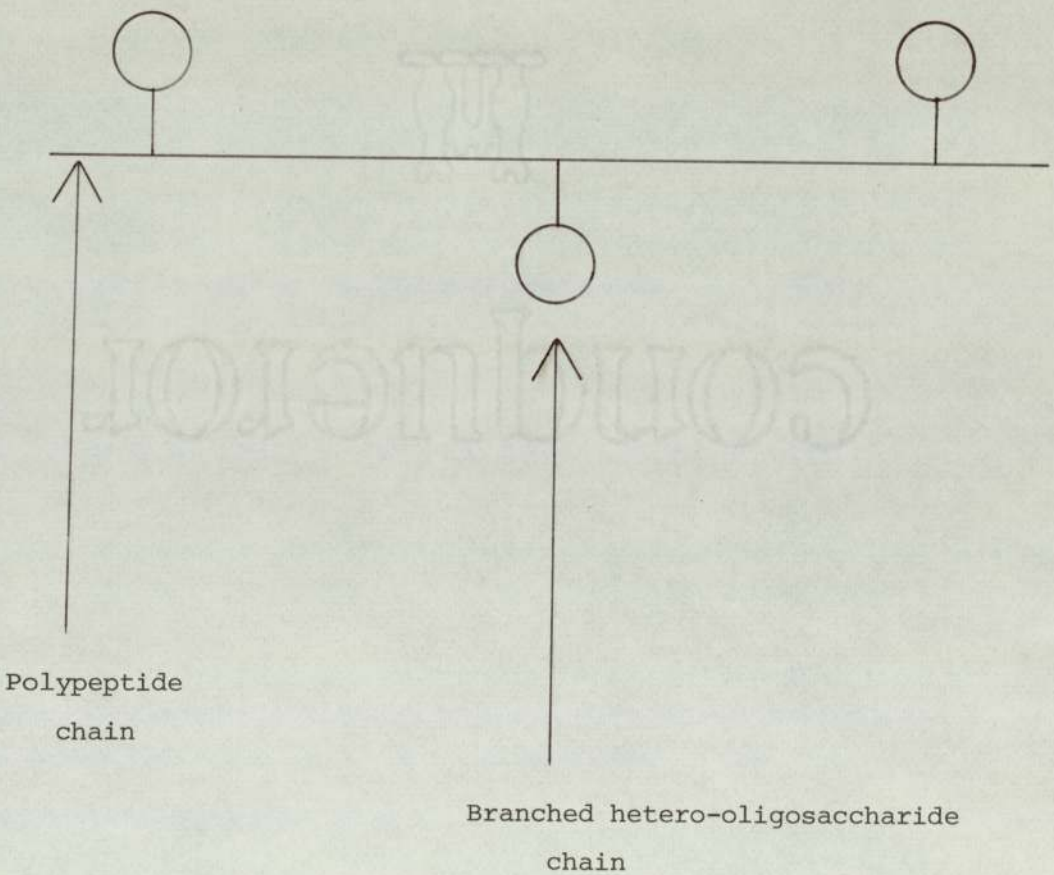
Rambourg, Neutra and Leblond (1966) demonstrated the existence of the surface coat on a large variety of rat tissues including epithelial cells, nerve cells and cells from mesenchymal origin such as lymphocytes and fibrocytes.

The glycocalyx is an integral part of the brush border membrane and it forms a continuous layer covering the microvilli of the epithelial cells of the small intestine (Ito, 1964; 1974). The degree of its development on the intestinal striated border is variable from species to species (Ito, 1965). The differences are not related to the taxonomic affinities or dietary habits of the animals (Ito, 1974).

The glycoprotein of the glycocalyx is composed of a polypeptide chain, which forms the back-bone of the glycoprotein, and oligosaccharide side chains (Fig. 1.1.). The oligosaccharide chain (10-15 residues long) are frequently branched and composed of different saccharide residues including, N-acetylgalactoseamine, mannose, galactose, fucose and the negatively charged residue N-acetylneuraminic acid, sialic acid (Winzler, 1970). Sialic acid is the usual monosaccharide unit which terminates the oligosaccharide side chains. It is the carboxyl groups of the sialic acid which contributes to the net negative charge on the surface of animal cells (Cook, 1976; Ambrose, 1975).

Autoradiographic studies indicated that the glycocalyx is a dynamic component of the cell membrane being continuously synthesised in the rough endoplasmic reticulum and Golgi region of an intact viable cell

Figure 1-1: General representation of glycoprotein structure (from Kennedy, 1973)



(Ito, 1965, 1969, Ito and Revel, 1964; Bennett, 1970; Leblond and Bennett, 1974). It has been suggested that the glycocalyx component moves to the exterior surface of the cell by means of vesicles (Michaels, 1977).

Crypt cells have few, short microvilli (Dognen, Visser, Daems and Galjaard, 1976) and the glycocalyx fibrils are shorter and less dense than that of the mature villous cells. In humans, the glycocalyx fibrils on the tips of the microvilli reach a length of 1.5 µm but between the microvilli the fibrils are less dense and shorter at 0.5 µm, while on the crypt cells they are only about 0.5 µm in length (Rifaat, Iseri and Gottlieb, 1965). Also the glycocalyx appears to be broken and poorly developed in the crypts compared to that of the villous cells (Rifaat, Iseri and Gottlieb 1965, Swanston 1978; Dognen, Visser, Daems and Galjaard, 1976). These differences seem to be due to the poorly developed Golgi apparatus and to the few strands of rough endoplasmic reticulum in the crypt cells which become more prominent during migration to the upper part of the crypt and onto the villi (Dognen, Visser, Daems and Galjaard, 1976), and to the slow rate of synthesis and incomplete structure of glycoproteins in the crypt cells (Weiser, 1976).

The glycocalyx reacts positively with the periodic acid Schiff (PAS). Carbohydrates which are positive to PAS include polysaccharides, mucopolysaccharides, mucoproteins, glycoproteins and glycolipids (Drury and Wallington, 1967). The reaction is almost entirely dependent upon the presence of sialic acid in their structure (Dahr, Uhlenbruck,

Schmalisch and Janssen, 1976).

Several functions have been attributed to the glycocalyx. Many investigators have suggested that the glycocalyx is the site of terminal digestion of carbohydrates and proteins (Ugolev, 1965; Greenberger, 1969; Fawcett, 1965). It is also established that a variety of terminal digestive hydrolyases (aminopeptidases, disaccharidases and alkaline phosphatase) are attached to the luminal surface of the brush border membrane and are embedded in the glycocalyx which provides them with a suitable environment for maximum activity (Louvard, Maroux, Vanner and Desnuelle, 1975; Maroux and Louvard, 1976). Other functions attributed to the glycocalyx include (i) joining adjacent cells (Ceralli, Familiari, Marinozzi and Muccioli-Casadei, 1976); (ii) cell protection (Martin and Louisot, 1976); (iii) as a diffusion barrier for mono and disaccharides (Hamilton and McMichael, 1968); (iv) Houston, Upshall and Bridges (1974; 1975) suggested that the glycocalyx might act as a hydrophilic barrier which determines the rate of absorption of molecules with relatively high partition coefficient such as pentyl, hexyl, heptyl or octyl carbamates; (v) as a diffusion barrier of hydrogen ions which creates an acid microclimate at the surface of the small intestine (Blair and Matty, 1974).

Alteration and damage to the glycocalyx has been observed in several well-defined disease conditions including coeliac disease and Crohn's disease (Swanston, Blair, Matty, Cooper and Cooke,1977; Swanston, 1978); ulcerative colitis (Ceralli, Familiari, Marinozzi and

Muccioli-Casadei, 1976); cow's milk intolerance (Iancu and Elian, 1976); and intestinal micro-organism infestations (Erlandsen and Chase, 1974). Rapidly dividing cells also produce a broken and incomplete glycocalyx (Swanston, 1978; Mondal and Heidelberger, 1980; Ishimura, Hiragun and Mitsui, 1980).

1.2. PROPOSED MODELS FOR WEAK ELECTROLYTE ABSORPTION :-

Several theories have been proposed to explain the mechanism of absorption of weak electrolytes. The non-ionic diffusion theory of Schanker, Tocco, Brodie and Hogben (1958) was one of the earliest models proposed. In their *in vivo* studies on the mechanism of drug absorption, they demonstrated a relationship between the rate of absorption and the dissociation constant of weak electrolytes. They also proposed that the mechanism involved was a passive diffusion of unionized species across a predominantly lipoidal and negatively charged membrane. Hogben and his colleagues (1959) later observed that the highly-lipid soluble drugs (i.e. drugs with relatively high partition coefficient) such as thiopental, were rapidly absorbed, while the less lipid soluble drugs although uncharged, such as sulfaguanidine, were slowly absorbed. This persuaded them to suggest that not only the availability of the unionized species of the weak electrolyte at the absorptive site is the rate limiting step, but that the lipid solubility of this form is also an important factor in determining the rate of absorption. The proposed model was called the pH-partition hypothesis.

The importance of lipid solubility in determining the rate of absorption of drugs was supported by the work of Houston, Upshall & Bridges (1974, 1975) and Bridges, Sargent & Upshall (1979).

The pH-partition hypothesis was widely applied to explain the mechanism of absorption of a variety of drugs across different parts of the gastrointestinal tract (Hogben, 1960; Kakemi, Arita, Hori, Konishi, Nishimura, Matsui and Nishimura, 1969; Crommelin, Modderkolk and Blaey, 1979; Yasuhara, Kobayashi, Kurosaki, Kimura, Moranishi and Sezaki, 1979), and in other systems such as rat and rabbit bladders (Borzelleca 1963, 1965).

Although the pH-partition hypothesis can explain the mechanism involved in the absorption of a variety of weak electrolytes, it was found difficult to reconcile the theory with the rapid absorption of moderately strong electrolytes, such as salicylic acid (pka 3.0) and nitro-salicylic acid (pka 2.3), since such acids are completely ionized at the pH of the intestinal chyme.

In an attempt to explain such deviation from the pH-partition hypothesis, a series of experiments to determine the distribution of the weak electrolytes between the plasma and the intestinal fluid at the steady state was carried out (Hogben, Tocco, Brodie and Schanker 1959). A suggestion was put forward that an acid microclimate pH 5.3 in the proximal jejunum, existed at the surface of the intestine which became less acidic further down the intestine. Hogben, Tocco, Brodie,

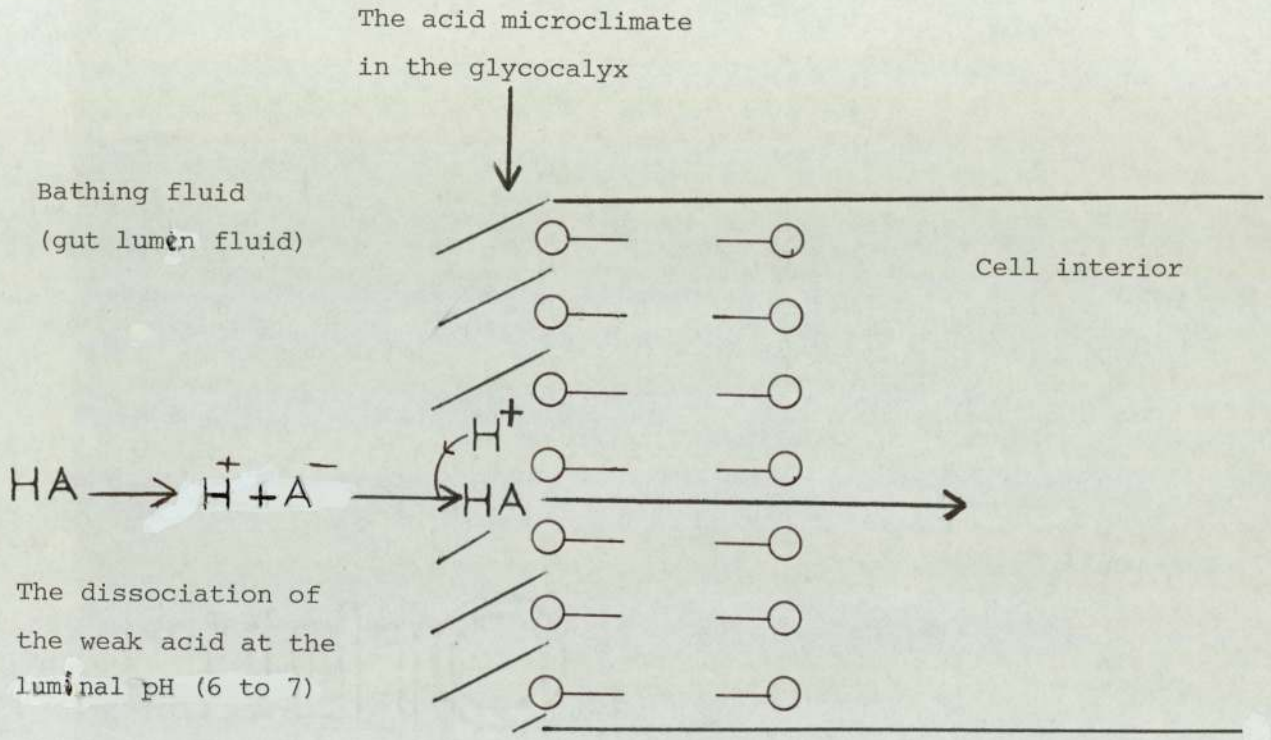
and Schanker (1959) assumed that the mechanism for the creation of the acid microclimate was related to the asymmetrics in the acid-base metabolism of the intestine. Also, the pH of the acid microclimate to some extent but not completely, independent of the pH of the intestinal chyme.

In the acid microclimate the ionized species of the weak electrolytes is converted into an unionized form which diffuses passively through the membrane (Figure 1.2).

Another theory to describe weak electrolyte transport was proposed by Kakemi, Arita, Hori, Konishi, Nishimura, Matsui, and Nishimura (1969). They suggested that the binding of both ionized and unionized forms of weak electrolytes (including moderately strong acids such as salicylic acid, and bases such as ephedrine pKa 9.6) onto the brush border membrane is an important step in their absorption and that the ionized form has the same adsorption ability as the unionized form. The adsorbed forms then diffuse easily and rapidly into the blood stream. They specify that this absorption mechanism is unique to the small intestine while absorption across the colon and rectum is consistent with the pH-partition hypothesis. No information was given about the nature of the binding site(s) involved.

An alternative theory for the absorption of weak electrolyte was proposed by Jackson and his colleagues, (Jackson, Shiau, Bane and Fox, 1974; Jackson and Morgan, 1975; Jackson & Kutcher, 1975).

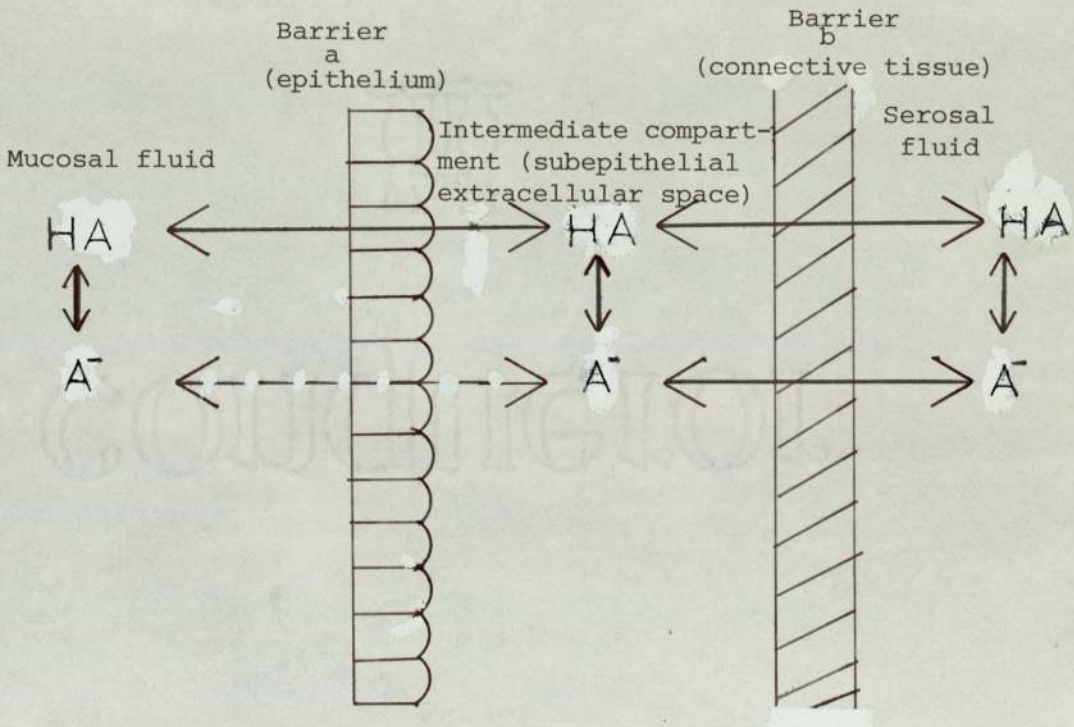
Figure 1-2 :- Schematic representation of the intestinal surface acid microclimate and its role in the absorption of weak electrolytes.



They confirmed the previous findings of Hogben, Tocco, Brodie and Shanker (1959) that the brush border membrane is impermeable to the ionized forms of weak electrolytes, but added that the ionized forms cross the mucosa through extracellular channels between the epithelial cells (the tight junctions). The structural basis of their model is that the mucosal bulk phase, the subepithelial extracellular space and the serosal bulk phase may be represented as three aqueous compartments (Figure 1.3). The model proposes that the serosal movement of metabolic bicarbonate anions from the epithelium into the subepithelial extracellular space maintains the pH of this compartment above that of the bulk phases, and that the created pH difference provides the driving force for the transport of weak electrolytes. Moreover Jackson et al., postulated that the alkalization of the serosal compartment is associated with acidification of the mucosal compartment and that low sodium ion concentration and metabolic inhibitors, inhibit both weak electrolyte absorption and serosal alkalization without affecting mucosal acidification.

The experimental observations concerning the elevation in the serosal compartment pH, the effect of low sodium concentration and metabolic inhibitors on mucosal acidification have been contradicted by a number of other published observations (Barry, Jackson and Smyth, 1966; Blair, Lucas and Matty, 1975, Lucas, 1974; Lei, Lucas, and Blair, 1977). Moreover it is difficult to relate the proposed model to the in vivo situation in which absorbed species move from the

Figure 1-3 :- The structural basis of weak electrolyte transport in rat jejunum (from Jackson and Kutcher, 1975)



mucosal bulk phase directly into the blood stream without crossing the other proposed compartment..

1.3 SURFACE PROPERTIES OF THE SMALL INTESTINE :-

(i) Characteristics of biological interfaces :-

It has long been realized that the behaviour of small particles at a physical interface is usually different from that when they are inside any of the phases sharing that interface. Deutch (1928) showed that when aqueous solutions of sulphonated acidic dyes are shaken with benzene there was a colour difference at the benzen-water interface indicative of a lower pH. Stearic acid in monolayers behaves as if its pKa value was three units higher than that recorded in the bulk phase (Bagg, Haber and Gregor, 1966) and demonstrates that there is a higher concentration of hydrogen ion at the interface than in the bulk phase of the solution.

Few enzymes exist as free protein molecules in an aqueous environment. They are either membrane bound or present in gel-like surroundings. The catalytic properties of bound enzymes can be quite different from those in free solution. The experimental observations of Michaels and Morelos (1955) revealed that when chymotrypsin was adsorbed onto kaolin particles it acted as if it was in an environment some two pH units lower than the bulk phase. Papain impregnated into a synthetic membrane yielded similar observations (Goldman, Silman, Caplan, Kedem and Katchalski, 1965; Goldman,

Kedem, Silman, Caplan and Katchalski, 1968). Goldman and his colleagues accounted for these differences by assuming that the enzyme in the membrane acts in a microenvironment different from that prevailing in the external solution. The extent of the deviation of the optimal pH for bound enzymes from the normal pH dependency profiles depends upon the rate of substrate hydrolysis and the rate of diffusion of the products away from the membrane (Goldman, Silman, Caplan, Kedem and Katchalski, 1965; Goldman, Kedem, Silman, Caplan and Katchalski, 1968). The higher concentration of hydrogen ion at the interface has been shown experimentally and theoretically to be due to the physical characteristics of the interface and increased hydrolylic activity of the enzyme (Beem, Zabusky and Sternlicht, 1978; Naparstek, Thomas and Caplan, 1973; Chary, 1980).

(ii) Intestinal surface acid microclimate :-

The proposal of an acid microclimate at the surface of the small intestine was put forward by Hogben, Tocco, Brodie and Schanker (1959). They suggested that the surface acid microclimate would have a pH of 5.3 at the proximal jejunum which decreases down the intestine by not more than one pH unit.

The concept of the acid microclimate has now been widely accepted and used to explain the features of the mechanism of absorption of a variety of drugs and nutrients in different systems, e.g. intestinal absorption of folate (Blair and Matty, 1974; Blair, Johnson and Matty, 1974; Blair, Matty and Razzaque, 1975; Kesavan and

Noronha, 1978; Noronha and Kesavan, 1979); propranolol and atenolol across the human oral mucosa (Schürmann and Turner, 1978), ammonia across the trout gill membrane (Lloyd and Herbert, 1960), theophylline across the rat rectal mucosa (Crommelin, Modderkolk and Blaey, 1979) and short chain fatty acids from human ileum (Schmitt, Soergel, Wood and Steff, 1977).

Recently the existence of the acid microclimate at the surface of rat and man intestine in vitro has been demonstrated by direct measurement using a pH-microelectrode (Lucas, Schneider, Haberich, and Blair, 1975; Lucas, Cooper, Lei, Johnson, Holmes, Blair and Cooke, (1978) with a maximum acidity in the proximal jejunum and less acid at the distal ileum. Aerobic conditions, glucose and sodium ion are essential requirements for its creation and maintenance (Lucas and Blair, 1978).

Blair and Matty (1974) proposed an enzymatic model for the creation of the intestinal surface acid microclimate. They excluded an electrostatic model in which the negatively charged phospholipid membrane accumulated high concentration of hydrogen ions by attraction, because such a model could not increase the transport rate of a weak acid such as folic acid which would be present in its ionized form in the intestinal chyme (pH 6 to 7).

Blair and Matty (1974) suggested the pH of the surface microclimate to be less than 4.0 in the proximal jejunum. However, due to the size of pH-microelectrodes used to measure the surface

acidity, it has not been possible to record a surface pH of less than 5 (Lucas, 1980).

The model proposed by Blair and Matty requires (i) an aqueous phase at the intestinal mucosal surface not in equilibrium with the intestinal chyme. (ii) a source of hydrogen ions arising from enzymatic hydrolysis or by phosphorylation reactions at the mucosal surface and (iii) a slow diffusion of the hydrogen ions within the aqueous phase. An acid microclimate of such kind would require for its effective functioning, three conditions. Firstly, the maintenance of the glycocalyx; secondly, the presence of appropriate enzyme(s) in adequate amounts, and lastly, the supply of substrates to these enzymes in adequate amounts.

The existence of an unstirred water layer at the cell surface is now well established (Dietschy, Sallee and Wilson, 1971; Parsons and Subjeck, 1972). Further the glycocalyx at the intestinal surface can retain hydrogen ions and slow their diffusion into the intestinal chyme as well as stabilize the unstirred layer at the cell surface which fulfils one of the requirements of the model.

The enzyme(s) have been suggested to be the mucosally located ATPase (Blair and Matty, 1974; Blair, Lucas and Matty, 1975) and that the external hydrolysis of metabolically produced ATP is the source for hydrogen ions.

Most of the evidence concerning the involvement of ATP and

mucosally located ATPase came from *in vitro* studies on the luminal acidification in the rat proximal jejunum (Blair, Lucas and Matty, 1975; Lucas, 1974). It has been suggested that ATPases are the major enzymes responsible for the production of hydrogen ion and that addition of ATP to the mucosal solution of everted sacs from rat proximal jejunum caused a greater acidification in the incubation medium than that observed with glucose though ATP does not enter the everted sac preparation in any appreciable quantity (Blair and Matty, 1974; Blair, Lucas and Matty, 1975).

The existence of ATPase in the brush border of the epithelial cells of the small intestine is well established (Noronha and Kesavan, 1979; Forstner, Sabesin and Isselbacher, 1968; Humphreys and Chou, 1978). Incubation *in vitro* of everted intestinal sacs causes an increase in the level of phosphate ions in both the serosal and the mucosal fluids (Wilson, 1954). Both these experimental observations support the Blair and Matty (1974) model. The proposal of Hogben, Tocco, Brodie and Schanker (1959) that the acidity of the surface acid microclimate decreases down the intestine can therefore be explained by decreased ATP and ATPase activity down the intestine (Hanninen, Hartiala and Nurmikko, 1964).

Several other models have been suggested to explain the luminal acidification seen in the jejunum. Lactic acid production in the intestinal epithelial cells through anaerobic glycolysis with a subsequent preferential migration of hydrogen ions mucosally and lactate

anions serosally together with serosal movement of bicarbonate anions has been proposed to explain the mucosal medium acidification seen after incubating the rat proximal jejunum in vitro (Wilson, 1953; 1954; Wilson and Kazyak, 1957). This mechanism of hydrogen ion production would also fit the original proposal of Hogben, Tocco, Brodie and Schanker (1959) of an acid microclimate with a maximum acidity in the proximal jejunum and becoming less acid in the ileum. This is because the jejunum has a higher rate of lactic acid production than the ileum (Wilson and Wiseman,1954b) which instead relies on oxidative metabolism (Gilman and Koelle, 1960). Also bicarbonate anions are secreted in very small amounts in the ileum (Wilson and Kazyak, 1957; Turnberg, Fordtran, Carter and Rector, 1970).

Another potential source of hydrogen ions could arise from the hydration of metabolically produced carbon dioxide with subsequent dissociation of the produced carbonic acid into hydrogen ions and bicarbonate anions. The hydration of carbon dioxide and the subsequent dissociation of the carbonic acid into hydrogen ions and bicarbonate anions might occur in the mucosal compartment with the hydrogen ions remaining in the mucosal side and the bicarbonate anions move serosally. This model has been used to explain the acidification in the turtle bladder (Schilb and Brodsky, 1972). Alternatively, the surface acidity may be a result of intracellular hydration of carbon dioxide involving the enzyme carbonic anhydrase with a subsequent intracellular dissociation followed by a preferential movement of hyd-

rogen ions mucosally and bicarbonate anions serosally (Turnberg, Fordtran, Carter and Rector, 1970; Turnberg 1978). A third possibility is that carbonic acid formed intracellularly through enzymatic hydration of carbon dioxide, is transported and subsequently ionized in the mucosal compartment with the hydrogen ions staying there and bicarbonate anions moving serosally.

(iii) Aims of the present studies on the surface properties of the small intestine :

1. To investigate the distribution of the surface acid microclimate along the length of the small intestine and colon.

2. To investigate whether or not a stomach surface microclimate exists.

3. To measure the intestinal surface acid microclimate pH in vivo in rats.

4. To elucidate the mechanism involved in the creation of the surface acid microclimate.

5. To determine the necessary requirements for the maintenance of normal surface acid microclimate if it exists.

6. To investigate the role of adenyl cyclase-phosphodiesterase system, in creating and maintaining the surface acid microclimate.

7. To investigate the role of the surface acid microclimate in the absorption of nutrients with particular reference to folate absorption.

8. To investigate the variations of the surface acid microclimate in human disease conditions and the impact of this on nutrient absorption, particularly folate.

1.4. INTESTINAL ABSORPTION OF FOLATES :-

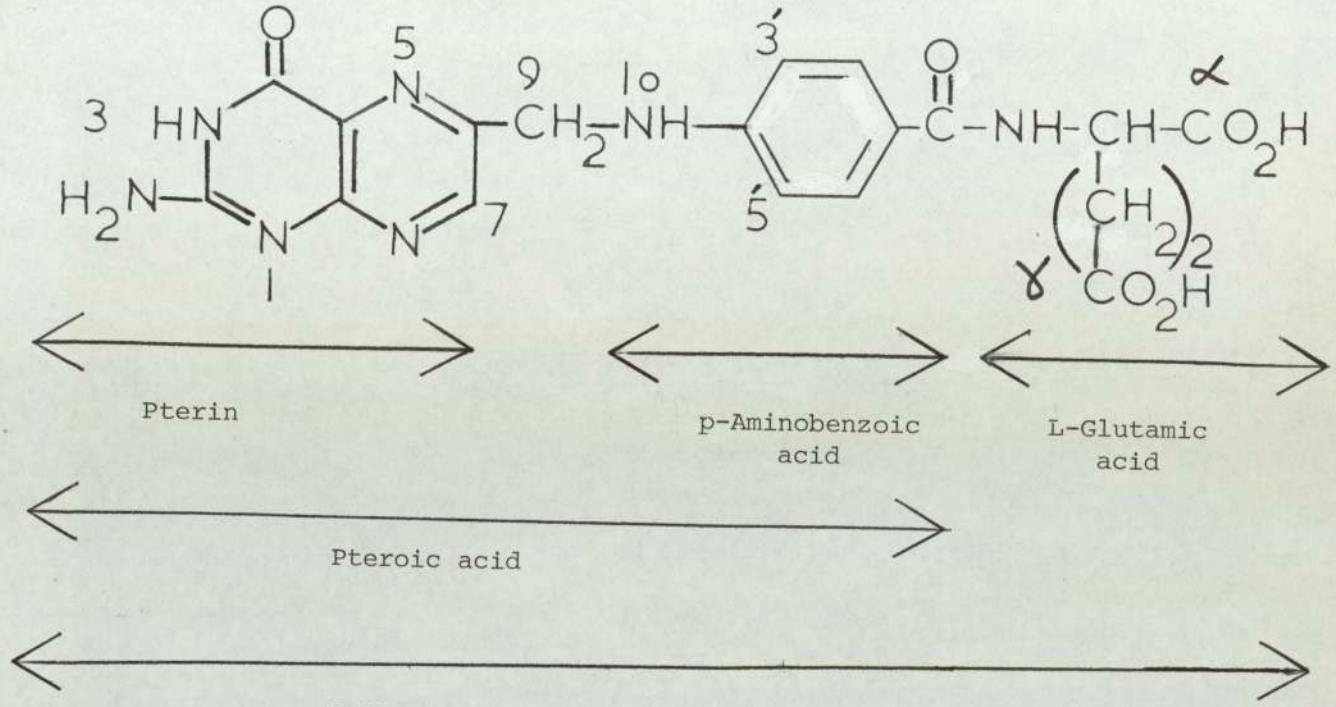
(i) Chemistry of folates :-

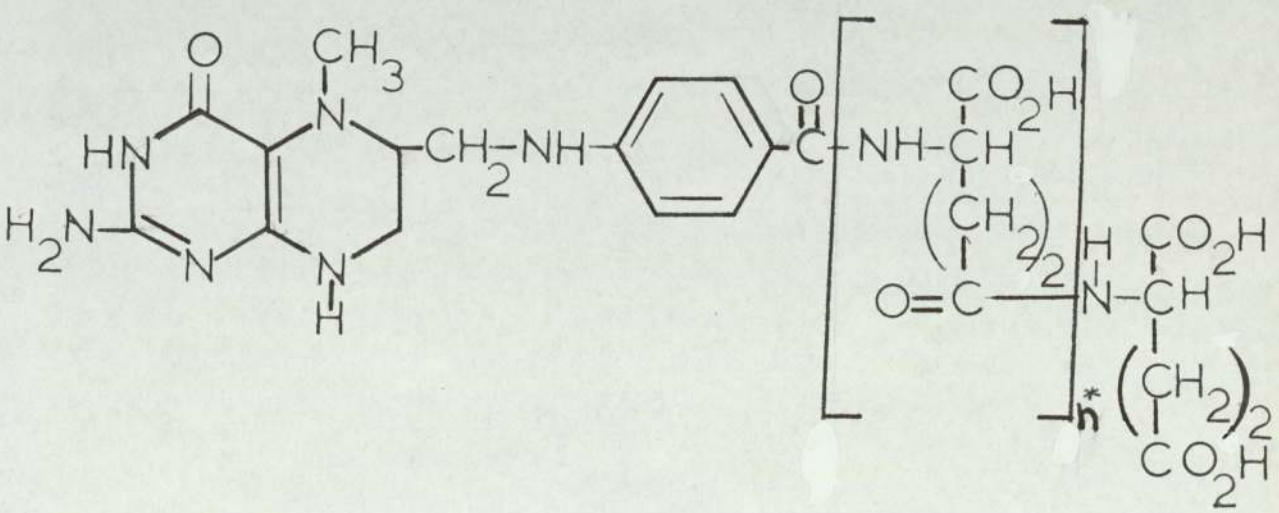
Dietary folates are a complex mixture of pteroylglutamates of various chain length with a variety of substitutions on the pteridine ring. More than 75-90% of dietary folates are in the form of pteroylpolyglutamates, principally 5-methyltetrahydrofolic acid (5-meTHF), while the remaining pteroylmonoglutamates are principally 10-formyltetrahydrofolic acid (Butterworth, Santini and Formmeyer, 1963; Perry, 1971; Stokstad, Shin and Tamura, 1977).

Folic acid is not found in the body, instead the predominant circulating and storage form of folate is the reduced form, principally 5-meTHF (Herbert, Larrabee and Buchanan, 1962). Figure 1.4 shows the structure of some folates and pteridines.

Normal metabolism in man and other animals necessitates certain derivatives of folic acid participating as co-enzymes in many metab-

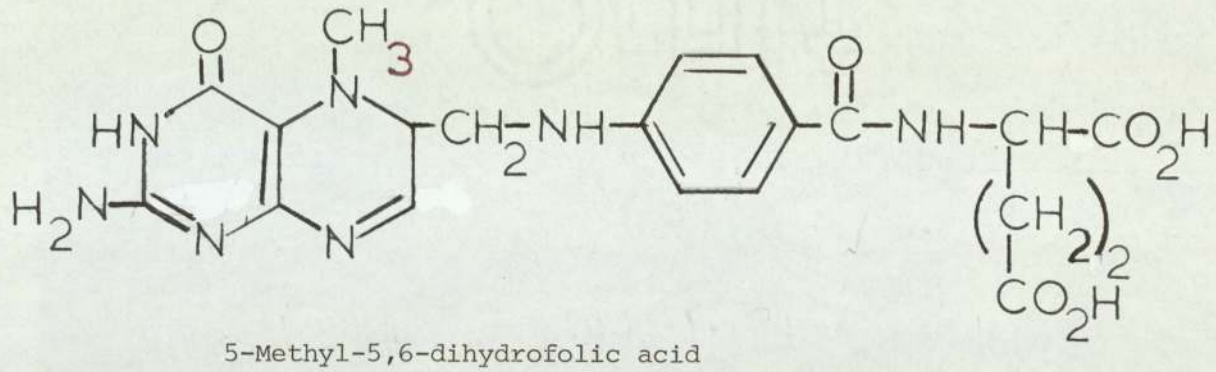
Figure 1-4: Chemical Structure of some folates and pterins



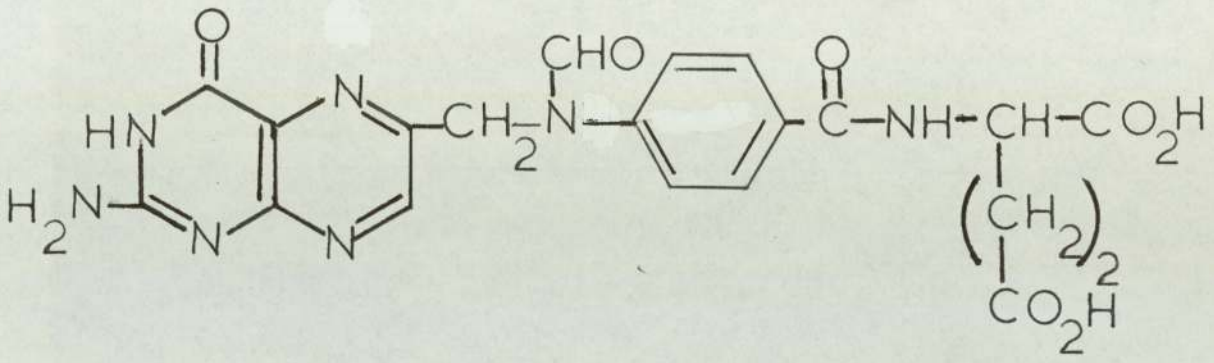


5-Methyltetrahydrofolate polyglutamate

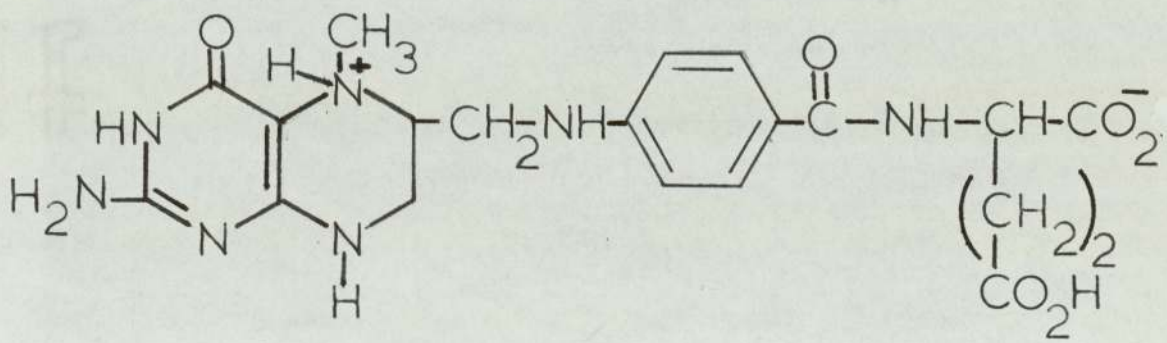
* n = 1 to 7.



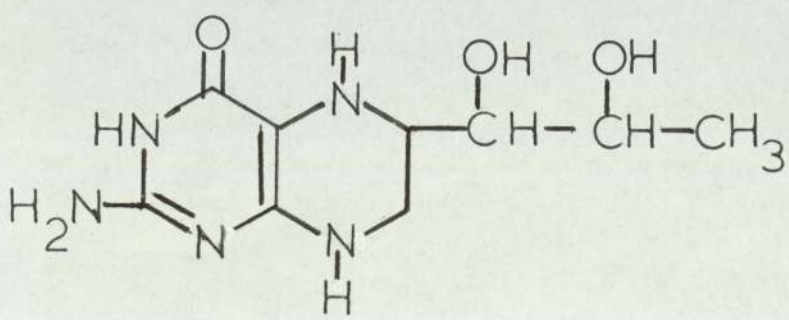
5-Methyl-5,6-dihydrofolic acid



10-Formylfolic acid



The neutral zwitterion form of 5-methyltetrahydrofolic acid.



Tetrahydrobiopterin

olic reactions and in the synthesis of DNA precursors. As there is no biosynthesis of folates within the body, they must be obtained from the diet by absorption.

Folate deficiency leads to a condition known as megaloblastic anaemia, in which less red blood cells are synthesised, and those synthesised are often mis-shapen or fragile. Other developing cells such as cervical, vaginal and gastrointestinal epithelia are also affected due to the inhibition of DNA biosynthesis; while RNA synthesis is unaffected (Stebbins and Bertino, 1976). Folic acid is successfully employed in treating the conditions associated with folate deficiency.

Both folic acid and 5-meTHF are weak acids with pKa values of 3.5 for the α-carboxyl group (Kallen and Jencks, 1966) and 5 and 4.8 for the γ - carboxyl groups of folic acid and 5-meTHF respectively (Pohland, Flynn, Jones and Shive, 1951; Kallen and Jencks, 1966). The neutral form of 5-me THF is the zwitterion (Figure 1.4). The saturation solubility (Table 1.1) and the percentage of the neutral form of folic acid and 5-meTHF (Figure 1.5 and Table 1.2) in aqueous media are pH dependent. At the physiological pH of the small intestine, folic acid and 5-meTHF are present completely in their ionized forms.

Although folic acid is relatively stable in aqueous media, 5-meTHF is an unstable compound and is readily oxidised by air (Blair, Pearson and Robb, 1975) to a yellow compound designated to be a

TABLE 1-1 : Saturation solubility data, in water at various pH values for folic acid at 30°C and 5-meTHF at 25°C (from Blair and Matty, 1974).

pH	Folic acid	5-meTHF
6.0	3.5×10^{-3}M	5.6×10^{-2}M
5.0	1.9×10^{-4}M	5.6×10^{-2}M
4.0	2.1×10^{-5}M	3.3×10^{-2}M
3.0	2.3×10^{-6}M	3.2×10^{-2}M
2.0	-	6.2×10^{-2}M

TABLE 1-2

Percentage of folic acid and 5-methyltetrahydrofolic acid as neutral species at various pH values. (From Coleman, Hilburn and Blair, 1979).

pH	Folic Acid (%)	5-Methyltetrahydrofolic Acid (%)
1.0	4.3	0.3
2.0	30.6	3.1
3.0	64.8	23.9
3.2	60.6	33.1
3.4	52.5	43.5
3.5	50.5	51.5
3.6	42.3	53.8
3.7	36.0	60.0
3.8	31.7	62.3
4.0	22.2	67.4
5.0	1.6	27.7
6.0	0.0	0.9
7.0	0.0	0.0

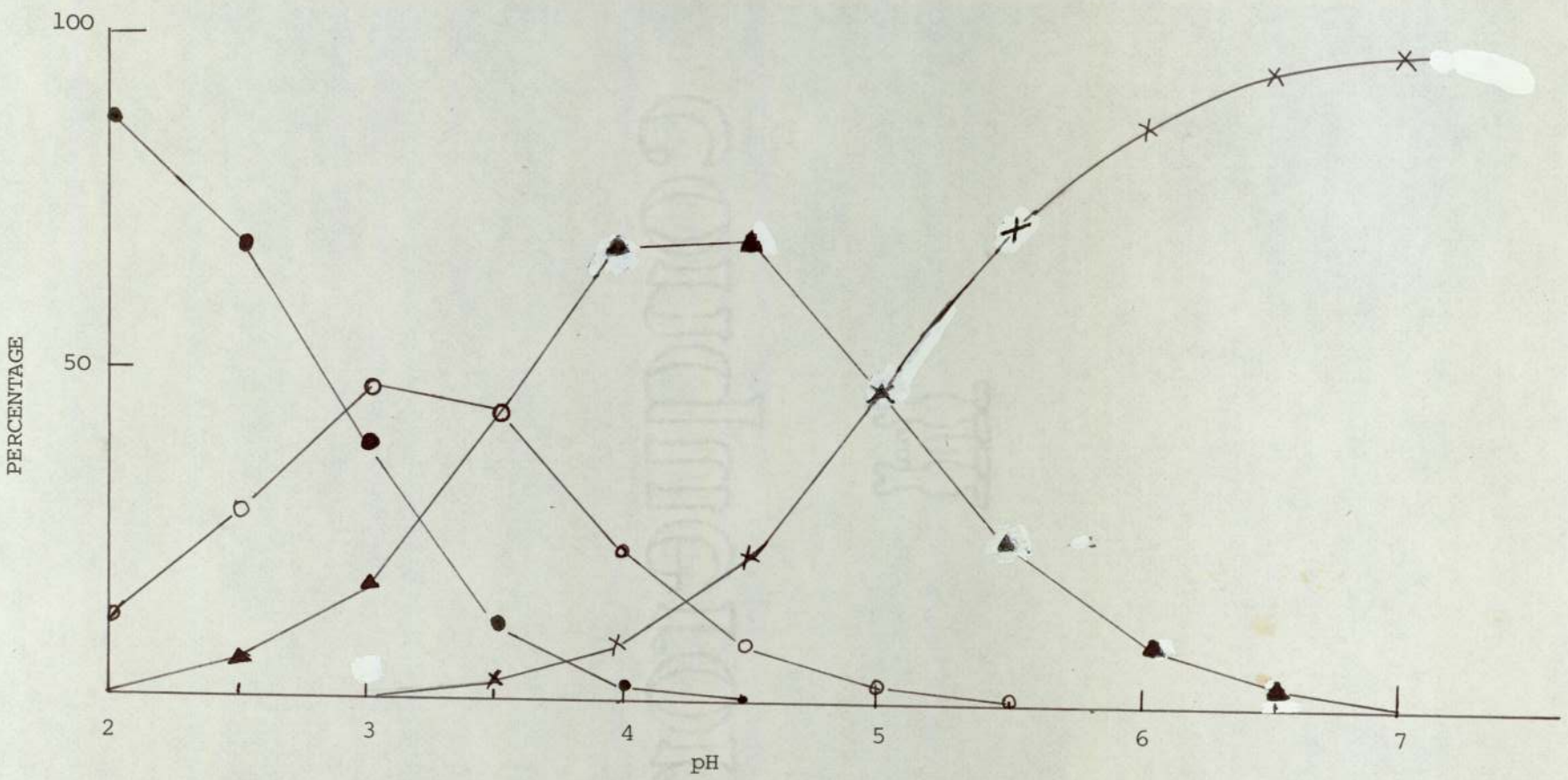


Figure 1.5: Relative concentrations of neutral and ionised species of folic acid at acidic pH values. (From Lucas 1977)

(x) = dianion; (▲) = monoanion; (o) = neutral molecule; (●) = cation.

pyrazino-s-triazine derivative (Jongejan, Mager and Berends, 1979). However, the addition of a suitable antioxidant such as ascorbic acid is able to minimize the oxidation reaction (Blakley, 1969).

(ii) Intestinal absorption of folates:-

The ingested polyglutamates are broken down in a stepwise fashion to monoglutamates by the hydrolytic enzyme conjugase (γ-glutamyl carboxy peptidase) at the cell surface. Most workers however, have reported that enzyme activity in intestinal cells is largely localized to the lysosomal fraction (Hoffbrand and Peters, 1969; Rosenberg and Godwin, 1971). This problem appears to have been resolved recently by the report of Reisenauer and colleagues (Reisenauer, Krumdieck and Halsted, 1977) of two separated enzymes with a pH optimum of 4.5 (presumably lysosomal) and the other localised at the brush border membrane, with a pH optimum of 7.5. The rate-limiting step in the absorption of the folate monoglutamates produced from the enzymatic hydrolysis of polyglutamates is not the hydrolysis step, but rather the absorption mechanism of the folate monoglutamate (Halsted, Baugh and Butterworth, 1975; Rosenberg, 1976).

The site of folic acid absorption is principally the proximal jejunum (Smith, Matty and Blair, 1970; Coleman, Hilburn and Blair, 1979). Conflicting reports exist as to the principal site of 5-meTHF absorption. Strum and his colleagues (Strum, Nixon, Bertino and Binder, 1971) reported that 5-meTHF is absorbed equally well across the jejunum as across the ileum while Coleman, Hilburn, and

Blair, (1979) reported a marked decline in the absorption from the jejunum down to the ileum.

No other topic in the field of folate absorption has produced more conflicting observations than has the inquiry into the mechanism of folate uptake by the intestinal epithelial cells. However, the pteridine ring, the para-aminobenzoyl moiety and at least one glutamate unit, together are the structural requirements for absorption, as neither pteroic acid nor tetrahydrobiopterin (Figure 1.4) are taken up by the intestine to any significant extent (Blair, Ratanasthien and Leeming, 1974).

Controversy still exists as to whether or not the transport process for folic acid and 5-meTHF is active or passive. Several reports using different techniques support the existence of an active mechanism for the absorption of folic acid from the small intestine of both man and experimental animals (Russell, Dhar, Dutta, and Rosenberg, 1979; Dhar, Selhub, Gay and Rosenberg, 1977; Halsted, Bhanthumnavin and MeZey, 1974; Rose, Koch and Nahrwold, 1978; Strum, 1979; Chungi., Bourne and Dittert, 1979). On the other hand, many other reports support a passive diffusion mechanism (Turner and Hughes, 1962; Smith, Matty and Blair, 1970; Yoshino, 1968). Blair and Matty (1974) have suggested a facilitated passive diffusion of the unionized folate forms with the aid of an acid microclimate at the surface of the intestine. The apparent saturation kinetics sited as evidence for active transport has been explained by Blair and Matty on the basis of the limited sol-

ubility of folate in the acid microclimate. Further, the requirement for energy and sodium, rather than supportive evidence for active transport was thought by the same workers to be essential for the maintenance of a low surface pH.

Similarly the transport of 5-meTHF has been suggested to be passive diffusion (Strum, Nixon, Bertino and Binder, 1971) facilitated passive diffusion through the intestinal acid microclimate (Blair and Matty, 1974; Blair, Matty and Razzaque, 1975; Blair, Ratanasthien and Leeming, 1974; Kesavan and Noronha, 1978; Noronha and Kesavan, 1979) or an active transport process (Weir, Brown, Freedman and Scott, 1973).

The rate of absorption of folic acid (Mackenzie and Russell, 1976; Russell, Dhar, Dutta and Rosenberg, 1979; Benn, Swan, Cooke, Blair, Matty and Smith, 1971; Smith, Matty and Blair, 1970) and 5-meTHF (Kesavan and Noronha, 1978) is maximal at pH 5 to 6 and decreases at more alkaline pH values. This observation can be explained equally well using the concept of active transport or facilitated passive diffusion through the acid microclimate. This is because it is possible to suggest that there is an increase in the affinity of the carrier towards its substrate with increased acidity, or that the acidity of the microclimate decreases as the pH increases (Lei, Lucas and Blair, 1977) so that the amount of undissociated folates available for absorption decreases.

(iii) Folate metabolism by the intestinal tissue :-

Although 5-meTHF traverses the small intestine metabolically unaltered (Strum, Nixon, Bertino and Binder, 1971), conflicting reports have been presented concerning folic acid. Thus, two groups who sampled the human portal blood by umbilical vein cathetarization found the molecule largely unchanged after oral administration of pharmacological doses (0.5 to 3 mg) of folic acid (Whitehead and Cooper, 1967; Melikian, Paton, Leeming and Portman-Graham, 1971). This has been confirmed in rats in vitro (Smith, Matty, and Blair, 1970). However, Olinger, Bertino and Binder (1973) demonstrated the appearance of 5-meTHF in serosal fluid after placing folic acid on the mucosal side of rat mucosal sheets. This has been confirmed by Perry and Chanarin (1973) and Selhub, Brin and Grossowicz (1973) who used everted sacs of rat proximal jejunum and demonstrated the production of both 5-meTHF and 10-formyltetrahydrofolic acid (10-FTHF) in the serosal fluid. Strum (1979) in his study in rat using the everted sac technique, found that enzymatic reduction and methylation of folic acid was extensive at pH 6 and negligible at pH 7.5. In man evidence from studies comparing the fate of orally and parenterally administered reduced folate suggested that 5-FTHF was substantially converted to 5-meTHF during the process of intestinal absorption (Nixon and Bertino, 1972).

The transport rate of folic acid can proceed however, without metabolic alteration (i.e. neither reduction nor one carbon substitution of monoglutamate are obligatory requirements in the transport process), as it has been demonstrated that transport occurs in the presence of methotrexate which will inhibit metabolism of folic acid (Olinger, Bertino and Binder, 1973; Rosenberg, 1976).

(iv) Folate malabsorption :-

Folate deficiency is encountered clinically as a primary result of impaired intestinal absorption in patients with marked villous damage or atrophy of the upper small intestine. Table 1.3. shows disease conditions associated with folate malabsorption. The explanation for the malabsorption of folates is dependent upon which model is accepted as the mechanism of transport.

In patients with coeliac disease, absorption of both folate polyglutamate and folate monoglutamate is impaired, (Halsted, Reisenauer, Romero, Cantor and Ruebner, 1977) while the amount of intestinal conjugase is increased (Jägerstad, Lindstrand, Norden, Westesson and Lindberg, 1974). Although it has been shown, using a jejunal perfusion technique, that the rate of hydrolysis of folate polyglutamate was depressed, the main disturbance appears to be in the uptake of folate monoglutamate (Halsted, Reisenauer, Romero, Cantor, and Ruebner, 1977). It has been suggested that in coeliac disease and in other diseases in which folate malabsorption was reported (see Table 1.3) the main disturbance is in the mechanism of transport. Weir (1974)

TABLE 1-3 :- Diseases associated with folate malabsorption

Disease	Citation
Coeliac disease	Halsted, Reisenauer, Romero, Cantor and Ruebner, (1977)
	Hoffbrand, Douglas, Fry and Stewart (1970)
Tropical sprue	Corcino, Coll and Klipstein (1975)
Crohn's disease	Franklin and Rosenberg (1973)
Ulcerative colitis	Franklin and Rosenberg (1973)
	Elsborg and Larsen (1979)
Small intestine resection	Booth (1961)
Dermatitis herpetiformis	Hoffbrand, Douglas, Fry and Stewart (1970)
Diabetic enteropathy	Klipstein (1966)
Small intestine lymphoma	Klipstein (1966)
Amyloid	Klipstein (1966)
Whipple's disease	Klipstein (1966)
Systematic bacterial infections	Cook, Morgan and Hoffbrand (1974)
Cardiac failure	Hyde and Loehry (1968)
Gastroectomy	Elsborg (1974a)

and Weir, Brown, Freedman and Scott (1973) assumed that a decrease in the affinity of the carrier transport system is the cause of the malabsorption of folates, while Blair and Matty (1974) hypothesised a decrease in the acidity of the acid microclimate at the intestinal surface to be the cause.

Low serum folate levels during pregnancy is not due to the malabsorption of the vitamin but is a result of the increased demands on the maternal folate stores by the growing foetus (Swanston, 1978).

Drugs which have been shown to inhibit the absorption of folic acid are listed in Table 1.4. The long term administration of the anticonvulsant drug phenytoin causes low serum folate levels either due to the inhibition of gastrointestinal conjugase (Hoffbrand and Necheles, 1968) or because folic acid metabolismin the body is altered (Krumdieck, Fukushima, Fukushima, Shiota and Butterworth, 1978) or because the luminal pH is altered (Benn, Swan, Cooke, Blair, Matty and Smith, 1971).

Methotrexate can produce folate malabsorption through either elevating the intestinal surface acid microclimate pH (Lucas, Swanston, Lei, Mangkornthong and Blair, 1978), or because the methotexate completes with a carrier system (Chungi, Bourne and Dittert, 1979) or alternatively, because methotrexate is toxic towards the intestine (Robinson, Antonioli and Vannotti, 1966).

Ethanol is reported to decrease serum folate level (Herbert,

TABLE 1-4 : Drugs associated with folate malabsorption

DRUG	CITATION
Methotrexate	Hepner (1969)
Diphenyl hydantoin (Phenytoin)	Elsborg (1974b); Hoffbrand and Necheles (1968); Benn, Swan, Cooke, Blair, Matty, and Smith (1971).
Ethanol	Halsted, Robles and Mezey (1973).
Oral contraceptives	Shojania and Hornady (1973)
Antituberculosis drugs	Klipstein, Berlinger and Reed (1967)
Paramino salicylate	Longstreth, Newcomer and Westbrook (1972)

Zalusky and Davidson, 1963) and impair folate absorption (Halsted, Robles and MeZey, 1973) and produce megaloblastic anaemia (Herbert, Zalusky and Davidson, 1963). The effect of ethanol is due to either the elevation in the intestinal acid microclimate, (Blair, Lucas and Swanston, 1979), abnormality in the intestinal mucosa, or to the various toxic effects of ethanol on cellular metabolism.

(v) Aims of the present studies on the intestinal absorption of folate :-

1. To determine the rate of uptake of folic acid and 5-meTHF by the intestinal tissue and their subsequent transfer to the serosal compartment in the presence of different mucosal concentrations of folic acid and 5-meTHF at different incubation pH values.

2. To examine the role of solvent drag with water movement in folate absorption.

3. To define a preferential site(s), if any, of absorption.

4. To attempt to elucidate the mechanism of folic acid and 5-meTHF absorption and whether or not they share the same mechanism of transport.

5. To study the effect on folate absorption of altering the integrity of the intestinal surface, i.e. glycocalyx.

6. To determine the possible metabolism of folic acid and 5-meTHF by the gut tissue during the absorption process with

emphasis on the products found in the tissue compartment.

