ACIDIFICATION IN THE RAT PROXIMAL JEJUNUM

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SUMMARY

Acidification of solutions by rat proximal jejunum was investigated in everted sacs, segments and in perfused in vivo loops. Acidification which declines down the jejunum, increased with increasing mucosal glucose concentration, reaching a maximal value. Derived Michaelis-Menten data gave an apparent Km of 0.05mM and a Vmax of 1.01 ($ugH^+/mg/hr$) Acidification was uninhibited by 10mM mucosal L-β-phenylalanine and unstimulated by mucosal 10mM pyridoxal phosphate: it was stimulated by glucose-1- and -6-phosphate.

Acidification was unaffected by 10mM mucosal acetazolamide and 10mM ouabain which excluded bicarbonate movement and made active sodium transfer unlikely, as causing acidification. Acidification was diminished by anoxia, by phlorrhizin and by DNP at concentrations that prevent oxidative processes, indicating the metabolic dependency and excluding anoxia as a source of acidification. Unaffected by histamine (10mM) and EDTA (10mM), acidification is distinct physiologically, from gastric acid production. The ineffectiveness of acetazolamide and the inability of mucosal lactic acid, pyruvic acid production and amino acid loss to fully account for acidification in sacs, segments and loops, makes less likely the existing theory of acidification being caused by bicarbonate movement and lactic acid production.

The non-metabolisable sugars 3-O-methylglucose and galactose do not stimulate, the metabolisable sugars glucose, mannose and fructose stimulate acidification, indicating the metabolic nature of the origin of the protons. Similarly, mucosal ATP (10mM) which does not enter the tissues causes an increase in acidification, not due to the chelating ability of ATP. Acidification is most markedly inhibited by aminophylline (10mM) implicating the importance of ATP turnover via the adenyl cyclasephosphodiesterase system. Imidazol (10mM) has no effect. It is proposed that acidification is not a passive process but the result of external hydrolysis of ATP at the surface of epithelial cells. This concept is examined in relation to the micro-climate hypothesis and the implications of acidification in the transfer of weakly-ionising substances discussed.

The research documented in this thesis was carried out between October 1969 and June 1972; it was done independently and has not been submitted for any other degree.

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INTRODUCTION

Since Claude Bernard's observations, which defined physiological processes in terms of self-regulatory systems, much research has been done into physiological homoeostasis, yet the questions implicit in the maintainence of levels of acidity in both intracellular and extracellular fluid have often been neglected. Various kinds of phenomena occurring in animal tissues will function only within distinct levels of acidity. This requirement for a limited pH range can easily be demonstrated by experiment, but the reasons why systems exhibit these requïrements for certain pH values remains often without adequate explanation.

In common with many other types of biochemical and biological phenomena inherent in animal tissues, it would seem that the properties and characteristics of processes occurring in the small intestine, from the molecular to the physiological level of hierarchy, are critically dependent on the concentration of hydrogen ions in the milieu in which these processes occur. The biochemical basis for the effect of acidity on living processes is manifested as the pH-dependence of enzymically controlled reactions in that an optimal pH exists for maximal rates of reactions occurring biological tissues. With specific regard to the mucosal cells present in the small intestine, a similar pH dependency exists for many transport processes occurring across the epithelium as well as for the biochemical reactions

connected with intracellular metabolism. The level of acidity that epithelial membranes are exposed to, can affect the movement of passively transported inorganic compounds, e.g. phosphate, ammonia (McHardy & Parsons, 1956; Swales, Tang & Wrong, 1971;), passively transported organic non-electrolytes, e.g. thiourea (Storelli, Pesente, & Lippe, 1969;) substances, usually organic non-electrolytes, whose transfer mechanism is unknown or disputed, e.g. benzoic acid and folic acid (Smith, Matty & Blair, 1970; Jackson, Shiau & Cassidy, 1970) and substances for whom a transport mechanism is known to exist, e.g. glucose, amino acids and some cations (Laszt, 1935; Ponz & Larralde, 1950; Iida, Moore, Broitman & Zamcheck, 1968; Thompson, Levin & Jackson, 1970; Greenberg & Gunther, 1932; Leaf, Keller & Dempsey, 1964). As with the maintenance of whole-body pH, it might well be thought that the maintenance of intestinal pH is a conditio sine qua non for the adequate functioning of intestinal absorption and other accessory processes.

Extreme levels of acidity or alkalinity denature most enzymic proteins, causing a collapse of tertiary structure and a loss in catalytic effectiveness. It may be that such denaturation by extremes of acidity accounts for the pH-dependent-transfer of some actively transported substances whose transfer mechanism might involve some enzymically controlled processes. Alternatively, variations in pH could alter the amount of energy available for these active transport processes, since external pH variations are known to affect the amount of glucose

metabolised in rat intestine (Barry, Jackson & Smyth, 1965). Where the movement of passively transported substances is affected by pH changes, these alterations in the amount transferred are thought to reflect variations in the amount of uncharged species of the substance that is available for non-ionic diffusion (Schanker, Tocco, Brodie & Hogben, 1958). Whatever the mechanism, the immediate control of environmental, extracellular acidity would seem to be of considerable importance to transporting epithelia.

Such a homoeostatic system is thought to exist for the rat jejunum, where the rates of transport of substances are often higher than in other portions of the intestine (Borgstrom, Dahlquist, Lundh & Sjovall, 1957; Baker, Searle & Nunn, 1961; Booth, Read & Jones, 1961). A mechanism for acidification exists in the small intestine that keeps the intraluminal pH at a value below that of neutral pH. Although the concept of gastric acidification is a familiar one dating back to the early 18th Century work of Reamur and Spallanzani, and was established without doubt when Prout in 1824 found gastric juice to be rich in hydrochloric acid, the concept of the small intestine being able to acidify under certain conditions is a new and relatively little discussed phenomenon. The clinical aspect of intestinal acidification and its possible role in some malabsorption syndromes is almost completely unknown. In the case of rat jejunum, it has been proposed (Hogben, Tocco, Brodie & Schanker, 1959) that a stagnant layer exists

juxtaposed to the intestinal wall, such that the pH in the region in close contact with the gut wall is possibly as much as two pH units lower than the bulk luminal phase, when the luminal phase is neutral. If this were so, then the secretion of hydrogen ions by jejunal tissues would play an important role in the maintenance of this microclimate.

Very few studies exist that deal with the production of acid by biological tissues, or with the mechanism for the maintenance of pH as a controlled variable, despite the fact that many varieties of animal tissues can cause acidification of surrounding incubation media. However, much data exists as a consequence of investigations into other phenomena, so that some insight into the general principles behind acidification can be gained by a comparative study of the literature over the complete range of vertebrate animals.

Where variations in the levels of acidity can be shown to occur in tissue compartments separated by biological membranes, it is often impossible to distinguish clearly between one or other of several mechanisms that might cause these variations to occur. Acidification might be caused by the net transfer of certain ions involving imbalances in the total ion concentration on one side of the membrane or by exchange mechanisms of certain ions, involving no such ion imbalances. The net flow of hydrogen ions in one direction, electrogenically or with an accompanying anion, could cause acidification. It could also be accomplished by the net flow of hydroxyl

ions in the reverse direction, electro genically, or with an accompanying cation. Alternatively, net transfer of compounds potentially able to buffer hydrogen ions could cause acidification or alkalinisation, with no movement of hydrogen or hydroxyl ions whatsoever.

With respect to exchange mechanisms, cationic exchange involving protons or anionic exchange involving hydroxyl ions might be the causes of changes in acidity; however, the simultaneous movement of ions in tissues which acidify, is no guarantee of their participation in any acidification mechanism. Similarly, where tissues both acidify and are capable of transport of anions or substances capable of buffering hydrogen ions, e.g. bicarbonate ions, as in the rat jejunum (Parsons, 1956; Wilson & Kazyak, 1957; Powell, Leif, Solberg, Plotkin, Catlin, Maenza & Formal, 1971) it is often impossible to distinguish between transport of the buffering anion, e.g. bicarbonate, as an effect consequent on the secretion of hydrogen ions or as a cause per se of variations in the hydrogen ion concentration. Nevertheless, acidification or alkalinisation seems to be an event fundamental to biological processes. All cells maintain intracellular acidity within definite limits and where cells or tissues do not actually affect the external pH of their suspending media, often considerable evidence has been amassed which points to the existence of apparent proton fluxes in order to maintain internal pH. Tissues that will affect the acidity of their suspension medium

directly include, plant chloroplasts (Baltschevsky & Von Stedingk, 1966), bacteria (Schultz, Epstein & Solomon, 1963), mitochondria (Brierly, Murer, Bachman & Green, 1963), yeasts (Lundegardh, 1945), turtle bladder (Schilb & Brodsky, 1966), bullfrog colon (Perheentupa, Harrison & Harrison, 1972), frog skin (Fleming, 1957), mammalian kidney (Pitts & Alexander, 1945), all mammalian gastric mucosa including frog, rat, dog and man (Janowitz, Colcher & Hollander, 1952; Davenport, 1962; Tsukamoto, 1961; Ihre, 1938) cortical membranes (Barrene, McCulloch & Nimms, 1937), rabbit gallbladder (Whitlock & Wheeler, 1969), dog, human, hamster and rat proximal jejunum (Robinson, 1935; Wilson, 1953; Wilson & Kazyak, 1957; Klotz & Schloerb, 1971), pancreas (Rothman & Brooks, 1965) and erythrocytes (Warburg, 1922). Tissues which do not seem to alter the acidity of their suspension medium but yet provide evidence of regulation of internal hydrogen ion levels, include, muscle (Waddell & Butler, 1959), mollusc ganglion nerve cells (Sorokina, 1965) and canine lung (Effros & Chinard, 1969).

It will be convenient to review the literature as if two distinct and mutally exclusive systems existed, capable of acidification. From a review of the empirical data available, a common set of principles governing acidification will be derived from the apparently more commonly occurring of the two systems. The existing and present experimental data for acidification in the small intestine will be examined in the light of these derived postulates and a model for intestinal acidification proposed.

The Mitochondrial Model.

In an attempt to formulate an alternative hypothesis that would explain the way in which oxidative phosphorylation is coupled to the electron transport chain, Mitchell (1966, 1968) proposed a chemi-osmotic theory of coupling which included basic postulates relevant to acidification in general. Classical theories of oxidative phosphorylation coupling infer the existence of chemical intermediates common to both the phosphorylative and the oxidative pathway. Since mitochondria, e.g. rat heart muscle mitochondria (Brierley et al., 1963) and rat liver mitochondria (Judah, Ahmed, McClean & Christie, 1965) will acidify their external suspension media during respiration, Mitchell postulated that the reverse flow of protons across the mitochondrial membrane could provide the energy for ADP phosphorylation. There are three basic postulates of the chemi-osmotic coupling hypothesis that are relevant to acidification: -

a) Mitochondrial membranes contain membrane-bound ATP-ase whose active centre is specifically accessible to protons from one side of the membrane and to hydroxyl groups from the other side. In this way, ATP hydrolysis is stoichiometrically linked to the translocation of either one proton or one hydroxyl molecule per ATP molecule hydrolysed, via the asmmetrical ATPase, which causes in effect, charge separation.

b) The membrane also contains exchange-diffusion systems that allow reversible cation flow exchange against protons and anion exchange against hydroxyl ions, in order to compensate for any alterations in the overall osmotic balance.

c) The mitochondrial membrane has a low permeability to ions in general and can be regarded as proton-impermeable.

Evidence suggests that yeast, bacteria and mitochondria have the same mode of acidification. Yeast cells, during anion translocation (Lundegaerdh, 1945) and bacteria e.g. E. coli, (Schultz, Epstein & Solomon, 1963) acidify their suspension medium. In yeast cells (Conway, Brady & Carton, 1950) and in the bacterium S. faecalis (Harold, Pavlasova & Baarda, 1970), the cytoplasm, during acidification, was demonstrated to be alkaline and the acidification reaction itself was shown to be confined to the cell periphery in yeast cells (Conway & Downey, 1950). In mitochondria, alkalinisation of the outer membrane occurs (Chance, Britten & Mela, 1966). These observations underlie the fact that the acidification reaction occurs at the cell periphery and that subsequent charge separation occurs, as has been proposed for mitochondria (Rossi, Stefano, Bielawski & Lehninger, 1966).

Some alternative sources of acid are possible as have been postulated for the yeast cell, notably succinic acid formation via intracellular metabolism and

also consequent carbon dioxide production (Conway & Brady, 1947, 1950). However, both succinic acid and carbonic acid are unlikely candidates for acid production or at least are not the major cause of acidification since the cytoplasm becomes slightly alkaline and not acid. The converse might be expected to occur if intracellular acid production was the cause of external acidification. Also lysing mitochondrial membranes with Triton X-100 produces little change in extra-mitochondrial pH (Mitchell & Moyle, 1967) whereas one might expect an increase if organic acids, e.g. carboxylic acid cycle intermediates were the cause.

With respect to Mitchell's postulate of ion exchange diffusion systems, it is thought that acidification occurs with compensatory movements of potassium ion in yeast (Conway & Brady, 1947, 1950), in the bacterium E. coli (Schultz et al., 1963) and S. faecalis (Harold et al., 1970) and in the mitochondrion (Judah et al., 1965; Palmieri & Quagliariello, 1969). When protons flow back into the mitochondrion, extracellular hydrogen ions exchange for intracellular potassium ion so that ATP synthesis is coupled with a subsequent phase after respiration, when potassium efflux occurs (Mitchell & Moyle, 1969; Massari & Azzone, 1970). This cation transport of potassium into the cell is mediated via mitochondrial ATPase (Gomez-Puyou, Gijon & Tuena, 1965) by way of ATP hydrolysis (Mitchell & Moyle, 1969). In S. faecalis, it has been shown that membrane ATPase

inhibitors inhibit hydrogen ion movements and that in both bacterial and mitochondrial hydrogen ion extrusion, a metabolic-dependency has been found, in that either a glucose (Harold et al., 1971) or a lactate (Reeves, 1971) source of substrate is required. This implies the involvement of the products of intracellular metabolism in the acidification process.

The bacterial and mitochondrial membrane is very similar to other membranes previously studied in that electrically neutral species traverse the membrane easily, whereas charged species hardly penetrate the membrane at all (Schanker et al., 1962; Mitchell & Moyle, 1969). The membrane is postulated to be totally impervious to protons but that proton-conducting pathways exist which under certain circumstances allow protons to cross the membrane. These proton-conducting pathways correspond to an ATPase system and an electron transport chain system; this is most likely since both electron chain inhibitors and uncouplers of oxidative phosphorylation will attenuate transient pH changes in E. coli (Reeves, 1971). Hydrogen ions are extruded so as to provide an electromotive force down which external cations can diffuse. Proton-conducting anti-biotics will reduce acidification in mitochondria (Mitchell, 1966) and in S. faecalis (Harold et al., 1970); in S. faecalis, antibiotics known to affect potassium diffusion, have no effect on acidification. This is a further piece of evidence showing that potassium ion movement is dependent

on an initial hydrogen ion translocation step and not the reverse.

In addition to the basic postulates for the mitochondrial model of acidification, acidification can be regarded as the result of a carrier-mediated active transport system (Rossi & Azzone, 1970) and not due to a passive realignment process of hydrogen ions because of existing transmembrane potential differences, as would be predicted by the Nernst equation. Finally, it can be postulated that the accompanying anion produced by charge separation during acidification is retained within the cytoplasm and is buffered by cell buffers.

Little information is available as to the chemical nature of the accompanying anion which is widely assumed to be an hydroxyl ion. This is most probably buffered by intracellular carbon dioxide formation and the whole process mediated at high rates of acid production by the enzyme, carbonic anhydrase. At low rates of acidification, the involvement of the enzyme may not be necessary.

The main points behind the 'mitochondrial' model of acidification can be summarised as follows: -

a) The membrane at which acidification occurs is impermeable to all ions, especially to protons, except where special transport mechanisms exist.

b) Acidification is an active energy-dependent process occurring by charge separation across the membrane and not by intracellular organic acid production.

c) This acidification process is not mediated via the transmembrane potential gradients, and involves the passive movement, in the opposite direction, of a counterion, most likely potassium.

d) The charge separation involves complementary alkali production, probably hydroxyl ion via ATP hydrolysis; the hydroxyl ion is buffered by intracellular carbon dioxide production which is enzyme-mediated if the rate of cytoplasmic alkalisation is high.

The Alternative Model.

It is often impossible, under classical 'in vitro' experimental conditions, to distinguish between acidification caused by proton secretion and that caused by base depletion of hydroxyl ion or bicarbonate. Often it cannot be said unequivocally whether or not membranes are permeable to hydrogen ions, hydroxyl ions or bicarbonate ions. An alternative system for acidification is proposed to be the exchange of an anion with hydroxyl or bicarbonate ions. This anion exchange system might cause acidification by an anion being transported against its concentration gradient, e.g. chloride ion and a basic anion being sequestered out of the compartment to be acidified, by its compensatory movement to preserve electroneutrality. Alternatively, one might postulate active transport of the basic anion itself, e.g. bicarbonate ion or hydroxyl ion.

It will be convenient to review the literature as if the anion exchange model and the mitochondrial model were mutually exclusive although where acidification occurs, the anion exchange system cannot be ruled out as acting complementarily. The following sections discuss the validity of the mitochondrial model in its application to other acidifying tissues.

LITERATURE REVIEW.

The mitochondrial model and empirical observations.

a) Membrane Permeability.

It is a fundamental tenet of membrane biology that the classical plasma membrane comprising of the bimolecular leaflet is a considerable barrier to diffusion (Davson & Danielli, 1940). Permeability of substances is dependent on their solubility in lipids and their molecular volume unless some specific transfer mechanism exists for their transport. It is further postulated that unionised, i.e. uncharged species move through membranes at a faster rate than ionised, i.e. charged species and that ionisation at cellular pH is a mechanism for the retention of substances within the cell (Davis, 1958).

It seems that most, if not all, biological membranes are impermeable to hydrogen ions. Often, the intracellular pH of various tissues can be altered by variations in the extracellular carbon dioxide tension but not by non-carbonic acidosis (Halpern & Binaghi, 1959; Kibler, O'Neil & Robin, 1964). Erythrocytes were thought to be permeable to protons since in the presence of chloride ion, red blood corpuscles will make their suspension medium alkaline (Warburg, 1922; Van Slyke, 1923). As this has been shown to be more likely due to bicarbonate hydroxyl ions exchanging for extracellular chloride and need not involve the movement of hydrogen ions, it is unnecessary to invoke permeablility of erythrocyte membranes to protons.

Little experimental evidence exists for the movement of hydrogen ions across membranes as a passive process, except possibly in some bacteria. In E. coli, the intracellular pH is linearly related to the external pH over the range 5.0 - 9.0 with some buffering towards more neutral values (Kashket & Wong, 1969). Some evidence exists for proton permeability in muscle membranes. Direct measurements of intracellular muscle cell pH with glass microelectrodes have shown a linear relationship between internal and external pH (Paillard, Sraer & Ardaillou, 1971). This suggests that the protons are distributed according to the transmembrane potential in a passive manner. However, indirect methods based on the distribution of weakly ionising compounds, e.g. 5,5-dimethyl 2-4 oxazolidinedione (DMO) give values for intracellular pH of 6.0 and suggest an independence of intracellular and extracellular pH. As noted by Paillard and his co-workers (1971), it is possible that both methods measure different parts of an inhomogenous cytoplasm. In conclusion, therefore, it can be said that all biological membranes are impermeable to hydrogen ions and that evidence to the contrary is very slight.

b) Membrane permeability and the carbonic acid system.

As a general rule it can be postulated that biological membranes are impermeable to the ionised species of the carbonic acid system: -

$$H_2O + CO_2 = H_2CO_3 = HCO_3^- + H^+$$

but remain freely permeable to the unionised species (Jacobs, 1920). The differential permeability of membranes to carbon dioxide and bicarbonate ion is a consequence of 'non-ionic' diffusion.

This general scheme has been demonstrated to be valid in diverse biological membranes including: - flower petal cells, Ehrlich ascites tumour cells, sheep placenta, blood-brain barriers in mammals, carp swimbladder, rabbit gall bladder, rat muscle and rat distal tubules (Jacobs, 1920; Breathnach, 1966; Bradley & Semple, 1962; Halpern & Binaghi, 1951; Poole et al., 1964; Curet, 1971).

Bicarbonate allegedly permeates freely through red blood cells, vascular capillary beds (Scarpa, Ceccheto & Azzone, 1970) and muscle (Adler, Roy & Relman, 1965), presumably because these tissues accommodate bicarbonate during whole body pH regulation (Nichols, 1958) or transport bicarbonate during respiration. The cause of this latter additional selectivity in being permeable to bicarbonate is not known and may involve specialised transport mechanisms. With these three exceptions, biological membranes are not permeable to bicarbonate ion.

c) Acidification and the membrane potential.

It is possible that variations in the hydrogen ion concentration on either side of a membrane might be caused by the passive realignment of hydrogen ions along the existing membrane potential as dictated by the Nernst

equation. Where an ion is transported across a membrane against its electrochemical gradient, a potential difference is generated across the membrane due to the differences in the chemical potential of that ion in the tissue compartments separated by the membrane. Other ions present in the two compartments tend to passively realign because of the potential difference, in accordance with the equation: -

$$\text{Potential (mV)} = \frac{RT}{nF} \ln \frac{(a1)}{(a2)}$$

i.e. the equilibrium concentrations of ion A in the two compartments will be dictated by the membrane potential and that the sign of the potential will determine which compartment has the greatest concentration of ion A at equilibrium.

Assuming a neutral internal pH for cells, it can be calculated that a change in the external pH caused by intracellular acidification, which involved a concentration ratio across the membrane of ten, would require a trans-membrane potential of approximately 60 mV. In very many biological tissues in which a transmembrane potential can be detected, the sign of the potential is in the opposite direction to that which would support passive hydrogen movement. Muscle tissue is characterised by the existence of very large transmembrane potentials of the order of 90 mV (Fatt & Katz, 1953). Muscle cell pH has been estimated as being between 6.8 - 7.2 (Caldwell, 1958; Waddell & Butler, 1959; Sorokina, 1961; Adler et al, 1965; Walker, Goodwin & Cohen, 1969; Williams,

Withrow & Woodbury, 1971). Although no empirical observations seem to exist demonstrating acidification of the incubation medium by metabolically active muscle, the existence of hydrogen ion pumps can be inferred by the direction of the transmembrane voltage. With the large potential difference as is present in muscle (Hill, 1955), hydrogen ions ought to diffuse into muscle, at neutral pH until an intracellular pH of 5.9 was reached. The sign of the potential is such that the outer surface of muscle membrane is positive to the inner surface and the net tendency would be for protons to be attracted inwards. The pH does not rise and it can be concluded that active hydrogen ion extrusion does occur. Where tissues have the transmembrane potential polarised in the same direction as muscle cells and yet acidify, clearly the transmembrane potential cannot be thought of as the cause of acidification.

Often the potential, if not in the reverse direction from that needed to cause passive realignment, is too small to effect the changes, e.g. renal tubules (Rector, Carter & Seldin, 1965) turtle bladder (Gonzalez & Schilb, 1969; Schilb & Brodsky, 1966), frog skin (Emilio, Machado & Menano, 1970), frog gastric tissue (Rehm, Hokin, Graffenried de Bajandas & Coy, 1951). Finally, it has been shown that cases exist of tissues that acidify but demonstrate no transmembrane potentials, e.g. in all gallbladders so far investigated, i.e. fish, frog, bullfrog, toad, turtle, guinea pig, rabbit, cat and dog gallbladders are capable of acidifying bile but little or no transmural potential exists (Diamond, 1968).

Conversely in the bladder of the toad Bufo marinus, a 70 - 90 mV transmural potential exists but no variations in the mucosal pH are ever seen (Leaf, 1965). It seems that the explanation of passive realignment of protons in accordance with the Nernst potential must be ruled out as a cause of acidification and the converse process considered, that hydrogen ion extrusion is itself an active component of the membrane potential.

Where many different ions are transferred and are capable of generating potential differences due to active transport, it is impossible to equate hydrogen ion movements with the potential difference or with the short circuit current, since the resulting potential difference and the overall short circuit current is the algebraic sum of many potentials and currents, all differing in direction and magnitude. Unless knowledge of the flux rates exists of only those ions causing potentials, at best it can only be inferred whether hydrogen ion movement is electrogenic or not.

From studies on frog gastric mucosa (Hogben, 1955, 1960) which is probably the most active acid secreting tissue known and capable of concentrating protons to a level of one million times that of the plasma level, chloride ion is actively transported to the mucosal surface. This process is electrogenic and when acid production occurs, the resting potential of the stomach falls (Rehm, Hokin, et al., 1951). A reverse potential can be obtained during active acid secretion by incubations in solutions containing the impermeant anion sulphate

(Heinz & Durbin, 1959) which offers evidence of an active hydrogen ion pump. Two active pumps are postulated, an active chloride and an active hydrogenion pump, which are both electrogenic. Consequently, the resting potentials merely reflect the discrepancy in activity between the two pumps.

It was often thought that active sodium transport accounted for most of the membrane potential differences as is the case in frog skin (Ussing & Zerahn, 1951), however this idea is being increasingly questioned. In the turtle bladder, discrepancies between the measured short circuit current and total serosal sodium movement have been interpreted as being both due to active bicarbonate movement (Gonzalez, Shamoo, Wysbrod, Solinger & Brodsky, 1967) and as being due to active hydrogen transport in the reverse direction (Steinmetz, Omochi & Frazier, 1967). Also, in the absence of sodium, the sign of the potential is normally reversed, demonstrating that other ions cause potential differences and that acidification is a system not subject to the constraints of the sign of the existing potential differences (Steinmetz et al., 1967). Similarly in the colon of the other amphibian species Bufo arenarum, which acidifies its incubation medium (Lew & Carlisky, 1967), potential differences in the absence of sodium reveal an ouabain-insensitive component of the short circuit current that is rapidly inhibited by acetazolamide (Lew, 1970). Although the authors favour bicarbonate as being the cause of the non-sodium short

circuit current component, they also state that active mucosal hydrogen ion extrusion in the opposite direction to the postulated active bicarbonate transport would also account for the discrepancies in the total measured current and the total sodium flux (Carlisky & Lew, 1970).

With respect to cerebrospinal fluid whose pH is maintained between very small limits, a homoeostatic mechanism is proposed, involving active transport of unspecified ions (Severinghaus, Mitchell, Richardson & Singer, 1963; Mitchell, Carman, Severinghaus, Richardson, Singer & Schnider, 1965). It has been proposed that active transport of hydrogen ions occurs from the arterial blood into the cerebrospinal fluid (Leusen, 1972). The existence of a suitable transmeningeal potential has been detected (Severinghaus, 1964) with the cerebrospinal fluid positive to arterial blood. The potential difference has been shown to be pH dependent. It is also ouabain sensitive (Held, Frencl & Pappenheimer, 1964) and responsive to potassium concentration (Cameron & Kleeman, 1970) and is therefore quite as likely to be active sodium transport as proton transport into the cerebrospinal fluid causing the transmeningeal potential (Welch & Sadler, 1965).

In tissues where sodium ion is transported and also acidification occurs, it is difficult to establish which of the ions is the primary cause of any established potential difference. The relationship of sodium to hydrogen flux is considered in a later section. In all events, it can be concluded that rather than being a

consequence of passive realignment as dictated by the Nernst equation, hydrogen ion movements are quite likely candidates themselves for causing electrogenic fluxes in biological tissues.

d) Charge separation and intracellular acid production.

One of the fundamental questions raised by the acidification phenomenon is whether it is caused by the production of organic acids intracellularly or as the 'mitochondrial model' requires, charge separation at some outer membrane. Where charge separation occurs at a biological membrane, i.e. positive and negative ions are separated, possibly enzymically by some electrogenic process, it may be difficult to demonstrate its occurrence by physical techniques. However, where tissues acidify suspending media, it is reasonable to assume the corollary of charge separation, that on disrupting the membrane at which charge separation occurs, little acidification should be seen since the equivalent amounts of separated acids and base would recombine. On the other hand, if acidification were due to intracellular production of organic acids, e.g. lactic acid, then membrane disruption should not seriously affect acidification and might even enhance it, since a permeability barrier, potentially preventing free diffusion of protons, has been removed.

As has been noted previously, convincing disruption experiments exist for mitochondria but data available from other tissues is scant. Organic acid production has been shown to be unable to account for all the acid production that occurs, as judged by measuring

lactic acid concentrations in the incubating media in turtle bladder, bullfrog colon from Rana catesbiana and from frog skin (Green, Steinmetz & Frazier, 1970; Perheentupa, Harrison & Harrison, 1972; Emilio et al., 1970). It was further noted in frog skin that when the epidermal surface produces acid, the reverse corial surface produces an equivalent amount of base (Fleming, 1957). This would seem to indicate if not actual charge separation at the epidermal membrane then some mechanism whereby there is differential migration of the anion from intracellular organic acid production. Little information seems to be available on simultaneous lactate production and acidification in mammalian tissues, apart from that in the small intestine, which will be discussed later.

In the mammalian stomach, the amount of acid appearing mucosally is equivalent to an amount of base produced serosally, since on immersing strips of gastric tissue in incubation media, no acidification is seen (Davies, 1951). This type of experiment is formally equivalent to disrupting as in the mitochondrial experiments, since the serosal and mucosal products are allowed to recombine. Clinically, in man it is a well known fact that after meals there is an 'alkaline tide' of bicarbonate present in the venous blood return demonstrating that some sort of complementary base production occurs (Davis, 1951; Teorell, 1951). By vital staining techniques, Bradford and Davies (1950) showed that acidification occurred at the boundary

between the cytoplasm and the canaliculi of the secreting parietal cells. Since they also saw an alkalinisation within the cytoplasm of these cells, it is very unlikely that hydrogen ions are derived from organic acids within these cells. In rabbit gall bladder, similarly, an equivalent amount of serosal alkalinisation occurs when the mucosal medium is being acidified, although alkalinisation can occur in the absence of corresponding acidification (Whitlock & Wheeler, 1969).

In summary, it can be said that such evidence as exists seems to preclude a significant role for the organic acids and does not contradict the principle of charge separation at membranes.

e) Cation: Hydrogen exchange.

i) Hydrogen with potassium.

In accordance with the concept of compulsory potassium: hydrogen ion cation exchange in the mitochondrial model, frog skin will not acidify potassium-free media (Fleming, 1957). No information is available on the turtle bladder; however, in the colon of the frog Rana catesbiana, hydrogen ions and potassium ions move in the same direction (Perheentupa, Harrison & Harrison, 1972). It is possible that the cation exchange occurs and then potassium moves in the same direction as the hydrogen ions, passively down the concentration gradient, thereby obscuring the initial hydrogen exchange.

In the rabbit gall bladder, the absence of potassium causes a separation of the acidification and the alkalinisation processes that occur, in that mucosal acidification is inhibited whereas serosal alkalinisation continues (Whitlock & Wheeler, 1969). A measure of criticism may be levelled at 'ion depletion' experiments because removal of one specific ion may have effects on metabolism in general and may metabolically hinder acidification without actually participating in any exchange mechanism.

In muscle tissue there is impressive evidence of a compulsory potassium : hydrogen ion exchange mechanism. Acute respiratory acidosis in dogs will cause plasma hyperkalaemia (Scribner, Fremont-Smith & Burnell, 1955); the converse is also true, that intraperitoneal injection of potassium causes extracellular acidosis in the dog and transient alkalosis in dog skeletal muscle which is then buffered by succinate production (Hudson & Relman, 1962). Furthermore, a direct relationship has been found between extracellular pH and muscle potassium content; a decrease in extracellular pH will cause a loss of potassium from muscle tissue (Adler et al., 1965). This compulsory potassium : proton exchange is not seen in cardiac muscle (Young, Sealy & Harris, 1954; Brown & Mowlem, 1965) and has not been found in renal distal tabules (Malnic, de Mello Aires & Giebisch, 1971).

In frog gastric tissue, gastric secretion varies directly with serosal potassium concentration (Harris &

Edelman, 1960) and falls to zero in its absence (Davis Rutledge, Keese, Bajandas & Rehm, 1965). Net potassium flux has been found to be passive in frog skin (Villegas, 1963) yet in rat stomach the mucosal potassium concentration is slightly higher than plasma levels (Webster, Toovey & Skoryna, 1958) and as such the prevailing potassium movement is in the same direction as the hydrogen ions. It seems then that if there is a relationship between acidification in gastric tissue and potassium levels, then it is not one of simple ion exchange. Since potassium flux is passive, yet there is a mucosal:plasma concentration ratio greater than unity, a more likely explanation is that serosal potassium is exchanged for intracellular sodium ion in the acid secreting cell. Potassium ion then subsequently having being transported into the cell, diffuses out mucosally down its concentration gradient in the same direction as the hydrogen ions. This dependency of acidification on the conventionally postulated Na^+/K^+ATPase-mediated sodium pump would explain the dependency on serosal potassium and also the direction of the net potassium flux. The situation in the gut gastric tissues may represent a more complex one than that occurring in muscle.

ii) Hydrogen with sodium.

Although not postulated in the mitochondrial model for acidification, some evidence exists for a

sodium:hydrogen ion coupling arrangement in mammalian tissues and some evidence exists against the principle of such an arrangement in amphibian tissues.

When turtle bladder is incubated in sodium-free buffers in vitro a reversal of the sign of the normal potential is seen, yet acidification proceeds as usual (Steinmetz, Omochi & Frazier, 1967). The authors interpreted this as demonstrating that acidification was occurring in the absence of sodium flux which would cause the potential to be in the opposite direction, thus precluding an absolute need for sodium flux to be involved in acidification. Furthermore, in turtle bladder, sodium transport has an energy source derived from anaerobic metabolism and is inhibited by dinitrophenol (Bricker & Klahr, 1966) whereas acidification is completely reduced by anaerobiosis and is unaffected by dinitrophenol (Steinmetz, 1967). The converse is true in frog skin where, unlike turtle bladder, acidification will proceed under anoxic conditions whereas sodium transport is abolished (Fleming 1957). Also, in frog skin, acidification occurs at too low a rate to be involved in a obligatory exchange mechanism, if there is to be tight coupling between the proton flux and the sodium flux (Fleming, 1957).

As regards mammalian tissues, rabbit gall bladder in vitro shows a requirement for sodium ion during incubation, as demonstrated by incubation in sodium-free media (Whitlock & Wheeler, 1969). However, as noted previously, experiments involving buffers deficient in

certain ions must be viewed with caution, especially in the case of sodium ion where not only are substantial reductions seen in the oxygen uptake of the tissue and hence, by inference, overall metabolism and general tissue viability, (Jordana & Igea, 1970; Ponz & LLuch, 1971), but also on incubation in sodium-free media, the passive permeability of membranes is known to decrease (Esposito, Faelli & Capraro, 1969). In this way it is possible that both a reduction in metabolism and a decreased membrane permeability could cause reductions in the appearance of hydrogen ion in the buffer without actually affecting any sodium:proton exchange mechanism.

In muscle tissue, ouabain will reduce the intracellular pH and will reduce the pH gradient across the muscle membrane (Williams et al., 1971) which again suggests coupling between hydrogen ion extrusion and the sodium pump. Similarly, serosal ouabain affects acidification in the rabbit gall bladder (Whitlock & Wheeler, 1969). The problem in interpreting the effects of such inhibitors on whole organs can best be illustrated in cerebral cortex. As in many other tissues, Na^+/K^+ATPase has been demonstrated to occur, as in cat brain (Vates, Bonting & Oppelt, 1964), in which ouabain causes reductions in the total amount of cerebrospinal fluid produced. It is difficult, therefore, to distinguish by the use of inhibitors between inhibition of the sodium pump causing a reduction in cerebrospinal fluid production with resultant loss of buffering power, and inhibition of some primary acid-base regulatory

system or other physiological mechanisms that altered the ion content and buffered or affected hydrogen ion concentration.

In gastric tissue, attempts to relate net sodium flux with acid secretion have not been successful and many workers have ruled out obligatory proton:sodium exchange (Linde, Teorell & Obrink, 1947; Cummings & Vaughan, 1965; Moody & Durbin, 1965). Yet in the frog, gastric secretion is inhibited by ouabain (Cooperstein, 1959; Davenport, 1962). Furthermore, although mucosal sodium levels in gastric acid are approximately the same as the plasma levels (Webster, Toovey & Skoryna, 1958), and the net flux is blood to lumen, the lumen to blood flux is strongly influenced by the acidity in the gut, being maximal when the stomach contents are neutral and minimal when the stomach contents are acid (Code, Higgins, Orvis & Scholer, 1963).

More convincing evidence for a compulsory sodium:hydrogen ion exchange mechanism comes from the kidney, where it is postulated that active secretion of protons occurs. Carbon dioxide from tubular cell metabolism, the extracellular fluid, and blood is hydrated by the enzyme carbonic anhydrase, bicarbonate moves into the extracellular fluid and simultaneously, the proton derived from the carbon dioxide hydration exchanges for sodium ion at the tubular cell-lumen interface (Brazeau & Gilman, 1953). This proton decomposes the bicarbonate in the tubular lumen causing migration of bicarbonate

into the tubular cell. The net result is the transfer of bicarbonate out of the tubular lumen along with sodium and an acidification of the luminal contents.

If active secretion of hydrogen ions occurs in the proximal and distal tubules, protons should combine with bicarbonate ion to form carbonic acid which would then slowly form carbon dioxide and water. In this case, there is reason to believe that a disequilibrium pH should exist which would then change if the system came to rest, to the equilibrium pH, since the dehydration of carbonic acid is a slow reaction. This disequilibrium pH would be difficult to detect if the slow reaction were catalysed by carbonic anhydrase. Direct in situ measurements with microelectrodes have been made in the proximal tubules of diamox treated rats (Vieira & Malnic, 1968) and a disequilibrium pH has been detected.

As with the jejunum (Powell et al., 1971) the sodium transport is related to tubule cell luminal bicarbonate concentration (Ullrich, Radkte, Rumrich & Kloss, 1971). The authors also found that bicarbonate transport was related to luminal bicarbonate concentration and that the effect was not specific for bicarbonate but could be imitated by luminal glycodiazine (pKa 5.7) and sulfamerazine (pKa 7.1), weak acids that are also capable of buffering. These buffers had the same effect on the rate of sodium transport as did bicarbonate (Ullrich et al., 1971). The authors postulated that the

concentrations of anion in the lumen promoted higher rates of acidification so that the anion of the weak buffer could be transported as the neutral species. Acidification will reach a maximal level but is also coupled with sodium transport which also will reach a maximal level of coupled transfer. This would explain the apparently unrelated observation that the addition of buffering compounds to the luminal surface of the tubular cell causes an increased uptake of sodium. What has really occurred is that the anion of the buffer requires acidification to occur as a prerequisite of non-ionic diffusive transfer and that this acidification is coupled with sodium movement in the reverse direction.

In the jejunum also there is evidence for a sodium:hydrogen ion exchange pump. It seems that there are two forms of sodium transport, an electrogenically neutral sodium pump in which sodium is transported with an accompanying chloride anion, and an electrogenic sodium pump which exchanges the sodium ion for a cation, presumed to be potassium (Munck, 1972). Since this electrogenic flux is very much smaller than the neutral flux, it is possible that here is a link between acidification and sodium movement in that possibly protons and not the presumed potassium ion, are exchanged with the electrogenic portion of the sodium flux. This would explain why acidification in gastric tissue could not be equated with the net sodium flux, yet remained sensitive to inhibition by ouabain, because the ouabain-sensitive portion of the sodium flux was also the

electrogenic portion and this alone was involved in a compulsory cation exchange.