7. To determine whether or not an isotopic effect exists in the absorption of ^{3}H- and ^{14}C- labelled folates.

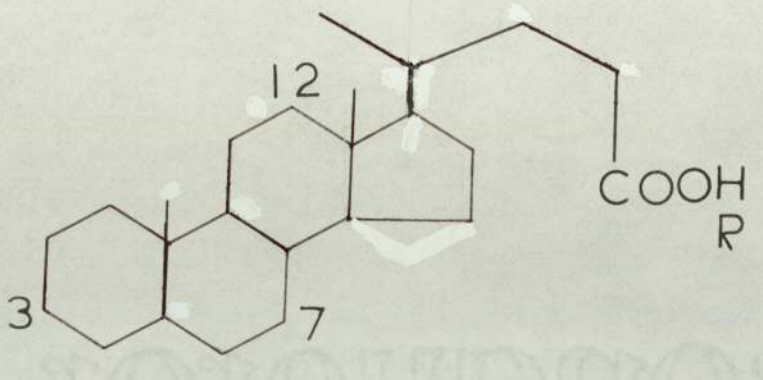
1.5. BILE ACIDS AND SALTS AND THEIR EFFECT ON THE SURFACE PROPERTIES OF THE SMALL INTESTINE :-

Bile is a fluid secreted from the liver, rich in potentially membrane damaging physiological detergents. All bile acids, which are the end product of the catabolism of cholestrol by liver, share the same basic structure ofC-24 carboxylic acids with a steroid nucleus. Attached to this at certain positions are hydroxyl groups (see Figure 1.6). Bile acids and salts contain both hydrophobic and hydrophilic regions, i.e., they are amphipathic, and possess many of the properties of surface active agents or detergents.

Bile acids are secreted as mixed micelles with phospholipids and cholestrol, from the liver as glycine or taurine conjugates, bile salts. The conjugation and micellular formation minimise the damaging effect of bile acids on the hepatobiliary system (Coleman, Holdsworth and Vyvoda, 1977; Coleman, Iqbal, Godfrey and Billington, 1979).

Bile enters the intestinal tracts midway down the duodenum and is actively re-absorbed as bile salts at the ileum (Dietschy, 1968; Beesley and Faust, 1979). On the other hand, some of the free bile acid produced through bacterial deconjugation and reduction (Hill, 1975) are absorbed passively along the entire length of the small intestine

Figure 1-6: The structure of some bile acids and salts.



Cholanic acid

	3	7	12
Cholic acid	OH	OH	OH
Deoxycholic acid	OH	H	OH
Chenodeoxycholic acid	OH	OH	H
Lithocholic acid	OH	H	H

When R is $-NH-CH_2-COOH$ it is glyco-bile acid;

When it is $-NH-CH_2-CH_2SO_3H$ it is tauro-bile acid.

and colon (Dietschy, 1968).

The important physiological role of these surfactants is to emulsify dietary triglycerides and to bring fatty acids and monoglycerides into micellar solution during the intraluminal phase of fat absorption. It has been recently suggested that bile salts help to regulate the fluidity of the contents of both the small and the large intestine (Saunders, 1975; Wingate, 1974; Binder, Filburn and Volpe, 1975; Forth and Rummel, 1975), as well as regulating colonic cell proliferation (Deschner and Raicht, 1979).

Several lines of evidence suggest that certain bile acids, particularly deoxycholate may be involved in the pathogenesis of large bowel cancer (Reddy and Wynder, 1977; Narisawa, Magadia, Weisburger and Wynder, 1974; Reddy, Watanabe, Weisburger and Wynder, 1977) and a good statistical correlation has been found between increased concentration of deoxycholate in the colon and raised incidence of colonic cancer (Hill, 1975; 1977 and 1978). Phorbol ester derivatives, the synthetic detergents, are tumour promoting and differentiation inhibitor agents (Mondal and Heidelberger, 1980; Yamaski, Fibach, Nudal, Weinstein, Rifkind and Marks, 1977; Ishimura, Hiragun and Mitsui, 1980).

Coleman and Holdsworth (1976) and Vyvoda, Coleman and Holdsworth (1977) have shown that dihydroxy bile acids, deoxycholic acid and its conjugates, are more damaging to biological membranes than trihydroxybile acids, cholic acid and its conjugates. Also de-

oxycholate at low concentrations has only a surface damaging effect as it was found to remove the surface bound enzymes from isolated hepatocytes without affecting the integrity of the cell (Coleman, Iqbal, Godfrey and Billington, 1979).

The effect of some bile acids, mainly deoxycholate, and bile salts on the integrity of the mucosal epithelial cells and their brush border membrane, the surface acid microclimate pH and the intestinal absorption of folates will be examined.

CHAPTER TWO

MATERIALS AND METHODS

2.1 ANIMALS AND CHEMICALS :-

Male albino rats of the Wistar strain weighing between 180-220g obtained from Bantin and Kingman Limited, were used throughout these studies. They were maintained in an animal house at 20°C and starved of food but not water for 18 hr prior to sacrifice to clear the intestine of its contents.

Radioactive (3',5',7,9-^{3}H) folic acid potassium salt (specific activity 500 mCi/mmole) and 2-^{14}C-folic acid potassium salt specific activity 55 mCi/mmole) and 5-^{14}C-methyltetrahydrofolic acid (5-^{14}C-meTHF) barium salt (specific activity 58 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, U.K. Unlabelled folic acid and 5-meTHF were obtained from Koch-Light Laboratories, Colnbrook, Bucks, U.K., and Eprova Research Institute, Basle, Switzerland respectively. ^{14}C-absorber-P, ^{3}H-scintillation cocktail, toluene, emulsifier No. 1 mix, 2,5-diphenyloxazole (PPO), and 1,4-[2-(5-phenyloxazolyl)]-benzene (POPOP) were supplied by Fisons Ltd. The scintillation cocktail consisted of toluene (1 litre), emulsifier No. 1 mix (500 ml), PPO (5g) and POPOP (0.1g).

All other chemicals, enzymes and reagents were of Analar quality and supplied by Sigma Company Limited, Fisons Limited, BDH Chemical Limited, and Fluorochem Limited. Inactin was supplied by Promanta.

2.2. PHYSIOLOGICAL BUFFER SOLUTIONS :-

Bicarbonate buffer and phosphate buffer solutions (Krebs and Henseleit, 1932) were used in this work. Krebs-Henseleit bicarbonate buffer contained sodium chloride (112mM), potassium chloride (4.5 mM), calcium chloride (2.4mM), potassium dihydrogen phosphate (1.12mM) magnesium sulphate (1.12mM), glucose 10 or 20 mM and was buffered to pH 7.4 with sodium bicarbonate (24mM). The sodium bicarbonate solution was treated with carbon dioxide until acid to phenolphthalein before mixing to prevent formation and precipitation of calcium carbonate. In this text, Krebs-Henseleit bicarbonate buffer will be referred to as bicarbonate buffer.

Krebs-Ringer phosphate buffer contained sodium chloride (112mM), potassium chloride (4.5mM), calcium chloride (2.4mM), potassium dihydrogen phosphate (1.12 mM), magnesium sulphate (1.12mM), glucose (10mM) and was buffered to pH 7 with 0.1M phosphate buffer (15.3 mM Na_2HPO_4).

In certain experiments the buffer pH was adjusted with 1N-hydrochloric acid (HCl) or 1N sodium hydroxide (NaoH).

2.2. PREPARATION OF THE EVERTED SACS :-

The *in vitro* everted sac technique of Wilson and Wiseman (1954a) was used in the absorption studies. Animals were killed by a single blow on the head followed by immediate decapitation. The abdomen was opened by a mid-line incision and the intestine from the point of

entry of the bile duct to the ileo-caecal junction was removed and immediately placed in a beaker containing 50 ml oxygenated bicarbonate buffer at 0-4°C. The lumen was flushed through with the same buffer and divided into 12 equal sections each about 8 cm in length. In these studies, section 2 and 3 represent the proximal jejunum, section 5 represents the distal jejunum, section 8 represents the proximal ileum and section 11 the distal ileum. A maximum of three sacs were prepared from each animal.

Each section selected for investigation was everted over a glass rod (1.5mm diameter), ligated at one end and gently blotted on Whatman's No. 1 filter paper saturated with ice-cold bicarbonate buffer. Care was taken to ensure that the everted sac never came into contact with dry filter paper as this could cause damage to the mucosal surface. The sac was weighed (W1) on a torsion balance (Whites Elec. Inst.Co.) and filled with 0.5 ml of bicarbonate buffer (serosal solution) using a 1 ml disposable syringe with a blunt needle. A second ligature was tied to seal the sac. The sac was re-weighed (W2) and incubated in a 25 ml Erlenmeyer flask containing 10 ml of bicarbonate buffer (mucosal solution) continuously gassed with 5% CO_2 in O_2 via a polythene tube. The flasks were maintained at 37°C in a Techne Temperor shaking water bath running at 80 oscillations/min. At the end of incubation, the sac was removed, carefully washed with the same buffer at room temperature, gently blotted and re-weighed (W3). The serosal contents were allowed to drain into a sample vial. The

empty sac was then reblotted and finally weighed (W4).

Fluid movement into the sac was assessed from the four weighings :

W_3 - W2 = Total fluid uptake

W_4 - W1 = Tissue fluid uptake

(W3- W2 - (W4 - W1) = Serosal fluid appearance (uptake).

The weight of the cotton threads used to ligate the sac was very small compared to the weight of the sac (1%) and so was ignored in calculation of water movement.

2.4 POTENTIAL DIFFERENCE MEASUREMENT :-

The method of Barry, Dikstein, Matthews, Smyth and Wright (1964) was used to measure the transmural potential difference. An everted sac 8 cm in length was cannulated over a hollow glass tube and the lower end of the tissue tied with a cotton ligature. A small weight was attached to the lower end in order that the sac remained vertical during the experiment. Bicarbonate buffer was introduced into the sac with a syringe via the glass cannula, care being taken to exclude bubbles and to ensure a continuous column of fluid. The sac was then immersed in 30 ml of bicarbonate buffer continuously gassed with 5% CO_2 in O_2, at 37°C. Electrical contact between serosal and mucosal solutions was maintained via a 3M-KCl-agar salt bridge lead away to calomel half cells. The salt bridges were made using polythene tubing

of constant internal diameter filled with 3M-KCl-agar gel. The potential difference reading was measured in millivolts from a Pye Unicam digital pH-meter (PW 9409 digital pH-meter).

2-5 GLUCOSE ESTIMATION :-

Glucose in the serosal and mucosal solutions was estimated colorimetrically using the glucose oxidase method and a Technicon autoanalyzer at the Clinical Biochemistry Department, Birmingham General Hospital. A calibration curve was plotted from glucose standards and used to estimate the concentration of glucose in serosal and mucosal solutions. The ratio of serosal glucose concentration to mucosal glucose concentration (S/M ratio) was used as expression of the amount of glucose transported. The principle of the reaction is :-

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \delta\text{-Gluconolactone} + 2H_2O_2$$

$$2H_2O_2 + HBS^{*} + \text{4-aminophenazone} \longrightarrow \underset{\text{(Purple colour)}}{\text{Quinoneimine}}$$

* HBS is 3,5-dichloro-4-hydroxybenzene sulphonate.

2.6 HISTOLOGICAL STUDIES :-

These studies were designed to examine the integrity of the intestinal tissue of everted sacs from rat proximal jejunum during preparation and after incubation. The damaging effect of a physiological

detergent was also examined.

After incubation, a small piece of an everted sac tissue was immediately immersed in 20 ml of a fixative (10% formalin saline).

The preparation and staining of the slides with the periodic acid Schiff reagent stain was carried out in the histology laboratory, Birmingham General Hospital and is summarized here :-

The tissue was dehydrated gradually over 24 hr using several changes of alcohol (50%, 70% and 100%) and then washed twice for 2hr with chloroform. The tissue was subsequently embedded in paraffin wax (melting point 56°C-57°C) for at least 4 hr and a thin section 0.4 µm cut with a sledge microtome (Type 1400, Leitz, West Germany). The sections were mounted on clean microscopic slides, the paraffin wax removed with xylene and stained with periodic acid schiff (McManus, 1946). The reaction acts by cleaving C-C bond of 1,2-glycol groups (- $\overset{OH}{\underset{H}{C}}$ - $\overset{OH}{\underset{H}{C}}$ -) and oxidising them to aldehydes (-CHO) with Schiff's reagent to give a red colouration.

After staining, photomicrographs were taken using an Orthomat Leitz photomicroscope (Leitz, West Germany).

2.7. ENZYME ASSAYS :-

(i) Alkaline phosphatase :-

Alkaline phosphatase activity in the incubation medium was

estimated as described by Boehringer (1973a). The principle of the reaction is :

$$\text{p-nitrophenylphosphate} + H_2O \xrightarrow[\text{pH 8}]{\text{alkaline phosphatase}} \text{p-nitrophenol} + Pi$$

The increase in the optical density at 405 nM per unit time was used as a measure of alkaline phosphatase activity. Readings of the optical density of the samples were performed against a blank at 25°C in a Pye Unicam spectrophotometer using 1 cm cuvettes (Unicam SP 1700 ultraviolet spectrophotometer). Enzyme activity was expressed in international units (I.U)/g initial tissue wet weight/10 ml of incubation medium (1 international unit is the enzyme activity which transfers 1 μmole of substrate per min. under optimal conditions).

(ii) Lactate dehydrogenase (LDH) :-

The appearance of LDH activity in the incubation medium was estimated as described by Boehringer (1973b). The principle of the reaction is :-

$$NADH + \text{pyruvate} + H^+ \xrightarrow{LDH} \text{Lactate} + NAD^+$$

The decrease in the optical density at 366 nM per unit time was used as a measure of LDH activity. Reading of the optical density of samples were performed against air at 25°C in a Pye Unicam spectrophotometer using 1 cm cuvettes. Enzyme activity was expressed in international units (I.U)/g initial tissue wet weight/10 ml of mucosal medium.

2.8 SURFACE pH MEASUREMENTS :-

The *in vitro* surface pH measurements in rats and man were carried out as described by Lucas and Blair (1978).

(i) pH-electrode details and experimental protocol :-

A surface pH-microelectrode from Pye Unicam, York Street, Cambridge, was used throughout these investigations. It was connected through a coaxial wire to a Pye Unicam pH-meter (PW 9409 digital pH meter). The reference electrode consisted of an agar-salt bridge (3M-KCl: 4% agar) dipping into the bathing medium and connected via a calomel half-cell to the pH meter. All pH measurements were carried out inside an earthed copper Faraday cage.

The time response and the linearity of response of the pH-microelectrode was tested routinely before use in buffers of pH 4, 7 and 9. The response time was found to be within 30-45 seconds and a linear pH response was obtained. The pH-microelectrode was then calibrated at 37°C in buffer pH 7. A Leitz micro-manipulator was used to hold and control the movement of the pH-microelectrode during the experiments.

(ii) *In vitro* surface pH measurements in rats :-

A flat 1 cm^2 strip of tissue from the section under investigation was held by dissecting pins onto a cork base of a glass vessel with the mucosal surface facing the incubation medium. In measuring the surface pH of the stomach, the 1 cm^2 strip used was taken from

the body of the stomach (Figure 2.1). In the intestine, the duodenum was divided into two sections by severing at the pyloric sphincter the ligature of Trietz and the point of entry of the bile duct. These sections were referred to as the proximal and distal duodenum. The rest of the intestine was sectioned as mentioned in section 2.3. In the colon the measurements were carried out on the first 8 cm section. In the intestine and colon the 1 cm^2 strip of tissue used was taken from the middle part of each section. Only one strip was taken from each animal.

The incubation medium used was either bicarbonate buffer or Krebs-Ringer phosphate buffer. Unless otherwise specified, the incubation temperature was $37^{o}C$.

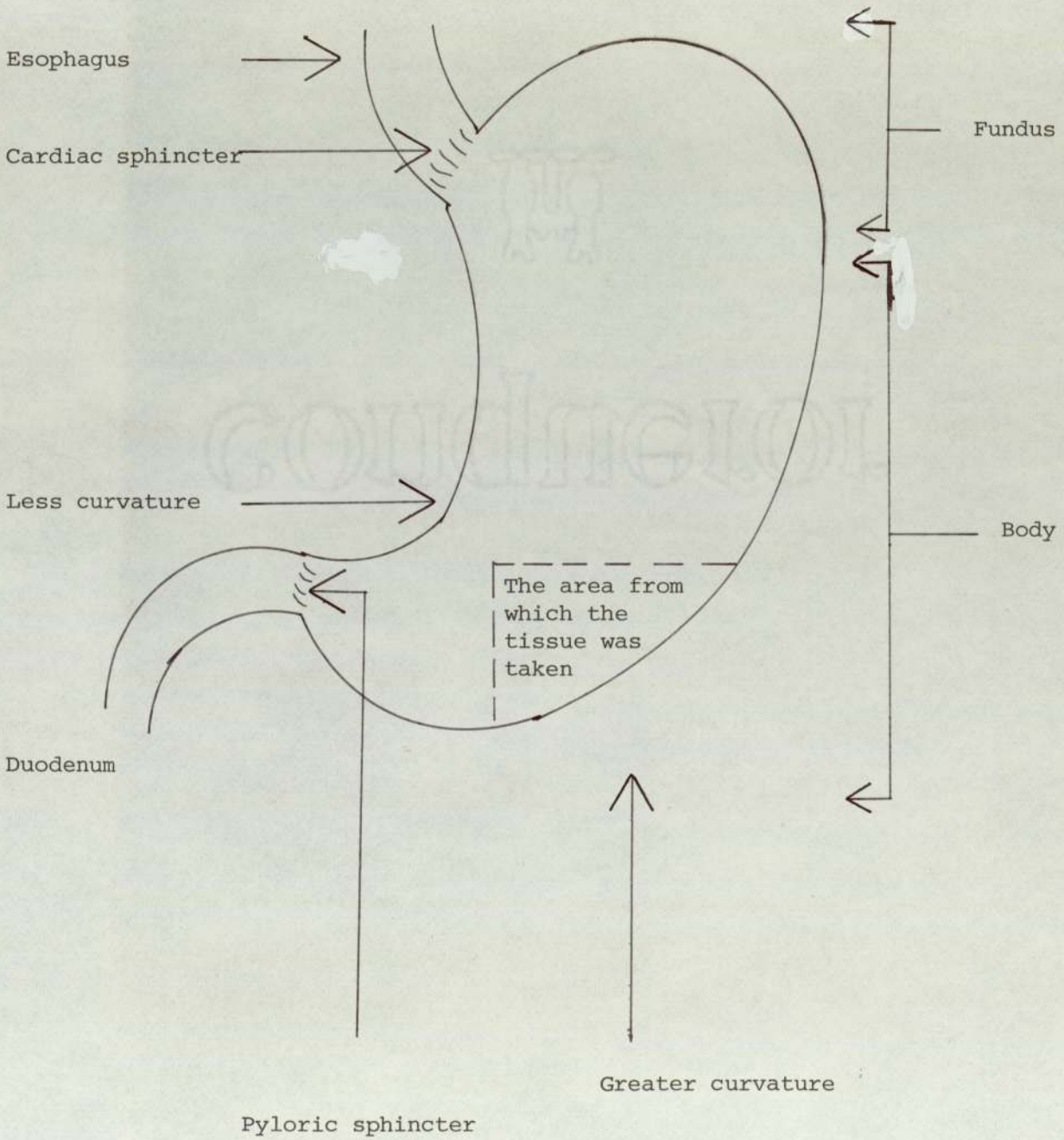
After checking the pH of the incubation medium, the surface pH was measured by racking down the pH-microelectrode onto the surface of the tissue until the tissue could just be seen to distort. Stable pH values, as judged by a reading not differing by more than 0.02 pH unit at a one minute interval, were achieved within four minutes. This pH value is referred to as the initial or onset pH. The pH-microelectrode was then taken off the surface of the tissue. The procedure was repeated after an interval of 30 min. to obtain the final pH values.

(iii) In vivo surface pH measurements in rats :-

Wistar rats were anaesthetized by intraperitoneal administration of Inactin (10 mg/100 g body weight) and the abdomen was

Figure 2-1 :-

The site from which flat strip of tissue for stomach surface pH measurements was taken.



opened by a mid-line incision. The proximal jejunum was identified and a longitudinal opening of 2-3 cm in length was made very carefully. The opened part was slightly moved aside, great attention was paid to avoid possible damage to the blood and lymph vessels, and held with the mucosa upward by dissecting pins onto a cork base of a glass vessel containing 10 ml of bicarbonate buffer continuously oxygenated with 5% CO_2 in O_2 at 37°C. Only the onset pH values were recorded.

(iv) In vitro surface pH measurements in man :-

The surface pH of the proximal jejunum was measured from peroral jejunal biopsies taken by clinicians with a suction biopsy capsule (Roye-Choudhury, Nicolson and Cooke, 1964) from subjects who have fasted overnight. These investigations were carried out at Birmingham General Hospital.

The capsule used was a hollow structure made of stainless steel that could be separated into two parts. On one side of the capsule, there was an aperture of 4 mm in diameter such that the lower part provided a sharp edge to cut the mucosal tissue when traction was applied. The inside of the capsule was connected with a hollow and flexible radio-opaque polyvinyl tube through which suction can be applied by means of a syringe. To speed the passage of the capsule through the stomach and upper intestine, a small bag containing mercury was attached to act as a weight. With some subjects local pharyngeal anaesthesia provided by a lignocaine spray was necessary before

capsule intubation.

The capsule was introduced over the tongue and swallowed, if necessary with the aid of some water. The position of the capsule was checked fluoroscopically. To facilitate the passage of the capsule through the pyloric sphincter, the subject was positioned on their right side, and if the capsule had not passed the pylorus sphincter within 20 minutes, 10-20 mg of metoclopramide was given intravenously. When the capsule was seen fluoroscopically in the duodenum, the subject was positioned on the left side to facilitate its passage to the jejunum through the duodenal-jejunal flexure.

Having confirmed the capsule was in the upper part of the proximal jejunum suction was applied by means of the syringe which invaginated part of the jejunal mucosal tissue into the inside of the capsule. Whilst maintaining suction, the capsule was withdrawn with a small piece of jejunal mucosal tissue. Part of the jejunal biopsy was taken immediately for surface pH measurements and part was sent for histology examinations.

A blood sample was also taken from the subject and sent to the haematology unit of the hospital for serum folate estimation using a microbiological assay.

Samples of human stomach were obtained from surgery and transferred immediately into oxygenated phosphate buffer. The delay between obtaining the intestinal or stomach tissue and obtaining a

steady onset surface pH was less than five minutes. All the surface pH measurements were carried out at the same time of the day.

2.9. ESTIMATION OF PURITY AND STABILITY OF 5-meTHF UNDER STORAGE AND USUAL EXPERIMENTAL CONDITIONS :-

The purity of the stock 5-^{14}C-meTHF present in 0.05M phosphate buffer containing 2% sodium ascorbate as antioxidant and stored in a sealed vial at -20^{o}C, was checked after a month of storage. Thin layer chromatography (T.L.C) was performed in glass tanks (23 cm x 23 cm x 8 cm) saturated with solvent using cellulose pre-coated plates (MN 300 "Polygram", sheets, Macherey-Nayel and Co.). The chromatography solvent used was 0.1 M phosphate buffer pH 7 and the experiment was carried out at room temperature ($\simeq$21^{o}C). Samples and standards were applied as spots using glass micropipettes and were observed as dark absorbing spots by viewing under U.V. light at 254 nm and by measurement of the radioactivity by cutting up the plates into appropriately sized pieces and placing them into vials to which was added 10 ml of scintillant (toluene (1 litre) containing PPO (5 g) and POPOP (0.1 g)). Radioactivity was measured in a Nuclear Enterprises liquid scintillation counter type NE 8310. Correction for quenching were made using the external standard ratio method related to a quench curve drawn up using increasing concentration of 2,4-dichlorophenol-indophenol against a standard amount of isotope used.

In a similar way the stability of 10^{-6}M 5-meTHF (containing 0.1 µCi of 5-^{14}C-meTHF) in bicarbonate buffer pH 7.4 containing 3 mg/ml

sodium ascorbate and kept under the usual experimental conditions of oxygenation and temperature for different periods of time was checked. The standard used was pure 5-^{14}C-meTHF.

2.10 ESTIMATION OF THE AMOUNT OF FOLATE TRANSPORTED INTO THE SEROSAL AND TISSUE COMPARTMENTS OF EVERTED SACS :-

(i) Folate in serosal compartment :-

Folate transport was measured by incubating everted sacs in bicarbonate buffer containing 20 mM glucose and 10^{-7}M - 10^{-4}M of either 5-meTHF (with 0.1 μCi of 5-^{14}C-meTHF as tracer) or folic acid (with 0.1 μCi of (3′,5′,7,9-^{3}H) folic acid as tracer either alone or with 0.1 μCi of 2-^{14}C-folic acid in addition) for 0-60 minutes. The amount of folate transported into the serosal compartment was estimated by pipetting 0.4 ml of the serosal fluid and 0.6 ml of distilled water into a counting vial containing 10 ml of scintillation cocktail. Samples together with appropriate standards and controls were counted for either 10 minutes or up to 10,000 counts in a Nuclear Enterprises Scintillation Counter type 8310. Appropriate corrections were made for quenching and background. When a dual labelled species was used, ^{14}C spillover into ^{3}H channel was corrected using the external standard ratio method. The amount of folate transferred to the serosal compartment was then calculated (assuming the serosal volume to equal W3-W4) by comparing the total serosal counts with that of standard.

(ii) Folate in tissue compartment :-

The amount of folate taken up by the tissue during the

incubation (tissue uptake) was estimated by measuring the total radioactivity present. The tissue was dried in an oven at 80°C for 12 hr, and oxidized in a Beckman biological material oxidizer. When the tissue contained ^{3}H -labelled species, the oxidation product was passed through a cold trap to collect water and $^{3}H_2O$. When it contained ^{14}C-labelled species, the oxidation product was passed through a trap containing 15 ml of ^{14}C-absorber-P (Fisons, Loughborough, Leics, U.K), a scintillation cocktail designed for the collection of CO_2. In experiments when dual labelled folate was used the oxidation product was first passed through a cold trap to collect water and $^{3}H_2O$ and then into a trap containing ^{14}C-absorber-P for CO_2 collection. Appropriate standards and controls were also made. Samples were then counted for radioactivity and corrections were made for quenching and background as before.

2.11 IDENTIFICATION OF FOLATE SPECIES PRESENT IN MUCOSAL, SEROSAL AND TISSUE COMPARTMENTS OF EVERTED SACS :-

(i) 5-meTHF :-

T.L.C. was used to identify the folate species present in the mucosal and serosal compartments of everted sacs prepared from the proximal jejunum and incubated in bicarbonate buffer containing 10^{-6}M 5-meTHF and 3 mg/ml sodium ascorbate for different periods of time at 37°C. The chromatography solvent used was 0.1 M phosphate buffer pH 7 containing 2% sodium ascorbate. The distribution of the radioactivity on the T.L.C. chromatogram was estimated as described in section 2.9.

(ii) Folic acid :-

After incubating everted sacs prepared from the proximal jejunum in bicarbonate buffer pH 7.4 containing 10^{-6} M folic acid (with dual labelled folic acid as tracers) and 20 mM glucose for 30 minutes at 37°C, folate species in the mucosal compartment and those transported into the serosal compartment were identified using DEAE cellulose ion exchange columns chromatography. Folate species appeared in the tissue compartments at the end of the incubation were identified using gel filtration i.e. Sephadex G.15, and ion exchange i.e. DEAE cellulose chromatography.

a. Folate species in serosal compartment :-

Diethylaminoethyl(DEAE)cellulose (DE 52, Whatman, Limited, Maidstone, Kent, U.K) (80 g) was washed with distilled water and equilibrated in 0.05 M phosphate buffer pH 7 containing dithiothreitol (5 mg %) until the washings were of constant ionic strength and at pH 7. After decanting off the fine particles and degassing, the prepared DE 52 was packed into a glass column (2 cm X 50 cm) plugged with glass wool. Four serosal samples from four everted sacs, were pooled together with appropriate standards and loaded onto the column. Standard linear gradients (0-1.2M NaCl in starting buffer) were eluted automatically using a LKB Ultra Grad attached to a peristaltic pump (LKB Instruments, Croydon, Surrey, U.K). The eluant was passed through two detectors in series, a purpose built scintillation flow cell for detecting radioactivity (Nuclear Enterprises Limited, Edinburgh, U.K)

and a U.V. monitor (LKB Uvicord II, LKB Instruments). Fractions (5 ml) were collected using an LKB Ultrarac fraction collector (LKB Instruments). The gradient, usually eluted over eight hours was determined by measuring the conductivity of every 10th fraction with a Mullard conductivity cell. Aqueous samples from each fraction were made up to 1 ml with water and 10 ml of the scintillation cocktail added and the radioactivity measured. Corrections for quenching, background and ^{14}C spillover into ^{3}H channel were carried out as mentioned before.

b. Folate species in tissue compartment :-

After 30 minutes incubation of four everted sacs from the proximal jejunum in bicarbonate buffer, the tissue was immediately cut into small pieces and dropped into a 50 ml boiling 0.05 M phosphate buffer pH 7 containing 2% sodium ascorbate. After 10 minutes boiling, the mixture was allowed to cool and then homogenized. The homogenate was centrifuged in a bench centrifuge and the supernatant solution collected and kept at -20 ^{o}C until the chromatographic studies were carried out.

A Sephadex G.15 (Pharmacia) slurry was prepared in 0.05 M phosphate buffer pH 7 containing dithiothreitol (5 mg %) and left to swell. After degassing the slurry was packed into a 2cm x 60 cm perpex column (Wright Scientific Ltd. Surrey, U.K). After loading the supernatant to which appropriate standards were added, elution was achieved using 0.05 M phosphate buffer containing 5 mg % dithiothreitol.

The eluent was examined, collected, and counted as before.

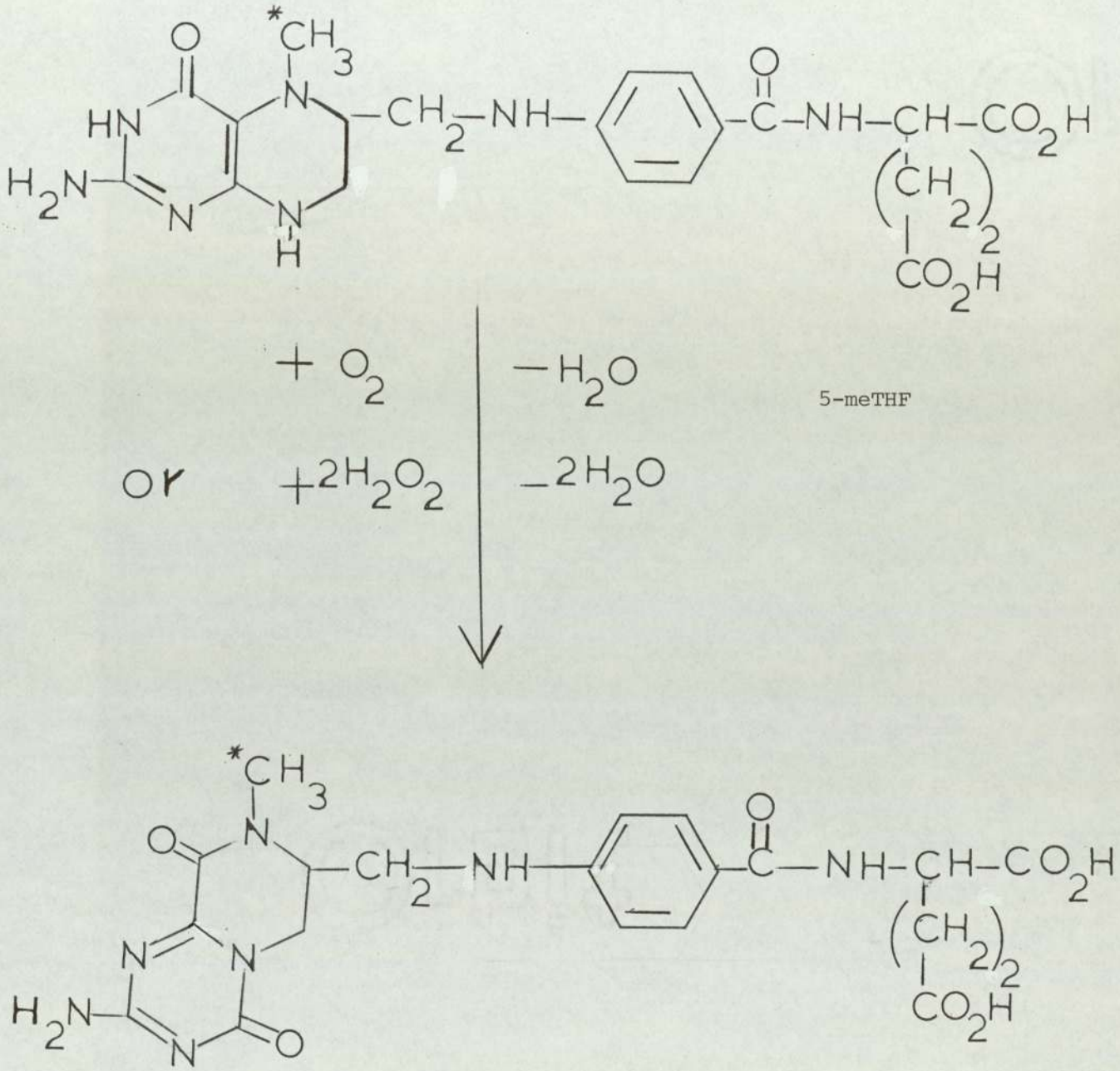
The peak fractions were combined and applied to a DEAE cellulose column as described in section 2-11.

2.12 PREPARATION, PURIFICATION, IDENTIFICATION AND TRANSPORT STUDIES OF A PYRAZINO-s-TRIAZINE DERIVATIVE :-

Oxidation of 5-meTHF by air (Blair, Pearson and Robb, 1975) or H_2O_2 (Gapski, Whiteley and Huennekens 1971; Jongejan, Mager and Berends, 1979) produces a yellow compound identified as a pyrazino-s-triazine derivative (Jongejan, Mayer and Berends, 1979). The principle of the reaction is shown in Figure 2.2 .

The ^{14}C-labelled and unlabelled form of the oxidation product of 5-meTHF were prepared by the method of Gapski,Whiteley and Hunnekens (1971). 0.1 M ammonium acetate (5 ml) pH 6 containing 0.5 ml of H_2O_2 (30%) was added to 5 ml of 0.1M ammonium acetate pH 6 containing 150 mg of unlabelled 5-meTHF. The two solutions were adjusted to pH 6 using IN glacial acetic acid. The reactions were allowed to proceed for 1 hr at room temperature ($\simeq 21^{\circ}C$) with continuous stirring. The reaction mixtures were then freeze-dried and the residue dissolved in 10 ml of cold distilled water. Purification was performed on a DEAE cellulose column (2 cm X 37 cm) eluted with 250 ml of distilled water and 500 ml of 0.1 M ammonium acetate. The 5 ml fraction (number 53-81) eluted with the 0.1 M ammonium acetate were collected, freeze dried and dissolved in 5 ml of 0.01 M phosphate buffer pH 7. The U.V

Figure 2-2:- The oxidation of 5-meTHF into a pyrazino-s-triazine derivative.



pyrazino-s-triazine derivative

* Site of ^{14}C labelling.

spectrum of the unlabelled compound agreed with published data and the ^{14}C-labelled compound co-chromatographed with the unlabelled compound on a cellulose pre-coated thin layer chromatogram using a mixture of n-propranol : water: aqueous ammonia (200:100:1) as solvent system.

The absorption studies of this compound were performed using everted sacs prepared from rat proximal jejunum and incubated for 30 minutes at 37°C in bicarbonate buffer pH 7.4 containing 20 mM glucose.

2.13 STATISTICAL ANALYSIS :-

Unless otherwise mentioned, all the results presented in this text are the mean of at least five separated experiments and are expressed as the mean ± standard error of the mean (S.E.M). Students "t" test was used to analyse the results and regression lines were achieved using the method of least squares. All statistical analyses were performed on an Olivetti Programma 101 Computer.

CHAPTER THREE

THE SURFACE PROPERTIES OF THE RAT SMALL INTESTINE

3.1. INTRODUCTION :-

The existence of an acidic microclimate on the luminal surface of the small intestine has been well established (Lucas, Schneider, Haberich and Blair, 1975; Lei, Lucas and Blair, 1977; Lucas and Blair, 1978). However, no investigation has been performed to attempt to define the mechanism of creation and the method of maintenance of the intestinal acid microclimate. Moreover, its possible role in the absorption of nutrients is also not fully defined.

The investigations reported in this chapter were designed to examine the intestinal acid microclimate (i) at the intracellular level by affecting the intracellular metabolic activities such as glycolysis and oxidative phosphorylation; (ii) at the extracellular level by changing incubation pH and by addition of substrates such as ATP, and (iii) at the level of the membrane by affecting the glycocalyx and the membrane permeability and bound enzymes.

An *in vitro* technique was employed to examine the surface properties of the intestinal tissue, its viability was assessed by measuring a number of metabolic processes and active transport mechanisms and by histological studies.

3.2. MATERIALS AND METHODS :

For specific details of procedures and chemicals see Chapter 2. Unless otherwise specified the incubation temperature of the surface acid microclimate studies was 37^{o}C, the incubation time was 30 minutes and the data presented are the mean $\pm$ the standard error of the

mean (S.E.M.) of at least five experimental observations.

3.3. RESULTS

3.3.1 ASSESSMENT OF TISSUE VIABILITY :-

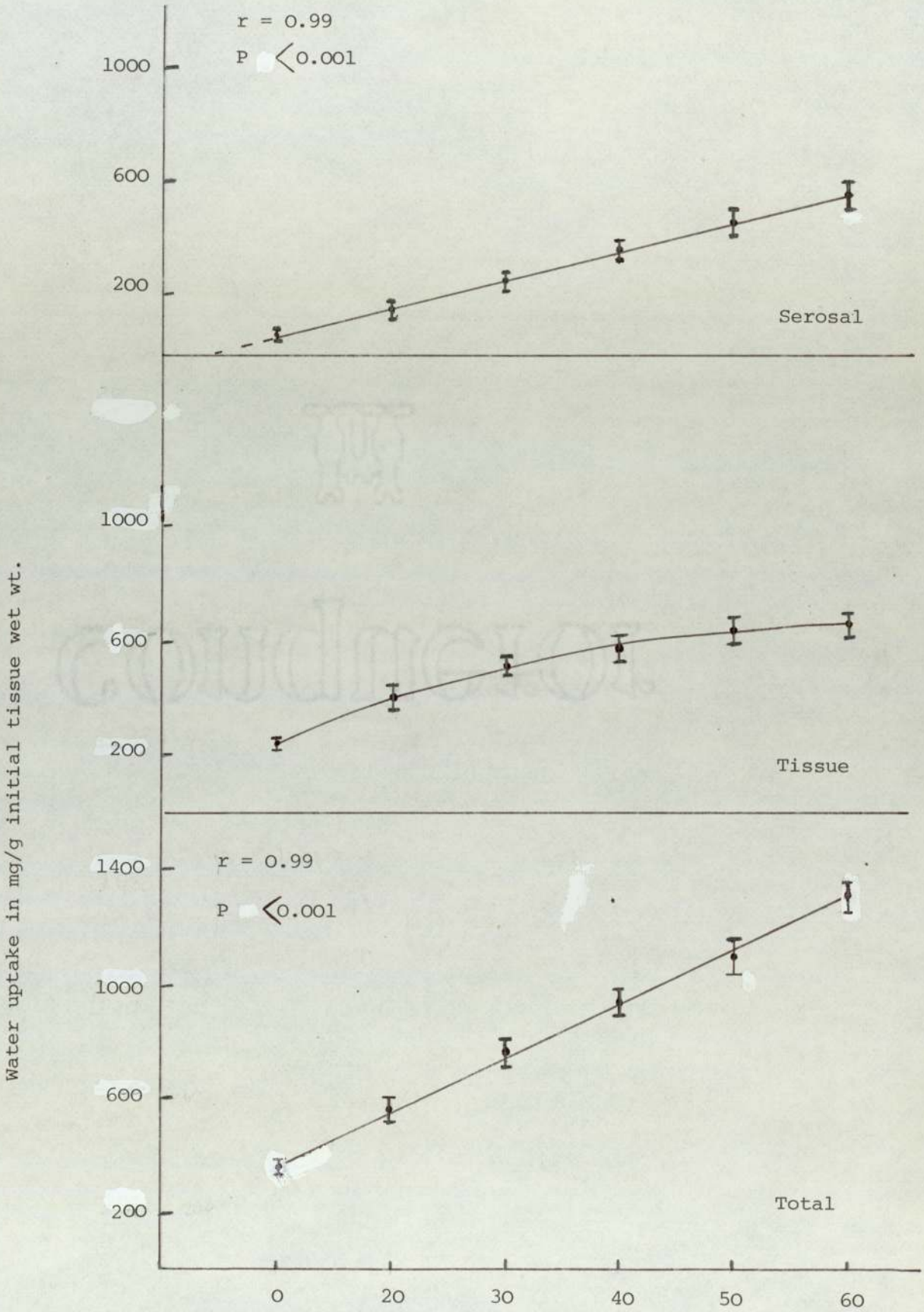
(i) Water transport :-

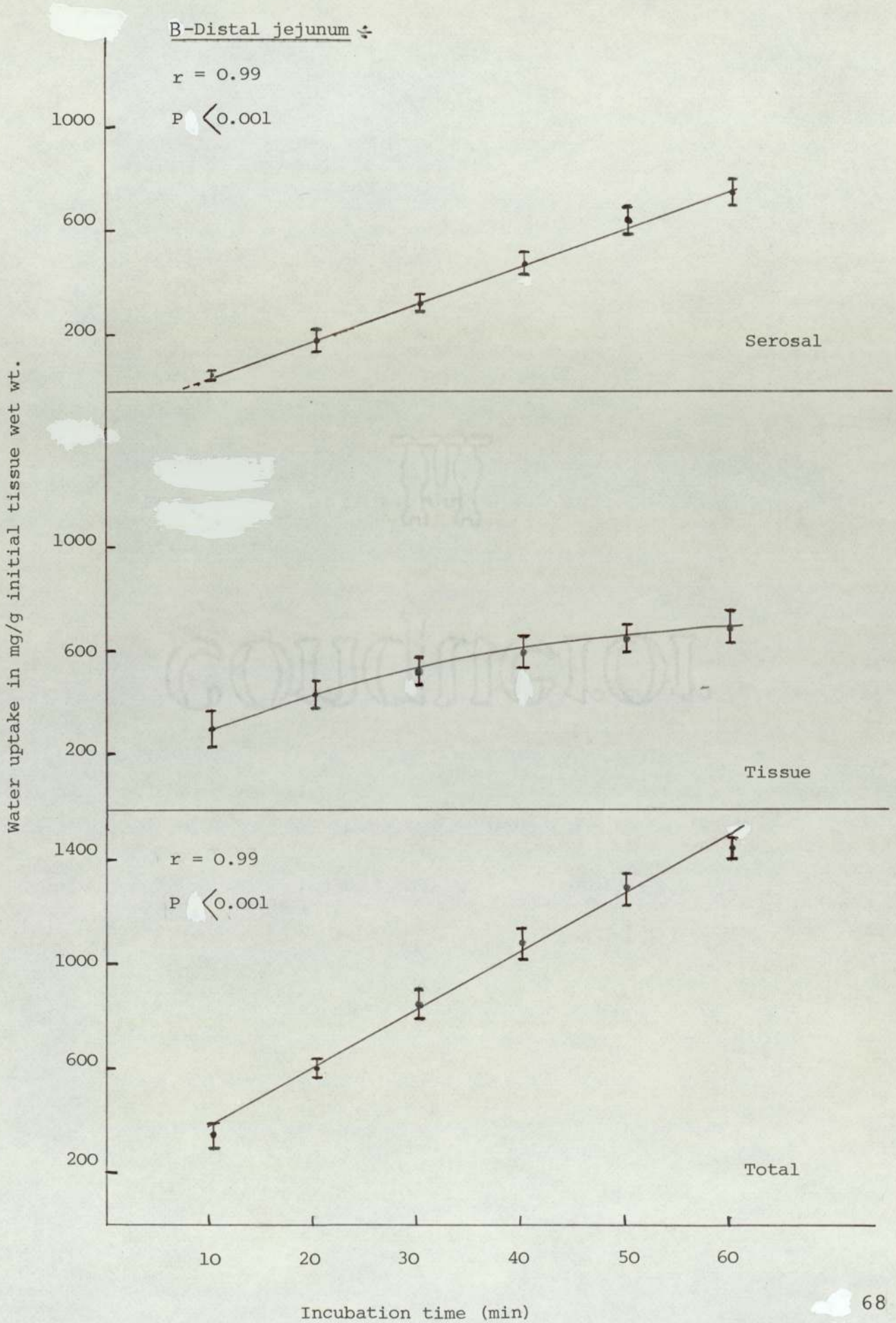
Water absorption is initiated by the active accumulation of solutes within the epithelial tissue, most probably in the epithelial interstitial fluid, which creates a local osmotic pressure and provides the force for water to move from the mucosal compartment to both the tissue and the serosal compartments. The movement of water in turn creates a hydrostatic pressure which provides the force for further transport of water and solutes. Movement of water can therefore be used as a criteria of tissue viability.

The absorption of water was measured across everted sacs prepared from the proximal jejunum, distal jejunum and proximal ileum after various periods of incubation (Figure 3.1). In all the regions examined, the rate of serosal uptake increased with the increase in the incubation time up to 60 minutes, while the rate of tissue uptake was rapid for the first 20-30 minutes, but decreased as the incubation period proceeded. The total water uptake was higher in the jejunum and less

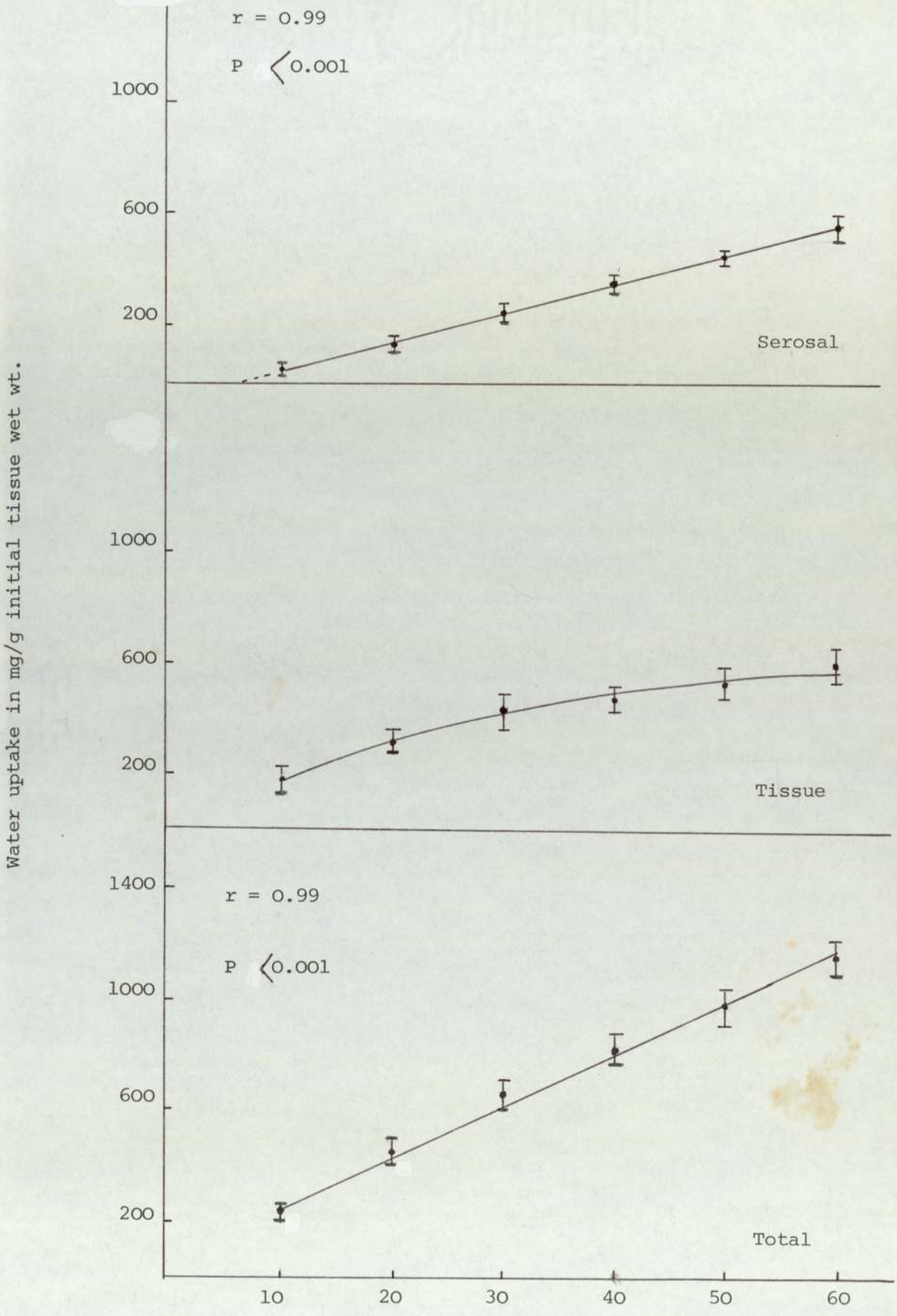
FIGURE 3.1 : The serosal, tissue and total uptake of water (mg/g initial tissue wet wt.) by everted sacs prepared from different regions of the small intestine and incubated in bicarbonate buffer containing 20 mM glucose at 37°C.

A. Proximal Jejunum :-




B-Distal jejunum ÷
r = 0.99
P < 0.001
1000
600
200
Serosal
1000
600
200
Tissue
r = 0.99
P < 0.001
1400
1000
600
200
Total
Water uptake in mg/g initial tissue wet wt.
10
20
30
40
50
60
Incubation time (min)

C - Proximal ileum :-



down the intestine. The results obtained were similar to that of Barry, Matthews and Smyth (1961) and Coleman, Hilburn and Blair, (1979).

(ii) Glucose transport :

Glucose transport, expressed as the ratio of serosal glucose concentration to mucosal glucose concentration, was measured across the wall of everted sacs prepared from the proximal jejunum, distal jejunum and proximal ileum and incubated in bicarbonate buffer for different periods of time at 37°C. The initial glucose concentration was 20 mM on both sides of the everted sacs.

The results (Table 3.1) indicate that as the serosal: mucosal ratio was greater than one, an active movement of glucose against a concentration gradient had taken place. The results are in good agreement with that of Barry, Matthews and Smyth (1961), Coleman (1979) and Lucas and Blair (1978).

(iii) Potential difference :-

Barry, Dikstein, Matthews, Smyth and Wright (1964) have shown that a difference in electrical potential exists across the intestinal wall, maintained by the flow of ions across the epithelial sheet. Potential difference is stimulated by the presence of glucose and is a readily measured feature of a viable in vitro preparation.

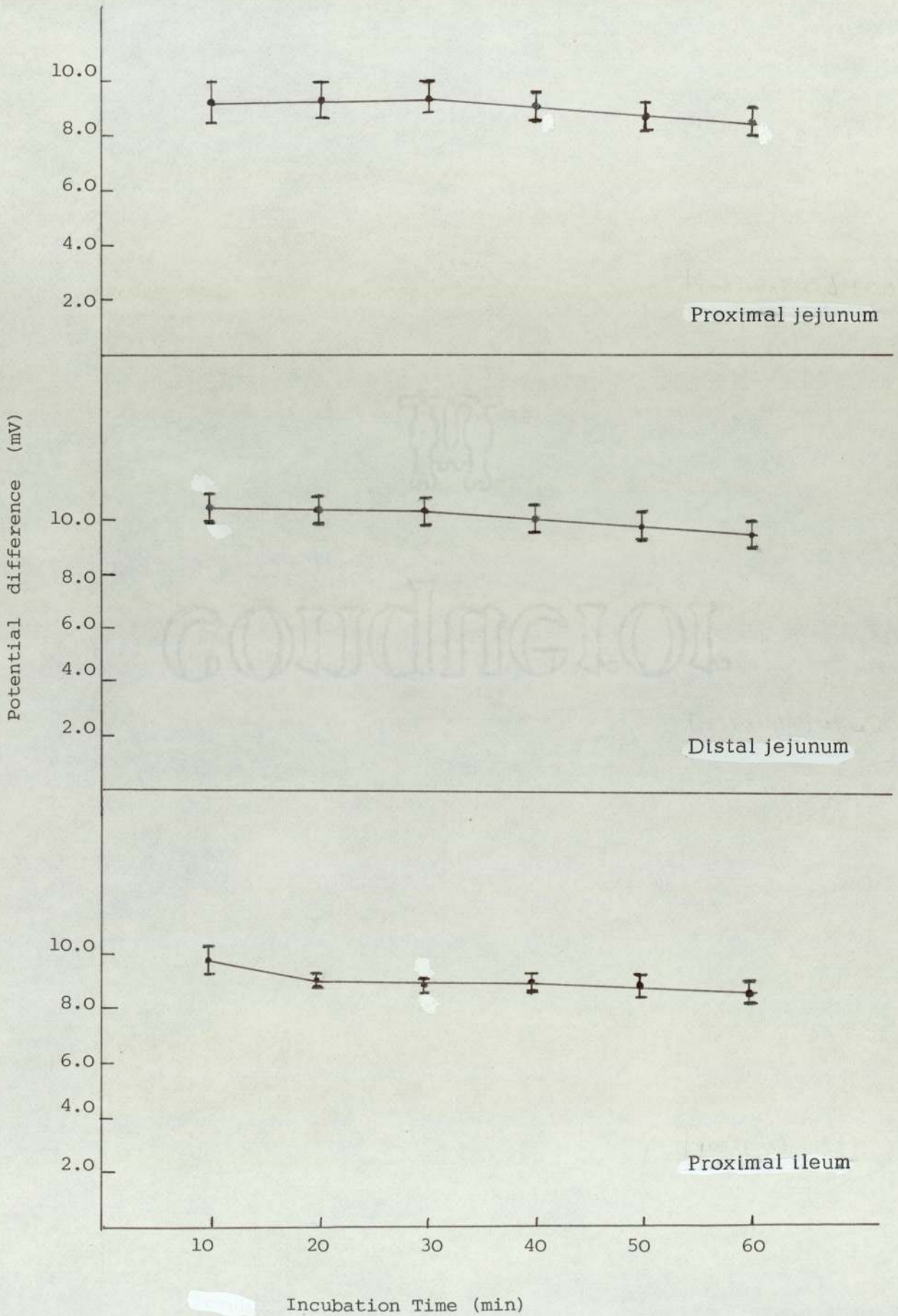
Cannulated everted sacs were prepared from the proximal jejunum, distal jejunum and proximal ileum. Potential difference (mV) measurements were made every 10 minutes for a period of 1 hour during

TABLE 3-1

Glucose transport (expressed as the ratio of serosal glucose concentration to mucosal glucose concentration) across the wall of everted sacs prepared from different regions of the small intestine and incubated in bicarbonate buffer pH 7.4 in the presence of 20 mM glucose on both sides of the preparation.

Time (min)	Proximal Jejunum	Distal Jejunum	Proximal ileum
10	1.05 ± 0.05	1.10 ± 0.03	1.05 ± 0.03
20	1.10 ± 0.06	1.20 ± 0.10	1.10 ± 0.03
30	1.15 ± 0.05	1.40 ± 0.04	1.30 ± 0.04
45	1.30 ± 0.07	1.60 ± 1.10	1.50 ± 0.10
60	1.40 ± 0.08	1.60 ± 0.20	1.50 ± 0.20

Figure 3-2: The change in potential difference across different regions of the small intestine with time.



incubation in bicarbonate buffer containing 20mM glucose at 37°C.

The potential difference (Figure 3-2) did not decline substantially over a 1 hour period of incubation and supported a mean of 9.00 ± 0.20 mV, 9.90 ± 0.20 mV and 8.90±0.20mV across the proximal jejunum, distal jejunum and proximal ileum respectively.

The results are in a good agreement with the published data (Coleman, 1979; Lucas ,1974).

(iv) Histological studies :-

Slides were prepared and stained with periodic acid Schiff from tissue taken from the proximal jejunum after eversion and incubation for 5 or 30 minutes in bicarbonate buffer containing 20 mM glucose at 37°C.

The everted sac preparations appeared to be structurally intact, both after eversion (plates 1 & 2) and after incubation for 5 and 30 minutes (plates 3 & 4 respectively). Some odema and swelling in the villi was observed after 30 minutes incubation. The glycocalyx appeared as a continuous intact layer covering the villi and remained intact even after 30 minutes incubation. These observations are similar to that of Taylor (1963) and Swanston (1978).

(v) Conclusion :-

The lack of histological damage to the preparation together with the steady transmural potential difference recorded across the everted sac preparation, water absorption and active glucose transport

PLATE 1 — Rat proximal jejunal villi after evertion. +

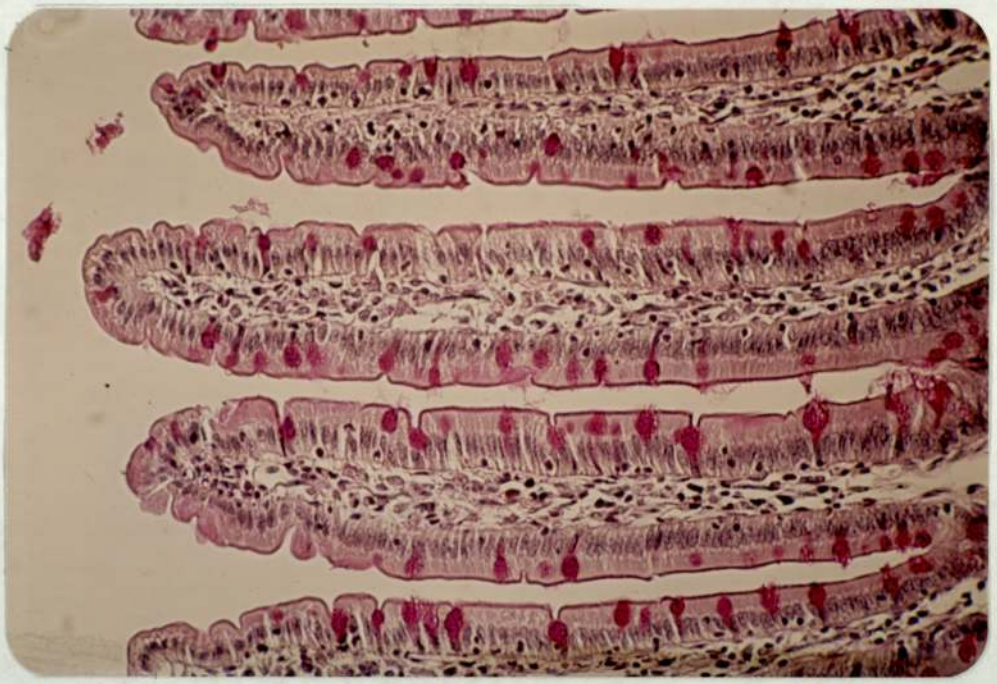
PLATE 2 — Rat proximal jejunal villi after evertion. ++

PLATE 3 — Rat proximal jejunal villi of everted sac after 5 minutes incubation in bicarbonate buffer containing 20 mM glucose at 37°C. ++

PLATE 4 — Rat proximal jejunal villi of everted sac after 30 minutes incubation in bicarbonate buffer containing 20 mM glucose at 37°C. ++

+ Magnification X 125

++ Magnification X 312



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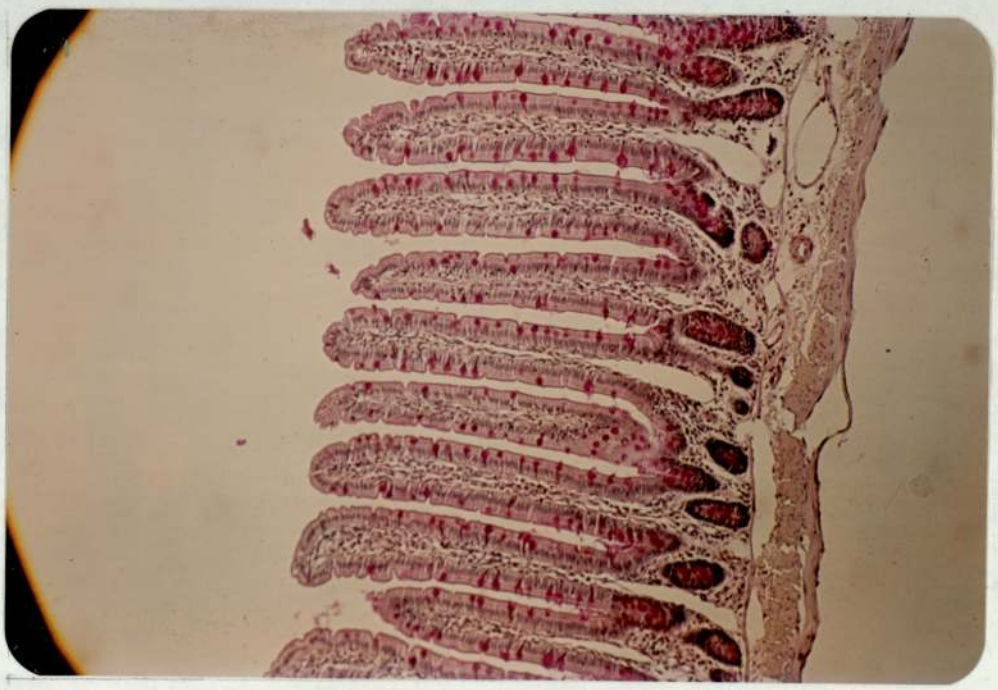

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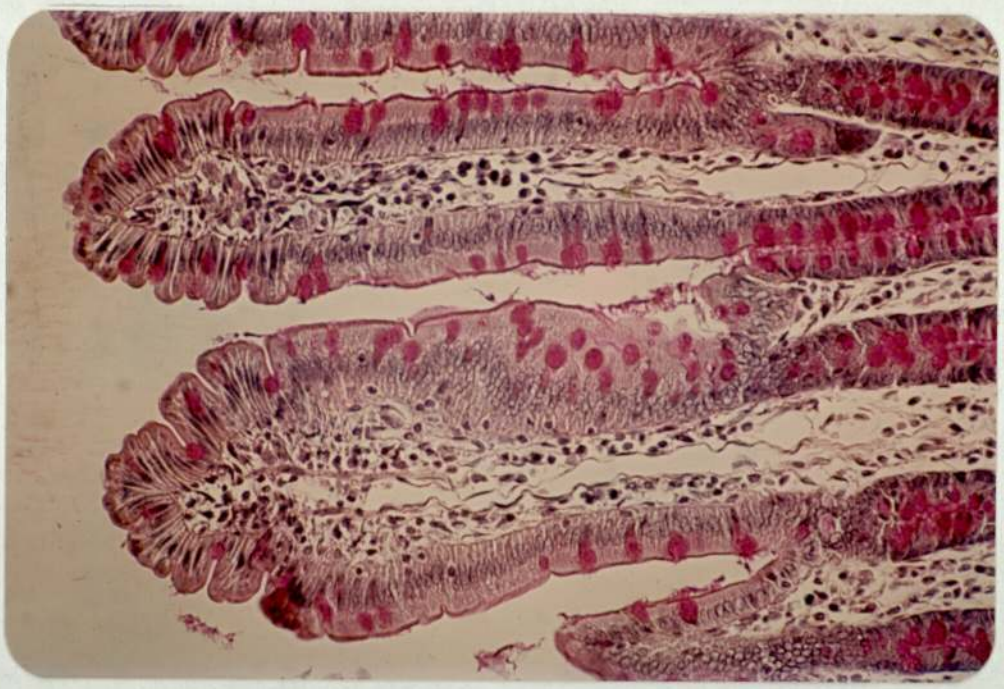

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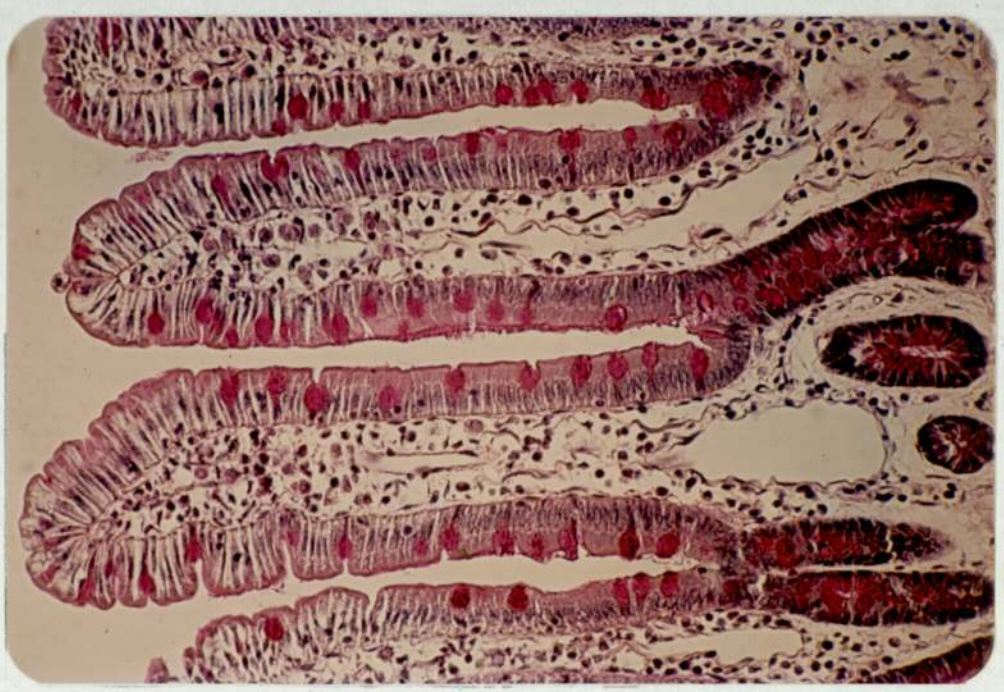


3

suggested that the tissues were viable and capable of carrying out normal physiological functions. The flat strips of tissue used in surface pH measurements were judged to be viable as they were incubated under identical conditions and the time between sacrificing the animal and incubating the tissue was similar.

3.3.2. INTESTINAL SURFACE ACID MICROCLIMATE INVESTIGATIONS :-

PRELIMINARY STUDIES :-

(i) Effect of glucose on the intestinal surface pH :-

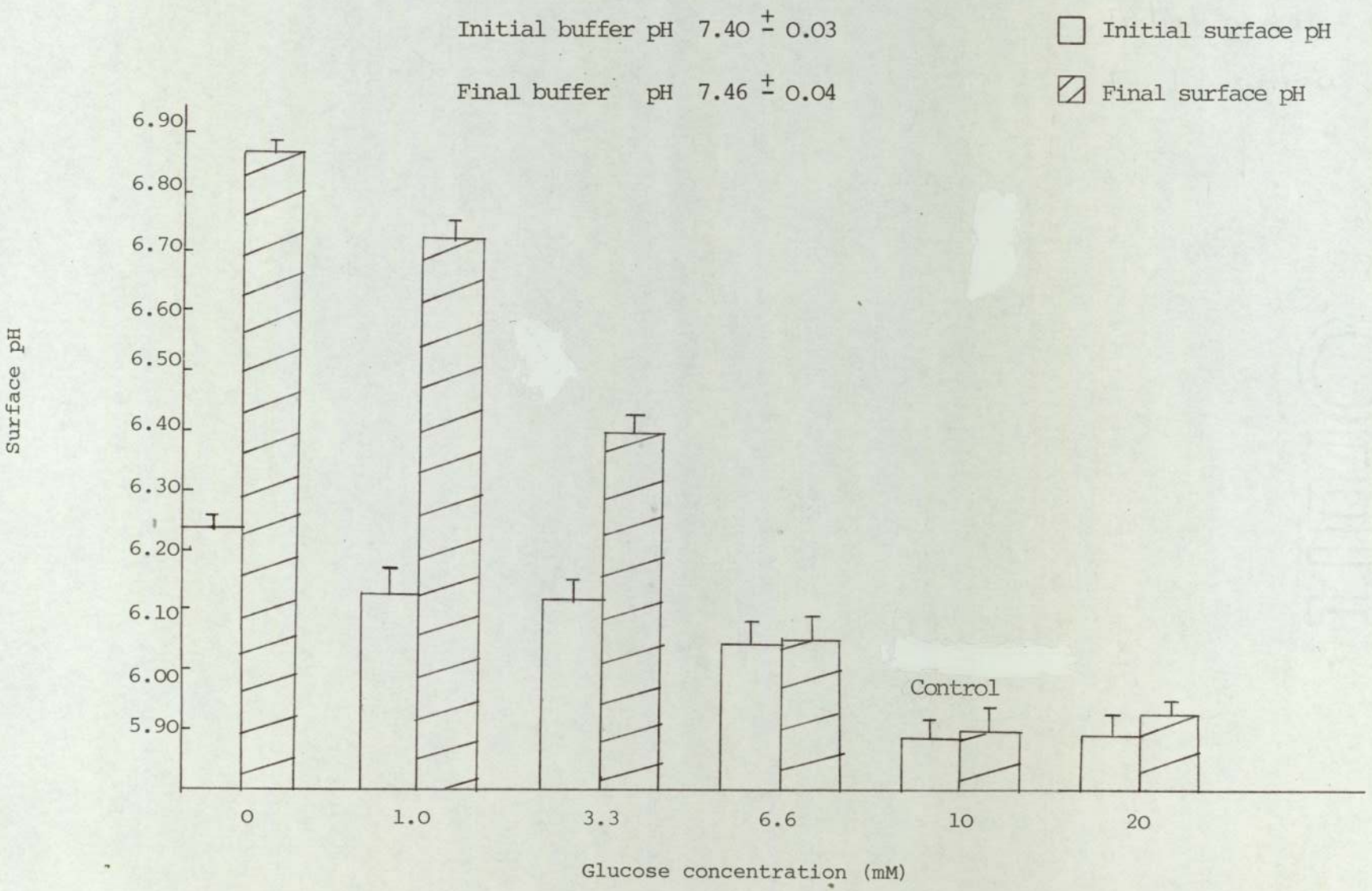
Glucose is an actively transported monosaccharide (Crane, 1965) and can be easily metabolised and used as a fuel by living cells both aerobically and anaerobically. Its role in the creation and maintenance of the intestinal acid microclimate was investigated. This was performed by measuring the surface pH of rat proximal jejunum in bicarbonate buffer at 37^{o}C, containing different concentrations of glucose. Table 3.2 shows typical successive recordings of the surface and buffer pH measurements at the onset and the end of incubation in the presence of 10 mM glucose. The pH-microelectrode used tended to have a response time similar to that of a standard commercially available electrode. This almost certainly is related to the relatively large surface area of sensitive glass.

In all the cases examined, no significant changes in buffer pH was observed (Figure 3.3). The most acidic surface, both at the onset and the end of incubation was found in the presence of 10 mM glucose. At higher concentration of glucose, there was no increase

TABLE 3-2 :- Typical successive recordingsofrat proximal jejunum surface pH in bicarbonate buffer pH 7.4 containing 10 mM glucose at 37°C (Control).

	Incubation time (min.)	
	t_0	t_{30}
Buffer pH	7.40	7.45
Surface pH	6.98	6.93
	6.50	6.42
	5.96	5.97
	5.94	5.95
Buffer pH	7.40	7.46

Figure 3-3:- The effect of different concentrations of glucose in the incubation medium (bicarbonate buffer) on the surface pH of the proximal jejunum at 37°C.



in surface acidity. At glucose concentrations 6.6 - 20 mM the low surface pH maintained itself during the incubation period, i.e. no time dependent elevation was observed. At lower glucose concentrations (0-6.6mM) there was an elevation in the surface pH which became significant at 6.6 mM ($P > 0.02$ and $P < 0.05$ for the onset and final surface pH respectively). A time dependent elevation in the surface pH was observed at glucose concentration of 0, 1 and 3.3 mM. The elevation is presumably due to the exhaustion of the intracellular glucose reservoir. The most alkaline onset and final surface pH values were observed in the absence of glucose although the surface pH remained lower than the pH of the incubation medium. 10 mM glucose was chosen as an appropriate concentration to be used for further investigations on surface pH.

(ii) The effect of the method of sacrifice on the intestinal surface pH :-

Surface pH measurements were carried out using a tissue from the proximal jejunum of rats sacrificed by either stunning or anaesthetisation with Inactin (20 mg/100 g body weight intraperitoneally). Significantly lower onset ($P < 0.05$) and final ($P = 0.02$) surface pH values were observed in animals sacrificed by stunning rather than by anaesthetisation (Table 3.3). No significant changes in buffer pH were observed. As the effect of anaesthetisation on surface pH might be related to the known inhibitory effect on the intracellular metabolic activities (Hulme and Krantz, 1955; Levine, Mcnary, Kornguth and Leblanc, 1970), stunning was preferred as the

TABLE 3-3 :-

The effect of the method of sacrifice on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose at 37°C.

	Surface pH		Buffer pH	
	Initial	Final	Initial	Final
1. Animals Sacrificed by stunning.	5.88±0.03	5.84±0.02	7.42±0.02	7.44±0.02
2. Animals sacrificed by anaesthatisation (20mg Inactin intra-peritoneally/100 g, body weight).	5.98±0.03	5.94±0.03	7.40±0.02	7.42±0.02

method of sacrifice.

(iii) The influence of the incubation buffer on the surface pH :-

The choice of the buffer system and its possible effect on surface pH was examined by measuring the surface pH of rat proximal jejunum in bicarbonate buffer pH 7.4 and in Krebs-Ringer phosphate buffer (pH adjusted to 7.4). In both cases 10 mM glucose was added. No significant change in surface and buffer pH during incubation was observed (Table 3.4). The data indicate that the type of buffer has no effect on the surface pH providing that sufficient amounts of nutrient and electrolytes are available for the tissue.

(iv) The effect of incubation temperature on the surface pH :-

The surface pH of rat proximal jejunum was measured in bicarbonate buffer containing 10 mM glucose at 27°C and 37°C. Significantly higher onset ($P < 0.001$) and final ($P < 0.001$) surface pH values were observed when the incubation was carried out at the lower temperature (Table 3.5).

(v) In vivo surface pH measurements :-

The surface pH of rat proximal jejunum was measured in vivo as described in Chapter 2 and the results are shown in Table 3.6. It is clear that a surface acid microclimate exists on the surface of the proximal jejunum in vivo. The in vivo surface pH is significantly higher ($P > 0.01$) than the onset surface pH of the in vitro preparation measured in bicarbonate buffer containing 10 mM glucose at 37°C.

TABLE 3-4:-

The influence of incubation buffer on the surface pH of the proximal jejunum. The measurements were performed in the presence of 10mM glucose at 37°C.

	Surface pH		Buffer pH	
	Initial	Final	Initial	Final
Bicarbonate buffer	5.95±0.03	5.97±0.04	7.41±0.03	7.45±0.04
Krebs-Ringer phosphate buffer	5.94±0.04	5.93±0.03	7.40±0.03	7.41±0.02

TABLE 3-5:-

The effect of the incubation temperature on the surface pH of rat proximal jejunum incubated in bicarbonate buffer containing 10mM glucose.

Incubation temperature	Surface pH		Buffer pH	
	Initial	Final	Initial	Final
27°C	6.07±0.04	6.09±0.03	7.42±0.02	7.43±0.02
37°C (control)	5.88±0.03	5.89±0.02	7.40±0.03	7.44±0.02

TABLE 3-6 :-

In vivo surface acid microclimate. The surface pH of the proximal jejunum was measured in vivo in bicarbonate buffer containing 10 mM glucose at 37°C. Only the initial surface pH was recorded.

1. In vivo measurements :-

Surface pH	Buffer pH
6.13 ± 0.06	7.40 ± 0.03

2. In vitro measurements :- (Animals killed by stunning)

Surface pH		Buffer pH	
Initial	Final	Initial	Final
5.93±0.03	5.94±0.02	7.40±0.04	7.46±0.04

The difference, is most probably, due to the technical difficulties involved with the in vivo method and the effect of the anaesthetic. The results contradict the observations of Rechkemmer, Wahl, Kuschinsky and Engelhardt(1979).

(vi) The distribution of the surface acid microclimate along the entire length of the small intestine and colon :-

Moving from the proximal duodenum towards the proximal jejunum an increase in the surface acidity was observed (Figure 3.4). The most acid surface was found in the proximal jejunum. Distally a severe decline in the surface pH towards neutrality was observed. The most alkaline surface pH was observed in the distal ileum and colon, although the surface pH remained lower than the pH of the incubation medium. In all the regions investigated, no significant change in the surface pH and buffer pH during 30 minutes incubation was observed.

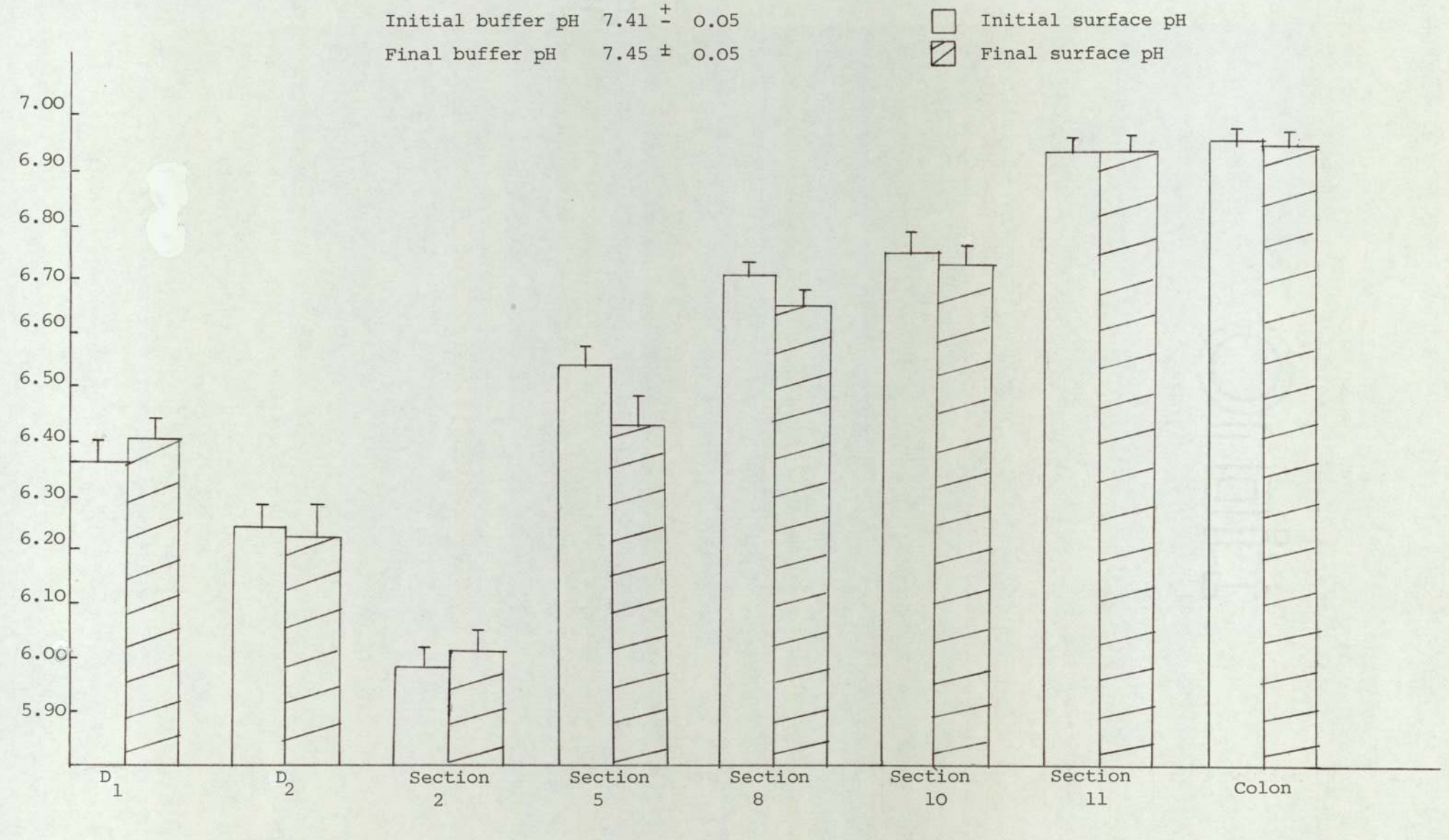
3.3.3. AN ASSESSMENT OF THE REQUIREMENTS FOR THE MAINTENANCE OF THE SURFACE ACID MICROCLIMATE :-

(i) The effect of other saccharides on the intestinal surface acidity :-

(a) Monosaccharides :-

The effect of hexoses was investigated to establish whether metabolised sugars could provoke low intestinal surface pH or whether low intestinal surface pH was a consequence of the active transport of sugars. The surface pH of rat proximal jejunum was

Figure 3-4:- The distribution of the surface acid microclimate along the small intestine and colon. Measurements were performed in bicarbonate buffer containing 10 mM glucose at 37°C



measured in bicarbonate buffer in the absence and presence of either 10 mM glucose, galactose, fructose or mannose. In all the cases examined, no significant changes in buffer pH during incubation was observed (Figure 3.5). In the presence of galactose, an actively transported but unmetabolised hexose of the small intestine (Wilson and Vincent, 1955; Goldner, Sarracino and Estep, 1977), similar onset and final surface pH to that recorded in the absence of saccharides were observed.

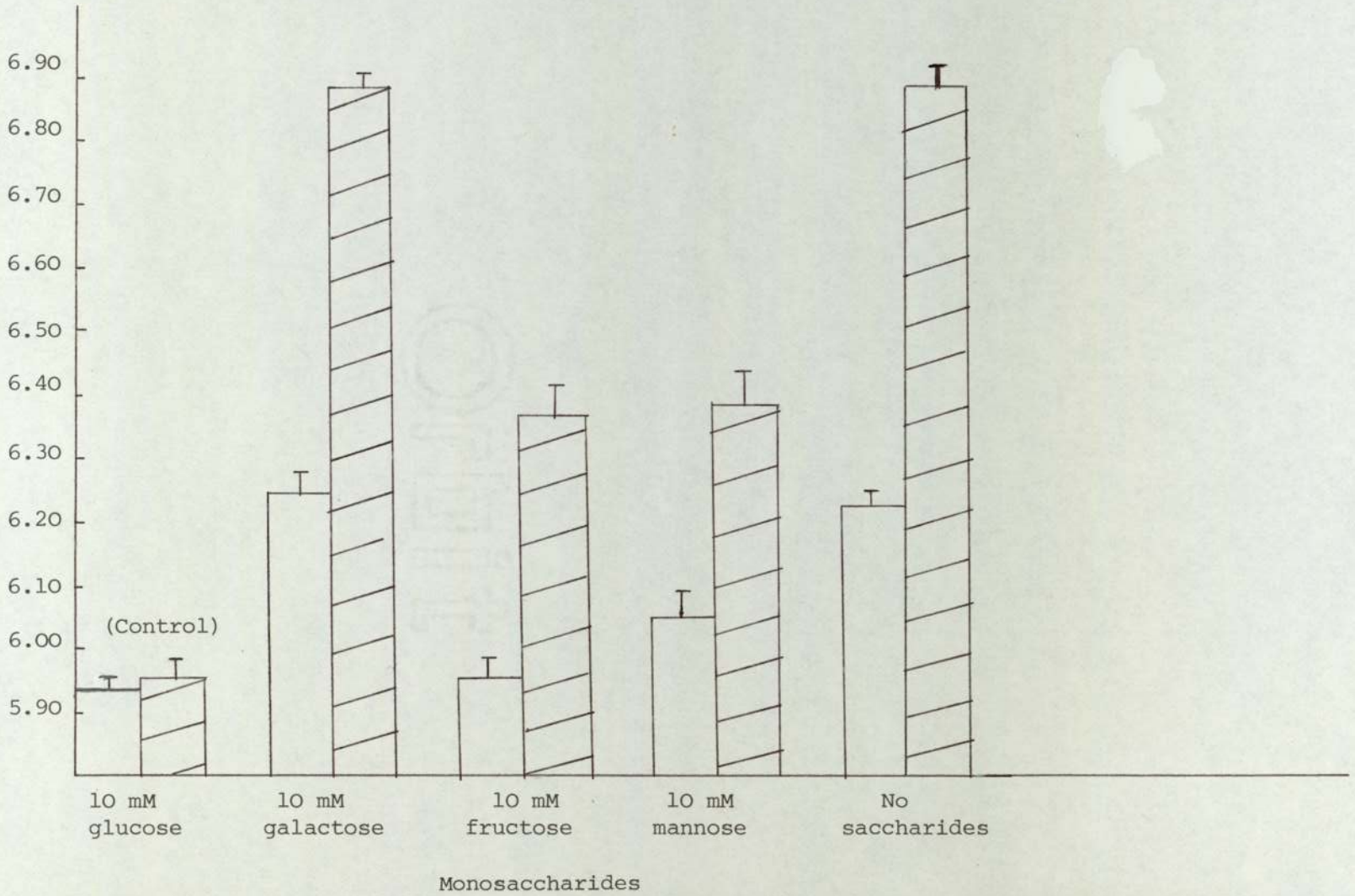
Fructose is a metabolised hexose (Wilson and Vincent 1955) for which some workers have proposed a specific transport system by the small intestine (Gracey, Burke and Oshin, 1970). Its presence made the onset surface pH insignificantly different from that of the control (10 mM glucose). However, the final surface pH was significantly higher ($P < 0.001$) than that of the control, but much lower than that observed in the absence of saccharides.

Mannose, on the other hand, is a passively transported hexose (Wilson and Vincent 1955) and can be phosphorylated and metabolised by the small intestine (Hele, 1953). It brought about a more acidic surface pH compared to that observed in the absence of any saccharide, however, it was significantly higher both at the onset ($P < 0.05$) and the end ($P < 0.001$) of incubation compared to control values (10 mM glucose).

Figure 3-5:- The effect of the presence and absence of either 10 mM glucose, galactose, fructose, or mannose, in the incubation medium on the surface acid microclimate of the proximal jejunum. Measurements were performed in bicarbonate buffer at 37°C.

Initial buffer pH 7.38 ± 0.05

Final buffer pH 7.43 ± 0.03



(b) Disaccharides :-

The surface pH of rat proximal jejunum was also measured in bicarbonate buffer in the absence and presence of either 10 mM glucose, maltose, sucrose or lactose. In all the cases examined, no significant change in buffer pH was observed (Figure 3-6). The replacement of 10 mM glucose by 10 mM maltose had no effect on the initial or final surface pH values.

In the presence of sucrose, although the initial and final surface pH were much lower than that observed in the absence of saccharide, it remained significantly higher than that of the control ($P < 0.001$ and > 0.001 for the initial and final surface pH respectively).

When lactose was added, the onset and final surface pH were insignificantly different from that obtained in the absence of saccharides.

(ii) The effect of ATP on the intestinal surface pH :-

Enzymatic hydrolysis of ATP produces hydrogen ions (Figure 3-7). It has been suggested by Blair and Matty (1974) that external hydrolysis of ATP by the mucosally located ATPase is the mechanism involved in the production of hydrogen ion of the intestinal acid microclimate and luminal acidification. To test this hypothesis and to investigate the role of ATP in creating and maintaining the low surface pH , measurements of the rat proximal jejunum surface pH were carried out in bicarbonate buffer in the absence and presence of 10 mM glucose and 10^{-2} M ATP. In the presence of 10^{-2} M-ATP and 10 mM glucose,

Figure 3-6: The effect of different disaccharides (10mM) on the surface pH of the proximal jejunum incubated in bicarbonate buffer at 37°C

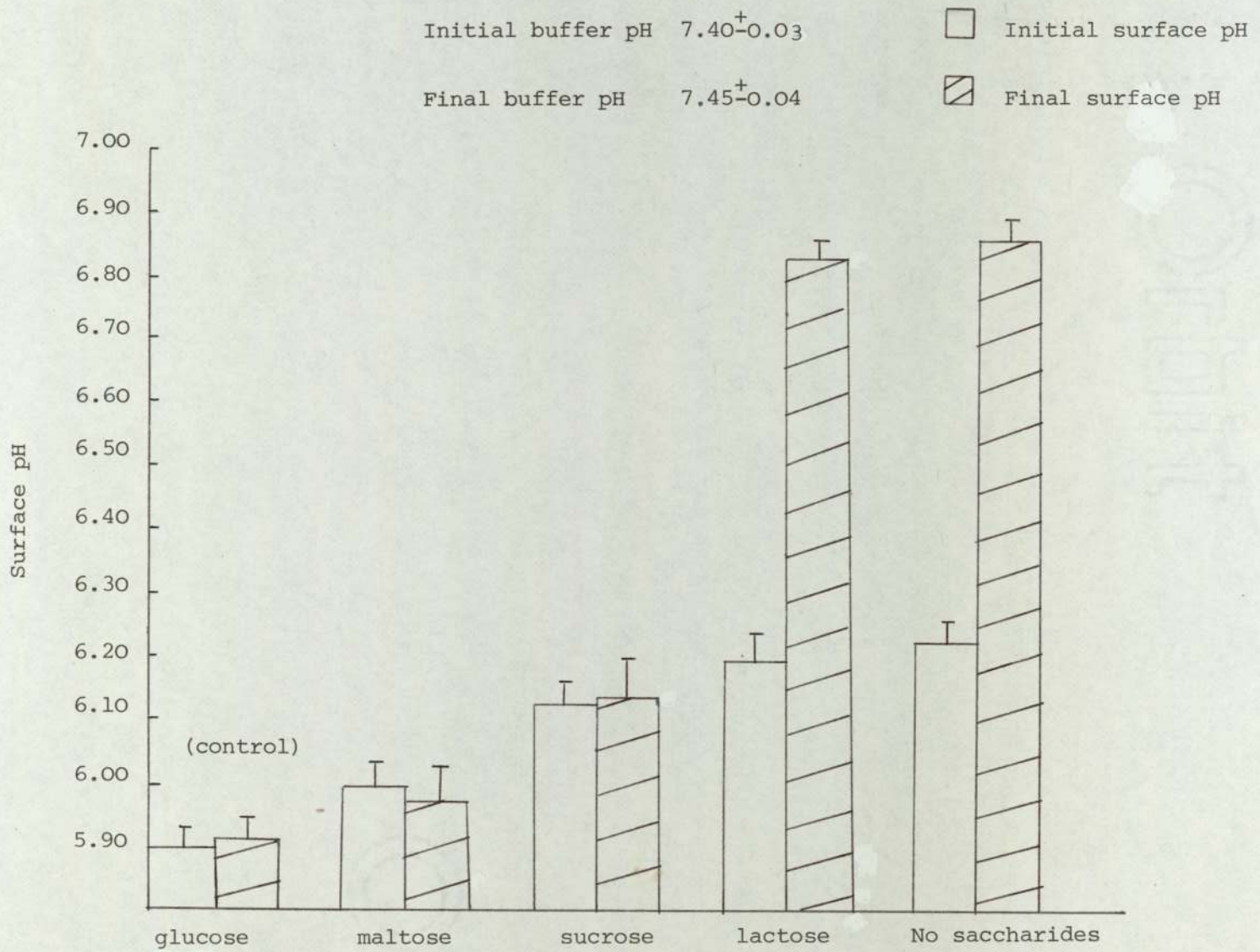
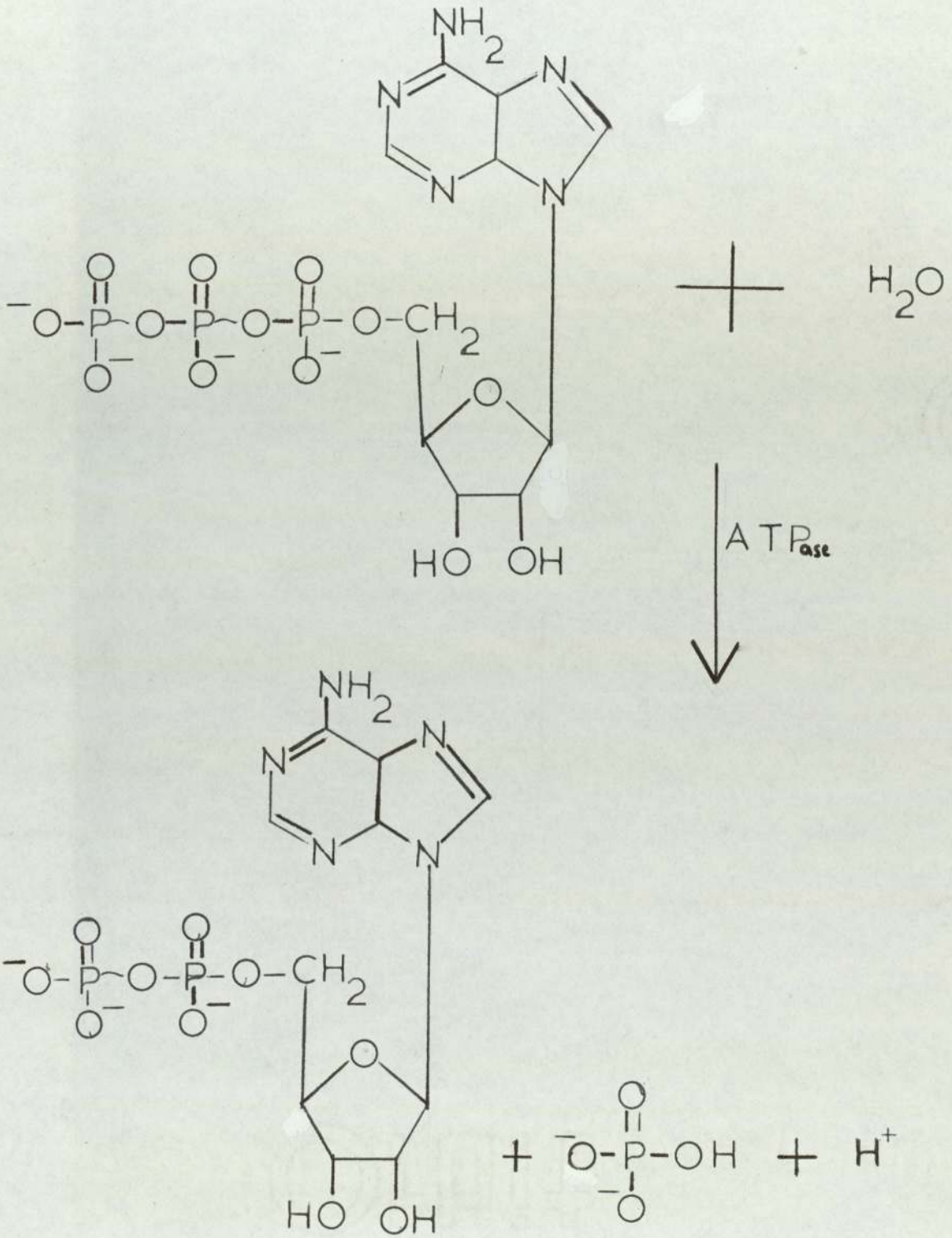
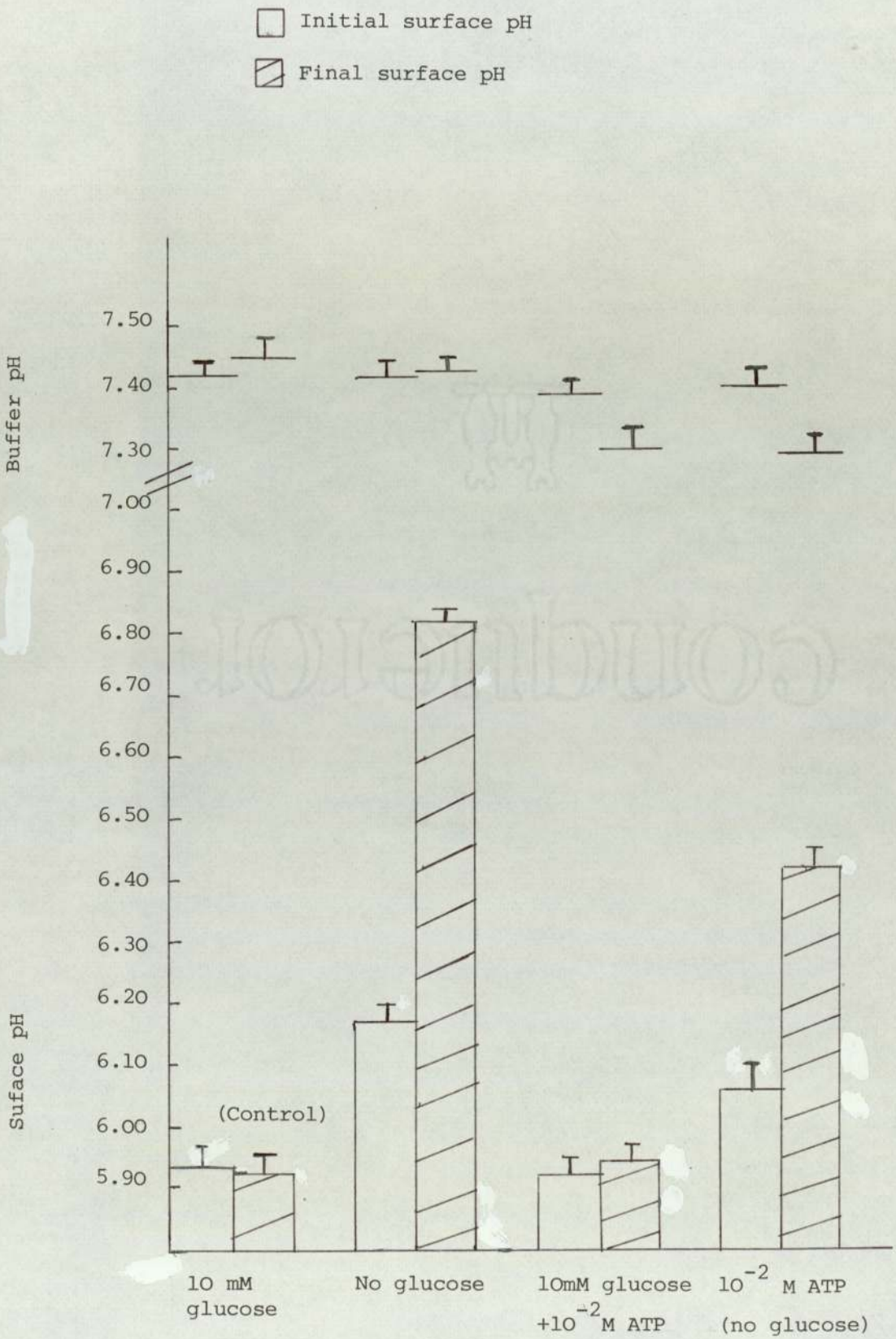


Figure 3-7 : Enzymatic hydrolysis of ATP



j.e. $ATP^{4-} + H_2O \xrightarrow{ATP_{ase}} ADP^{3-} + HPO_4^{2-} + H^+$

Figure 3.8: The effect of ATP on the surface acid microclimate of the proximal jejunum incubated in bicarbonate buffer at 37°C in the presence and absence of 10 mM glucose.



the onset and final surface pH were similar to that of control (10 mM glucose only) (Figure 3-8). On the other hand, when 10^{-2} M ATP was added alone (no glucose present) significantly more acidic onset ($P > 0.02$) and final ($P < 0.001$) surface pH values were observed compared to those in the absence of glucose. However, both values were significantly higher than that of the control ($P < 0.02$ and < 0.001 for the initial and final surface pH respectively). The time dependent elevation in the surface pH contrasted with a decrease in buffer pH during incubation. The change in buffer pH is possibly due to the chemical hydrolysis of ATP.

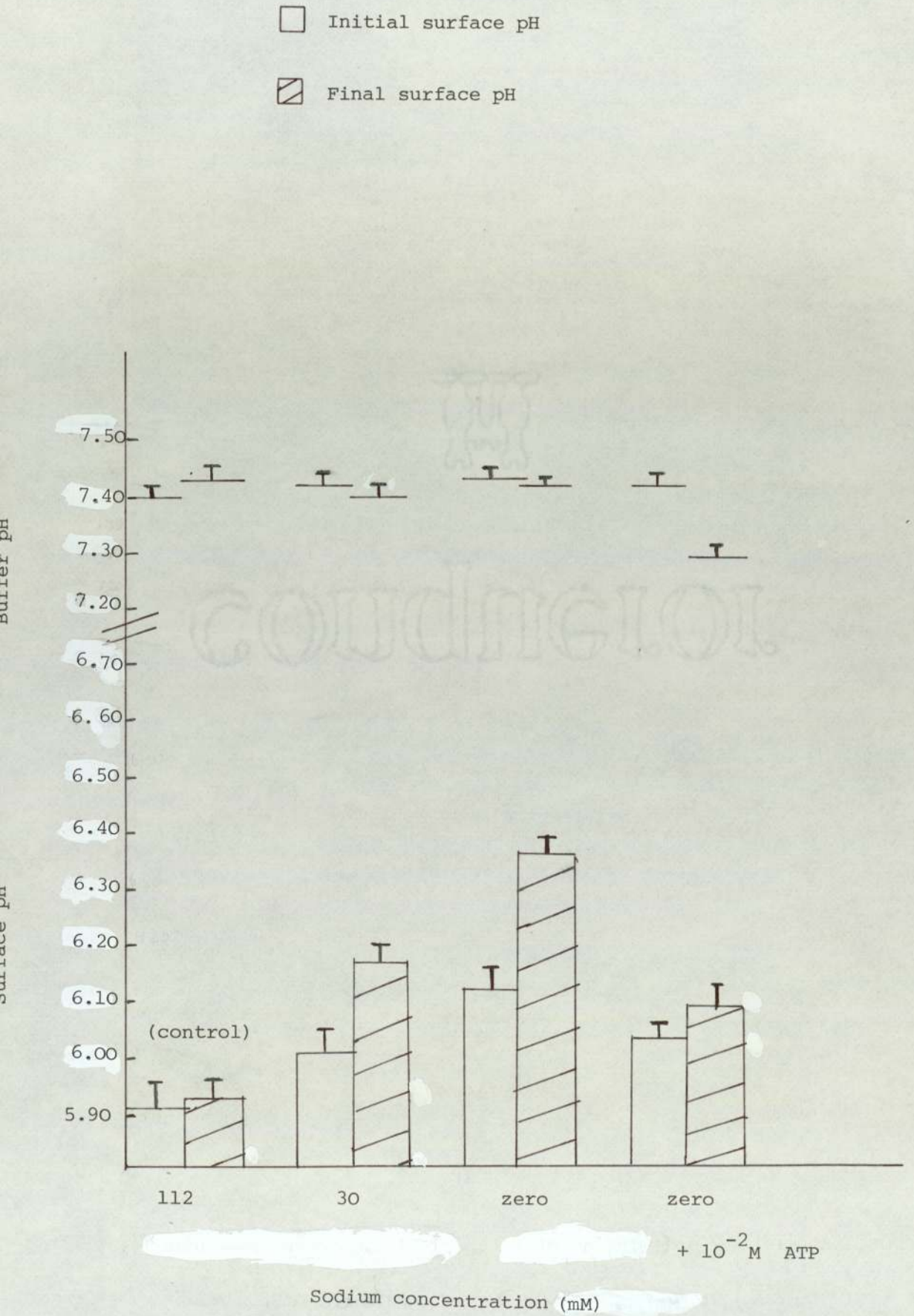
(iii) The effect of buffer electrolytes on the surface pH :-

(a) Sodium ion :-

The effect of sodium on the surface acid microclimate was investigated by measuring the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose and different concentrations of sodium ion . The osmolarity of the buffer was compensated with potassium.

No change in buffer pH during incubation occurred except in the presence of 10^{-2} M ATP (Figure 3-9). In the presence of 30 mM sodium, the initial surface pH was not significantly higher than that of the control value. However, the final surface pH was found to be significantly higher ($P < 0.001$). In the absence of sodium, significantly higher initial ($P < 0.001$) and final ($P < 0.001$) surface pH values were obtained . The time dependent elevation in the surface pH was

Figure 3-9: The effect of different concentrations of sodium in the incubation medium (bicarbonate buffer containing 10 mM glucose) on the surface acid microclimate of the proximal jejunum at 37°C.



even higher than that observed in the presence of 30 mM sodium.

In an attempt to differentiate whether the elevation in the surface pH in the absence of sodium ions is related to the known reduction in the oxygen uptake and metabolic activity of the gut tissue in vitro (Levin and Syme, 1975) and restriction of glucose transport (Faust, 1962) or whether it is an indication of a decrease in Na^+:H^+ ion exchange process as suggested by Turnberg, Fordtran, Carter and Rector (1970) and Turnberg (1978), the surface pH was measured after adding 10^{-2} M ATP to the sodium free incubation medium. The results are shown in Figure 3-9. An insignificant decrease in the onset surface pH was observed in the presence of 10^{-2}M ATP. On the other hand, significant decrease ($P < 0.001$) in the final surface pH was observed.

(b) Magnesium ion:

Magnesium (Mg^{2+}) is an important electrolyte for the activity of many enzymes including those participating in glycolysis and oxidation phosphorylation reactions. It is also essential for the activity of ATPase (Kesavan and Noronha, 1978).

The effect of magnesium ions on the surface acidity was examined by measuring the surface pH of proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose in the presence and absence of 1.12 mM Mg^{2+}. A significant elevation in the onset ($P < 0.001$) and the final ($P < 0.001$) surface pH was observed in the absence of Mg^{2+} (Table 3-7). However, both in the absence and presence of

TABLE 3 - 7 :

The effect of magnesium ions on the surface pH of the proximal jejunum. Measurements were performed in bicarbonate buffer containing 10 mM glucose in the presence and absence of 1.12 mM magnesium ion.

Mg^{2+} Concentration (mM)	Surface pH		Buffer pH	
	Initial	Final	Initial	Final
0	6.06 ± 0.03	6.05 ± 0.03	7.43 ± 0.03	7.43 ± 0.02
1.12 (control)	5.87 ± 0.02	5.92 ± 0.02	7.43 ± 0.02	7.43 ± 0.02

Mg^{2+}, no time dependent changes in the surface and buffer pH were observed during the 30 minutes incubation period.

3.3.4 METABOLIC INHIBITORS AND THEIR EFFECT ON THE INTESTINAL ACID MICROCLIMATE :-

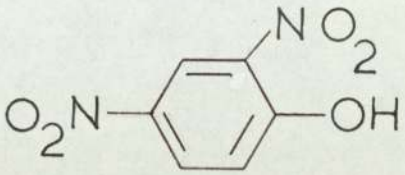
The relationship between the surface acidity of the intestine and the intracellular activities of the mucosal epithelial cells such as glycolysis, citric acid cycle and oxidative phosphorylation, was investigated using a variety of metabolic inhibitors. The metabolic inhibitors used were 2,4-dinitrophenol, iodoacetate, sodium fluoride, sodium azide, hydroxylamine, atractyloside and sodium vanadate (Figure 3-10).

(i) 2,4-Dinitrophenol (DNP) :-

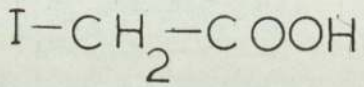
DNP is a known uncoupler of oxidative phosphorylation (Loomis and Lipmann, 1948). The surface pH of rat proximal jejunum was measured in bicarbonate buffer containing 10 mM glucose and different concentrations of DNP. The results are shown in Figure 3-11. In the presence of 10^{-4} M DNP the surface pH at the onset of incubation was not significantly higher than that of the control value, while at the end of incubation the value was significantly higher ($P < 0.001$). In the presence of 10^{-3} M DNP, significantly higher surface pH values at the onset ($P < 0.001$) and the end ($P < 0.001$) of incubation were recorded. In both cases there was a time dependent elevation in the surface pH but not in the buffer pH.

Figure 3-10 :- The chemical structure of the metabolic inhibitors used.

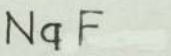
2,4-Dinitrophenol



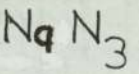
Iodoacetate



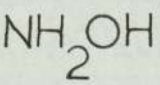
Sodium fluoride



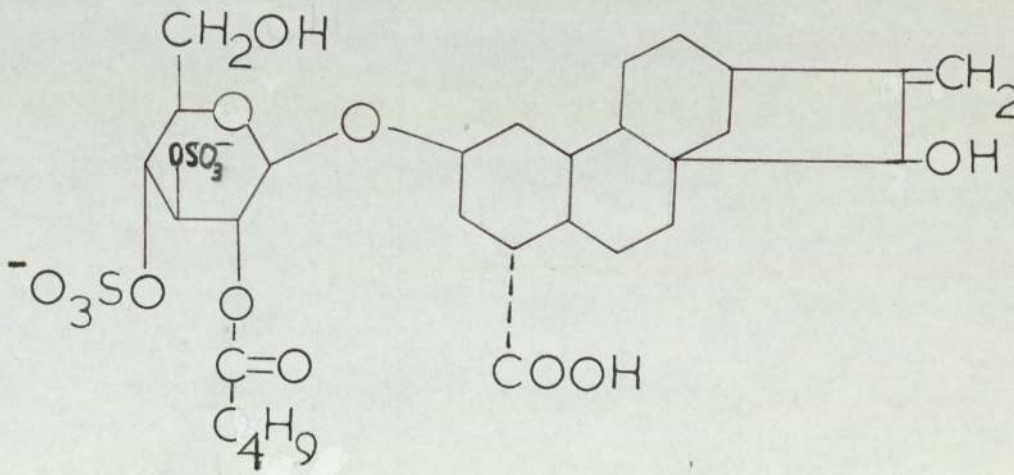
Sodium azide



Hydroxylamine



Atractyloside



Sodium meta vanadate

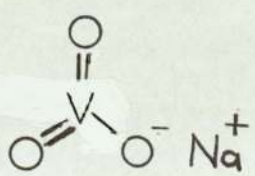
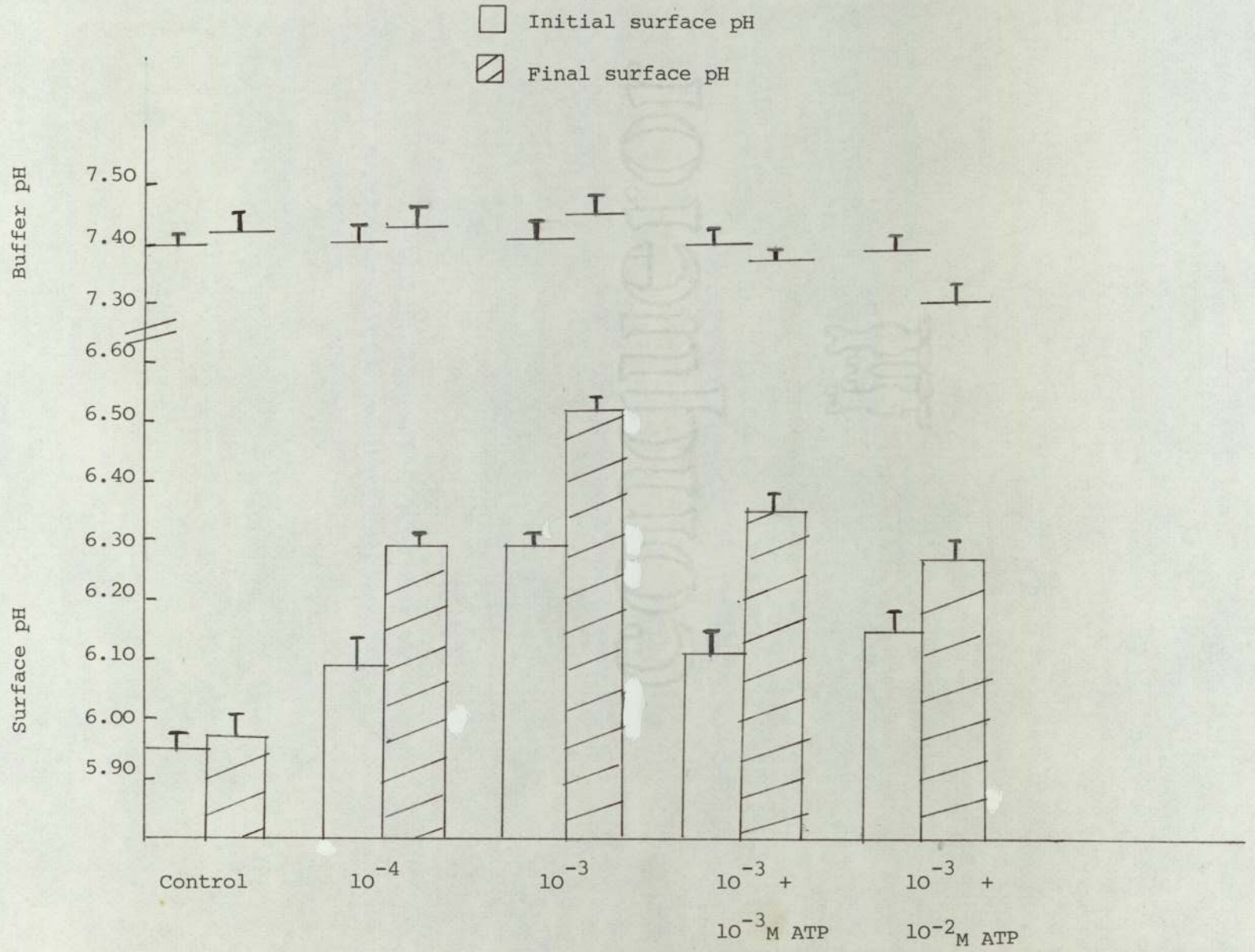


Figure 3-11:- The effect of DNP at different concentrations on the surface acid microclimate of the proximal jejunum incubated in bicarbonate buffer containing 10mM glucose at 37°C.