Recent work on acidification in the lizard gastric mucosa has shown that ouabain will reduce the transmural potential, the short-circuit current and also acid secretion, suggesting a role for Na^+ /K^+ ATPase in the secretory process. It may well be that Na^+/K^+ATPase enzyme, normally assumed to be the enzyme associated with sodium transport, may have to be regarded primarily as the enzyme responsible for the proton pump and that the cation specificities merely reflect the need for an ion exchange mechanism to preserve electroneutrality. This may be especially relevant in view of the evidence that suggests that the bulk of sodium transport is a neutral (Munck, 1972) and a passive (Turnberg et al., 1970) process. Therefore it must be concluded that a compulsory cation exchange involving sodium rather than potassium as predicted by the mitochondrial model, cannot be ruled out.

f) Acidification and the involvement of carbon dioxide.

The failure of intracellular acid production to account for acidification causes many workers to propose that bicarbonate transfer, either active or passive, is one of the causes of acid production in various tissues. Bicarbonate would be sequestered from out of a fluid compartment and would in this way cause acidification. This mechanism has been proposed for turtle bladder (Schilb & Brodsky, 1966) where the

bicarbonate levels at the end of incubation are inevitably lower than those at the beginning. Specifically, in turtle bladder, the mucosal carbon dioxide tension is higher than the serosal tension (Green, Steinmetz & Frazier, 1968) which indicates that proton secretion rather than bicarbonate depletion has occurred since on bicarbonate depletion one would expect a complimentary lowering of the mucosal carbon dioxide tension and an elevation in serosal tension, i.e. the exact reversal of what is actually seen. The relative levels of the carbon dioxide tensions indicates that proton secretion has occurred, that the mucosal bicarbonate has been decomposed by acid and consequently the mucosal carbon dioxide tension has risen. Acidification has also been shown to occur in bicarbonate-free incubation media, in the absence of an exogenous source of carbon dioxide (Steinmetz, 1967; Perheentupa et al., 1972; Emilio et al., 1970). More exact and elegant work on the turtle bladder has shown that the concept of bicarbonate transfer causing acidification cannot as yet be ruled out (Schwartz & Steinmetz, 1971; Schilb & Brodsky, 1972). Mucosal ouabain will reduce acidification, implying some involvement of the sodium pump, yet this inhibition can be reversed by increasing the availability of carbon dioxide in the mucosal solution and suggests that the action of ouabain is to reduce sodium transport, in agreement with orthodox views, but that there is also reduced energy turnover for the pump and a reduction in

intracellular carbon dioxide production. This involves a reduction in carbon dixide available for hydration to carbonic cell for acidification or for mucosal bicarbonate production which is then subsequently transferred from out of the mucosal fluid compartment in the ensuing acidification process. Schilb and Brodsky have further shown that when the correct experimental values for serosal and mucosal carbon dioxide tension have been chosen, acidification can be seen not only with concomitant reduction in mucosal bicarbonate level but also with parallel reductions in mucosal carbon dioxide tension. The authors argue that carbon dioxide production inside the cell via intracellular metabolism is so great that when metabolic carbon dioxide diffuses out of the cell as is inevitable, this diffusion process will mask any changes in carbon dioxide tension caused by acidification. It has been noted, however, that acidification in the turtle bladder will take place in the absence of an exogenous source of carbon dioxide, in bicarbonate-free solutions (Steinmetz, 1967; Emilio et al., 1970). This would seem to preclude bicarbonate as a cause of acidification. However, in these same amphibian tissues, in the absence of external carbon dioxide and bicarbonate, acetazolamide will inhibit acidification. Acetazolamide (Diamox) has been characterised pharmacologically as inhibiting only the enzyme carbonic anhydrase (Maren, 1967). Acetazolamide will also reduce acidification occurring in the proximal and distal convoluted tubules of the mammalian kidney (Pitts &

Alexander, 1944, 1945; Litchfield & Bott, 1960; Gottschalk, Lassiter & Mylle, 1960) and reduces the acidification seen in rat gastric mucosa (Tsukamoto, 1961). These obvervations led to the proposal that acidification was caused by intracellular hydration of metabolically produced carbon dioxide, mediated by the enzyme carbonic anhydrase. Further evidence in support of this explanation of acidification is that carbonic anhydrase enzyme has been found in all tissues that acidify, even in turtle bladder (Scott, Shamoo & Brodsky, 1970) which was previously reported to possess no carbonic anhydrase (Gonzalez, 1969). Conversely, a similar tissue which does not acidify, toad bladder from Bufo marinus, has no detectable carbonic anhydrase activity (Leaf, 1965).

Alternatively, as proposed by Schilb and Brodsky, respiring turtle bladder would quickly cause increases in both bicarbonate and carbon dioxide levels in an initially carbon dioxide-bicarbonate free mucosal solution which upon their sequestration back into the cell would cause acidification. This has certain theoretical difficulties since turtle bladder can acidify to pH values approaching 4 and the pKa of carbonic acid is approximately 6. This means that below a pH of about 5 most of the carbonic acid thus secreted would be in an unionised form and some form of removal of the accompanying bicarbonate anion must be engineered by the cells, e.g. bicarbonate: chloride exchange. This might then fit in with Brodsky and Schilb's concept of carbonic acid moving across the acidifying membrane in one direction and the anion minus proton moving back across the membrane unaccompanied.

It would be extremely difficult to distinguish thermodynamically between that and simple hydrogen ion extrusion and also difficult to verify experimentally.

Evidence against the concept of acidification being mediated by this mechanism is more convincing in various mammalian studies. Acetazolamide will leave muscle cell pH, demonstrated to be susceptible to ouabain, completely unaffected in the in vivo dog, both when the kidneys are left intact or when nephrectomised to prevent renal clearance of the inhibitor (Rollins, Withrow & Woodbury, 1970). Also, ethoxyzolamide, another potent inhibitor of carbonic anhydrase, failed to inhibit acidification in the rabbit gall bladder (Whitlock & Wheeler, 1969). The bicarbonate model for absorption is not thought to be applicable for the mammalian kidney, another tissue that acidifies. Hydrogen ion extrusion has been related to tubular bicarbonate concentration (Ullrich et al., 1971) but this effect has been mimicked by buffers installed in the tubular lumen and it may be that the explanation put forward for this effect in the kidney, applies to the turtle bladder as well, i.e. that of extracellular buffers and not just bicarbonate alone, stimulating acidification. There is, however, convincing evidence that proton secretion does occur in the mammalian kidney (Vieira & Malnic, 1968) but even these authors concluded that intra-tubular bicarbonate load was a key factor in determining the rate of distal hydrogen ion secretion in the distal tubules (Malnic, de Mello Aires, & Giebisch, 1972). One of the difficulties in characterising

the mechanism rather than just recording its properties, is the uncertainty of the actual site of action of inhibitors, e.g. as in the action of ouabain outlined above, which although it may act at its well-documented site of action, the sodium pump, may also have other allosteric effects. This may also be true of acetazolamide.

Although acetazolamide is thought to have only one function, i.e. exclusively, the inhibition of carbonic anhydrase enzyme, some instances have been found where acetazolamide shows an ability to inhibit net rates of secretion of cerebrospinal fluid (Tschirigi, Frost & Taylor, 1954; Oppelt, Maren, Owens & Rall, 1963) and gastric juice (Janowitz, Colcher & Hollander, 1952). Also, acetazolamide has been shown to affect chloride transfer in the stomach (Durbin & Heinz, 1958) at concentrations very much higher than those needed to inhibit carbonic anhydrase activity (Hogben, 1967). It is difficult to reconcile acetazolamide action into any mechanism for homoeostasis of brain pH, since the exact relationship of the cerebrospinal fluid, the blood vascular system and the brain tissue remains unclear. Changes in extracellular and blood plasma pH, induced by hyperventilation, are reflected in the brain pH, yet changes induced by non-carbonic acidosis are not. (Kibler, O'Neil & Robin, 1964). Also, little variations in total brain tissue carbon dioxide will occur, despite wide fluctuations in the plasma bicarbonate (Siesjoe, 1964). In this respect the

blood-brain barrier acts like most other tissue membranes in being impermeable to protons and bicarbonate ions, whilst remaining freely permeable to carbon dioxide. Yet acetazolamide causes a fall in extracellular pH due to non-carbonic acidosis in the intact rat and a simultaneous rise in brain pH (Rollins et al., 1970). In humans, acetazolamide causes increases in the cerebrospinal fluid:plasma bicarbonate ratio and also in the total cerebrospinal fluid bicarbonate concentration with a complementary reduction in chloride content (Maren & Robinson, 1960). These authors provisionally regarded this as being due to inhibition of chloride and hydrogen ion transport rather than inhibition of any carbonic anhydrase activity.

Some of these contradictions can be resolved if it is proposed that carbonic anhydrase does take part in the acidification process but not in the manner conventionally proposed. Instead of regarding acidification as caused by the intracellular enzymic hydration of metabolic carbon dioxide (which would cause a decrease in the cytoplasmic level of pH unless the reaction were located at the membrane surface), the involvement of carbonic anhydrase can be harmonised with the mitochondrial model of acidification by charge separation, by postulating that carbon dioxide buffers the hydroxl radical produced at the membrane surface. Furthermore, it can also be postulated that this buffering effect is only necessary at levels of cytoplasmic alkalinisation where normal cell

buffering is inadequate. This would explain why acetazolamide affects acid production in the stomach (Powell, Robbins, Boyett & Hirschowitz, 1962) and turtle bladder (Schilb & Brodsky, 1966)(which can also acidify media to very acid values) but does not affect gall bladder acidification where only comparatively small amounts of acid are produced. Where it is thought that anion exchange exists between chloride ions and bicarbonate anions, the apparent effect of carbonic anhydrase inhibitors on chloride transfer (Durbin & Heinz, 1958; Kithara, Fox & Hogben, 1967)) can also be reconciled by this scheme. The inhibition of carbonic anhydrase activity would mean a failure to provide adequate buffering of intracellular alkali, with the result that intracellular metabolism is prevented and the net energy dependent extrusion of chloride ion is stopped. In this way a physiological effect uncharacteristic of carbonic anhydrase inhibition might be caused, which had biochemically, carbonic anhydrase inhibition as its cause, i.e. a biochemical reaction having physiologically disparate effects.

It is interesting to speculate that if acidification were caused by some bicarbonate transfer mechanism as proposed for the turtle bladder, a natural corollary would seem to be that the accompanying anion should have to be sequestered away or replaced by the anion of a strong acid with a lower pKa in order to achieve the low pH values seen in some biological tissues.

It would also seem to have to be a surface reaction and with the sequestration of the accompanying anion, it would be almost indistinguishable from proton production via surface hydrolysis of ATP.

g) Acidification and metabolism.

From the mitochondrial model of acid production it was assumed on the evidence from mitochondria, bacteria and yeast, that the source of acidification was intermediate metabolism and that ultimately, acidification was caused by the hydrolysis of ATP. Little experimental evidence has been produced to link acidification with ATP hydrolysis in the tissues cited in which acidification occurs: most of the work done relates to gastric tissue.

An early theory propounded to explain how acidification was achieved in the stomach was the 'redox' theory of acid production, in which the acidification was caused by the electron transfer chain. In this system, the cytochrome groups would remove electrons from substrate molecules containing hydrogen atoms and in this way, form protons. The transferred electrons would be combined with water and oxygen to form an equivalent number of hydroxyl groups at another site. The scheme is as follows: -

$$4\ Fe^{3+} + 4H \leftrightharpoons 4\ Fe^{2+} + 4H^{+} \quad \text{..... eqn 1.}$$

$$4\ Fe^{2+} + O_2 + 2\ H_2O \leftrightharpoons 4Fe^{3+} + 4OH^{-} \quad \text{..... eqn 2.}$$

---

i.e. $4H \longrightarrow 4H^{+} + 4e^{-}$

$4e^{-} + O_2 + 2H_2O \longrightarrow 4\ OH^{-}$

---

overall $4H + O_2 + 2H_2O \longrightarrow 4\ H^{+} + OH^{-}$ .... eqn 3.

It can be seen that there is a theoretical limiting ratio of the number of protons formed per molecule of oxygen used. Although this has been experimentally verified (Bannister, 1965a, 1965b), ratios greater than four equivalents of hydrogen ion per equivalent of oxygen ion have been recorded (Forte & Davies, 1964). The redox theory has lain dormant recently, yet would explain the coupling of gastric acidification with oxygen requirements and might also explain the high incidence of mitochondria in gastric oxyntic cells. More recently the redox theory has been invoked in studies involving non-destructive optical methods involving difference spectra of redox components in the aerobic and anaerobic state (Hersey, High & Jobsis, 1972). Changes in the difference spectra seem to be closely linked with acidification in the frog and rabbit and not merely explained by redistributions in the amounts of energy substrates available. When histamine is added to their preparations, hydrogen ion is produced, but the difference spectra are those characteristic of a transition in mitochondria from state 3 to state 4, i.e. from an active to a resting state.

An alternative scheme, (Davies & Ogston, 1950) is one where ATP hydrolysis occurs, i.e. high energy phosphate compounds generate a proton gradient (the reverse of mitochondrial chemi-osmotic coupling). In this context, dinitrophenol, an uncoupler of oxidative phosphorylation, has been shown to inhibit acid secretion in the stomach (Davies, 1951; Heinz & Durbin, 1957) and

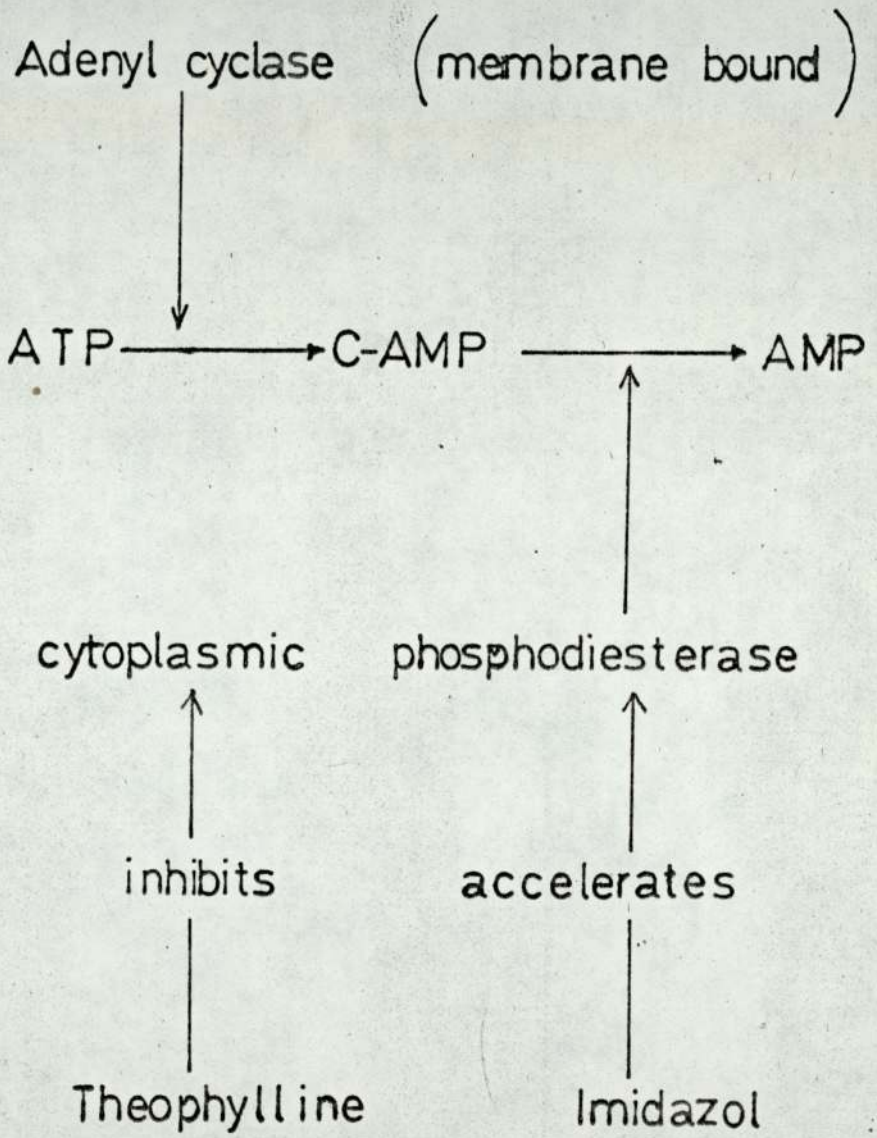
secretory rates have been directly correlated with nucleotide triphosphate levels in gastric tissue (Forte, Adams & Davies, 1965). It might be expected, on theoretical grounds, since acidification has been implicated with the potassium ion in the mitochondrial model and a case can be made for coupling with the sodium flux, that acidification is intimately connected with the sodium pump. The enzyme reported to be responsible for active sodium transport, Na^+/K^+ ATPase has not been detected in frog gastric mucosa (Kasbekar & Durbin, 1965; Sachs, Mitch & Hirschowitz, 1965) but has been detected in lizard gastric mucosa (Hansen, Bonting & Sleegers & dePont, 1972) rat (Cummings & Vaughan, 1965) and human gastric mucosa (Mosznik, 1969). Also a ouabain-insensitive ATPase without requirements for sodium and potassium but whose activity is greatly stimulated by the presence of bicarbonate has been detected in frog (Kasbekar & Durbin, 1965) and a similar potassium and magnesium dependent, ouabain-insensitive but bicarbonate stimulated ATPase found in rabbit (Forte, Forte & Saltman, 1967). However, the presence of these enzymes in gastric tissue is no guarantee that these enzymes participate in the acidification process in gastric mucosa.

More evidence exists for a biochemical link between acidification and the adenyl cyclase-phosphodiesterase system. This is a system that controls the level of cyclic AMP present in tissues and has been implicated as being the possible mode of action of hormones when they exert their effects on a cellular level,

as dictated by the "second messenger" hypothesis. Briefly, membrane-bound adenyl cyclase will convert ATP to a cyclic form of AMP with the resultant production of pyrophosphate. This cyclic form of AMP can be broken down by phosphodiesterase enzyme to form the usual variety of AMP. The breakdown of cyclic AMP is a cytoplasmic event and can be influenced by various compounds. Methyl xanthines such as theophylline (Butcher & Sutherland, 1962) will inhibit the action of phosphodiesterase and lead to elevated levels of cyclic AMP in tissues. Imidazol has the converse action and will accelerate the action of diesterase causing an increase in the breakdown of cyclic AMP, consequently leading to a decrease in the level of cyclic AMP in the tissues (Butcher & Sutherland, 1962). (See diagram I).

Cyclic AMP will cause an increase in hydrogen ion secretion in frog gastric mucosa (Harris & Silen, 1964). Theophylline will also increase the rate of hydrogen ion production (Alonso & Harris, 1965; Harris, Nigon & Alonso, 1969), and imidazol which has the converse action on phosphodiesterase, inhibits acid secretion (Alfonso 1963; Harris & Silen, 1964). This seems to indicate that acidification is related to a high level of cyclic AMP in tissues and that high rates of turnover of ATP as must be assumed occurs when tissues are incubated with imidazol, are not related to acidification. This work in frog is confirmed in humans in vivo where theophylline also increases the hydrogen ion secretion (Dehyle, Miederer & Stedelman, 1971).

DIAG.1


Adenyl cyclase
(membrane bound)
ATP
C-AMP
AMP
cytoplasmic
phosphodiesterase
inhibits
accelerates
Theophylline
Imidazol

However, more recent work in humans produces exactly contradictory results and the exact reversal of effects, also in canine gastric mucosa in vivo, from that seen in frog. In vivo work in both dog and man indicates that cyclic AMP caused inhibition of gastric acid secretion (Levine & Wilson, 1971). In rat gastric mucosa, cyclic AMP has been shown to inhibit the transport ATPases, such as Na^+/K^+ ATPase and in this way high cyclic AMP levels could prevent ion transport, e.g. proton transport (Moszik, 1969) if it is mediated by a phosphatase.

It might be postulated that acidification is due to ATP hydrolysis by adenyl cyclase in mammalian tissues but that the reverse process, also mediated by adenyl cyclase, is associated with acid production in amphibian gastric mucosa. This would also explain why exogenous ATP tends to inhibit acid secretion in the frog gastric mucosa(Kidder, 1971) and yet is thought of as having a function in mammalian gastric acidification (Forte, Adams & Davies, 1965). It must be concluded that a relationship exists between acidification and metabolism but that it is not at all clear how this is achieved biochemically.

The Anion Exchange Model.

Principally, this model of acidification relies on a bicarbonate:chloride ion exchange or a hydroxyl: chloride ion exchange and can be thought of as occurring in erythrocytes, cerebrospinal fluid membranes, rat colon and ileum and in the pancreas.

It had long been thought that red cell membranes were freely permeable to hydrogen ions (Warburg, 1922; Van Slyke, 1923) which were not actively transported but were distributed according to the membrane potential in a passive manner. It was further thought that the distribution of other ions was dependent on the initial passive alignment of the hydrogen ions (Maizels, 1952). More recently, suspensions of rat, human and guinea pig red blood corpuscles have been shown to acidify in sucrose media and to alkalinise in the presence of saline without additional metabolisable substrates (Scarpa, Cechetto & Azzone, 1970). The acidification in sucrose media which indicates the metabolic nature of the phenomenon, is caused by a bicarbonate:chloride anion exchange. Where external chloride anion is present, alkalinisation occurs. In the presence of acetazolamide, chloride anion will exchange for hydroxyl anion rather than bicarbonate anion (Scarpa et al., 1970) although it is impossible to distinguish between hydroxyl anion and hydrogen ion movements (Funder & Wieth, 1966; Crandall, Klocke & Forster, 1971) across red blood corpuscle membranes.

In cerebrospinal tissues, the situation is very similar. Normally the cerebrospinal fluid has less buffering power than the blood. However, during acute respiratory acidosis in cats, the cerebrospinal fluid pH does not decrease as much as the blood pH. This is due to the existence of an increased bicarbonate and a decreased chloride level in the cerebrospinal fluid (Swanson & Rosengren, 1962) and implies the

existence of a chloride:bicarbonate anion pump. Acetazolamide seems to mimic the effect of respiratory acidosis in humans. It causes an increase in the CSF:plasma bicarbonate ratio and an increase in the total CSF bicarbonate concentration, with a reduction in the CSF chloride ion content, again indicating a compulsory bicarbonate:chloride exchange (Maren & Robinson, 1960). The authors regarded this as being due to inhibition of chloride transport at the choroid plexus.

In rat colon, where bicarbonate secretion takes place and consequently some degree of alkalinisation occurs, this secretion occurs only when chloride ion is present mucosally and in its absence bicarbonate ion is absorbed (Phillips & Schmalz, 1970). Acetazolamide is only effective in reducing bicarbonate secretion when chloride ion is present (Phillips & Schmalz, 1970) indicating the importance of the position that chloride ion has in the compulsory bicarbonate:chloride ion exchange. It may be that active chloride secretion as is known to occur in the stomach (Hogben, 1955) is the primary force in an anion exchange with bicarbonate, in the production of acid. In this case all the postulates for the mitochondrial model of acidification would be valid, except that chloride anion would be extruded in exchange for an alkali anion.

A case in point would be pancreatic secretion where substantial amounts of bicarbonate are secreted mucosally. As in the jejunum (Powell et al., 1971), there is a reciprocal relationship between the relative

levels of bicarbonate and chloride ion in the exudate and the sum of the two ions remains constant (Solomon, 1952; Birnbaum & Hollander, 1965). No secretion occurs in the absence of chloride ion in rabbit pancreas in vitro (Rothman & Brooks, 1965). Acetazolamide reduces the bicarbonate content of the secretion in dogs (Pak, Hongs, Pak & Hongs, 1966) and in humans (Dreiling, Janowitz, & Halpern, 1955). Pancreatic secretion in these respects resembles closely the previously cited anion-exchange systems in its chloride and bicarbonate levels and in its sensitivity to acetazolamide.

With increased serosal pH, there is an increase in mucosal bicarbonate secretion in the in vitro rabbit pancreas (Swanson & Soloman, 1971). It is possible that this decrease in serosal pH stimulates serosal hydrogen ion secretion which then accelerates bicarbonate transport in that the bicarbonate traverses the membrane in the neutral form. In this context a suitable bicarbonate-stimulated ATPase has been detected in the pancreas (Simon, 1972) which might power bicarbonate transport in classical carrier terms or in the presence of bicarbonate, provide protons on hydrolysis in order to facilitate bicarbonate transport.

In essence, the problems involved in defining exactly how bicarbonate is transported in the pancreas are exactly those that exist in jejunal and renal bicarbonate movement. In the pancreas also, bicarbonate levels in the pancreatic secretion and by inference amounts of bicarbonate transported, are related to bicarbonate

levels in the serosal perfusates of in vitro preparations. As in the kidney (Ullrich et al., 1971), a weakly ionising buffer, e.g. sulphamerazine, can replace the bicarbonate in the serosal perfusate with the same relationship being maintained between the perfusate level and the rate of transport mucosally, as in preparations with bicarbonate perfusate (Schulz, 1972). In this case the rate of undissociated acid is rate limiting. It was postulated that at one cell membrane charge separation must occur and that the serosal migration of a proton causes transfer of base in the reverse direction.

The distinction between two alleged systems of acidification, i.e. active cation extrusion and active anion extrusion is a convenience of category and is not meant to imply systems which are mutually exclusive when they occur in tissues. Anion exchange may be restricted to tissues where bicarbonate is transported and where by implication, a corresponding acidification occurs elsewhere. The question still remains whether the proton extrusion causes anion movement as the neutral form or does anion movement lead to alterations in the hydrogen ion level, i.e. it is not clear whether bicarbonate movement is a product of active anion extrusion or whether initial hydrogen ion secretion causes bicarbonate movement. This can be restated in terms of the acidification phenomenon, whether it is caused by actual proton extrusion or merely by anion exchange. In the rat jejunum both anion exchange simultaneously with proton secretion have been invoked as the cause of acidification.

Acidification in the small intestine.

The existence of regional differences in the luminal pH of rat small intestine has been known to physiologists for a long time (Redman, Willimot & Wokes, 1927; McRobert, 1928) when it was demonstrated by in vitro methods that the jejunal luminal pH is slightly acid and becomes increasingly less acid down the length of the small intestine until it reaches alkaline values in the distal ileum. The significance of these observations remains unclear. Later work with canine intestine in vivo revealed that a fluid is secreted into the empty intestine that had a pH value lower than that of blood plasma and a carbon dioxide tension higher than that of blood plasma (DeBeer, Johnston & Wilson, 1935; Robinson, 1935). These experimental findings were confirmed to occur in the human jejunum (McGee & Hastings, 1942; Robinson, Luckey & Mills, 1943) and implied some degree of selectivity of the content of the secreted fluid, especially as regards hydrogen ion concentration.

* Morishita et al., 1971

It was further discovered that rat intestine both in vivo and in vitro, could modify the pH of solutions instilled in the lumen so as to shift acid and alkaline incubation media towards more neutral values (Ponz & Larralde, 1950; McHardy & Parsons, 1956; Foerster, Erdlenbruch & Mehnert, 1967; Smith, 1971). This seems to imply the existence of a definite homoeostatic mechanism designed to maintain the luminal pH at a distinctive level. Later in vitro work using everted

sacs and Fisher-Parson's loops showed that the acidification phenomenon, as estimated from the distribution of bicarbonate in the incubation media via the Henderson-Hasselbalch equation, might be accounted for by the movement of bicarbonate serosally, along with the mucosal production of lactic acid (Wilson, 1953; 1954). Wilson could detect no variation in the serosal pH of everted sacs. This was confirmed in vivo by direct measurements with pH electrodes; however, no observations were made on the pH of the serosal solution (Wilson & Kazyak, 1957). Since up to 85% of the acidification could be accounted for by the appearance of lactic acid mucosally (Wilson & Kazyak, 1957) it was proposed that the pH changes were caused by intracellular lactic acid production with consequent differential migration of the proton to the mucosal surface and the lactate ion to the serosal surface. This simple scheme would explain the high serosal concentrations of lactate found in the everted sac preparation (Wilson & Wiseman, 1954) in the absence of a demonstrable lactic acid active transport system (Newey, Smyth & Whaler, 1955), the lack of serosal pH change and also the mucosal acidification. It would also explain why this acidification phenomenon was associated only with the jejunum; the jejunum has a high rate of lactic acid production by way of aerobic glycolysis (Dickens & Weil-Malberbe, 1941; Wilson & Wiseman 1954; Srivastava & Huebscher, 1966) whereas the ileum relies on oxidative metabolism and produces comparatively little

lactic acid (Gilman & Koelle, 1960). These metabolic observations would explain the occurrence of the acidification phenomenon in the jejunum only, if it were related completely to lactic acid production. A further difference of significance to the acidification phenomenon is that the direction of bicarbonate movement in the ileum is the reverse of what it is in the jejunum (Wilson & Kazyak, 1957) in that in the ileum, bicarbonate is secreted into the lumen in very small amounts. Both the direction of movement of bicarbonate and the regional distribution of lactate production agree very well with the difference in acidity between the ileal and jejunal lumen.

These experimental observations have recently been confirmed in vivo and acidification demonstrated to occur in a wide variety of electrolyte media (Powell, Solberg, Plotkin, Catlin, Maenza & Formal, 1971). These authors showed an acidification of the jejunal contents with an elevation of the carbon dioxide tension where bicarbonate buffer was used. Similar data has been derived from triple-lumen perfusion studies in human subjects. Human jejunum also acidifies the mucosal medium, involving increased mucosal carbon dioxide levels in the gut lumen. Because a greater elevation in luminal carbon dioxide tension occurs where bicarbonate media are used as jejunal perfusates, it was proposed that acidification was not the result of carbon dioxide secretion but that hydrogen ion secretion occurred as the initiating step in bicarbonate absorption (Turner, Fordtran, Carter & Rector, 1970). The authors postulated the occurrence of a sodium:hydrogen cation exchange pump to explain these ion movements.

It is difficult to confirm whether jejunal acidification can be validly described by the mitochondrial model for a acidification, since little empirical data exists on the acidification phenomenon per se and simultaneously occurring ion movements. The mitochondrial model requires charge separation at a membrane with a corresponding anion being produced, which Wilson (1953, 1954) postulated as being lactate anion, in the case of the jejunum. Other workers, using direct measurements with pH electrodes, in contrast to the lack of variation in serosal pH seen by Wilson (1953, 1954), have managed to demonstrate in vitro a greater fall in the serosal pH than that seen in the mucosal fluid (Barry, Jackson & Smyth, 1966). In which case, it would seem that lactate production must be ruled out as a source of protons, as the mitochondrial model predicts. Lactate can still be implicated as a source of protons if it is assumed that lactic acid is produced in the cell and that on migration out of the cell, both mucosally and serosally the lactic acid dissociates to form lactate anion and a corresponding proton; in all events Wilson's model of the differential migration of lactate anion serosally and the proton mucosally becomes a more unlikely explanation. Measured ion fluxes indicate that in everted sacs there is both mucosal and serosal potassium ion loss (Wilson, 1953, 1954) which is what would be expected if acidification occurred at both the serosal and mucosal surfaces and involved the compulsory coupling of potassium ion to hydrogen ion transfer.

When incubated in media containing no bicarbonate, water sodium, potassium and bicarbonate are secreted by the rat jejunum into the lumen: under these circumstances, no information on pH is available. In the presence of bicarbonate ion in the incubation medium, the converse flow of water and ions is seen. In both the presence and absence of bicarbonate, chloride is transported serosally (Powell et al., 1971). This movement of chloride was also seen in vitro by Wilson (1953; 1954). At low rates of serosal sodium transfer, extracellular chloride ion is exchanged for intracellular bicarbonate ion, which itself moves serosally at higher rates of sodium transfer. At the normal rates of sodium transfer, the chloride:bicarbonate exchange mechanism is obscured by the ensuing serosal bicarbonate flux. This means that there is an anion exchange occurring as required by the mitochondrial model but that it is obscured by other ion movements occurring in the jejunum.

Recent potential and short-circuit current studies have shown that under certain circumstances, the polarity of the potential, normally seen in the jejunum, can be reversed, particularly in buffers with a low sodium content (Faelli & Garotta, 1971a; 1971b). The absence of sodium ensured that the sodium flux usually associated with the intestinal potentials was not present and the authors tentatively identified the resulting negative potentials as being caused by active electrogenic bicarbonate transport. Since, however, bicarbonate has been shown

to be secreted when the jejunum is incubated in media having a low level of sodium (Powell et al, 1971) a more likely explanation is that the reversal of potential is caused by the active transport of hydrogen ions in the opposite direction to that of active sodium transport, i.e. the appearance of hydrogen ion mucosally is an electrogenic active transport process, as required by the mitochondrial model for acidification. Further support for this view comes from the triple-lumen perfusion studies, where it was shown that, in the human jejunum, osmotically induced water flow markedly increases the transfer of sodium (Fordtran, Rector, Ewton, Soler & Kinney, 1965; Fordtran, Rector & Carter, 1968); it was concluded that sodium transfer was passive in the human jejunum and dependent on solvent flow. Under these same experimental conditions, bicarbonate was absorbed at a constant rate (Turnberg, Fordtran, Carter & Rector, 1970) during which time the potential did not alter. The potential did not alter in the presence or absence of bicarbonate. The authors concluded that bicarbonate transport was not the electrogenic step, neither was the sodium transport electrogenic but that bicarbonate absorption was mediated by hydrogen ion transport which was the active electrogenic step.

The final piece of evidence implicating the mitochondrial model in acidification is that on incubating everted sacs, there is an increase in the level of phosphate ion in both the serosal and the mucosal fluid which might be expected if surface hydrolysis of ATP at some outer

membrane occurred which caused acidification, with the phosphate fragments appearing extracellularly and the nucleotide portion retained within the cells. This appearance of phosphate mucosally does not account for all the acidification if it is assumed that there is a one-to-one relationship between phosphate groups and protons; in fact, the phosphate could only account for some 5% of the acidification (Wilson, 1953, 1954). There is, however, a transport mechanism for phosphate in the rat jejunum (McHardy & Parsons, 1956) and it may well be that phosphate as soon as it is produced in any hydrolytic reaction, is immediately transported back into the cell.

The question remains whether acidification in the jejunum is an event of physiological significance since it might be caused solely by some sort of autolytic process in the in vitro preparations and by incubation in certain buffers in in vivo preparations, though having no role in normal intestinal function. If jejunal acidification is clearly a genuinely significant phenomen, there remains also the question of whether it can be adequately explained in terms of lactate production and and bicarbonate transport or whether it conforms to the mitochondrial model for acidification. Finally, as the production of lactic acid by intestinal epithelial cells has been proposed as having a possible role in the maintenance of local intercellular pH (Ugolev & Mitiushova, 1971) in that very localised concentrations of lactic

acid should cause a lowering of pH near the surface of the jejunum, the function of acidification and its participation in the workings of a surface microclimate, as proposed by Hogben and his co-workers (1959) must be made clear. The evidence for the existence of an acid surface microclimate is reviewed in the following section.

The microclimate hypothesis.

It has long been realised that the characteristics and properties of natural phenomena relating to small particles occurring at physical interfaces are often different from those observed in the absence of any interface, even with reference to a simple physical occurrence such as diffusion (Sears, 1970). This has important consequences for biological processes since many activities necessary for the maintenance of life are connected with membrane systems, which as such constitute solid interfaces in an otherwise fluid environment. Enzymes that are bound to solid matrices often have properties varying from enzymes in solution and facts of minimal importance in free solution may become of critical importance when an interface is involved (Crook, 1968).

With respect to hydrogen ions, this often means differences in the pH of the bulk phase when compared to the pH of the solution near an interface. This has been demonstrated convincingly by experiments not involving any biological material. When aqueous solutions of

sulphonated acidic dyes are shaken up with benzene to form an emulsion there is a colour change visible, indicative of a lower pH at the benzene-water interface than in the bulk phase (Deutsch, 1928). Equations have been derived which can relate this concentration of hydrogen ions at a negatively charged interface, to the bulk pH (Hartley & Roe, 1940). These equations show that depending on the zeta potential of the charged surface, these differences could theoretically be of the order of one pH unit difference. However, experimental observations on the enzyme chymotrypsin reveal that when the enzyme is adsorbed on to kaolin particles, as if it were in an environment some two pH units lower than the bulk phase, i.e. as if the hydrogen ion concentration were one hundred times that of the bulk phase (Michaels & Morelos, 1945). It has also been observed in experiments on stearic acid in monolayers, that an interfaced stearic acid behaves as if the pKa value was three units higher than the bulk phase, i.e. as if the acid were in an environment of pH much lower than the bulk phase. (Bagg, Haber & Gregor, 1966).

In studies on the hydrolytic enzyme papain, impregnated into an artificial collodion membrane, this fact was confirmed, together with the conclusion that the enzyme milieu in such membranes might well be determined also by the products of the enzymes own catalytic activity. The extent of the deviation of the optimal pH for bound enzyme from the normal pH-dependency

profiles seemed to depend on the rate of substate hydrolysis in the membrane (Goldman, Silman, Caplan, Kedem & Katchalski, 1965; Goldman, Kedem, Silman, Caplan & Katchalski, 1968). In this respect, one might expect where bound hydrolytic enzymes are concerned, an increased concentration of hydrogen ions at an interface due to both physical factors as predicted by the Hartley & Row equation and also an increase due to enzymic hydrolysis itself.

Physiologically, this phenomenon has been invoked to explain apparent discrepancies in the transport of weakly ionising substances. It was established that the transport of drugs across the intestinal barrier is achieved mainly by passive diffusion of the unionised forms (Schanker, Tocco, Brodie & Hogben, 1958; Dietschy, Sallee & Wilson, 1971). However, the steady state distribution of these drugs was consistent with the concept of a microclimate of hydrogen ions, juxtaposed to the mucosal surface of the jejunum, some two pH units lower than the bulk luminal phase (Hogben, Tocco, Brodie & Schanker, 1959). Since similar discrepancies exist between the amount of ammonia transferred across fish gill and the amount that ought to be transferred as predicted by non-ionic diffusion, the idea of a micro-climate has also been proposed for the trout gill membrane (Lloyd & Herbert, 1960). This concept has been incorporated into several models for active transport and its theoretical implications are well known (Stehle & Higuchi, 1967). Independent studies on the absorption of a range of acidic and basic drugs having very varied

pKa values, managed to inadvertently confirm the observations of Hogben and his co-workers (1959). Using a range of passively absorbed drugs, a relationship between the theoretical unionised fraction and the absorption characteristics was shown over a range of pH values (Kakemi, Aritea, Hori, Konishi, Nishimura, Matsui & Nishimura, 1969). The rate of transfer at the pH values taken was imcompatible with the amount of the drug that was theoretically unionised: the drug was absorbed across the rat jejunum as if the milieu pH were about between two and three units more acid than the measured pH values. In the colon and rectum, the pH-absorption profiles showed no such discrepancies between what ought to have been transferred according to the theoretically unionised fraction at a given pH and what actually was absorbed at the same given pH. The authors attributed the discrepancy between the theoretical unionised fraction and the actual fraction that would be required to achieve the amounts of absorption seen, as being due to some unidentified binding factor. This discrepancy has been confirmed in vivo in the rat jejunum for salicylic acid, independently, and the same explanation offered (Kunze, Rehbock & Vogt, 1972).

Recently, the existence of 'unstirred' or stagnant layers of fluid of about between 40-60 microns in depth have been demonstrated to occur in frog skin, corneal tissue and also in red blood corpuscles, where they are estimated to be of about 10 microns in depth (Dainty & House, 1966; Green & Otori, 1970; Naftalin, 1971).

The act as barriers to diffusion (Kidder, 1970) particularly to particles above a certain size(Wilson, Sallee & Dietschy, 1971) and can effect cellular permeability (Sha'afi, Rich, Siddert, Sidel, Bossert & Solomon, 1967), and have been evoked to explain the failure of Crane's hypothesis for sugar transport in vivo in its attempt to link sodium ion gradients with hexose transport (Saltzman, Rector & Fordtran, 1972).

Electron microscopical studies have shown the existence of an extracellular layer of mucopolysaccharide that coats the apical villi of the intestinal epithelial cells (Ito, 1965; Rambourg, Neutra & Leblond 1966; Rubin, 1971). This means that a morphological basis exists to explain the empirical phenomenon that on vigorous stirring of incubating solutions, rates of transport are often accelerated. This mucopoly-saccharide coat, called the 'glycocalyx', has been invoked as a diffusion barrier (Prichard, 1969) functioning in the extracellular digestion of disaccharides. It is very possible that these structural features provide the milieu for the retention of hydrogen ions at the mucosal surface of epithelial cells.

The microclimate hypothesis has been invoked to explain the transport of folic acid (Matty & Blair, 1968; Smith, Matty & Blair, 1970). In this simple model, substances that are ionised at the bulk phase neutral pH would become unionised in the microclimate and be transferred in the unionised form. This scheme of facilitated transport would require a source of

protons at the transporting membrane. It is interesting to relate here that in the case ofsalicylic acid, a compound thought to be passively transferred yet known to be transferred at a rate higher than is compatible with the concept of non-ionic diffusion, its rate of transfer is inhibited by the metabolic inhibitor, dinitrophenol (Kunze, Rehbock & Vogt, 1972). This microclimate has never been demonstrated directly in situ, although it may have been demonstrated in crab muscle fibre (Caldwell, 1958). This postulated microclimate would control the transfer of all weakly ionising substances, e.g. vitamins, cofactors, amino acids, long chain fatty acids, that are ionised at physiological pH, unless some specific active transport mechanism existed for that particular compound, e.g. propionate (Barry, Jackson & Smyth, 1966) glycerol (Howard, 1966; 1971) choline (Sanford & Smyth, 1971). Further evidence for the microclimate theory of transport is seen in studies of the effect of lowering jejunal pH on the transfer of amino acids. A lowering of the jejunal pH causes an increased transfer through the gut wall of all amino acids whose isoelectric point is below that of the usual jejunal pH. The amount of transfer is directly related to the increased amounts of unionised amino acid available, as might be expected from the concept of non-ionic diffusion (Thompson, Levin & Jackson, 1970). These observations show that in principle an addition of acid to a milieu can in practice alter the rates of transfer of certain compounds

as is depicted in the microclimate theory of transport. These increased rates of transfer were found not to occur in the ileum indicating again that the jejunal microclimate or jejunal acidification has a significant role in this effect.

An alternative way in which acidification and unstirred layers might interact to cause accelerated rates of transport is as follows. Jejunal mucopolysaccharide which is thought of as acting as a diffusion barrier, has been shown to become more viscous in acid media (Heatley, 1959). It is possible that acidification causes increased viscosity of the mucosubstance next to the jejunal fuzzcoat and allows greater rates of transport, solely by the ensuing mechanical changes.

The following experiments were designed to characterise jejunal acidification biochemically and physiologically so that the acidification process could be altered. In this way the possible microclimate could be manipulated by biochemically inhibiting and accelerating acidification with various biologically active compounds so as to abolish and alternatively resynthesise a microclimate. It is hoped that the following experiments will act as a prelude to further studies involving direct measurements to establish the existence of a microclimate.

METHODS

a) Chemical Estimations.

b) Physiological Methods.

a) Chemical Estimations.

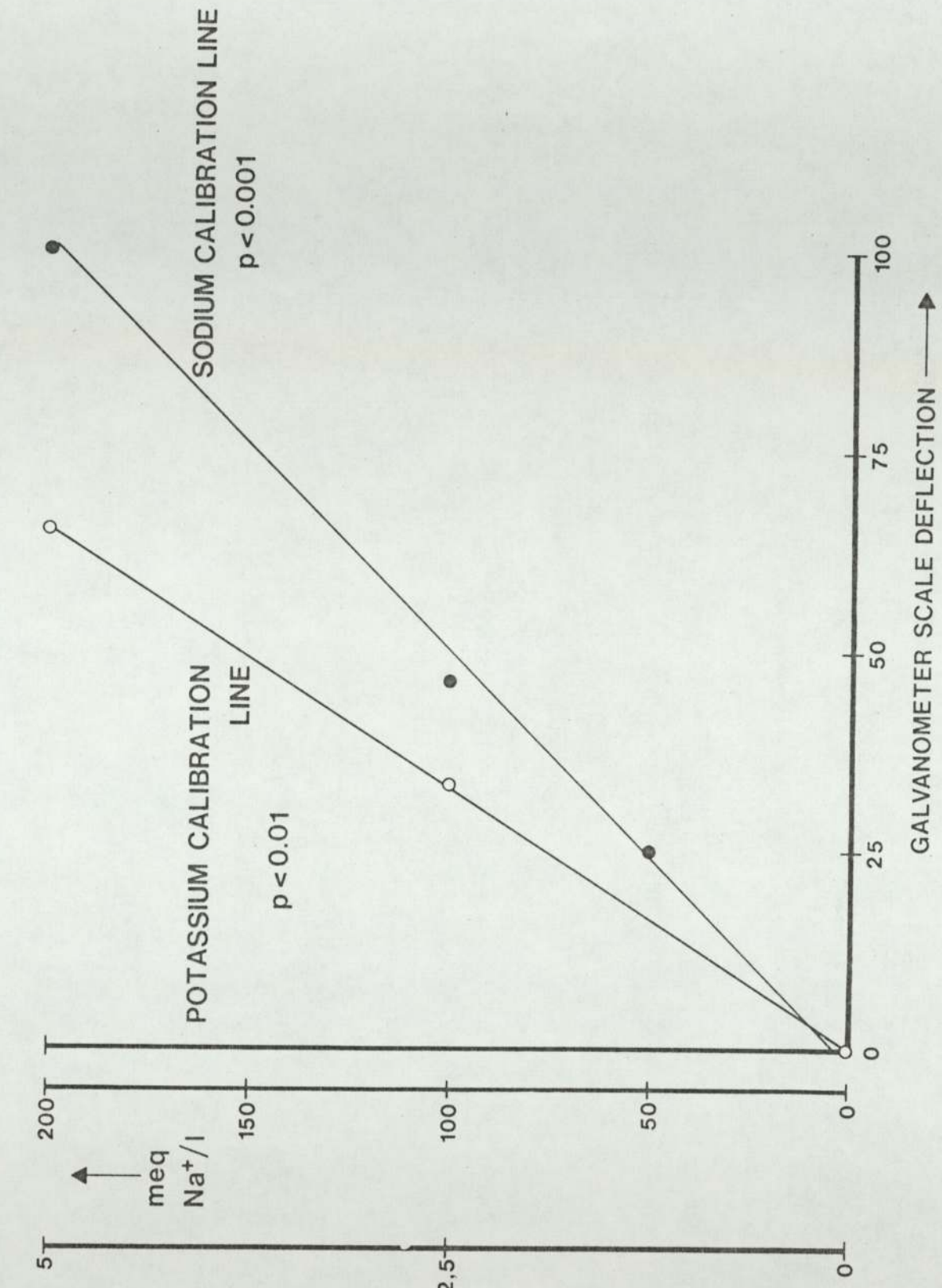
1) Sodium and potassium estimation.

Samples of buffer solution taken from experiments were assayed directly for sodium and potassium content by flame photometry using an EEL flame photometer. The method (Domingo & Klyne, 1949) makes use of the fact that metals give characteristic emission spectra (sodium and potassium give emission peaks at 820 and 345 manometres respectively) and that at the metal's emission peak, the intensity of emission will be directly proportional to the concentration of metal ion present. In the case of both metal ions, a suitable range of standards were made up spanning the concentration range approximately of the unknown samples (see figure 1). Unknown samples were then read off against the standard calibration curve.

2) Phosphate estimation.

Total phosphate in solution was measured by an automated procedure (Lundgren, 1960) based on the reaction by which phosphomolybdic acid is reduced by aminonapthol-sulphonic acid (Fiske & Subbarow, 1925). Phosphates are first converted to orthophosphate by hydrolysis with sulphuric acid,a process which is 98% efficient (Lundgren, 1960). Orthophosphate reacts with molybdic acid to form phosphomolybdic acid. This then reacts with the aminonapthol sulphonic acid to form an intense blue coloured product. Since molybdic acid is in excess, the intensity of coloration is proportional to the concentration of phosphate

FIGURE 1.

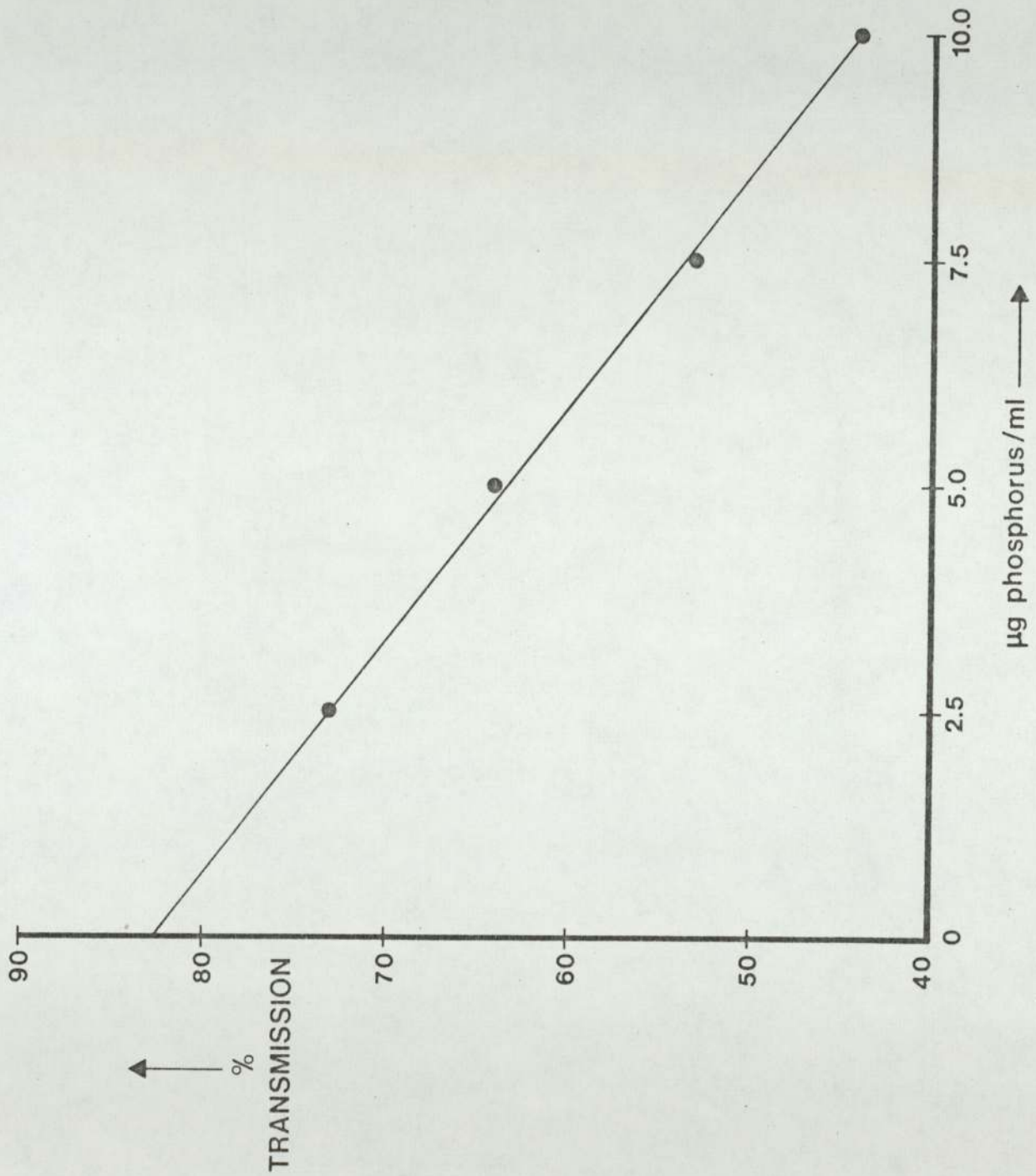

SODIUM CALIBRATION LINE
p < 0.001
POTASSIUM CALIBRATION LINE
p < 0.01
meq Na+/l
meq K+/l
200
150
100
50
0
5
2,5
0
0
25
50
75
100
GALVANOMETER SCALE DEFLECTION

originally present and consequently can be colorimetrically measured as in the original method (Fiske & Subbarow, 1925). Since 5 mls of sample solution are required, a 1 ml aliquot of buffer solution taken from the experiment was made up to five mls with distilled water. The automated technicon procedure incorporates a dialysis phase which obviates the need for deproteination. A simple calibration curve is shown (see figure 2) in which photometric transmission is shown to be proportional to the total medium phosphorus present. This means that an arithmetical adjustment must be made to express results in terms of phosphate present.

3) Chloride estimation.

Chloride was measured by coulombimetric titration (Cotlove, 1961) on an EEL chloride titration meter. Samples taken directly from experiments, were added to glacial acetic acid, 0.2 mls of sample to 13 mls of acetic acid buffer. Conventionally, thymol blue indicator is added to the gelatine based buffer since it will change colour if bacterial contamination is present, due to a lowering of pH. Silver electrodes dipping into the solution measure the current due to the flow of silver ions. The sample is added containing the chloride and immediately silver chloride is precipitated on to the electrodes. After a certain time no more chloride is left and the resumption of the deposition of silver ions occurs. The change from silver deposition to chloride deposition causes a change in the conductivity of the solution as registered by the electrodes. The length of time of this period of change in the conductivity of the

FIGURE 2.



solution can be directly related to the amount of chloride ion present in the original solution. The timing device is calibrated in milligrams of chloride ion per litre. A range of known standards was also made up to check the linearity of response (see figure 3).

4) Estimation of the chelating ability of ATP.

This was estimated by a micro-adaptation (Copp, Cheney, & Stokoe, 1963) based on an earlier method (Fales, 1953). Murexide dye is added to a standard calcium solution to form a characteristically coloured mauve ligand in solution. Aliquots of 1M EDTA are titrated into this solution with the result that calcium is sequestered away from the ligand complex by chelation and a colour change occurs that can be measured photometrically. Molar ATP was then similarly titrated against the dye solution and the chelating ability of ATP relative to equimolar EDTA was thereby calculated.

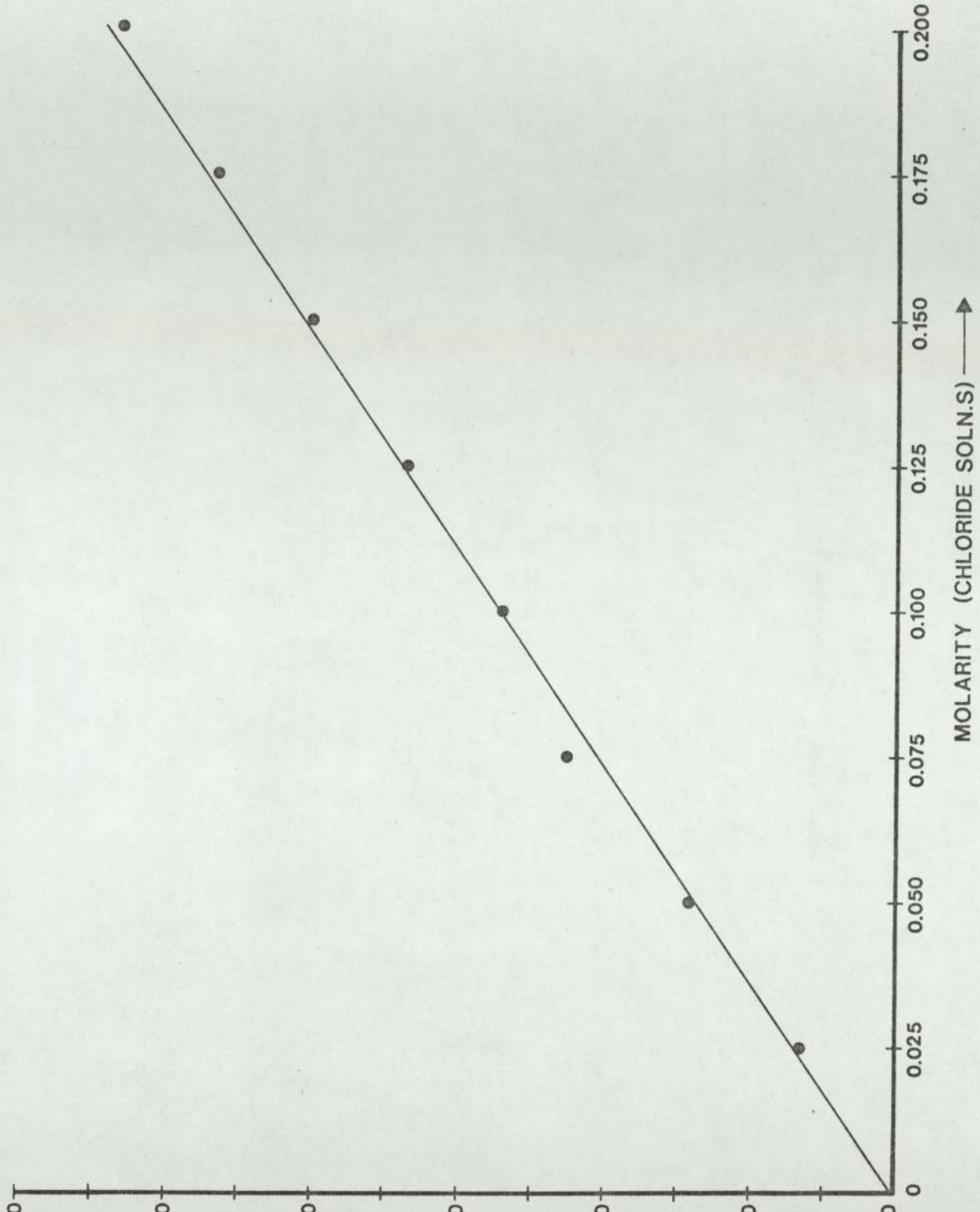
5) Lactate estimation.

Lactate was estimated by an enzymatic method (Hohorst, 1957) based on the enzymically controlled oxidation of L-lactate by NAD to pyruvate and $NADH^+$: -

$$\text{L-lactate} + NAD^+ = \text{pyruvate} + NADH^+ + H^+$$

When a hydrazine buffer is used and the pyruvate formed is trapped, the reaction proceeds to the right. When NAD is in excess the $NADH^+$ formed is equivalent to the amount of L-lactate originally present.

FIGURE 3.


meq Cl⁻ per vol. sample
240
200
160
120
80
40
0
0
0.025
0.050
0.075
0.100
0.125
0.150
0.175
0.200
MOLARITY (CHLORIDE SOLN.S)

Sample aliquots of 0.5 ml. were first deproteinated by centrifuging with 0.1 ml. of 0.6N ice-cold perchloric acid for ten minutes at 3000 r.p.m. 0.2 ml aliquots of the supernatant were incubated for one hour at 25 degrees Centigrade in 2 mls of hydrazine buffer, together with 0.2 mls of NAD and 0.02 mls of LDH enzyme. After one hour the solutions were transferred to cuvettes which had a one centimetre light path and the absorption of the sample and a corresponding blank read at 366 nm against air, either on a Beckman DB or a Pye-Unicam SP 800 spectrometer. As well as the samples to be measured, a 1N solution of lactate was assayed as a standard each time so that each batch of samples was compared with a known standard of lactate.

6) Pyruvate estimation.

In the estimation of pyruvate the same principle is used as in the estimation of lactate except that the substitution of the hydrazine buffer for a conventional buffer means that the reaction proceeds in the reverse direction. NADH consumed during the reaction is equivalent to the original quantity of pyruvate.

4.0 mls aliquots of test solution are mixed with 4.0 mls of 1N ice-cold perchloric acid and centrifuged for ten minutes at 3000 r.p.m. 4.0 mls of the supernatant are mixed with 2.0 mls of 0.7M tripotassium phosphate buffer and left to stand for ten minutes in an ice bath, at the end of which the solution is filtered and 2 mls taken for analysis. 2 mls of the filtered solution are transferred to a cuvette of 1 cm light path, 0.2 mls of NADH are added and the

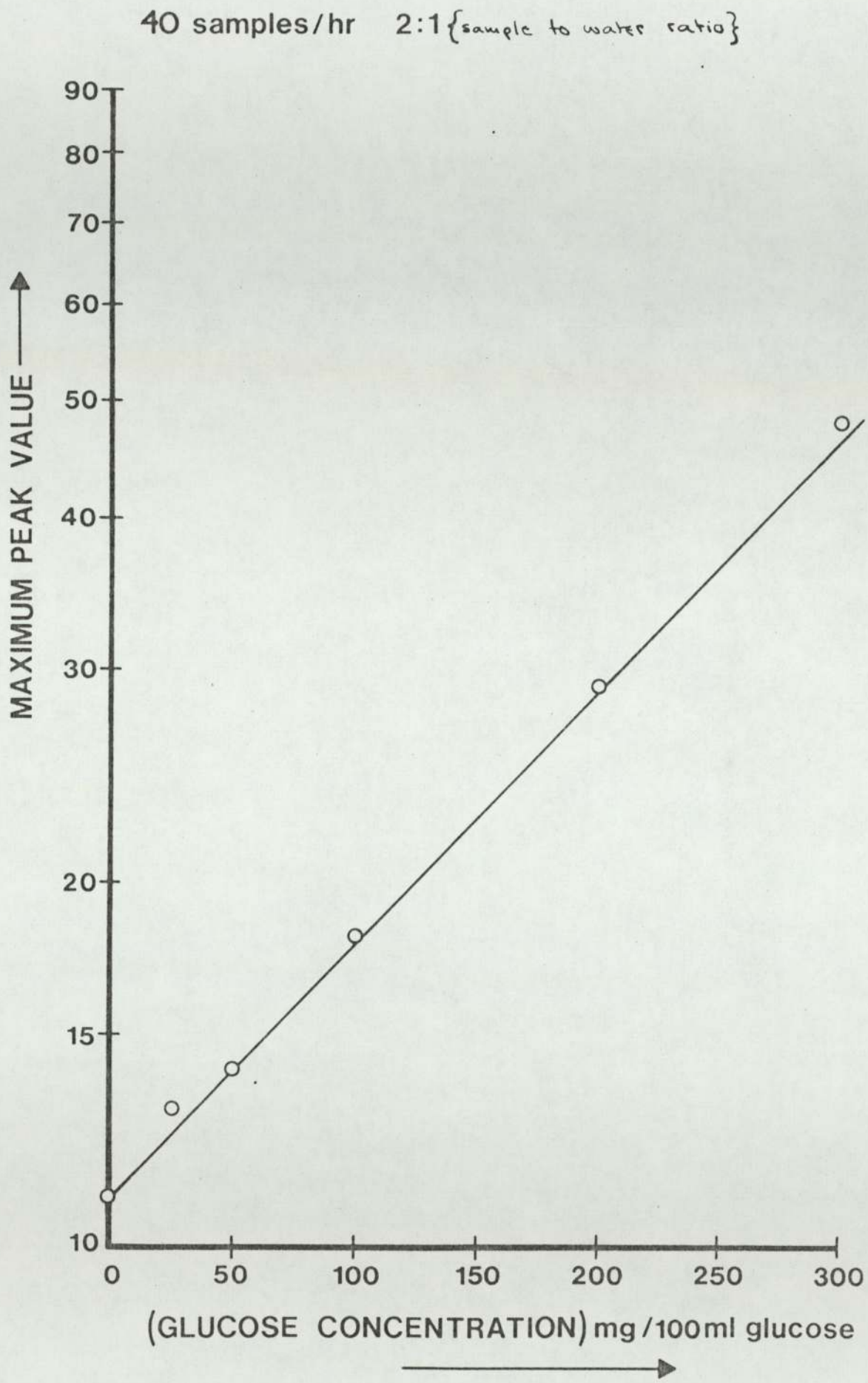
absorbance measured at 366 nm. Then, 0.02 mls of NADH enzyme are added and after the reaction is complete, the change in absorbance is measured at 366 nm against air. The change in absorbance is proportional to the original concentration of pyruvate.

7) Hexose estimations.

Glucose was estimated colormetrically by an automated version (Salway, 1969) of the ferricyanide reduction method (Hoffman, 1937) which measures total reducing sugar. Aliquots were sampled at the rate of 40 samples per hour and the ratio of sample to water was 2:1. From a test series, the coefficient of variation which included pippetting and chart read-off errors, was about 1%. Sample peaks from the chart were read off against a calibration curve (see figure 4) that was constructed from glucose standards.

Since in the course of following experiments, other sugars were used besides glucose, this method was also used to estimate them. Various reducing sugars reduce the reagent to differing degrees; five 10 mM samples were tested of each of the five reducing sugars, glucose, galactose, 3-O-methylglucose, mannose and fructose against five commercially prepared (Technicon) standards. An analysis of variance (Fisher, 1971) revealed a significant difference degree ($p < 0.01$) of heterogeneity and by chi-squared tests it was shown that galactose and 3-methylglucose were the significant ($p < 0.001$) causes of variation.

FIGURE 4.


40 samples/hr 2:1 {sample to water ratio}
MAXIMUM PEAK VALUE
90
80
70
60
50
40
30
20
15
10
0
50
100
150
200
250
300
(GLUCOSE CONCENTRATION) mg/100ml glucose

Consequently, fructose and mannose values were read off from the standard calibration curve (figure 4) and values for galactose and the methylglucose read also off the standard calibration curve but then were corrected by the following correction factors derived from regression equations: -

$$\frac{\text{read off value} - 5.5033}{0.8460} = \text{real value for galactose (mgs \%)}$$

$$\frac{\text{read off value} - 7.5033}{0.6460} = \text{real value for methyl-glucose (mgs \%)}$$

The correction formulae were derived from linear regression data which calculated the way in which equimolar galactose and 3-O-methylglucose standards regressed on the glucose standards.

b) Physiological Methods.

1) The Everted Sac preparation.

There are several techniques available for investigations into intestinal physiology (Parsons, 1968) involving in vitro and in vivo preparations, each of which have inherent advantages and disadvantages. In the present experiments, mucosal scrapes, segments and in situ in vivo perfused loops were used but the routine experimental procedure was the everted sac. The everted sac preparation has become a standard technique in intestinal physiology since its initiation for research into intestinal function (Wilson & Wiseman, 1954). The preparation is simple and is a technique which allows reproducibility. The technique allows collection of the serosally transferred solutes and adequate oxygenation if the sac is slightly distended. It is free of the disadvantages of in vivo methods which include uncontrollable extraneous, e.g. hormonal, influences and false assumptions about site of action where pharmaceutical agents have other physiological actions at sites other than in the intestine, e.g. renally active agents. The everted sac technique has been described in detail (Wiseman, 1961) and a modification of this method (Turner, 1959) was used in order to produce five or more sacs from the same animal. The various parameters that can be varied in the preparation of sacs, inter alia, injected serosal volume, method of injection of serosal volume, temperature of flushing solutions and time taken to produce sacs, were made to coincide with values known to be optimal for the everted sac (Perris, 1964) so as to incorporate the least damaging

handling procedures and to obtain optimal conditions in which the sacs could function.

Choice of buffer.

One of the prerequisites for a successful in vitro method is the use of solutions isotonic to plasma, which contain the electrolytes found in the plasma in similar quantities. Unfortunately the solutions are also capable of buffering pH changes. Two physiological buffers are available (Krebs & Henseleit, 1932) a bicarbonate buffer with a volatile buffering component and a phosphate buffer with a non-volatile buffering component. Bicarbonate buffer has been shown to cause the least loss in structural integrity (Benet, Orr, Turner & Webb, 1971). Also, greater water, sodium and chloride transfer occurs in this buffer (Parsons, 1971) and present studies indicate (see Table 1) that mucosal glucose transfer is greatest in bicarbonate buffer. Accordingly this buffer was used most of all.

Bicarbonate buffer cannot be used in an open system if pH changes are to be measured. In the case of experiments in bicarbonate buffer, a procedure has been devised, employing a period of time on ice, which monitors pH changes in a closed system. The closed system means that the buffer has to be gassed prior to incubation and that once the system is closed no further oxygenation can occur. The alternative buffer was used in an open system and oxygenated continuously with the same gas component as the

TABLE No. 1

Mucosal glucose transfer in everted sacs incubated in phosphate and bicarbonate buffers: rats were ether anaesthetised. Results are given as mean and standard error of the mean (number of animals).

	mucosal glucose transfer (μg/mg tissue dry weight)
a) bicarbonate buffer + 10mM glucose	138.9 ± 31.3 (5)
b) phosphate buffer + 10mM glucose	51.2 ± 17.3 (5)

$p < 0.05$

bicarbonate buffers. Although some workers (Robinson & Alvarado, 1971) have suggested a possible harmful effect of hyperoxygenation, the buffer has the advantage of continuous oxygenation and it provides a medium in which the carbon dioxide tension is constant. A comparison of acidification values for the proximal jejunum, incubated in the standard conditions in both the buffers, reveals that acidification is similar (see Table 2) in the two buffers. This underlines the fact that the bicarbonate buffer procedure was adequate for measuring acidification and was preferred to the phosphate buffer because of the reasons previously mentioned.

A criticism that can be levelled against in vitro methods in both buffers is that if bicarbonate or phosphate transfer occurs, i.e. if the buffering anion is transferred, pH changes might occur without any movement of hydrogen ions as such. For this reason as well as to confirm the in vitro studies, in vivo experiments were done using perfusates with almost no buffering power.

A) Experiments in bicarbonate buffer.

Male Wistar rats of between 200-250 grams weight were stunned by a blow on the neck and decapitated. In later experiments, animals were ether-anaesthetised. The abdomen was opened by a median incision and the intestine cut at the duodenum just below the pylorus. A cannula was inserted and the gut flushed in situ with chilled 0.154M saline coming from a wash bottle, at a

TABLE No. 2

Acidification by everted sacs in phosphate and bicarbonate buffer in the presence and absence of 10mM mucosal glucose. Results are given as mean and standard error of the mean (number of animals). Acidification is expressed as micrograms of hydrogen ion per milligram tissue dry weight.

	phosphate buffer	bicarbonate buffer
without glucose	0.350 ± 0.071 (11)	0.274 ± 0.043 (32)
10mM glucose	0.606 ± 0.081 (12)	0.632 ± 0.047 (51)

TABLE No. 3

Ether anaesthesia contrasted with stunning prior to sac preparation in its effect on acidification. Everted sacs in bicarbonate buffer containing 10mM mucosal glucose. Details as for Table 2.

Condition	Acidification	Statistical significance.
stunned	0.646 ± 0.048 (51)	
ether anaesthesia	0.441 ± 0.107 (6)	$p < 0.001$

pressure head of about 20 cms of water. Whilst under slight hydrostatic pressure, the small intestine was manually freed of gut mesentery and fat material. Finally, the distal end of the small intestine was cut just above the ileo-caecal junction and was flushed of all faecal contents.

The gut was transferred to a dish of chilled saline and slipped onto a 30 cm polished stainless steel rod of 1.5 mm diameter. The duodenal end was tied with a ligature 5 cm from the end of the rod and the rest of the intestine was everted over the ligature and slipped off the end of the rod. The existing ligature was cut and the everted section of the intestine was suspended from a new ligature in chilled saline kept continuously oxygenated by a 95:5%6 (v/v) O_2:CO_2 gas mixture. Whilst suspended, five or more ligatures were made at 3.5 cm intervals from the original ligature, the excess gut cut away and the preparation was reimmersed in the chilled saline. When required, the section of jejunum was lifted out of the saline, a segment cut off and the rest of the gut replaced until needed.

Water transport measurements.

Where water transport measurements were being made, a 3.5 cm long segment was taken, a second ligature loosely tied at the free end and the tissue weighed on a torsion balance. Any excess saline clinging to the sac was blotted away and the sac was weighed. The sac was then transferred to a petri dish containing a moist filter

paper base and 0.2 ml of bicarbonate buffer was injected. This was done via a syringe with a very blunt hypodermic needle. The needle was introduced into the free end of the sac and the loose ligature was tied around the sac with the needle in it. The serosal contents were injected, then the needle was slipped out and the ligature tightened. The sac was then reweighed on a torsion balance and then transferred to 25 ml conical flasks containing 10 mls of bicarbonate buffer that had been gassed at 0 degrees C for one hour with the usual gas mixture.

After incubation, each everted sac was suspended from the torsion balance, lightly blotted and this weight taken as the final sac weight after incubation. When this had been weighed, the sac was cut and allowed to drain. The serosal fluid was collected in a tube of known weight. The cut end of the sac was blotted and the cut sac reweighed. Initially, the blotting paper used to blot the sac was itself reweighed in order to add to the accuracy of the weighing procedure. This accounted for another portion of the error and left finally only about 9% of the serosal volume that had been injected initially, unaccounted for.

This meant that when water transport was being measured, four weighings were available: -

sac empty	W1
sac + injected serosal volume	W2
sac on incubation	W3
sac cut	W4

The injected initial serosal volume was calculated as W2-W1, the increase in a sac weight on incubation after the serosal contents had been drained off, also called the gut fluid uptake, was calculated as W4-W1 and this measured the amount of fluid that was taken up but not transferred. The total amount of water transferred mucosally was calculated as W3-W2. Although it was possible to calculate the serosal transfer of water as W4-W3 it was preferred to use the actual amount of serosal fluid that was measured on drainage and collection in test tubes. Occasionally this led to negative values due to the error in the method but was more useful when other estimations were made on the serosal fluid and related to concentration.

Measurement of pH changes.

To rule out differences in the time taken to produce each individual sac, all flasks were gassed at zero degree C to reduce metabolism in the tissues and then all the flasks transferred simulaneously to a shaking water bath for incubation. The gassing at zero degrees C necessitated a slightly more complex measuring procedure since a decrease in temperature leads to an increase in the solubility of carbon dioxide in water with a change in pH as a result. Measuring the pH at room temperature and gassing at a subsequent cooler temperature meant that the initial pH was lower than that recorded and acidification would occur after the hour on ice whose origins were solely physical. In view of this difficulty, bicarbonate was gassed for one hour at zero degrees C

prior to incubation and the pH measured. The sacs were then made and transferred to the waiting flasks. When all the sacs were made, the stoppered flasks were transferred to a shaking water bath and incubated at 37 degrees for one hour. Then after incubation they were transferred to iced water for another hour and left to equilibrate to the physical conditions at that temperature. In this way any carbon dioxide that had come out of solution during the hours of incubation at 37 degrees would be taken up by the medium at the original temperature that the pH was measured at.

The increase in hydrogen ion concentration represented by equilibrating the medium with carbon dioxide at room temperature and then at zero degrees C is enough to shift the pH from 7.36 to 7.20. The mean change in pH was determined by a series of blank runs through the entire experimental procedure where it was found that there was an overall shift enough to change the pH from 7.00 to 7.09. In view of this shift to the alkaline values, owing to carbon dioxide loss inherent in the procedure, it was decided to run a 'blank' flask containing Krebs-Ringer only to act as an index of carbon dioxide loss. Blanks can be seen to vary from experiment to experiment but are internally consistent, i.e. two blanks in the same experiment will give similar pH changes, whereas blanks from two experiments will vary.

The decrease in hydrogen ion concentration in the blank was added on to the other flasks values for the concentration change so that their acidification values

always became positive quantities. In effect, if the pH value in a flask with a sac remained constant it was assumed that the tissue had produced that amount of hydrogen ion, necessary to counteract changes due to physical causes as determined by the blank. Tissues are deemed not to have produced hydrogen ion if they fall to the blank's final value. i.e.

$$\left[H^+\right] \text{ tissue} = \left[H^+\right] \text{ in flask} - \; - \left[H^+\right] \text{ blank}$$

$$= \left[H^+\right] \text{ flask} + \left[H^+\right] \text{ blank}$$

After one hour on ice, the stoppered flasks were opened and the mucosal fluid transferred to test tubes. The pH of the mucosal fluid was determined using a pye-Dynacap 11087 pH meter converted for use with the standard Eo7 Pye 401 glass membrane electrode.

It might be argued that the pouring of solutions containing carbon dioxide into containers prior to the measurement of pH is an unnecessary source of error and that transference under oil would have been better. However, there is the possibility that oil would affect the pH electrode response. When the electrode was introduced into the test tube, there was almost no room for the gas to escape and the actual measurement was over in a matter of seconds. The pH electrode was kept moist with buffer itself and was wiped prior to use. This meant that as the electrode was dipped into the test solution it was registering a slightly alkaline pH of about 7.4. On being dipped into the test solution the

electrode quickly moved to the more acid value that the test solution had (approximately 7.0 - 7.1). This pH remained stable and then after about thirty seconds a drift to alkaline values could be seen which was of a very slow nature. This meant that the pH value that was initially measured, i.e. the most acid value that the electrode registered was the final pH and was not affected at all be carbon dioxide loss. Carbon dioxide loss only became of importance on leaving the test solutions standing for protracted lengths of time.

The initial and final pH values so attained represent changes in hydrogen ion concentration in buffers and are only an estimate of the real changes in hydrogen ion since bicarbonate will buffer acidity. Nomograms were constructed so that changes in buffer pH could be correlated with actual changes in the level of hydrogen ion. Saturated bicarbonate buffer with the pH adjusted to 8.0 had 1N HCl titrated against it and the changes seen in 100 mls of buffer followed with a pH electrode. In this way a change in buffer pH would be related to actual changes in hydrogen ion production. Also 1N lactic acid was used as a comparison but as both regression lines were similar, the 1N HCL nomogram was adopted for routine use. (see figure 5).