To see whether the change in surface acidity in the presence of DNP is due to a reduction in the intracellular ATP level, with subsequent reduction in its availability at the surface, different concentrations of ATP were added to the incubation medium in the presence of 10^{-3} M DNP and the surface pH measured (Figure 3-11). On adding 10^{-3} M ATP, significantly lower surface pH values at the onset ($P > 0.001$) and the end ($P < 0.001$) of incubation were obtained. Adding more ATP (10^{-2} M) produced no further increase in the surface acidity.

(ii) Iodoacetate :-

Iodoacetate is a glycolytic inhibitor (Webb, 1966). The effect of different concentrations of iodoacetate on the surface pH of the rat proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose was examined. The results are shown in Figure 3-12a. In the presence of 10^{-4} M iodoacetate, significantly higher onset ($P = 0.02$) and final ($P < 0.001$) surface pH values were observed. When 10^{-3} M iodoacetate was added, a greater elevation in the onset and the final surface pH was observed. In both cases, no significant change in buffer pH during incubation occurred.

In another series of experiments, 10^{-2} M ATP was added to the incubation medium in the presence of 10^{-3} M iodoacetate and the surface pH measured. Although there was a significant drop in the onset ($P < 0.01$) and the final ($P > 0.001$) surface pH values compared to that recorded in the presence of 10^{-3} M iodoacetate, the values

Figure 3.12a :- The effect of iodoacetate at different concentrations on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose at 37°C.

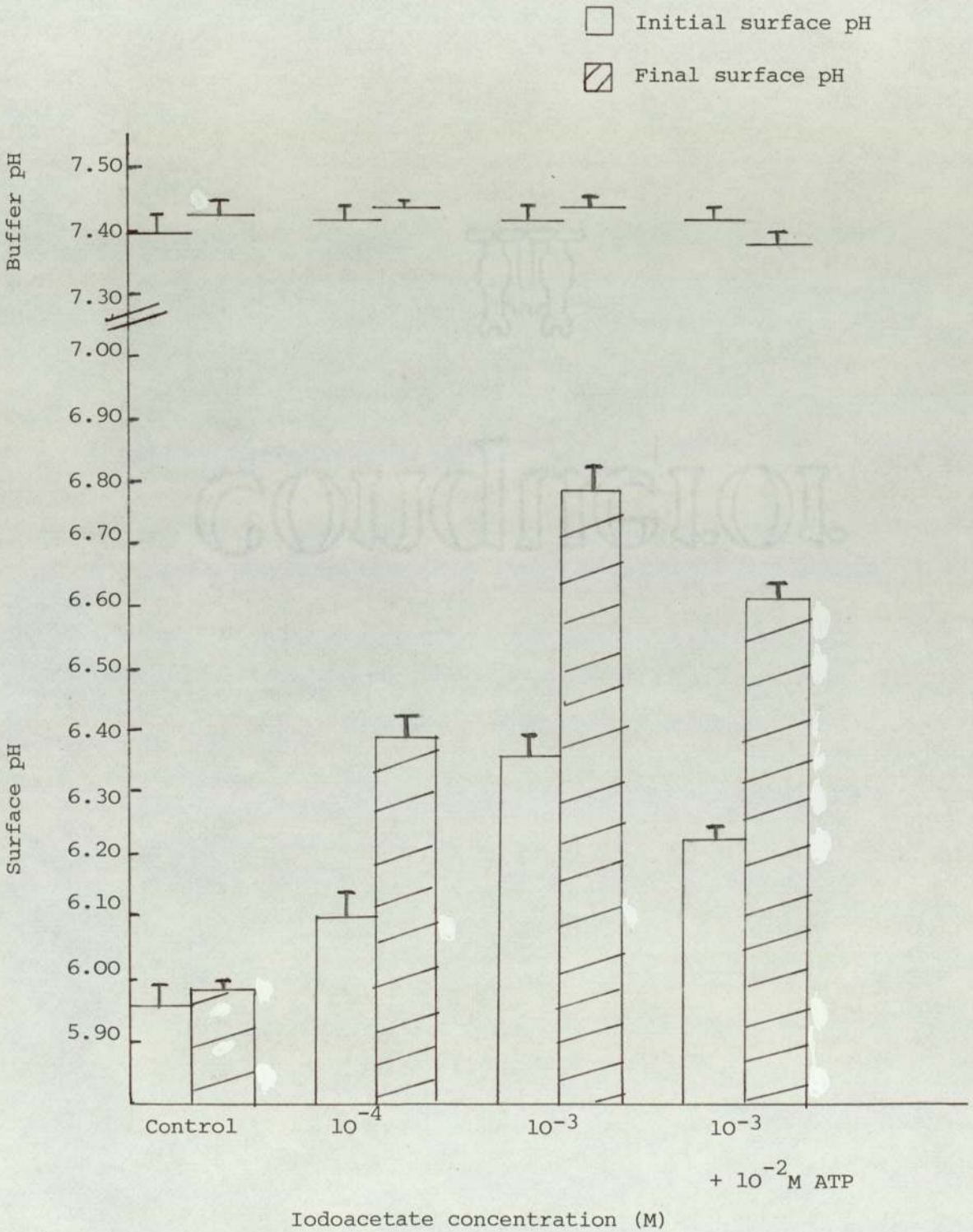


Figure 3-12b: The effect of 10^{-3} M iodoacetate on the surface pH of the proximal jejunum incubated in bicarbonate buffer in the absence of glucose at 37°C.

Initial buffer pH 7.41±0.03

Final buffer pH 7.45±0.03

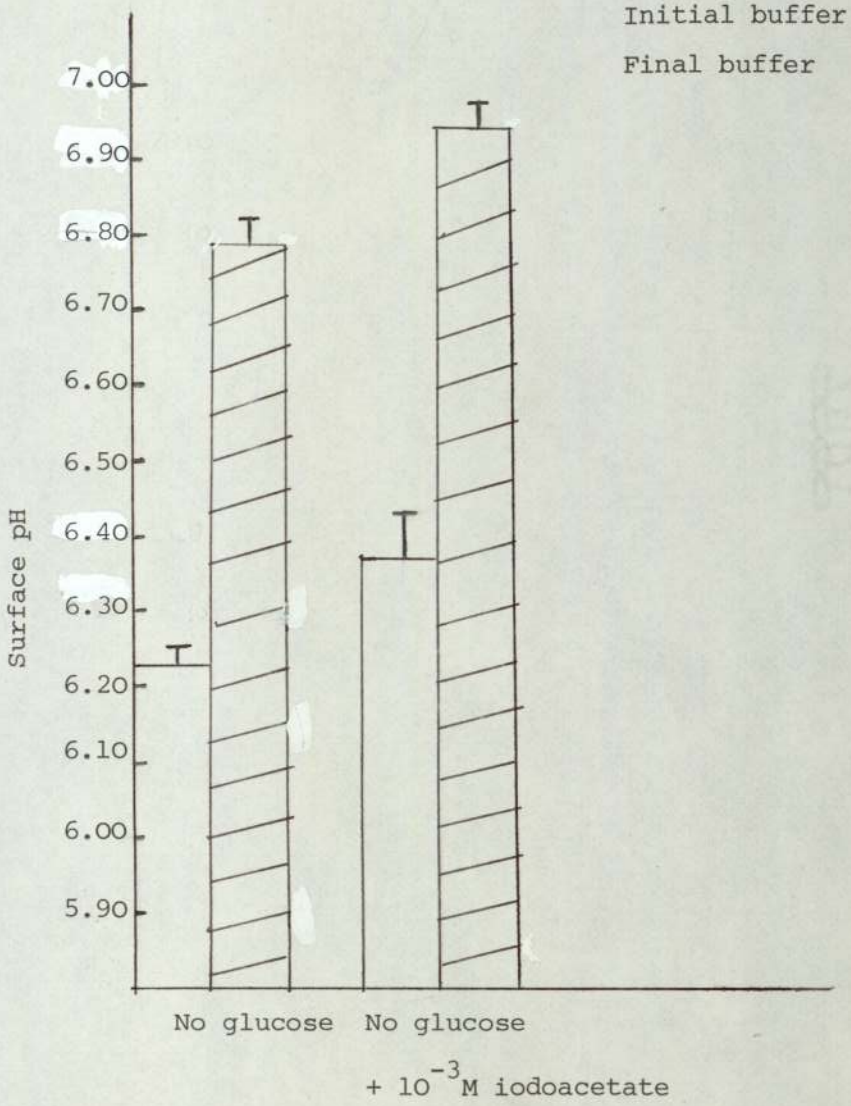
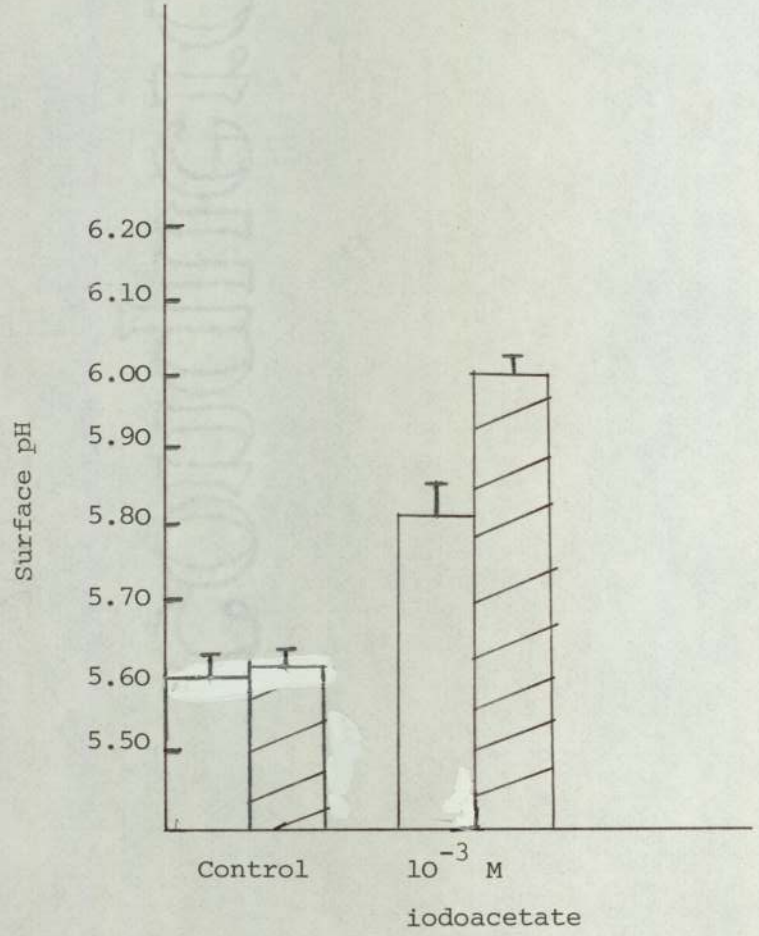


Figure 3-12C: The effect of 10^{-3} M iodoacetate on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose at 37°C.

Initial buffer pH 6.02±0.03

Final buffer pH 6.21±0.02



were significantly higher than that of the control ($P < 0.001$ for both the initial and final surface pH values) (Figure 3-12a).

10^{-3} M iodoacetate also caused a significant increase in the initial ($P < 0.05$) and final ($P < 0.01$) surface pH when added to a glucose - free medium (Figure 3-12b).

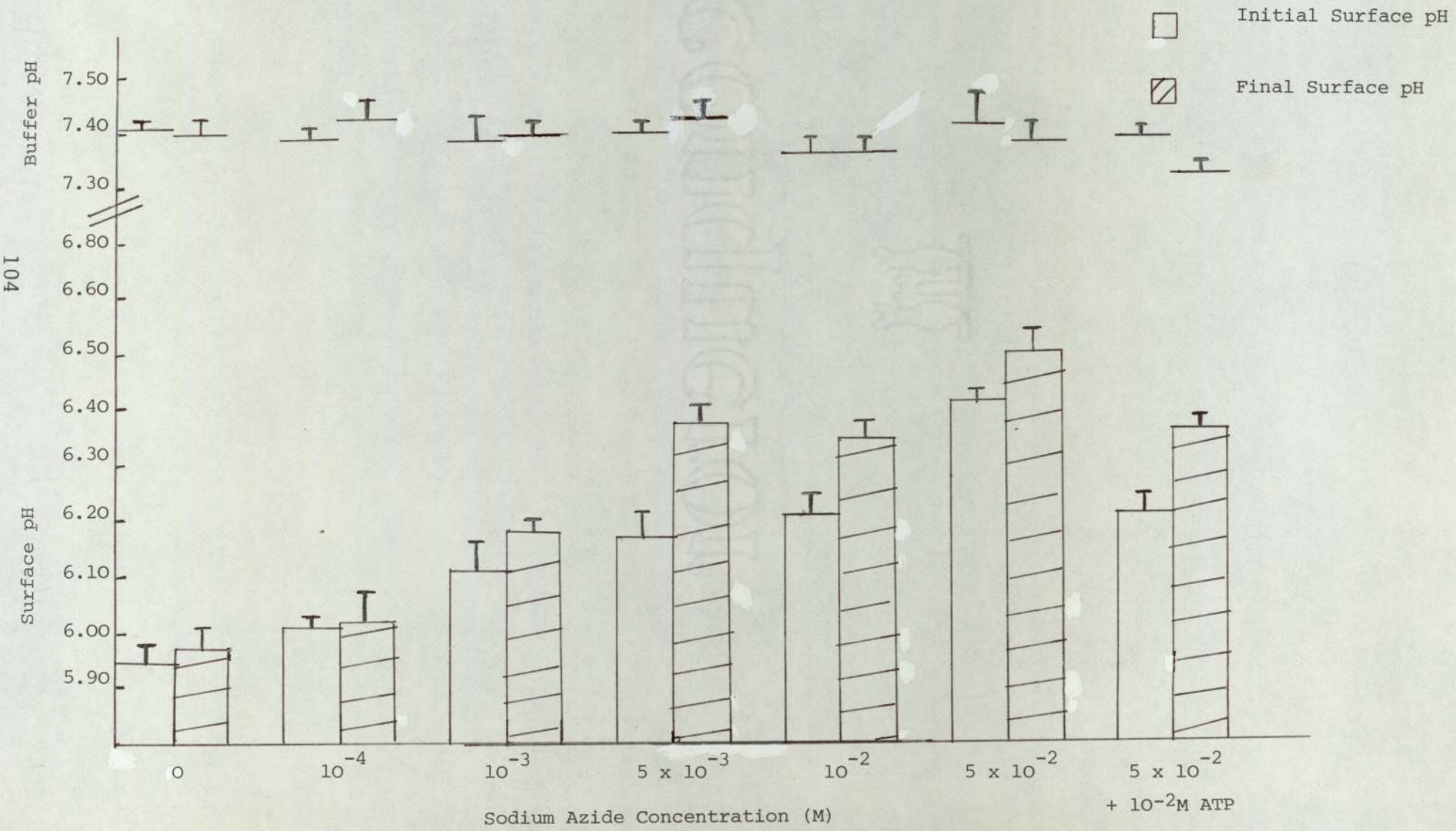
The effect of 10^{-3} M iodoacetate was also examined when the incubation buffer was adjusted to pH 6. It was again found that iodoacetate caused a significant increase in the intial ($P < 0.001$) and final ($P < 0.001$) surface pH compared to that of the control values at the same buffer pH (Figure 3-12c).

(iii) Sodium azide :-

Sodium azide inhibits the activity of a large variety of metallo-enzymes, including Mg^{2+}-stimulated ATPase and cytochrome oxidase (Kesavan and Noronha, 1978; Mahler and Cordes, 1966).

The effect of sodium azide on the surface acidity was investigated by measuring the surface pH of rat proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose and different concentrations of the inhibitor. An elevation in the onset and the final surface pH was observed as the concentration of the inhibitor was increased in the incubation medium (Figure 3-13). The elevation became significant in the presence of 10^{-3}M sodium azide ($p < 0.05$ and < 0.01 for the initial and final surface pH respectively).

Figure 3-13 :- The Effect of sodium azide in different concentrations on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose at 37°C.



In another experiment, the surface pH was measured in the presence of 5×10^{-2} M sodium azide and 10^{-2} M ATP. The effect of ATP was again to make the surface more acid ($P < 0.001$ and > 0.01 for the initial and final surface pH values respectively), but the addition of ATP to the sodium azide incubation medium was not able to reduce the surface pH back to the control value. In all the cases examined, no change in the buffer pH during incubation were observed except in the presence of 10^{-2} M ATP.

(iv) Sodium fluoride :-

Sodium fluoride also inhibits the activity of a large variety of metallo-enzymes including enolase, Mg^{2+}-ATPase and cytochrome oxidase (Mahler and Cordes, 1966). In contrast it stimulates the activity of adenyl cyclase (Coyne, Bonorris, Chung, Conley and Schoenfield, 1977). The effect of this inhibitor, at different concentrations, on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose was investigated. Elevation in the onset and the final surface pH were observed as the concentration of sodium fluoride increased (Figure 3-14a). The elevation became significant both at the onset ($P < 0.001$) and the end ($P < 0.001$) of incubation in the presence of 5×10^{-3} M sodium fluoride.

The effect of adding ATP (10^{-2} M) to the sodium fluoride (10^{-2}M) medium was also investigated. The presence of ATP did not significantly alter the effect of sodium fluoride (Figure 3-14a).

Figure 3-14a:- The effect of sodium fluoride at different concentrations on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10mM glucose at 37°C

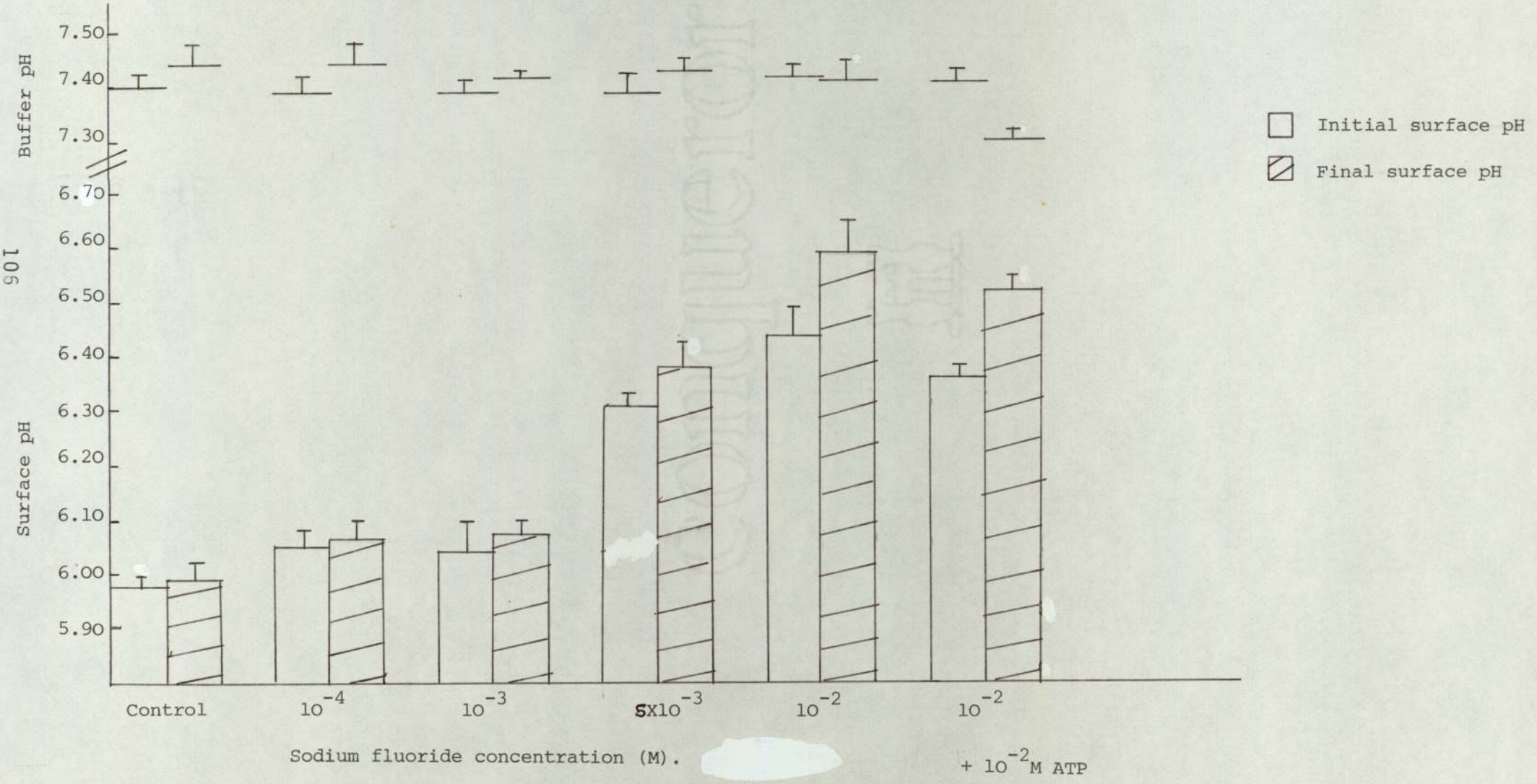


Fig. 4.1 Effect of sodium fluoride on the surface pH of the proximal jejunum incubated in bicarbonate buffer in the absence of glucose at 37°C.

Initial buffer pH 7.40 ± 0.03

Final buffer pH 7.44 ± 0.04

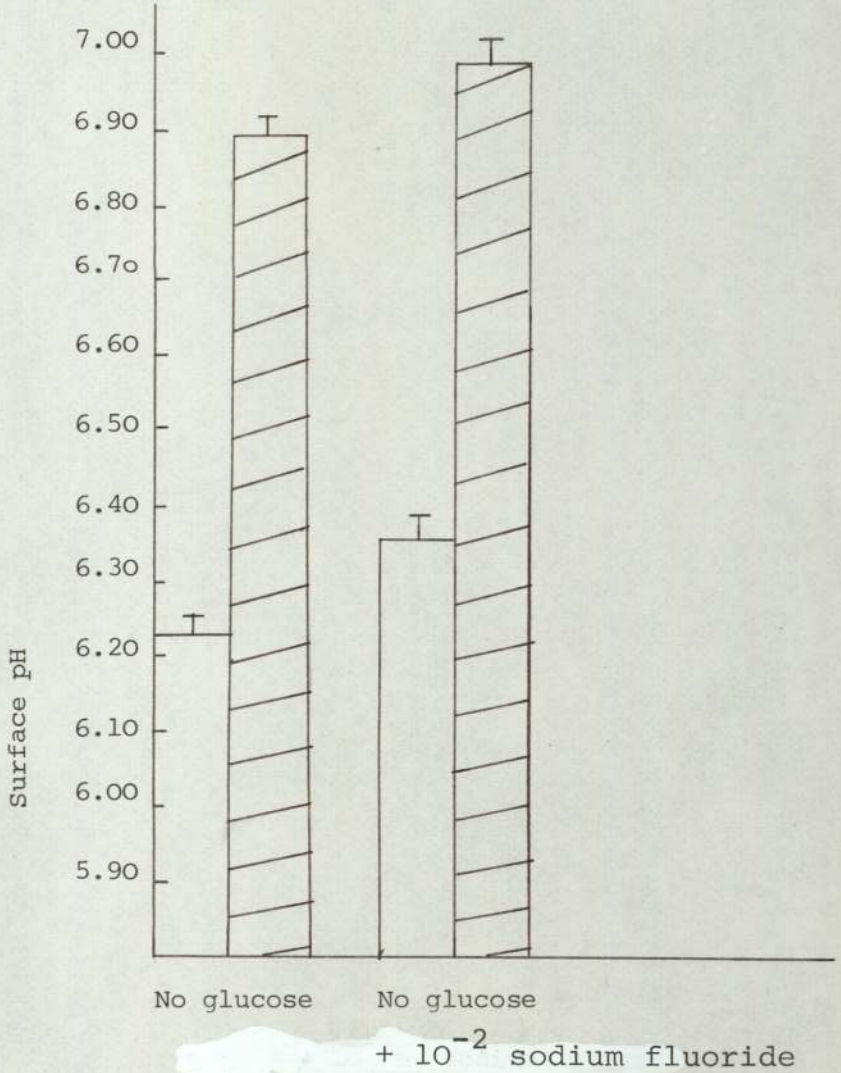
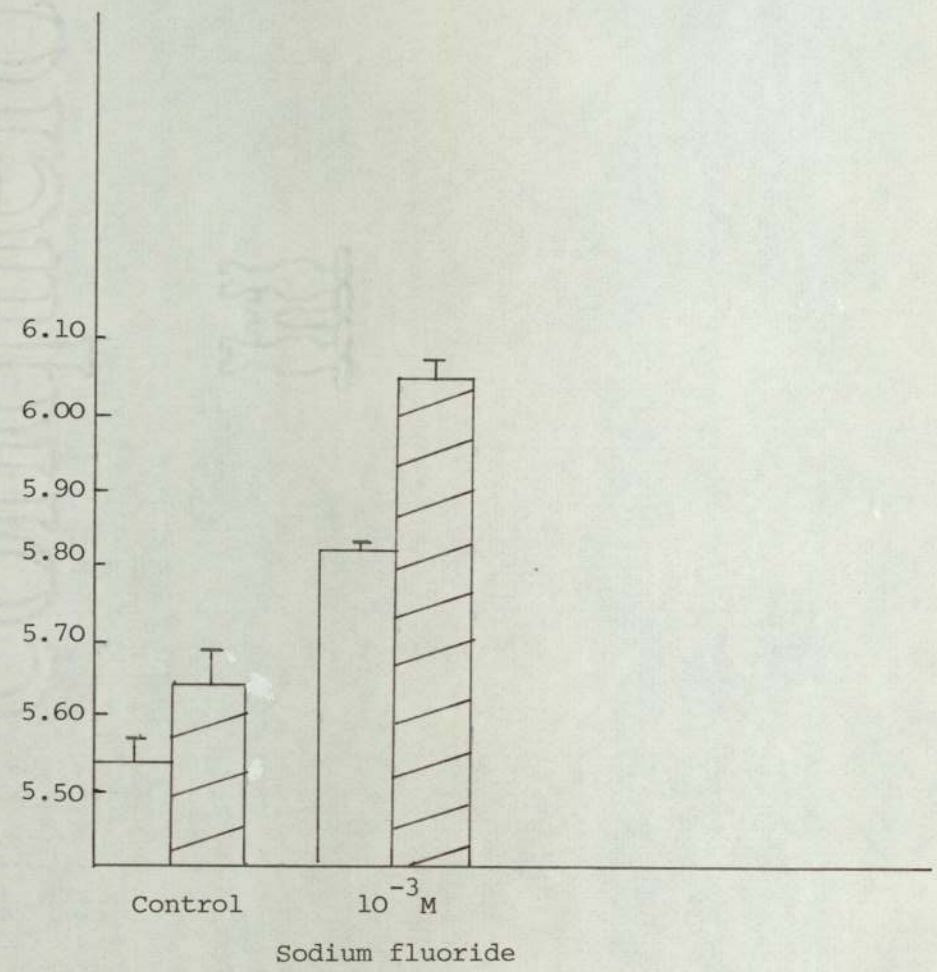


Fig. 4.2 Effect of sodium fluoride on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10mM glucose at 37°C.

Initial surface pH 6.03 ± 0.04

Final surface pH 6.18 ± 0.07



In the absence of glucose from the incubation medium (Figure 3-14b), sodium fluoride (10^{-2} M) caused a significant elevation in the onset ($P < 0.01$) and the final ($P < 0.05$) surface pH over that observed in the absence of glucose.

The effect of sodium fluoride (10^{-3}M) in an incubation buffer adjusted to pH 6 was also examined (Figure 3-14c). Significant elevation in the onset ($P < 0.001$) and the final ($P < 0.001$) surface pH values was observed compared to the control at the same buffer pH.

(v) Hydroxylamine :-

Hydroxylamine is a chemical mutagen (Mahler and Cordes, 1966). Its effect on the surface pH of rat proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose was examined. In the presence of 10^{-3} M hydroxylamine, there was an insignificant elevation in the initial and the final surface pH compared to control values (Figure 3-15). 10^{-2} M hydroxylamine caused a significant elevation in the onset ($P < 0.001$) and the final ($P < 0.001$) surface pH compared to control values.

(vi) Atractyloside :-

Atractyloside specifically inhibits adenine nucleotide translocase, the carrier responsible for the movement of nucleotides across the mitochondrial membrane (ATP:ADP exchange)(Chappell and Crofts, 1965; Meisner, 1971).

The effect of different concentrations of atractyloside on the

Figure 3-15:- The effect of hydroxylamine at different concentrations on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose, at 37°C

Initial buffer pH 7.42 ± 0.03

Final buffer pH 7.40 ± 0.02

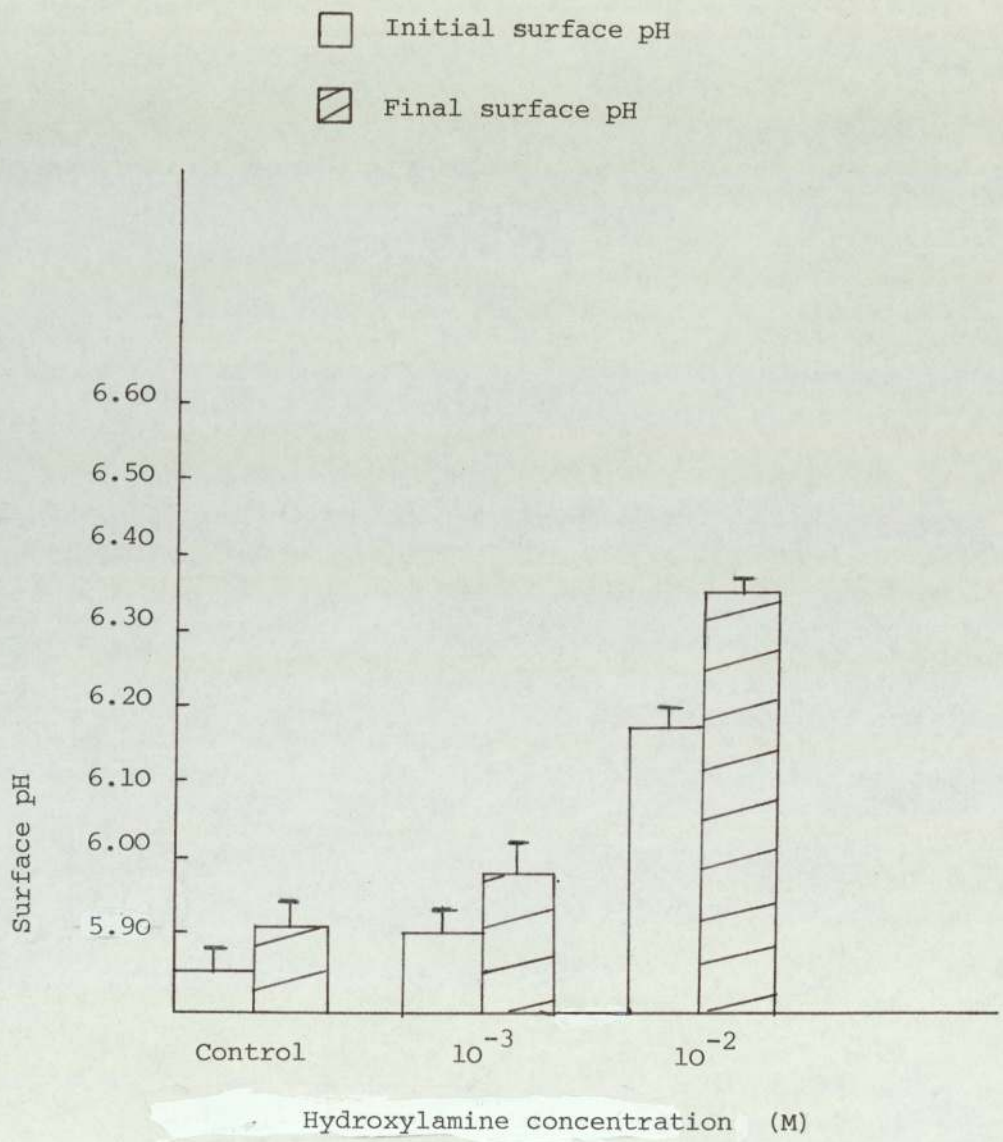
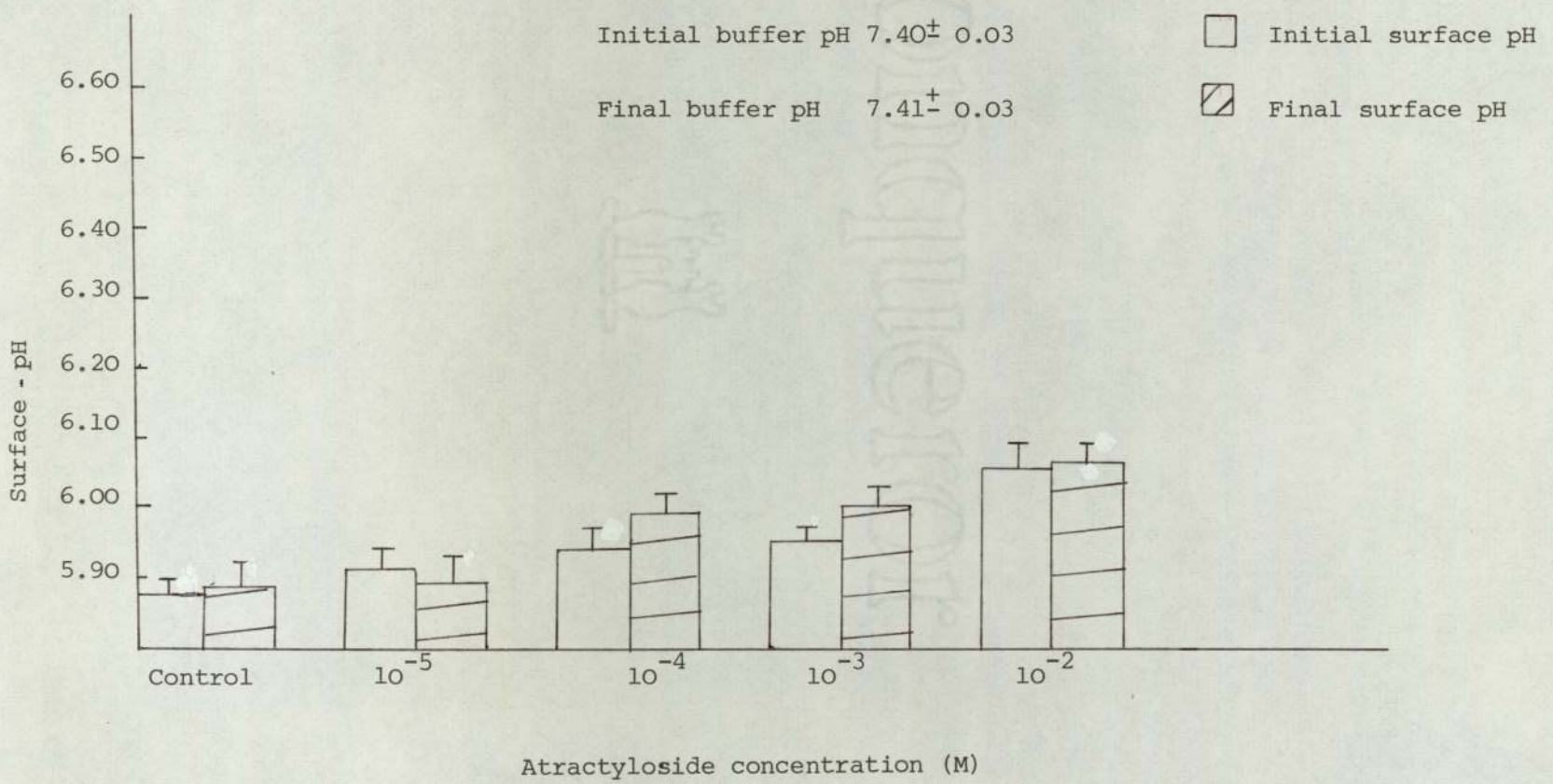


Figure 3-16: The effect of atractyloside at different concentrations on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10mM glucose at 37°C.



surface pH of rat proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose was investigated. 10^{-4} M atractyloside, a concentration shown to produce complete inhibition of adenine nucleotide translocase (Chappell and Crofts,1965), caused no significant elevation in the onset surface pH, but caused a significant elevation ($P > 0.02$) in the final surface pH (Figure 3-16). In the presence of 10^{-3} M atractyloside, there was significant elevation in the intial ($P > 0.02$) and final ($P < 0.02$) surface pH values. More elevation in the surface pH was observed at 10^{-2} M atractyloside ($p < 0.01$ for the initial and final surface pH values).

(viii) Sodium meta vanadate :-

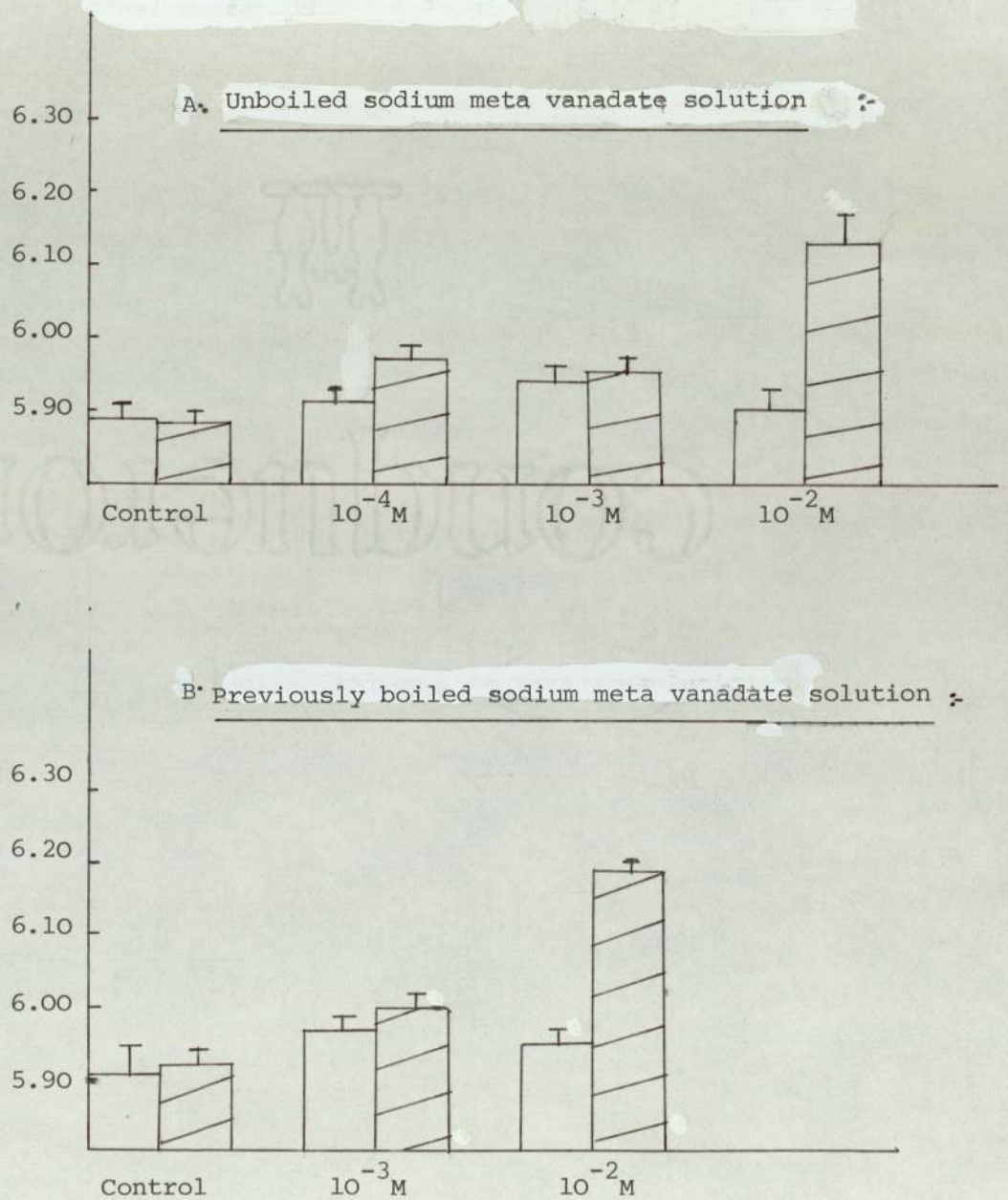
Vanadium has long been recognized as an essential trace element for normal growth. Two recent reviews (Simons, 1979; Macara, 1980) exphasize the wide variety of its effects on cellular metabolism and its possible involvement in regulation. Many enzymes were found to be inhibited by vanadium at high concentrations, these include almost all phosphohydrolases such as Na^{+}-K^{+} ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase and alkaline phosphatase (Macara, 1980). On the other hand, vanadium activates adenyl cyclase (Schwabe, Puchstein, Hannemann and Söchtig, 1979).

Vanadates at physiological pH tend to aggregate to form complexes. The dissociation rates of these polymers are very slow at room temperature and for this reason vanadate solutions should be boiled or left to stand for several days to ensure that equilibrium has been attained (Macara, 1980).

Figure 3.17:- The effect of sodium meta vanadate at different concentrations on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose, at 37°C.

Initial buffer pH 7.38±0.04 □ Initial surface pH

Final buffer pH 7.42±0.05 ▨ Final surface pH



The effect of different concentrations of sodium meta vanadate on the surface pH of rat proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose was investigated. In the presence of unboiled sodium meta vandate (10^{-4}M-10^{-2}M) no significant change in the onset surface pH was observed however, the final surface pH was significantly higher at all the three concentrations tested ($P < 0.02$, < 0.05 and < 0.001 for the 10^{-4}M, 10^{-3}M and 10^{-2}M respectively). Similar effects on the initial and final surface pH were observed when previously boiled sodium meta vanadate solution was applied (Figure 3-17).

3.3.5 THE INTESTINAL ACID MICROCLIMATE AND ITS RELATIONSHIP WITH THE ADENYL CYCLASE-PHOSPHODIESTERASE SYSTEM :-

The level of cyclic-AMP (c-AMP) in the living cells is controlled by the adenyl cyclase -phosphodiesterase system. This system has been implicated as being the possible mode of action of hormones when they exert their effects at a cellular level. The membrane bound adenyl cyclase (Murer, Ammann, Biber and Hopfer, 1976) converts ATP into c-AMP with the resultant production of inorganic pyrophosphate. The c-AMP is then converted in the cell cytoplasm into AMP by the action of phosphodiesterase (see Figure 3-18).

The level of c-AMP in the intestinal epithelial cells is thought to play a key role in regulating the absorption of water and electrolytes, i.e. it controls the permeability of the intestinal mucosal barrier (Binder, Filburn and Volpe, 1975; Coyne, Bonorris, Chung, Conley and Schoenfield, 1977; Corazza, Ciccarelli, Caciagli and Gasbarrini, 1979).

FIGURE 3-18 :-

Adenyl cyclase - phosphodiesterase system.

The formation of c-AMP.

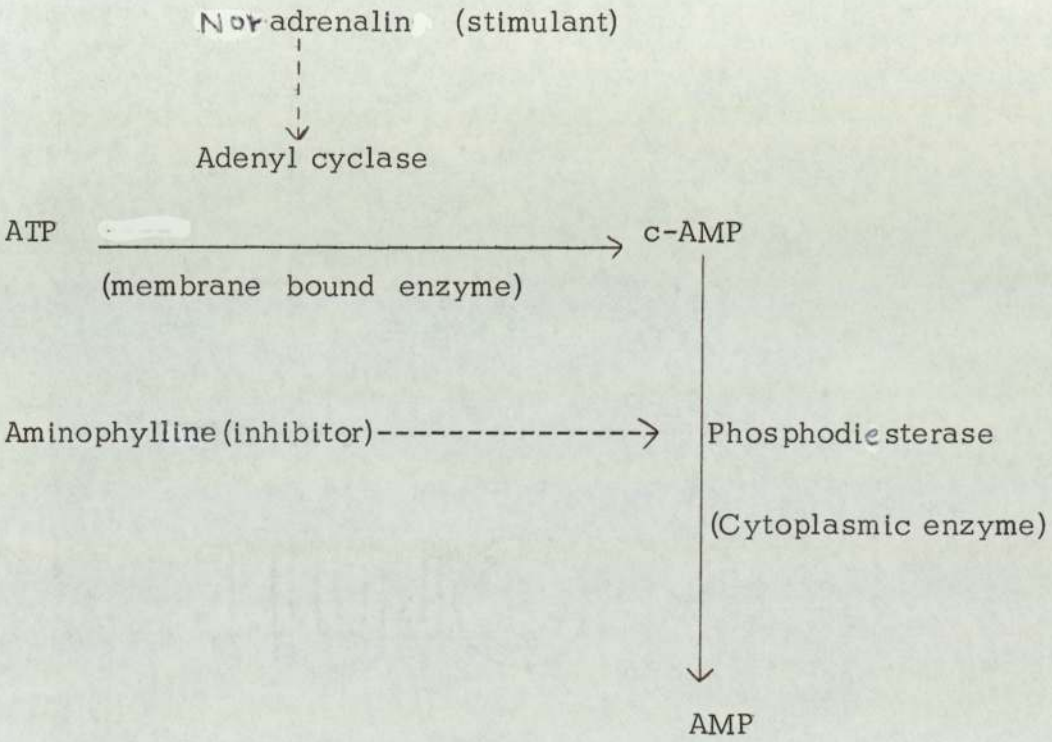


TABLE 3-8 :- The effect of different concentrations of c-AMP, dibutyryl c-AMP, nor-adrenalin and aminophylline on surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10mM glucose at 37°C.

	Surface pH		Buffer pH	
	Initial	Final	Initial	Final
Control	5.90 ± 0.03 (8)*	5.91 ± 0.03 (8)	7.40 ± 0.02 (8)	7.42 ± 002 (8)
c-AMP				
10^{-3}M	5.97 ± 0.02	5.94 ± 0.03	7.43 ± 0.01	7.42 ± 0.01
10^{-2} M	5.97 ± 0.03	5.98 ± 0.02	7.44 ± 0.01	7.42 ± 0.01
Dibutyryl c-AMP				
10^{-2}M	5.87 ± 0.03	5.86 ± 0.06	7.42 ± 0.02	7.38 ± 0.01

Continued.....

TABLE 3-8 (Continued...)

	Surface pH		Buffer pH	
	Initial	Final	Initial	Final
Noradrenalin				
10^{-2}M	5.94 ± 0.02	5.92 ± 0.02	7.42 ± 0.02	7.41 ± 0.02
Aminophylline				
10^{-2}M	5.99 ± 0.05	5.97 ± 0.05	7.41 ± 0.02	7.44 ± 0.02

* Number of animals (experiments).

The effect of increasing the level of c-AMP on the intestinal surface acidity was investigated. This was performed by measuring the surface pH of rat proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose and different concentrations of either c-AMP, dibutyryl c-AMP, noradrenalin or aminophylline. None of the compounds applied showed any significant effect on surface acidity (Table 3-8).

3.3.6 THE EFFECT OF DETERGENTS ON THE SURFACE ACIDITY, INTEGRITY AND VIABILITY OF THE SMALL INTESTINE :-

(i) The effect of detergents on surface acidity :-

The effect of physiological and synthetic detergents on the surface pH of the rat proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose was examined. The physiological detergents used were sodium deoxycholate, glycodeoxycholate and sodium cholate. The synthetic detergent used was phorbol-12-myristate-13-acetate.

In all the conditions examined, no significant change in buffer pH during incubation was observed. However, time dependent elevation in the surface pH was observed when high concentrations of the detergents were used.

The unconjugated dihydroxy bile acid sodium deoxycholate caused an elevation in the onset and final surface pH as the concentration of the detergent increased. The elevation became significant at 5×10^{-5} M

Figure 3-19:- The effect of sodium deoxycholate at different concentrations on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose, at 37°C.

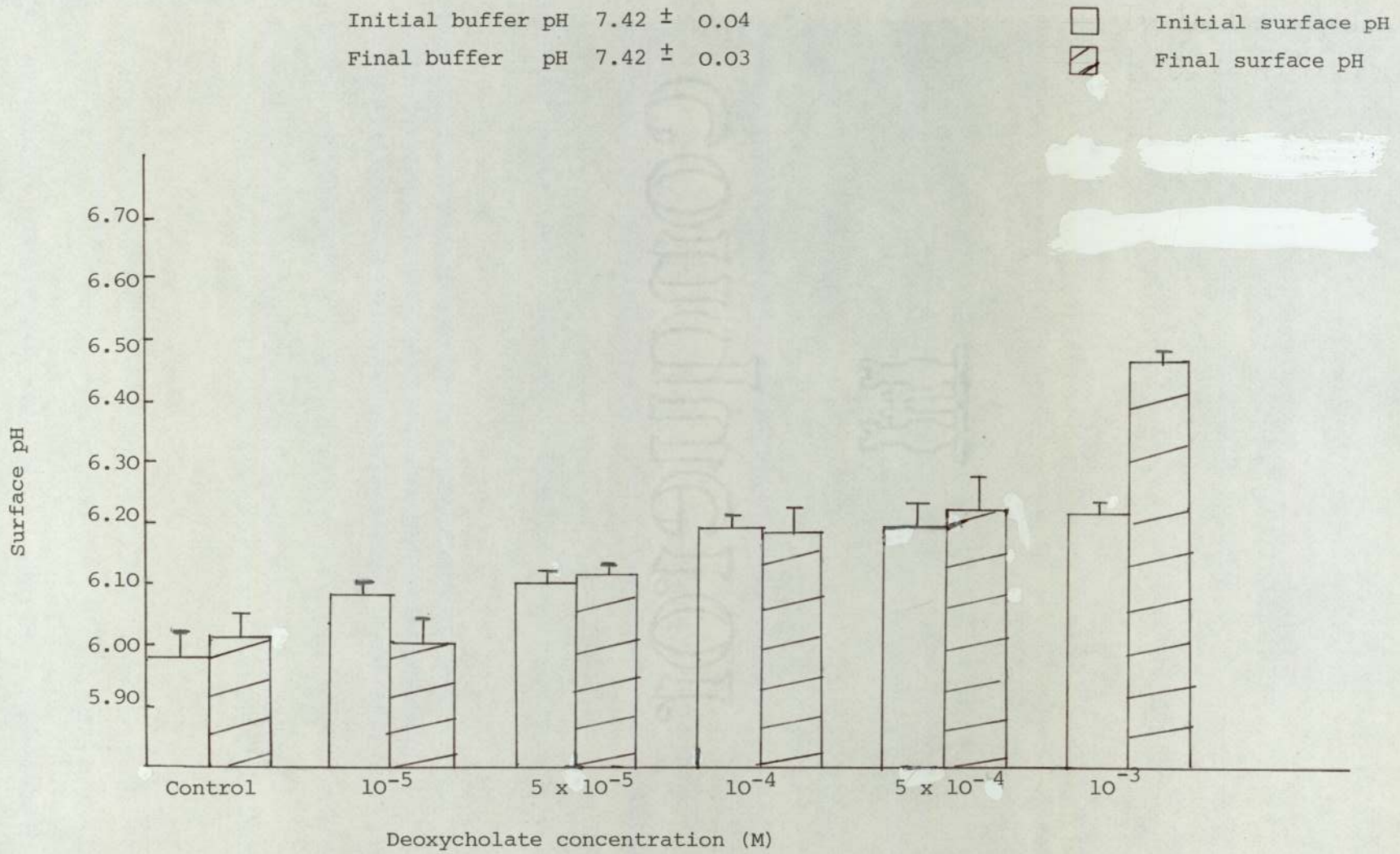


Figure 3-20:- The effect of glycodeoxycholic acid at different concentrations on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10mM glucose at 37°C.

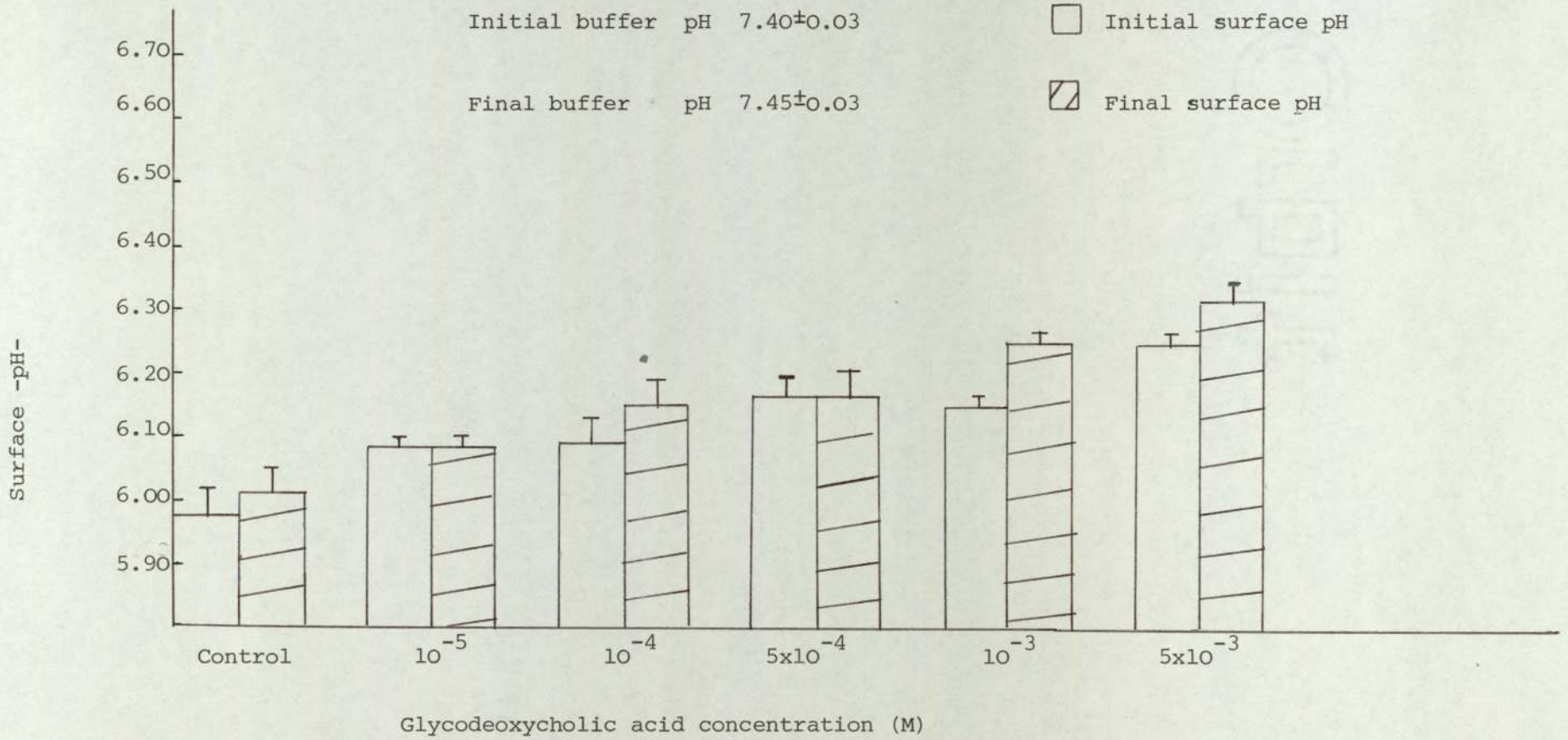


Figure 3-21: The effect of cholic acid at different concentrations on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose, at 37°C.

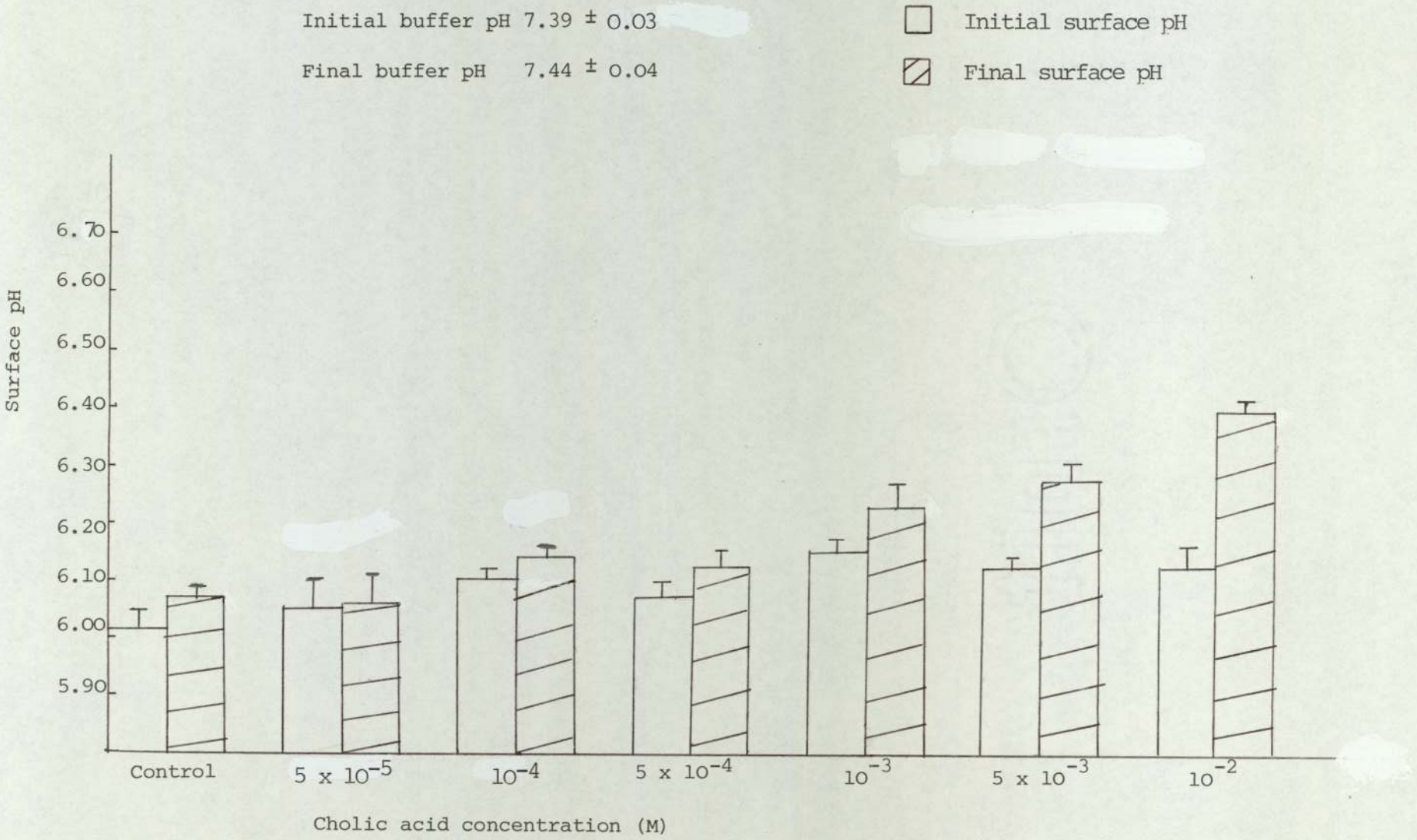


TABLE 3-9:

The effect of 10^{-4} M phorbol-12-myristate-13-acetate on the surface pH of the proximal jejunum incubated in bicarbonate buffer containing 10 mM glucose, at 37°C.

	Surface pH		Buffer pH	
	Initial	Final	Initial	Final
Control	5.91 ± 0.04	5.92 ± 0.02	7.43 ± 0.02	7.45 ± 0.02
10^{-4} M of the detergent	5.97 ± 0.06	5.96 ± 0.02	7.45 ± 0.02	7.42 ± 0.02

deoxycholate ($P < 0.05$ for both the initial and the final surface pH) (Figure 3-19).

Similarly there was an elevation in the onset and final surface pH values in the presence of glycodeoxycholate, which became significant at 5×10^{-4} M ($P < 0.01$ and > 0.01 for the initial and final surface pH respectively) (Figure 3-20).

The effect of sodium cholate on the surface acidity is shown in Figure 3-21 . Again, an elevation in the onset and the final surface pH was observed as the concentration of the detergent increased, which became significantly higher than that of control at 10^{-3}M ($P < 0.01$ for both the initial and final surface pH).

The presence of 10^{-4} M phorbolacetate, a synthetic detergent, caused an insignificant elevation in the surface pH compared to control values (Table 3-9).

In general the unconjugated dihydroxy bile acid, deoxycholate, was found to be more effective in elevating the surface pH than its conjugated form, glycodeoxycholate and the unconjugated trihydroxy bile acid, cholate.

(ii) <u>The effect of detergents on the integrity and viability of the intestinal preparation</u> :-

To examine the effect of the detergents on the integrity of the brush border membrane and its epithelial cells and to specify the

possible site(s) of action of the detergents, several parameters were examined, (i) the release of a brush border marker enzyme, alkaline phosphatase and a cytoplasmic marker enzyme, lactate dehydrogenase, in the incubation medium; (ii) histological examination of the tissue with periodic acid Schiff reagent and (iii) the potential difference across the tissue.

Everted sacs prepared from rat proximal jejunum were incubated in bicarbonate buffer containing 10 mM glucose and different concentrations of the most powerful physiological detergent, deoxycholate (Coleman and Holdsworth, 1976; Vyvoda, Coleman and Holdsworth, 1977).

(a) The release of marker enzymes :-

In the absence of sodium deoxycholate increasing alkaline phosphatase activity was normally detected in the incubation medium as the incubation time increased (Table 3-10A). Similar activity was determined when 10^{-5} M deoxycholate was added to the incubation medium. However, at higher concentrations of deoxycholate i.e., 10^{-4}M and 10^{-3}M there was an increase in the activity of the enzyme in the incubation medium over that of the control values.

Similarly, in the absence of sodium deoxycholate, increased lactate dehydrogenase activity was detected in the incubation medium as the incubation time increased. However, the amount was very small (Table 3-10B). A similar activity was estimated in the presence

TABLE 3-10 :- The activities of alkaline phosphatase and lactate dehydrogenase in the mucosal medium of everted sacs from the proximal jejunum incubated in bicarbonate buffer containing 10mM glucose at 37°C, in the absence and presence of different concentrations of sodium deoxycholate.

A - Alkaline phosphatase activity in I.U/g initial tissue wet weight/10 ml of mucosal medium :-

Time (min)	Control	Sodium deoxycholate concentration (M) 10^{-5}	10^{-4}	10^{-3}
5	0.10 ± 0.01	0.10 ± 0.02	0.20 ± 0.02	0.30 ± 0.01
10	0.18 ± 0.01	0.20 ± 0.02	0.30 ± 0.02	0.50 ± 0.10
15	0.30 ± 0.02	0.30 ± 0.10	0.40 ± 0.03	1.00 ± 0.10
20	0.40 ± 0.03	0.40 ± 0.10	0.50 ± 0.05	1.50 ± 0.10
25	0.50 ± 0.10	0.50 ± 0.10	0.70 ± 0.10	2.30 ± 0.20
30	0.60 ± 0.03	0.70 ± 0.10	0.90 ± 0.10	2.80 ± 0.30
60	1.10 ± 0.05	1.20 ± 0.10	1.60 ± 0.20	3.30 ± 0.30

B - Lactate dehydrogenase activity in I.U./g initial tissue wet weight/10 ml of mucosal medium:-

5	3.80 ± 0.08	5.00 ± 0.50	4.0 ± 0.80	6.30 ± 0.50
10	7.50 ± 0.40	8.30 ± 0.80	8.0 ± 0.80	13.30 ± 0.60
15	10.70 ± 1.0	10.30 ± 1.00	10.80 ± 0.50	20.80 ± 0.80
20	15.70 ± 0.83	15.00 ± 1.20	13.30 ± 2.50	30.80 ± 1.30
25	20.0 ± 1.0	18.8 ± 1.30	19.50 ± 2.70	43.30 ± 1.50
30	23.30 ± 0.67	20.4 ± 1.20	22.70 ± 2.0	50.0 ± 1.0
60	33.30 ± 3.30	33.20 ± 1.30	33.30 ± 2.0	84.0 ± 2.80

TABLE 3 - 10 (Continued....)

C - The percentage inhibitory effect of 10^{-3} M sodium deoxycholate on the activity of alkaline phosphatase and lactate dehydrogenase :-

Enzyme	Incubation time (min.)	% Inhibition
Alkaline phosphatase	30	0
	60	0
Lactate dehydrogenase	30	0
	60	15

of 10^{-5}M and 10^{-4}M sodium deoxycholate. In contrast, 10^{-3}M sodium deoxycholate caused an increase in the enzyme activity over that of the control, after only 5 minutes incubation.

The possible inhibition of the enzymes by sodium deoxycholate was investigated by measuring the activity of the same amounts (units) of enzyme in the presence and absence of 10^{-3}M sodium deoxycholate. The degree of inhibition was expressed as a percentage. Sodium deoxycholate (10^{-3}M) was found not to inhibit either alkaline phosphatase or lactate dehydrogenase (Table 3-10C).

(b) Histological studies :-

Everted sacs from rat proximal jejunum were incubated for either 5 or 30 minutes in bicarbonate buffer containing 20 mM glucose and different concentrations of sodium deoxycholate at 37°C.

No obvious difference was noticed between the appearance of the control preparation and that after incubation for 30 minutes with 10^{-5}M sodium deoxycholate (see plates 5 and 6 respectively). Similarly, intact villi were observed after incubating everted sacs with 10^{-4}M sodium deoxycholate for 5 and 30 minutes (plates 7 and 8 respectively). On the other hand, incubating everted sacs with 10^{-3}M sodium deoxycholate for 5 minutes caused clear damage to the tip of the villi, while the side of the villi were less damaged (plates 9 and 10). Extending the incubation time to 30 minutes with 10^{-3}M sodium deoxycholate caused severe distruction to the villi (plates 11 and 12).

PLATE 5 — Rat proximal jejunal villi of everted sac after 30 minutes incubation in bicarbonate buffer containing 20 mM glucose at 37°C. ++

PLATE 6 — Rat proximal jejunal villi of everted sac after 30 minutes incubation in bicarbonate buffer containing 10^{-5}M sodium deoxycholate and 20 mM glucose at 37°C. ++

PLATE 7 — Rat proximal jejunal villi of everted sac after 5 minutes incubation in bicarbonate buffer containing 10^{-4}M sodium deoxycholate and 20mM glucose at 37°C. ++

PLATE 8 — Rat proximal jejunal villi of everted sac after 30 minutes incubation in bicarbonate buffer containing 10^{-4}M sodium deoxycholate and 20mM glucose at 37°C. ++

PLATE 9 — Rat proximal jejunal villi of everted sac after 5 minutes incubation in bicarbonate buffer containing 10^{-3}M sodium deoxycholate and 20 mM glucose at 37°C. +

PLATE 10 — Rat proximal jejunal villi of everted sac after 5 minutes incubation in bicarbonate buffer containing 10^{-3}M sodium deoxycholate and 20 mM glucose at 37°C. ++

PLATE 11

Rat proximal jejunal villi of everted sac after 30 minutes incubation in bicarbonate buffer containing 10^{-3}M sodium deoxycholate and 20 mM glucose at 37°C. +

PLATE 12

Rat proximal jejunal villi of everted sac after 30 minutes incubation in bicarbonate buffer containing 10^{-3}M sodium deoxycholate and 20 mM glucose at 37°C. ++

+ Magnification X 125

++ Magnification X 312

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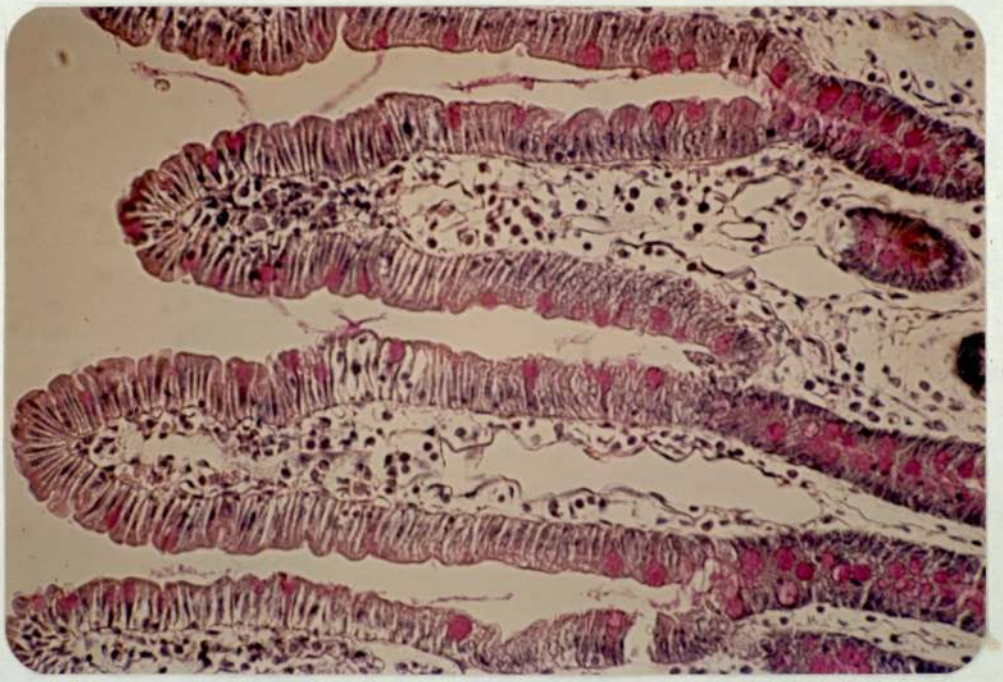

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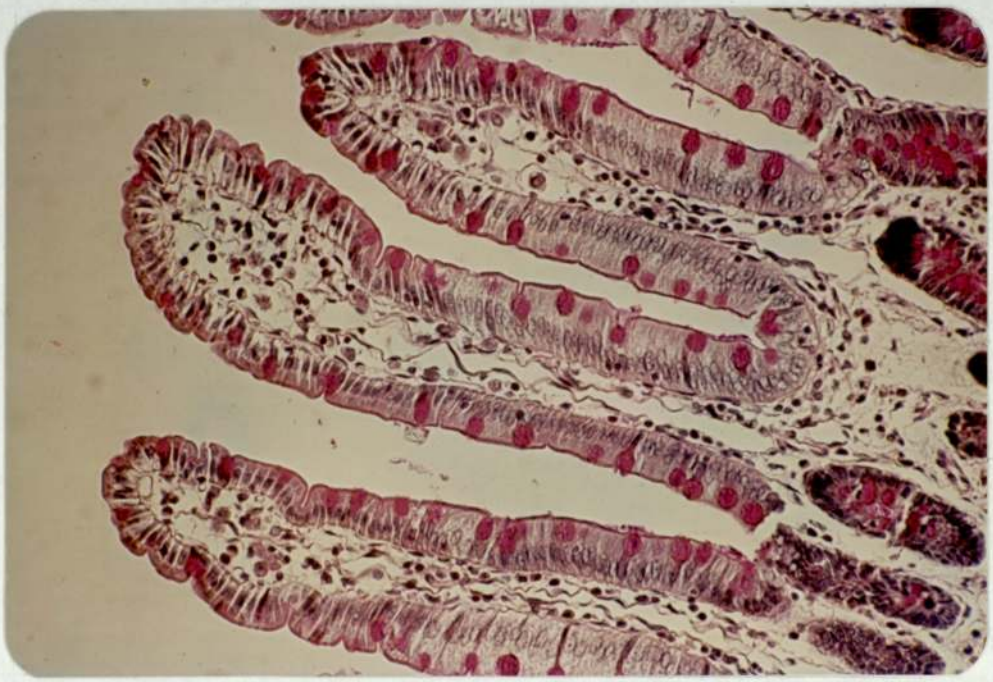

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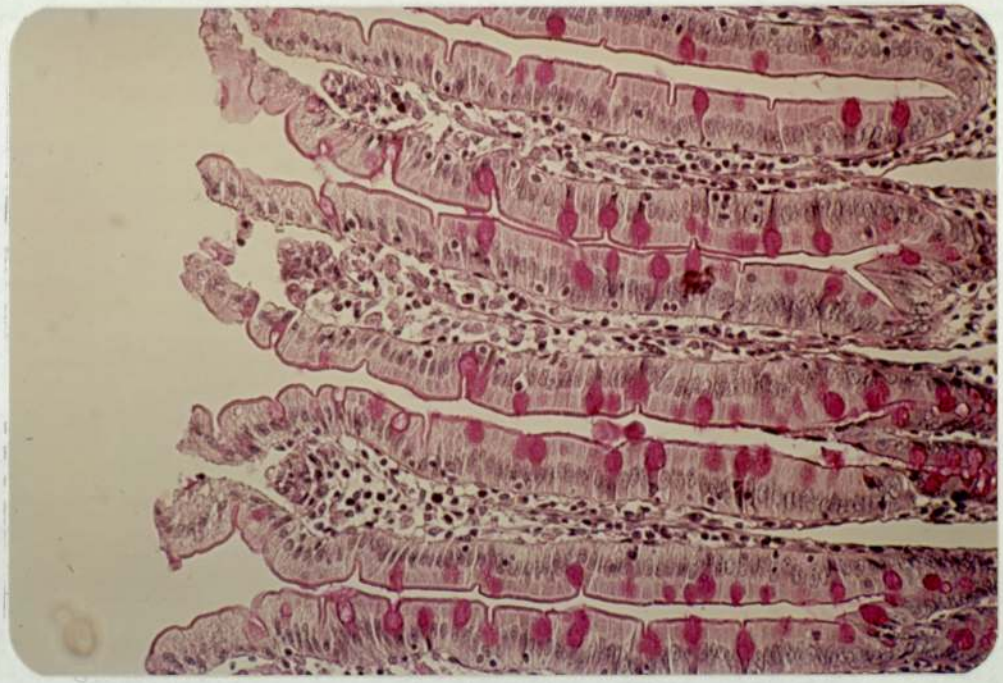

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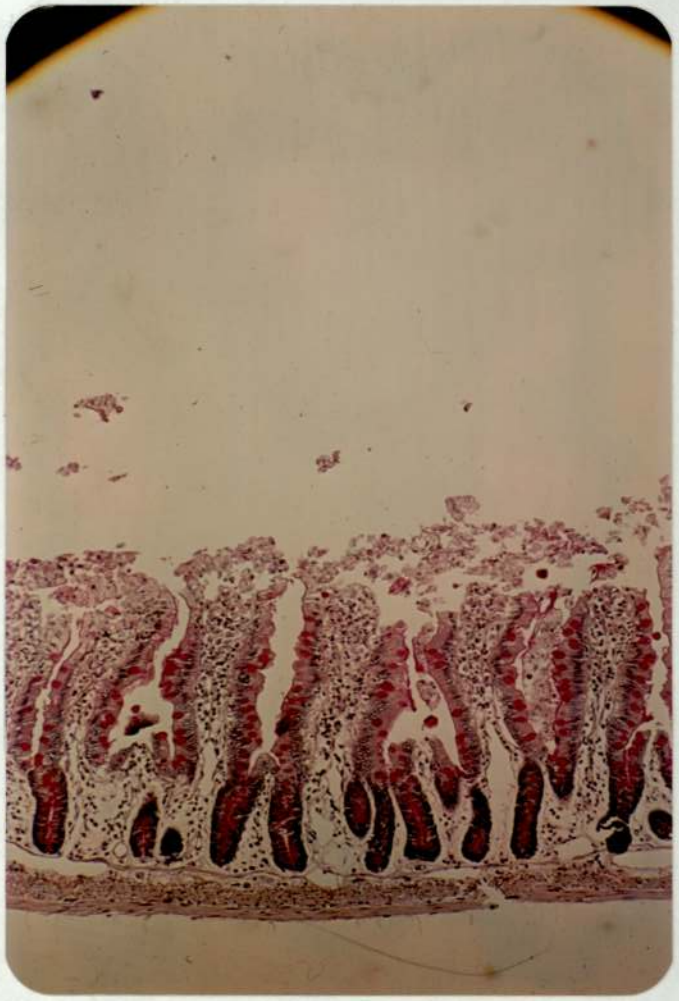

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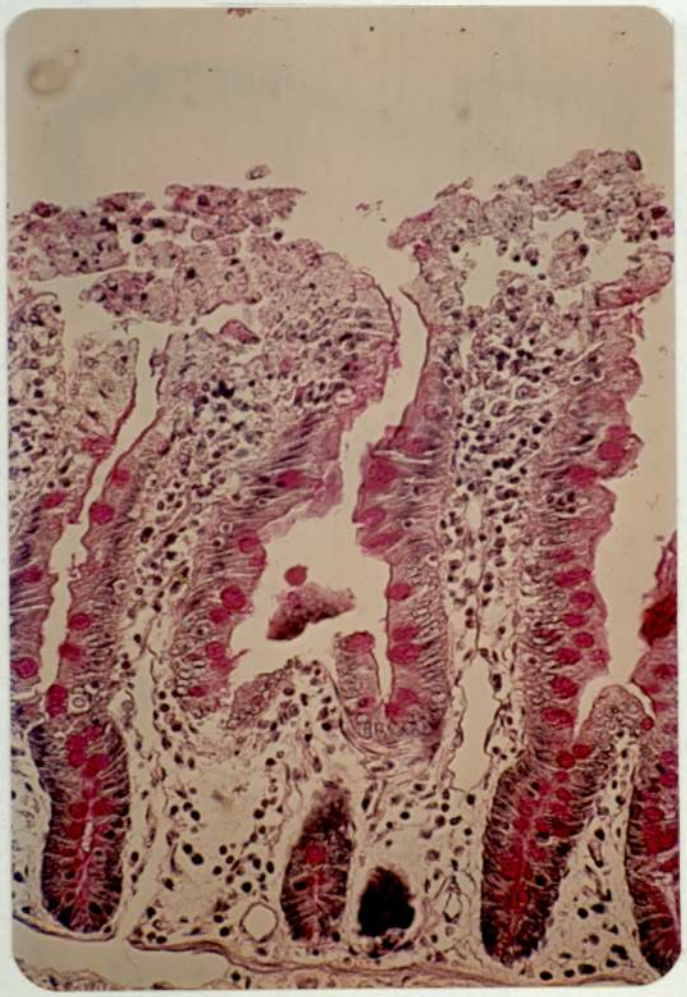

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(c) The effect of detergents on the transmural potential difference :-

The effect of sodium deoxycholate (10^{-5} - 10^{-3}M) on the transmural potential difference across the wall of everted sacs prepared from the proximal jejunum, distal jejunum and proximal ileum and incubated in bicarbonate buffer containing 20 mM glucose at 37°C was investigated.

In all the regions examined, the everted sac preparations were found to be able to support a considerable and steady potential difference in the presence of 10^{-5} M and 10^{-4} M deoxycholate, although it was lower than that of the control (Figure 3-22). On the other hand, 10^{-3} M sodium deoxycholate caused a severe and rapid decline in the potential difference.

The release of marker enzymes, the potential difference data and the histology results taken together suggested that the in vitro preparation was viable in the presence of 10^{-5}M and 10^{-4}M sodium deoxycholate while it had lost most of its viability characteristics after approximately 5-8 minutes in the presence of 10^{-3}M sodium deoxycholate.

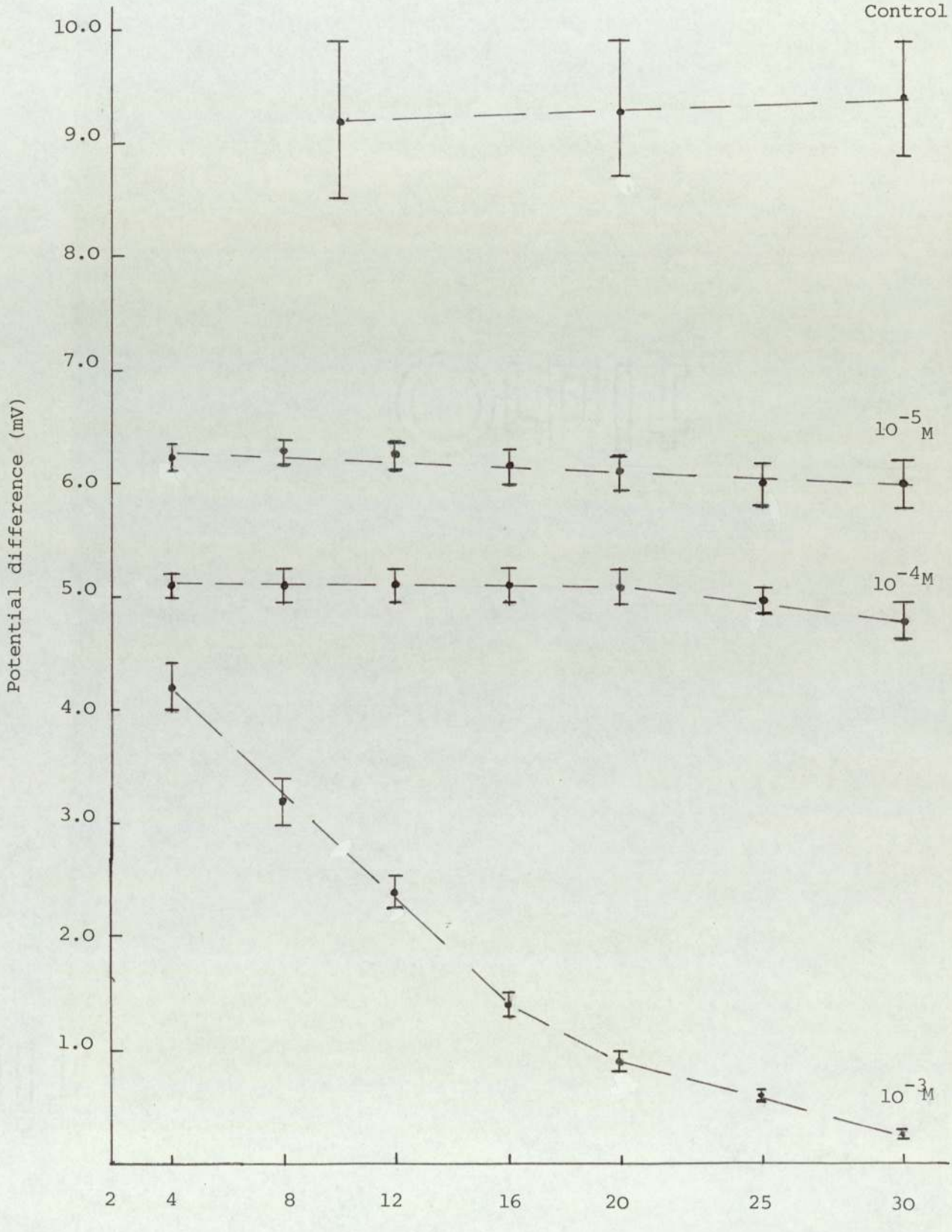
3.3.7 RAT STOMACH SURFACE pH MEASUREMENTS :-

(i) Stomach surface pH and the effect of glucose :-

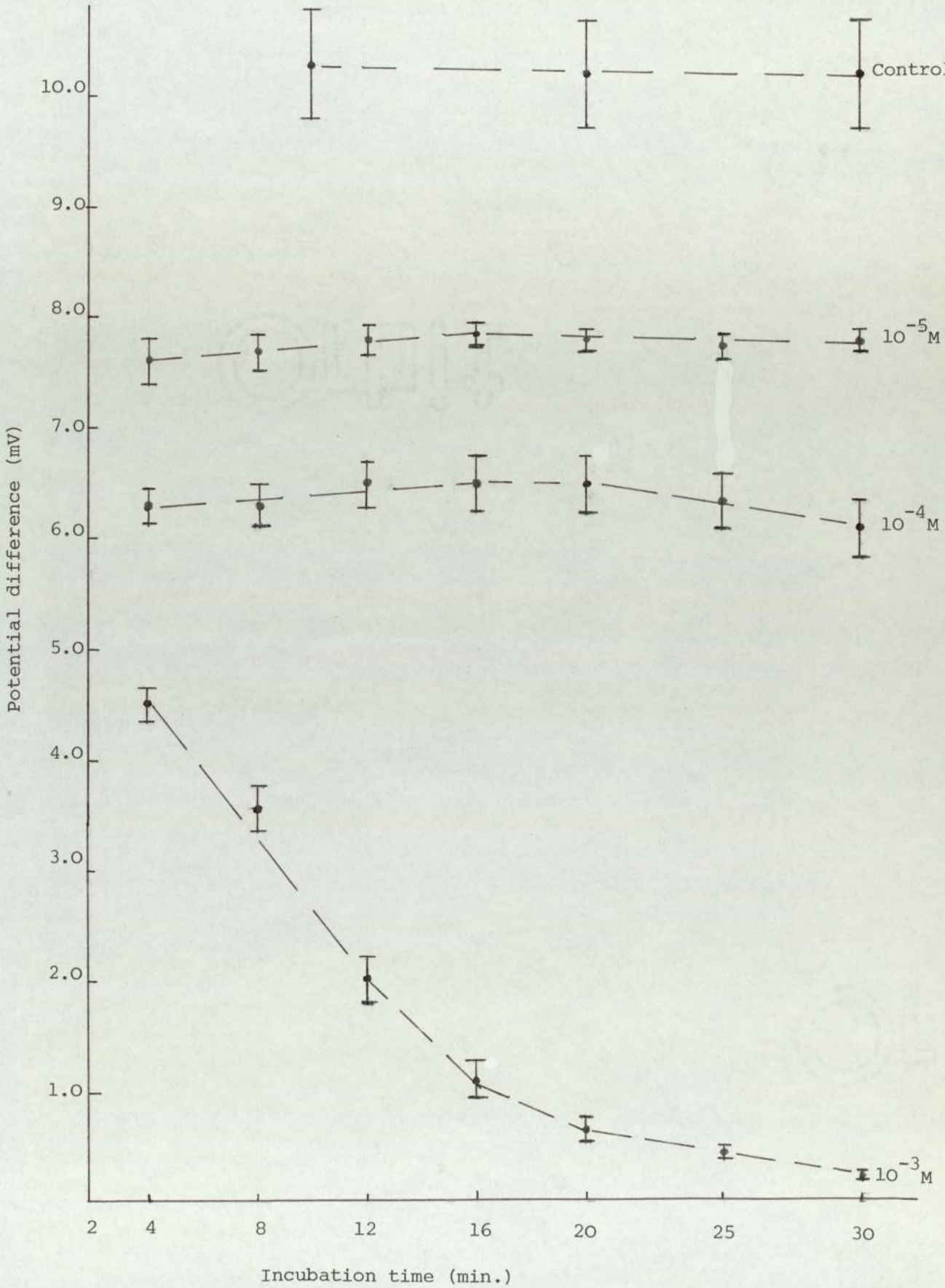
Stomach surface pH was measured in Krebs-Ringer phosphate buffer pH 3 in the presence and absence of 10 mM glucose. Typical successive recordings of stomach surface and buffer pH measurements

FIGURE 3-22 :- The effect of sodium deoxycholate at different concentrations on the potential difference (mV.) across different regions of the small intestine. Measurements were carried out in bicarbonate buffer containing 20 mM glucose, at 37°C.

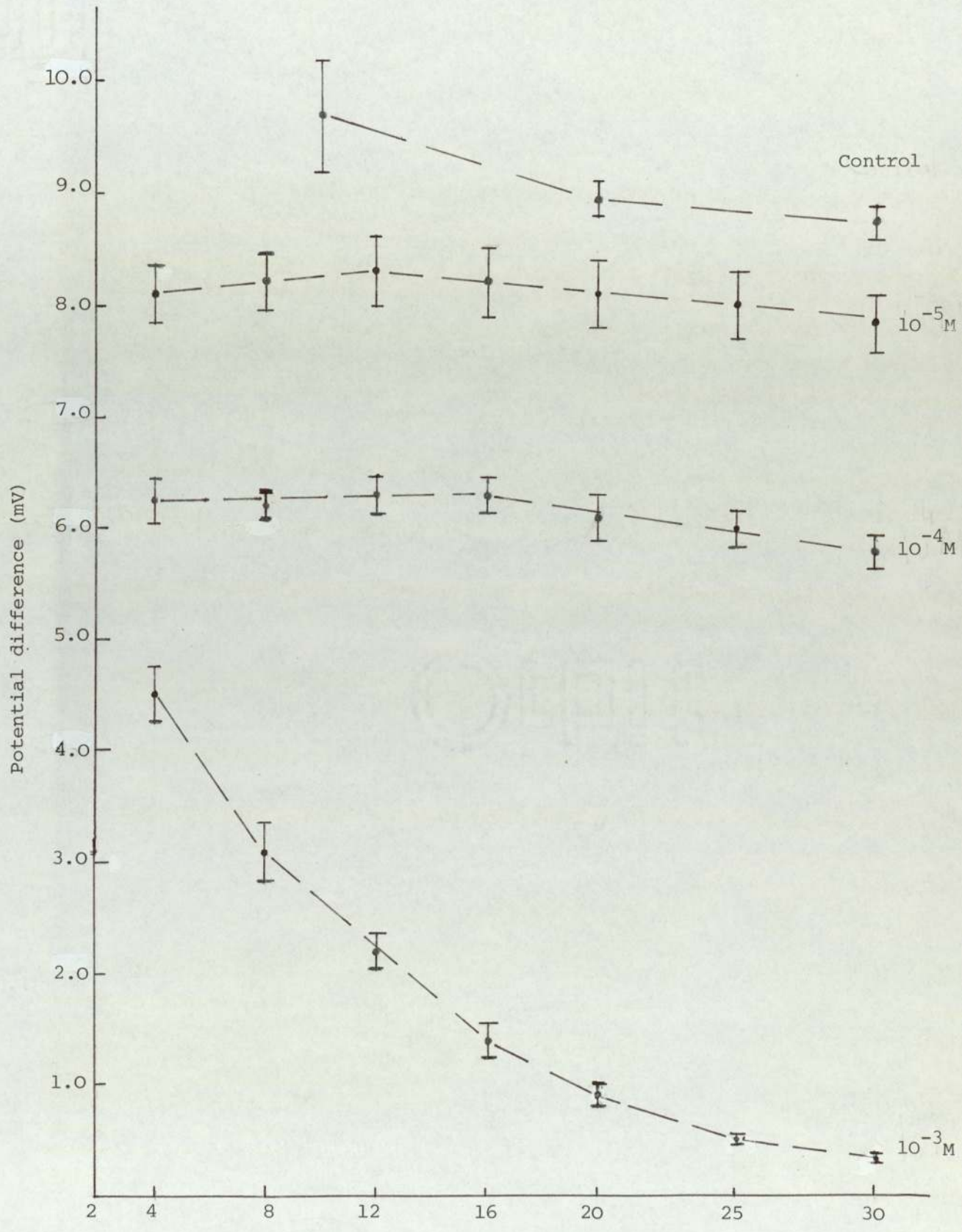
A-Proximal Jejunum :-



B. Distal jejunum :-



C. Proximal ileum:-



at the onset and the end of incubation in the presence of 10 mM glucose are shown in Table 3.11.

In the presence of glucose the stomach surface pH both at the onset and the end of incubation was much higher than the buffer pH. There was no significant change in the surface pH during incubation (Table 3-12). On the other hand, in the absence of glucose although the surface pH was higher than that of the incubation medium, it was significantly less than that observed in the presence of glucose both at the onset ($P < 0.05$) and the end ($P < 0.001$) of incubation. Also a significant decrease ($P > 0.001$) in the surface pH during incubation was observed. In both cases, no change in buffer pH during incubation was observed.

The results demonstrate the existence of an alkaline microclimate relative to the lumen, covering the rat stomach surface in vitro and that glucose is important for its creation and maintenance. A similar less acidic surface microclimate has been observed in rabbit stomach in vitro (Williams and Turnberg, 1980) and in rat stomach in vivo (Ross, Bahari and Turnberg, 1980).

(ii) The influence of buffer pH on the stomach surface pH :-

Stomach surface pH was measured in Krebs-Ringer phosphate buffer adjusted to either pH 1.8, 3.0, 3.5, 5.2, or 7.0 in the presence of 10 mM glucose.

In all the cases examined no change in surface pH during incub-

TABLE 3-11 : Typical successive recordings of stomach surface and buffer pH measurements at the onset and the end of incubation in Krebs-Ringer phosphate buffer containing 10 mM glucose at 37°C.

	Incubation time (min)	
	t_0	t_{30}
Buffer pH	2.99	3.05
Surface pH	3.20	3.50
	3.90	4.30
	4.45	4.58
	4.61	4.60
	4.62	
Buffer pH	3.00	3.04

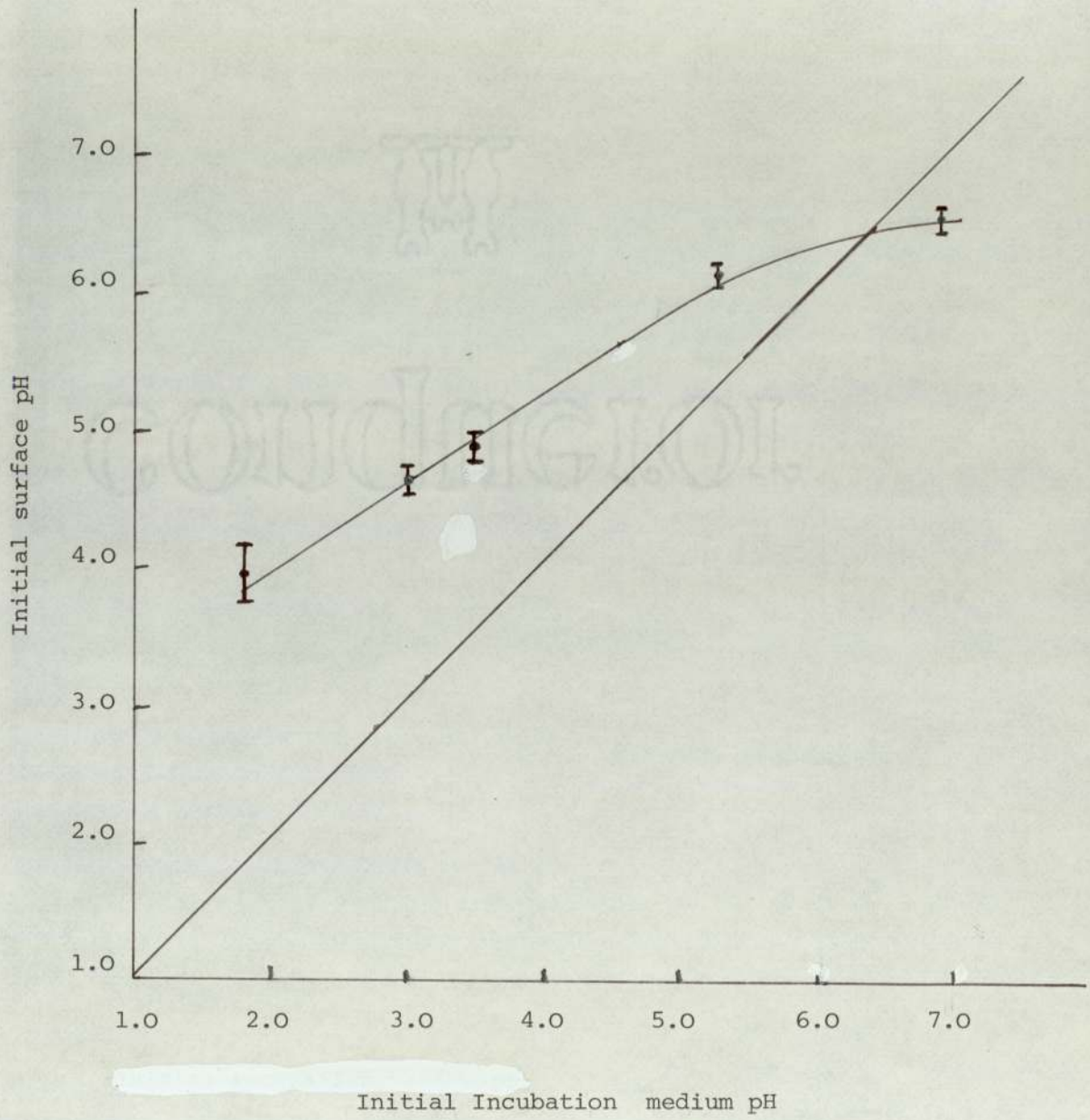
TABLE 3-12: The effect of the presence and absence of 10 mM glucose on the rat stomach surface pH measured in Krebs-Ringer phosphate buffer at 37°C.

Glucose Concentration (mM)	Surface pH		Buffer pH	
	Initial	Final	Initial	Final
10	4.63 ± 0.10	4.60 ± 0.10	2.99 ± 0.02	3.06 ± 0.03
0	4.26 ± 0.10	3.67 ± 0.05	2.96 ± 0.02	3.01 ± 0.03

Table 3-13 :- Rat stomach surface pH measured in Krebs-Ringer phosphate buffer adjusted to different pH values and containing 10 mM glucose, at 37°C.

Surface pH		Buffer pH	
Initial	Final	Initial	Final
3.97 ± 0.20	3.80 ± 0.20	1.80 ± 0.03	1.90 ± 0.03
4.63 ± 0.10	4.60 ± 0.10	2.99 ± 0.02	3.06 ± 0.03
4.90 ± 0.10	4.94 ± 0.13	3.50 ± 0.01	3.70 ± 0.05
6.14 ± 0.07	6.00 ± 0.09	5.20 ± 0.02	5.30 ± 0.03
6.54 ± 0.05	6.60 ± 0.04	6.90 ± 0.01	6.90 ± 0.01

Figure 3.23:- The relationship between rat stomach initial surface pH and the pH of the incubation medium. Measurements were performed in Krebs-Ringer phosphate buffer containing 10mM glucose at 37°C.



ation was observed (Table 3-13). At buffer pH 6.9 significantly more acidic initial ($P < 0.001$) and final ($P < 0.001$) surface pH values were observed (Table 3-13 and Figure 3-23). When the pH of the incubation medium was adjusted to more acidic values (5.3, 3.5, 3.0 or 1.8) the onset and final surface pH values were relatively more alkaline. The equilibrium pH, when the bulk and surface have the same pH value was estimated to be approximately pH 6.0-6.6.

3.4. DISCUSSION :

3.4.1. GENERAL OBSERVATIONS :

From the previously described experiments, it may be concluded that an acid microclimate at the surface of the small intestine is not created by a moment of transient anoxia in preparation, as the tissue was shown to be viable as assessed by a number of criteria. Further, immersion of the tissue in oxygenated buffer containing glucose did not restore a neutral pH at the tissue surface. Finally, if the absence of substrate and loss of cellular function during incubation *in vitro* was responsible for the creation of an acid surface pH one would expect the surface pH to become more acidic in the absence of glucose, this was not the case. The demonstration of the surface acidity *in vivo* excludes the possibility that the lack of blood supply is the cause of the observed surface microclimate *in vitro*. In conclusion, the data suggest that the surface acid microclimate is a real and normal physiological phenomena and not an artefact or a characteristic of the *in vitro* conditions.

3.4.2. THE EFFECT OF GLUCOSE AND OTHER SACCHARIDES ON SURFACE pH :-

Glucose was found to be essential for the maintenance of surface acidity. Increasing the concentration of glucose in the incubation medium up to 10mM, was associated with a decrease in the surface pH. The maximum surface acidity was measured in the presence of 10mM glucose. At higher concentration of glucose there was no increase in the surface acidity. In the absence of glucose, the surface became significantly more alkaline although lower than the buffer pH. The fact that surface acidity, albeit small, exists when glucose is absent from the incubation medium, suggests that the endogenous tissue substrates are responsible for this phenomena. This suggestion is supported by the observations that addition of iodoacetate or sodium fluoride to a glucose free medium neutralizes the surface acidity further (Figures 3-12 and 3-14). It also suggests that the surface microclimate *in vitro* has a mucosal glucose-dependent component and a mucosal glucose-independent component.

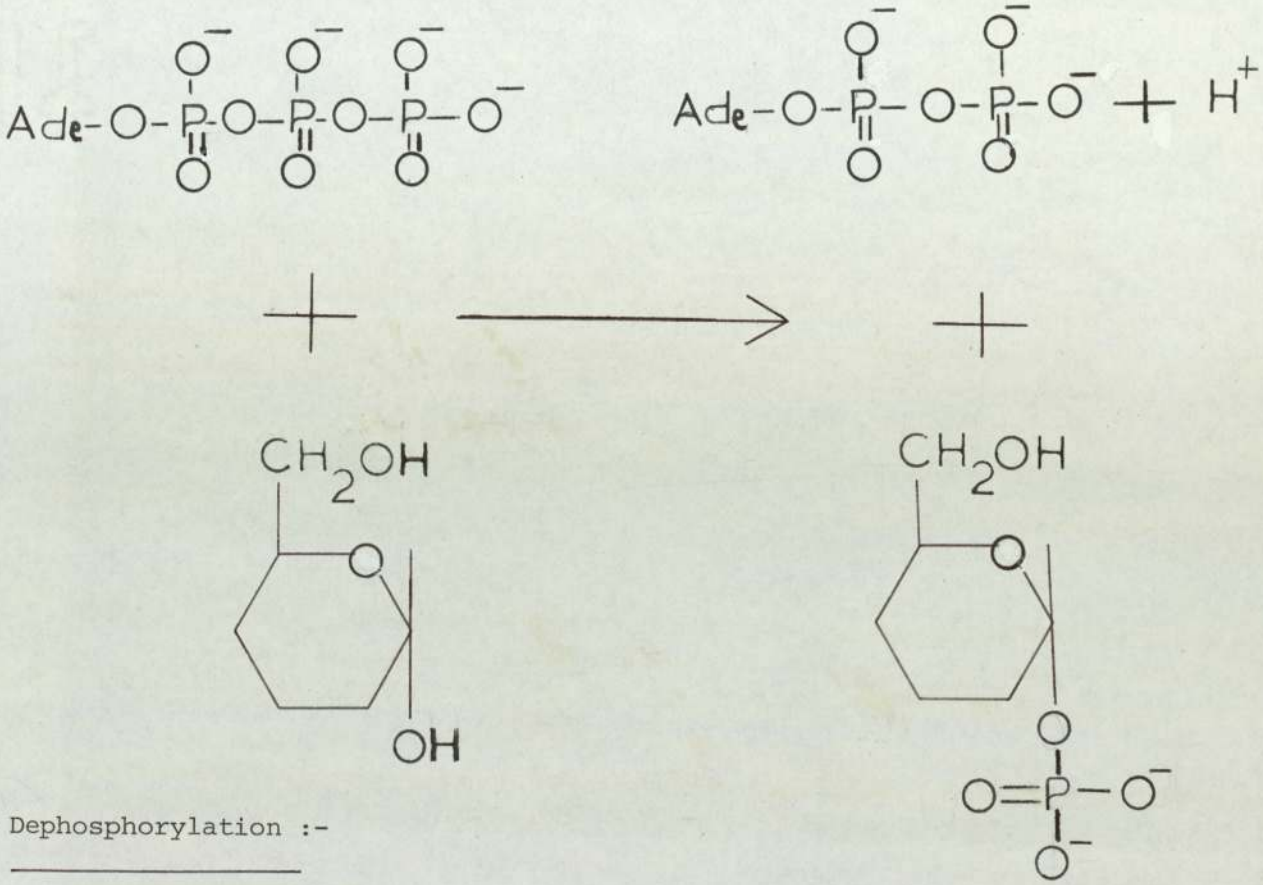
The effect of glucose on surface acidity is either related to its active transport mechanism or to its intracellular metabolism subsequent to transport. Two main theories have been advanced to explain active transport of glucose. Wilbrandt and Laszt's (1933) suggested a phosphorylation of glucose on the luminal side of the brush border membrane and a subsequent dephosphorylation on transfer across the membrane into the epithelial cell. Hydrogen ions would be produced if glucose

was phosphorylated at the brush border membrane of the epithelial cells (Figure 3-24). The alternative theory, which is widely accepted today, was proposed by Crane (1965) and is known as the sodium gradient theory. It suggests that a link exists between glucose transport and active sodium transfer. Hydrogen ions would be available at the surface if some obligatory cation exchange of proton for sodium ion took place. $Na^+ : H^+$ ion exchange process has been suggested to occur in the small intestine (Turnberg, Fordtran, Carter and Rector, 1970; Turnberg, 1978).

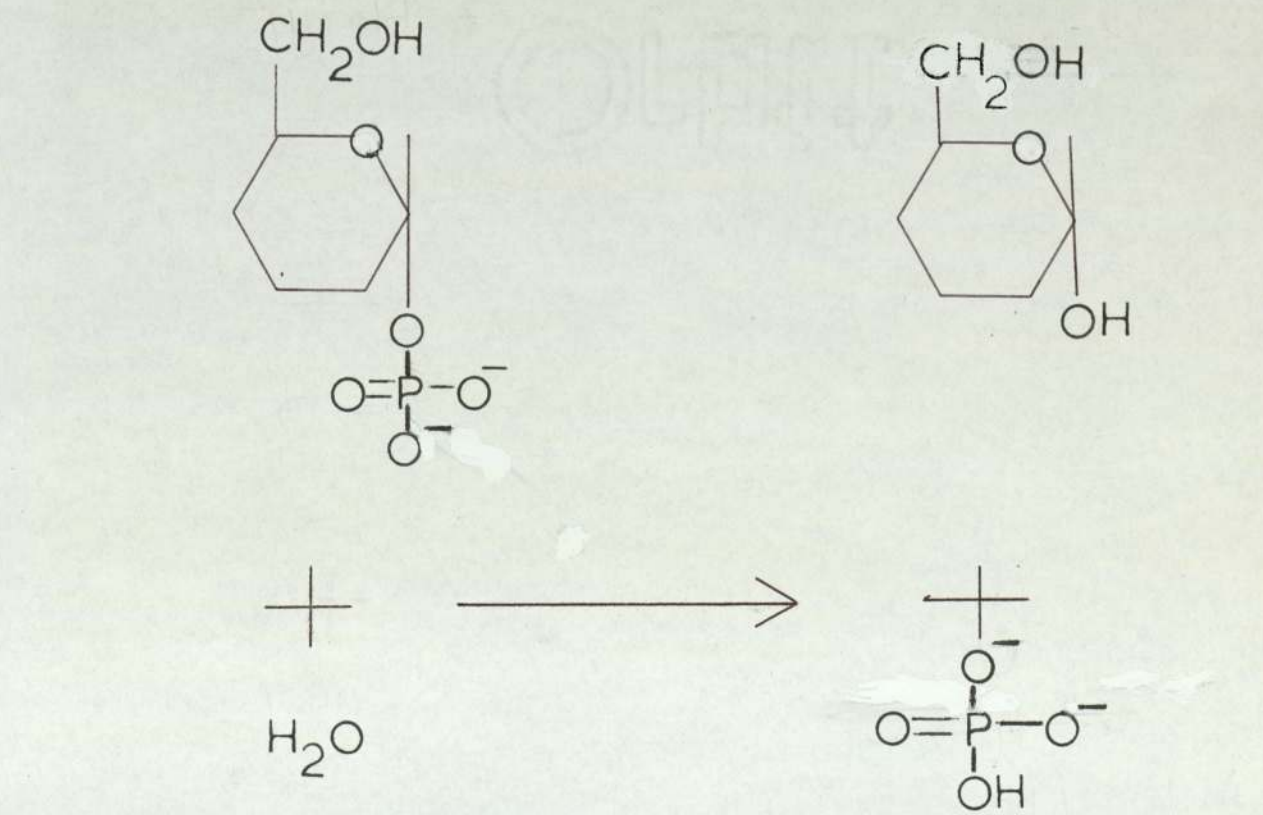
Glucose transport appeared to have no role in maintaining the surface acid microclimate. This was demonstrated when it was found that addition of glucose at concentrations above 10mM produced no further decrease in surface pH (Figure 3-3) while glucose transport continues in a linear fashion and is not saturable until much higher concentrations than 10mM (Atkinson, Parsons and Smyth, 1957). Secondly, galactose the unmetabolisable hexose of the small intestine (Goldner, Sarrocino and Estep, 1977) although actively transported by a common carrier with glucose (Crane 1960, McMichael, 1975) failed to support the acidity of the surface. Thirdly, mannose a passively transported hexose (Wilson and Vincent, 1955) which can be metabolised by the small intestine (Hele, 1953) and fructose a metabolisable hexose (Wilson and Vincent 1955; Fridhandler and Quastel, 1955) for which some workers have proposed specific active transport system (Gracey, Burke and Oshin, 1970) were found to support a considerable

Figure 3-24: Wilbrandt and Laszt's model (1933) of glucose transport.

a - Phosphorylation :-



b- Dephosphorylation :-



surface acidity.

The more alkaline surface pH observed when 10mM mannose or fructose was added as a substrate compared to that observed with 10mM glucose is possibly because comparatively little mannose enters the epithelial cells (Duerdoth, Newey, Sanford and Smyth, 1964) and that the rate of metabolism of fructose is slow compared to that of glucose (Sherratt, 1968).

The disaccharides maltose and sucrose which can be enzymically hydrolysed by the small intestine and give metabolisable subunits (Deren, 1968) also support a low surface pH. The more alkaline surface pH observed in the presence of 10mM sucrose is possibly due to its limited rate of hydrolysis. On the other hand, the dissacharide lactose which cannot be hydrolysed by the small intestine of adult rats due to the low activity of the hydrolysing enzyme lactase (Deren, 1968) failed to support normal surface acidity.

In conclusion, the effect of glucose on the surface acidity is solely related to its metabolism inside the intestinal epithelial cells.

3.4.3. SOURCE(S) OF HYDROGEN IONS OF THE INTESTINAL SURFACE ACID MICROCLIMATE :

Several potential sources for the generation of hydrogen ions for the acid microclimate have been suggested; lactic acid (Wilson 1953, 1954; Wilson and Kazyak, 1957), carbon dioxide (Turnberg, Fordtran, Carter and Rector, 1970; Turnberg, 1978) and ATP (Blair and Matty 1974).

Lactic acid production in the epithelial cells through anaerobic glycolysis and a subsequent preferential migration of hydrogen ions mucosally and lactate anions serosally, together with the serosal movement of bicarbonate anions is not the mechanism involved in the creation of the surface acid microclimate. This is because:-

(i) Lactic acid production only accounts for a small percentage of the total amount of hydrogen ions produced mucosally (Blair and Matty, 1974; Lucas and Blair, 1978).

(ii) The sign of the transmembrane potential across the brush border membrane (Barry, 1967) is unfavourable for the mucosal movement of hydrogen ion from the cell, i.e. the luminal side of the brush border membrane is positively charged.

(iii) The surface acid microclimate has been measured in a system where no spatial separation barrier exists between the mucosal and the serosal compartments (i.e. a flat strip rather than an everted sac), therefore, what is transported through the cell tissues re-enters the buffering medium. The fact that no significant change in incubation buffer pH was observed, argues against the preferential movements of bicarbonate and lactate anions serosally.

The same three points also argue against the involvement of metabolically produced carbon dioxide in the creation of hydrogen ions for the surface acid microclimate.

The third model suggested for the production of hydrogen ions is

the external hydrolysis of ATP by mucosally located ATPase. ATP is provided in high quantities inside cells through glycolysis, the citric acid cycle and oxidative phosphorylation. One glucose molecule, if it is completely metabolised produced 36 molecules of ATP.

When ATP was added to the incubation medium as a substrate instead of glucose, the surface maintained a considerable acidity (see Figure 3.8). Since ATP cannot enter the cell (Blair, Lucas and Matty, 1975; Rorive and Kleinzeller, 1972) this observation can only be explained on the basis that mucosally located ATPase is responsible for the external hydrolysis of ATP.

It might be argued that the effect of ATP is due to its ability to chelate calcium ions, (Kimizuka and Koketsu, 1962) remove them from the intestinal epithelial cell membrane and cause increase in the permeability of the cells to cations and thereby specifically cause a leakage of protons from the cell. However, several important observations argue against this proposal. Firstly, in the absence of glucose the epithelial cells have limited sources for producing hydrogen ions, and therefore, even if there is an increase in the cell permeability, it is doubtful whether the production of hydrogen ions could account for the observed surface acidity in the presence of ATP. Secondly, the addition of ATP to a glucos containing incubation medium caused no further reduction in the surface pH. Thirdly, it has been found that ethylenediaminetetracetic acid (EDTA) with its strong chelating capability has no effect on the luminal acidification of rat proximal jejunum (Blair, Lucas and Matty, 1975).

3.4.4. THE EFFECT OF METABOLIC INHIBITORS ON THE SURFACE ACIDITY :-

More insights on the nature of the relationship between the surface acid microclimate and the metabolic activities of the intestinal epithelial cells came from studying the effect of different metabolic inhibitors on the surface acidity.