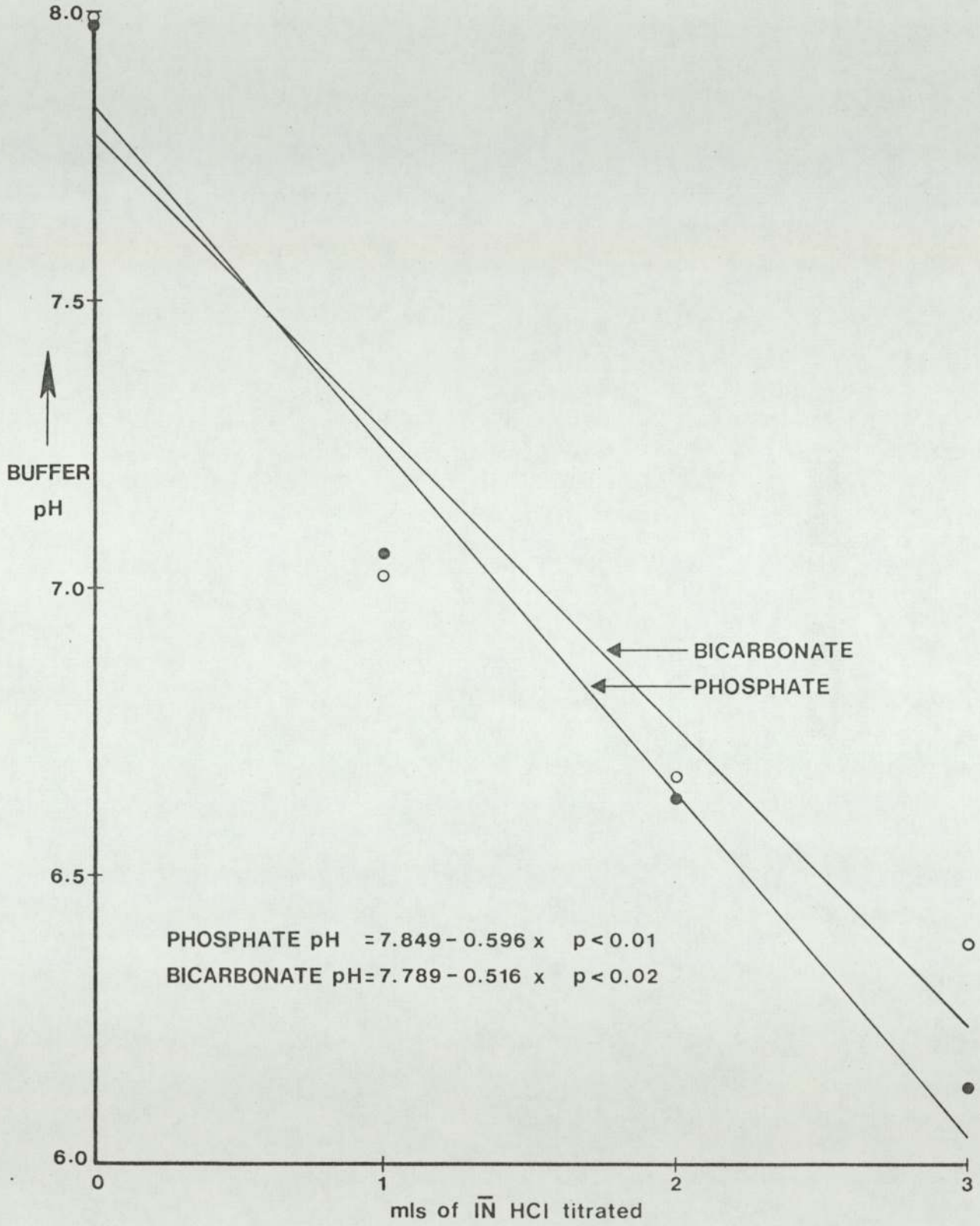
Finally, after the pH had been measured, the ligatures were removed from the sacs and the sac was weighed to a constant weight at 80 degrees C. Tissues dried at 120 degrees C for two hours will give a dry weight: wet weight ratio of 14% (Baker & George,1971). The present

data gave a value of 15%. Sacs were left to dry for 48 hours beyond which little change was seen although variations of $\pm$ 2 mgs did occur, probably dependent on the humidity in the laboratory at the time. The accuracy of the torsion balance was checked against a conventional Mettler gravimetric balance. There were no differences in the weighing values. Acidification was expressed as micrograms of hydrogen ion produced per milligram tissue dry weight.

B) Experiments in phosphate buffer.

In some experiments acidification was measured in an open system using phosphate buffer. The buffer was gassed at 37 degrees C. with the same gas mixture as the bicarbonate buffer for one hour prior to incubation, the pH was then measured and the sacs deposited into the buffer. The buffer was continuously gassed and the need for any period on ice was obviated. At the end of the experimental period, the sacs were removed and the pH measured. As with the bicarbonate buffer, a nomogram was constructed of buffer pH against titrated acid (see figure 5) from which the actual change in hydrogen ion concentration could be calculated. The sacs were dried in the usual way and the results expressed as micrograms of hydrogen ion per milligram tissue dry weight.

FIGURE 5.


8.0
7.5
BUFFER
pH
7.0
6.5
6.0
BICARBONATE
PHOSPHATE
PHOSPHATE pH = 7.849 - 0.596 x p < 0.01
BICARBONATE pH = 7.789 - 0.516 x p < 0.02
0
1
2
3
mls of $\bar{1}N$ HCl titrated

Statistical Comments on the Everted Sac Experiments.

It was found to be more economical, in terms of time and number of animals, to use up to six sacs from each rat and to apply five treatments and one control. Six sacs could easily be made before the onset of deterioration on storage. This meant that methodological and biological variation had to be reduced as much as possible. Six sacs were made sequentially and treatments allocated in a definite order. In subsequent experiments, the first sac would be allocated to the next treatment in the treatment sequence, i.e. a Latin square arrangement of treatment allocations. This was preferred to random allocations, since the time taken to make a sac and any variation in acidification down the length of the proximal jejunum (see Table 12a), although completely confounded in a Latin square, might only be partially confounded in totally random allocations. Rather than an overall analysis of variance, statistical comparisons were made using paired values. In this way, the Latin square design produced five treatments for comparison with one control from six rats, one sac per rat per treatment in each of the six positions down the length of the gut in a way compatible with paired statistical tests, which would minimise innate biological variability as a source of error.

Results were expressed as micrograms of hydrogen ion per milligram tissue dry weight as it was felt that this was more realistic and susceptible to arithmetical

manipulations, rather than pH values as such, which are recommended because they are normally distributed (Bates, 1967). The distribution of a population of the present hydrogen ion production values from 10 mM glucose 'control' conditions was investigated for deviations from Gaussian normality. The data was compared by the chi-squared statistic with an expected histogram from a normal population and found to be significantly different ($p < 0.01$). However, the normalising transformations log x, root x and log 10 root x made the fit significantly worse ($p < 0.001$) in all cases. Product moments were calculated (Mounsey, 1952) to see whether significant differences were due to skewdness in the shape of the curve, since this could seriously invalidate t-tests. The product moment derivative gamma 1, an index of skewdness was not significant in this population but the product moment derivative gamma 2, an index of spread, showed that the original differences were due to the original population being flatter than normal. Where the number of observations exceeds three, there is no possibility of over estimation of 't' in t tests (Ratcliffe, 1968) and it can be concluded that it is legitimate to apply the t statistic to the present experimental values.

2) <u>The Mucosal Scrape Preparation</u>

In an attempt to separate the tissue components responsible for acidification and any other associated phenomena, mucosal scrapes were taken of jejunal material excised in the normal manner. This resolved the

acidification into a muscle component and a mucosal component which includes the actively absorbing epithelial cells. Of the many other methods available for producing cellular preparations, including crude cell 'breis' (Wilbrandt & Laszt, 1933; Sols, 1956; Dickens & Weil-Malherbe, 1941; Newey, Smyth & Whaler, 1955, and isolated cell preparations (Paterson & Zbarsky, 1958; Harrer, Stern & Reilly, 1964; Huang, 1965), some lead to poorly respiring cells (Clark & Porteous, 1965) and the most recent methods which successfully produce adequately respiring cells (Kimmich, 1970; Reiser & Christiansen, 1971; Evans, Wrigglesworth, Burdett & Pover, 1971) invariably involve pre-incubation at physiologically significant temperatures for varying periods of up to one half hour. Adoption of a mucosal cell isolation method would have made direct comparison with results derived from the everted sac procedure impossible. In order to facilitate comparison with the routine everted sac procedure, a technique not involving pre-incubation was selected from the literature and slightly modified to incorporate the pH measurements (Rasmussen, Waldorf, Dziewatkowski & DeLuca, 1963).

Male wistar rats weighing between 150 - 200 g were killed by stunning and decapitation or ether anaesthesia. The jejunum was cut some 4 cm past the pylorus and a cannula inserted in order to flush the proximal jejunum free of any material. After washing, the jejunum was pulled free of mesentery and a 4 cm section

excised at a point approximately 10 cm below the pylorus. This was stored in ice-cold 0.154M saline solution oxygenated with 5% CO_2: 95% O_2 (V/V) gas mixture. Segments were taken from five rats and stored on ice; the time taken to excise five segments was about five minutes.

An intestinal segment was then slit open, laid out on a chilled glass plate, (mucosa upwards) and the mucosal tissue scraped away with a microscope slide. Five such scrapes were put into 10 mls of buffer kept oxygenated with the conventional gas mixture. The scrapes were dispersed by taking them up and expelling them from a 10 ml. plastic syringe. As in the everted sac procedure, weighed experimental flasks containing buffer of known pH had been outgassed on ice with a similar gas mixture for one hour prior to experiment. In some cases an anaerobic gas mixture of 5% CO_2:95% N_2(V/V) was used. To these experimental flasks 2 ml. aliquots of dispersed mucosal scrapings were added, the flasks then being sealed. The flasks were then transferred to a water bath at 37°C shaking at 74 oscillations per minute, for one hour. After this period of time had elapsed, flasks were transferred to an ice bath for a further hour and the pH then recorded as in the everted sac technique.

In an attempt to make the mucosal scrape technique as compatible as possible with the everted sac technique, recommended additives, e.g. 1% Bovine Serum Albumen (Perris, 1966) were not used. After the

pH determinations were made, the mucosal fluid containing the scrapes was returned to the weighed experimental flasks and dried for 48 hours in a drying oven at 100°C to constant weight. The whole solution was evaporated off leaving behind any cellular debris. It was thought that this would be preferable to centrifuging down the mucosal scrapings from the buffer solution and then drying the remains since large amounts of protein and DNA are lost into the medium (Stewart & Zbarsky, 1963). This could affect dry weight measurements. All parameters studied with this technique were expressed per milligram tissue dry weight.

3) The Segment Preparation.

As with the mucosal scrapes, a 4 cm section of proximal jejunum was excised in the normal manner from rats killed with ether anaesthesia. This section of the jejunum was not everted as in previous methods (Agar, Sidhu & Hird, 1954; Crane & Mandelstam, 1960) but merely cut into ten approximately equal 4 mm segments and stored in a petri dish containing 0.154 M saline solution. From the petri dish two segments, selected at random, were placed in each of five 25 ml conical flasks containing gassed buffer of known pH. Five rats were used per experiment so that at the end of the distribution of segments each flask contained ten segments, two from each of the five rats, with no differences between flasks due to time taken for preparation.

After one hour's incubation at 37°C the incubation fluid was removed and the pH measured. The segments were dried on Whatmans No. 1 filter paper and left to dry at 100°C to constant weight. Depending on which buffer was used, pH values were converted via one of the titration nomograms (see Figure 5) and hydrogen ion production expressed as μg per mg tissue dry weight.

Statistical comment on the segment and mucosal scrape preparations.

In the segment and mucosal scrape preparations, biological variation as a source of error could be kept to a minimum; this meant that statistically these were better preparations although possibly less viable than the everted sac. Pooling of the mucosal scrapes and subsequent division of the homogenate into control and experimental groups meant that innate biological variation between the control and experimental group was ruled out. In the case of segments there is less randomisation possible so that some biological variation still exists. However, the standard errors in these preparations are always smaller than those derived from sac experiments. Where the results from one batch of five rats were combined with the results from a second batch, the variance ratio of these independent samples (F-test) was computed. This was done to establish whether the pooling of data was legitimate and hence whether t-tests between control and experimental groups were justificable (Colquhoun, 1971).

4) The 'in vivo' perfused jejunal loop.

In this in vivo method (Fullerton & Parsons, 1956) the basic method of Sols and Ponz (1947) is adapted to a continuously recirculating perfusion system. Unfasted male rats were anaesthetised with nembutal (45 mg/kg body weight) intraperitoneally and tracheotomised to prevent respiratory obstruction during the period of experiment. The trachea was cleared of mucus intermittently by suction via cannula tubing, using a 10 ml syringe. The abdomen was opened and the upper end of the proximal jejunum was cannulated at the duodenal loop. A corresponding incision was made approximately 10 cms distally and this loop flushed out with double-distilled water to remove any food material. After flushing the distal end of the proximal jejunal loop was cannulated, the loop put back into place in the abdomen and the abdominal wall clipped back together again.

The jejunal loop was perfused with 20 mls of double-distilled water, whose pH was adjusted to about 7.2, from an open reservoir, recirculated at 3 mls per minute. After five minutes, this was replaced with 20 mls of double-distilled water of known pH. Although there is some evidence that double-distilled water damages the intestine (Reid, 1898; Dennis, 1940; Williams 1963) and causes increased permeability to passively moving anions, it was decided to use double-distilled water rather than saline because in comparison it had little buffering power and since no chloride ion was present, it might be possible to see whether chloride ion also

TABLE No. 4

Rectal temperature in the rat and the percentage contamination by blood of luminal perfusate during in vivo perfusion of proximal jejunal loops in the rat.

	1st half hour incubation	2nd half hour incubation
initial rectal temperature (C)	33.0 ± 0.64 (5)	36.40 ± 0.65 (5)
final rectal temperature (C)	36.60 ± 0.70 (5)	37.24 ± 0.22 (5)
Volume % blood contamination (ml blood/100 ml perfusate)	0.364	0.272

moves mucosally during acidification. Two one-half hour periods of perfusion occurred with replacement of the 20 mls of luminal fluid after the first half hour. After this and the second half hour, the initial and final pH was recorded with a glass electrode in the usual way and the solutions were then assayed for various electrolytes.

Body temperature as shown by a rectal thermometer, registered a drop to around 33 degrees centigrade, despite the animal being kept covered with cotton wool and warmed with a table lamp. Rectal temperature climbed steadily during the first half hour and was at normal values for the second half-hour incubation period. Some samples of perfusate seemed to be contaminated with blood and accordingly were compared with known standards of blood in double-distilled water. The samples were checked colorimetrically on a DB spectrophotometer at a fixed wavelength of 539 nonometres, the absorption peak of haemoglobin. Although it was impossible that blood contamination would lead to artefactual acidification, it was likely that it might obscure ion movement occurring in the gut. However, blood contamination was shown to be only of the order of less than one third of a volume per cent and as such insignificant (see Table 4). Rectal temperature data show that the second perfusion period is the better half hour for making observations (see Table 4).

Finally, 10 mls of perfusion medium (1 ml from each experiment) were titrated against 20 microlitre aliquots of normal hydrochloric acid to quantify the average

buffering capacity acquired by the double-distilled water, after perfusion through the small intestine proximal jejunum. Estimates of hydrogen ion production were made from the constructed nomogram, for each individual experiment. At the end of the perfusion period, the loop was removed and dried to constant weight at 100 degrees C, after all the fat and mesentery has been manually removed. Results were expressed as micrograms of hydrogen ion produced per milligram tissue dry weight.

Manometric determination of tissue oxygen uptake.

Tissue oxygen uptake was determined for the everted sac preparation in bicarbonate buffer using standard manometric methods (Umbreit, Burris & Stauffer, 1951). Everted sacs were made as described in the previous sections and placed into Warburg flasks of known volume (approximately 15 mls). The flasks contained 4.5 mls of buffer that had been gassed and kept on ice until all the other sacs were made. After the final sac had been made and introduced into the flasks, each flask centre well was filled with 0.5 mls of 3N KOH, injected by syringe, together with a rolled portion of filter paper to provide a large surface area for carbon dioxide absorption. Six flasks so prepared were transferred to the Warburg apparatus and allowed to equilibrate for fifteen minutes with the incubation temperature of 37 degrees C, prior to the assessment of oxygen uptake.

At zero time, the six flasks and also a thermobarometer flask were sealed and then shaken for one hour

at 74 oscillations per minute. After each hour the difference in the initial and the final level was noted and each manometric unit was reset. In this way the oxygen uptake could be studied over a long period of time. At the end of the experiment, after dissecting away the ligatures, the sacs were dried to a constant weight at 80 degrees C. Sacs needed at least 48 hours drying at this temperature; drying beyond this length of time led to no further significant reductions in the dry weight: wet weight ratio which reached a figure of 15.65% $\pm$ 0.42%. The oxygen uptake (Q_{O_2}) was expressed as microlitres of oxygen per milligram tissue dry weight.

Polarographic determination of tissue oxygen uptake.

In the case of the mucosal cell scrapes, tissue oxygen uptake was measured by a polarographic method (Bronk & Parsons, 1965) using an EIL Bishop SOH 33 oxygen electrode, connected via an EIL pO_2 accessory unit to an EIL 33B-2 Vibron voltmeter. This measured the electrode potential caused by variations in the amount of dissolved oxygen in solution and displayed it on a Chandos recorder.

The electrode was calibrated with a zero oxygen standard of saturated sodium dithionate and a maximum standard of saturated bicarbonate buffer. The actual oxygen content of saturated buffer was determined by the colormetric Winkler titration (Evans, 1923) and in this way, the electrode potential correlated with definite levels of oxygen in solution.

The oxygen content is determined at time zero, then 1 ml aliquots of mucosal scrapes are added to 25 ml flasks containing 10 mls of buffer. After one hours incubation at 37 degrees C, the oxygen content is measured again with the electrode and the changes in uptake of oxygen related to mucosal scrape tissue dry weight, exactly as in the manometric method.

Isotopic Methods involving ^{14}C-ATP.

In the isotopic experiments, two five cm long sacs were taken from each rat after ether anaesthesia. The sacs were incubated in bicarbonate buffer which was continually gassed with 95:5% & O_2:CO_2 (v/v) in contrast to the closed everted sac experiments. The sacs were incubated in this medium which also had present 10mM ATP containing ^{14}C-ATP isotope, uniformly labelled in the sugar ring, of specific activity 10 microcuries per millimole. Sacs were incubated for thirty minutes without agitation since the gas stream caused enough agitation. One sac was incubated at the usual temperature of 37 degrees C, the other sac was incubated at 0 degrees C so as to provide a control situation in which diffusive processes could be estimated.

Mucosal aliquots (0.2 ml) of the buffer medium were taken for counting prior to the experiment. After the incubation period, the sac was washed in three sequential 50 ml changes of saline and then 0.2 ml of serosal fluid taken for counting. The entire sac was homogenised in one ml of saline solution and deproteinised by boiling for

ten minutes at 90 degrees C with subsequent centrifugation at 3000 r.p.m. A 0.2 ml homogenate aliquot was taken from the deproteinised fluid and added to 10 mls of NE220 liquid scintillator. All such radio-active samples were counted on a Nuclear Enterprises 8305 scintillation spectrometer operating at 0 degrees C. Counting efficiency was determined by internal standardisation using n-1-^{14}C hexadecane (1.10 microcuries per gram) as a standard. The tissue concentration of ATP was calculated on the basis of tissue water assuming that this amounted to 85% of the tissue wet weight, a figure derived from the drying experiments mentioned in the section on the studies on oxygen uptake.

Tissue Viability in in vitro Preparations.

The incorporation of in vitro methods into intestinal physiology provides conditions where experimental variables can be more precisely controlled in the investigation of their effects on normal intestinal function. Incubation conditions can be standardised and other physiological parameters, e.g. blood flow, that affect gut function in the intact animal, are excluded. A further consequence of in vitro techniques is that the usual source of oxygen provided by the blood-vascular system is not present and the possibility exists of anoxic conditions, which have been shown to reduce or abolish many transport processes in the proximal jejunum (Baker, Searle & Nunn, 1961; Faust, 1962; Bihler, Hawkins & Crane, 1962; Kirchenberger, 1966). In the in vitro

case, the preparations must be shown to be viable under the artificial conditions provided and demonstrated to be still functional, during the experimental period. To this end, certain parameters commonly accepted as demonstrating viability were investigated, including oxygen uptake, transmural potential, water transport, glucose transport, potassium loss and some histological criteria.

a) Oxygen uptake.

Oxygen uptake measured manometrically gave results between 3.0 and 4.0 for the Q_{O_2} of jejunal sacs in the presence of 10 mM mucosal glucose in bicarbonate buffer. This was maintained at an almost linear rate for three hours and was taken as evidence that the sacs were viable after eversion and remained so for up to three hours (see Table 5 and Figure 6). Fully saturated bicarbonate buffer has a unit volume oxygen content of about half that seen in vivo in normal conditions (Maggi, Brue, Brousolle, Bensimon & Peres, 1970) and represents a poor approximation to in vivo conditions. Despite the fact that oxygen content of buffers declines on incubation (Pietra & Cappelli, 1968) and that the Q_{O_2} is dependent on the oxygen content (Pietra & Cappelli, 1970) the relatively linear oxygen uptake is proof of continuing viability.

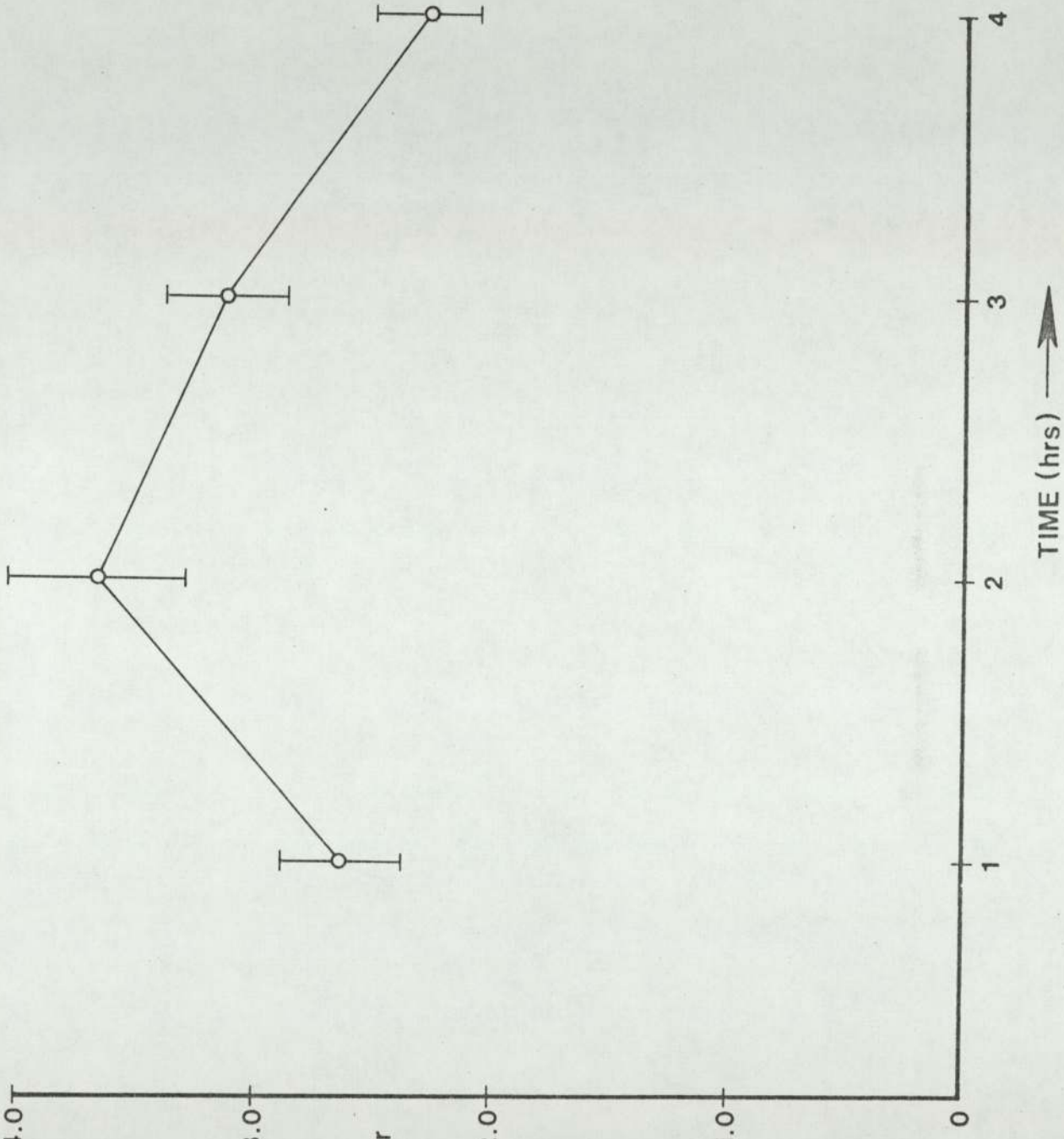
The estimation of oxygen uptake by tissues has led to varying values for the intestine. Some workers (Wilson & Wiseman, 1954; Turner, 1959; Bamford, 1966; Bamford & Holmes, 1971) cite values in the 10-20 range,

TABLE No. 5

Oxygen uptake of everted sacs incubated in bicarbonate buffer with 10mM mucosal glucose. Results are given as mean and standard error of mean (number of animals). Results are expressed as microlitres oxygen per milligram tissue dry weight.

Time	1st hour	2nd hour	3rd hour	4th hour
Q_{O_2}	2.63 ± 0.26 (5)	3.66 ± 0.37 (5)	3.12 ± 0.26 (5)	2.25 ± 0.20 (5)

FIGURE 6.



others quote lower values, although comparison is often difficult (Dorman, 1957 (cat); Faust, 1962; Bronk & Parsons, 1965; Jordana & Ponz, 1969; Smith, Matty & Blair, 1971; Wahle, Weekes & Sherratt, 1972 (sheep). There are naturally occurring parameters that affect the oxygen demand in intestinal tissue, notably, position in the gut (Bronk et al., 1965; Bamford et al., 1971), the state of nutrition (Evans, 1969), the age of the rat (Bamford, 1966). Other factors are inherent in the methods of preparation and the prevailing in vitro conditions, e.g. buffer and gas mixture used (Sherratt, 1968), reduction of traumatic anoxia by anaethetics (Bounous, Hampson & Gurd, 1963), the type of anaesthetic used (Levine, McNary, Kornguth & Leblanc, 1970) and the size of the sac (Bamford et al., 1971). Although higher Q_{O_2} values seem to indicate better preparations, it is also possible that cellular damage has occurred, with resultant loss of structural integrity and that very high values are a measure of mitochondrial respiration. However, variations in the measured Q_{O_2} probably do not reflect the quality of preparations: age of rat (Bamford, 1966) and size of sac (Bamford et al., 1971) seem to be the greatest source of variation. It is probably the size of sac in the present studies which is the cause of lower Q_{O_2} values and not viability.

In the case of the adapted mucosal scrape technique (Rasmussen et al., 1963), suspensions gave values of 2.13 in similar conditions. Occasional cell counts involving a differential staining technique, using a vital dye (Phillips

& Terryberry, 1957) indicated that on average about 65% of cells derived from this technique were living. This figure corresponds with the Q_{O_2} value in showing that the mucosal scrape preparations did respire actively but were not as active as the everted sac preparations. Cell suspensions from the rat have been shown to have Q_{O_2} values of between 1.0 and 15.0, depending on the type of buffer and substrate present (Perris, 1966; Stern, 1966).

b) Transmural potential.

Further measurements on viability included measuring transmural potential, the method is described in detail by Barry et al., (1964). Under the standard conditions of incubation, i.e. bicarbonate buffer with 10mM mucosal glucose, the transmural potential was determined to be 8.9 ± 4.20 (6) which is in agreement with that found by other workers (Barry, Dikstein, Matthews, Smyth & Wright, 1964; Smith, Blair & Matty, 1970). The transmural potential is closely associated with active transport of sodium and is reduced by anoxia (Barry et al., 1964) and is further evidence that the sacs were viable.

c) Water transport criteria.

Water transport was also measured in the everted sac in the presence and absence of glucose in bicarbonate buffer (see Figure 7 and Table 6). Water transport was seen to occur and also the usual increase in gut fluid uptake (Parsons & Wingate, 1961) attributable to epithelial cell

TABLE No. 6

Water transport in the everted sac in the presence of 10mM mucosal glucose in bicarbonate buffer. Rats stunned. Units are milligrams per 200 milligram tissue wet weight. Details as for Table 2.

	zero glucose	10mM glucose	statistical significance.
mucosal water transport	76.7 $\pm$ 6.5 (14)	67.6 $\pm$ 9.3 (15)	NS
gut fluid uptake	88.2 $\pm$ 7.6 (14)	53.8 $\pm$ 7.7 (15)	$p < 0.01$

FIGURE 7.

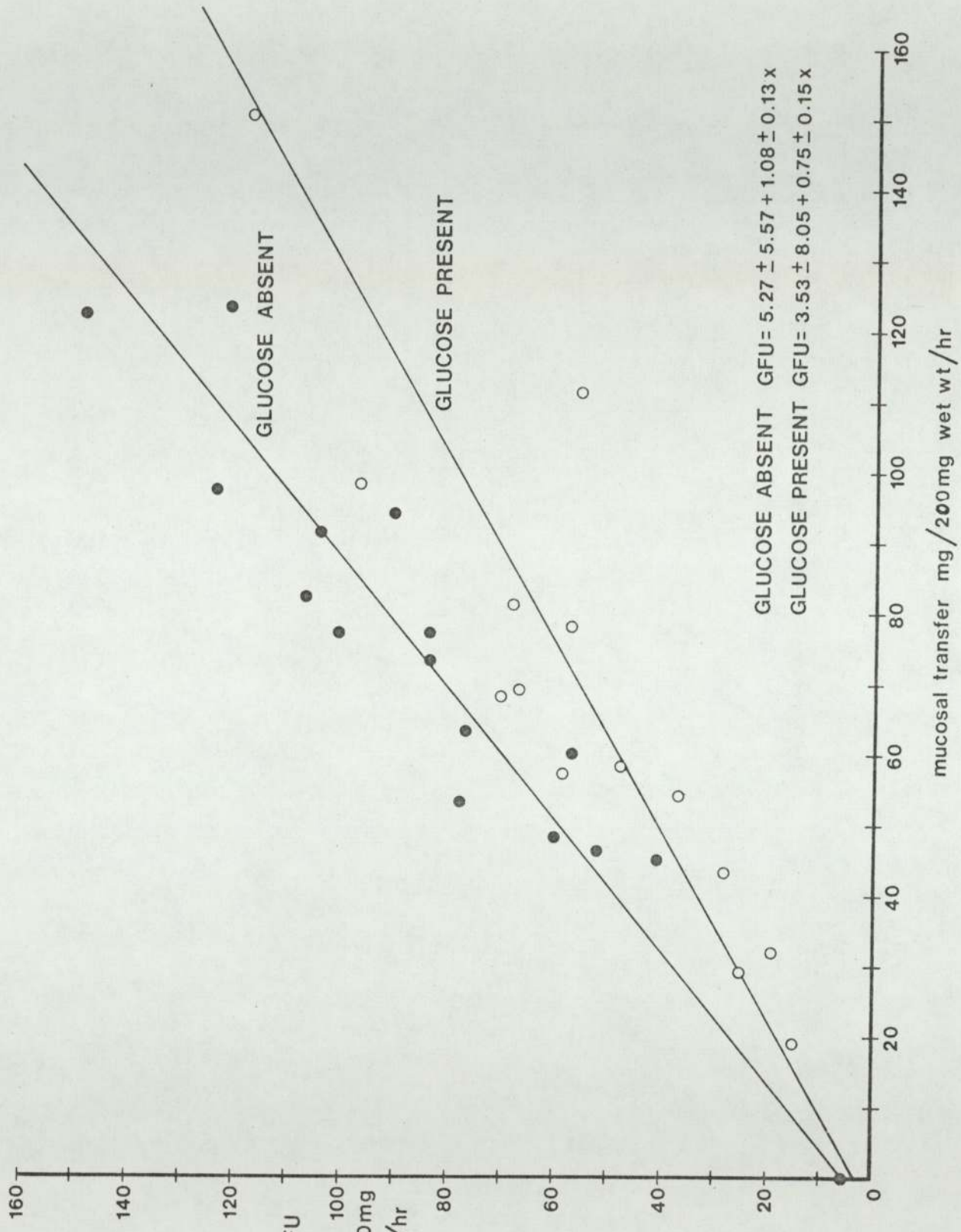

GLUCOSE ABSENT
GLUCOSE PRESENT
GFU mg/200mg wet wt/hr
mucosal transfer mg/200mg wet wt/hr
GLUCOSE ABSENT GFU= 5.27 ± 5.57 + 1.08 ± 0.13 x
GLUCOSE PRESENT GFU= 3.53 ± 8.05 + 0.75 ± 0.15 x
0
20
40
60
80
100
120
140
160

TABLE No. 7

Regression equations derived from water transport data in the everted sac (see Table No. 5). The regression of gut fluid uptake on mucosal water transfer in the presence and absence of glucose.

a) zero glucose.

gut fluid uptake = $5.27 \pm 5.57 + 1.08 \pm 0.13$ x mucosal water transfer

n = 15 (data points) $r = 0.922$ $p < 0.001$

b) 10mM glucose.

gut fluid uptake = $3.53 \pm 8.05 + 0.75 \pm 0.15$ x mucosal water transfer

n = 14 (data points) $r = 0.897$ $p < 0.001$

swelling (Jackson, 1968; Jackson & Cassidy, 1969; 1970). Parsons and Wingate did not show any hydration of their jejunal segments in the absence of glucose in isotonic conditions, which has been seen in the present investigations. The present data (see Figure 7) also confirms the linear relationship between gut fluid uptake (increase in sac weight) with mucosal water transport derived by Jackson (1968, 1970). Estimates of the slope of the regression lines (see Table 7) in the presence and absence of mucosal glucose do not significantly differ and indicate that glucose is not very effective in this region, in increasing water transport. This fact and the water transport values indicate that water transport, typical of the proximal jejunum is occurring (Barry, Matthews & Smyth, 1961).

d) Histological data.

Slides were made of the everted sac just after eversion and after an hours incubation in the bicarbonate buffer containing mucosal glucose at 10mM concentration, i.e. the standard incubation conditions. The jejunal material was fixed and stained by the usual haemotoxylin and eosin method and specimen slides are shown in the Figure 8; photograph 1 shows jejunal material after eversion, photograph 2 after two hours incubation. Histologically, the everted sac preparation looked structurally intact after eversion; although after the incubation period, shedding of mucosal sheets and disintegrating villi could be observed. This was as had been previously noted in the literature (Levine et al., 1970;

1

× 200

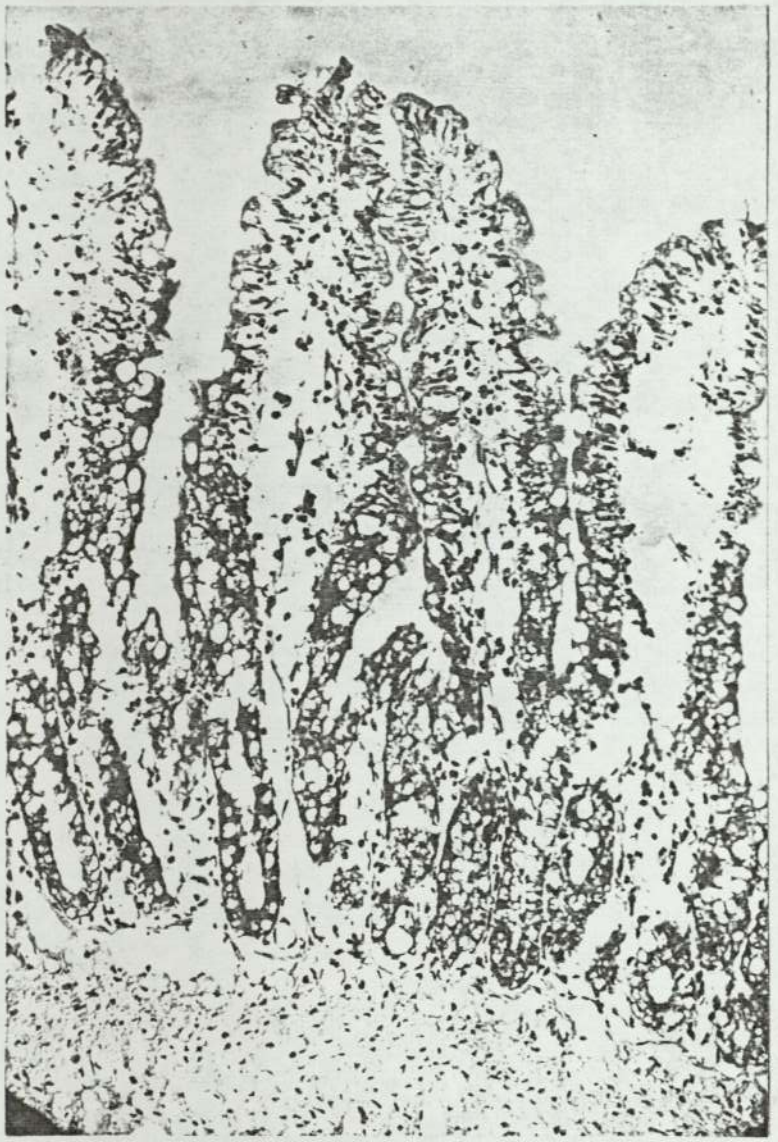
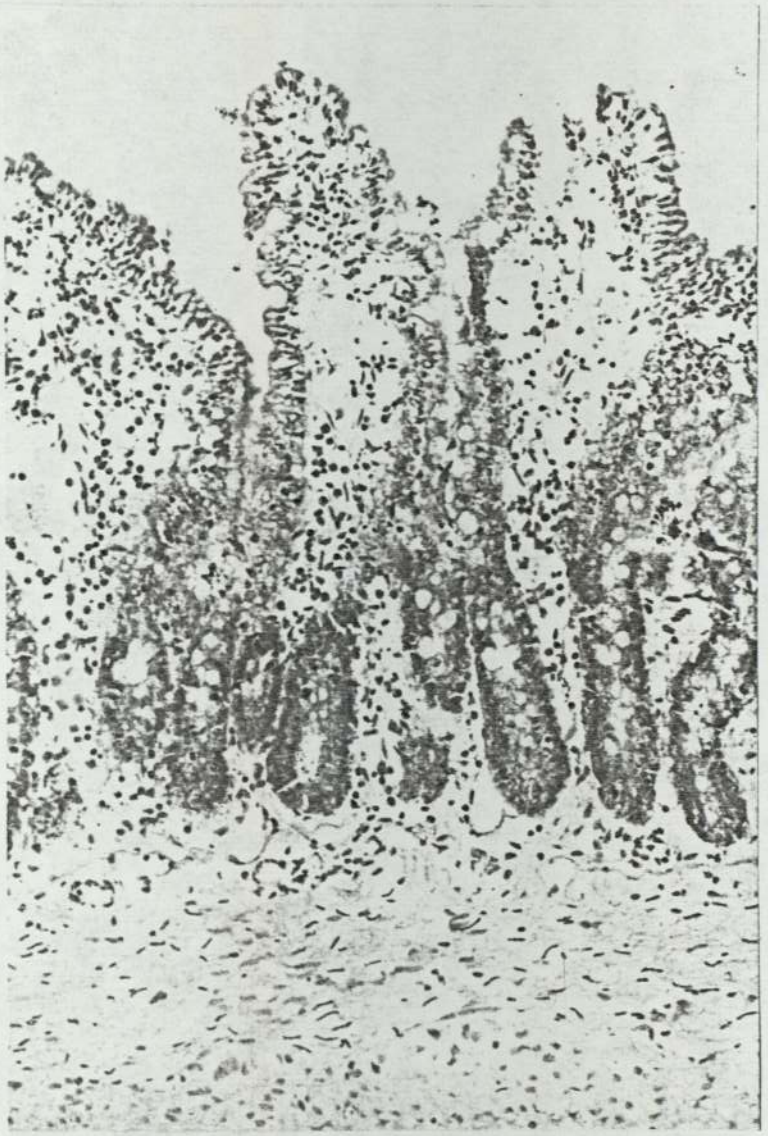


FIG: 8

2

× 200



Mohiuddin & Olubi, 1970; Plattner et al., 1970), but on the whole the everted sacs still looked fairly intact after incubation and was taken as further evidence that the sac was a viable preparation.

e) Glucose transport.

Under the standard incubation conditions, glucose transport was measured, both the mucosal transfer, i.e. amount of glucose passing the mucosal barrier, and the glucose concentration ration, i.e. the ratio of serosal to mucosal glucose concentration. In order to measure the glucose concentration ratio, glucose was also included in the serosal fluid in this experiment. The mucosal glucose transfer was found to be 99.51 $\pm$ 5.70 (10)micrograms of glucose per milligram tissue dry weight, which compares favourably with other estimates of this parameter by other workers (Barry et al., 1961). The glucose concentration ratio was found to be 1.66 $\pm$ 0.34 (4) and was conclusive proof that active transfer of glucose was occurring since ratios greater than one indicate that transport against a concentration gradient has taken place. It was concluded that sacs were both viable and capable of carrying out normal functions.

f) Potassium loss.