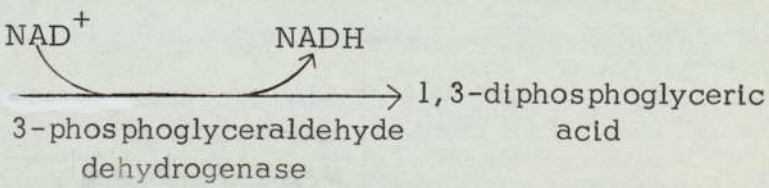
DNP is a known uncoupler of the oxidative phosphorylation (Loomis and Lipmann, 1948) which will cause a reduction or even a depletion in the intracellular levels of ATP of the intestine (Fridhandler and Quastel, 1955). The presence of DNP caused a sharp elevation in the surface pH which became more significant in the presence of higher concentrations of inhibitor (see Figure 3-11). Anoxia caused a similar elevation in the surface pH (Lucas and Blair, 1978). When ATP was added externally, in the presence of DNP, a significant decrease in the surface pH was observed. It is possible therefore that the elevation in the surface pH is due to uncoupling of oxidative-phosphorylation with a subsequent decrease in ATP production and availability at the surface. The inhibitory effect of DNP on the surface acidity and the counter effect of externally added ATP confirms that the surface acid microclimate is dependent on the availability of ATP produced through aerobic metabolism and that the external hydrolysis of ATP is the mechanism involved in the production of hydrogen ions.

The time dependent elevation in the surface pH observed in the presence of DNP is either due to the limited rate of entry of the inhibitor into the cells and then into the mitochondria or is due to an endogenous

ATP reservoir or a combination of both factors.

Anaesthetics such as ether and barbiturates have been shown to uncouple oxidative phosphorylation (Hulme and Krantz, 1955; Brody and Bain, 1954) and therefore, probably explain the observed inhibitory effect of anaesthetization on surface acidity (see Table 3-3).

Iodoacetate is an inhibitor of the aerobic metabolism of glucose and other carbohydrates to pyruvate, and the anaerobic metabolism to lactate. It blocks the glycolysis irreversably by inhibiting the enzyme 3-phosphoglyceraldehyde dehydrogenase. Iodoacetate reacts with the sulphydryl groups present in the active site of the enzyme forming a stable complex ($E\text{-}S\text{-}CH_2\text{-}COOH$) (Webb, 1966).



The inhibition of the glycolysis will subsequently lead to a decrease in the rate of oxidative phosphorylation.

The time dependent elevation in the surface pH observed in the presence of iodoacetate is either due to its limited rate of entry into the cell or is due to an endogenous ATP reservoir or a combination of both factors. The presence of iodoacetate in the incubation medium caused a sharp elevation in the surface pH which can be attributed to the reduction of ATP synthesis and subsequent availability at the surface. The effect of externally added ATP in depressing the elevation of surface

pH caused by iodoacetate confirms this suggestion.

The higher elevation caused by iodoacetate compared with equimolar DNP has several interesting implications. Assuming the rate of penetration of both inhibitors into the cell to be the same, then the surface acid microclimate appears to be dependent upon aerobic glycolysis which occurs at an exceptionally high rate in the intestinal epithelium (Weil-Malherbe, 1938). Secondly, it may indicate that 10^{-3}M iodoacetate has some other moderate inhibitory effect on some other site(s), most probably ATPase. An inhibitory effect of iodoacetate (10^{-3}M) on ATPase would explain the elevation demonstrated by the inhibitor in a glucose free incubation medium (see Figure 3-12b).

The inhibitors sodium azide and sodium fluoride, had severe inhibitory effects on surface acidity particularly at high concentrations ($> 10^{-3}$M). Azide and fluoride inhibit a variety of metallo-enzymes such as Mg^{2+}-ATPase, enolase and cytochrome oxidase by interacting with specific groups in the active site of the enzyme such as -SH, $-CO_2H$ or histidine or by removing the metal (co-factor) from the enzyme (Mahler and Cordes, 1966). However, the preferential site(s) at which these inhibitors exert their effect on the surface pH appears to be different. This is because externally added ATP caused a significant decrease in the surface pH in the presence of sodium azide (5×10^{-2}M) but had no effect in the presence of sodium fluoride (10^{-2}M). This indicates that sodium fluoride exerts relatively more of its inhibitory effect on the surface microclimate by inhibiting mucosally located ATPase. This effect of

sodium fluoride therefore, explains the inhibitory effect on the mucosal glucose-independent component of the surface acidity.

The inhibitory effect on surface acidity caused by the inhibitors hydroxylamine, atractyloside and vanadate was due to their effect on cellular metabolic activities. It further supports the existence of a direct relationship between the intracellular metabolism, mucosally located ATPase and the surface microclimate.

From the presented data it is clear that the acid microclimate is created when ATP produced intracellularly by aerobic metabolism of glucose and other substrates, is subsequently hydrolysed externally by mucosally located ATPase.

The physiological mechanism by which ATP with four negative charges crosses the negatively charged membrane of the epithelial cell is not known. However, evidence does exist that ATP may be translocated from the cytosol to the exterior of the cell (Trams, 1974; Rorive and Kleinzeller, 1972). Also the existence of nucleotide pyrophosphatase (Evans, 1974) ATPase (Ronquist and Agren, 1974; Kesavan and Noronha, 1978) and ATP requiring protein kinases (Mastro and Rozengurt, 1976) on the surface of some animal cells almost make it obligatory that some mechanism for the provision of external ATP should exist.

3.4.5 THE EFFECT OF SODIUM AND MAGNESIUM IONS ON SURFACE ACIDITY:

Sodium ions have been found to play an important role in the absorption process of many nutrients such as glucose, amino acids and

folic acid (Crane, 1965; Rosenberg, Coleman and Rosenberg, 1965; Rose, Koch and Nahrwold, 1978). Their removal from the incubation medium caused a significant elevation in the surface pH. This elevation could be indicative of either (i) a reduction in glucose transfer, oxygen uptake and general metabolic activities of the tissue (Faust, 1962; Levin and Syme, 1975) which causes a reduction in ATP production and subsequent availability at the cell surface or alternatively, (ii) a Na^{+}:H^{+} ion-exchange mechanism in the jejunum as suggested by Turnberg, Fordtran, Carter and Rector (1970) and Turnberg (1978).

The addition of ATP to a sodium-free incubation medium caused a significant increase in the surface acidity of the tissue surface (see Figure 3-9) and indicates that sodium affects the normal metabolic status of the tissue cells and hence the surface acidity.

Magnesium is an important co-factor for the activity of many enzymes including phosphotransferase and phosphohydrolases such as the mucosally located ATPase (Lehninger, 1975; Noronha and Kesavan, 1979). The maintenance of surface acidity is sensitive to the presence of this electrolyte in the incubation buffer as a significant elevation in surface pH occurred upon its removal. However, the elevation was not as sharp as that observed in the absence of sodium possibly because the amount of Mg^{2+} present inside the cell is enough to maintain normal metabolism.

3.4.6 THE DISTRIBUTION OF THE SURFACE ACID MICROCLIMATE ALONG THE SMALL INTESTINE AND COLON :-

It has been shown that many intestinal activities decrease distally. These include (i) the acidification of luminal contents (Lucas, 1974), (ii) glycolysis and lactate production (Wilson and Wiseman, 1954b); (iii) oxygen uptake (Sherratt, 1968, Wilson and Wiseman, 1954b) the absorption of many substances such as glucose, sodium and chloride, and (v) the level of certain enzymes such as alkaline phosphatase (Lafont and Morett, 1970) and ATPase (Hanninen, Hartiala and Nurmikko, 1964).

Surface acidity was also found to vary with position along the intestine (see Figure 3-4). Maximum acidity was found at the proximal jejunum. The relatively high surface pH in the duodenum is possibly due to the wide distribution of Brunner's glands in the proximal duodenum which decrease towards the duodenal-jejunal flexure and secrete an alkaline bicarbonate-rich fluid (Florey and Harding, 1934). Distal to the duodenal jejunal flexure the limited distribution of these glands together with the high metabolic activity of the intestinal mucosa alters the surface acidity so that it renders its maximum value at the proximal jejunum. Distally a non-linear decline of surface acidity towards neutrality at the distal ileum was observed. The measured decline in the surface acidity supports the Blair and Matty (1974) model in which a non-linear decline in ATPase activity and ATP content (Hanninen, Hartiala and Nurmikko, 1964) may be correlated with the decline in

surface acidity. At the ileum, bicarbonate anions are secreted into the lumen which would tend to attenuate the surface acidity (Turnberg, Fordtran, Carter and Rector, 1970; Turnberg 1978). These observations confirm the earlier prediction of Hogben, Tocco, Brodie and Schanker (1959) of an acid microclimate with maximum acidity in the proximal jejunum which decreases down the intestine.

At the colon the surface pH is of the same order of magnitude to that of the distal ileum. This observation may be expected as the mucosa of both regions has the same activity of ATPase, the same content of ATP (Hanninen, Hartiala and Nurmikko, 1964) and a limited absorption of glucose (Cordero and Wilson, 1961; Parsons and Paterson, 1965).

3.4.7 THE SURFACE ACID MICROCLIMATE AND THE ADENYLCYCLASE-PHOSPHODIESTRASE SYSTEM :-

Neither exogenous c-AMP, dibutyryl c-AMP, noradrenalin or aminophylline showed any significant effect on the surface acidity. Therefore no relationship between the surface acid microclimate and the adenyl cyclase-phosphodiasterase system appears to exist.

Cyclic-AMP induces secretion of sodium chloride and water by inhibiting net sodium and water absorption and by inducing active transport of chloride (Field, Brasitus, Sheerin and Kimberg, 1976; Dennhardt, Lingelbach and Haberich, 1979; Binder, Filburn and Volpe, 1975). Therefore, the experimental data further indicates that the availability

of the hydrogen ions of the acid microclimate at the surface does not involve a Na^+ : H^+ ion exchange mechanism.

3.4.8. THE EFFECT OF DETERGENTS ON THE SURFACE ACID MICROCLIMATE :

All the physiological detergents examined inhibited the surface acidity of rat proximal jejunum in a concentration dependent manner. The extent of inhibition varied with the damaging potential of the detergent and decreased in the following order, deoxycholate $>$ glyco-deoxycholate $>$ cholate (Coleman and Holdsworth, 1976; Vyvoda, Coleman and Holdsworth 1977).

Deoxycholate at its critical micellular concentration, approximately 10^{-3}M (Heaton, 1972), caused a considerable decrease in the surface acidity of the intestinal preparation. However, the tissue had lost most of the characteristics associated with a viable in vitro preparation.

The decrease in the potential difference observed in the presence of 10^{-5}M and 10^{-4}M deoxycholate is possibly related to the increase in the intracellular c-AMP level which subsequently leads to an alteration in electrolyte movement, particularly that of sodium (Binder, Filburn and Volpe, 1975; Coyne, Bonorris, Chung, Conley and Shoenfield, 1977; Salden and Harris, 1972).

In the presence of 10^{-5}M sodium deoxycholate, the tissue was viable and there was no significant increase in the release of alkaline phosphatase or lactate dehydrogenase from the preparation nor was there any structural damage to the preparation. Under these conditions, the

surface acid microclimate was not affected. However, at 10^{-4}M deoxycholate a significant elevation in surface pH was observed. The glycocalyx appeared to be slightly altered as there was an increase in the rate of release of alkaline phosphatase but not lactate dehydrogenase from the preparation. As 10^{-4}M deoxycholate has no effect on glucose absorption, oxygen uptake or oxidative phosphorylation (Salden and Harris, 1972; Faust and Liu-Wu, 1966), the elevation in surface pH could only be explained by the effect of the detergent on the glycocalyx. Alteration to the structural integrity of the glycocalyx, external to the epithelial cell, will lead to a more rapid diffusion of hydrogen ions away from the surface and into the lumen. Hence the acid microclimate becomes less acidic.

3.4.9 RAT STOMACH SURFACE pH :-

A microclimate with a relatively higher pH exists on the surface of rat stomach despite excessive luminal acidification (see Figure 3-23 and Table 3-13). The surface pH of the stomach was less acidic than that of rat proximal jejunum when both were measured in Krebs-Ringer phosphate buffer pH 7. Glucose is an important requirement for the creation and the maintenance of this stomach surface microclimate.

It has been suggested that active secretion of bicarbonate anions from the stomach epithelial cells is the mechanism responsible for the creation of this surface microclimate (Flemström, 1977, Garner and Flemström, Allen and Garner, 1980). The mechanism suggested is one in which the stomach mucus slows down both the rate of diffusion of bicarbonate

anions away from the surface into the lumen, and the back diffusion of hydrogen ions from the lumen into the surface (Allen and Garner, 1980; Williams and Turnberg, 1980; Ross, Bahari and Turnberg, 1980). Agents which interfere with the mucus structure and integrity, or with bicarbonate production and secretion would abolish this micro-climate at the stomach surface (Rose, Bahari and Turnberg, 1980; Allen and Garner, 1980).

Similarly, a higher surface pH compared to the luminal pH was measured at the surface of human stomach preparation in vitro (see Chapter 6).

CHAPTER FOUR

THE INTESTINAL ABSORPTION OF FOLATES

4.1. INTRODUCTION :-

Folates are known to be readily absorbed by man and other mammalian species. The exact mechanism involved in their transport across the intestine however, has not been established. Many workers suggested that the mechanism involves a facilitated passive diffusion through the intestinal surface acid microclimate, while others suggested an active transport mechanism. This study was designed to clarify the site(s) and the mechanism of absorption of two folate species, folic acid and 5-methyltetrahydrofolic acid (5-meTHF) and the factors that affect their rate of transport. The metabolic status of folate species present in the tissue and the serosal compartments after incubation was also investigated. The everted sac preparation was chosen for this purpose as it is a convenient technique by which the molecular mechanism of transport can be determined and a variety of factors can be examined.

4.2. MATERIALS AND METHODS :-

Unless otherwise stated, everted sacs were incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 20 mM glucose at 37°C. 5-^{14}C-meTHF and [3',5',7,9-^{3}H] - or 2-^{14}C-labelled folic acid was used as tracers. The results are expressed as the mean ± S.E.M. for six experiments.

Further details of the experimental procedure and materials are described in Chapter 2.

The viability of the in vitro preparation was estimated by measur-

ing water and glucose transport, potential difference, histological appearance and the release of brush border and cytosol marker enzymes (see Chapter 3).

4.3 RESULTS

The transport of folate expressed as nmole/g initial tissue wet wt. was determined in the whole tissue (tissue uptake) which represents the amount of folate present in the tissue compartment after a specific incubation period; and in the serosal compartment which represents the amount of folate which has entered into the serosal compartment and has therefore passed through the tissue compartment. The total uptake represents the summation of serosal and tissue uptake.

4.3.1 PRELIMINARY STUDIES :-

(i) The purity and stability of 5-meTHF under storage and experimental conditions :

5-meTHF is an unstable compound in aqueous medium under aerobic conditions compared to folic acid (Blakely, 1969). It oxidizes to 5-methyl-5,6-dihydrofolate (see Figure 1-4) and subsequently to a pyrazino-s-triazine derivative (see Figure 2-2) (Gapski, Whiteley and Huennekens, 1971; Jongejan, Mager and Berends, 1979; Blair, Pearson and Robb, 1975). Both of the oxidation products are biologically inactive (Ratanasthien, Blair, Leeming, Cooke, and Melikian, 1976; Kennelly, Blair and Pheasant, 1979a). 5-methyl-5,6-dihydrofolate is easily reduced to 5-meTHF by a variety of antioxidants including ascorbate (Blakely, 1969).

TABLE 4-1 :-

The stability of 5-meTHF under experimental conditions. 10^{-6}M 5-meTHF was added to the bicarbonate buffer pH 7.4 containing 3mg/ml sodium ascorbate and continuously oxygenated with a mixture of 95% O_2 and 5% CO_2 at 37°C. Folate was identified on a cellulose precoated thin layer chromatograms eluted with 0.1M phosphate buffer pH 7 containing 2% sodium ascorbate.

Incubation Time (min.)	Percentage identified as 5-meTHF
30	89
45	94
60	88

The purity and stability of the radioactive species of 5-meTHF (5-^{14}C-meTHF) was checked as described in Chapter 2. It appeared that no oxidation or decomposition occurred during storage conditions for one month in the presence of 2% sodium ascorbate at -20^{o}C as 98% of the radioactivity co-chromatographed with the standard unlabelled 5-meTHF. The Rf value for 5-meTHF in 0.1M phosphate buffer pH 7 was 0.88.

Similarly, 5-meTHF appeared to be stable under the experimental conditions in the presence of 3 mg/ml sodium ascorbate (Table 4-1). For this reason 3 mg/ml sodium ascorbate was added to the incubation medium in all the 5-meTHF absorption studies.

(ii) The effect of sodium ascorbate on folic acid transport :-

Folic acid is a relatively stable derivative of folate (Blakely, 1969). Sodium ascorbate (3mg/ml) was routinely added to all experiments in which 5-meTHF transport was measured. In order to assess the effect of sodium ascorbate on folic acid transport, everted sacs were prepared from the proximal jejunum and incubated in bicarbonate buffer containing 10^{-6}M folic acid in the presence and absence of 3mg/ml sodium ascorbate.

The transport rate of folic acid in the presence and absence of sodium ascorbate was within experimental measuring error, identical (Table 4-2). This indicates that sodium ascorbate has no effect on the rate of folic acid transport.

TABLE 4-2 :- The effect of 3mg/ml sodium ascorbate in the incubation medium on the uptake of 10^{-6}M folic acid by everted sacs from the proximal jejunum incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 20 mM glucose, at 37°C.

A. The effect on folic acid uptake :-

Sodium ascorbate concentration (mg / ml)	Folic acid uptake (n mole / g initial tissue wet weight)		
	Serosal	Tissue	Total
3	0.32 ± 0.02	1.88 ± 0.12	2.20 ± 0.12
0	0.33 ± 0.02	2.00 ± 0.06	2.33 ± 0.05

B. The effect on water uptake :-

Sodium ascorbate concentration (mg / ml)	Water Uptake (mg / g initial tissue wet weight)		
	Serosal	Tissue	Total
3	456 ± 17	565 ± 43	1021 ± 51
0	492 ± 28	571 ± 36	1063 ± 38

(iii) The role of solvent drag with water flow in folic acid transport :-

Solvent drag with water flow is a mechanism by which considerable amounts of passively transported substance may cross the intestinal barrier. The role of this mechanism in folic acid absorption was examined by increasing the osmolarity of the mucosal fluid and thereby decreasing water movement. This was achieved by adding mannitol to the mucosal bicarbonate buffer and measuring the uptake of 10^{-6}M folic acid. Dual labelled folic acid (2-^{14}C-folic acid and [3',5',7,9-^{3}H]folic acid) was used as a tracer.

As the concentration of mannitol in the incubation medium was increased there was a decrease in water movement to the serosal compartment but not the tissue compartment (Table 4-3). The serosal water uptake was diminished when 200 mM mannitol was present. Simultaneously there was a small decrease in serosal folic acid uptake and a small increase in tissue folic acid uptake. The data implies that water movement plays only a minor role in carrying folic acid from the tissue to the serosal fluid. This results together with the *in vitro* observations of Blair, Lucas and Swanston (1979) that methotrexate induced water uptake is associated with a significant decrease in folate uptake indicates that solvent drag with water flow is not the mechanism for folate transport.

In all the conditions examined more ^{3}H-labelled folic acid was taken up by the tissue and the serosal compartments than the ^{14}C-labelled

TABLE 4-3:- The role of solvent drag with water flow in the uptake of 10^{-6}M folic acid (dual labelled) by everted sacs prepared from the proximal jejunum and incubated for 30 minutes in bicarbonate buffer pH 7.4. containing 20mM glucose at 37°C

Mannitol Concentration (M)	[3',5',7,9-^{3}H]-Folic acid uptake (nmole/g initial tissue wet wt.)			2-^{14}C-Folic acid uptake (nmole/g initial tissue wet wt.)			Water uptake (mg/g initial tissue wet wt.)		
	Serosal	Tissue	Total	Serosal	Tissue	Total	Serosal	Tissue	Total
0	0.48±0.03	1.45±0.07	1.93±0.12	0.35±0.05	1.37±0.06	1.72±0.09	245±77	550±29	795±63
50	0.45±0.07	1.47±0.10	1.92±0.15	0.33±0.04	1.38±0.06	1.71±0.09	234±77	513±19	746±80
100	0.41±0.04	1.56±0.03	1.96±0.07	0.31±0.03	1.40±0.01	1.71±0.08	159±73	577±33	737±41
200	0.29±0.03	1.60±0.08	1.88±0.07	0.22±0.02	1.46±0.08	1.68±0.07	-26±34	524±40	497±21

species. This observation is examined further in Chapter 5.

4.3.2 THE UPTAKE OF 5-meTHF AS A FUNCTION OF INCREASING INCUBATION TIME :-

Everted sacs from the proximal jejunum were incubated in bicarbonate buffer for up to 60 minutes in the presence of 10^{-5}M 5-meTHF.

The serosal uptake of 5-meTHF increased linearly with time (Figure 4-1A and Table 4-4). However, there was a delay in the appearance of 5-meTHF in the serosal compartment represented by an intercept of the best straight line with the x-axis. The rate of tissue uptake of 5-meTHF was more rapid during the initial 20 minutes of incubation and declined to a slower but constant rate thereafter. Tissue uptake was higher than the serosal uptake so that after 60 minutes approximately three times more folate appeared in the tissue compartment than in the serosal compartment. The results demonstrate no saturation during the 60 minute incubation period. Similar observations have been reported with folic acid (Swanston, 1978).

Water uptake was measured simultaneously (Figure 4-1B and Table 4-4). The total and serosal water uptake increased linearly with time, while tissue uptake reached saturation after approximately 30 minutes incubation.

4.3.3 THE TRANSPORT OF FOLIC ACID AND 5-meTHF ACROSS DIFFERENT REGIONS OF THE SMALL INTESTINE :-

Everted sacs prepared from different regions of the small intestine

TABLE 4-4 : The serosal, tissue and total uptake of 10^{-5}M 5-meTHF with time. Everted sacs from the proximal jejunum incubated for different periods of time in bicarbonate buffer pH 7.4 containing 20 mM glucose and 3 mg/ml sodium ascorbate at 37°C.

A. The uptake of 10^{-5}M 5-meTHF :-

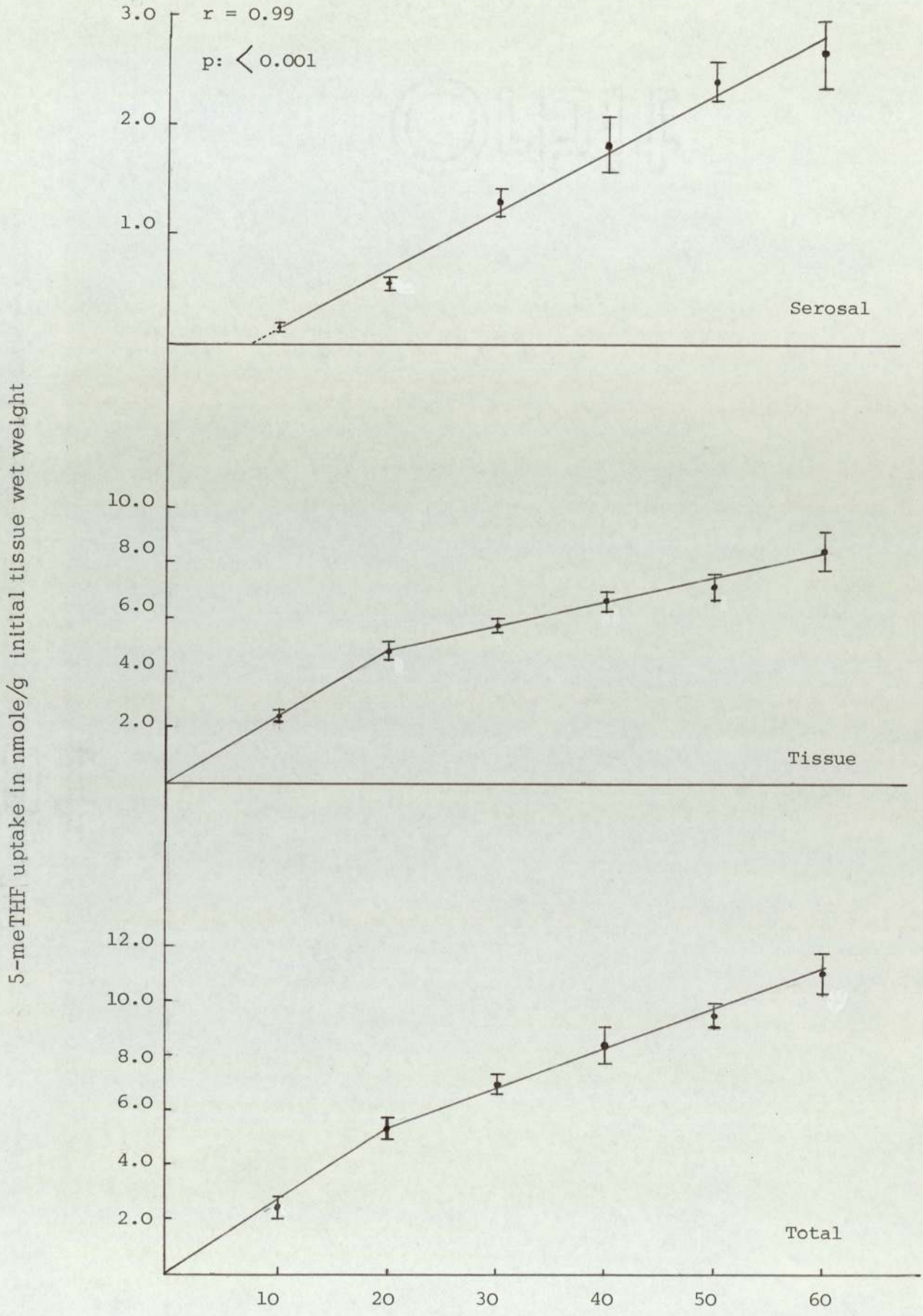
Incubation Time (min.)	5-me THF Uptake (n mole / g initial tissue wet weight)		
	Serosal	Tissue	Total
10	0.17 ± 0.03	2.23 ± 0.40	2.40 ± 0.40
20	0.55 ± 0.06	4.8 ± 0.32	5.35 ± 0.40
30	1.28 ± 0.13	5.73 ± 0.20	7.01 ± 0.25
40	1.83 ± 0.26	6.60 ± 0.35	8.43 ± 0.64
50	2.40 ± 0.17	7.1 ± 0.45	9.5 ± 0.41
60	2.67 ± 0.31	8.50 ± 0.70	11.17 ± 0.70

B. The uptake of water :-

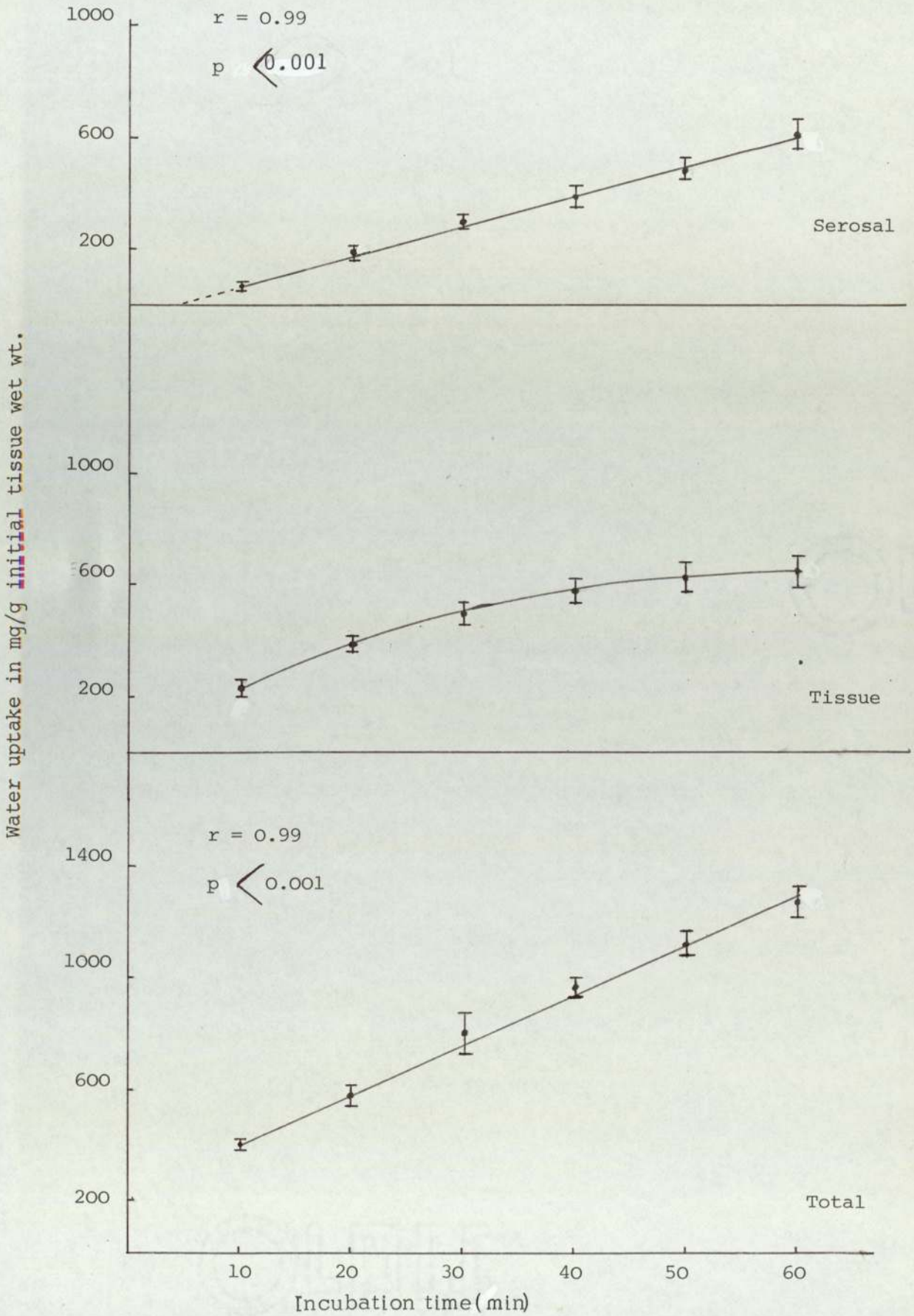
Incubation Time (min.)	Water Uptake (mg / g initial tissue wet weight)		
10	70 ± 9	235 ± 23	304 ± 13
20	190 ± 20	387 ± 24	577 ± 36
30	300 ± 24	500 ± 43	800 ± 74
40	390 ± 41	580 ± 42	970 ± 27
50	490 ± 34	635 ± 45	1125 ± 44
60	620 ± 49	649 ± 50	1269 ± 59

FIGURE 4-1: The serosal, tissue and total uptake of (A) 5-meTHF and (B) water, by everted sacs from the proximal jejunum incubated in bicarbonate buffer pH 7.4 containing 20 mM glucose at 37°C.

A- 5-meTHF Uptake :-



B- Water uptake



were incubated for 30 minutes in bicarbonate buffer at 37°C containing either 10^{-6}M 5-meTHF or 10^{-6}M folic acid.

Equal amounts of 10^{-6}M folic acid and 10^{-6}M 5-meTHF were transported across the proximal jejunum. Across the distal regions of the small intestine there was a marked decline in the transport of both folic acid and 5-meTHF. However, the decline in folic acid transport was steeper than that of 5-meTHF, so that at any particular region distal to the proximal jejunum significantly more 5-meTHF than folic acid was transported (Table 4-5). Water uptake, which was measured simultaneously, also declined down the intestine.

Similar trends of decline in the total uptake of 5-meTHF down the intestine was observed when 10^{-5}M of the compound was added to the incubation medium (Table 4-6).

The results confirm the observations of Coleman, Hilburn and Blair (1979), and contradict those of Strum, Nixon, Bertino and Binder, (1971).

4.3.4 THE UPTAKE OF 5-meTHF AND FOLIC ACID AS A FUNCTION OF INCREASING MUCOSAL CONCENTRATION :-

Everted sacs prepared from the proximal jejunum were incubated for 30 minutes in bicarbonate buffer adjusted to various pH values (7.4 to 5.0) in the presence of a range of concentrations of 5-meTHF (10^{-7} to 10^{-4}M). Similar preparations were incubated in bicarbonate buffer pH 7.4 in the presence of a range of concentrations of folic acid (10^{-7}

TABLE 4-5:- The Uptake of 10^{-6}M folic acid and 10^{-6}M 5-meTHF across different regions of the small intestine. Everted sacs were incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 20 mM glucose at 37°C.

A. (i) 10^{-6}M Folic acid uptake :-

Section	Folic acid uptake (n mole / g initial tissue wet weight)		
	Serosal	Tissue	Total
2	0.33 ± 0.02	1.90 ± 0.06	2.23 ± 0.05
5	0.19 ± 0.01	1.17 ± 0.09	1.36 ± 0.09
7	0.17 ± 0.02	1.00 ± 0.1	1.17 ± 0.10
8	0.13 ± 0.01	0.45 ± 0.05	0.58 ± 0.05
9	0.08 ± 0.01	0.36 ± 0.04	0.44 ± 0.04
10	0.05 ± 0.01	0.24 ± 0.02	0.29 ± 0.02

(ii) Water uptake :-

Section	Water Uptake (mg / g initial tissue wet weight)		
	Serosal	Tissue	Total
2	492 ± 28	571 ± 14	1062 ± 28
5	263 ± 12	539 ± 42	802 ± 43
7	225 ± 9	480 ± 22	705 ± 30
8	192 ± 16	394 ± 31	586 ± 43
9	167 ± 7	427 ± 24	594 ± 26
10	128 ± 8	410 ± 17	538 ± 18

B. (i) 10^{-6}M 5-meTHF uptake :-

Section	5-me THF uptake (n mole / g initial tissue wet weight)		
	Serosal	Tissue	Total
2	0.48 ± 0.05	1.67 ± 0.03	2.15 ± 0.07
5	0.33 ± 0.03	1.83 ± 0.14	2.15 ± 0.16 (> 0.001) *
7	0.27 ± 0.04	1.53 ± 0.24	1.80 ± 0.20 (< 0.05)*
8	0.18 ± 0.03	0.82 ± 0.09	1.0 ± 0.10 (< 0.01)*
9	0.13 ± 0.02	0.72 ± 0.08	0.85 ± 0.10 (< 0.01)*
10	0.05 ± 0.002	0.32 ± 0.02	0.37 ± 0.02 (< 0.01)*

(ii) Water uptake :-

Section	Water Uptake (mg / g initial tissue wet weight)		
	Serosal	Tissue	Total
2	409 ± 72	500 ± 37	909 ± 49
5	369 ± 29	514 ± 46	883 ± 43
7	358 ± 67	592 ± 90	749 ± 67
8	243 ± 16	387 ± 45	630 ± 53
9	167 ± 28	377 ± 46	544 ± 67
10	128 ± 17	320 ± 23	448 ± 24

* Probability expressed as the difference in transport rates between 10^{-6}M 5-meTHF compared to 10^{-6}M folic acid for the same region of the small intestine.

TABLE 4-6 :- The uptake of 10^{-5}M 5-meTHF across different regions of the small intestine. Everted sacs were incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 20 mM glucose at 37°C.

A. 10^{-5}M 5-meTHF uptake :-

Section	5-meTHF Uptake (n mole/g initial tissue wet weight)		
	Serosal	Tissue	Total
2	1.28 ± 0.13	5.73 ± 0.19	7.01 ± 0.25
5	0.83 ± 0.06	6.27 ± 0.23	7.10 ± 0.22
7	0.78 ± 0.11	5.69 ± 0.76	6.46 ± 0.8
8	0.70 ± 0.05	4.95 ± 0.16	5.65 ± 0.16
9	0.58 ± 0.06	3.50 ± 0.42	4.08 ± 0.45
10	0.40 ± 0.02	3.04 ± 0.26	3.44 ± 0.27

B. Water uptake :-

Section	Water uptake (mg / g initial tissue wet weight)		
	Serosal	Tissue	Total
2	300 ± 29	500 ± 43	800 ± 74
5	321 ± 16	508 ± 11	830 ± 16
7	249 ± 32	450 ± 23	699 ± 28
8	268 ± 21	379 ± 13	647 ± 17
9	157 ± 10	411 ± 26	568 ± 31
10	140 ± 27	297 ± 24	437 ± 28

to 5×10^{-5}M).

There was a linear increase in the serosal, tissue and total uptake of 5-meTHF as its concentration in the incubation medium increased (Figure 4-2 and Table 4-7). The linear relationship was observed whether the incubation buffer was adjusted to pH 7.4, 6.0, 5.5 or 5.0, however the amount transported was different. There was significant increase in the total uptake of 5-meTHF when the initial buffer was adjusted to pH 6 compared to pH 7.4 except at 10^{-4}M 5-meTHF where the increase was insignificant. Adjustment of the buffer to pH 5.5 or 5.0 caused no further increase in the total uptake of 5-meTHF. Changes in buffer pH were observed during incubation (see Table 4-2).

Total water uptake which was measured simultaneously, decreased as the pH of the incubation medium decreased, mainly due to the decrease in serosal uptake which was severely inhibited at pH 5.0. This observation further indicates that solvent drag with water flow has no role in the serosal uptake of 5-meTHF.

Serosal, tissue and total uptake rates of folic acid increased linearly with concentration upto about 5×10^{-6}M (Figure 4-3 and Table 4-8). At higher concentrations the increase in the rate was sharply depressed and became about zero at 10^{-5}M indicating a saturation in the transport mechanism.

The data presented on the uptake of 5-meTHF and folic acid as a function of mucosal concentration and pH are in agreement with that

TABLE 4-7: The serosal tissue and total uptake of 5-meTHF as a function of increasing mucosal concentration at different initial incubation buffer pH values. Everted sacs from the proximal jejunum were incubated for 30 minutes in bicarbonate buffer adjusted to pH 7.4, 6, 5.5 and 5, and containing 20 mM glucose at 37°C.

A. At initial buffer pH 7.4 :-

(i) 5-meTHF uptake:-

5-meTHF mucosal concentration (M)	5 - me THF uptake (n mole / g initial tissue wet weight)		
	Serosal	Tissue	Total
10^{-7}	0.06 ± 0.005	0.28 ± 0.02	0.34 ± 0.02
5×10^{-7}	0.17 ± 0.01	0.47 ± 0.04	0.65 ± 0.05
10^{-6}	0.28 ± 0.02	1.60 ± 0.02	1.87 ± 0.04
10^{-5}	0.99 ± 0.06	4.90 ± 0.70	5.89 ± 0.35
5×10^{-5}	2.11 ± 0.10	7.24 ± 0.40	9.35 ± 0.40
10^{-4}	5.30 ± 0.50	22.20 ± 0.80	27.50 ± 1.2

(ii) Water uptake :-

5-meTHF mucosal concentration (M)	Water Uptake (mg / g initial tissue wet weight)		
	Serosal	Tissue	Total
10^{-7}	283 ± 12	480 ± 22	763 ± 22
5×10^{-7}	253 ± 23	552 ± 31	805 ± 29

Continued..

5-meTHF mucosal concentration (M)	Water Uptake (mg / g initial tissue wet weight)		
	Serosal	Tissue	Total
10^{-6}	270 ± 12	494 ± 11	764 ± 21
10^{-5}	290 ± 30	486 ± 17	776 ± 36
5×10^{-5}	281 ± 28	465 ± 36	746 ± 40
10^{-4}	270 ± 16	491 ± 27	761 ± 28

The buffer pH at the end of incubation was 7.20 ± 0.07

B. At intial buffer pH 6 :-

(i) 5-meTHF uptake :-

5-meTHF mucosal concentration (M)	5 - meTHF Uptake (n mole / g intial tissue wet weight)		
	Serosal	Tissue	Total
10^{-7}	0.10 ± 0.01	0.53 ± 0.05	0.63 ± 0.05 (<0.001)*
5×10^{-7}	0.24 ± 0.01	1.03 ± 0.04	1.27 ± 0.05 (<0.001)*
10^{-6}	0.24 ± 0.08	2.25 ± 0.30	2.50 ± 0.30 (>0.02)*
10^{-5}	1.05 ± 0.09	6.49 ± 0.50	7.54 ± 0.60 (>0.02)*
5×10^{-5}	2.71 ± 0.30	12.0 ± 11.60	14.70 ± 1.70 (<0.01)*
10^{-4}	5.40 ± 0.60	25.40 ± 1.40	30.8 ± 1.40 (>0.05)*

(ii) Water uptake :-

5-meTHF mucosal concentration (M)	Water Uptake (mg / g initial tissue wet weight)		
	Serosal	Tissue	Total
10^{-7}	160 ± 12	423 ± 20	583 ± 15
5×10^{-7}	188 ± 9	441 ± 18	629 ± 26
10^{-6}	203 ± 28	429 ± 63	632 ± 69
10^{-5}	188 ± 19	473 ± 35	661 ± 49
5×10^{-5}	142 ± 6	435 ± 23	577 ± 21
10^{-4}	229 ± 42	409 ± 4	638 ± 40

The buffer pH at the end of incubation was 6.30 ± 0.12.

* Probability expressed as the difference in the transport rates of 5-me-THF at incubation buffer medium pH 6 and 7.4.

C. At initial buffer pH 5.5 :-

(i) 5-meTHF uptake :-

5-meTHF mucosal concentration (M)	5-me THF Uptake (n mole/ g initial tissue wet weight) Serosal	Tissue	Total
10^{-7}	0.08 ± 0.002	0.52 ± 0.03	0.60 ± 0.02
10^{-6}	0.45 ± 0.07	3.20 ± 0.20	3.65 ± 0.25
10^{-5}	0.93 ± 0.12	6.86 ± 0.33	7.79 ± 0.38
10^{-4}	6.20 ± 0.64	22.93 ± 1.20	29.13 ± 1.70

(ii) Water uptake :-

5-meTHF mucosal concentration(M)	Water Uptake (mg / g initial tissue wet weight) Serosal	Tissue	Total
10^{-7}	195 ± 20	415 ± 19	610 ± 24
10^{-6}	198 ± 34	468 ± 29	666 ± 50
10^{-5}	164 ± 16	415 ± 22	579 ± 35
10^{-4}	165 ± 13	427 ± 22	592 ± 29

The buffer pH at the end of incubation was 6.30 ± 0.10

D. At initial buffer pH 5.0 :-

(i) 5-meTHF uptake :-

5-meTHF mucosal concentration (M)	5-meTHF uptake (n mole / g initial tissue wet weight)		
	Serosal	Tissue	Total
10^{-7}	0.06 ± 0.01	0.55 ± 0.04	0.61 ± 0.04
10^{-6}	0.44 ± 0.05	3.48 ± 0.14	3.92 ± 0.16
10^{-5}	1.43 ± 0.12	6.75 ± 0.20	8.18 ± 0.32
10^{-4}	7.20 ± 0.8	23.10 ± 0.82	30.30 ± 1.10

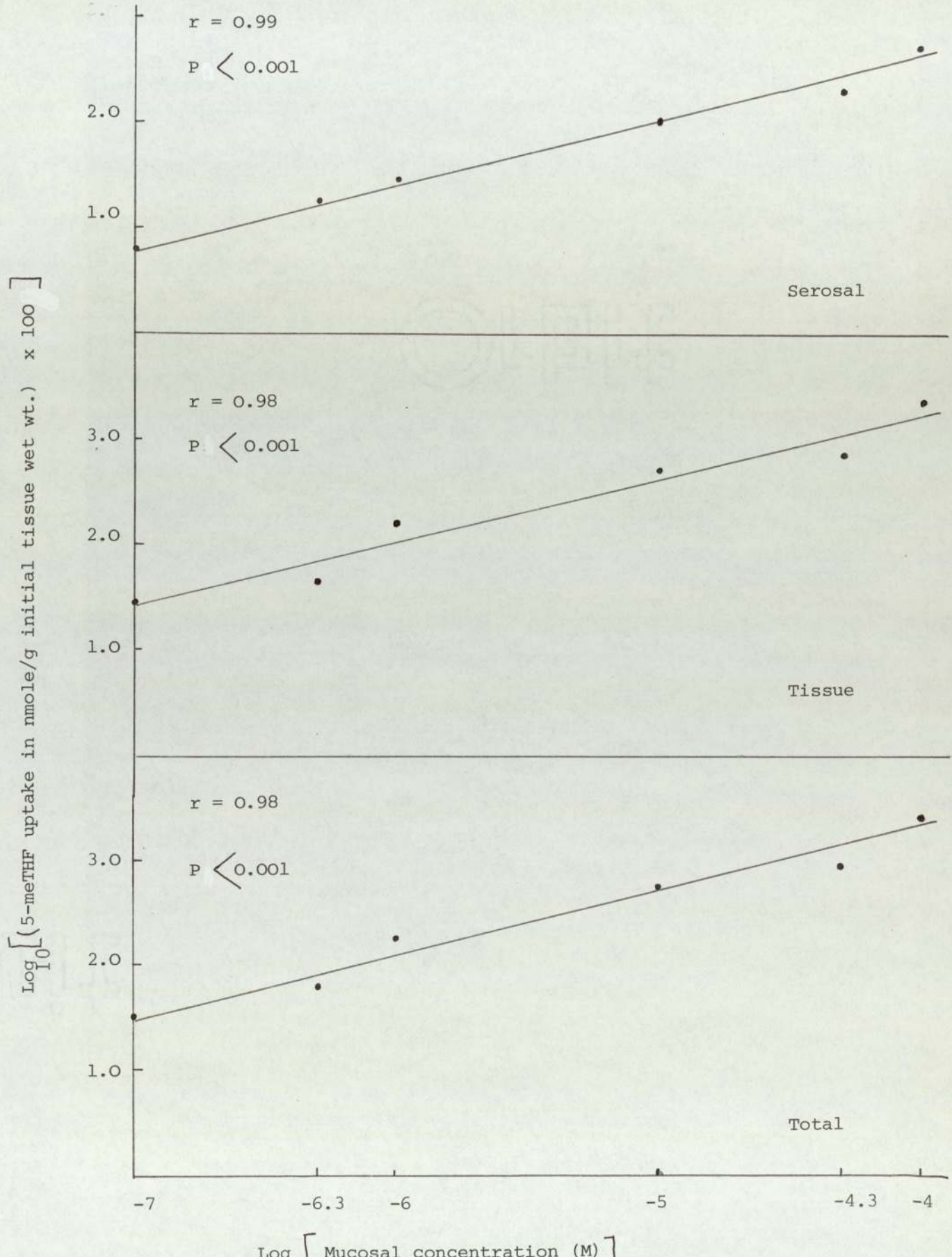
(ii) Water uptake :-

5-meTHF mucosal concentration (M)	Water Uptake (mg / g initial tissue wet weight)		
	Serosal	Tissue	Total
10^{-7}	-19 ± 26	423 ± 17	404 ± 24
10^{-6}	-73 ± 29	389 ± 15	316 ± 16
10^{-5}	49 ± 41	404 ± 90	453 ± 32
10^{-4}	66 ± 28	430 ± 13	496 ± 26

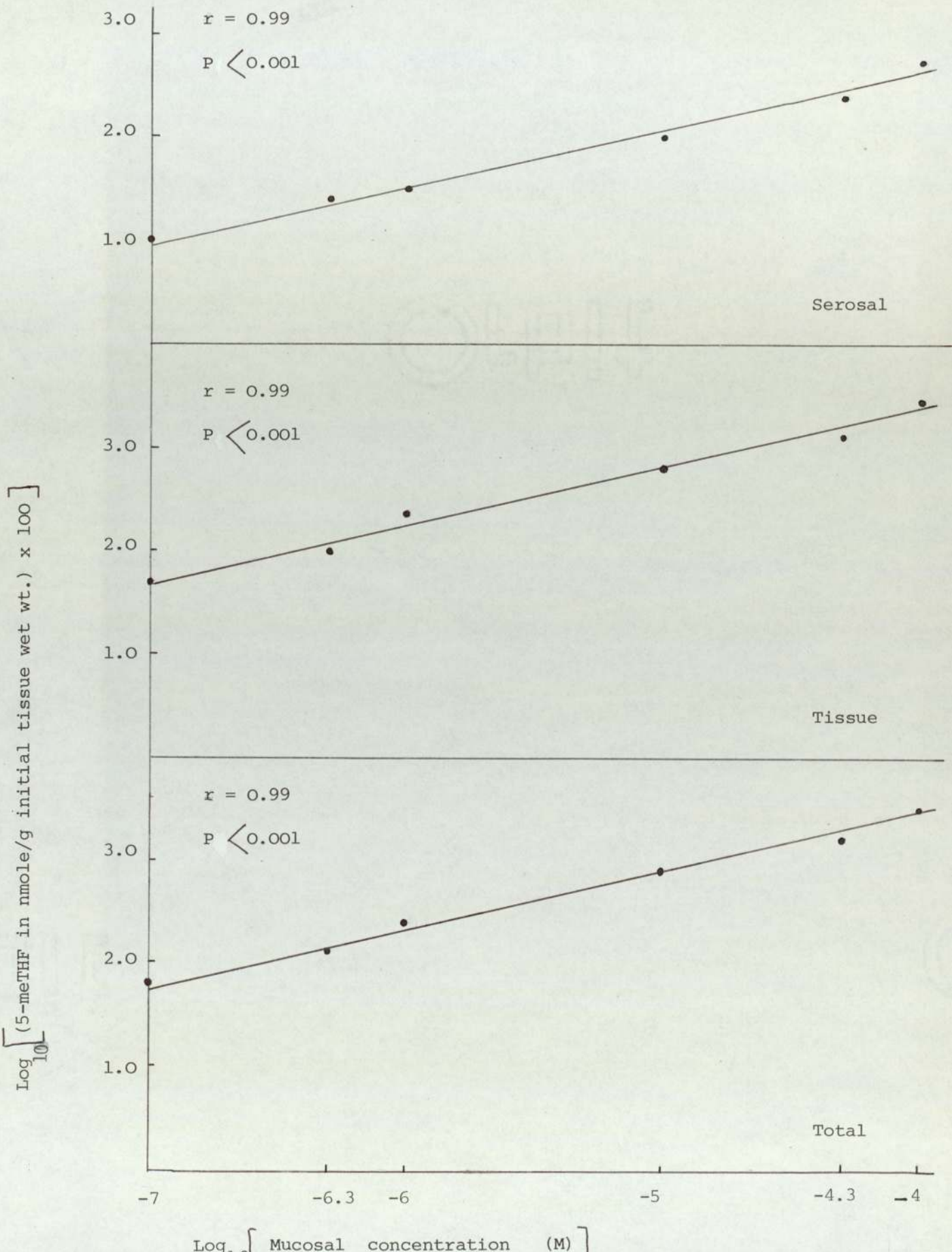
The buffer pH at the end of incubation was 5.98 ± 0.04

FIGURE 4-2:- The serosal, tissue and total uptake of 5-meTHF as a function of increasing mucosal concentration at different initial incubation buffer pH values. Everted sacs from the proximal jejunum were incubated for 30 minutes in bicarbonate buffer adjusted to pH 7.4, 6, 5.5 and 5 containing 20 mM glucose at 37^{o}C.

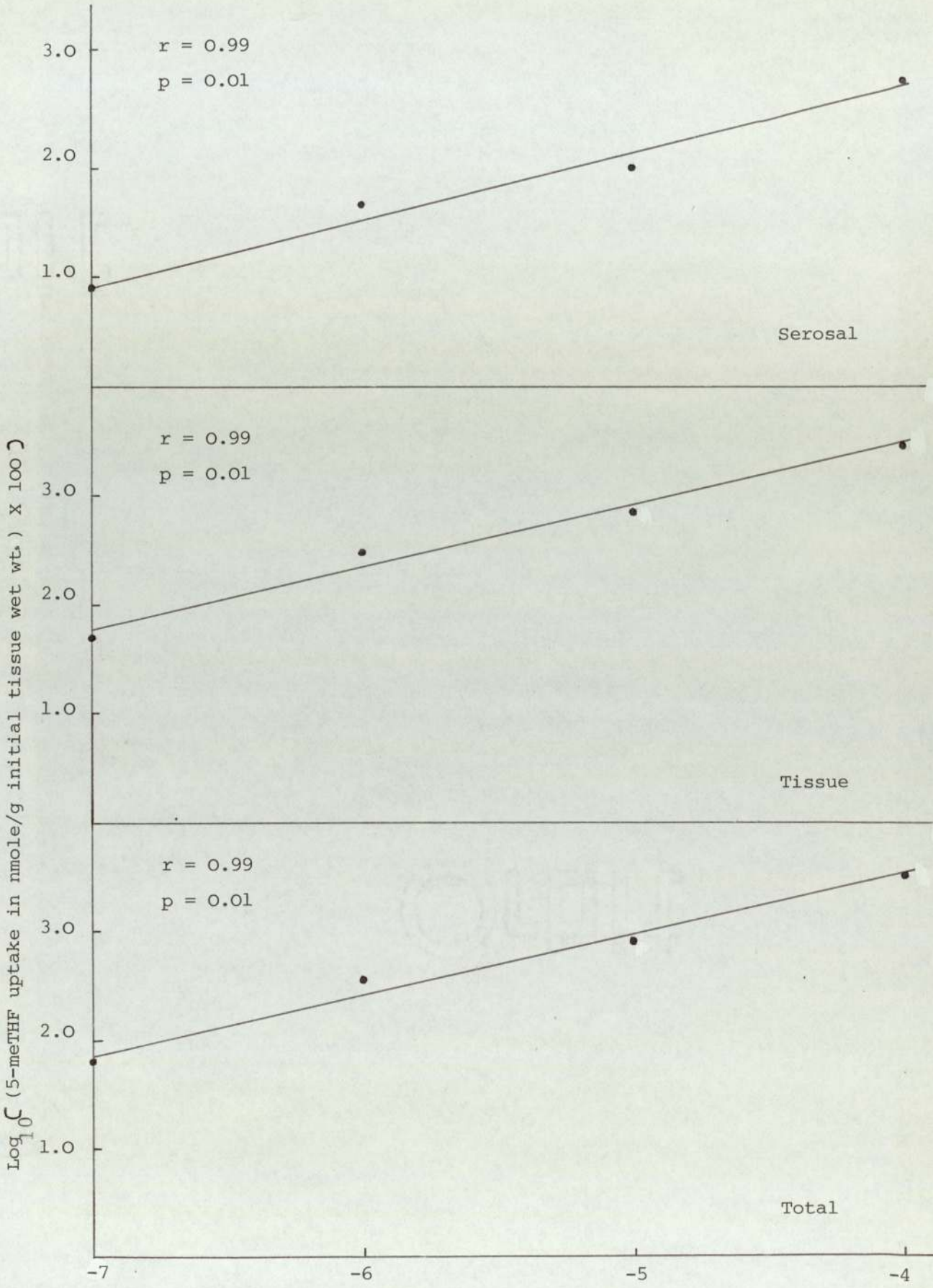
A - At initial buffer pH 7.4 :-



B- At initial buffer pH 6.0 :-



C. At initial buffer pH 5.5 :-



D - At initial buffer pH 5 :-

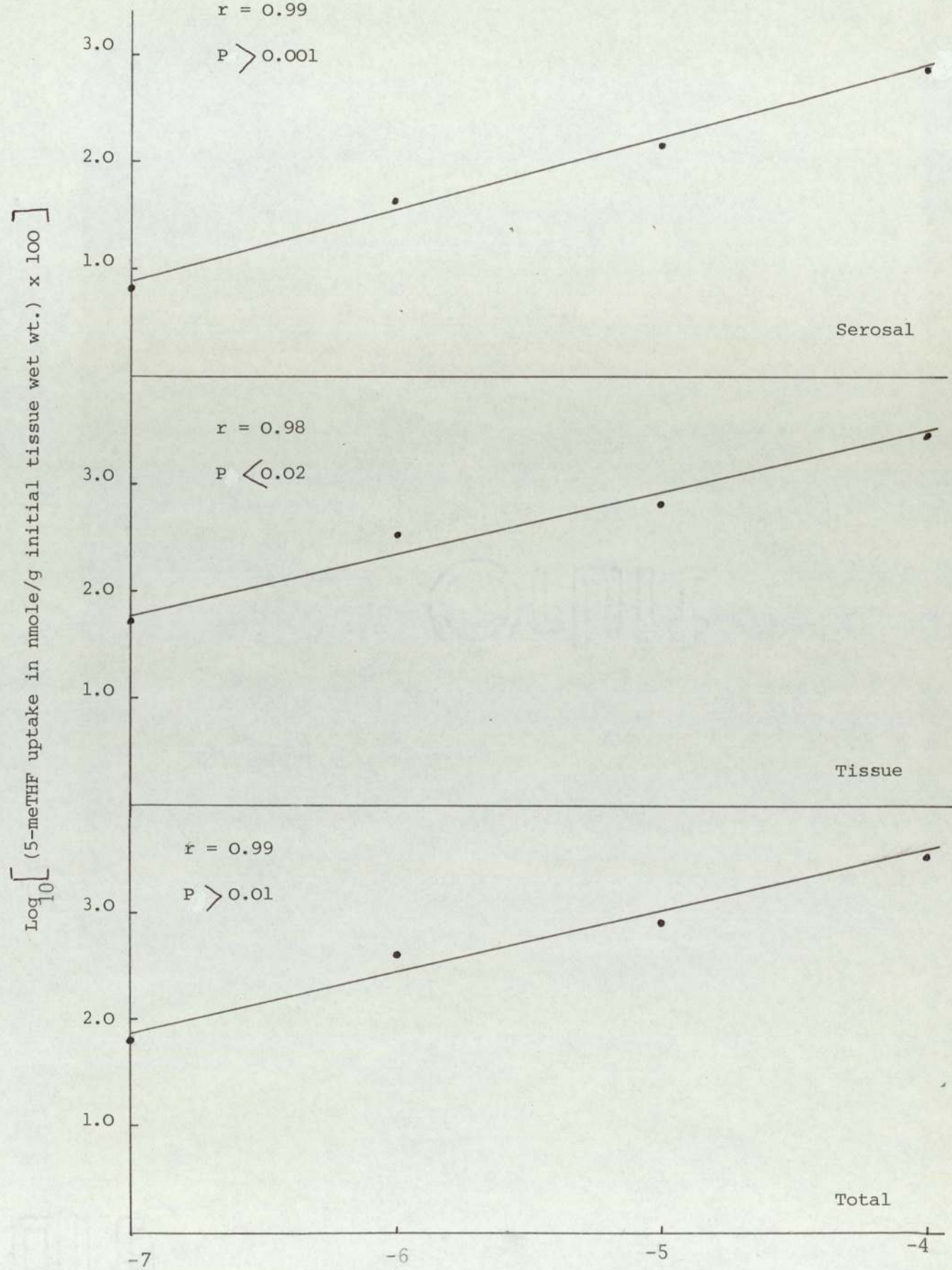


TABLE 4 -8:- The serosal, tissue and total uptake of folic acid as a function of increasing mucosal concentration. Everted sacs from the proximal jejunum were incubated for 30 minutes in bicarbonate buffer pH 7.4, containing 20 mM glucose at 37°C.

(i) Folic acid uptake :-

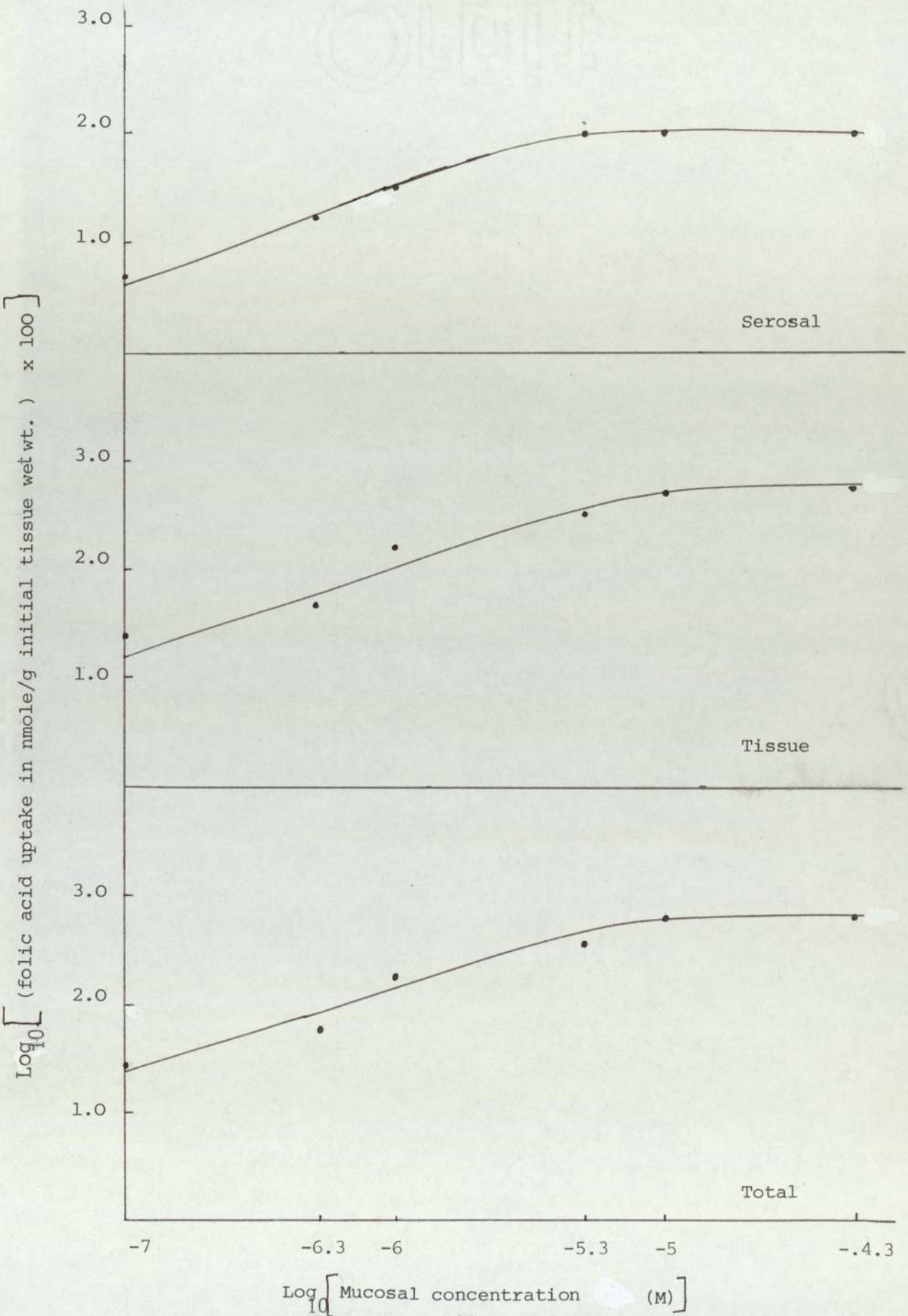
Folic acid mucosal concentration (M)	Folic acid uptake (n mole / g initial tissue wet weight)		
	Serosal	Tissue	Total
10^{-7}	0.05 ±0.006	0.25 ± 0.02	0.30 ± 0.02
5×10^{-7}	0.15 ±0.10	0.45 ± 0.05	0.60 ± 0.06
10^{-6}	0.33 ± 0.02	1.51 ± 0.12	1.84 ± 0.15
5×10^{-6}	1.06 ± 0.05	3.12 ± 0.15	4.18 ± 0.15
10^{-5}	1.00 ± 0.07	5.20 ± 0.30	6.20 ± 0.35
5×10^{-5}	1.14 ± 0.05	6.10 ± 0.23	7.24 ± 0.30

(ii) Water uptake :-

Folic acid mucosal concentration (M)	Water Uptake (mg / g initial tissue wet weight)		
	Serosal	Tissue	Total
10^{-7}	461 ± 28	547 ± 22	1008 ± 33
5×10^{-7}	417 ± 35	506 ± 23	923 ± 40
10^{-6}	445 ± 48	556 ± 38	1001 ± 60
5×10^{-6}	542 ± 17	566 ± 26	1108 ± 30
10^{-5}	406 ± 37	493 ± 26	899 ± 48
5×10^{-5}	377 ± 25	496 ± 27	873 ± 45

The buffer pH at the end of incubation was 7.15 ± 0.08 .

Figure 4-3 : The serosal, tissue and total uptake of folic acid as a function of increasing mucosal concentration. Everted sacs from the proximal jejunum were incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 20 mM glucose at 37°C.



published by other investigators (Strum, Nixon, Bertino and Binder, 1971; Blair, Matty and Razzaque, 1975; Noronha and Kesavan, 1979; Blair, Johnson and Matty, 1974; Rose, Koch and Nahrwold, 1978; Chungi, Bourne and Dittert, 1979; Kesavan and Noronha, 1978; Smith, Matty and Blair, 1970).

4.3.5 TRANSPORT OF FOLIC ACID AGAINST A CONCENTRATION GRADIENT :-

Everted sacs prepared from the proximal jejunum were incubated in bicarbonate buffer adjusted to either pH 7.4 or pH 6, with equal amounts of folic acid (10^{-7}M or 10^{-6}M) on both sides of the preparation.

When 10^{-7}M folic acid was initially added to both the mucosal and the serosal sides of the preparation, the serosal/mucosal folic acid concentration ratio (S/M) after 30 minutes was 0.84 at pH 7.4 and 1.2 at pH 6 (Table 4-9). Tissue folic acid uptake, which occurred mainly from the mucosal side, was slightly higher at pH 6 than pH 7.4. On the other hand, less water moved into the everted sac preparation at pH 6 than pH 7.4.

When 10^{-6}M folic acid was initially present on both sides, the ratio after 30 minutes was 0.9 when the buffer was pH 7.4 or pH 6.0. Tissue folic acid uptake was again higher at pH 6 than pH 7.4. and less water moved into the preparation at the lower incubation buffer pH.

In the experiments, water movement from the incubation medium into the everted sac introduces another variable. It causes the serosal folic acid concentration to be diluted while the mucosal concentration of folic acid become slightly increased. In order to minimize the variation

TABLE 4-9 : Transport of folic acid against a concentration gradient.

Everted sacs from the proximal jejunum were incubated for 30 minutes in bicarbonate buffer pH 7.4 or pH 6.0 containing 20 mM glucose at 37°C. Equal concentrations of folic acid (10^{-7}M or 10^{-6}M) were added to both the mucosal and serosal fluid.

A. Folic acid movement :-

Initial folic acid concentration(M)	Incubation buffer pH	Serosal folic acid conc. at the end of incubation (nmole/g initial tissue wet wt.)	Tissue folic acid uptake (nmole/g initial tissue wet wt.)	S/M ratio
10^{-7}	7.4	0.114±0.006	0.20±0.01	0.84±0.05
	6.0	0.106±0.005	0.23±0.01	1.2 ±0.03
10^{-6}	7.4	1.46 ±0.11	1.50±0.04	0.9 ±0.03
	6.0	1.14 ±0.06	1.75±0.12	0.9 ±0.03
(200mM Mannitol)	7.4	1.05 ±0.03	2.11±0.06	1.2 ±0.02

B. Water uptake :-

Initial folic acid concentration(M)	Incubation buffer pH	Water Uptake (mg / g initial tissue wet wt.)		
		Serosal	Tissue	Total
10^{-7}	7.4	387±32	702±48	1089±59
	6.0	106±9	589±31	695 ±23
10^{-6}	7.4	555±57	651±48	1206 ±95

Cont/d

TABLE 4-9 : Continued.....

Initial folic acid concentration(M)	Incubation buffer pH	Water Uptake (mg / g initial tissue wet wt.)		
	6.0	276 ± 35	453 ± 28	729 ± 22
(200 mM Mannitol)	7.4	5.0 ± 34	425 ± 21	430 ± 23

caused by water movement, transport of 10^{-6}M folic acid against a concentration gradient was measured in the presence of 200 mM mannitol at pH 7.4. The S/M folic acid concentration ratio at the end of incubation was found to be equal to 1.2 (Table 4-9). Tissue folic acid uptake was also appreciable.

4.3.6. COMPETITION BETWEEN FOLIC ACID AND 5-meTHF :-

Experiments were designed to see whether folic acid and 5-meTHF affected each others transport. This was performed by incubating everted sacs from the proximal jejunum in bicarbonate buffer in the presence of equimolar concentrations of mucosal folic acid and 5-meTHF at 10^{-6}M, 5×10^{-6}M and 10^{-5}M. Controls of folic acid or 5-meTHF alone at the same concentrations were also performed. [3',5',7,9-^{3}H] Folic acid and 5-^{14}C-meTHF were used as tracers.

At all the concentrations examined, neither compound appeared to interfere with the transport characteristics of the other; as no significant changes occurred in the total uptake of either of them when added together compared to control values (Table 4-10).

4.3.7. THE EFFECT OF SODIUM DEOXYCHOLATE ON THE UPTAKE OF 5-meTHF :-

The addition of sodium deoxycholate to the incubation medium was found to cause an elevation in the surface pH of the rat proximal jejunum (see Figure 3-19). It has been suggested that the surface acid microclimate is responsible for the absorption of folates from the intestine and

TABLE 4-10: Competition between folic acid and 5-meTHF. Everted sacs from the proximal jejunum were incubated for 30 minutes in bicarbonate buffer pH 7.4 containing equimolar concentrations, of folic acid and 5-meTHF and 20mM glucose at 37°C

	Folic acid uptake (nmole/g initial tissue wet wt)			5-meTHF uptake (nmole/g initial tissue wet wt.)			Water uptake (mg/g initial tissue wet wt.)		
	Serosal	Tissue	Total	Serosal	Tissue	Total	Serosal	Tissue	Total
A- At 10^{-6}M :-									
1. 10^{-6}M folic acid and 10^{-6}M 5-meTHF	0.27 ± 0.04	1.54 ± 0.06	1.81 ± 0.08	0.38 ± 0.04	1.95 ± 0.06	2.33 ± 0.04	239 ± 45	554 ± 17	793 ± 45
2. 10^{-6}M 5-meTHF				0.40 ± 0.04	2.01 ± 0.11	2.41 ± 0.14	344 ± 36	569 ± 36	914 ± 59
3. 10^{-6}M folic acid	0.45 ± 0.06	1.68 ± 0.13	2.13 ± 0.19				385 ± 69	597 ± 21	983 ± 82
B- At 5×10^{-6}M :-									
1. 5×10^{-6}M folic acid and 5×10^{-6}M 5-meTHF	0.62 ± 0.03	1.75 ± 0.05	2.37 ± 0.06	0.52 ± 0.03	2.17 ± 0.12	2.69 ± 0.12	234 ± 0.30	553 ± 32	787 ± 33
2. 5×10^{-6}M 5-meTHF				0.62 ± 0.13	2.35 ± 0.14	2.97 ± 0.19	246 ± 21	499 ± 18	745 ± 37
3. 5×10^{-6}M folic acid	0.90 ± 0.10	1.57 ± 0.12	2.47 ± 0.09				286 ± 43	576 ± 11	862 ± 43

TABLE 4-10 (continued..)

	Folic acid uptake (nmole/g initial tissue wet wt.)			5-meTHF uptake (nmole/g initial tissue wet wt.)			Water uptake (mg/g initial tissue wet wt.)		
	Serosal	Tissue	Total	Serosal	Tissue	Total	Serosal	Tissue	Total
C- At 10^{-5}M:-									
1. 10^{-5}M folic acid and 10^{-5}M 5-meTHF	$1.17^{+}0.20$	5.49 ± 0.50	6.66 ± 0.67	1.05 ± 0.16	5.51 ± 0.50	6.55 ± 0.60	389 ± 33	523 ± 18	912 ± 47
2. 10^{-5}M 5-meTHF				0.96 ± 0.10	5.57 ± 0.30	6.53 ± 0.40	281 ± 32	511 ± 28	792 ± 27
3. 10^{-5}M folic acid	1.29 ± 0.10	5.90 ± 0.51	7.19 ± 0.60				265 ± 10	554 ± 37	820 ± 32

TABLE 4-11 : The effect of sodium deoxycholate at different concentrations on the uptake of 5-meTHF and water. Everted sacs from the proximal jejunum were incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 10^{-6}M 5-meTHF, different concentrations of sodium deoxycholate and 20 mM glucose at 37°C.

A - The effect on 5-meTHF uptake :-

Region	Deoxycholate concentration (M)	5-me THF Uptake (nmole / g initial tissue wet weight) Serosal	Tissue	Total
Proximal Jejunum	0	0.48 ± 0.05	1.67 ± 0.03	2.15 ± 0.07
	10^{-5}	0.42 ± 0.04	1.58 ± 0.08	2.00 ± 0.08
	10^{-4}	0.15 ± 0.01	0.91 ± 0.07	1.06 ± 0.07
	10^{-3}	0.04 ± 0.01	0.25 ± 0.02	0.29 ± 0.03
Distal Jejunum	0	0.39 ± 0.04	1.55 ± 0.04	1.94 ± 0.07
	10^{-5}	0.31 ± 0.02	1.34 ± 0.12	1.65 ± 0.14
	10^{-4}	0.12 ± 0.01	0.76 ± 0.05	0.88 ± 0.06
	10^{-3}	0.04 ± 0.01	0.26 ± 0.02	0.30 ± 0.02
Proximal ileum	0	0.21 ± 0.02	0.94 ± 0.09	1.15 ± 0.10
	10^{-5}	0.11 ± 0.01	0.45 ± 0.04	0.56 ± 0.04
	10^{-4}	0.09 ± 0.01	0.48 ± 0.04	0.57 ± 0.04
	10^{-3}	0.04 ± 0.01	0.27 ± 0.01	0.31 ± 0.02

TABLE 4.11 (Continued)

B. The effect on water uptake :-

Region	Deoxycholate Concentration (M)	Water Uptake (mg / g initial tissue wet weight)		
		Serosal	Tissue	Total
Proximal jejunum	0	409 ± 72	500 ± 37	909 ± 49
	10^{-5}	380 ± 32	514 ± 32	894 ± 41
	10^{-4}	359 ± 19	496 ± 29	855 ± 32
	10^{-3}	9.2 ± 7	216 ± 26	225 ± 26
Distal jejunum	0	398 ± 46	366 ± 27	764 ± 39
	10^{-5}	273 ± 20	500 ± 11	773 ± 26
	10^{-4}	296 ± 15	435 ± 17	731 ± 15
	10^{-3}	48 ± 12	142 ± 13	190 ± 7
Proximal ileum	0	275 ± 31	469 ± 28	744 ± 57
	10^{-5}	287 ± 17	369 ± 32	656 ± 29
	10^{-4}	246 ± 10	415 ± 16	661 ± 21
	10^{-3}	46 ± 2	78 ± 8	124 ± 9

that elevation in the surface pH will subsequently lead to a decrease in the amount of folate absorbed (Blair and Matty, 1974). To examine this hypothesis, the effect of sodium deoxycholate on the uptake of 5-meTHF was examined. This was performed by incubating everted sacs from the proximal jejunum, distal jejunum and proximal ileum in bicarbonate buffer pH 7.4 in the absence and presence of either 10^{-5}M, 10^{-4}M and 10^{-3}M sodium deoxycholate.

In the presence of 10^{-5}M sodium deoxycholate, an insignificant reduction in the total uptake of 5-meTHF occurred in the proximal and distal jejunum, while a significant reduction occurred in the proximal ileum ($p < 0.001$) (Table 4-11). The presence of 10^{-4}M sodium deoxycholate on the other hand, reduced the total uptake of 5-meTHF to half its normal value in all regions examined. The reduction was in both the serosal and tissue uptake of 5-meTHF. In the presence of 10^{-3}M sodium deoxycholate, a concentration shown to cause structural damage to the intestinal mucosa (see Chapter 3), there was a severe reduction in the serosal, tissue and total uptake of 5-meTHF in all the three regions. The amount of 5-meTHF taken up by the three regions was no longer different.

In all the regions examined, total water uptake, which was measured simultaneously, was not significantly reduced in the presence of 10^{-5}M or 10^{-4}M sodium deoxycholate, but was significantly reduced in the presence of 10^{-3}M deoxycholate ($P < 0.001$ for all the regions). These obervations further indicate that in the presence of 10^{-5}M and 10^{-4}M sodium

deoxycholate the tissue preparations were viable and capable of performing their normal physiological functions, while they had lost most of their viability characteristics when 10^{-3}M sodium deoxycholate was present.

4.3.8. IDENTIFICATION OF THE SPECIES PRESENT IN THE MUCOSAL, SEROSAL AND TISSUE COMPARTMENTS DURING TRANSPORT OF 5-meTHF AND FOLIC ACID :-

Folate species in the serosal and mucosal compartments of everted sacs prepared from the proximal jejunum after incubation for different periods of time in bicarbonate buffer pH 7.4 containing 10^{-6}M 5-meTHF, 3 mg/ml sodium ascorbate and 20 mM glucose at 37°C, were identified as described in Chapter 2. The major band in the mucosal and serosal solutions co-chromatographed, on cellulose pre-coated thin layer chromatograms with 5-meTHF(standard) (Table 4-12). Between 85-94% and 79-94% of the starting material was identified in the mucosal and serosal solutions respectively. It appears that no identified metabolic or chemical alterations occurred to the 5-meTHF molecule during its presence in the mucosal medium and during its transport into the serosal compartment. These conclusions are similar to that reported by other workers (Strum, Nixon, Bertino and Binder, 1971; Razzaque, 1975).

When everted sacs were incubated for 30 minutes in bicarbonate buffer at pH 7.4 containing 20 mM glucose and 10^{-6}M folic acid, no other species were identified in the mucosal compartment at the end of the incubation. Only one peak was eluted from the DE 52 column

TABLE 4-12 : Folate species present in the mucosal and serosal compartments of everted sacs from rat proximal jejunum incubated for different periods of time in bicarbonate buffer pH 7.4 containing 10^{-6} M 5-meTHF, 3mg/ml sodium ascorbate and 20 mM glucose at 37°C. Folate identified on cellulose pre-coated thin layer chromatograms eluted with 0.1 M phosphate buffer pH 7 containing 2% sodium ascorbate.

Incubation time (min)	Percentage identified as 5-meTHF in the mucosal compartment	Percentage identified as 5-meTHF in the serosal compartment
15	94%	89%
30	93%	79%
45	85%	81%
60	85%	94%

TABLE 4-13: Folate species present in the tissue and serosal compartments. Everted sacs from the proximal jejunum were incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 10^{-6}M folic acid [3',5',7,9-^{3}H]- and [2-^{14}C] -labelled folic acid were added as tracers) and 20 mM glucose at 37°C. DEAE cellulose and Sephadex G-15 columns chromatography were used to separate the folate species. The solvent system was 0.05M phosphate buffer pH 7 containing 5 mg% dithiothreitol.

A - In the tissue compartment :-

	Percentage of ^{14}C-labelled Species	Percentage of ^{3}H-labelled Species
Folic acid	39	42
5-meTHF	28	30
10-Formylfolic acid	4.0	8.0
Unidentified species	29.5	20

B - In the serosal compartment :-

	Percentage of ^{14}C-labelled Species	Percentage of ^{3}H-labelled Species
Folic acid	62.6	59
5-meTHF	16.7	15.7
10-Formylfolic acid	13.6	10
$^{3}H_2O$	0	2.4
Unidentified Species	7.1	12.9

which co-chromatographed with a folic acid (standard). However, in the tissue compartment not only folic acid was identified, but also a considerable amount of 5-meTHF together with a small amount of 10-formylfolic acid (Table 4-13). In the serosal compartment, again folic acid was not the only folate species present. Considerable amounts of 5-meTHF and 10-formylfolic acid were also identified. The data is similar to that reported by Olinger, Bertino and Binder (1973) and Selhub, Brin and Grossowicz (1973).

4.3.9. TRANSPORT STUDIES OF THE OXIDATION PRODUCT OF 5-meTHF (A PYRAZINO-s-TRIAZINE DERIVATIVE):-

The prepared unlabelled pyrazino-s-triazine (see Chapter 2) had a maximum absorbance (λ max) at 275 nm and 279 nm at pH 1 and pH 7 respectively, which is in good agreement with the published data (Gapski, Whiteley and Huennekens, 1971). The ^{14}C-labelled pyrazino-s-triazine co-chromatographed with the unlabelled species on cellulose pre-coated thin layer chromatograms using a mixture of n-propanol : water: aqueous ammonia (200:100:1) as the solvent system and gave a calculated R_f value for the compound of 0.34.

The transport of this biologically inactive compound (Gapski, Whiteley and Huennekens, 1971; Kennelly, Blair and Pheasant, 1979a) as a function of increasing mucosal concentration was studied by incubating everted sacs from the proximal jejunum for 30 minutes in bicarbonate buffer at pH 7.4 containing 20mM glucose at 37°C.

TABLE 4-14 :- The serosal, tissue and total uptake of a pyrazino -s- triazine derivative by everted sacs from the proximal jejunum incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 20mM glucose, as a function of increasing mucosal concentration, at 37°C.

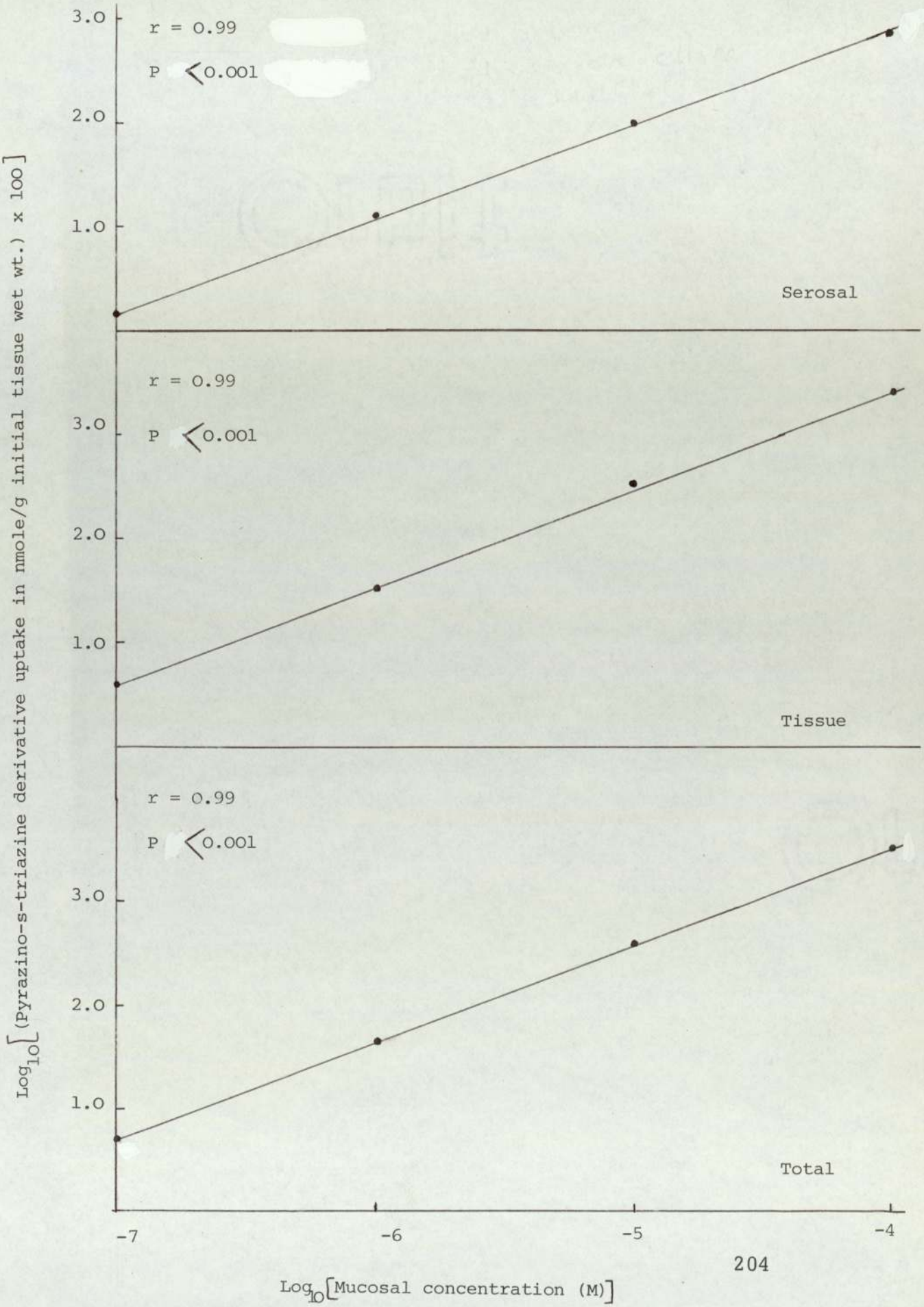
A - Pyrazino-s-triazine derivative uptake :-

Initial mucosal concentration (M)	Pyrazino-s-triazine Uptake (n mole / g initial tissue wet weight)		
	Serosal	Tissue	Total
10^{-7}	0.01 ± 0.006	0.04 ± 0.01	0.05 ± 0.01
10^{-6}	0.13 ± 0.01	0.31 ± 0.02	0.44 ± 0.02
10^{-5}	0.98 ± 0.03	3.0 ± 0.07	3.98 ± 0.1
10^{-4}	7.67 ± 0.6	25.0 ± 1.9	32.67 ± 2.4

B - Water uptake :-

Initial mucosal concentration(M)	Water Uptake (mg/g initial tissue wet weight)		
	Serosal	Tissue	Total
10^{-7}	178 ± 6	548 ± 28	726 ± 29
10^{-6}	180 ± 16	443 ± 40	623 ± 31
10^{-5}	196 ± 14	472 ± 30	668 ± 25
10^{-4}	178 ± 15	459 ± 19	637 ± 26

Figure 4.4: The serosal, tissue and total uptake of pyrazino-s-triazine derivative as a function of increasing mucosal concentration. Everted sacs from the proximal jejunum were incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 20 mM glucose at 37°C.



There was a linear increase (r = 0.99) in the serosal, tissue and total uptake of pyrazino-s-triazine with increased mucosal concentration (Table 4-14 and Figure 4-4).

4.4. DISCUSSION :-

4.4.1. KINETIC STUDIES ON THE ABSORPTION OF 5-meTHF AND FOLIC ACID :-

No conclusive evidence has been presented to date concerning the mechanism of folate absorption by the mammalian intestine. Two transport mechanisms have been proposed, a facilitated passive diffusion through a surface acid microclimate and an active specific carrier mechanism.

The serosal, tissue and total uptake of 10^{-5}M 5-meTHF increased with time up to 60 minutes. The tissue uptake was higher than the serosal uptake and indicates that the tissue has the ability to accumulate 5-meTHF.

The tissue uptake of 5-meTHF occurred in two stages; an initial rapid binding stage followed by a relatively slower accumulation process. The initial rapid binding of 5-meTHF to the tissue preparation might imply a binding onto a specific protein in the brush border membrane of the epithelial cells as has been suggested by Leslie and Rowe (1972). Recently a folate specific binding protein has been identified and isolated from the brush border membrane of the intestinal epithelium (Selhub, Gay and Rosenberg, 1979; Noronha and Kesavan, 1979).

Oral ingestion of 5-meTHF results in maximum serum folate levels after 30 to 45 minutes followed by a subsequent drop 1 hour after ingestion. The brush border protein bound folate activity also increased rapidly after ingestion of 5-meTHF reaching a maximum value after 30 minutes. However, it remained unaltered for at least 5 hours after folate ingestion (Noranha and Kesavan, 1979). The data suggests that the brush border folate binding protein has no role in the transport mechanism of folate. Folate brush border binding therefore might represent a mechanism for its efficient uptake when the tissue or body is folate deficient or it might represent a mechanism for its storage when food is rich in folate.

Tissue accumulation of 5-meTHF can also be interpreted to suggest a binding onto a specific protein(s) or macromolecule(s) within the cells. A support for this suggestion comes from the observation that methotrexate the inhibitor of dihydrofolate reductase, decreases the concentration of folate within the intestinal epithelial cells while increasing the transmural transport across the epithelium (Selhub, Brin and Grossowicz, 1973; Olinger, Bertino and Binder, 1973). Such observations have been interpreted by a model in which methotrexate saturates intracellular binding sites and facilitates the movement of folate across the epithelium (Rosenberg, 1976).

The appearance of 5-meTHF in the serosal compartment was slow as the folate has to traverse the tissue compartment before reaching the serosal fluid, a process which takes approximately 8 minutes. After

this lag, the uptake was found to increase linearly with time up to 60 minutes.

The intestinal transport of 5-meTHF therefore appears to involve three stages; an initial rapid binding stage; an accumulation stage within the tissue, and a slower transfer from the tissue into the serosal compartment.

The serosal, tissue and total uptake of 5-meTHF was found to increase linearly with increasing mucosal concentrations (10^{-7}M to 10^{-4}M) over the pH range 5 to 7.4 (Figure 4-2). The lack of saturation supports the concept of a passive diffusion mechanism for 5-meTHF.

The kinetic observations concerning folic acid showed that serosal, tissue and total uptake of folic acid saturated at a concentration between 5×10^{-6}M and 10^{-5}M. These observations are consistent with previous reports (Blair, Johnson and Matty, 1974; Rose, Koch and Nahrwold, 1978; Smith, Matty and Blair, 1970). Other workers have demonstrated saturation in the uptake at either lower or higher concentrations (Russell, Dhar, Dutta and Rosenberg, 1979; Burgen and Goldberg, 1962). Others who failed to report saturation in the uptake of folic acid (Yoshino, 1968; Hepner, Booth, Cowan, Hoffbrand, and Mollin, 1968) worked in the low concentration range at which a linear increase has been observed. The demonstration of saturation kinetics of a solute over a wide range of luminal solute concentrations is frequently cited as evidence for a specific carrier mediated absorption process.

However, an alternative explanation which correlates with the experimental data is based on the limited solubility of folic acid in the surface acid microclimate (Blair and Matty, 1974).

The tissue compartment was shown to accumulate folic acid and 5-meTHF with respect to the mucosal and the serosal compartments when the concentration of folic acid and 5-meTHF in the bathing medium was 10^{-6}M or less. This observation cannot be taken as evidence for the involvement of an active transport system because the folate molecule is known to bind to different sites in the tissue, e.g. the specific protein in the brush border membrane (Noronha and Kesavan, 1979) and the intracellular protein(s) and macromolecular sites (Rosenberg, 1976).

The total uptake of 5-meTHF was significantly higher at pH 6 than at pH 7.4 principally as a result of increased tissue uptake. This observation is consistent with that reported by Russell, Dhar, Dutta and Rosenberg (1979) and Kesavan and Noronha (1978).

No net movement of folic acid against a concentration gradient was observed (Table 4-9). On the other hand folic acid accumulated in the tissue when there was equal concentration in the mucosal and serosal compartment. Many factors should be taken into account when interpreting the observations. Firstly, water movement will cause a greater change in the serosal concentration of folic acid than the mucosal concentration. Secondly, the binding of folic acid onto specific sites in the tissue was found to occur preferentially from

the mucosal side. Thirdly, folic acid metabolism by the intestinal epithelial cells into other folate species has been shown to occur (Table 4-13). These factors explain why that with a zero concentration gradient at pH 6 or in the presence of 200 mM mannitol of pH 7.4 the serosal/mucosal folic acid concentration ratio after 30 minutes incubation was slightly higher than unity. Other workers have also reported the lack of movement of folic acid against a concentration gradient (Smith, Matty and Blair, 1970; Turner and Hughes, 1962). Hepner et al., (1968) and Hepner (1969) measured the increase in serum folate level in man and rat after perfusing folic acid in the small intestine and claimed to have demonstrated absorption of folic acid against a concentration gradient. However, because the liver converts folic acid to 5-meTHF (Whitehead and Cooper, 1967), it is likely that the folic acid was transported into the serum against a 5-meTHF gradient rather than a folic acid gradient.

Other workers have shown that 5-meTHF does not move against a concentration gradient (Strum, Nixon, Bertino and Binder, 1971; Blair, Matty and Razzaque, 1975).

The fact that there is no competition between folic acid and 5-meTHF (Table 4-10) suggests that either (i) the two compounds do not share a common carrier, or (ii) that the transport mechanism is different, or (iii) that they share the same transport mechanism but that this mechanism is not affected by their presence together.

Similar rates of transport of folic acid and 5-meTHF were observed in the proximal jejunum (Table 4-5). This indicates that the transport is not affected by (i) a change in the stereochemistry at the C-6 site from a planar trigonal to a tetrahedral configuration, (ii) a reduction of the pyrazine ring to tetrahydro form and (iii) a change from planar pyrazine ring to the non-planar tetrahydropyrazine ring.

4.4.2. THE POSSIBLE INVOLVEMENT OF A SPECIFIC CARRIER AND WATER MOVEMENT IN THE TRANSPORT MECHANISM OF FOLIC ACID AND 5-meTHF :-

The observations presented in this study on the transport of 5-meTHF which include (i) the lack of saturation in its serosal, tissue and total uptake over a wide range of concentrations in the mucosal medium and (ii) the lack of an inhibitory effect on the uptake by another folate analogue (folic acid) together with the observations of other workers of (iii) a low Q_{10} (Blair, Matty and Razzaque, 1975), (iv) no transport against a concentration gradient (Strum, Nixon, Bertino and Binder, 1971; Blair, Matty and Razzaque, 1975) and (v) the lack of stereospecificity around the C-6 atom of the pteridine ring (Kennelly, Blair and Pheasant, 1979b) all argue against the concept of the involvement of an active transport mechanism and favour a passive diffusion transport mechanism.

There are also a number of experimental observations which are inconsistent with the involvement of a specific transport carrier for folic acid; (i) the lack of folic acid transport against a concentration gradient, (ii) the similarity in the rate of transport of folic acid and

5-meTHF across the proximal jejunum, (iii) the lack of competition between folic acid and its analogue 5-meTHF, and (iv) a low Q_{10} (Blair, Johnson and Matty, 1974).

Weir, Brown, Freedman and Scott (1973) after an oral administration of both active and inactive diastereoisomers of 5-meTHF to human subjects found that a greater secretion of the active than the inactive form in urine. On this basis they suggested that a carrier transport mechanism is involved in the absorption of 5-meTHF. However, in their experiments, the body was presented with a large dose of active diastereoisomer (300 µg orally plus metabolites from 15 mg parenteral folic acid) but only with a small dose of the inactive diastereoisomer (300 µg orally; no contribution from parenteral folic acid). This consequently results in more excretion of the active diastereoisomer as Blair and Dransfield (1971) showed that the percentage urinary excretion of folic acid was very much greater with large doses than with small doses.

Solvent drag with water movement also appeared to have no role in the transport of 5-meTHF . Decreasing the pH of the incubation medium from 7.4 to 5 caused an increase in the serosal uptake of 5-meTHF while the serosal water uptake was severely inhibited. Tissue uptake also increased but water transport to the tissue was not affected. Sodium deoxycholate (10^{-4}M) caused a severe inhibition in the serosal and tissue uptake of 5-meTHF without significantly reducing water uptake. Both of these observations together with the fact that

there was a very poor correlation between tissue uptake of 5-meTHF and water movement (Figure 4-1) suggest that water movement played no role in the transport of 5-meTHF.

Addition of 200 mM mannitol to a 10^{-6}M folic acid incubation medium diminished serosal water uptake and significantly decreased serosal folic acid uptake. However, because tissue uptake of folic acid increased, the total uptake was not affected. Therefore, water movement may participate in the transport of small amounts of folic acid from the tissue compartment into the serosal fluid but not from the mucosal to the serosal fluid. Similar conclusions have been drawn by Blair, Lucas and Swanston (1979).

4.4.3. THE POSSIBLE ROLE OF THE SURFACE ACID MICROCLIMATE IN FOLIC ACID AND 5-meTHF ABSORPTION :-

Folic acid and 5-meTHF exist in aqueous solution at the pH of the intestinal chyme(pH 6 to 7) in their anionic forms. (Table 1-2 and Figure 1-5). Movement of these anions across the negatively charged phospholipid membrane would be expected to be prevented by strong electrostatic repulsion. Repulsion could be avoided by conversion to a neutral species (Zwitterion in case of 5-meTHF) (Figure 1-4) in the surface acid microclimate of the small intestine. The surface acid microclimate has been assumed to have a pH of less than 4 in the proximal jejunum (Blair and Matty, 1974). At this pH approximately 50% of 5-meTHF and folic acid would be present in its neutral forms.

The surface acid microclimate may therefore play an essential role in the mechanism of folate absorption. The observed saturation of folic acid transport, but linear transport of 5-meTHF could be explained on the solubility basis of these compounds in aqueous media at the pH of the surface acid microclimate (Table 1-1). The inhibitory effect of both sodium removal from the bathing medium and metabolic inhibitors, on the uptake of folic acid and 5-meTHF (Rose, Kach and Nahrwold, 1978; Strum, 1979; Razzaque, 1975) can therefore be explained by their inhibitory action on the surface acid microclimate. An elevation in the surface pH has been demonstrated under these conditions (see Chapter 3) which therefore reduces the amount of folate species in their neutral form.

A support for the involvement of the surface acid microclimate in the absorption of folates also comes from the work of Kesavan and Noronha (1978). These workers showed that the uptake of 5-meTHF in rat in vitro is inhibited by whole body X-irradiation and by the addition of 50 mM sodium azide to the incubation medium at pH 6.5. They also showed that the inhibitory effect of X-irradiation could be restored by the addition of ATP to the incubation medium. 50 mM sodium azide has been shown to inhibit the surface acid microclimate (see Figure 3-13). Further, X-irradiation depletes intracellular ATP levels (Kesavan and Noronha, 1971) and such conditions have been shown to lead to an elevation in the surface pH (see Chapter 3). The data strongly supports a role of the surface acid microclimate in the mechanism of absorp-

tion of folate.

4.4.4. THE SITE OF FOLATE ABSORPTION :-

The preferential site of folic acid and 5-meTHF absorption was found to be the proximal jejunum though considerable amounts of 5-me-THF were also absorbed across the distal jejunum. The observations explains why proximal jejunum diseases or resection are usually associated with folate malabsorption, while distal intestinal resection has far less influence on folate absorption (Booth, 1961; Rosenberg, 1976).

Equal amounts of folic acid and 5-meTHF were transported across the proximal jejunum (Table 4-5). This observation further supports the involvement of the surface acid microclimate in folate absorption as equal amounts of the neutral forms of folic acid and 5-meTHF are present at the surface acid microclimate pH of the proximal jejunum (less than pH 4) (see Table 1-2).

In the more distal regions of the small intestine a decline in the uptake of 5-meTHF and folic acid was observed. The decline was more sharp and rapid in the case of folic acid compared to that of 5-meTHF. This can be attributed to the observed decline in the surface acidity down the intestine (Figure 3-4)and that aslight increase in surface pH (for example from pH 3.5 to 4.5) will cause a great decrease in the amount of the neutral form of folic acid compared to that of 5-meTHF (Table 1-2).

The decrease in the surface area down the intestine and its part-

icipation in the observed decline in the absorption of folate should not be ignored.

4.4.5. THE EFFECT OF SODIUM DEOXYCHOLATE ON THE ABSORPTION OF FOLATE :-

As the surface acid microclimate plays an important role in the mechanism of folate absorption, any factor that causes an elevation in its pH would eventually lead to a decrease in the absorption of these compounds. 10^{-4}M deoxycholate, the unconjugated dihydroxy bile acid, was found to cause a significant elevation in the surface pH by damaging the glycocalyx without affecting the viability of the preparation (Chapter 3). The same concentration of this bile acid was found to reduce the total uptake of 5-meTHF to half its normal control value by decreasing both the tissue and the serosal uptake of the vitamin without significantly affecting the total water uptake. These observations further support the important role the surface acid microclimate plays in the absorption of folate and that the transport of folate is unrelated to water movement.

The effect of 10^{-3}M deoxycholate on the uptake of 5-meTHF and water is due to the severe damaging effect on the tissue integrity which made the tissue lose most of the characteristics of a viable preparation.

These effects of sodium deoxycholate on the intestinal function and integrity, particularly that of the mucosal surface, are of great clinical importance. This is because, if bacterial invasion to the proximal small intestine occurs, deconjugation and reduction of the

predominant conjugated trihydroxy bile salt would be extensive (Hill, 1975; 1977; 1978). Therefore, the concentration of the unconjugated dihydroxy bile acid, which is the most powerful damaging physiological detergent, would increase. This increase will lead to structural damage to the intestinal surface (i.e. the glycocalyx) and a subsequent elevation in the surface pH which leads to malabsorption of folates. This suggestion would explain the malabsorption of folates which occurs in tropical sprue (Corcino, Coll and Klipslein, 1975; Lindenbaum, 1979) and ulcerative colitis (Franklin and Rosenberg, 1973) as jejunal colonization by coliform bacteria occurs in almost all patients with tropical sprue (Tomkins, James and Drasar, 1975; Klipstein, Engert and Short, 1978) and in many patients with ulcerative colitis (Dickinson, Varian, Axon and Cooke, 1980).

4.4.6. INTESTINAL METABOLISM OF FOLATES :-

No metabolic alteration to 5-meTHF occurs during its transport to the serosal compartment (Table 2-12). By contrast considerable amounts of folic acid undergo metabolic alterations (Table 4-13).

The first and essential step in the metabolism of folic acid is the enzymatic reduction of the pyrazine ring of the pteridine unit of the molecule into dihydrofolic acid and then into tetrahydrofolic acid (Figure 4-5). The enzyme resonsible for such reduction is dihydrofolate reductase which is present in the intestinal epithelial cells (Olinger, Bertino and Binder, 1973). Following reduction a series of enzymatic

reactions through which different folate co-enzymes including 5-meTHF and 10-formyltetrahydrofolic acid could be produced (see Figure 4-5).

10-Formylfolic acid is one of the folate derivatives which is not present naturally in the body (Blakely, 1969). Its appearance in the tissue and serosal compartment is most probably the result of atmospheric oxidation of the metabolically produced 10-formyltetrahydrofolic acid.

Experiments with methotrexate demonstrated that folate transport occurs in the presence of a fully inhibited metabolising system (Olinger, Bertino and Binder, 1973, Selhub, Brin and Grossowicz, 1973). Since it is clear that transport of folic acid can proceed without metabolic alteration, neither reduction nor one carbon substitution of monoglutamyl folate are obligatory for its transport across the intestine.

Reduction by dihydrofolate reductase and one carbon substitution are both saturable systems and therefore a high concentration of folic acid will saturate the reduction system. For this reason most of the folic acid transported is unmodified. At concentrations within the capacity of the enzymic system a substantial portion of the transported folic acid will be reduced and methylated. This would explain why some workers were unable to show metabolic alteration to folic acid during transport (Whitehead and Cooper, 1967; Pratt and Cooper, 1971), while others did (Olinger, Bertino and Binder, 1973; Selhub, Brin and Grossawicz, 1973; Perry and Chanarin, 1973).

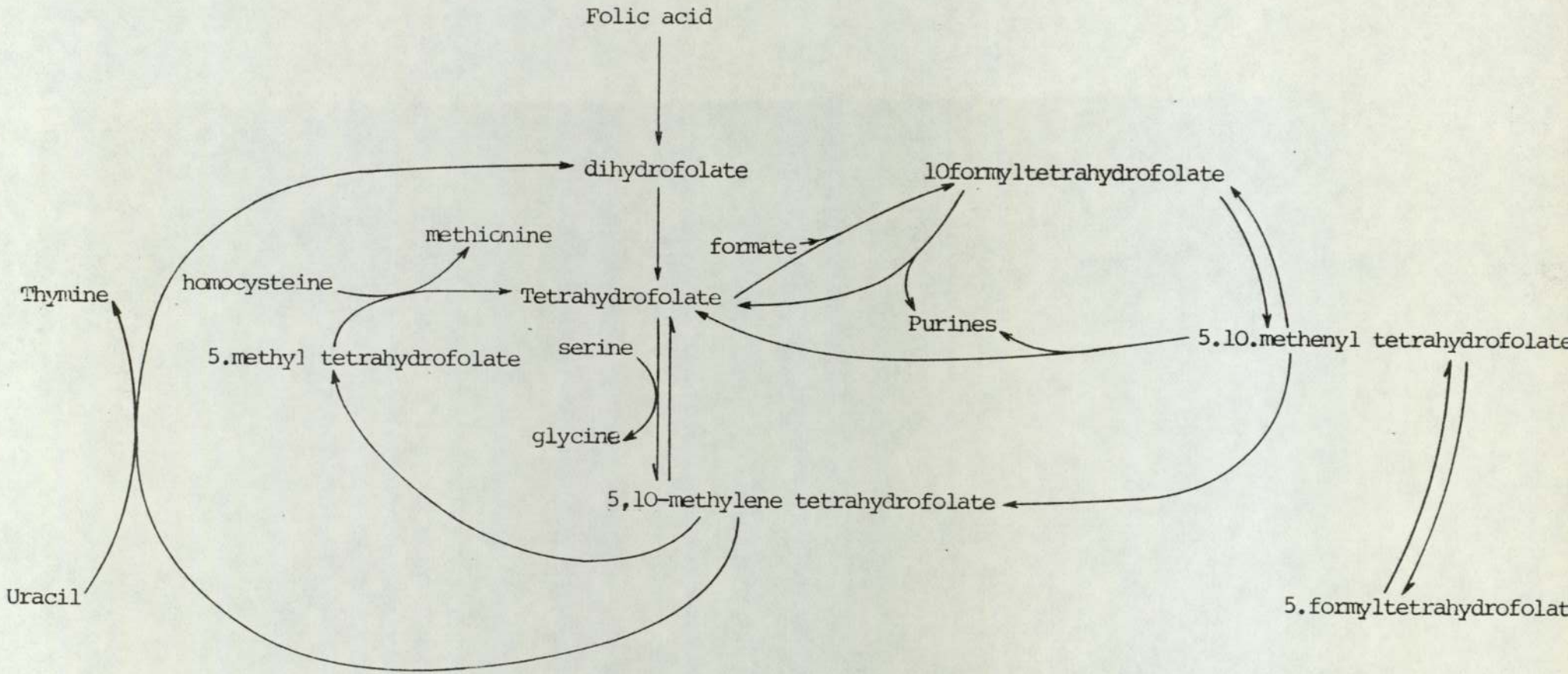


Figure 4-5 Interconversions of the folate coenzymes

(From Rowe, 1978)

CHAPTER FIVE

ISOTOPIC EFFECT IN STUDIES USING RADIOLABELLED FOLATE TRACERS

5.1 INTRODUCTION

The use of isotopically labelled compounds as metabolic tracers for *in vivo* and *in vitro* studies is particularly important when biological materials occur in transient and/or trace amounts as is the case for the folate co-enzymes. However, the chemical or physical behaviour of isotopically labelled compounds is not always identical to that of the corresponding unlabelled compounds. The difference in the behaviour is called an isotopic effect. The isotopic effect could be either primary, where there is breaking or formation of isotopically different bonds, or secondary, where the isotopically different chemical bonds are not directly involved, i.e. not broken or formed during the rate process being studied.

Secondary isotope effects have been reported for tritium in biological and non-biological systems. 1,2-^{3}H testosterone has a higher binding affinity for the sex-binding globulin than 4-^{14}C-testosterone (Caputo, Hosty and Melton, 1973). There is a relatively greater rate of urinary excretion of ^{3}H-folic acid and ^{3}H-5-meTHF after oral doses of ^{3}H and ^{14}C-labelled folic acid and 5-meTHF (Connor, Blair and Said, 1980; Kennelly, 1980).

In non-biological systems, again many examples are available such as the difference in the chromatographic behaviour of ^{3}H and ^{14}C-labelled folic acid on DEAE-cellulose and Sephadex G-15 columns (Connor, Blair and Said, 1980); Several ^{3}H and ^{14}C-labelled amino acids (Klein

and Szczepanik, 1967) and ^{3}H and ^{14}C-labelled 2-aminopurine (Gottschling and Freese, 1962). More examples of an isotopic effect for tritium both in biological and non-biological systems were reported by Evans (1974).

In these studies the absorption rate of ^{3}H and ^{14}C-labelled and unlabelled folic acid and other folate species by everted sacs were investigated in order to determine whether an isotopic effect existed in these systems.

5.2. MATERIALS AND METHODS :-

Folic acid ^{3}H-labelled (at the 3',5',7,9 positions with 32% at C-9, 25.5% at C-7 and 42.5% at C-3' and C-5'; and at the 7,9 positions only with 41% at C-9 and 59% at C-7) and ^{14}C-labelled folic acid at the C-2 position of the pteridine ring, were obtained from the Radiochemical Centre - Amersham. The 2-^{14}C-10-formylfolic acid, [3',5',7,9-^{3}H]-10-formylfolic acid, [2-^{14}C]-pterin-6-carboxylic acid (pterin-6-COOH) and [7-^{3}H]-pterin-6-COOH were prepared and purified before use by DEAE-cellulose and Sephadex G-15 columns (Connor, 1979). The serosal content of ^{3}H and ^{14}C-labelled species was examined by high performance liquid chromatography (HPLC) as described by Connor, Blair and Said (1980).

All other details concerning the materials and methods are mentioned in Chapter 2.

5.3. RESULTS :-

5.3.1. THE UPTAKE OF ^{3}H and ^{14}C-LABELLED FOLIC ACID AS A FUNCTION OF INCREASING MUCOSAL CONCENTRATION :-

Everted sacs prepared from rat proximal jejunum (sacs 2 and 3) were incubated for 30 minutes in bicarbonate buffer pH 7.4 at 37°C containing 20 mM glucose and different concentrations of folic acid (0.1 μCi each of [2-^{14}C] and [3',5',7,9-^{3}H] labelled folic acid was added). The purity of the radioactively labelled folic acid was checked on DEAE-cellulose columns before use.

As folic acid uptake versus a wide range of concentrations had been previously performed (see Figure 4-3 and Table 4-8) and because the only aim of this study is to compare the rate of uptake of ^{3}H and ^{14}C-labelled folic acid, only two concentrations within the linear and one within the saturated uptake range were used.

Over the concentration range 10^{-7} - 10^{-5}M there was more serosal, tissue and total uptake of ^{3}H-labelled than the ^{14}C-labelled species (Table 5-1). The ratio of the $^{3}H/^{14}C$ labelled species appearing in the serosal compartment increased as the concentration of folic acid in the incubation medium increased. This increase in the serosal $^{3}H/^{14}C$ ratio lead to an increase in the total $^{3}H/^{14}C$ ratio while the tissue ratio remained the same (Table 5-1).

The Km and Vmax of the transport process for both labelled species was calculated using the Lineweaver-Burke plot. The best straight line

TABLE 5-1: A-The uptake of dual labelled folic acid at different concentrations by everted sacs prepared from the proximal jejunum and incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 20mM glucose at 37°C. 0.1 μCi of 2-^{14}C labelled and 0.1 μCi of [3',5',7,9-^{3}H] labelled folic acid was added as tracer.