Potassium loss is sometimes taken as an index of viability since the active cell maintains a low sodium concentration intracellularly and a high potassium concentration. On cellular breakdown or disfunction the

concentration gradients maintained by the viable cell should decay and one might expect an increase in medium potassium. As with rat jejunum (Brown & Parsons, 1962) and rabbit ileum (Koopman & Schultz, 1969) mucosal levels were shown to decline slightly on incubation, indicating that not only was cellular potassium content stable but that some potassium was actually taken up from the buffer (see Table 9).

Investigations into the effect of the post-incubation period on ice.

As the routine experimental procedure for the everted sac preparation in bicarbonate buffer included an hour on ice after incubation, to redissolve carbon dioxide, the effects of this hour were investigated with respect to glucose and water transport and cell potassium loss into the medium. A group of ten everted sacs were incubated at 37 degrees C; at the end of the first hour, five had samples of mucosal fluid removed and were incubated on ice for a further hour, so that five sacs that had been incubated at normal temperatures could be compared with five that had been incubated for one hours normal incubation plus one hour on ice. This investigation was carried out because of the possibility that acidification could be a consequence of some sort of autolytic or anoxic phenomenon occurring during the second hour on ice. Alternatively, the low temperature might affect the normal ion extrusion mechanism and potassium loss might occur, i.e. passive ion movements which might cause acidification by their being linked to passive proton movements.

TABLE No. 8.

A comparison of glucose and water transfer in the everted sac incubated at 37°C with and without a subsequent period on ice. Further details as for Table No.3.

	incubation alone (one hour total)	incubation with one hour on ice (two hours total)	statistical significance
a) water transfer (mg/mgs tissue wet weight)			
i) mucosal transfer	89.7 ± 16.1 (5)	183.7 ± 22.2 (5)	$p < 0.001$
ii) gut fluid uptake	73.2 ± 4.7 (5)	101.3 ± 9.2 (5)	$p < 0.001$
iii) serosal transfer	3.5 ± 4.7 (5)	61.7 ± 14.9 (5)	$p < 0.001$
iv) gut fluid uptake) mucosal transfer)	0.85 ± 0.04 (5)	0.57 ± 0.03 (5)	$p < 0.01$
b) glucose transfer (µg/mg tissue dry weight)			
i) mucosal glucose transfer	99.51 ± 5.70 (10)	7.61 ± 6.43 (5)	$p < 0.001$
ii) glucose concentration ratio *	1.66 ± 0.34 (4)	2.58 ± 0.25 (5)	$p < 0.01$

* after arcsine transformation.

TABLE No. 9.

Potassium loss in the everted sac, incubated at 37C with and without a subsequent period on ice.
Details as for Table No. 7.

Potassium loss into the mucosal medium

(mg K^+/ml mucosal solution)

		incubation at 37C	incubation at 37C plus period on ice
i)	initial mucosal concentration	0.463 ± 0.072 (10)	0.424 ± 0.044 (10)
ii)	concentration at end of first hour	0.455 ± 0.025 (10)	0.500 ± 0.029 (10)
iii)	concentration end of further hour on ice		0.485 ± 0.061 (5)

As can be seen (Table 8) potassium ion movement does not change significantly over the first hours incubation or over the period on ice. This indicates that cell potassium content remains constant for the entire period of incubation and that no rapid breakdown of structural integrity occurs during the second hour on ice.

Very little mucosal glucose transfer is seen during the second hour on ice and indicates that the active transfer mechanism is inhibited (see Table 8). Water transport (see Table 8) across the mucosal barrier was maintained during the second hour on ice as well as during the first hour. The glucose concentration ratio actually rises during the second hour, even though there is a greater serosal exit of water.

These facts can be reconciled with the known facts about active glucose transfer (Wilson, 1962). During glucose transfer the intracellular concentration of glucose is very much elevated. When the sacs are placed on ice, mucosal transfer of water continues because of osmotic pressure. Despite the serosal exit of water, the serosal concentration of glucose continues to rise because of the large amounts of glucose then moving into the serosal compartment passively down its concentration gradient. The active transfer of glucose as judged by the mucosal transfer of glucose is abolished during the second hour and decay of intracellular glucose concentration gradients causes the increase in the serosal:mucosal glucose concentration ratio.

It can also be seen (see Table 8) that although

the gut fluid uptake and mucosal water transfer predictably differ in the two conditions, the ratio of gut fluid uptake to total mucosal water transfer also varies ($p < 0.01$). Since mucosal water transfer is continuing in the second hour of incubation on ice the rate of gut fluid uptake must be slowly decreasing during that period. This seems to indicate that gut fluid uptake reaches a maximal value sometime and that any direct relationship between gut fluid uptake and mucosal transfer of water is apparent only in the initial stages of water transfer. It may well be that although gut fluid uptake has been shown to relate directly to epithelial cell swelling (Jackson, 1969) this may be of little consequence in the water transport mechanism and dependent on osmotic effects subservient to other phenomena.

In any event the sacs have been shown to transport glucose and water in amounts comparable to that previously reported in the literature for the proximal rat jejunum (Barry, Matthews & Smyth, 1961). The period of incubation on ice to redissolve carbon dioxide is seen to all but stop mucosal glucose transfer and by implication metabolic processes, since active transport of glucose is metabolically dependent.

As a final check to find out whether the period on ice had any effect on acidification per se, four 7 cm sacs were made and incubated individually under normal incubation conditions in 20 mls of buffer contained in 50 ml conical flasks with sidearms. At the end of the incubation period the sacs were put on ice for a further

hour, but two of the series had the incubation fluid transferred via the sidearm to a connecting 20 ml flask; the other sacs were also in flasks connected via a sidearm to a 20 ml conical flask but the incubation medium was not transferred to the side arm. This meant that in one case, the incubation fluid re-equilibrated with the sacs in it and in the other case it re-equilibrated in the absence of sacs so that subsequent biological phenomena could not affect its final pH. It was found that at least 75% of the acidification occurred during the first hour as judged from these simple experiments.

These results seem to indicate that the period on ice does reduce the active functions associated with jejunal metabolism and also that this cooling does not have any untoward effects on acidification. This meant that the routine procedure involving everted sacs was a legitimate one for studying acidification in vitro.

Starvation and Acidification.

Rats were fasted so that the gut would be free of food material, making the eversion and preparation of the sacs easier. Also, all sacs would be in a uniform state of nutrition. Early work suggests that starvation reduces glucose transport (Cori, 1927), however, later work suggests the exact converse, also in the case of amino acid and sodium transport (Kershaw, Neame & Wiseman, 1961; Esposito, Faelli & Capraro, 1967; Wright & Barber, 1970; Madge, 1970). It is certain that gut weight and metabolism declines on starvation (Newey, Sanford & Smyth, 1970;

McManus & Isselbalcher 1970) as does lactate production (Cappelli, Pietra, & Panagia, 1968), glycolytic activity (Srivastava & Huebscher, 1968; Srivastava, Shakespeare & Huebscher, 1968) and mitosis (Brown, Levine & Lipkin, 1963; McManus & Isselbalcher, 1970). As gut weight declines on starvation, it was argued that elevated transport levels were artefactual since when related to gut length, which does not change on starvation (Cappelli et al., 1970) transport rates fall and even when related to gut weight alone, on persistent starvation transport rates eventually fall (Lis, Crampton & Matthews, 1972). Against these 'artefactual' explanations, L-glucose active transport occurs in the fasted rat (Neale & Wiseman, 1968) and also D-glucose transport in the ileum (Hindmarsh, Ross Kilby & Wiseman, 1967), a region where active transport has not previously been shown to occur (Barry et al., 1961). The effect of 24 and 48 hour starvation on acidification was investigated (see Table 10a) both lengths of starvation give rise to no obvious structural damage (Williams, 1963). In the absence of mucosal glucose, acidification did decline, but was not statistically significant at this number of observations. In the presence of glucose, acidification was minimal after 24 hours starvation, the highest value was seen at 48 hours starvation. These results can be related to the decline in metabolism seen on starvation. Fasting probably reduces acidification in both the presence and absence of glucose, but it is elevated in the presence of glucose after 48 hours starvation when related to gut weight. Fasting this long produced the greatest difference

between acidification, that is to say acidification in the absence of glucose and made the effect of substrates more pronounced. For this reason as well as the physical advantages already mentioned, 48 hour starvation was incorporated into the experimental procedure.

The validity of the everted sac technique.

Recently papers have appeared in the literature that criticise the everted sac as a preparation for assessing intestinal function (Levine et al., 1970; Plattner et al., 1970; Mohiuddin & Olubi, 1970). From electron and light microscopy data, these authors have noted that after some time, large complexes of epithelial cells are desquamated into the lumen and villi begin to disintegrate: this process was accelerated when metabolisable substrate was present (Levine et al., 1970). Extrusion zones began after the first half hour and the authors concluded that incubation times should be restricted to the first half hour after preparation (Plattner et al., 1970; Mohiuddin et al., 1970). These results have been confirmed in the frog (Loeschke, Hare & Csaky, 1971).

Some factors which affect sac integrity are known (Perris, 1964) and it has been shown that the preparative stage can be left in unoxygenated saline at room temperature for up to 24 minutes before any reduced fluid transfer capacity becomes evident (Barry, Matthews & Smyth, 1961). It would seem that hypoxic anoxia during the preparative stage is not as vital a factor as might be imagined. The use of anaesthetics in preparing everted sacs causes

less structural damage (Levine et al., 1970); however, since anaesthetics cause substantial reductions in sodium movement and transmural potential via the uncoupling of oxidative phosphorylation (Dinda & Beck, 1968) the lesser amount of structural damage is probably only a consequence of less cellular activity and as such is not a reliable index of functional integrity. A factor of apparently greater significance, detrimental to optimal functioning and obviated by anaesthetics, is the appearance of shock and traumatic anoxia rather than hypoxic anoxia in the preparative stage. Vasoconstriction occurs in shock (Fine, 1967); oxygen consumption and ATP synthesis are reduced (Bounous, Hampson & Gurd, 1963; Bounous, Scholefield, Hampson & Gurd, 1964) yet the prolonged hypovolaemic component of shock reduced fluid and sodium transport in everted sacs only after three hours of incubation (Balcazo, Parkins, Miller & Parkins, 1972). Also, rats rendered docile by maintenance in the dark prior to experiment provide sacs that give superior transport values (Porteous & Pritchard, 1972). These facts and the observation that ten minutes of in situ ischaemia caused by ligating intestinal arteries almost abolishes transport in the jejunum (Robinson & Mirkovitch, 1972) indicate that any disturbed intestinal function is less the result of hypovolaemia and hypoxic anoxia than the direct initial effect of intense vasoconstriction which might occur during handling.

RESULTS.

i) Preliminary data

ii) Effect of glucose

iii) Derivative data from glucose concentration curve.

iv) Variation in acidification with position in jejunum.

v) Acidification and phosphate hydrolysis.

vi) Effects of compounds active in gastric tissue.

vii) Anaerobiosis, and various inhibitors.

viii) Effect of diamox.

ix) Lactate and pyruvate production in composite in vitro preparations.

x) The effects of metabolisable hexoses.

xi) Effects of aminophylline and imidazol.

xii) Acidification in sacs, pyruvate production, lactate production, mucosal hexose, phosphate and sodium transfer in the presence of actively transported hexoses and ATP.

xiii) Distribution of isotopic ^{14}C ATP across jejunum.

xiv) Acidification in 'in vivo' loops.

i) Preliminary data.

As mentioned in the Methods Section, the effect of fasting on acidification, mucosal water transfer and gut fluid uptake was investigated (see Table 10a). Everted sacs will acidify the incubation medium significantly in the absence of any substrate, an ability that declines slightly on starvation, although not significantly. There was no correlation between acidification and mucosal water transfer or gut fluid uptake over the various experimental conditions. When summed for the various states of nutrition (see Table 10b), there was a significantly greater acidification ($P < 0.01$) when 10 mM mucosal glucose was present, also when glucose-1-phosphate was given ($p < 0.05$). 1 mM ATP had no effect.

Acidification was also measured in germ-free rats to see whether acidification was a consequence of the enteric bacterial flora and fauna. From the two available animals, a total of fourteen sacs were made which gave an average value of 0.740 in the presence of 10 mM glucose in bicarbonate buffer. This was some 28% more than that seen in the control rats. Since the fresh weight of jejunal mucosa is significantly lower in SPF rats, which are comparable animals (Hietanen & Haenninen, 1971) this is probably just a consequence of expressing acidification on a weight basis. These results showed that acidification did occur in germ-free rats and that acidification was not just dependent on the bacterial flora.

TABLE 10a. Starvation, acidification and the effect of some substrates. Everted sacs in bicarbonate buffer. H^+ production in μg/mg tissue dry weight.

	No glucose	10 mM glucose	10 mM glucose -1-phosphate	1 mM ATP
Acidification				
No starvation	0.388 ± 0.128 (7)	1.089 (2)	0.571 (2)	0.784 ± 0.620 (3)
24 hrs starvation	0.354 ± 0.108 (9)	0.603 ± 0.112 (7)	0.428 (2)	0.422 (2)
48 hrs starvation	0.328 ± 0.085 (8)	1.155 (2)	1.023 (2)	0.468 ± 0.457 (3)
Water transfer (mucosal) mgs water/200 mgs tissue wt. weight.				
No starvation	63.6 ± 7.5 (7)	96.8 (2)	50.2 (2)	78.6 ± 7.8 (3)
24 hrs starvation	78.7 ± 8.5 (9)	79.8 ± 10.0 (7)	78.3 (2)	72.0 (2)
48 hrs starvation	56.6 ± 2.4 (8)	59.83 (2)	83.0 (2)	57.6 ± 6.7 (3)
Gut fluid uptake mgs water/200 mgs tissue wet weight.				
No starvation	61.9 ± 7.4 (7)	67.2 (2)	45.0 (2)	65.6 ± 9.5 (3)
24 hrs starvation	67.5 ± 4.0 (9)	64.4 ± 9.3 (7)	70.8 (2)	70.4 (2)
48 hrs starvation	63.8 ± 5.1 (8)	54.0 (2)	71.8 (2)	72.3 ± 8.0 (3)

TABLE 10b. Starvation, acidification and the effect of some substrates. Statistical analysis. Details as for Table 10a.

Acidification (All starved states summed: see previous table)

Zero glucose	10 mM glucose	10 mM glucose -1-phosphate	1 mM ATP.
0.467 ± 0.086 (24)	1.039 ± 0.152 (11)	0.885 ± 0.163 (6)	0.603 ± 0.264 (8)

Statistical significance

Zero glucose v. 10 mM glucose $p < 0.01$

Zero glucose v. 10 mM glucose -1- phosphate $p < 0.05$

ii) The effect of glucose on acidification.

Since glucose and glucose-1-phosphate caused significant increments in the acidification of the mucosal medium by sacs from animals in various nutritional states, the effect of glucose was investigated over a range of concentrations in the forty-eight hour starved animal. Glucose caused a significant increase in acidification ($p < 0.05$) at 1 mM concentration in the mucosal medium, higher concentrations of glucose caused significantly ($p < 0.001$) greater acidification (see Table 11a). The glucose-dependent increment in acidification tended to a maximum value; acidification at 100 mM glucose concentration differed ($p < 0.05$) from that at 1 mM but not 10 mM glucose.

Mucosal water transport and gut fluid uptake were also measured in this experiment; there was significant correlation ($p < 0.001$) between increasing mucosal water transfer and increasing glucose concentration but no correlation between the latter and gut fluid uptake. Over the concentration range studied, mucosal water transfer and acidification (see Table 11c) were significantly correlated, an observation not seen in the previous section where animals in various nutritional states were used.

Further regression analyses show that acidification, when correlated with the log of glucose concentration, showed a significant linear relationship ($p < 0.01$). However, a log of acidification versus log

TABLE 11a. Acidification at various mucosal glucose concentrations. Sacs from stunned rats in Krebs-bicarbonate (see also Figure 9).

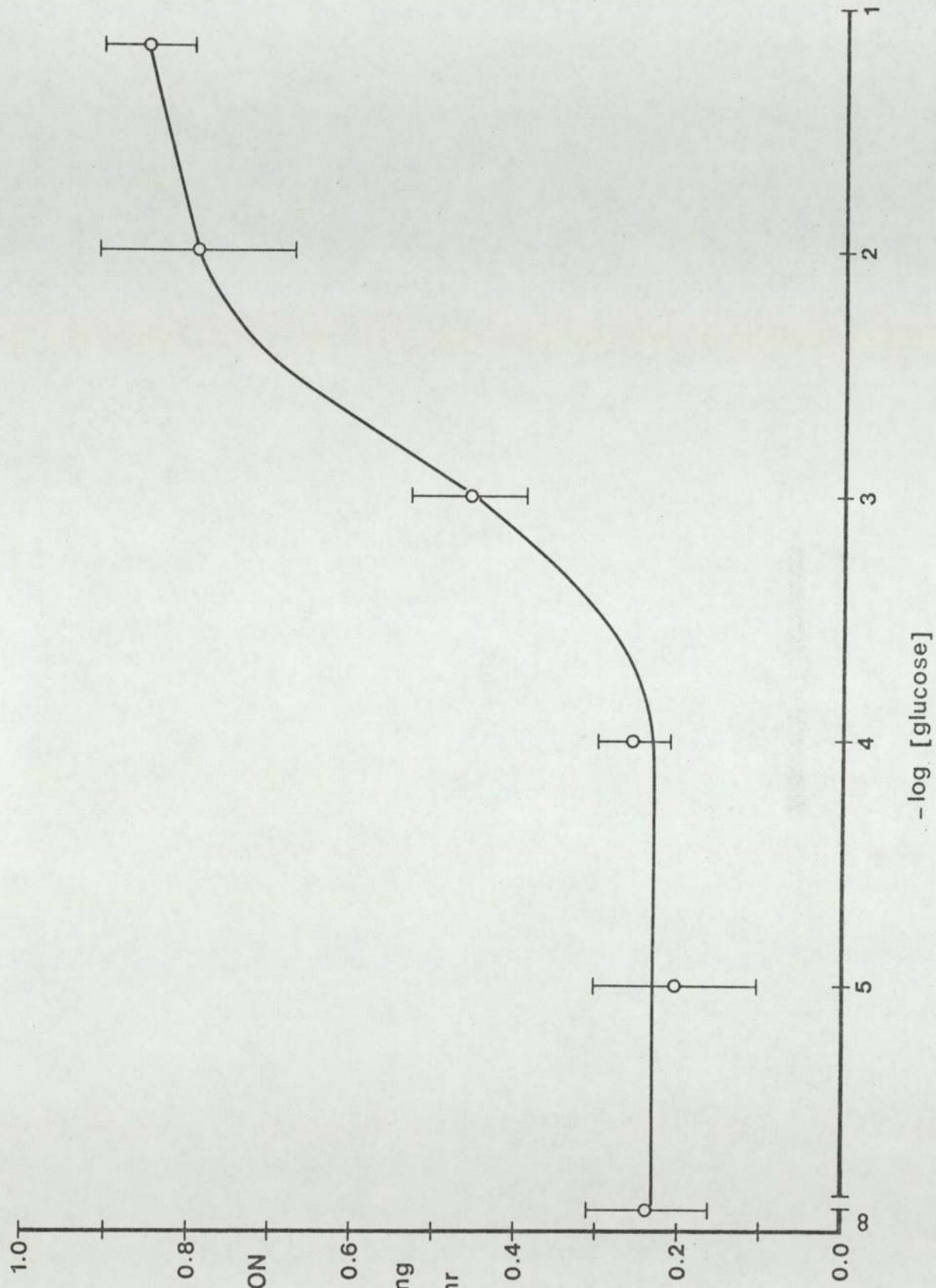
Mucosal glucose concentration	Acidification (μgH^+/mg dry wt/hr)	Mucosal water transfer (mgs/200 mg tissue wet wt/hr)	Gut fluid uptake (mgs/200 mg tissue wet wt/hr)
100 mM	0.848 ± 0.054 (12)	73.11 ± 16.66 (12)	62.68 ± 10.31 (12)
10 mM	0.790 ± 0.120 (12)	67.92 ± 14.62 (11)	62.93 ± 10.57 (10)
1 mM	0.453 ± 0.076 (12)	67.55 ± 8.96 (11)	69.82 ± 9.51 (12)
0.1 mM	0.257 ± 0.041 (12)	57.35 ± 8.51 (12)	69.29 ± 6.74 (11)
0.01 mM	0.203 ± 0.102 (12)	55.51 ± 8.70 (10)	59.20 ± 5.20 (11)
No glucose	0.235 ± 0.075 (12)	57.49 ± 5.36 (12)	60.85 ± 5.55 (12)

TABLE 11b

Statistical significance of data from Table 11a, variation in acidification with mucosal glucose concentration. P values from t-tests.

Concentrations	0.01mM	0.10mM	1.00mM	10mM	100mM
0.0 mM	NS	NS	0.05	0.001	0.001
0.01mM		NS	NS	0.001	0.001
0.10mM			0.05	0.001	0.001
1.00mM				0.05	0.001
10.00mM					NS

FIGURE 9.


1.0
0.8
0.6
0.4
0.2
0.0
ACIDIFICATION
µg H+ / mg
dry wt / hr
1
2
3
4
5
∞
- log [glucose]

of glucose concentration plot gives a better ($p < 0.001$) line of best fit, indicating the exponential relationship between acidification and glucose concentration, of the form: -

$$x = A(1 - e^{-y})$$ where x = glucose concentration
y = acidification
A = asymptotic constant.

Over the range of concentrations studied the log-log plot (see Table 11c) line of best fit goes through the origin indicating that acidification did reach its asymptotic maximum.

iii) Derivative data from the glucose concentration curve.

Since the glucose concentration - acidification curve was shown to be a logarithmic one, and the reciprocals of both functions were (see Table 11c) linearly related ($p < 0.05$), it was assumed that this represented an approximation to Michaelis-Menten (1913) kinetics. 'Apparent' kinetic constants were derived as acidification data was in rate form, i.e. weight of hydrogen ion produced per hour. The Michaelis-Menten kinetic equation: -

$$v = \frac{V \max (S)}{Km + (S)} \quad \ldots\ldots\ldots\ldots \quad (1)$$

can have various linearising transformations, the most common proposed by Lineweaver and Burke (1934) being the double-reciprocal plot of $\frac{1}{V}$ against $\frac{1}{(S)}$ giving: -

TABLE 11c.

Regression details from Table 11a (see also figure 9).

Independent variable	Dependent variable	Intercept (a)	Slope (b)	Correlation coefficient (r)	Significance ($p <$)
glucose conc.	water transport	78.02	4.58	0.9609	0.001
gut fluid uptake	acidification	-	-	-	NS
water transport	acidification	-	-	0.9333	0.01
log glucose conc.	acidification	-	-	0.9688	0.01
log glucose conc.	log.acidification	-	-	0.9777	0.001
reciprocal glucose conc.	reciprocal acidification	-	-	0.8101	0.05
log glucose conc.	ratio $\frac{\text{water transport}}{\text{gut fluid uptake}}$			0.8883	0.02

$$\frac{1}{V} = \frac{Km}{Vmax} \quad \frac{1}{(S)} + \frac{1}{Vmax} \quad \ldots\ldots\ldots (2)$$

where the slope of the equation is $\frac{Km}{Vmax}$ and the intercept = $\frac{1}{Vmax}$ In the case of diffusion kinetics where the rate of appearance of hydrogen ion would depend on its concentration in the system, a double-reciprocal plot would also give a straight line (Smyth, 1971) so that it is necessary to establish that the $\frac{1}{Vmax}$ intercept is statistically significant. From the double-reciprocal plot data (see Table 11c) the significance of the intercept for the acidification rate axis is established ($p < 0.05$) indicating that the rate of acidification in its relationship with glucose is determined by something other than simple diffusion.

An alternative linearising transformation, the Woolf plot (Hanes, 1932) was also used, being a plot of $\frac{(S)}{V}$ against (S) giving : -

$$\frac{(S)}{V} = \frac{1}{Vmax} \quad (S) + \frac{Km}{Vmax} \quad \ldots\ldots\ldots (3)$$

of slope $\frac{1}{Vmax}$ and intercept $\frac{Km}{Vmax}$

For the present data, plotting $\frac{(S)}{V}$ against (S) gave a significantly better correlation than the Lineweaver-Burke plot. The Lineweaver-Burke double-reciprocal plot has been shown to be one of the worst linearising transformations for kinetic parameters (Dowd & Riggs, 1965). The Woolf plot has been shown to be the best transformation (Colquhoun, 1969) with the proviso that it under-estimates the parameters if the experimental errors are at all large. This plot gave

the following estimates: -

K_m = 0.0355 mM, V_{max} = 1.011 (μgH^+/mg dry wt /hr).

iv) The variation in acidification with position in the jejunum.

From the glucose concentration data (see figure 9) it can be seen that acidification reaches a maximum value. Acidification in the absence of mucosal glucose was chosen as the standard against which to compare possible acidification-stimulating substrates. 10 mM mucosal glucose was adopted as the control for comparing acidification inhibitors since this concentration gave a high glucose-dependent increment in acidification, yet would be without the possible osmotic complications of adopting a 100 mM mucosal glucose solution. As these two control treatments were adopted in most subsequent experiments and also their position down the jejunum varied from trial to trial, it became eventually possible to derive additional data on the variation in acidification with the position in the jejunum.

Acidification can be seen to vary down the length of the proximal jejunum (see Table 12a). The seven sequential jejunal sacs span a length of approximately 22 cms beginning 10 cms below the pyloric sphincter. Acidification in the absence of mucosal glucose declines down the length of the proximal jejunum as shown by linear regression ($p < 0.05$) whereas acidification in the

TABLE 12a.

Variation in acidification down the length of the proximal jejunum. Data from 48 hr. starved rats, stunned, with sacs incubated in bicarbonate buffer. H^+ production is μg/mg tissue dry wt/hr.

Sac position	Acid Production (10 mM glucose)	Acid Production (glucose absent)
1	0.494 ± 0.071 (17)	0.273 ± 0.142 (6)
2	0.581 ± 0.066 (19)	0.372 ± 0.134 (5)
3	0.590 ± 0.086 (15)	0.255 ± 0.059 (7)
4	0.596 ± 0.059 (17)	0.305 ± 0.183 (6)
5	0.549 ± 0.099 (17)	0.269 ± 0.080 (6)
6	0.517 ± 0.051 (17)	0.179 ± 0.143 (7)
7	0.454 ± 0.073 (9)	0.139 ± 0.023 (6)

Regression details.

a) 10 mM glucose present in mucosal medium.

position 4 distally	$r = 0.9925$	$p < 0.001$
ditto corrected for surface area	$r = 0.9847$	$p < 0.01$

b) glucose absent

complete data	$r = 0.7624$	$p < 0.05$
position 4 distally	$r = 0.9858$	$p < 0.02$
complete data corrected for surface area	$r = 0.7109$	NS
position 4 distally corrected for surface area	$r = 0.9844$	$p < 0.02$

presence of 10mM mucosal glucose reaches a peak and then declines. In the presence and absence of glucose the decline in acidification distally down the jejunum is more acute ($p < 0.001$ in the presence of glucose, $p < 0.02$ in the absence of glucose) from sac position 4 onwards.

Equations derived from the literature, which relate variations in mucosal surface area to distance from the ileo-caecal valve (Wood, 1944; Fisher & Parsons, 1950) were used to correct the present data for variations in mucosal surface area. After surface area corrections, there were still significant correlations in acidification with respect to position in the jejunum from position 4 onwards, both in the presence ($p < 0.01$) and absence of ($p < 0.02$) of glucose. It can be seen that these variations in acidification with respect to site in the jejunum are not just a diffusion phenomenon and a corollory of Fick's Law, since on surface area correction, these variations persist.

From other pooled data, although from ether-anaesthetised rats, glucose transfer as measured by final serosal fluid glucose concentration (mgs %) when the mucosal fluid contained 180 mgs % increased down the length of the proximal jejunum (Table 12b) and lactate production remained constant. (Table 12c). This meant that acidification did not correlate with glucose transport or lactic acid production with respect to positional data.

TABLE 12b.

Variation in glucose concentration (mgs %) in serosal fluid after incubation down the length of the proximal jejunum. Sacs in bicarbonate buffer from rats ether anaesthetised. Initial mucosal glucose concentration was 180 mgs %.

Sac position	serosal glucose concentration mgs %.
1	112.8 ± 34.0 (5)
2	175.6 ± 63.2 (5)
3	172.0 ± 51.8 (5)
4	160.0 ± 50.1 (5)
5	221.6 ± 47.7 (5)
6	276.8 ± 52.6 (5)
7	302.8 ± 64.8 (5)

10 mM glucose present in mucosal solution. Paired 't' test between sac position 1 and 7 gives $p < 0.05$.

Regression equation: -

mgs % concentration = 85.71 + 29.36 x sac position

correlation coefficient $r = 0.9382$ $p < 0.01$

TABLE 12c.

Variation in lactate production with position down the jejunum. Data from ether anaesthetised rats, sacs incubated in 10 mM glucose - bicarbonate buffer.

Sac position	Lactate production μg/mg tissue dry wt/hr.
1	43.0 ± 14.0 (6)
2	50.4 ± 16.5 (5)
3	39.1 ± 10.0 (6)
4	44.0 ± 10.0 (3)

v) Acidification and phosphate hydrolysis.

As glucose-1-phosphate caused a significant increase ($p < 0.05$) in acidification (see Table 10b), some compounds were included in the mucosal medium to see whether hydrolysis of phosphates would contribute to acidification. L-β-phenylalanine, an inhibitor of intestinal alkaline phosphatase (Huebscher & West, 1965) was included in the mucosal medium to test for any inhibitory effects. Over the three-fold concentration range, 1mM up to 100 mM, L-β-phenylalanine (see Table 13) had no inhibitory effect on glucose-stimulated acidification. At 100 mM concentration of phenylalanine, water transport was significantly lower ($p < 0.05$) and also tissue Q_{O2} was reduced, indicating some impairment of function but even at this high toxic level phenylalanine-inhibition of alkaline phosphatase had no effect on acidification.

Pyridoxal phosphate and glucose-6-phosphate were included as substrates in the mucosal medium. Pyridoxal phosphate did not cause any increase in acidification, however 10mM glucose-6-phosphate (see Table 14) did cause a significant ($p < 0.05$) increase. The lesser significance of glucose-1-phosphate and glucose-6-phosphate in causing acidification as compared with equivalent amounts of mucosal glucose, and the failure of pyridoxal phosphate to acidity seems to suggest that acidification is not caused by simple hydrolysis of phosphates; the glucose phosphates are probably hydrolysed to glucose first and then subsequently acidify in the same manner as glucose does.

TABLE 13

The effect of L-β-phenylalanine on acidification and water transport in everted sacs incubated in bicarbonate containing 10 mM glucose. Rats stunned and decapitated.

	Acidification (μgH^+/mg dry wt/hour)	Water transport (mg/200 mg wet weight/hour)	Qo_2 ($\mu l O_2$/mg dry/wt/hr)
Control	0.498 ± 0.082 (6)	86.33 ± 6.51 (4)	2.19
100 mM Phe-ala	0.532 ± 0.038 (6)	62.60 ± 5.94 (6)	1.16
Control	0.482 ± 0.026 (6)	85.00 ± 19.00 (6)	
10 mM Phe-ala	0.458 ± 0.053 (6)	77.00 ± 17.50 (6)	
Control	0.522 ± 0.053 (6)	69.00 ± 5.39 (6)	
1 mM Phe-ala	0.673 ± 0.046 (6)	65.66 ± 9.12 (6)	

TABLE 14.

The effect of phosphate compounds in the mucosal medium on acidification. Sacs were incubated in bicarbonate buffer. Rats were stunned and decapitated. Values are given as means and standard error of the mean (number of animals). Acidification is expressed as μg/mg dry weight.

Compound	Acidification	Statistical Significance.
10 mM pyridoxal phosphate	0.519 ± 0.109 (10)	NS
10mM glucose-6-phosphate	0.831 ± 0.161 (8)	$p < 0.05$
No substrate	0.489 ± 0.082 (12)	control

vi) The effects of compounds known to influence gastric acidification.

Histamine, a compound which stimulates acid production in the rat gastric mucosa (Tsukamoto, 1961), was included in the mucosal medium. The chelating agent, ethylene diamine tetra acetic acid (EDTA) which sequesters calcium ion and reduces acid output in the gastric mucosa (Chung, Sum, Goldman, Field & Silen, 1970) was also given mucosally, as well as aminophylline, a compound which blocks the action of phosphodiesterase (Butcher & Sutherland, 1962) and stimulates parietal acid output in the gastric mucosa (Dehyle, Miederer & Stadelman, 1971). All these compounds were present in 10 mM concentration. Histamine had no effect on acidification (see Table 15) nor did EDTA on acidification in the absence of glucose. 10 mM aminophylline, however, substantially reduced acidification (p 0.001) in the absence of glucose (see Table 15).

vii) The effect of anaerobiosis and various inhibitors.

The effect of anoxia on acidification in everted sacs was studied. Sacs were incubated in buffer containing 10 mM mucosal glucose with an anaerobic gas mixture (95.5% N_2:Co_2 v/v). Under these anoxic conditions acidification was significantly reduced ($p < 0.001$) (see Figure 11). This makes it extremely unlikely that acidification is a phenomenon induced by temporary anoxia during the preparation of sacs as acidification seems to depend on oxidative metabolism and did not increase on induced anoxic conditions.

TABLE 15.

The effect of EDTA, histamine and aminophylline on acidification in everted sacs. Details as for Table 14. Aminophylline data see Figure 10.

Substrate	Acidification	Statistical Significance.
Zero glucose control	0.230 $\pm$ 0.103 (7)	
10 mM mucosal EDTA	0.290 $\pm$ 0.109 (7)	NS
10 mM mucosal histamine	0.162 $\pm$ 0.079 (7)	NS
10 mM mucosal aminophylline	0.035 $\pm$ 0.056 (7)	$p < 0.001$

FIGURE 10

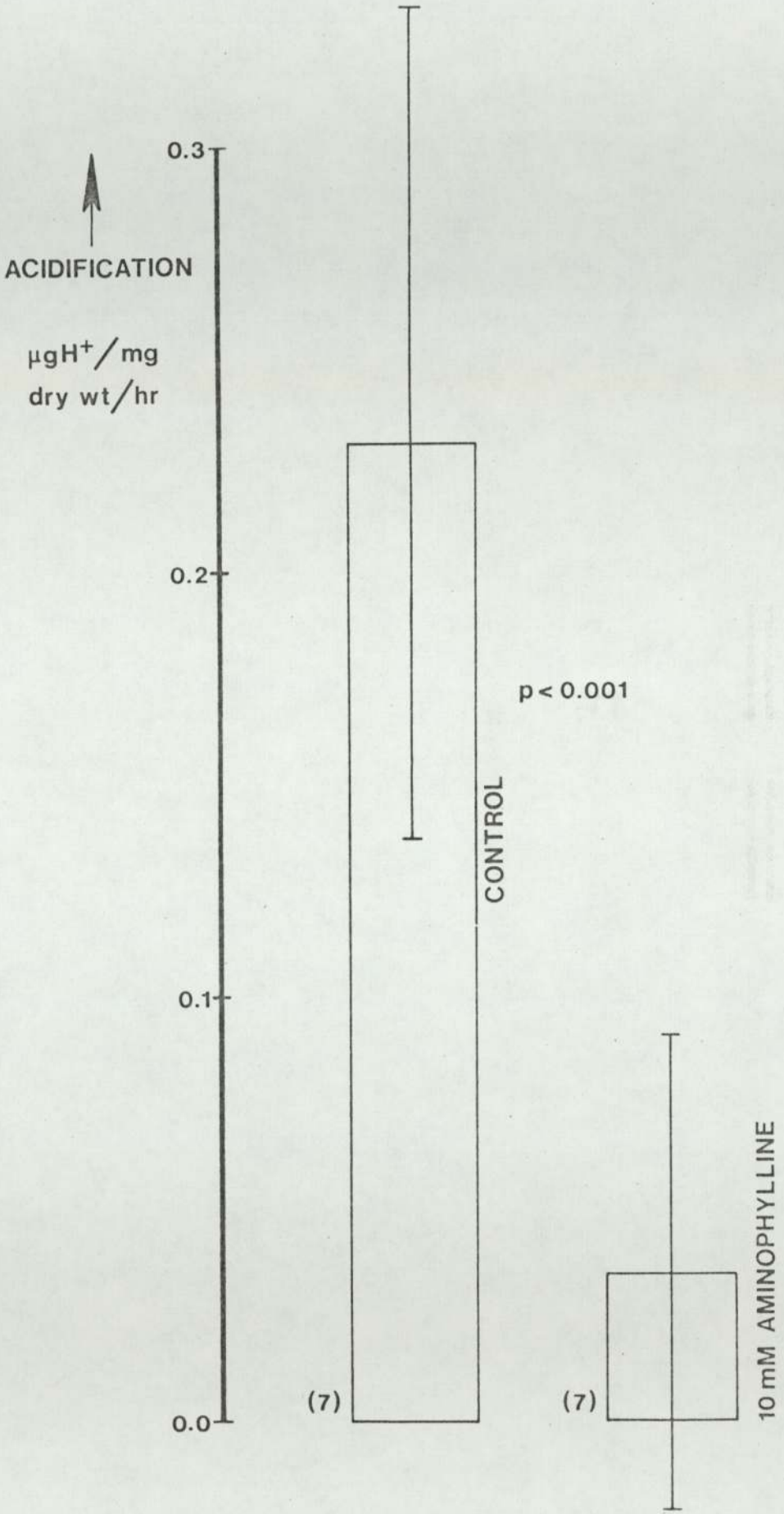

0.3
0.2
0.1
0.0
ACIDIFICATION
µgH+/mg
dry wt/hr
p < 0.001
CONTROL
(7)
(7)
10 mM AMINOPHYLLINE

FIGURE 11.

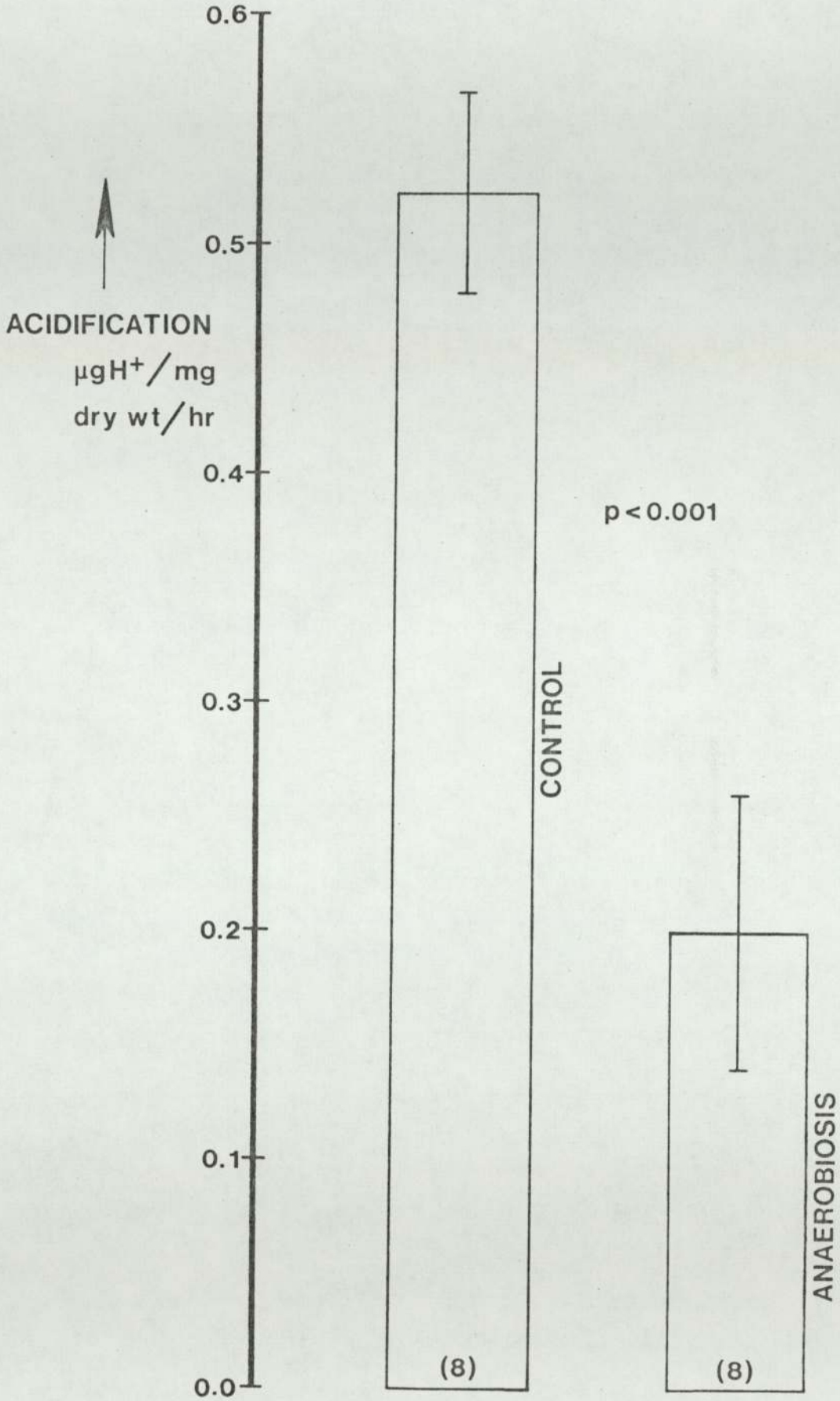

0.6
0.5
0.4
0.3
0.2
0.1
0.0
ACIDIFICATION
µgH+/mg
dry wt/hr
p<0.001
CONTROL
ANAEROBIOSIS
(8)
(8)

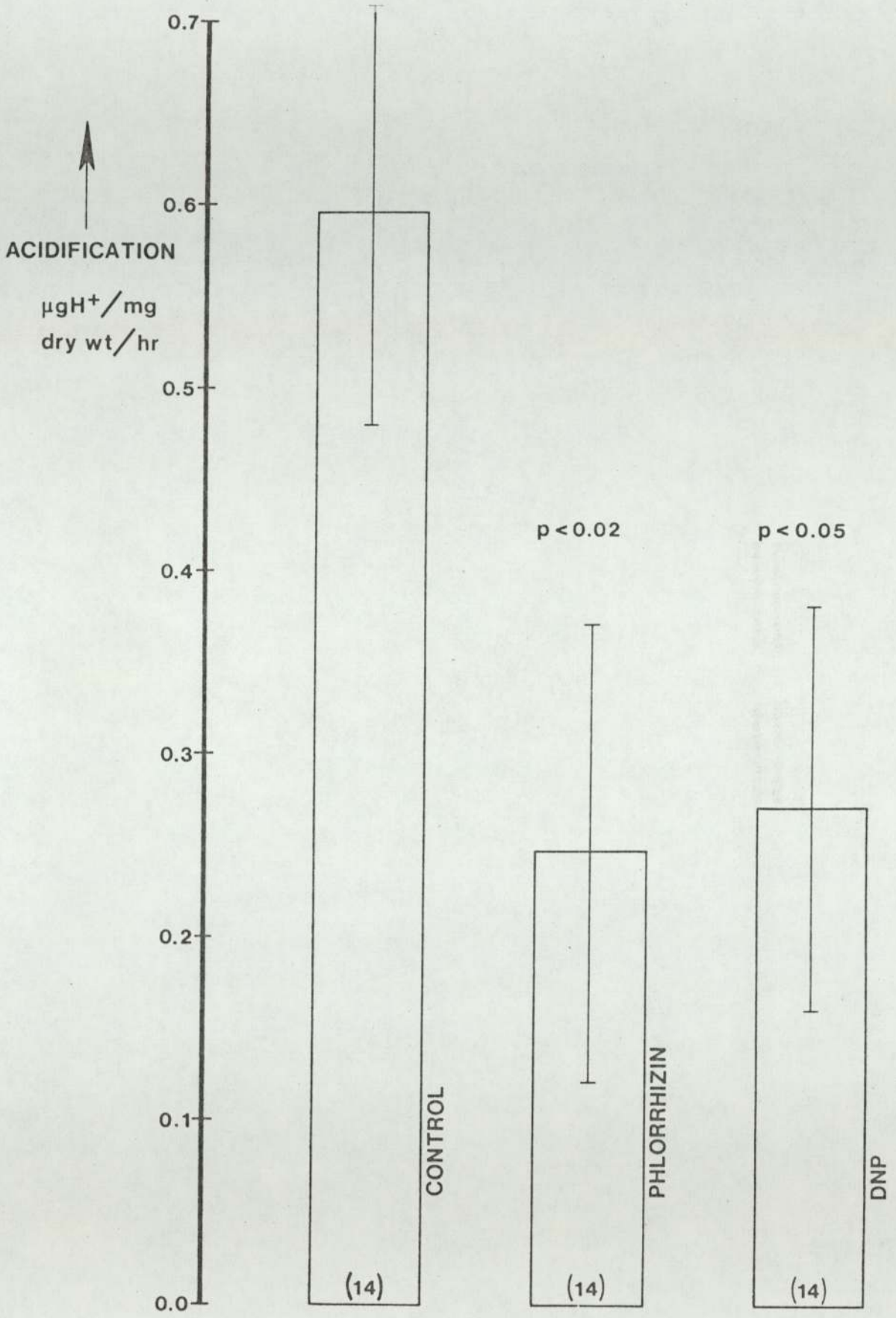
TABLE 16.

The effect of potential inhibitors on acidification in everted sacs incubated in bicarbonate.
Details as for Table 14.
(see Figure 12).

Inhibitor	Acidification	Statistical Significance
1 mM serosal ouabain	0.405 ± 0.094 (14)	NS
10 mM mucosal phlorrhizin	0.248 ± 0.125 (14)	$p < 0.02$
10 mM mucosal 2:4 DNP	0.271 ± 0.110 (14)	$p < 0.05$
10 mM mucosal glucose control	0.597 ± 0.111 (14)	
10 mM mucosal ouabain	0.428 ± 0.114 (10)	
10 mM glucose control	0.489 ± 0.082 (12)	NS

Buffers with inhibitors also contained 10 mM glucose.

FIGURE 12


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ACIDIFICATION
µgH+/mg
dry wt/hr
p < 0.02
p < 0.05
CONTROL
PHLORRHIZIN
DNP
(14)
(14)
(14)

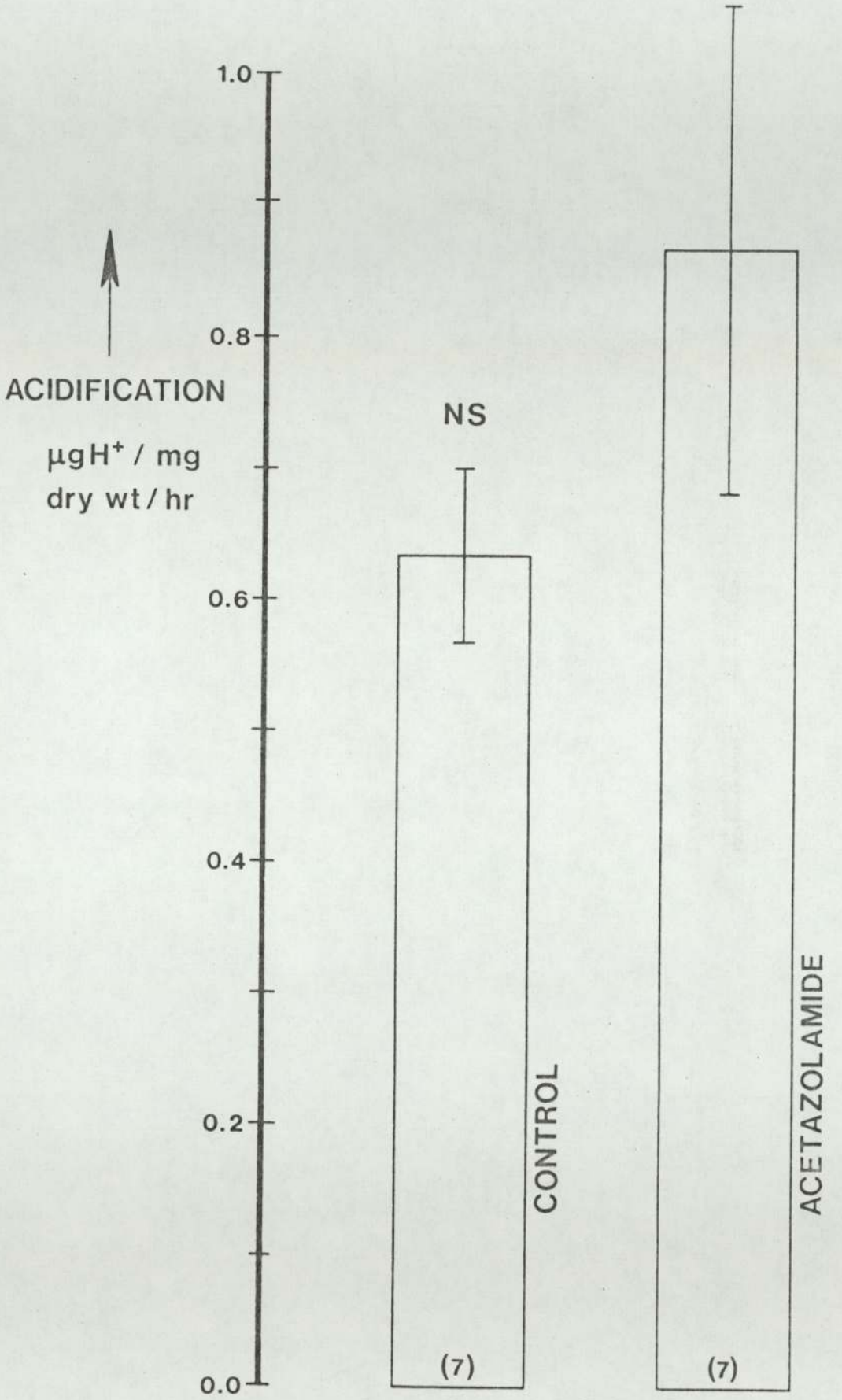
Dinitrophenol and phlorrhizin at 10 mM concentration were tested to see if their inhibitory effects affected acidification. Phlorrhizin abolishes selective absorption in the rat as well as reducing oxidative metabolism (Parsons, Smyth & Taylor, 1958) probably as in the kidney cortex by preventing the phosphorylation of ADP (Lotspeich & Keller, 1956). Dinitrophenol uncouples oxidative phosphorylation as well as having an effect on selective sugar absorption. Dinitrophenol abolishes selective sugar absorption in the guinea pig (Darlington & Quastel, 1955;) whether it does this in rat without reducing metabolism by its toxicity is doubtful (Brueckner, 1951). Both (see Table 16) substances caused reductions in glucose-stimulated acidification, phlorrhizin caused a greater ($p < 0.02$) inhibition than dinitrophenol ($p < 0.05$), and at these concentrations it is almost certainly because of their inhibitory effect on metabolism. Ouabain, a cardiac glycoside that inhibits active sodium transport in rat intestine was included in the serosal volume at 1 mM concentration. This concentration of ouabain had no statistically significant effect, although acidification was reduced; similarly 10 mM mucosal ouabain (see Table 16b) was also without demonstrable effect statistically. It was concluded that ouabain had little effect on acidification.

TABLE 17

Hexose Transports.

	µg hexose transferred mucosally per mg tissue dry wt.	hexose transferred as % of total amount of hexose available mucosally.
10 mM glucose present	133.8 ± 31.1 (6)	26.0
10 mM glucose + 10 mM Diamox	182.2 ± 31.9 (7)	25.6

FIGURE 13.


1.0
0.8
0.6
0.4
0.2
0.0
ACIDIFICATION
µgH+ / mg
dry wt / hr
NS
CONTROL
(7)
ACETAZOLAMIDE
(7)

viii) The effect of carbonic anhydrase inhibitor.

Acetazolamide (2:acetylamino-1:3:4-thiadiazole-5: sulphonamide), an inhibitor of carbonic anhydrase was included in the mucosal medium. This inhibitor will cause variations in rat ileal pH (Schnell & Miya, 1970) though not in the duodenum. Since acetazolamide will cause drastic inhibition of jejunal bicarbonate transport serosally, if acidification were to be mediated via bicarbonate transport, acetazlolamide ought to have some effect on acidification.

Acetazolamide had no effect on acidification at the concentration used in sacs incubated under the standard incubation conditions of bicarbonate buffer containing 10 mM mucosal glucose (see Figure 13). Mucosal glucose transfer was also measured (see Table 17) and did not differ in the control and experimental situations. It was concluded that acidification was not mediated by the enzymically controlled movement of bicarbonate.

ix) Acidification and concurrent lactate and pyruvate production in various in vitro preparations under aerobic and anoxic conditions.

a) Acidification.

Further 'in vitro' preparations were used to see which position of the intestine contributed most to the acidification phenomenon. Mucosal scrapes (Rasmussen et al, 1963) were made of jejunal material and the

TABLE 18.

Acidification in the various in vitro preparations in aerobic and anaerobic conditions from stunned and ether anaesthetised rats; preparations incubated in bicarbonate with 10 mM glucose present.

a) ether.	mucosal scrapes	everted sacs	ratio
stunned and decapitated	0.103 ± 0.011 (5)	0.648 ± 0.048 (51)	15.9%
ether anaesthetised	0.086 ± 0.016 (10)	0.442 ± 0.098 (6)	17.9%

values are μgH^+/mgm dry wt/hr. Scrape values are significantly less than whole everted sacs ($p < 0.001$)

b) anoxia

preparation	aerobic conditions	anaerobic conditions
mucosal scrape	0.086 ± 0.016 (10)	0.070 ± 0.011 (10)
mucosal sac	0.192 ± 0.036 (12)	0.151 ± 0.028 (12)

remaining "mucosaless" everted tissue made into 'muscle sacs' consisting of little more than everted muscle (Newey, Smyth & Whaler, 1955). Having found that significant reductions ($p < 0.001$) occurred in acidification when rats were ether-anaesthetised (see Table 4), mucosal scrapes were taken of both anaesthetised and stunned rats. Acidification by these mucosal scrapes in the presence of 10 mM glucose was very much less than acidification in the integral everted sac preparation ($p < 0.001$) both in the ether anaesthetised and stunned rat. In the case of the 'muscle sacs' (see Table 18) acidification produced by muscle sacs incubated with 10 mM mucosal glucose was also less ($p < 0.05$) than that produced by the integral everted sacs. The sum of the component parts for acidification, i.e. the sum of the mucosal scrape value and the muscle sac value for acidification, total to a value approximately 40% of the acidification value for the entire everted sac. This deficiency that appears on totalling the acidification of the component parts indicates that in the entire everted sac there is either the transport of a substance causing pH changes or some alignment of a vectorial component to cause acidification.

In an experiment using an anoxic nitrogen mixture (95:5% N_2: CO_2) as the gas phase, acidification was measured in the mucosal scrape and muscle sac preparations (see Table 18). Both in the mucosal scrape and muscle sac preparations acidification in the presence of 10 mM mucosal glucose did not increase and, if anything,

slightly declined. This confirms that acidification is a phenomenon dependent on the oxidative metabolism occurring in tissues.

b) Lactate production.

In the case of the (see Table 19) mucosal scrapes there was no difference in lactate production under aerobic or anaerobic conditions. Significantly higher anaerobic lactate production occurred ($p < 0.05$) serosally in the muscle sacs. This indicates the existence of a small Pasteur effect in the jejunal muscle layers that manifests itself in serosal lactate production. The mucosae do not contribute to this Pasteur effect although they are a significant source of lactate. The sum of lactate production in the mucosal scrape and muscle sac more than equals the lactate production in the integral sac.
The acidification that could be accounted for by measured lactate was calculated as a percentage of the total acidification; this consisted in estimating how much hydrogen ion the measured lactate would account for if it was assumed that all mucosal lactate was produced as the neutral species and had subsequently ionised. In these preparations (Table 19) lactate could account for a substantial amount of acidification, however in the muscle sac under anoxic conditions there is much more lactate produced than that predicted on a stoichiometric 1:1 relationship between hydrogen ion and lactate. It is probable that lactate appears as the dissociated anion in the mucosal solution.

TABLE 19.

Lactate production in mucosal scrapes and muscle sacs from stunned rats; all incubations in bicarbonate containing 10 mM glucose, units are µg/mg dry wt/hr.

Preparation	Aerobic Conditions	Anaerobic Conditions.
Mucosal scrapes	6.62 ± 1.81 (10)	5.96 ± 2.68 (8)
Muscle sac (mucosal)	24.79 ± 7.36 (10)	35.54 ± 9.32 (6)
Muscle sac (serosal)	1.07 ± 0.26 (8)	3.12 ± 1.18 (4)

Lactate contribution to Acidification.

Preparation	Aerobic Conditions	Anaerobic Conditions.
Mucosal scrapes	80%	100%
Muscle sac (mucosal)	87%	240%
Muscle sac (serosal)	4%	25%

TABLE 20.

Pyruvate production and acidification in various intestinal preparations from ether anaesthetised animals in bicarbonate buffer containing 10 mM glucose. Details as for Table 14. Results are expressed as μg/mg tissue dry weight/hour.

Preparation.	Mucosal pyruvate production	Acidification	Pyruvate contribution to acidification.
muscle sac (aerobic)	7.05 ± 0.74 (4)	0.282	21.6%
muscle sac (anaerobic)	7.19 ± 1.53 (3)	0.166	44.0%
mucosal scrapes	2.21 ± 0.16 (7)	0.036	55.8%

c) Pyruvate production.

As well as lactate production mucosally, the appearance of pyruvate was measured in the various in vitro preparations in the presence of 10 mM mucosal glucose. Higher quantities of mucosal pyruvate are produced by the muscle sac (see Table 20) than by the mucosal scrapes ($p < 0.001$). This production of pyruvate is not affected by anoxic conditions. If the percentage contribution of pyruvate to acidification is calculated and added to the estimations for the lactate percentage contribution, these two compounds more than account for acidification in the muscle sac and mucosal scrapes in aerobic conditions.

Acidification and lactate production in 'in vitro' segments.

In experiments with everted sacs incubated in bicarbonate buffer, acidification of the mucosal medium occurs. In bicarbonate buffer in a closed system, this acidification could be caused by respiratory carbon dioxide being produced mucosally and forming carbonic acid which will ionise in the mucosal fluid. Also, transport of the buffering anion, bicarbonate, could occur causing pH shifts as predicted by the Henderson-Hasselbalch equation without any movement of hydrogen ions whatsoever. From the mucosal scrape and muscle sac experimental data (see Table 18) there is cause to believe that such a vectorial or transport component to acidification exists. By using 'in vitro' segments in phosphate buffer saturated at a

constant carbon dioxide tension at equilibrium with the buffer, any increase in carbon dioxide from the tissues metabolic sources will only displace buffer carbon dioxide into the atmosphere. Metabolic carbon dioxide appearing in the mucosal medium will not dissociate but be displaced out of the medium without causing a change in pH. With respect to the vectorial aspect of acidification, if this occurs because of a secretion of protons mucosally with the accompanying anions moving serosally, this should not cause acidification in segment preparations where there is no separation of mucosal and serosal compartments.

An experiment was done using segments incubated in phosphate buffer that was continually gassed with the usual gas stream. Segments were incubated in the presence and absence of 10 mM glucose and subsequently assayed for the appearance of lactate in the mucosal medium and also the appearance of amino acids. The amino acid content of the mucosal medium was assayed after incubation to check for amino acid leakage as another possible artefactual source of acidification.

Both in the presence and absence of added glucose there is significantly less (see Table 21) acidification in the second hour of incubation. In the presence of 10mM mucosal glucose a greater amount of acidification occurs than is seen with everted sacs; the converse is true in segments incubated in the absence of added glucose. Segments incubated in the absence of glucose provide acidification value reminiscent of mucosal

TABLE 21.

Acidification and lactate production in segments from ether anaesthetised animals; sacs incubated in phosphate buffer. Details as for Table 14.

	Acidification 1st hour	2nd hour
10 mM glucose present	1.703 ± 0.086 (5)	1.081 ± 0.112 (5)
glucose absent	0.135 ± 0.023 (5)	0.005 ± 0.019 (5)

	Lactate Production (total 2 hours)	Lactate contribution to acidification
10 mM glucose present	35.015 ± 11.841 (5)	33.5%
glucose absent	1.559 ± 0.522 (5)	13.0%

Statistical significance

Acidification	Significance
Between 1st & 2nd hour	
a) in presence of glucose	$p < 0.02$
b) in absence of glucose	$p < 0.001$
Between presence and absence of glucose	
a) in 1st hour	$p < 0.001$
b) in 2nd hour	$p < 0.001$

Lactate production.

Between presence and absence of glucose over entire 2 hr. period $p < 0.02$

scrapes, indicating once more the vectorial component in acidification in everted sacs.

The heightened acidification occurring in segments which may be a consequence of increased availability of glucose to tissues is reflected by very much greater amounts of lactate appearing in the medium that is seen in the integral everted sac. In the absence of glucose, lactate appearance in the medium as a % of acidification is similar to that occurring in the everted sac. If the possible lactate contribution to the acidification is calculated, this lactate contribution could not account for the total amount of acidification, it accounts for even less acidification (see Table 21) in the absence of glucose.

Amino acid loss into the medium does not occur where segments are incubated in 10 mM glucose. In the absence of glucose, amino acid loss does occur. Since neutral amino acids would appear as the zwitterion in solution around pH 6 these can be neglected. Most of the remaining amino acids would cause an alkalinisation of approximately 0.10μg of hydroxyl ion per milligram segment dry weight. (see Table 22).

The segment experiment indicates that acidification in the presence and absence of exogenous glucose substrate is caused by a process other than metabolic carbon dioxide production, vectorial transport of a buffering anion, e.g. bicarbonate or phosphate, or amino acid leakage, but that a portion of that acidification might be accounted for by intracellular

TABLE 22.

Amino acid loss from segments incubated in Krebs-phosphate buffer in the absence of glucose: sacs from ether anaesthetised animals. Details as for Table 21.

Amino acid	amount (μ mole/ml)	molecular weight	Type of amino acid.
Tryptophane	0.09	204.23	
Lysine	1.52	149.21	basic
Histidine	0.19	155.16	basic
Arginine	1.15	174.20	basic
Aspartic	0.41	133.10	acidic
Threonine	0.53	119.12	
Serine	0.56	105.09	
Glutamic	1.40	147.13	acidic
Proline	0.59	115.13	acidic
Glycine	3.17	75.07	
Alanine	1.85	89.09	
Cystine	-	240.03	
Valine	1.66	117.15	
Methionine	0.61	149.00	
Isoleucine	0.98	131.18	
Leucine	2.60	131.18	
Tyrosine	0.76	18.19	
Phenylalanine	0.92	165.19	

lactate production with subsequent transport into the mucosal medium.

x) The effect of metabolisable hexoses on acidification.

Mannose and fructose, two metabolisable sugars, were tested to see whether metabolisable sugars in general could provoke acidification or whether acidification was a consequence of the active transport of sugars, particularly the active transport of glucose.

Mannose is a sugar which preferentially enters intestinal cells from the serosal side (Duerdoth, Newey, Sanford & Smyth, 1964) and is not actively transported (Crane & Krane, 1956, 1959). At very high serosal concentrations (222 mM) mannose stimulates metabolism but not fluid transfer (Duerdoth et al, 1964). In the present experiments (see Table 23) 100 mM serosal mannose caused an increase in acidification that did not significantly differ from that caused by 10mM mucosal glucose. 10 mM serosal glucose caused no acidification and was significantly less ($p < 0.001$) than 10 mM mucosal glucose control and also less than ($p < 0.05$) that caused by 100 mM serosal mannose. This was taken to mean that mannose can support acidification.

Fructose, another metabolisable monosaccharide, for which some workers have proposed specific active transport systems (Gracey, Burke & Oshin, 1970; Guy & Deren, 1971), was investigated for its effect on acidification. Fructose caused a significant ($p < 0.05$)

TABLE 23.

The effect of hexoses in the serosal fluid on acidification in everted sacs. Details as for Table 14.

Hexose	Acidification	Statistical Significance.
100 mM serosal mannose	0.473 ± 0.090 (14)	NS
10 mM serosal glucose	0.203 ± 0.085 (14)	$p < 0.001$
10 mM mucosal glucose control	0.598 ± 0.111 (14)	control

Serosal mannose v. serosal glucose $p < 0.05$

TABLE 24.

The effect of 10 mM mucosal fructose on acidification in everted sacs. Details as for Table 14.

Hexose	Acidification	Statistical Significance
zero glucose control	0.230 ± 0.103 (7)	
10 mM mucosal fructose	0.377 ± 0.056 (7)	$p < 0.05$
10 mM mucosal glucose	0.637 ± 0.067 (7)	$p < 0.01$

Mucosal hexose transfers μg/mg tissue dry wt/hr

Hexose	Mucosal hexose transfer
Glucose	133.8 ± 13.6 (6)
Fructose	22.3 ± 12.9 (7)

increase (see Table 24) when present in the mucosal solution at 10mM concentration. Fructose provoked approximately 35% of the acidification achieved with equimolar glucose. The amount of monosaccharide transferred mucosally was also measured in this experiment (see Table 24): the amount of fructose transferred from a 10mM solution was approximately 16% of the amount of glucose transferred in the 10 mM glucose-control situation.

These two experiments show that mannose and fructose, two alternative monosaccharides to glucose that can be metabolised, both stimulate acidification. Since mannose does this at concentrations which fail to provoke fluid transfer in the intestine (Duerdoth et al., 1964) it seems that the correlation between mucosal water transfer and acidification (Table 11c) reflects only their mutual dependence on metabolic phenomena. The fact that fructose also causes acidification and yet little transport of fructose occurs when compared with glucose indicates that acidification is not linked with general hexose transfer, at least not in a stoichiometric fashion.

xi) <u>The effect of Aminophylline and Imidazol on acidification.</u>

Since aminophylline was seen to produce a highly significant depression of acidification in the absence of metabolisable substrate (see Table 15) the effect of aminophylline was more closely examined in the

TABLE 25

The effect of various hexoses and aminophylline on acidification; data from everted sacs incubated in phosphate buffer. Rats were ether anaesthetised. Details as for Table 14. (See figure 14).

Substrate	Acidification	Statistical Significance.
zero glucose	0.563 ± 0.100 (4)	
10 mM glucose	0.808 ± 0.081 (5)	
10 mM glucose + 10 mM aminophylline	0.258 ± 0.098 (5)	$p < 0.001$
10 mM mannose	0.598 ± 0.099 (5)	
10 mM mannose + 10 mM aminophylline	0.070 ± 0.046 (5)	$p < 0.001$
10 mM methylglucose	0.364 ± 0.081 (5)	
10 mM methylglucose + 10 mM aminophylline	0.034 ± 0.028 (5)	$p < 0.001$

glucose v 3 methylglucose $p < 0.001$

glucose v zero glucose $p < 0.05$

zero glucose v methylglucose $p < 0.05$

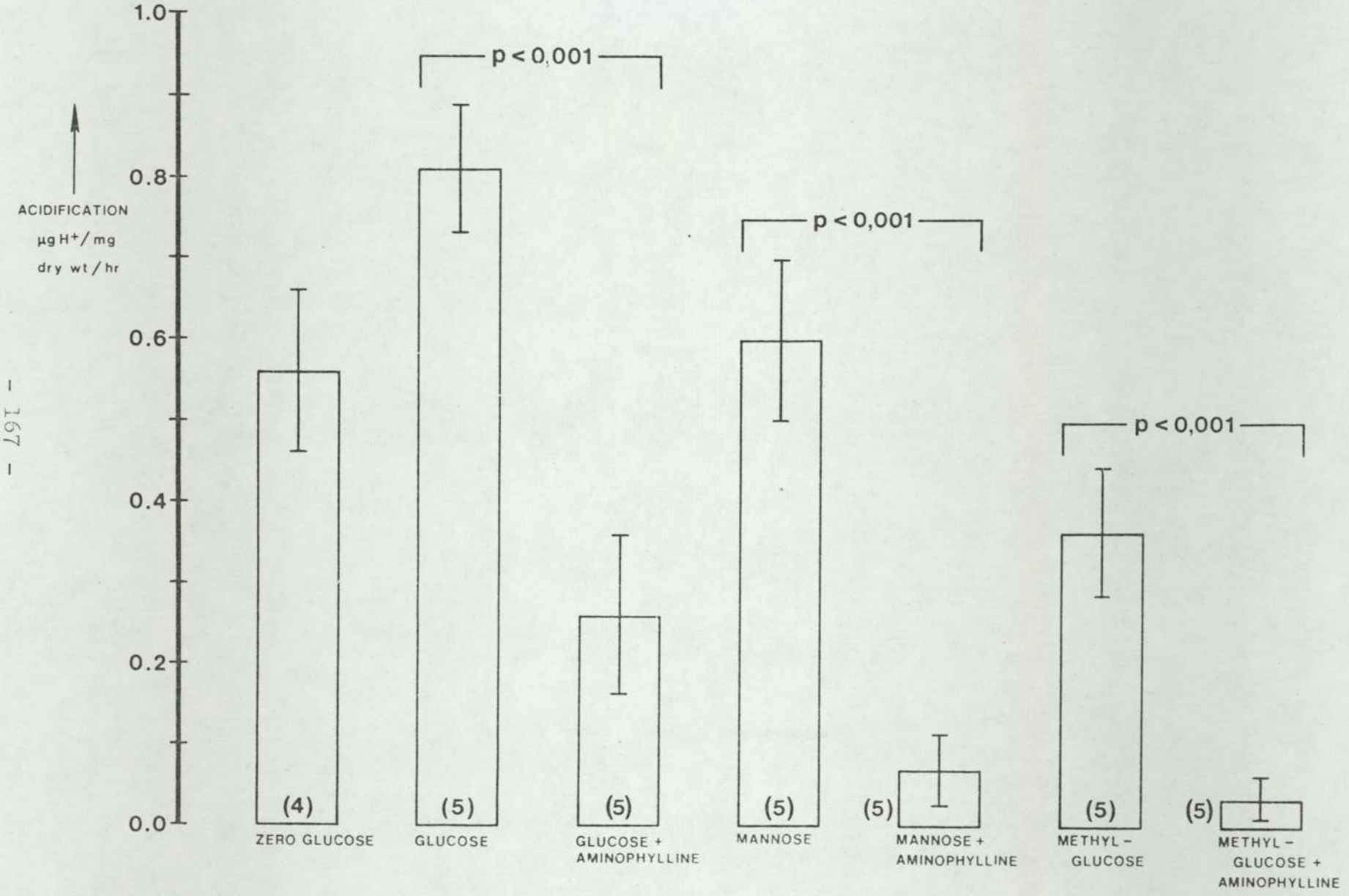

1.0
0.8
0.6
0.4
0.2
0.0
ACIDIFICATION
µg H+/ mg
dry wt / hr
p < 0,001
p < 0,001
p < 0,001
(4)
(5)
(5)
(5)
(5)
(5)
(5)
ZERO GLUCOSE
GLUCOSE
GLUCOSE + AMINOPHYLLINE
MANNOSE
MANNOSE + AMINOPHYLLINE
METHYL - GLUCOSE
METHYL - GLUCOSE + AMINOPHYLLINE

FIGURE 14.

presence of metabolisable and unmetabolisable hexoses. 10 mM aminophylline was included in the mucosal medium and containing 10 mM of hexose either glucose, mannose or methylglucose and compared to control situations where sacs were incubated with hexose only. Mucosal hexose transfer was also measured in this experiment. The buffer was gassed with 95:5% O_2:CO_2 (v/v). As in bicarbonate buffer, 10 mM glucose as substrate in the mucosal medium caused a greater ($p < 0.05$) acidification than in the absence of glucose (see Table 25). The unmetabolisable sugar, methylglucose caused an acidification not only less than that seen in the presence of glucose ($p < 0.001$) but less than that seen in the absence of glucose ($p < 0.05$).

In each case, where aminophylline was present there is a very highly significant ($p < 0.001$) reduction in acidification. This reduction was almost total where mannose and methylglucose were used (Table 25), but where glucose was used as the substrate some acidification persists.

Mucosal hexose transfer was also measured in this experiment in phosphate buffer and gave some very erratic results: in some cases there was more hexose present at the end of the experiment than at the beginning and it is probable that the significantly reduced hexose transfer that occurs in this buffer (see Table 1) explains this phenomenon. It is well known that some efflux of glucose occurs during active transport (Foerster & Hoos,

TABLE 25a.

The effect of aminophylline on mucosal hexose transfer. Details as for Table 25.

Substrate	Hexose transfer (μg/mg tissue dry weight)	Significance.
glucose	51.2 ± 17.3 (5)	NS
glucose + aminophylline	33.8 ± 37.4 (5)	
mannose	- 2.8 ± 54.7 (5)	NS
mannose + aminophylline	10.2 ± 38.7 (5)	
methyl glucose	-85.2 ± 83.6 (5)	NS
methyl glucose + aminophylline	-37.4 ± 45.8 (5)	

1972). All sugars were measured by the ferricyanide reduction method which measures total reducing sugar in solution. At low rates of influx one might in effect see some counter-transport of hexose inside the cells for hexose outside the cells. Counter-transport of methylglucose, a sugar which does not reduce as powerfully as glucose, (see Methods A) for extracellular glucose could lead to the artefactual situation of an apparent increase in mucosal fluid total reducing sugar, when estimated on the nomogram constructed for methylglucose. This assumption would also explain why including 10 mM methylglucose in the mucosal medium led to acidification values that were less than those in the absence of any added hexose. Methyglucose would counter-transport with intracellular glucose and lead to the substitution of a metabolisable sugar inside the cell with an unmetabolisable sugar, which as a consequence would mean a reduction in total substrate available for acidification. Despite the erratic transfer data leading to large standard errors and a net gain in mucosal hexose in the case of incubations with methylglucose (see Table 25a), aminophylline seems to have no effect on mucosal hexose transfer, this is clearly seen where glucose was present mucosally.

Imidazol, a compound shown to have the reverse effect on phosphodiesterase (Butcher & Sutherland, 1962) that aminophylline has, has no effect on acidification both in the presence and absence of glucose, in fact a slight reduction occurs. (See Table 26).

TABLE 26.

The effect of 10 mM Imidazol on acidification by everted sacs incubated in Krebs-phosphate buffer. Details as for Table 25.

Substrate	Acidification
zero glucose	0.266 ± 0.071 (6)
zero glucose + imidazol	0.144 ± 0.058 (6)
10 mM glucose	0.478 ± 0.111 (6)
10 mM glucose + imidazol	0.347 ± 0.067 (6)

xii) Acidification, lactate and pyruvate production, hexose, sodium and phosphate transfer in everted sacs: the influence of various hexoses.

Two unmetabolisable sugars, galactose (Diedrich & Anderson, 1960) and 3-O-methylglucose (Csaky & Wilson, 1956) which are actively transported (Csaky, 1943; Fisher & Parsons, 1953) were tested as substrates for acidification, as well as mannose which was shown to acidify when present in the serosal fluid (see Table 23) and ATP which did not acidify at 1 mM concentration (Table 10b).

Neither 10 mM mucosal galactose nor 10 mM mucosal 3-O-methylglucose (see Table 27) caused any significant increase in acidification. 10 mM ATP on the other hand caused an acidification that was significantly greater than that in the absence of added glucose ($p < 0.001$) and also greater than acidification in the presence of 10 mM mucosal glucose ($p < 0.02$). Mannose causes an acidification that is significant when compared to acidification in the absence of glucose ($p < 0.05$) and is indistinguishable from acidification in the presence of 10 mM mucosal glucose. This is in contrast to acidification in the presence of 10 mM galactose or 10 mM methylglucose, both of which are significantly less ($p < 0.01$ and $p < 0.02$) than the mucosal glucose control value.

TABLE 27.

The effect of various hexoses and ATP on acidification in everted sacs from ether anaesthetised rats incubated in bicarbonate buffer. Details as for Table 14. (see figure 15).

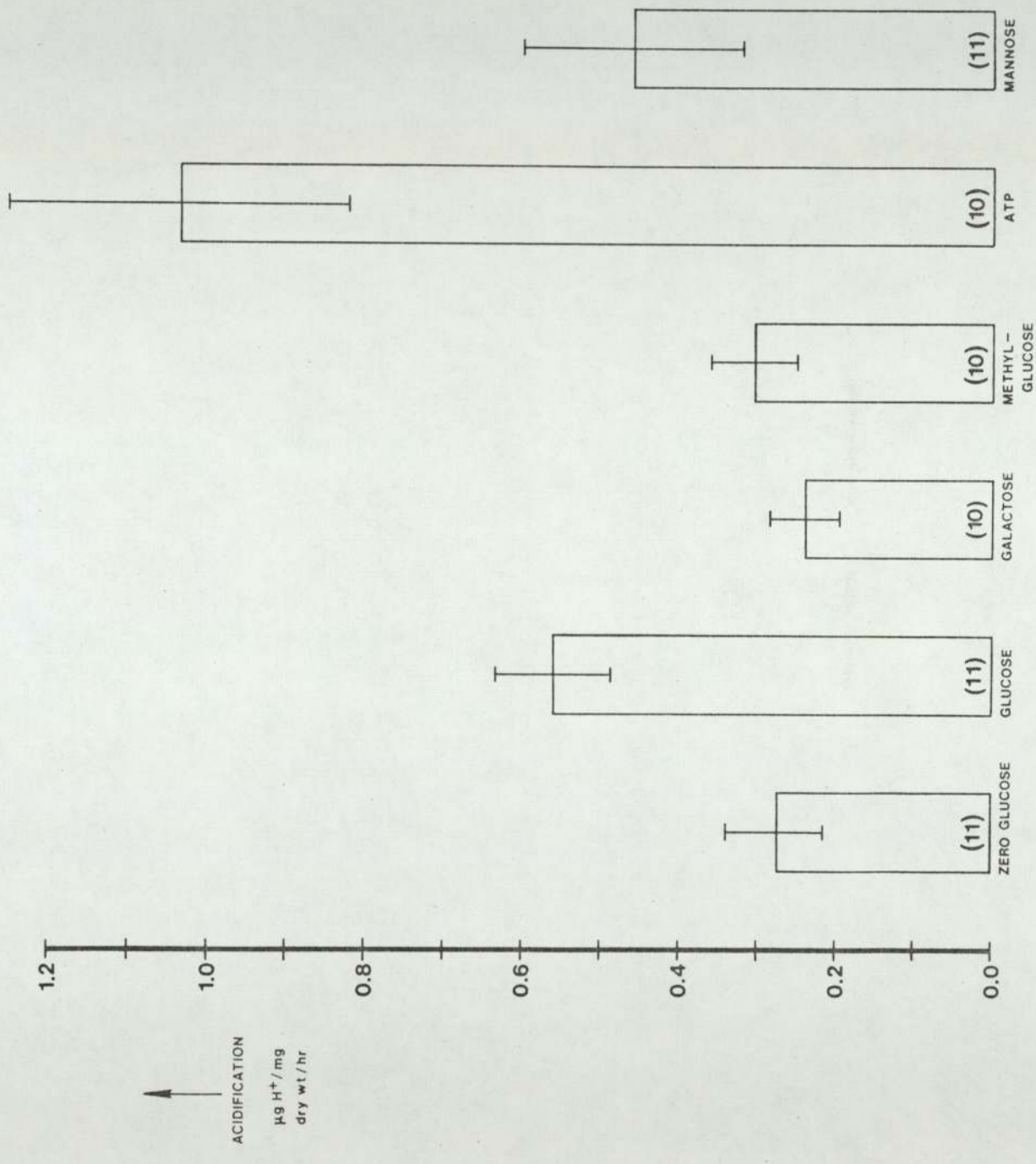
Substrate	Acidification	Lactate contribution to acidification(%)*
No glucose	0.274 ± 0.061 (11)	16.5 ± 12.0 (10)
10 mM glucose	0.563 ± 0.075 (11)	16.8 ± 11.0 (11)
10 mM galactose	0.241 ± 0.044 (10)	5.2 ± 3.0 (10)
10 mM methyl-galactose	0.307 ± 0.055 (10)	19.0 ± 7.0 (10)
10 mM ATP	1.075 ± 0.186 (10)	3.5 ± 1.3 (10)
10 mM mannose	0.511 ± 0.091 (11)	13.9 ± 6.6 (11)

* Standard errors of percentages calculated after arcsin transformation.