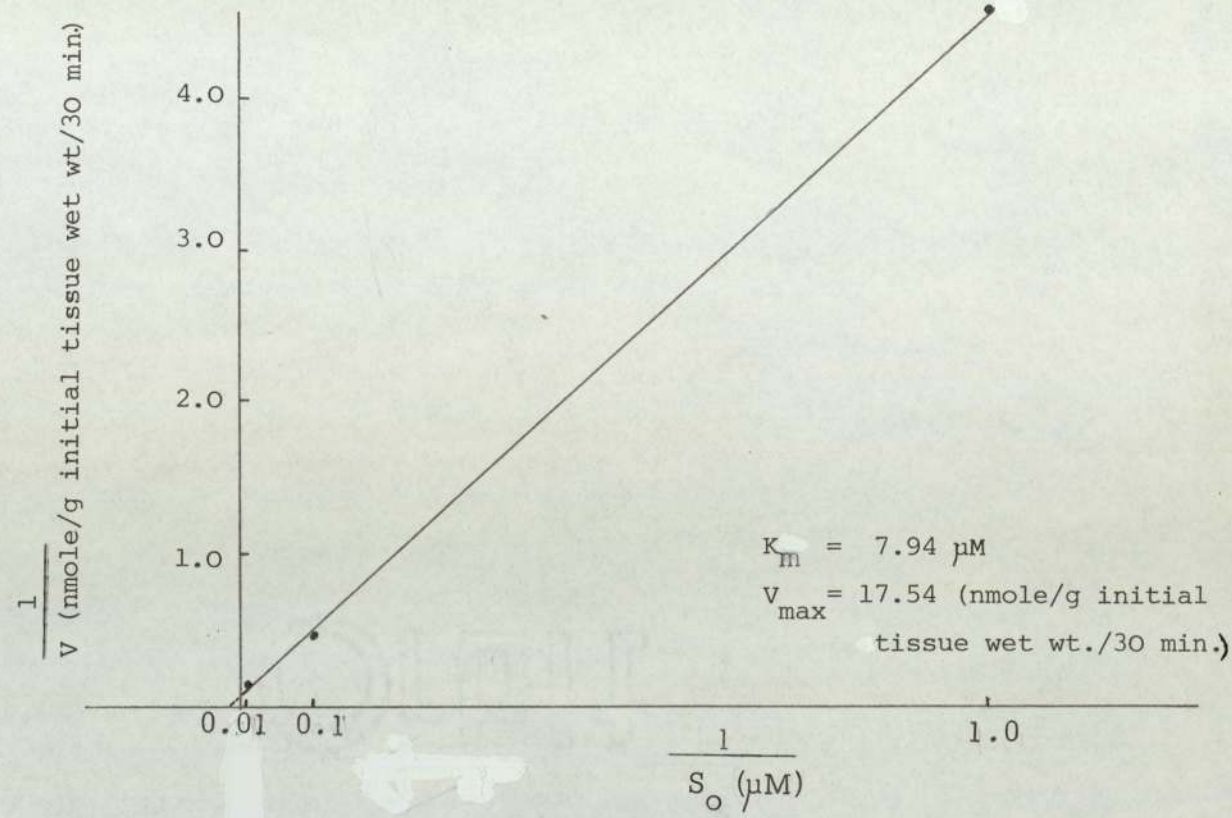
Mucosal concentration(M)	^{3}H-folic acid uptake (nmole/g initial tissue wet wt.)			2-^{14}C-folic acid uptake (nmole/g initial tissue wet wt.)			Water uptake (mg/g initial tissue wet wt.)		
	Serosal	Tissue	Total	Serosal	Tissue	Total	Serosal	Tissue	Total
10^{-7}	0.07±0.01	0.17±0.01	0.24±0.02	0.07±0.01	0.15±0.01	0.22±0.01	491±42	609±16	1100±43
10^{-6}	0.59±0.04	1.81±0.10	2.40±0.12	0.46±0.04	1.65±0.09	2.11±0.11	550±60	515±16	1065±66
10^{-5}	1.98±0.16	6.41±0.32	8.39±0.42	1.50±0.12	5.90±0.37	7.40±0.34	595±61	561±20	1155±60

B-The ^{3}H:^{14}C ratio for the serosal, tissue and total folic acid uptake :-

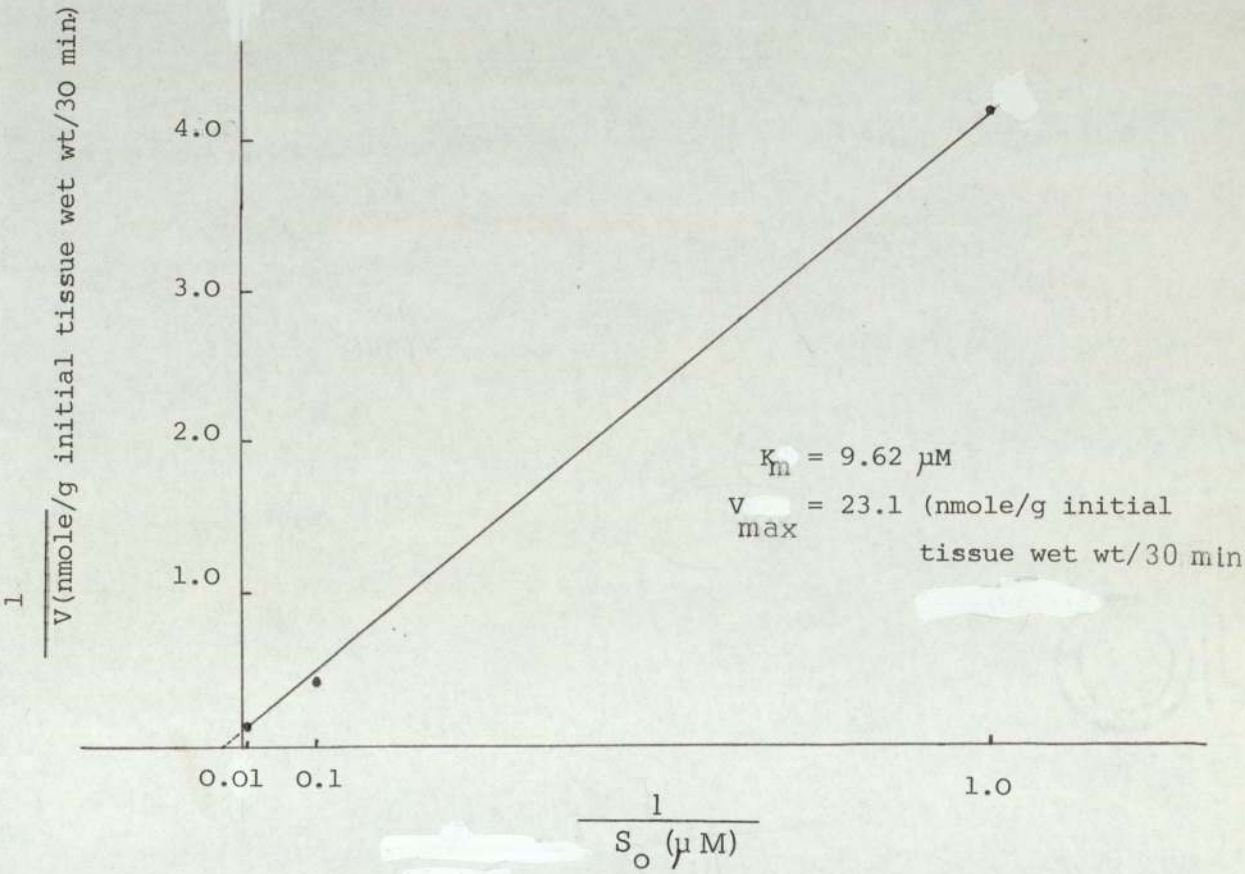
	Serosal	Tissue	Total
10^{-7}	1.08:1	1.10:1	1.10:1
10^{-6}	1.28:1	1.10:1	1.14:1
10^{-5}	1.32:1	1.10:1	1.14:1

Figure 5-1: Lineweaver-Burk plot for the total uptake of 2-^{14}C and [3',5',7,9-^{3}H] -labelled folic acid

A- 2-^{14}C-folic acid



B- [3',5',7,9-^{3}H]folic acid



was calculated by the least squares method (Figure 5-1). The Km and Vmax for the total uptake of ^{14}C-labelled folic acid was found to be 7.94 μM and 17.54 nmole/g initial tissue wet wt./30 minutes, respectively; while those of ^{3}H-labelled folic acid were 9.62 μM and 23.1 nmole/g initial tissue wet wt./30 minutes, respectively. The Km and Vmax observed in these studies were higher than that observed by Smith, Matty and Blair (1970) (0.7 μM and 2.3 nmole/g initial tissue wet wt./30 minutes, respectively).

5.3.2. THE RELATIVE AMOUNTS OF ^{3}H-LABELLED, ^{14}C-LABELLED AND UNLABELLED FOLIC ACID, 10-FORMYLFOLIC ACID AND PTERIN-6-COOH TRANSPORTED INTO THE SEROSAL COMPARTMENT OF EVERTED SACS :-

Experiments were designed in order to determine which isotopically labelled folic acid species behaved most like the unlabelled compound and to determine the site(s) of labelling important to the isotopic effect.

Everted sacs prepared from rat proximal jejunum (sacs 2 and 3) were incubated for 30 minutes at 37°C in bicarbonate buffer pH 7.4 containing 20 mM glucose and 10^{-6}M of either folic acid ([2-^{14}C] - folic acid and either [3',5',7,9-^{3}H] or [7,9-^{3}H] folic acid and unlabelled folic acid), 10-formylfolic acid ([2-^{14}C] - 10-formylfolic acid [3',5',7,9-^{3}H] 10-formylfolic acid and unlabelled 10-formylfolic acid , or pterin-6-COOH ([2-^{14}C] pterin-6-COOH, [7-^{3}H] pterin-6-COOH and unlabelled pterin-6-COOH). 0.1 μCi of each isotope was added.

The combined serosal fluid from three sacs was subjected to HPLC to separate the starting compound from any metabolites formed. The $^{3}H/^{14}C$ ratios and specific activities of the starting compound were then determined. The results are shown in table 5-2. The ^{14}C-specific radioactivities of all compounds were the same in both the serosal and the mucosal compartments after incubation. It is clear that more ^{3}H-labelled folic acid entered the serosal compartment than the ^{14}C-labelled species which entered at a similar rate to that of unlabelled folic acid.

Similarly more [3',5',7,9-^{3}H] 10-formylfolic acid entered the serosal compartment than [2-^{14}C] labelled species which entered at a similar rate to the unlabelled 10-formylfolic acid. However, the serosal $^{3}H/^{14}C$ ratio was less than that observed with folic acid (Table 5-2). In all the cases mentioned, i.e. with folic acid and 10-formylfolic acid experiments, the mucosal solutions were checked at the end of each experiment and found to contain only one significant peak (the starting compound) containing slightly excess ^{14}C over ^{3}H (about 91% ^{3}H-labelled species) (Connor, 1980).

On the other hand, pterin-6-COOH showed no sign of an isotopic effect, i.e. the rate of entry of 2-^{14}C-labelled, 7-^{3}H-labelled and the unlabelled species were the same. Also the mucosal solution at the end of experiment contained one peak (the starting compound) with similar amounts of ^{3}H and ^{14}C.

TABLE 5-2 : The relative amounts of ^{3}H-labelled, ^{14}C-labelled and unlabelled pterins transported into the serosal compartment of everted sacs prepared from the proximal jejunum and incubated for 30 minutes at 37°C in bicarbonate buffer containing 20 mM glucose and 10^{-6}M of each species. 0.1 μCi of each isotope was added. The Serosal content was determined by HPLC.

Compound and isotope mixture	^{3}H:^{14}C ratio		^{14}C:absorbance ratio	
	Starting Compound	Serosal compound	Starting compound	Serosal compound
[2-^{14}C] folic acid, [3',5',7,9-^{3}H]-folic acid. and unlabelled folic acid	1 : 1	1.25:1	1 : 1	1 : 1
[2-^{14}C]-folic acid[7,9-^{3}H] folic acid and unlabelled folic acid	1 : 1	1.29:1	1 : 1	1 : 1
[2-^{14}C] 10-formylfolicacid [3',5',7,9-^{3}H]-10 formylfolic acid and unlabelled 10-formylfolic acid	1 : 1	1.10:1	1 : 1	1 : 1
[2-^{14}C]-pterin-6-COOH, [7-^{3}H] pterin-6-COOH and unlabelled pterin-6-COOH	1 : 1	0.98:1	1 : 1	1 : 1

5.4. DISCUSSION :-

It has been reported that after an oral administration to rats of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (10-100 µg per Kg body wt.) the urine collected between 0-48 hours after administration contained a large excess of ^{3}H over ^{14}C, whereas faecal samples contained more ^{14}C than ^{3}H compared with the initial oral dose (Connor, Blair and Said, 1980; Connor, 1979). Chromatographic analysis on DEAE-cellulose and Sephadex G-15 revealed that the catabolites present in the urine that were labelled with ^{3}H - only ($^{3}H_2O$, p-acetamidobenzoate and p-acetamidobenzoyl-L-glutamate) accounted for only 50% of the ^{3}H excess, the residue being due to an enhanced ^{3}H to ^{14}C ratio of the intact folate excreted (largely 5-meTHF, 10-formyltetrahydrofolic acid and unmetabolised tracer with the latter representing 20% of the excess (Connor, 1979). The same experimental observations have been reported for man (Saleh, Pheasant, Blair and Allan, 1980).

Similarly excess of ^{3}H over ^{14}C radioactivity was recovered as intact folates excreted in the urine after dosing rats with 5-methyl-[2-^{14}C]-THF and 5-methyl-[3',5',7,9-^{3}H]-THF (8 µg/Kg body wt.) (Kennelly, 1980). The ^{3}H/^{14}C ratio of recovered 5-meTHF was between 1.3 - 1.5).

In the in vitro situation (Table 5-1) the serosal, tissue and total uptake of [3',5',7,9-^{3}H] folic acid was more than that of 2-^{14}C-folic acid. The serosal uptake of ^{3}H-labelled species was relatively

higher than the tissue uptake particularly at the higher concentration. This indicates that there were two sites at which discrimination between isotopes occurs. The first is at the entry of folate molecule into the everted sac, i.e. at the luminal cell surface or in the cell membrane, and the second is in the tissue being responsible for a further increase in $^{3}H/^{14}C$ ratio of folate appearing on the serosal side.

When everted sacs were incubated with mixtures of [2-^{14}C] [7-^{3}H] pterin-6-COOH and unlabelled pterin-6-COOH, the $^{3}H/^{14}C$ ratio remained constant in the serosal and mucosal compartments. This indicates that the important site of labelling seems to be position 9, although this assumes that the pterin-6-COOH is transported by a similar mechanism to that of folate.

In all the cases examined, the ^{14}C-labelled compounds were transported at a similar rate to the unlabelled species (Table 5-2).

The isotopic effect of folate is not confined to the biological system as ^{3}H-folic acid molecule was found to elute slightly faster than ^{14}C-labelled molecules from DEAE cellulose ion exchange and Sephadex G-15 columns (Connor, Blair and Said, 1980; Connor, 1979). Again the important site appeared to be the ^{3}H-labelling at C-9.

The isotopic effect observed with folate in biological and non-biological systems is a secondary isotopic effect as the C-^{3}H is not affected by any reaction, i.e. not broken.

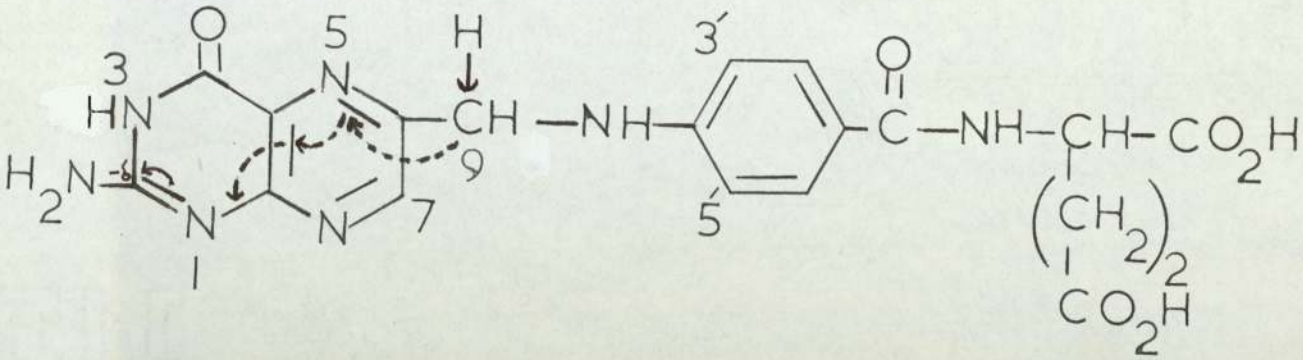
The mechanism(s) behind the higher rate of ^{3}H-folic acid uptake

compared to that of ^{14}C-labelled and unlabelled species needs more investigations to be fully elucidated. However, two theoretical possibilities might be responsible. The first is based on the electronegativity of ^{3}H and its subsequent effect on the amount of folic acid present in the neutral form at the pH of the surface acid microclimate. Hyperconjugation from C-9 is possible in the folic acid molecule (see Figure 5-2A). In hyperconjugation, tritium is less electron donating than hydrogen (Melander and Saunders, 1980; Jencks, 1969). In the 2-^{14}C folic acid molecule, the presence of hydrogen at position 9 will lead to a relatively greater change in the electron density around C-2 of the pteridine ring compared to the ^{3}H-labelled folic acid at position 9, i.e. a relatively greater partial negative charge will be created at C-2 in the presence of hydrogen than tritium at position 9 (Figure 5-2A). This effect will lead to a slight increase in the pKa of the amino group attached to C-2 in the case of 2-^{14}C folic acid compared to the ^{3}H-labelled folic acid at position 9, and subsequently to a decrease in the percentage of the neutral form of 2-^{14}C folic acid, compared to the ^{3}H-labelled folic acid at position 9, available at the pH of the surface acid microclimate (see Table 1-5). Therefore, more ^{3}H-folic acid will be absorbed than the ^{14}C-labelled and the unlabelled folic acid at all concentrations as found experimentally.

The second possibility assumes that a carrier system is involved in the transport of folic acid and that the uptake process follows the simplest form of Michaelis-Menten Kinetics:-

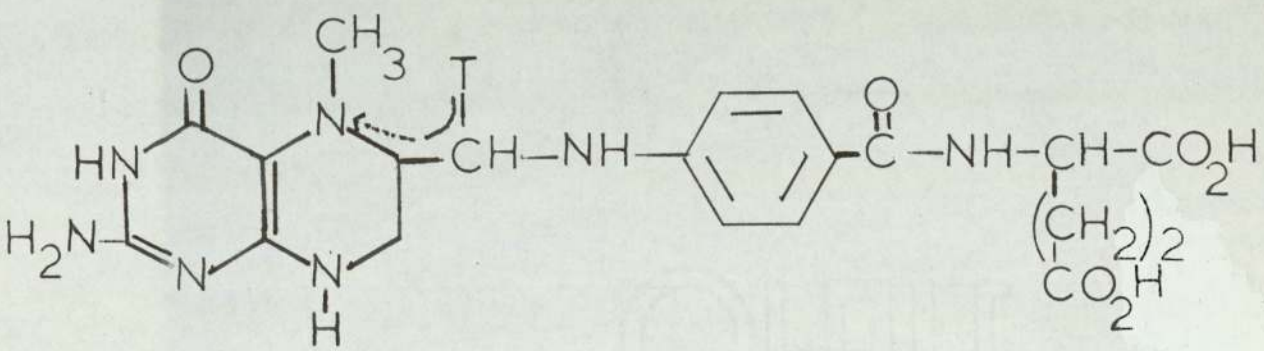
Figure 5-2 :-

A- The hyperconjugation effect of hydrogen attached to C-9, on the amino group attached to C-2 of the pteridine ring of folic acid molecule.



Folic acid

B- The inductive effect of tritium attached to C-9, on the N-5 of the pteridine ring of 5-meTHF molecule.



5-meTHF

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + S$$

(carrier) (folic acid)

$$V_{max} = k_{+2} \left[E_{total}\right] \qquad \text{---------- (1)}$$

$$\text{and} \quad Km = \frac{k_{-1} + k_{+2}}{k_{+1}} \qquad \text{---------- (2)}$$

As C9 - ^{3}H appeared to be the important site in the observed isotopic effect of folate, this according to a carrier model means that it will either directly or indirectly affect the binding of folic acid to the carrier. The direct effect could be the isotope steric effect due to the shorter C-^{3}H bond than C-^{1}H bond (Melander and Saunders, 1980; Jencks, 1969). Therefore, the binding of ^{3}H-folic acid to carrier is greater compared to that of ^{14}C-labelled and unlabelled folic acid. The stronger binding of ^{3}H - folic acid means that more energy is required to release the ^{3}H-folic acid from the carrier on both sites of the membrane, i.e. more stable ES complex, and therefore, lower k_{-1} and k_{+2}. These effects will lead to a decrease in the Km and Vmax values of ^{3}H-folic acid compared to that of ^{14}C-labelled and unlabelled folic acid (equation 1 and 2), which was not the case (Figure 5-1).

^{3}H at position 9 could also affect the binding to a carrier by altering the electronegativity of the pteridine ring. The pteridine ring of folate is electron deficient and is only attacked by a nucelophile (Hurst, 1980). ^{3}H at position 9 is less electron donating to the pteridine ring

by hyperconjugation than hydrogen (see Figure 5-2A). This means that the electron density of the pteridine ring of ^{3}H-folic acid is less than that of the ^{14}C-labelled and the unlabelled folic acid and therefore, easier and stronger binding of ^{3}H-folic acid with nucleophile should be expected. As this is the only possible interaction between the ^{3}H-folic acid molecule and carrier, again it means that lower k_{-1} and k_{+2} values, i.e. lower Km and V_{max} values for ^{3}H-folic acid compared to that of the ^{14}C-labelled and the unlabelled folic acid (equation 1 and 2) which was not the case (Figure 5-1).

In a carrier model k_{+2} for ^{3}H-folic acid, even when it is a composite of several rate processes, cannot be greater than that of k_{+2} for the ^{14}C-labelled and unlabelled compound but must be the same or less. Therefore, Vmax of ^{3}H-folic acid as a carrier model must be the same or less than that for the unlabelled folic acid. This is not found experimentally.

The fact that the isotopic effect of folate was also occurred in a non-biological system (Connor, Blair and Said, 1980; Connor, 1979) supports the possibility of a simple physical effect, i.e. the first possibility.

In the case of the passively absorbed folate derivative, 5-meTHF (see Chapter 4) the observed increase in the absorption of ^{3}H-labelled 5-meTHF over that of the ^{14}C-labelled species (Kennelly, 1980) could also be explained on the basis of the difference in the electronegativity of tritium and hydrogen. The ^{3}H at position 9 would increase the

pKa of the N-5 of the pteridine ring by an inductive effect (Figure 5-2B) as ^{3}H is more electron donating in such system than ^{1}H (Melander and Saunders, 1980; Jencks, 1969). This will lead to an increase in the percentage of the neutral Zwitterion form (Figure 1-4) of the ^{3}H-labelled 5-meTHF compared to the ^{14}C-labelled and the unlabelled species at the pH of the surface acid microclimate and subsequently to an increase in the absorption rate.

In conclusion, the isotope effect observed in the absorption of folate could be best explained using the concept of the surface acid microclimate as a mechanism of absorption.

CHAPTER SIX

HUMAN INTESTINAL SURFACE ACID MICROCLIMATE AND ITS VARIATION IN HEALTH AND DISEASE

6.1. INTRODUCTION

The surface acid microclimate has been shown to exist at the luminal surface of the rat small intestine both *in vitro* and *in vivo* (see Chapter 3). The mechanism of its creation is thought to be the external hydrolysis of ATP by mucosally located ATPase. An intact glycocalyx, adequate amounts of metabolisable substrates that produce ATP and an active mucosal ATPase in appropriate amounts are the necessary requirements for its existence and maintenance.

The mechanism of the intestinal absorption of folates was found to be the facilitated passive diffusion of the neutral species by the intestinal surface acid microclimate. Elevation in the surface acid microclimate pH was found to be associated with a decrease in folate absorption (see Chapter 4).

The object of these investigations was to examine the surface acid microclimate of the human intestine and to further investigate its variation in disease conditions particularly those associated with folate malabsorption, such as coeliac disease (Kitis, Lucas, Schneider, Bishop, Sargent, Blair and Allan, 1979; MacKenzie and Russell, 1976; Halsted, Reisenauer, Romero, Cantor and Ruebner, 1977; Hoffbrand, Douglas, Fry and Stewart, 1970), and Crohn's disease (Franklin and Rosenberg, 1973; Hoffbrand, Stewart, Both and Mollin, 1968; Eade, Cooke and Williams, 1972). Experiments were also carried out to examine the surface pH of normal stomach tissue.

Coeliac disease is a condition associated with gross morphological and functional abnormalities of the small intestine following an administration of gluten in the diet. These abnormalities include villous atrophy ie. flat mucosa, infiltration of chronic inflammatory cells in the lamina propria, elongation of the crypt, fewer and shorter microvilli, and lead to malabsorption and diarrhoea (Creamer, 1974; Padykula, Strauss, Ladmann and Gardner, 1961; Fordtran, Rector, Locklear and Ewton, 1967). At a cellular level there is a decrease in the activities of brush border and many intracellular enzymes (Peters, Johnes and Wells, 1978; Riecken, Stewart, Booth and Pearse, 1966) and a broken and incomplete glycocalyx (Swanston, 1978; Swanston, Blair, Matty, Cooper and Cooke, 1977). On withdrawal of gluten from the diet most of the abnormalities either disappear or are extensively improved.

The toxic moiety of the gluten was isolated and found to be a glycopeptide which binds to a brush border membrane component of the coeliac intestinal cells but binds only poorly to normal intestinal brush border membrane (Douglas, 1976).

Crohn's disease is an inflammatory condition most commonly affecting the colon and the ileum but can involve any part of the gastro-intestinal tract (Creamer and Lockhart-Mummery, 1974; Linder, Marshak, Wolf and Janawitz, 1963). There is increasing evidence to suggest that Crohn's disease is a diffuse lesion of the gastro-intestinal tract, i.e. even if the active disease is confirmed to the colon or the ileum,

abnormality of the proximal small intestine frequently occurs (Tootla Lucas, Bernacki and Tabor, 1976; Nugent, Richmond and Park, 1977; Arvanitakis, 1979). Associated with the disease is reduced levels of brush border enzyme activities (Dunne, Allan and Cooke, 1976; Arvanitakis 1979) as well as broken and an incomplete glycocalyx (Swanston, 1978).

6.2. MATERIALS AND METHODS :-

The proximal jejunum surface pH was measured in vitro in jejunal specimens obtained from subjects with normal and diseased small bowel, and from those whose small bowel disease had been treated. Biopsies were always taken at the same time of the day following overnight fasting.

The delay between obtaining the samples and immersion in the buffer medium was less than one minute. Samples were incubated for 30 minutes in bicarbonate buffer pH 7.4 containing 10 mM glucose at 37^{o}C.

Stomach surface pH was measured over a 30 minute period in Krebs-Ringer phosphate buffer pH 3 containing 10 mM glucose at 37^{o}C.

All other details on the methods and materials are described in Chapter 2.

6.3. CLINICAL NOTES :-

Jejunal biopsies were obtained from fifty five subjects and divided into groups according to histological evidence and case history record.

(i) Controls :-

The subjects classified as a control group were those whose jejunal biopsies yielded normal histology. They attended the clinic because of mouth ulcers, abdominal pain, loss in weight, constipation, diarrhoea, macrocytic anaemia due to vitamin B_{12} deficiency, indigestion and/or rectal bleeding. The surface pH investigations of this group involved 19 subjects, 14 females and 5 males (age range 14-54 years, mean 33 years).

(ii) Subjects with non-specific diarrhoea and abnormal jejunal histology :-

All subjects of this group had diarrhoea of unknown cause and abnormal jejunal histology. The abnormality ranged from slight inflammation of the lamina propria with normal columnar epithelial cells to broader and shorter villi with cuboidal epithelial cells and moderate inflammation of the lamina propria. Some subjects, in addition to diarrhoea, had abdominal pain, loss in weight, weakness and/or indigestion.

No subject of this group had a previous history of abnormal biopsies.

The surface pH investigation involved 19 subjects 10 females and 9 males (age range 24-76 years, mean 45 years).

(iii) Subjects with coeliac disease :

a. Active coeliac disease (untreated coeliacs on normal diet) :-

The subjects of this sub-group were those who had an abnormal jejunal biopsy of untreated coeliac disease characterised by villous atrophy ie. flat mucosa, crypts elongation, cuboidal mucosal epithelial cells and severe chronic inflammatory cellular infiltration of the lamina propria. Five female subjects had a diagnosed coeliac disease for more than fifteen years, whereas two subjects (1 male and 1 female) were newly diagnosed. All of them were on normal diets. The age of the subjects ranged between 42-57 years (mean 55 years).

(b) Inactive coeliac disease (treated coeliacs on gluten free diet):-

The subjects of this sub-group were those who had coeliac disease and were on a gluten free diet for a period ranging between 5 to 14 years. They had responded symptomatically to gluten free diets. However, their jejunal histology still showed some abnormalities including moderate inflammation of the lamina propria, shorter villi, slightly elongated crypt and inflammation of the lamina propria. There were 4 subjects, 2 males and 2 females in this group, ranging between 34-61 years (mean 48 years).

(iv) Crohn's disease :-

The surface pH of the proximal jejunum of four subjects, 2 men and 2 women (age range 34-48 years, mean 38 years), with Crohn's disease diagnosed according to conventional criteria (Linder, Marshak, Wolf and Janowitz, 1963) was measured.

The length of history of the disease varied from 1 to 19 years. One subject had involvement of the colon alone, in two subjects the disease had diffused to the ileum, and with one subject the disease had diffused as far as the proximal small intestine.

(v) Stomach surface pH :-

Stomach surface pH was measured from normal stomach tissue taken by surgery from subjects with stomach cancer involved either the antrum region (1 male aged 50 years) or the antrum and the proximal duodenum (1 male and 1 female aged 45 years and 36 years respectively).

6.4. RESULTS

6.4.1. INTESTINAL SURFACE pH IN HEALTHY AND DISEASED CONDITIONS :-

The surface pH of the proximal jejunum of all subjects was found to be significantly lower than the buffer pH both at the onset ($P < 0.001$) and the end ($P < 0.001$) of 30 minutes incubation. No significant changes in buffer pH was observed during the duration of the experiments (Table 6-1).

Control subjects showed no significant changes in the surface pH during incubation (Table 6-1 and Figure 6-1). The mean surface pH of the group did not alter with the age or sex of the subject nor with the seasonal time during which these investigations were performed (approximately two years). Therefore, alterations in mean age, sex and time

TABLE 6-1 : The proximal jejunum surface acid microclimate of control and diseased subjects. Surface pH was measured in bicarbonate buffer containing 10 mM glucose at 37°C.

Initial buffer pH	7.40 ± 0.05
Final buffer pH	7.46 ± 0.06

Subjects	Surface pH		Serum folate (µg/l)
	Initial	Final	
Control	6.16 ± 0.03 (19)*	6.17 ± 0.02 (19)	5.5 ± 0.6 (16)
Non-specific diarrhoea with abnormal jejunal histology	6.31 ± 0.06 (19) (P <0.02)	6.31 ± 0.05 (19) (P <0.02)	3.9 ± 0.6 (14) (P >0.05)

Continued....

TABLE 6-1 (continued...)

Subjects	Surface pH		Serum folate (µg/l)
	Initial	Final	
Active coeliac disease	6.57 ± 0.07 (7) (P <0.001)	6.58 ± 0.09 (7) (P <0.001)	2.9 ± 0.8 (4) (P >0.05)
Inactive coeliac disease	6.35 ± 0.10 (4) (P >0.02)	6.28 ± 0.10 (4)	3.8 (2)
Crohn's disease	6.40 ± 0.02 (4) (P >0.001)	6.29 ± 0.01 (4) (P >0.02)	–
Folate deficient anaemia	6.51 (1)	6.43 (1)	–
Chronic alcoholism	5.94 (1)	5.94 (1)	3.6 (1)

* Number of subjects (experiments).

Figure 6-1 : The proximal jejunum surface acid microclimate of control subjects, and subjects with active and inactive coeliac disease. Measurements were performed in bicarbonate buffer containing 10 mM glucose at 37°C.

Initial buffer pH 7.40 ± 0.05

Final buffer pH 7.46 ± 0.06

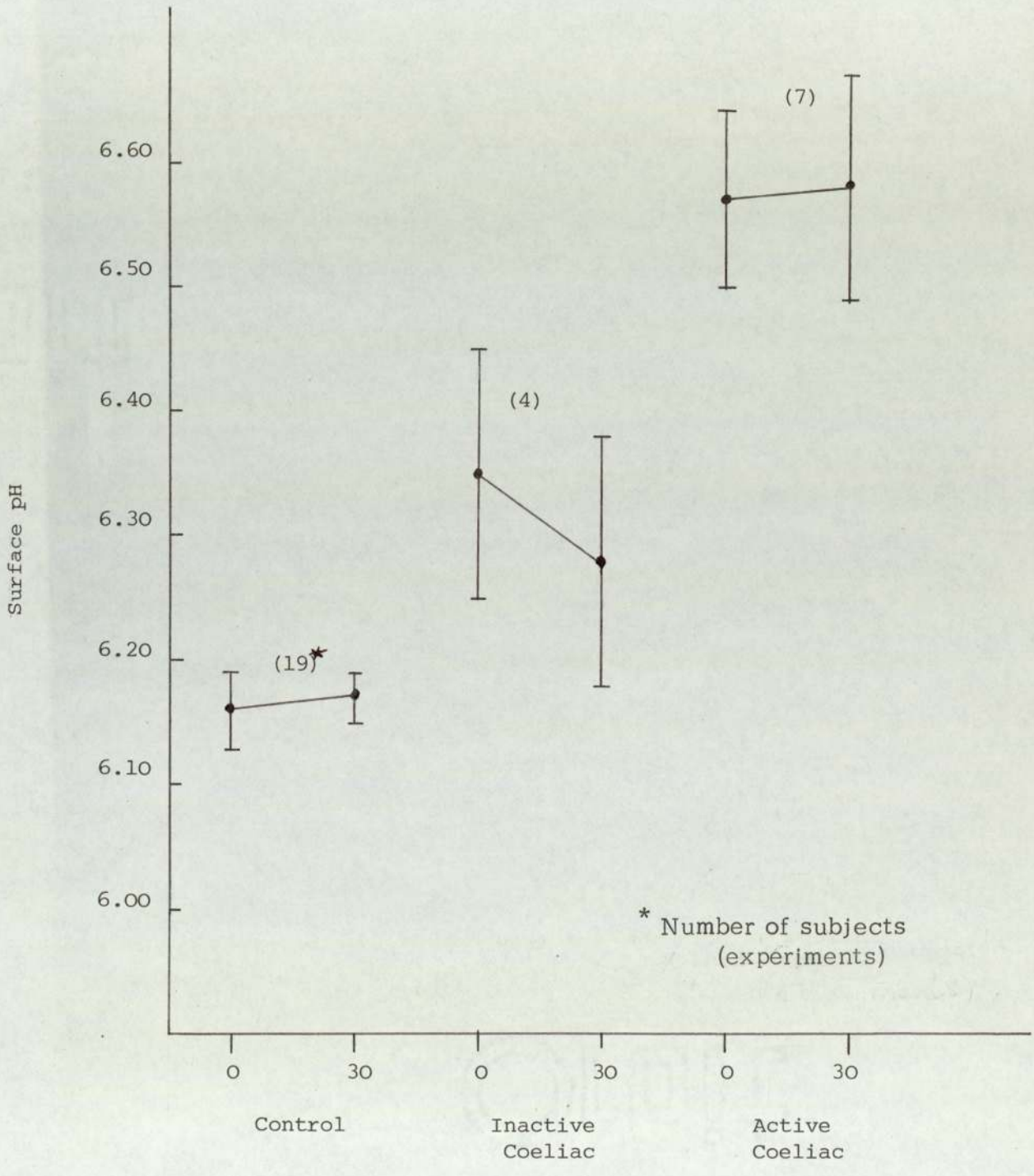
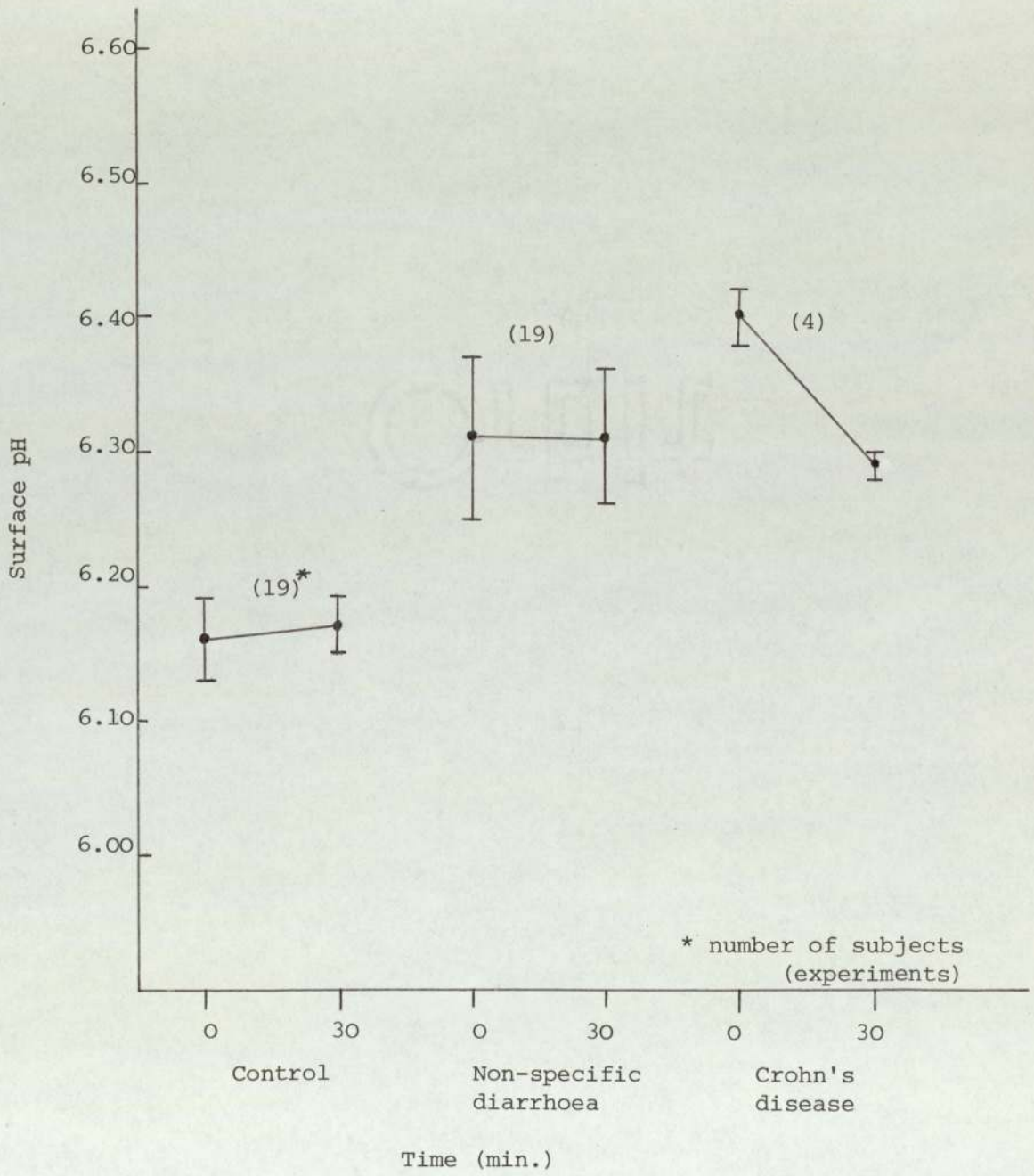


Figure 6-2 : The proximal jejunum surface acid microclimate of control subjects, and subjects with non-specific diarrhoea with abnormal jejunal histology and Crohn's subjects. Measurements were performed in bicarbonate buffer containing 10 mM glucose at 37°C.

Initial Buffer pH 7.39 ± 0.06

Final Buffer pH 7.45 ± 0.06



of the experiment were not a significant source of error.

The subjects with active coeliac disease showed the highest surface pH of all the groups investigated (Table 6-1). The surface pH was significantly higher than that of the control group both at the onset ($P < 0.001$) and the end ($P < 0.001$) of incubation (Table 6-1 and Figure 6-1). On the other hand, subjects with inactive coeliac disease showed a lower but insignificant surface pH compared to those of active coeliac disease. The onset surface pH of this sub-group was significantly higher ($P > 0.02$) than that of control but the final surface pH was not significantly higher. In both active and inactive coeliacs, no significant changes in the surface pH during incubation was observed.

In the subjects with non-specific diarrhoea and abnormal jejunal histology the surface pH was significantly higher than that of the control group both at the onset ($P < 0.02$) and the end ($P < 0.02$) of incubation (Table 6-1 and Figure 6-2). Again no significant alteration in the surface pH during incubation was observed. Crohn's subjects also had significantly higher surface pH values compared to the control group both at the onset ($P > 0.001$) and the end ($P > 0.02$) of incubation (Table 6-1 and Figure 6-2). In this case a significant ($P < 0.001$) decrease in the surface pH during incubation was observed.

6.4.2. CORRELATION BETWEEN SURFACE pH AND SERUM FOLATE LEVEL IN HEALTHY AND DISEASED CONDITIONS :-

Serum folate is a fluctuating variable dependent upon folate

Fig. 6-3 :- The regression of surface pH and serum folate levels in various clinical states.

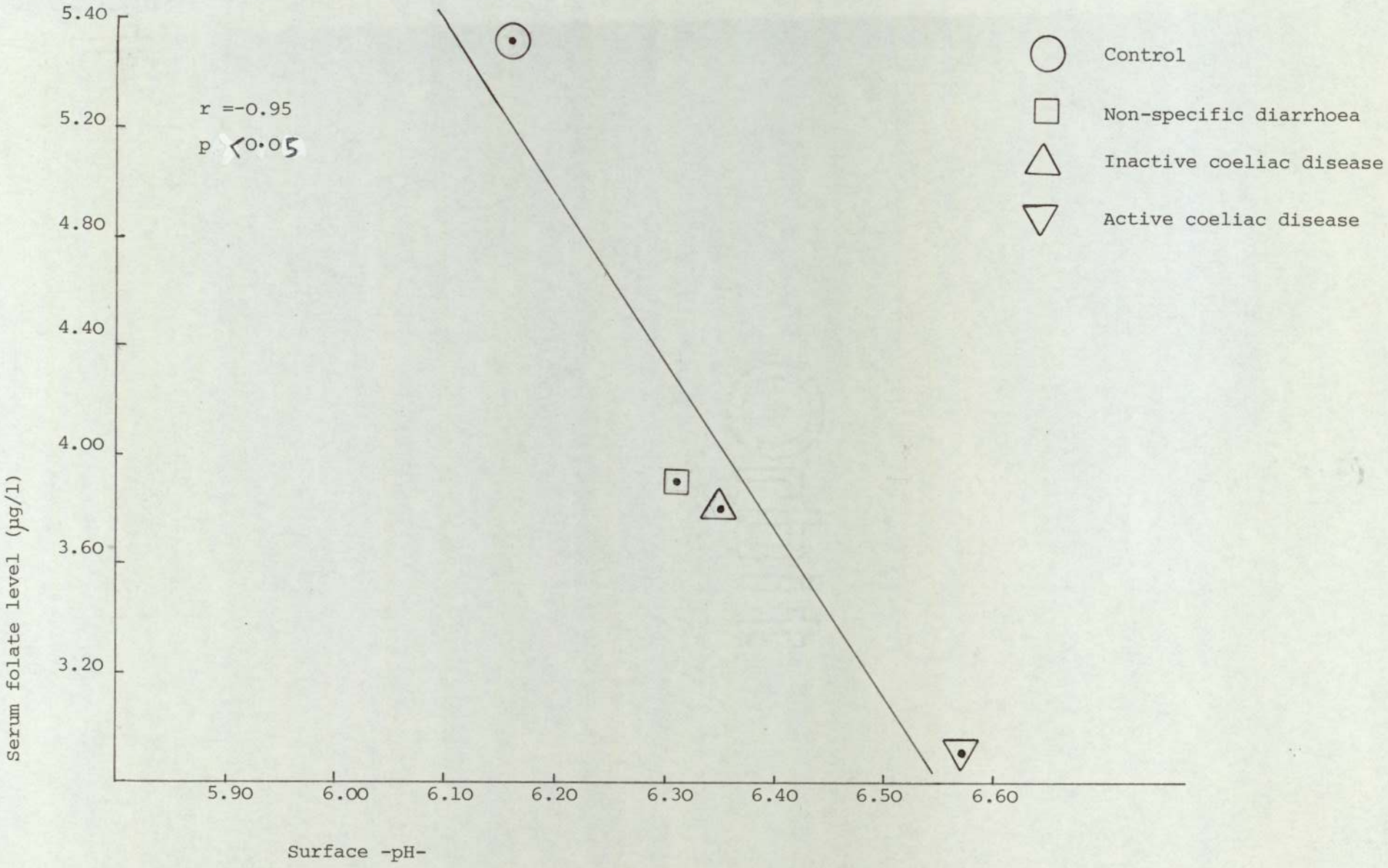


TABLE 6-2 : The surface pH of normal stomach tissue in man.

Surface pH was measured in Krebs-Ringer phosphate buffer containing 10 mM glucose, at 37°C.

Stomach Surface pH		Buffer pH	
Initial	Final	Initial	Final
5.61 ± 0.16	5.16 ± 0.09	2.93 ± 0.7	3.10 ± 0.05
(3)	(3)	(3)	(3)

* Number of subjects (experiments).

intake and absorption, degree of tissue uptake and elimination. However, provided other factors are constant, serum folate levels should reflect the degree of uptake of folate. On the basis of this assumption, and because folate malabsorption is known to occur in all the clinical conditions examined (see Table 1-3) an attempt to correlate the initial surface pH with serum folate level (μg/l) was performed.

Estimation of serum folate levels by a microbiological assay method was carried out on all subjects except those on folate supplements.

A negative linear correlation ($r = -0.95$, $P < 0.05$) between serum folate levels and initial surface pH was found to exist for all patients examined (Figure 6-3), i.e. an increase in the serum folate level (folate absorption) was found to be associated with a decrease in surface pH.

6.4.3. STOMACH SURFACE pH :-

Stomach surface pH was found to be significantly higher than the buffer pH both at the onset ($P < 0.001$) and the end ($P < 0.001$) of the incubation (Table 6-2). This observation confirms the earlier finding of a relatively alkaline stomach surface microclimate in rats (see Chapter 3).

6.5. DISCUSSION

6.5.1. THE INTESTINAL SURFACE ACID MICROCLIMATE IN HEALTH AND ITS VARIATION IN DISEASE CONDITIONS :-

An intestinal surface acid microclimate was found to exist at the

mucosal surface of the human proximal jejunum in vitro with a pH much lower than that of the incubation medium (Table 6-1). The surface pH maintained itself during incubation which indicates that the in vitro intestinal preparation is able to perform its normal physiological metabolic activities.

In the previous surface microclimate studies using rats (described in Chapter 3) it was found that the mechanism responsible for the creation of the surface acid microclimate is the external hydrolysis of ATP by mucosally located ATPase. An intact glycocalyx to retain the hydrogen ions at the surface by slowing their diffusion into the lumen, adequate amounts of ATP and an active brush border ATPase in adequate amounts are the necessary requirements for the creation and maintenance of normal intestinal surface acid microclimate.

In untreated (active) coeliac disease, the activity of the brush border ATPase is severely diminished (Riecken, Stewart, Booth and Pearse, 1966) and the glycocalyx is damaged and biosynthetically incomplete (Swanston, 1978; Swanston, Blair, Matty, Cooper and Cooke, 1977). Both of these abnormalities will lead to a decrease in the amount of hydrogen ion at the surface of the small intestine and would explain the increase in the surface pH observed in this condition (Table 6-1 and Figure 6-1). On treating coeliac subjects with a gluten-free diet, an improvement in the activities of the brush border enzymes (Riecken, Stewart, Booth and Pearse, 1966) and glycocalyx structure (Swanston, 1978) occurs. Both of these changes would lead

to an increase in the amounts of hydrogen ion present at the luminal surface of the intestine, and therefore, it explains the decrease in the surface pH observed with treated coeliac disease compared to the untreated condition.

The surface pH of the treated coeliac subjects although more acid than untreated subjects, did not recover to the control value; even though they were on gluten free diets for a period of 5 to 14 years. This was either because the subjects were not strictly adherent to their gluten free diets and/or because the activities of the brush border enzymes although improved, remained persistently defected (Peter, Jones and Wells, 1978).

In Crohn's disease, the glycocalyx has been found to be broken and damaged (Swanston, 1978). There is also a decrease in the brush border disaccharidases and alkaline phosphatase activities associated with the diseased state (Arvanitakis, 1979). A broken glycocalyx together with a defected brush-border ATPase will lead to a decrease in the amounts of hydrogen ion at the intestinal surface and therefore, would explain the significant increase in the surface pH over that of control values (Table 6-1 and Figure 6-2). The significant decrease in the surface pH during incubation is possibly due to the absence *in vitro* of an inhibitory factor acting either on the system that produces hydrogen ions and/or the imput of glucose into the system in Crohn's disease.

The significantly higher surface pH recorded in subjects with non-specific diarrhoea with abnormal jejunal histology is either related to abnormal glycocalyx and/or a defect in ATP production or hydrolysis.

In conclusion, in all the disease conditions examined with higher surface pH values than control values, the cause is either related to a damaged glycocalyx, a decrease in the activity of brush border ATPase and/or a decrease in ATP production.

6.5.2. INTESTINAL MALABSORPTION OF FOLATES IN DISEASE CONDITIONS

Folate malabsorption is known to occur in many disease conditions including coeliac disease and Crohn's disease (see Table 1-3 and 6-1). In coeliac disease, folate malabsorption is not related to a defect in the rate of deconjugation of dietary folate, but is due to a defect in the transport mechanism of the folate monoglutamate (Jägerstad, Lindstrand, Norden, Westesson and Lindberg, 1974; Rosenberg, Godwin, Russell and Franklin, 1974; Halsted, Reisenauer and Corcino, 1975).

In contrast to the decrease in the absorption of folic acid in coeliac disease, the absorption of propranolol a weak base (pKa, 9.45) was found to increase compared to normal control conditions (Kitis, Lucas, Schneider, Bishop, Sargent, Blair and Allan, 1979; Parsons and David, 1979; Parsons, Kaye, Raymond, Trounce and Turner, 1976). The increased rate of absorption of propranolol and decreased rate of folic acid absorption in coeliac disease is difficult to explain in terms of poor intestinal function or reduction in surface area in coeliac

disease since both these factors should affect weak acid and weak base absorption to the same extent.

In the previous studies using rats (Chapter 4) the mechanism of the intestinal absorption of folate was found to be the passive diffusion of the neutral forms of folates with the aid of the intestinal surface acid microclimate. An elevation in the surface pH caused a decrease in folate absorption. In coeliac disease the surface pH was elevated compared to that of normal conditions (Table 6-1 and Figure 6-1). This would provide a good explanation for the observed decrease in folate absorption and the increase in propranolol absorption in this diseased condition. This is because the elevation in the surface pH observed in coeliac disease would lead to a decrease in the proportion of the weak acid (folate) in neutral form and an increase in the proportion of the weak base (propranolol) in neutral form. In the same way, the elevated surface pH in coeliac disease will provide an explanation for the malabsorption of penicillin (Bolme, Eriksson and Stintzing, 1977) and the increased absorption of trimethoprim and cephalexin (Parsons and David, 1979) after oral administrations to coeliac subjects.

On gluten withdrawal the pH of the surface acid microclimate moves toward the normal values and folate absorption improves (Table 6-1).

Similarly the malabsorption of folate in Crohn's and other disease conditions (see Table 1-3 and 6-1) is possibly due to an elevation in the surface acid microclimate pH.

6.5.3. STOMACH SURFACE pH IN MAN :-

An alkaline surface pH relative to the incubation buffer was measured in vitro at the luminal surface of normal stomach tissue over the normal in vivo luminal pH range (pH 1.8 - 3.5) (see Table 6-2). This relatively alkaline surface pH is thought to be created by mucus retention of bicarbonate anions actively secreted from stomach epithelial cells (Allen and Garner, 1980). It has a physiological cyto-protection function by decreasing the back diffusion of hydrogen ions from the lumen back into the surface (Williams and Turnberg, 1980; Allen and Garner, 1980). Agents such as aspirin which are known to cause damage to the gastric mucosa inhibit epithelial cells bicarbonate anions secretion (Garner 1977, 1978). On the other hand, agents such as prostoglandin which have a gastric cytoprotective action, stimulate bicarbonate secretion and therefore, decrease the damaging action of luminal hydrogen ions (Garner and Heylings, 1979).

CHAPTER SEVEN

CONCLUSIONS AND FUTURE WORK

1. CONCLUSIONS :-

A. The intestinal surface acid microclimate has been measured at the luminal surface of the small intestine of both rat and man *in vitro* and the rat *in vivo*. The microclimate is not in equilibrium with the incubation buffer medium.

B. The surface acid microclimate is a normal physiological phenomena of the small intestine. It is affected by metabolic inhibitors, bile acids and bile salts and removal of glucose and sodium from the incubation medium and is related to the physiological status of the small intestine.

C. The surface pH of the intestinal surface acid microclimate varies along the entire length of the small intestine. The distribution of the surface acidity is directly related to the metabolic activities of a particular part of the small intestine. The surface acidity is maximum in the proximal jejunum and lowest in the distal ileum. In the colon the surface acidity is of a similar order of magnitude to that of the distal ileum.

D. The mechanism of the creation of the intestinal surface acid microclimate is the *external* hydrolysis of intracellularly produced ATP through aerobic metabolism by mucosally located ATPase.

E. The glycocalyx of the small intestine together with the unstirred water layer retain the hydrogen ions at the surface by slowing their rate of diffusion into the lumen.

F. The necessary requirements for normal surface acidity are, (i) normal intracellular metabolic activities, (ii) adequate amounts of metabolisable substrates, particularly glucose; that produce ATP, (iii) an active mucosally located ATPase in sufficient amounts to hydrolyse ATP, and (iv) a structurally intact and biosynthetically complete glycocalyx to retain the hydrogen ions at the surface.

G. The adenyl-cyclase phosphodiesterase system has no role in creating, maintaining or regulating the surface acidity of the small intestine.

H. The elevation in the surface acid microclimate which occurs in coeliac and Crohn's disease is due to a structurally damaged and biosynthetically incomplete glycocalyx and an inadequate amount of mucosally located ATPase.

I. The preferential site of 5-meTHF and folic acid absorption is the proximal jejunum at which equal amounts are absorbed. Malabsorption of folate therefore occurs when the proximal jejunum is diseased or damaged.

J. A considerable amount of folic acid undergoes metabolic alterations during transport, while 5-meTHF traverses without metabolic alteration.

K. The mechanism involved in the intestinal absorption of folate is not an active carrier transport mechanism as this model is unable to provide a good explanation for the available experimental observations which include (i) the failure to show transport of folate against a concentration gradient, (ii) the lack of competition between the folate analogues, i.e. folic acid and 5-meTHF, (iii) the equal rate of absorption of 5-meTHF and folic acid in the proximal jejunum and their variation down the intestine, (iv) the lack of saturation in the uptake of 5-meTHF over a wide range of mucosal concentrations, and (v) the lack of stereospecificity around C-6 of the pteridine ring of 5-meTHF.

L. Solvent drag with water movement has a limited role in folate absorption.

M. A model incorporating the essential role of the intestinal surface acid microclimate provides a better explanation for the experimental observations concerning folate absorption.

N. The mechanism of the intestinal absorption of folate therefore involves the facilitated passive diffusion of neutral forms created in the intestinal surface acid microclimate.

O. An increase in the intestinal surface microclimate pH will lead to a decrease in the amount of the neutral form of folate species and a lower absorption rate. This relationship was observed experimentally in the case of intestinal preparations treated with sodium deoxycholate or metabolic inhibitors and naturally as in the case of coeliac and Crohn's disease.

P. The saturation observed in the uptake of folic acid with increasing mucosal concentration and the lack of saturation of 5-meTHF is due to the limited solubility of folic acid in aqueous medium at the pH of the intestinal surface acid microclimate, while 5-meTHF is more soluble.

Q. The malabsorption of folate in coeliac and Crohn's disease is due to abnormally high intestinal surface acid microclimate pH. It is not related to the reduced surface area which occurs in coeliac disease.

R. Adherence to gluten free diets of subjects suffering from coeliac disease is essential if folate deficiency is to be avoided.

S. Bacterial invasion of the proximal jejunum could lead to a damaged glycocalyx either directly or indirectly by deconjugation and reduction of the predominant trihydroxybile salts. The resultant unconjugated dihydroxybile acid particularly deoxycholate exerts its damaging effect on the glycocalyx which subsequently leads to an elevation in the surface pH and a decrease in folate absorption.

T. Long term exposure of the small intestine to synthetic detergents will lead to an elevation in the surface pH and subsequent malabsorption of folates by either a direct damaging effect to the glycocalyx, or indirectly by increasing the rate of cellular proliferation which will lead to a biosynthetically incomplete glycocalyx.

U. The secondary isotopic effect in which the ^{3}H-labelled

folate species are absorbed at a faster rate than the ^{14}C-labelled and the unlabelled species is due to relatively higher amounts of ^{3}H-labelled folate present in its neutral form at the pH of the intestinal surface acid microclimate. For this reason it is advisable to use ^{14}C-labelled folate species as tracers in future studies.

V. A more alkaline surface microclimate relative to the incubation medium(pH 2-3.5) exists at the luminal surface of the rat and human stomach in vitro. It is not in equilibrium with the incubation medium and resists the fluctuation in buffer pH. Glucose is an important requirement for its creation and maintenance.

W. The role of the stomach microclimate is to protect the stomach mucosa against the high acidity of the lumen.

7.2. FUTURE WORK :-

A. To develop smaller pH microelectrodes than the one used in this study in order to determine the exact pH of the intestinal and stomach surface microclimates.

B. To measure the pH and to examine the distribution of the intestinal surface acid microclimate in other animal species.

C. To study further the role of the intestinal surface acid microclimate in the intestinal absorption of weak acids and weak bases such as propranolol.

D. To measure the pH of the intestinal surface microclimate in other disease conditions of the gastro-intestinal tract particularly those associated with folate malabsorption, e.g. tropical sprue , ulcerative colitis, Whipple's disease and gastroectomy.

E. To measure the stomach surface pH of man and rat in vivo and to determine the mechanism of its creation and the necessary requirements for its maintenance. This will be performed by investigating the effect of the metabolic inhibitors, mucolytic agents, bile salts and acids, cyclic-AMP, and by altering the constituent of the incubation medium.

F. To make a wider study on the secondary isotopic effect observed in folate absorption and to determine its exact origin and implications using different techniques such as NMR. Also to examine whether or not the isotope effect phenomena exists in the absorption of other important compounds.

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