Statistical significance of results.

Acidification		Lactate contribution	
no glucose v mannose	$p < 0.05$		
no glucose v glucose	$p < 0.01$	ATP v. 3 methylglucose	$p < 0.05$
no glucose v ATP	$p < 0.001$		
glucose v mannose	NS		
ATP v glucose	$p < 0.02$		
glucose v galactose	$p < 0.01$		
glucose v 3 methyl-glucose	$p < 0.02$		

FIGURE 15.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
ACIDIFICATION
µg H+/mg dry wt/hr
(11)
ZERO GLUCOSE
(11)
GLUCOSE
(10)
GALACTOSE
(10)
METHYL-GLUCOSE
(10)
ATP
(11)
MANNOSE

a) Lactate and pyruvate production.

Lactate production was measured in these experiments, both the mucosal and serosal lactate production and also the mucosal pyruvate production. (see Table 28). Mucosal lactate and pyruvate production in the everted sac was about the same as the lactate and pyruvate production in the mucosal scrapes, however, the percentage contribution of these metabolites to acidification in the everted sac is very much lower.

Mucosal lactate production was significantly ($p < 0.05$) lower in everted sacs incubated with galactose and serosal lactate production, i.e. the appearance of lactate in the serosal fluid, is less ($p < 0.02$) on incubating with galactose and 3-methylglucose (see Table 28), both non-metabolised sugars. It is precisely with these sugars that acidification is reduced as well (see Table 27).

The percentage contribution of mucosal lactate and pyruvate and serosal lactate to mucosal acidification was calculated; for serosal lactate it was assumed that the serosal fluid contained an anion and that the accompanying proton had migrated mucosally to contribute to mucosal acidification. Both pyruvate production and serosal lactate production would account for less than 4% each and as such can be neglected in the acidification phenomenon.

Mucosally, lactate production could account for a small proportion of acidification if it were produced as the undissociated form that ionised in the mucosal

TABLE 28.

The effect of various hexoses and ATP on mucosal lactate and pyruvate production and serosal lactate production in everted sacs from ether anaesthetised rats incubated in bicarbonate buffer. Results are μg/mg tissue dry weight/hour.

Substrate	Mucosal Lactate Production	Serosal Lactate Production	Mucosal Pyruvate Production
No glucose	3.48 ± 1.21 (10)	0.22 ± 0.10 (9)	0.216 ± 0.050 (10)
10 mM glucose	8.30 ± 2.40 (11)	0.70 ± 0.18 (9)	0.266 ± 0.063 (10)
10 mM galactose	2.38 ± 0.86 (11)	0.25 ± 0.17 (8)	0.390 ± 0.142 (9)
10 mM methylglucose	5.19 ± 1.75 (11)	0.26 ± 0.07 (9)	0.374 ± 0.139 (10)
10 mM ATP	3.93 ± 1.49 (11)	0.43 ± 0.16 (9)	0.210 ± 0.075 (9)
10 mM mannose	4.93 ± 1.16 (11)	0.41 ± 0.21 (8)	0.204 ± 0.059 (10)

Statistical significance.

Mucosal lactate production

glucose v. galactose $p < 0.05$

all others NS

Serosal lactate production

glucose v. methylglucose $p < 0.02$

glucose v. galactose $p < 0.02$

all others NS

TABLE 29

The effect of various hexoses on phosphate movement and sodium transport mucosally in everted sacs from ether anaesthetised rats incubated in bicarbonate buffer. Results are expressed as μg/mg tissue dry wt/hour.

Substrate	mucosal Na^+ transfer	phosphate uptake
Zero glucose	77.5 ± 25.2 (11)	- 3.42 ± 0.68 (10)
10 mM glucose	84.7 ± 23.0 (11)	- 0.10 ± 0.46 (10)
10 mM galactose	69.0 ± 22.8 (10)	0.76 ± 0.60 (9)
10 mM methylgalactose	101.0 ± 27.5 (11)	0.71 ± 0.64 (10)
10 mM ATP	133.1 ± 22.8 (10)	-
10 mM mannose	109.4 ± 29.4 (11)	0.46 ± 0.54 (8)

Statistical significance.

mucosal Na^+ transfer	phosphate uptake
ATP v galactose $p < 0.01$	All substrates v. zero glucose $p < 0.001$

solution. This lactate contribution to acidification is much the same where sacs are incubated with various hexoses; where ATP caused an acidification the mucosal lactate contribution, if it does contribute to acidification, is minimal.

b) Mucosal sodium, hexose and phosphate transfer.

The mucosal transfer of the various hexoses used in the previous experiment was measured; there was evidence of the usual selective uptake of sugars that occurs in functioning everted sacs (Cori, 1925; Wilbrandt & Laszt, 1933). Glucose was transported at a faster rate ($p < 0.01$) than mannose (see Table 30) with 3-methylglucose and galactose giving intermediate values. Although there was no direct correlation between mucosal hexose transfer and acidification, some theoretical calculations were done on the data to estimate the possible effect on acidification, if the active transport of a hexose molecule were associated with the production of one proton. It was assumed that mannose transfer represented an approximate estimate of the total passive hexose transfer. This was subtracted from the values for glucose, galactose and 3-methylglucose transfer and the percentage accountability of any stoichiometric coupling between hexose transport and acidification (see Table 31b).

Sodium uptake mucosally under these conditions shows a uniform uptake except for a slight elevation when ATP is given as substrate and a depression when galactose is present (see Table 29). These two values differ

TABLE 30

Mucosal hexose transfer in everted sacs. Units are μg/mg tissue dry weight/hour. Details as for Table 29.

Hexose Substrate	Mucosal Hexose Transfer
glucose	138.9 ± 31.3 (5)
mannose	53.0 ± 42.2 (5)
galactose	74.5 ± 30.9 (4)
3-methylglucose	99.9 ± 26.0 (5)
glucose v. mannose	$p < 0.01$

TABLE 31a.

Percentage of (H^+) production accountable solely from metabolic sources in the everted sac. See tables 27 and 28.

Substrate	Mucosal Lactate Production (%)	Serosal Lactate Production (%)	Mucosal Pyruvate Production (%)
Zero glucose	16.5 ± 12.0 (10)	0.7 ± 0.4 (8)	1.0 ± 0.3 (10)
10 mM glucose	16.8 ± 11.0 (11)	2.1 ± 0.5 (9)	0.6 ± 0.2 (10)
10 mM mannose	5.2 ± 3.0 (10)	1.6 ± 0.8 (10)	0.0 ± 0.9 (10)
10 mM galactose	19.0 ± 7.0 (10)	2.8 ± 1.2 (8)	4.02 ± 2.55 (9)
10 mM methylglucose	3.5 ± 1.3 (10)	1.5 ± 0.6 (8)	2.65 ± 1.16 (9)
10 mM ATP	13.9 ± 6.6 (10)	0.7 ± 0.2 (9)	0.2 ± 0.1 (9)

significantly ($p < 0.01$). There was no correlation between sodium transfer and acidification; however as with hexose transfer, the percentage contribution to acidification of a direct sodium hydrogen ion exchange mechanism was calculated from this data (see Table 31b). The passive transfer component was estimated from data taken from Schultz and Zalusky, (1964) on sodium movement in the presence of ouabain in rabbit ileum. This value was subtracted from the other values and then the percentage contribution of a sodium:hydrogen exchange pump to acidification (see Table 31b) was calculated.

With respect to phosphate movement all substrates led to an uptake of phosphate anion, except glucose where a moderate but insignificant loss occurred (see Table 29). In the case of acidification in the absence of glucose a significant increase in mucosal fluid phosphate content was detected ($p < 0.001$). There was no overall correlation between phosphate movement and acidification.

c) Percentage accountability data.

As previously indicated, cellular lactate production appearing mucosally could account for only approximately 15% of the acidification in all cases (see Table 31a). A tissue respiratory component from hydration of metabolic carbon dioxide was also calculated, based on the assumption that tissue Q_{O_2} values are 2.0 for sacs in the absence of substrate and 4.0 in the presence of metabolisable substrate. It was assumed that all carbon dioxide produced, assuming a respiratory

TABLE 31b.

Percentage of acidification accountable assuming stoichiometry between a) hexose transport and b) sodium transport. Data from Tables 27 and 29.

a) hexose/proton exchange.

substrate	percentage	percentage corrected for passive diffusion
glucose	136	84
mannose	73	-
galactose	165	47
methylglucose	189	87

b) sodium/proton exchange.

glucose absent	128	66
glucose	65	36
mannose	110	62
galactose	120	56
methylglucose	149	95
ATP	46	33

TABLE 31c.

Summary of possible sources of acidification and their contribution to acidification in everted sacs.

Substrate	Mucosal Lactate	Tissue Respiration	Hexose Transfer	Sodium Exchange	Total
Zero Glucose	16.5	34.0	0.0	66.0	116.5
glucose	16.8	32.0	84.0	36.0	168.8
mannose	13.9	44.0	0.0	62.0	119.9
galactose	15.2	36.0	47.0	56.0	194.2
3-methylglucose	19.0	21.0	87.0	95.0	213.0
ATP	3.5	5.0	0.0	33.0	41.5

quotient of one, formed carbonic acid which ionised in the mucosal medium. This could account for approximately one third of acidification. Stoichiometric hexose transfer or a sodium:hydrogen exchange pump, though both unlikely, could account for large amounts of acidification. The exceptional circumstance in these experiments is where ATP was given mucosally: all the above possible sources could only account for about one half of the total acidification (see Table 31c). It remained to be seen whether ATP could enter the cells and cause acidification by an intracellular action or whether ATP could provoke acidification and remain outside the intestinal tissues.

xiii) <u>The distribution of isotopic ^{14}C ATP across rat jejunum.</u>

In order to establish whether ATP could enter the everted sac preparation from the mucosal side, sacs were incubated with ^{14}C isotopic ATP. This was done at $0^{o}C$ and $37^{o}C$, the incubation at $0^{o}C$ providing a control comprising of errors due to carry-over of label, contamination on homogenisation and simple diffusion into the extracellular space. There was no significant difference (see Table 32) in the serosal concentration of ATP attained, but a difference did exist ($p < 0.025$) between ATP levels present in the homogenates of sacs incubated at the two temperatures. Little or no ATP was seen to penetrate into either the sac tissues or the serosal fluid as can be seen by the small percentage of total mucosal ATP present in these sac compartments. Label

did appear in the serosal fluid but this appearance was dependent on physical factors, e.g. diffusion, etc. and not on biological processes. The level of ATP present in the homogenates incubated at 37°C probably represents the uptake of ribose from the degraded ATP molecule since the isotope label was in the sugar ring. This uptake of label is probably similar to the way that adenosine rather than ATP is taken up by smooth muscle (Axelsson & Holmberg, 1968). Since no appreciable amounts of ATP entered the intestinal tissues, any acidification that occurs on incubating sacs with medium containing ATP, must be an external process due to surface hydrolysis of ATP by the epithelial cells.

The hydrolysis of ATP in solution.

In experiments involving mucosal ATP as a substrate there is the possibility that hydrolysis of ATP will occur forming ADP and an acidic phosphate group. Blank flasks containing no tissue were put through the entire incubation procedure to determine whether ATP at the high concentrations used, would cause acidification due to hydrolylic cleavage occurring in Krebs-Ringer alone.

Adding ATP to Krebs-bicarbonate buffer causes the pH to change to approximately 6.90. If these flasks (see Table 33) are incubated a significantly greater loss of carbon dioxide occurs from flasks containing ATP because of the differences in initial pH. If flasks containing

TABLE 32.

The distribution of ^{14}C - isotopically labelled 10 mM ATP (specific activity 10μCi/mmole) across rat jejunum at two temperatures. Sacs from ether anaesthetised animals and incubated in continually gassed bicarbonate for 30 min. Results expressed as mean and standard error of mean (number of animals).

Concentration data

Incubation temperature	Mucosal ATP concentration (mg/ml)	Serosal ATP concentration (mg/ml)	Homogenate ATP concentration (mg/ml/tissue water)
37°C	5.55 ± 0.57 (5)	0.90 ± 0.20 (5)	3.10 ± 0.82 (5)
0°C	5.55 ± 0.94 (5)	0.50 ± 0.40 (5)	1.41 ± 0.18 (5)

Total activity

Incubation temperature	Total mucosal ATP (mg)	Total serosal ATP (mg)	Homogenate ATP (mg)	Serosal as % mucosal	Homogenate as % mucosal
37°C	55.5 ± 5.7 (5)	0.18 ± 0.04 (5)	0.53 ± 0.14 (5)	0.34	0.96
0°C	55.5 ± 9.4 (5)	0.10 ± 0.08 (5)	0.24 ± 0.03 (5)	0.19	0.43

Statistical significance

serosal values	p is NS
homogenate values	$p < 0.025$

TABLE 33

10 mM ATP and pH shifts in flasks containing Krebs-bicarbonate buffer alone on incubation. Units are apparent loss in H^+ g/l x 10^{-4} calculated from pH values.

	1) buffer alone	2) buffer + 10m ATP	significance.
pH unadjusted	- 2.47 $\pm$ 0.19 (7)	- 3.64 $\pm$ 0.48 (7)	$p < 0.05$
pH adjusted	- 2.21 $\pm$ 0.18 (12)	- 2.21 $\pm$ 0.19 (12)	NS.

ATP have their pH readjusted with dilute KOH back to 7.4, there is no difference in pH changes between the flasks. Despite the possibility that some ATP is hydrolysed on adjusting the pH, this procedure of readjusting the pH of ATP-containing buffer was adopted.

The chelating ability of ATP.

Mucosal ATP (10 mM) has been shown to increase acidification (see Table 27) and demonstrated not to enter intestinal tissues (see Table 32). A possible effect of ATP might be a surface phenomenon such as chelating calcium ion from epithelial cell membranes and causing general increased permeability. In this way ATP could have an effect on acidification without entering tissues. To test this, the chelating ability of ATP was measured against an EDTA standard. An equimolar amount of ATP was shown to have 16% of the chelating ability of EDTA, however, since EDTA subsequently proved to be ineffectual in provoking acidification (see Table 15) the action of ATP is not via its chelating ability.

xiv) Acidification in 'in vivo' perfused loops.

Acidification was measured also in 'in vivo' loops where there can be little question of inadequate oxygenation and poor viability since 'in vivo' loops possess intact and functional blood-vascular systems. The cannulated segment of gut, ligated at the duodenum and consisting of the most proximal portion of the jejunum,

was perfused with double-distilled water whose pH had been readjusted to about 7.2. Double-distilled water was used instead of buffer medium as it could be argued that acidification was merely the consequence of incubation in buffers with subsequent transport of a buffering anion. Double-distilled water was not used in 'in vitro' experiments because it is a very much inferior medium and would quickly lead to tissue disintegration. With distilled water 'in vivo' there was initially little buffering power compared with physiological buffers; however, due to exorption of plasma solutes (Vogel, Passman & Meyering, 1970; Vogel, Stoeckert & Meyering, 1971) there was a significant increase in buffering ability over the incubation period. The use of double-distilled water containing minimal quantities of anions meant that the possibility existed of identifying any anions that might appear mucosally and be associated with acidification. The use of solutions containing anions, e.g. chloride to maintain osmolarity was precluded since any accompanying anion associated stoichiometrically with acidification would be at the same low concentration levels as the hydrogen ion and difficult to measure against a background of high concentration of that anion.

As in the everted sacs, in vitro muscle sacs, mucosal scrapes and segments, there was a significant acidification occurring in vivo in the proximal jejunum. Despite an increase in perfusate buffering power over the incubation period, little acidification was needed to shift the mucosal perfusion solution pH. The high rates

TABLE 34

Acidification and other ion movements in 'in vivo' perfused loops containing double-distilled water.

Acidification	mucosal Na^+ transfer	mucosal P transfer	mucosal Cl^- transfer
0.084 ± 0.002 (10)	112.70 ± 5.10 (10)	0.050 ± 0.006 (10)	0.021 ± 0.04 (10)

lactate production	lactate contribution to acidification (arcsin transformed)
0.020 ± 0.007 (10)	15.00 ± 7.50%

units are µg/mg tissue dry wt/hr.

of acidification 'in vitro' by everted sacs are a reflection of the buffering capacity of bicarbonate and phosphate medium. The contamination of jejunal perfusate by blood leaking out from the cut ends of the loops was measured, since this could be a source of extraneous anions etc. There was less than 0.5% volume contamination of perfusate by blood; however, as blood pH is a constant 7.4 this would not cause acidification.

Lactate production (see Table 34) was also measured; though this could have arisen from blood contamination there was no correlation between volume % blood contamination and amount of lactate present. A more probable explanation is that the lactate is either present in the plasma exsorbate or produced by intestinal metabolism. In all events the possible lactate contribution to acidification is only some 15%.

Movement of sodium, phosphate and chloride into the mucosal perfusate was also measured (see Table 34). Neither the appearance of sodium, chloride or phosphate could be correlated with the volume percentage contamination by blood. The movement of these inorganic ions from serosal to mucosal fluid contrasts with their movement in the everted sac preparation (see Table 29) which is from mucosal to serosal surface. No direct correlation could be found between movement of these ions and acidification. Furthermore, the amounts of these ions that are transferred would seem to preclude any direct stoichiometric relationship with acidification.

The 'in vivo' experiments also demonstrate that acidification is not just an artefactual occurrence dependent on intestinal tissues being incubated in physiological salines. Mucosal acidification is a naturally occurring phenomenon, apparently uncorrelated with sodium, phosphate and chloride movement, and has a possible very small component arising from the intracellular production of lactic acid.

DISCUSSION.

General Observations on Acidification.

From the previously described experiments, it must be concluded that proximal rat jejunum will acidify the suspension medium that it is incubated in, both in the presence and absence of available glucose. This effect was demonstrated directly with a glass electrode (and not calculated indirectly from the distributions of carbon dioxide and bicarbonate across the jejunum). This was preferred to other available indirect methods, e.g. distribution of bicarbonate and carbon dioxide or of DMO, since all of these substances are transported across the jejunum (Powell et al., 1971; Kleinzeller, Kostyuk, Kotyk & Lev, 1971) and the interpretation of results from experiments involving DMO would seem to be problematical. (Paillard, 1971). The acidification process has been shown in vitro in everted sacs, segments and mucosal scrapes, in alternative buffers and also in vivo in the absence of glucose, and confirms the work of many previous authors (Parsons, 1956; Wilson & Kazyak, 1957).

Acidification in the jejunum continues to occur in vitro despite initial ether anaethesia during the preparation of everted sacs. Ether anaesthesia would tend to reduce any adrenalin-mediated partial anoxia of the intestine in vivo (Robinson, Antonioli & Mirkovitch, 1966), that might be caused whilst handling the animals. Anoxic conditions brought about in vitro by incubating everted sacs in an atmosphere of nitrogen and carbon

dioxide reduced acidification by nearly half, yet significant acidification does persist under these anoxic conditions. Furthermore, phlorrhizin and dinitrophenol at concentrations affecting oxidative metabolism, also reduce acidification. Since acidification also occurs in intestinal loops in vivo with an intact and functioning blood supply, it is extremely unlikely that acidification represents some autolytic or anoxic phenomenon.

Calculations involving the relationship between oxygen uptake and acidification, both in the presence and absence of glucose, show that acidification does not seem to be related to the electron transfer chain as required by the 'redox' theory of gastric acidification (Davies, 1951). In the absence of glucose, the ratio between hydrogen ions produced mucosally and oxygen molecules taken up during respiration is within the 'redox' limits as dictated by the overall equation: -

$$4\ H + 2\ H_2O + O_2 \longrightarrow 4\ H^+ + 4\ OH^-$$

In the presence of external glucose, the theoretical limiting value of four protons per molecule of oxygen is exceeded, although only by 10%. The proposed redox mechanism would seem to be an unlikely basis for acidification on theoretical grounds.

The concept of acidification being solely due to the hydration of metabolic carbon dioxide produced by the tricarboxylic acid cycle can also be defaulted on similar

grounds. Assuming the respiratory quotient of the jejunum to be approximately unity, the total carbon dioxide produced, if it were all hydrated, would only account for about one quarter of the acidification seen. Certainly, the assumption of the respiratory quotient is questionable but its value is hardly likely to be greater than unity, since tissues from starved rats are more likely to be gluconeogenic and have a respiratory quotient of about 0.7. In this case, the carbon dioxide component to acidification would be even less than one quarter.

It might be argued that acidification is a phenomenon consequent on the passive redistribution of ions, including protons due to a Gibbs-Donnan equilibrium or a realignment as dictated by the transepithelial or the transjejunal Nernst potential. The bulk of the evidence on proton permeability is overwhelmingly in favour of biological membranes being impermeable to protons, which would seem to rule out passive movement of hydrogen ions ab initio. In terms of the direction of the potential across the transepithelial cell (Wright, 1966) hydrogen ions would tend to diffuse into the cell since the inside of the cell is negative to the outside and would passively attract protons into the cell. Also in this case, for a Gibbs-Donnan or Nernst equilibrium to occur, there would have to be a transepithelial cell potential of about 25 mV to achieve the acidification seen, if it is assumed that jejunal tissues have an internal pH of 7 and are

incubated in solutions of pH 7.4. The transmural potential in the proximal jejunum in the absence of glucose is 0.5 mV and in the presence of glucose the maximally evoked potential is usually about 6 mV (Barry, Dikstein, Matthews, Smyth & Wright, 1964). From these recorded potentials, it can be seen that acidification far exceeds that predicted by the NERNST equation, if it is assumed that the intracellular pH is neutral. The actual hydrogen ion production in the absence of glucose is estimated at approximately 0.2 micrograms of hydrogen ion per milligram of tissue dry weight, which in the case of a thirty milligram sac means some fifteen micrograms total per ten millilitres of buffer. This represents an increase in concentration of the order of 0.6 milligrams of hydrogen ion per litre, i.e. enough to cause a change in pH to about pH 4 in an unbuffered solution. Passive realignment clearly could not account for the changes in pH seen. Also in the case of buffered mucosal solutions, which would act as a proton sink, even a small change in pH from pH 7.4 to 7.2 which means a change in the intracellular to extracellular concentration ratio of two, would require a potential of some twenty millivolts which is not seen in this region of the intestine. Moreover, simple calculations show that insufficient hydrogen ion is present in the tissues in the everted sac preparation. If it is assumed that the average size of the everted sac is about 3.5 cm long, 1.5 cm wide and about 0.3 cm. thick, its fluid volume can be estimated at about 1.6

millilitres. If this volume of tissue were all fluid, it would contain at neutral intracellular pH approximately 1.6×10^{-8} milligrams of hydrogen ion. This is very much less than the total amount of hydrogen ion needed (40×10^{-8} milligrams) to lower the pH of ten millilitres of buffering fluid from pH 7.4 to pH 7.0. It must be assumed that the jejunal tissues have either very great buffering powers since their hydrogen ion content would be almost depleted and the tissues would become extremely alkaline or that some 'de novo' synthesis of hydrogen ion has occurred, either at the mucosal borders of the cell or intracellularly. Since mechanistically it is possible that variations in the level of acidity are produced by some sort of bicarbonate shuttle, as has been proposed for the turtle bladder (Schilb & Brodsky, 1972), 'de novo' synthesis of protons would also have to incorporate the concept of the secretion of hydrated carbon dioxide which as previously noted would seem an unlikely source of acidification because it could not account for all the acidification seen.

Starvation causes a decline in the rate of acidification, although this effect was never demonstrated to be statistically significant; this effect recalls McRobert's observations (1928) that on starvation the normally acid pH of the proximal jejunal lumen moves nearer to neutral and often to alkaline values. This decline of acidification on starvation points to the

metabolic dependency of the phenomenon, since metabolism (Newey et al., 1970) and glycolysis (Srivastava & Huebscher, 1966) decline on starvation when corrected to the same dry weight index as acidification. This is in contrast to transport rates calculated on a weight basis (Esposito et al., 1967; Madge, 1970) which increase implicates metabolic rather than transport processes as the cause of acidification.

Acidification also varies with position along the jejunum, irrespective of surface area corrections. Values, both in the presence and absence of glucose, tend to a maximal value which occurs at a position approximately fifteen centimetres distal to the pyloric sphincter. This diminution in the amount of hydrogen ion secretion in the region immediately distal to the first duodenal loop might be caused by the secretions of the Brunners glands, which continue as far as the duodenal-jejunal flexure and secrete an alkaline, bicarbonate-containing fluid (Florey & Harding, 1934). Past the duodenal-jejunal flexure, the lower limit of distribution for the Brunners glands, (Bensley, 1903) acidification reaches a maximum and then declines and it is possible that up to this point, acidification is masked by the bicarbonate secretion of the glands. Various other intestinal processes have been shown to decline distally, notably alkaline phosphatase activity (Jervis, 1963; Lafont & Morretti, 1970; Harrison & Webster, 1972), lactate production (Wilson & Wiseman, 1954; Sherratt, 1968),

oxygen uptake (Sherratt, 1968; Bamford & Holmes, 1971), and levels of both ATP hydrolytic activity and ATP tissue content (Hanninen, Hartiala & Nusuikko, 1964). Some transport processes are also maximal in the proximal jejunum e.g. water, sodium and chloride movement (Schedl, Miller & Wilson, 1968). This is in complete contrast to some other transport processes that are not maximal in this region, e.g. glucose whose transport characteristics are minimal in the distal jejunum and in the proximal ileum (Barry, Matthews & Smyth, 1961; Sayeed, McNally & Fallot, 1963).

The capacity of this region of the intestinal tract to acidify would seem to be related to the general metabolic tone of the tissues and could easily be reconciled into a system for the maintenance of jejunal luminal pH by way of acidification. This region of the intestine is sensitive to changes in the luminal pH and it has been demonstrated in humans that acid in the duodenum will stimulate the production of a bicarbonate-containing neutralising fluid secreted via a saturable system, in order to restore duodenal pH to its usual range (Winship, Caflish & Schultze, 1972). It is possible that acidification could be the next step in the control of jejunal pH within finer limits; acidification might regulate any duodenal overshoot and bring the pH down to slightly more acid values.

It can be concluded then that acidification is unlikely to be artifactual as demonstrated by simple

experiments involving anoxic conditions and inhibitors. Neither does it seem to be a passive alignment process consequent on the prevailing transmural potentials but on the contrary, part of a system actively functioning to maintain jejunal pH at set values and is a system very much influenced in vitro by the presence of glucose.

The Influence of Glucose on Acidification.

Although everted sacs acidify their mucosal incubation medium in the absence of glucose (an effect not previously demonstrated in vitro), the inclusion of glucose in the buffer causes significant increases in the acidification of the medium. Acidification shows a glucose-dependency that reaches a saturable level at about 100 mM mucosal glucose. There are two distinct ways in which glucose might cause increases in acidification. The increases might be a consequence of some glucose active transport process or of an active transport mechanism stimulated by glucose, involving the expenditure of metabolic energy. Alternatively, glucose might cross the tissue membranes and cause increases in acidification by way of intracellular metabolism.

Such data as can be derived from the glucose-dependency curve suggests that acidification is not a phenomenon dependent on the active transport of glucose. Acidification reaches a maximal value at approximately 100 mM mucosal glucose concentration whereas glucose transport has been shown to continue in a linear fashion

past this concentration and is saturable at far higher glucose concentrations (Atkinson, Parsons & Smyth, 1957). It is at precisely the same concentration that evokes the maximal acidification response that maximal lactate production has been shown to occur (Atkinson et al., 1957). Since the glucose-dependency curve has been shown to resemble Michaelis-Menten kinetics, V_{max} and Km data were derived, bearing in mind that the estimation of such data is a procedure which must be viewed with some circumspection unless certain conditions are fulfilled (Smyth, 1971). Although the glucose-dependency data are better described by an exponential relationship, the Michaelis-Menten kinetic data derived show that the Km value is very much less than that recorded for glucose transport in vitro (Fisher & Parsons, 1953; Crane, 1960) and in vivo (Foerster et al., 1972). The Km value resembles those found in the glycolytic sequence of reactions rather than that of glucose transport or oxidative metabolism and most resembles the Km characteristic of pyruvate kinase (Reynard, 1961). It is conceivable therefore that acidification is stoichiometrically related to glycolysis at the glycolytic step presided over by the enzyme pyruvate kinase, whose reaction proceeds: -

phosphoenolpyruvate + MgADP ---> pyruvate + MgATP

This is the first step in the glycolytic sequence where a net production of ATP occurs and this then might become

available for acidification. The calculated Km value is also characteristic for some ATPases (Sachs, Mitch, & Hirschowitz, 1965) and interestingly enough is the same as the 'apparent' Km found for the transient response of the transmural potential in the rat jejunum when exposed to ATP (Kohn Newey & Smyth, 1970). It is interesting to speculate that if glucose were responsible for making available more ATP for the acidification process at the cell surface, then this might explain the resemblance of the acidification Km value to those of ATP hydrolysis and also explain why the process was better explained by some exponential relationship. Since surface hydrolysis of ATP would produce protons that would then subsequently diffuse away, the rate of diffusion away from the site of production would be a process exponentially dependent on the rate of production of ATP from the glucose present. Whether this is the case or not, it must be concluded that the derived Km data, if valid, indicate the metabolic rather than the transport nature of the influence of glucose.

Two main theories have been advanced to explain active transport of glucose, Wilbrandt and Laszt's theory (1933) of phosphorylation with the subsequent dephosphorylation of glucose on transfer across the jejunal wall; the alternative theory is that of a link between glucose transport and active sodium transfer proposed by Crane (1966), called the sodium-gradient theory. Acidification would occur if glucose were to be initially phosphorylated

at the brush border of the intestinal epithelial cell with the phosphorylated glucose intermediate moving into the cell and the products of the phosphorylation process remaining mucosally. Alternatively acidification would occur if some obligatory cation exchange of a proton for a sodium ion took place during the sodium-dependent glucose transport. Although experimental evidence has been brought forward against both the phosphorylation theory (Jervis, Johnson, Sheff & Smyth, 1956; Sols, 1956; Landau & Wilson, 1959; Diedrich & Anderson, 1963; Nunn, Sayeed, McNally & Fallot, 1963; Leese & Bronk, 1972) and against the sodium-gradient theory (Kimmich, 1970; Jordana & Igea, 1971; Burdett & Schneider, 1971; Foerster & Hoos, 1972; Foerster & Menzel, 1972; Saltzman, Rector & Fordtran, 1972) as being unlikely mechanisms for active glucose transport, it is possible that the active transport of glucose could provide protons in a hitherto unspecified way. The fact remains that glucose is transported seemingly chemically unaltered (Taylor & Langdon, 1956; Hawkins & Willis, 1957) by a phlorrhizin-sensitive process (Parsons, Smyth & Taylor, 1958) located at the epithelial cell brush border (McDougal, Little & Crane, 1960), and as such could implicate both the phosphorylation-dephosphorylation theory and the sodium gradient theory both of which are further considered below.

Acidification and the movement of sodium.

According to the sodium-gradient hypothesis (Crane, 1966), the active transport of certain hexoses

involves the obligatory simultaneous movement of sodium and hexose in the same direction via a common carrier. The serosal sodium pump causes a flow of sodium into the cell passively down its concentration gradient from the mucosal surface; via a common glucose-sodium carrier, glucose is transported into the cell against its own concentration gradient. It is feasible that the increase in acidification seen on incubating rat jejunum with mucosal glucose, could result from the stimulation of a compulsory sodium:hydrogen ion exchange during increased glucose transfer. Alternatively, it is possible that glucose transfer could stimulate the sodium pump in a way unrelated to Crane's hypothesis but still be linked stoichiometrically to sodium movement, if sodium:hydrogen exchange occurs.

It has been established that transmural potential differences exist in the rat jejunum with the serosal side being positive to the mucosal surface (Sawadwa & Asano, 1963; Barry et al., 1964). Since Ussing's early work it has been generally assumed that potentials are caused solely, or at least mainly, by the active transfer of sodium. The enzyme usually associated with active transfer of sodium, i.e. Na^+/K^+ATPase, has been demonstrated in guinea pig (Taylor, 1962) and rat intestinal epithelium (Quiggley & Gotterer, 1969) and furthermore has been shown to be predominantly a jejunal enyzyme (Gnanaprakasam & Srivastava, 1972). Also in keeping with the accepted model of sodium transfer (Wilson, 1962) the enzyme is

thought to be located at the serosal surface (Fujita, Matsui, Nagano & Nakao, 1971). It is assumed to provide the enzymatic basis for active sodium transport in a wide variety of tissues although this concept has been questioned in the case of the rat jejunal mucosa (Robinson, 1970) and has been characterised with respect to ouabain inhibition (Berg & Szekerczes, 1966; Leopold, Furukawa & Forth & Rummel, 1970). However, a formal equivalence between the transmural potential and sodium transport, and the simultaneous inhibition of both by ouabain has only as yet been shown in the rabbit ileum (Schultz & Zalusky, 1964) together with one report that ouabain delays sodium transfer in vivo in the rat gut (Van rees, Woodbury & Noach, 1969).

Nevertheless, some insight into the relationship between acidification and the active transfer of glucose can be obtained from a consideration of the transmural potential. Mucosal glucose will both increase the transmural potential and the net sodium flux in the rat gut (Barry et al., 1964). Glucose in the presence of mucosal phlorrhizin, which will inhibit the active component of glucose transfer (Parsons, Smyth & Taylor, 1958; Newey, Parsons & Smyth, 1959) produces an increase in the sodium and fluid transfer with no effect on the transmural potential, a similar effect is seen with mucosal mannose, a hexose that is not apparently actively transported (Barry, Eggenton & Smyth, 1969). This would seem to indicate that the increase in transmural potential observed when jejunal

sacs are incubated with glucose, is closely associated with the active transfer of glucose but not with the increased transfer of sodium. Galactose in contrast produces elevations in the transmural potential with no change in sodium transfer. (Barry et al., 1969). Although these authors postulated the existence of two sodium pumps, an electrogenic pump stimulated by hexose transfer and a non-electrogenic pump stimulated by metabolism, it might be argued that although hexose transfer seems to stimulate some kind of electrogenic event, as judged by the increase in potentials, it is not clear that this is due to a sodium pump. Galactose has been shown not to produce any significant increase in acidification. Although galactose is reported to cause damage to intestinal tissues (Perry, Moore, Thomas & Hird, 1956; Casey & Felber, 1967), methylglucose, a hexose which will also cause increases in the transmural potential (Barry et al., 1964) also caused no significant increases in acidification. It seems unlikely, therefore, that acidification is caused by either active hexose transfer per se or by the electrogenic movement of sodium ions stimulated by hexose transfer.

Acidification was shown to be increased by the presence of serosal mannose and mucosal fructose. There is some evidence that fructose is transported actively in the rat intestine (Gracey, Burke & Oshin, 1970; Guy & Deren, 1971): active transport of mannose, thought to occur in the toad intestine (Csaky & Ho, 1966) has never

been demonstrated in the rat intestine although mannose transfer is retarded by iodoacetate (Wilbrandt & Laszt, 1933). Serosal mannose will cause increased sodium and water movement without significantly altering transmural potential (Barry et al., 1969) and similarly fructose also has no effect on transmural potential (Barry et al., 1964) which is always associated with active hexose transfer (Barry et al., 1969). The action of these sugars on potential and acidification confirms that acidification is not related to active hexose transfer or to the transfer-stimulated electrogenic movement of sodium ion. The possibility still remains that acidification could be connected with the neutral active movement of sodium ion, which is stimulated by hexose metabolism.

It is well known that ouabain, the cardiac glycoside that inhibits intestinal epithelial cell Na^+/K^+ATPase whilst leaving residual ATPase unaffected (Berg & Szekerczes, 1966; Leopold et al., 1971), also reduces the transmural potential difference (Sawada & Asano, 1963; Barry et al., 1964;). However, no evidence exists to show that ouabain actually causes a reduction in the amount of sodium ion transferred in the rat jejunum. Ouabain has even been shown to cause an increase in the transmural potential in the presence of glucose (Sawada & Asano, 1963). Ouabain is said to be effective from the side to which sodium is pumped (Dunham & Glynn, 1961), consequently ouabain was administered serosally at a concentration of 1 mM which will cause

90% inhibition of rat intestinal Na^+/K^+ATPase (Berg & Szekerczes, 1966) and had no effect on acidification.

It is possible that Na^+K^+ATPase activity is present in the apical brush border (Berg & Chapman, 1965; Quiggley & Gotterer, 1969). The administration of 10 mM ouabain mucosally was also without effect on the acidification process. Even from this surface at least 20% of the ouabain ought to reach the serosal surface as glycoside transport in rat gut is a passive non-saturable process (Greenberger, MacDermott, Martin & Dutta, 1969). This is still sufficient to cause approximately 75% inhibition of the Na^+/K^+ATPase activity. It is interesting to note that EDTA is also a potent inhibitor of rat intestinal Na^+/K^+ATPase activity, causing some 90% inhibition of the enzyme (Berg & Szekerczes, 1966) and also has no effect on the acidification phenomenon. In the absence of evidence implicating ouabain as inhibiting only the electrogenic movement of sodium, it can only be assumed that acidification is not associated with the active transfer of sodium. Since ouabain reduces carbon dioxide production from glucose and by implication oxidative metabolism in rat diaphragm (Torben, 1966) and further evidence shows that ouabain affects sodium transfer only at concentrations interfering with glucose metabolism (Robinson, 1970), the conclusions to be drawn from ouabain inhibition could be equivocal. Certainly there is no correlation in the net fluxes of sodium and hydrogen ions since in the everted sacs sodium ions move serosally, but in vivo loops perfused with

double-distilled water, sodium movement was in the same direction as acidification. There remains the possibility that subsequent ion movements dependent on other forces, obscure any initial relationship between the two ions. If it is assumed that there is a direct stoichiometric relation between mucosal equivalents of sodium ion transferred and equivalents of hydrogen ion appearing, it can be calculated that unless corrected for the amount of sodium ion passively transferred by diffusion, if tight coupling were to occur, more acidification ought to be seen. When corrected for passive diffusion, in some cases, e.g. in the presence of mucosal ATP, not all the acidification could be accounted for. It would seem unlikely, therefore, that acidification involves obligatory cationic exchange in a scheme involving hexose transfer or in the active transport mechanism for sodium. This is even more probable if ouabain can be shown to inhibit all sodium transport and not just electrogenic sodium movement.

Acidification and the Phosphorylation Theory.

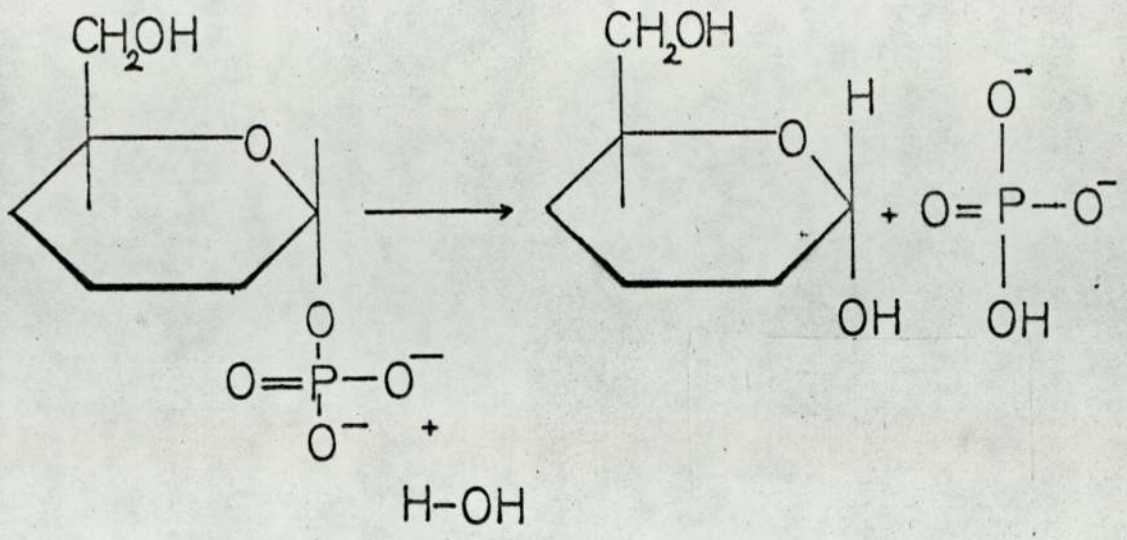
According to the phosphorylation-dephosphorylation theory, which is thought to account for hexose transport in bacteria (Gachelin & Kepes, 1970) but not in fungi (Brown & Romano, 1969), hexose transfer in the gut might be achieved by the mucosal phosphorylation of the hexose molecule at the brush border with subsequent dephosphorylation at a subsequent site in the epithelial

cell, possibly the cellular side of the brush border. By implication this would involve phosphorylating enzymes capable of phosphorylating hexoses, e.g. hexokinases and implies also the subsequent action of phosphohydrolytic enzymes capable of hydrolysing hexose monophosphate esters, e.g. acid phosphatase, alkaline phosphatase, glucose-6-phosphatase.

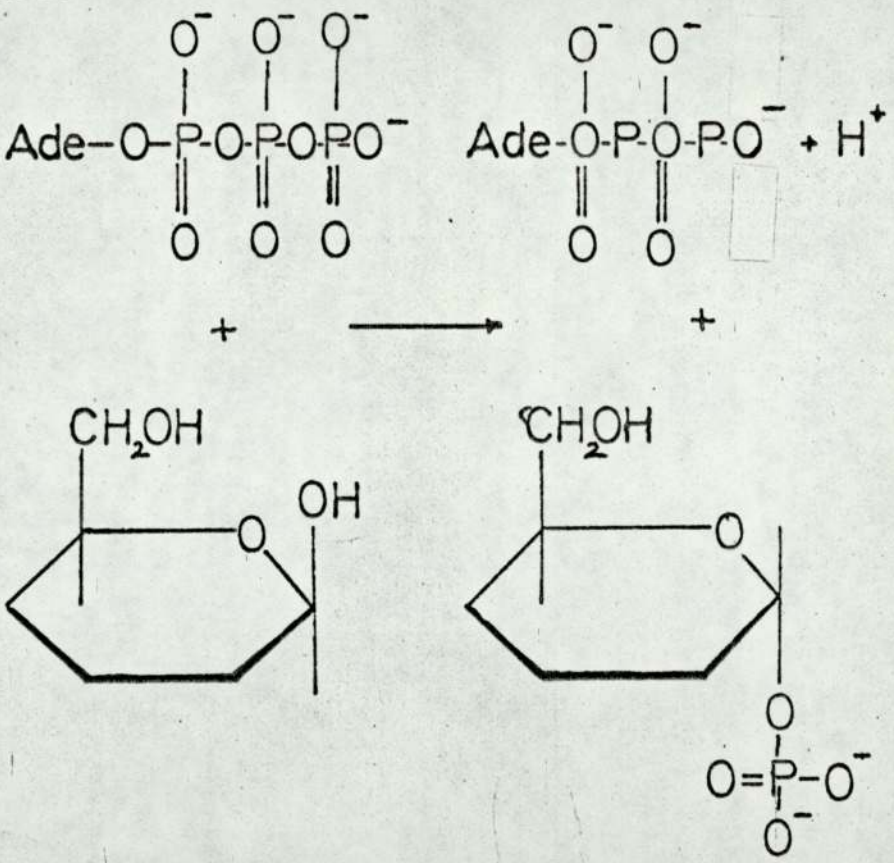
Previous authors have noted a parallel between the rates of absorption of various sugars and their ability to be phosphorylated (Hele, 1950; Bissegger & Laszt, 1951) and also that a uniform rate of dephosphorylation of the phosphates of glucose, galactose, fructose and mannose exists (Hele, 1953a; 1953b). One would not expect proton production during the hydrolysis of hexose phosphates because hydrolysis involves the transference of an ionisable hydroxyl group to a carbon atom in a position precluding subsequent ionisation (see Diagram 2).

This is verified experimentally by the fact that neither the inclusion of glucose-1-phosphate nor glucose-6-phosphate in the mucosal medium causes an appreciable increase in the acidification process. The slight increase that occurs on incubation with glucose-6-phosphate was probably owing to the hydrolysis of the monophosphate to the original sugar and then that having an effect just as glucose does. Pyridoxal phosphate, another phosphate compound, when given mucosally also fails to increase acidification. Further circumstantial evidence can be derived from the fact that the inhibitors

DIAG. 2



DIAG. 3



i.e. ATP^{4-} + Glucose → ADP^{3-} + H^{+} + Glucose-P^{2-}

of alkaline phosphatase and glucose-6-phosphatase had no effect on acidification. L-β-phenylalanine had no effect on endogenous acidification in the absence of glucose, even at toxic concentrations where water transport and oxygen uptake were significantly diminished. This precluded a role for alkaline phosphatase and also the absence of effect of EDTA on endogenous acidification precluded a role for glucose-6-phosphatase. It seems unlikely, therefore, that endogenous acidification in the absence of glucose involves the enzymic hydrolysis of phosphate compounds.

Despite circumstantial evidence to the contrary (Eiler et al., 1940; Jervis, Johnson, Sheff & Smyth, 1956; Nunn et al, 1963; Feher, 1966), the demonstration that only 9% of transported glucose passes through the intracellular pool (Landau & Wilson, 1959) and the lack of suitable phosphatases and enzymes that would act at the carbon-2 position of the 'Crane specific' sugars, it is still possible that phosphorylation could cause the selective absorption of glucose as in bacteria (Gachelin & Kepes, 1970) and cause acidification. The phosphorylation of hexoses would cause proton production in that a hydroxyl molecule group on the sugar molecule would be attacked and a proton associated with a carbon atom in an unionised context, would become associated with a phosphorus atom and would be free to ionise, (see Diagram 3). This has been verified experimentally in studies involving the use of Ehrlich Ascites tumour

cells, in which 2 deoxyglucose, a sugar that can be phosphorylated but not metabolised, will cause a fall in intracellular pH, even when glycolysis is blocked with oxamate or iodoacetic acid (Poole & Butler, 1969). Furthermore, in the Ehrlich Ascites tumour cells, the metabolisable sugars glucose, mannose and fructose, the same metabolisable sugars that have been used in the present studies, will cause a fall in external pH with only a slight fall in intracellular pH, in contrast to deoxyglucose. This means that phosphorylation definitely does not cause the changes in external pH since deoxyglucose, 3 O-methylglucose and galactose will not cause changes in external pH, yet can be phosphorylated (Poole, 1967).

As regards the jejunum, mannose is a sugar that can be phosphorylated but not actively transported (Crane & Krane, 1956) and will cause acidification. Methyl-glucose and galactose, both sugars that are actively transported (Csaky, 1943; Campbell & Davson, 1948; Fisher & Parsons, 1953), did not lead to increases in acidification. It must be postulated, as with the Ehrlich Ascites tumour cels that a phosphorylation mechanism to either transport a hexose molecule or initiate the hexose molecule into the glycolytic sequence, will not cause acidification of the external incubation medium. Furthermore, only the metabolisable sugars are capable of doing this. Evidence exists that galactose may interfere with normal intestinal function (Perry et al, 1956; Casey &

Felber, 1967) and may retard normal intracellular metabolism (Diedrich & Anderson, 1960) although none so far for the other actively transported but unmetabolisable sugar. It is therefore improbable that acidification occurs as a consequence of the phosphorylation of hexoses in the context of a transport mechanism and it is also unlikely that a phosphorylation mechanism is the cause of selective sugar absorption. In all events, acidification is unconnected with the hexose active transport mechanism whether caused by phosphorylation or in accordance with the 'sodium gradient' hypothesis.

Acidification and Hexose Metabolism.

A definite correlation exists between acidification and phosphorylation in that sugars which can be phosphorylated by hexokinases will cause increased acidification whereas sugars which are not able to be phosphorylated do not cause acidification. Glucose, fructose and mannose are all hexoses that can be phosphorylated (Hel, 1953; Sols, 1956), and hence can be metabolised: in contrast, galactose and methylglucose are hexoses which cannot be phosphorylated (Sols, 1956; Csaky & Wilson, 1956) or metabolised. Galactose-1-phosphate has been demonstrated in rat gut during galactose absorption (Diedrich & Anderson, 1960) and reports of limited metabolism do exist for galactose (Simon, Pesch & Topper, 1959) but the two sugars are thought to be unmetabolisable. More recently it has been shown that

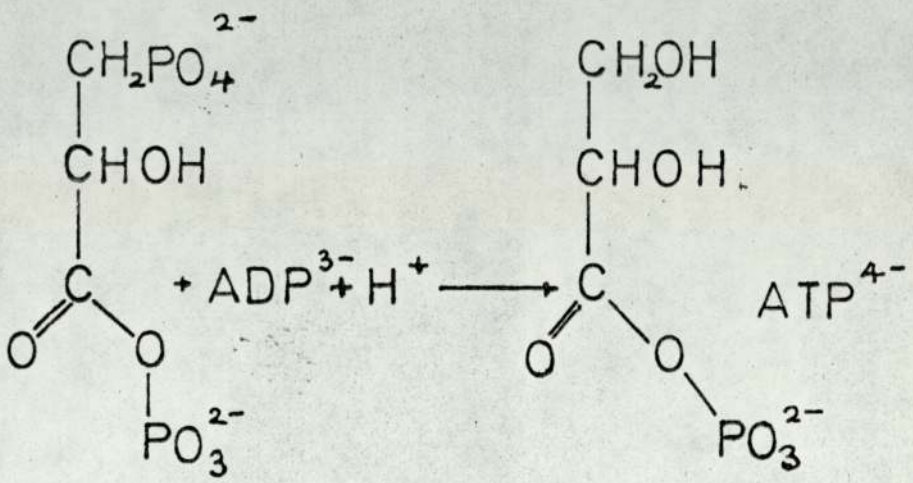
on incubation with 28 mM galactose, the tissue levels of ATP in rat jejunum are very much reduced, implying that the accumulated galactose-1-phosphate rather than being metabolised to a small degree (Simon et al., 1959), in fact blocks the production of ATP and can be thought of as being unmetabolisable. The metabolisable sugars are the ones that give rise to acidification and, in contrast, the unmetabolisable sugars do not give rise to acidification. Whether or not the unmetabolised sugars are phosphorylated, when they are included in the incubation medium, there is significantly less serosal lactate production and by inference, less overall metabolism than is the case when glucose is included in the incubation medium.

As noted previously, data from the glucose-dependency curve seem to implicate among other possible enzymic candidates, the enzyme pyruvate kinase in the glycolytic sequence. An alternative explanation is that, given the overall stoichiometry for glycolysis is: -

Glucose -------- 2 lactate anion + $2H^+$

the increase in hydrogen ions could occur at the first point in the glycolytic sequence where a carboxylic group is formed, i.e. the formation of 1,3 diphosphoglycerate from glyceraldehyde catalysed by glyceraldehyde phosphate dehydrogenase. This would mean the formation of a carboxylic group which would ionise at cellular pH. (see diagram 4). This last rearrangement of the phosphate group on to the second carbon atom means that a carboxylic

DIAG.4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CHOH
C
O
O
PO_3^{2-}
$+ ADP^{3-} + H^+$
CH_2OH
CHOH
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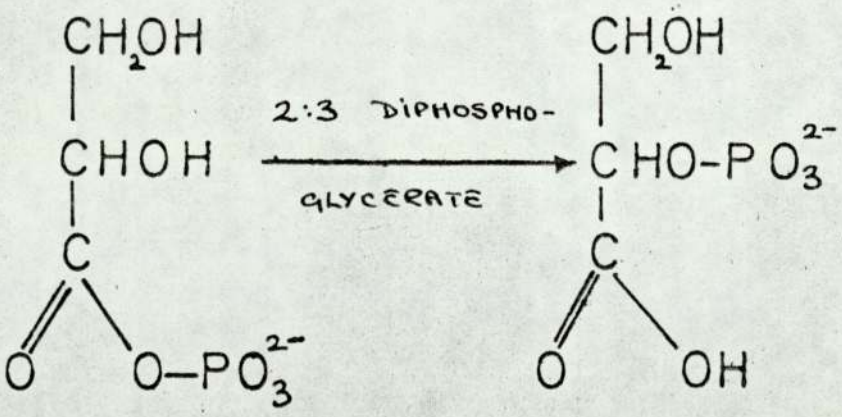
O
O
PO_3^{2-}
ATP^{4-}

then:-


CH_2OH
CHOH
C
O
$O-PO_3^{2-}$
2:3 DIPHOSPHO-
GLYCERATE
CH_2OH
$CHO-PO_3^{2-}$
C
O
OH

group is formed at the third carbon atom with a tendency to ionise. Whichever step does provide protons for acidification there should be a direct correlation between the lactic acid produced mucosally and the acidification phenomenon.

a) Lactic acid production.

In order to estimate the involvement of lactic acid in mucosal acidification, the lactic acid content of the mucosal fluid was assayed after incubation, in the everted sac, segment and in vivo loop preparations. Unlike previous authors (Wilson, 1953) pH was measured directly and was able to be directly correlated with hydrogen ion production since all buffers were back-titrated with hydrochloric acid and the change in hydrogen ion concentration measured rather than calculated from changes in buffer pH changes. In this way, it was possible to correlate lactic acid production with increases in the hydrogen ion concentration, something which had not previously been done.

In the everted sac preparations, lactic acid could only account for sixteen per cent of the total amount of hydrogen ion production, assuming that lactic acid was produced as the undissociated acid which then ionised in the mucosal solution. As the accompanying anion, lactate anion could only account for a fraction of the acid produced; even in the presence of glucose there is no significant increase in this proportion. In the

presence of the unmetabolisable hexoses, there is little increase in the serosal or mucosal production of lactic acid and also no significant increase in acidification. The proportion of acid accounted for as lactic acid is about the same, except for galactose. It is interesting to note that in this case the proportion falls to 5% and might indicate additional evidence for a possible deleterious effect of galactose on in vitro preparations. In the in vitro segment preparations, where the transport of substances with the maintenance of subsequent concentration gradients was precluded, acidification in the absence of added glucose was about the same as that in the mucosal scrap preparations and lower than the integral everted sac value. In the segment preparation, lactate could only account for about one tenth of the observed acidification, in the absence of glucose. In the presence of glucose, segment acidification was very much higher than that seen in the everted sacs, possibly indicating a greater accessibility of substrate to the biochemical components that cause acidification. The proportion of acidification that could be accounted for by lactic acid production was about one third, i.e. higher than in other preparations. Yet in this preparation with no spatial separation of the transported species and therefore no acidification of the medium due to transfer of exogenous buffering anion, e.g. bicarbonate or phosphate, lactate appearance could still not account for all of the observed acidification. These results were confirmed in vivo, where a segment perfused with double-distilled water containing no glucose also

acidified its perfusate and the amount of this acidification that could be accounted for as lactic acid was only about 15%. As with other intestinal preparations, the isolated loop perfusate was back-titrated with hydrochloric acid to establish the amount of buffering power that it had acquired. It is well known that exorption of solutes occurs in the intestine (Vogel, Stoeckert & Meyering, 1971; Vogel, Passman & Meyering, 1970) leading to the accumulation of substantial buffering power over a short period of time. In the in vivo preparation, the acid production was lower than all the previous in vitro methods and still the lactate appearance could only account for quite a small proportion of acidification.

b) Pyruvate acid production.

Incubation fluids were also analysed for pyruvate on the grounds that it was just as likely for this species of acid to occur mucosally as it was for lactic acid or any other of the acids involved in intracellular metabolism. Pyruvate was measured in the mucosal medium after incubation and was found to be present in such amounts as would account for an insignificant portion of acidification in the everted sac. This small fraction of pyruvate appearing in the mucosal medium was unaffected by the presence of hexoses such as mannose, galactose, methylglucose and glucose and also by ATP and meant effectively that the involvement of pyruvic acid in intestinal acidification could be discounted.

c) Lactic and Pyruvate acid production in component preparations.

Lactic acid production and simultaneous acidification were also measured in a mucosal scrape and in a 'mucosa-less' sac preparation. In effect, everted jejunal tissue had the mucosa scraped off the residual but intact muscle layers and serosal portions of the intestine were made into everted sacs in the usual manner. The scraped mucosa and the 'mucosa-less' sacs were both used in an attempt to determine which component of the whole intestine was of greater significance in the acidification process.

Acidification in the component tissues, basically mucosa and muscle, was very much less than that seen in the integral sac preparation. The sum total of mucosal scrape and 'mucosaless' sac acidification did not sum to the value normally seen in the everted sacs, however the appearance of lactate did. Furthermore, lactate in the component preparations seemed to account for significant fractions of the acidification. Lactate appearance in the mucosal solution on incubation with mucosal scrapes is about the same as that occurring on incubation with everted sacs, yet on incubation with 'mucosa-less' sacs much greater lactate production is seen. This seems to show, as other workers have indicated (Iemhoff, VandenBergh, Pjiper & Hulsman, 1970; Kimmich, 1970; Porteous & Pritchard, 1972) that the lactate appearing in the mucosal fluid, when everted sacs are incubated, has its origins in the mucosal epithelial cell metabolism; lactate having its origins in muscle metabolism does not diffuse into the mucosal fluid because

the epithelial cells act as a diffusion barrier, but rather diffuses preferentially into the serosal volume causing the high serosal:mucosal lactate concentration ratio seen on incubation.

Pyruvate was also measured in these component preparations and in both the mucosal scrapes and the 'mucosa-less' sacs a significant amount of pyruvate was formed that would account for acidification. However, the sum total of lactate and pyruvate anions in solution would more than account for the acidification present. Under anaerobic conditions, which caused a reduction in the acidification in the everted sac, the amount of acidification that these anions could account for greatly exceeded the acidification seen. In the case of the 'mucosa-less' sacs incubated under anaerobic conditions, lactic acid production alone would have accounted for two times the actual hydrogen ion production.

In conclusion, it seems that, on the basis of the experiments done on mucosal scrapes and 'mucosa-less' sacs, lactate, as it appears in the mucosal medium, does not exist as the undissociated acid but as the salt of lactic acid. The appearance of lactate in the mucosal medium must be regarded only as a guide to the extent and type of metabolism occurring within the tissues likely to cause acidification and not as a cause of acidification in itself. It is conceivable that glycolysis occurs within the tissues forming lactate and that the proton is somehow sequestered by the intracellular metabolic pathways while the lactate anion diffuses mucosally. More likely is that glycolysis and

lactic acid production accounts for only a small fraction of the hydrogen ions made available for the acidification process. Since acidification is reduced under anoxic conditions, the other major cause of acidification is more likely to be oxidative metabolism or some process dependent on the energy derived from oxidative metabolism. One obvious source of protons is that derivable from the decarboxylations of the tricarboxylic acid cycle. As noted previously it can be calculated that as with lactate production there is not enough carbon dioxide production to account for all of the acidification.

Since the acidification by the mucosal scrapes and the 'mucosa-less' sacs, when summed, did not equal the acidification of the whole everted sac, it is possible that a component in the acidification process depends on the spatial separation or unequal distribution that occurs when serosal and mucosal compartments exist that are not in connection with one another. This could for example be due to the transport of buffering anions or by the charge separation process implicit in the mitochondrial model of acidification if it were dependent on oxidative metabolism.

It can be assumed, as the mucosal scrapes respired and also produced lactic acid, that the failure of the mucosal scrapes and the 'mucosa-less' sac acidification values to sum to the everted sac value did not merely represent less viable preparations. If this is so, then since the everted 'mucosa-less' sacs, which had a spatial separation of mucosal and serosal compartments did not

acidify as much as the everted sac did, the greater portion of this vectorial component of acidification must rely on the correct alignment of the mucosal epithelial cells. It is unlikely that the muscle layer was so damaged that it did not produce the normal amounts of hydrogen ions, since in segments, which probably have a greater degree of relative damage, acidification increases rather than decreases. It would seem then in conclusion that lactate production is not a factor causing the observed mucosal acidification as is more in keeping with the mitochondria model for acidification. It is more likely that acidification is dependent on active transport mechanisms or biochemical activities consequent on oxidative metabolism occurring within the epithelial cells.

Acidification and the Movement of Phosphate and Bicarbonate.

Whether or not bicarbonate movement in the rat jejunum is active or passive is uncertain, but changes in luminal bicarbonate concentration occur on incubation whether in vivo or in vitro (Parsons, 1956; Wilson & Kazyak, 1957) although there is disagreement as to whether the total carbon dioxide in the lumen rises or falls. Recent in vivo work has shown that with bicarbonate-containing media, the carbon dioxide tension rises and the bicarbonate concentration falls (Powell et al., 1971), however, when bicarbonate-free incubation media are used, a small amount of bicarbonate moves into the luminal solution. In vitro, the bicarbonate concentration increases on the serosal side (Wilson &

Kazak, 1957) which seems to indicate that transfer of bicarbonate does occur: it is possible that this bicarbonate has its origins in intracellular metabolism. A component of the transmural potential has been provisionally identified with serosal bicarbonate movement (Faelli & Garotta, 1971a; 1971b) and can be inhibited with acetazolamide, as can bicarbonate movement (Parsons, 1956). Phosphate, the other buffering anion present in physiological buffers is also capable of being transported by the rat jejunum (McHardy & Parsons, 1956; Cramer, 1961) although it is uncertain whether this is an active or a passive process, would cause a loss in the overall buffering power of the mucosal medium and a gain in the serosal buffering power. This would cause an alteration in the mucosal medium pH without any movement of hydrogen ions whatsoever and could lead to the spurious conclusion that the mucosal fluid has gained in total hydrogen ion concentration. The evidence against this being the mode of action of acidification comes from the segment experiments. Where segments of jejunal tissue were used, and where the spatial separation of the two compartments no longer existed and what was transported through the cell tissues re-entered the bathing fluids, acidification still persisted. This excludes the possibility that acidification is due only to the transference of buffering anions.

Another possible way in which acidification might occur, invovling bicarbonate movement would be if carbonic acid were secreted mucosally and then ionised. Sacs,

however, that were incubated in phosphate buffer and gassed not with the usual 100% oxygen gas phase but with the alternative mixture containing 5% carbon dioxide, continued to acidify. This means that had carbonic acid been secreted from the tissues, it would have been secreted into a saturated system at a high and constant bicarbonate level and consequently would have displaced some medium bicarbonate at equilibrium, which would have formed carbon dioxide itself and bubbled off with no change in the medium pH. This fact supports the mitochondrial model of acidification in that acidification is not due to the intracellular production of carbonic acid from metabolic sources. In the same experimental situation the converse compensation of transferred bicarbonate by the tissues can be achieved by the continuous gas stream but not without alterations in the mucosal pH. This means that the Schilb & Brodsky model for acidification (1972) cannot be ruled out, i.e. that the shuttle of carbonic acid mucosally and the transfer of only the bicarbonate ion serosally, or even just the transfer of bicarbonate ion from the mucosal solution cannot be ruled out from these experiments; the bulk secretion of carbonic acid and also alterations merely due to changes in buffering ability can be ruled out as causes of acidification.

Some evidence against serosal transfer of buffering anions as being causative agents can be gained from a consideration of the direction of movement of these anions under certain circumstances. When everted sacs

are incubated in the absence of glucose, phosphate moved out of the sacs into the incubation medium. Since the converse was true in the presence of glucose, it seems unlikely that a relationship exists between phosphate transfer and acidification, especially since there was no overall correlation between transfer and acidification when sacs were incubated with the various hexose substrates, i.e. methylglucose, galactose, glucose and mannose. The reversal of direction of phosphate movement on the addition of glucose to the incubation media also indicates that secretion of some acid phosphate compound is unlikely to cause acidification unless its anion were immediately transported serosally. The same situation is true for bicarbonate ion. In the absence of mucosal bicarbonate ion, bicarbonate ion is secreted by the jejunum (Powell et al., 1971) and in vivo acidification still occurs despite the absence of mucosal bicarbonate ions. This would seem to preclude bicarbonate transport as a means of acidification. It is possible, however, as Schilb & Brodsky argue that bicarbonate secretion occurs into an initially bicarbonate-free medium and that thereafter bicarbonate transfer would cause acidification.

Schilb & Brodsky's model for acidification is based on the fact that acetazolamide will reduce acidification in the turtle bladder (Steinmetz, 1967). In the rat gut it has been shown that acetazolamide will reduce bicarbonate transport (Parsons, 1956) and will reduce transmural potentials associated with the active transfer of bicarbonate

(Faelli & Garotta, 1971a; 1971b). This same inhibitor has no effect at all on acidification. In effect, this meant that the acidification process was not dependent on the enzymic hydrolysis of carbon dioxide and made the transfer of bicarbonate or the refined bicarbonate 'shuttle' mechanism very unlikely causes of acidification, since it is difficult to conceive of either of these processes occurring without the intervention of the enzyme, carbonic anhydrase. The direct transfer of bicarbonate anion is very unlikely to cause acidification since this should have been almost fully inhibited at the concentration of acetazolamide used (Parsons, 1956), whereas acidification continued unaffected. The Schilb & Brodsky 'shuttle' model cannot actually be ruled out but as it would be difficult to envisage this process occurring without the involvement of carbonic anhydrase enzyme it is a not very probable mechanism for acidification in the proximal jejunum. It can be brought to mind at this stage that both the mechanisms invoked in the model for jejunal acidification brought forward by Wilson (1953; 1954; 1957), i.e. that a lactate production and of serosal bicarbonate movement, do not seem to adequately explain the phenomenon. It seems that acidification represents a genuine extrusion of hydrogen ions into the mucosal medium and is not merely the artefactual occurrence consequent on incubating intestinal tissues in physiological buffers. Though it may remain possible to associate the acidification process

stoichiometrically with an accompanying anion, e.g. phosphate or bicarbonate ion or both, acidification represents the movement mucosally of hydrogen ions, dependent somehow on oxidative metabolism and is not the consequence of the transport of anions in the reverse direction.

Acidification and the Involvement of ATP.

The greatest increase in acidification was seen when everted sacs were incubated with mucosal adenosine triphosphate as substrate in the mucosal medium. Acidification in the presence of ATP provoked an acidification significantly higher than that seen in the presence of mucosal glucose. This occurred without any significant increase in the mucosal or serosal lactate production which further underlines the insignificant role that lactate production has in acidification. The acidification caused by ATP also occurred in the absence of any mucosal glucose and indicates that acidification is not exclusively dependent on the transport of hexoses. With respect to sodium ion movement, the inclusion of ATP in the mucosal medium does not cause an increase in sodium transfer and further indicates that acidification is not associated with a compulsory sodium:hydrogen ion exchange mechanism. The combined concentration of lactate and pyruvate appearing mucosally on incubation, could only account for a very small percentage of acidification and metabolic carbon dioxide production, based on assumptions previously

stated could also only account for a similarly small percentage of acidification.

It is known that ATP will chelate calcium to form a ligand complex with a high stability constant. In artificial lecithin film experiments ATP has a chelating ability comparable with that of EDTA (Kimizuka & Koketsu, 1962). It was possible that ATP sequestered calcium ion away from intestinal cell membranes by way of chelation, causing a general increase in permeability of the cells to cations and specifically causing a leakage of protons from out of the cell. It is to be remembered that the incubation of a cell of internal pH 7.0 in buffer of pH 7.4 represents a concentration gradient across the cell membrane of about three, which under certain circumstances might cause protons to leak out of the cells. The chelating ability of ATP was estimated to be about 15% of that of equimolar EDTA. However, in the absence of glucose, equimolar EDTA which causes a reduction in gastric acidification due to enhanced back-diffusion of protons, (Chung, Sum, Goldman, Field & Silen, 1970) has no discernible effect on acidification. This made it improbable that acidification was caused by a leakage of protons down a concentration gradient and furthermore demonstrated that the effect of ATP was not mediated via its ability to chelate ions.

The experiments with isotopic ATP revealed that effectively ATP was not transferred into the intestinal cells. Some label did enter the tissue but this was

most probably a small fraction due to the uptake of portions of the degraded ATP molecule, i.e. labelled ribose, since the isotope was labelled in the sugar ring. Where ATP is presented externally to biological tissues, sometimes only the adenosine portion of the molecule has any effect as in smooth muscle relaxation (Axelsson & Homberg, 1969) or else the effects of ATP can be mimicked by ATP analogues such as sodium tripolyphosphate (Schwartz, Piro & Lajtha, 1971). The most likely explanation for the effect of ATP on intestinal acidification is that the ATP is hydrolysed and that this is exclusively an extracellular event. The empirical data so far presented will harmonise quite well with the concept that acidification is mediated by the hydrolysis of ATP and that substrates such as glucose and states such as anoxia merely serve to increase or reduce the amount of ATP available in the cell for the surface hydrolysis reaction. This is exactly as predicted by the mitochondrial model for acidification.

Acidification and the Adenyl Cyclase System.

By far the most striking effect seen in the acidification process, is its inhibition when everted sacs were incubated in the presence of mucosal aminophylline. The theophylline-containing compound blocks the action of the phosphodiesterase enzymes causing a reduction in cyclic-AMP breakdown and also, by inference, increased c-AMP levels within the tissues (Butcher & Sutherland, 1962). Aminophylline reduced acidification almost to nothing in

the absence of glucose, in the presence of the unmetabolisable sugar methylglucose and in the presence of the metabolisable but poorly penetrating mannose. Aminophylline also inhibited acidification in the presence of glucose to about one third of its usual value and in this respect was more inhibiting in equimolar quantities than the presence of phlorrhizin, DNP or anoxic conditions. However, some acidification persisted which may indicate the reversibility of the inhibition in the presence of adequate glucose. This inhibition in the presence of mucosal glucose was not merely the result of retarded glucose transport since hexose transfer did not vary when aminophylline was administered.

Imidazol which might be predicted from its action to have a stimulatory effect on acidification, on the contrary caused a slight but insignificant reduction. This might be because acidification as a consequence of c-AMP turnover was proceeding at a maximal rate and that the stimulation of phosphodiesterase activity by imidazol was of no consequence to acidification. Alternatively, as imidazol has been shown to have an inhibitory effect on adenyl cyclase in fat tissues (Allen & Clark, 1971), it might be reducing overall formation of c-AMP from ATP and hence overall ATP hydrolysis. An interesting speculation is that adenyl cyclase is a membrane-bound enzyme (Jost & Rieckenberg, 1971) present at the cell surface of the epithelial cells in rat jejunum (Kimberg, Field, Johnson, Henderson & Gershon, 1971) and more susceptible to exogenous

agents whereas phosphodiesterase is a cytoplasmic enzyme and consequently less accessible to imidazol. Another possible mode of action which is in keeping with the concept of acidification caused by ATP hydrolysis is that Imidazol has an inhibitory effect on mitochondrial ATPase, ATP terminal group phosphate exchange and on oxidative phosphorylation in general (Conover, Gonze & Estabrook, 1964).

The response of acidification in the jejunum to agents that affect the adenyl cyclase system is quite distinct from that in the gastric mucosa. Furthermore the response to agents such as EDTA, histamine and acetazolamide is also quite different. Histamine causes a marked increase in vitro in acidification by rat gastric mucosa and conversely acetazolamide causes a marked inhibition (Tsukamoto, 1961). In the rat jejunum, in contrast, neither histamine nor acetazolamide had any effect. The chelating agent EDTA was also without effect in the rat jejunum but it has been shown to have a large effect in causing a reduction in gastric acidification by loosening the gastric mucosal membranes and making them more permeable and in this way causing back-diffusion of hydrogen ion (Chung et al. 1970). Since acidification in the rat jejunum is of an order much less than that occurring in the gastric mucosa, it is quite possible that no hormonal stimulation is necessary vis-a-vis histamine action, that little back-diffusion of protons would occur in comparison with the back-diffusion possible with the concentration gradient of one million often seen in gastric mucosa, and

that swamping of the associated anion by enzymically stimulated carbon dioxide production is unnecessary in rat jejunal tissue. In this respect, it is possible for the mechanism behind acidification in both the gastric and the jejunal mucosa to be biochemically similar and yet physiologically distinctive in their reactions to the same circumstances.

As has been noted previously, gastric acidification has been linked with cyclicAMP levels but the fact is that in amphibian tissues it seems to be associated with increased AMP levels; theophylline causes increases in acidification and not the decrease seen in the present experiments (Harris, Nigon & Alonso, 1969). More recently in human and canine gastric mucosa theophylline caused reductions in acidification (Levine & Wilson, 1971) which would agree with the situation found in the jejunum. It is more than likely that mammalian gastric and jejunal mucosa acidify by the same biochemical system. Although the biochemical details of jejunal acidification are by no means clear at the present time, it can be postulated with confidence that jejunal acidification, involving the adenyl cyclase system, involves the surface hydrolysis of ATP.

Jejunal Acidification and the Mitochondrial Model.

It might be proposed that acidification in the jejunum has various sources of protons available, which are ultimately dependent on metabolism, i.e. lactate production, hydration of the carbon dioxide produced by citric acid cycle decarboxylations and transport of bicarbonate

dependent on oxidative metabolism, either as a consequence of its transport per se as proposed in the Schilb & Brodsky model (1972) or as a consequence of the chemical reactions associated with providing energy for bicarbonate transport. The increased production of carboxylic acid cycle intermediates, e.g. succinic acid or citric acid, can be discounted as a source of protons since there is no net turnover of carboxylic groups within the cycle or net production of carboxylic groups. Such carboxylic groups which might be introduced into the carboxylic acid cycle would ultimately have come from the glycolytic sequence and it is at this stage in the production of acidic groups from glucose that it is to be expected that protons become derivable from carboxylic groups.

All obvious conceivable sources of spurious acidification, such as tissue anoxia or autolysis and the leakage of certain organic acids, e.g. pyruvate and amino acids have been ruled out and also artefactual occurrences inherent in the incubation in certain buffers. Basically, acidification as it is influenced by glucose and other hexoses is a consequence of metabolism and not of transport processes and at first sight seems physiologically distinct from that occurring in gastric mucosa. Individually, lactate production and carbon dioxide production will not account for the phenomenon since acidification is seen under circumstances where it is impossible for them to have caused acidification. In both cases, neither lactate production as measured by empirical methods nor cellular

carbon dioxide production as calculated from available data could account for all the acidification observed. As has been stated previously, transfer of bicarbonate across the intestine has also been ruled out as a cause and the transfer of bicarbonate across a membrane equally unlikely because of the lack of acetazolamide inhibition which is characteristically seen where chloride:bicarbonate exchange occurs (Maren & Robinson, 1960; Rothman & Brooks, 1965; Scarpa et al. 1970). It is important to draw the distinction between transfer across the intestine, meaning movement of bicarbonate with an accompanying cation causing changes in the buffering ability of the solution and transfer across a membrane meaning transfer of a bicarbonate anion which leaves its accompanying hydrogen ion behind. Hydrolysis of simple phosphates, e.g. hexose phosphates, pyridoxal phosphates is also not the cause of acidification and in conclusion the most likely cause is the surface hydrolysis of ATP by membrane bound ATPases.
This means that the previous hypothesis for the cause of jejunal acidification (Wilson, 1953; 1954), that of bicarbonate transfer serosally and lactic acid production mucosally must be summarily rejected as inadequately explaining the phenomenon.

The concept of jejunal acidification having its cause in surface hydrolysis of ATP made available mainly by oxidative metabolism would explain all of the experimental data. Anoxia, which retards oxidative metabolism, DNP which uncouples oxidative phosphorylation

(Loomis & Lipman, 1948) and phlorrhizin, which also reversibly prevents phosphorylation of ADP (Lotspeich & Keller, 1956) would all reduce acidification by limiting the amount of ATP produced within the cells. The persistence of some acidification under anoxic conditions can also be explained by the fact that some ATP is still produced under anoxic conditions by way of anaerobic glycolysis. Similarly, metabolisable sugars will increase ultimately the availability of ATP and the unmetabolisable sugars might not make any ATP available, but in contrast may even compete for ATP with other processes within the cell, as proposed by other authors (Newey & Smyth, 1964; Saunders & Isselbalcher, 1965) and consequently prevent acidification by making ATP less available. ATP hydrolysis would also rationalise the effect of aminophylline on the adenyl cyclase-phospho-diesterase system, in which aminophylline would reduce c-AMP turnover and as a consequence, little ATP would be hydrolysed to give cyclic AMP. Acidification mediated directly as a consequence of chemistry of the adenyl cyclase-mediated reaction rather than in terms of the 'second messenger' hypothesis is physiologically unusual in this respect.

In terms of the mitochondrial model, therefore, jejunal acidification does conform quite reasonably well to the principles as described by Mitchell (1966; 1968): it is an active energy-dependent process and is not caused by intracellular organic or carbonic acid production.

Acidification is definitely not mediated via the trans-membrane potential gradient. If it involves the movement of a counterion, as predicted by the mitochondrial model, the counterion is probably not sodium: the role of potassium ion was not investigated. Acidification is mediated by the hydrolysis of ATP, although it remains unclear whether charge separation does occur. There is no need to invoke separation since the reaction: -

$$ATP + H_2O \dashrightarrow ADP + H_3PO_4 \dashrightarrow ADP + H_2PO_4^- + H^+$$

for the dissociation of phosphoric acid has a very low pKa. It is therefore possible for acidification to reach very low pH values without the problems previously discussed with the secretion of other acids, e.g. carbonic acid, which have relatively high pKa values. The presented experimental data do not exclude the possibility that the function of ATP is not to produce protons itself but to power bicarbonate transport, if this were to cause acidification, as proposed by Schilb & Brodsky (1972). Intthis model, the bicarbonate 'shuttle', in which endogenous carbon dioxide production forms carbonic acid which then diffuses into the region to be acidified and the transport of only the bicarbonate anion causes the acidification, would be powered conventionally by metabolic supplies of ATP. The only real evidence that can be brought against this model is the fact that external application of ATP will cause acidification far beyond the tissues capability to produce carbon dioxide as judged from normal respiratory data and that one might expect acetazolamide to have some

inhibitory action. In conclusion, it must be said that the only model that explains all the experimental facts is the mitochondrial model of jejunal acidification.

Acidification and the Microclimate.

The proposed model of acidification fulfils exactly one of the prerequisites of an acid microclimate, that of a source of protons at a surface, which could be retained in some microclimate layer. The microclimate has important consequences for the transfer of weakly-ionising substances that cross the membranes as the neutral species, by non-ionic diffusion, since an acid microclimate would alter the amount of hydrogen ions that are available for the anions to combine with and consequently non-ionic diffusion would be increased. This model may be applicable to the transfer of propionate (Barry, Jackson & Smyth, 1966), middle-chain length fatty acids (Bloch, Haberich & Lorenz-meyer, 1972), folic acid (Benn, Swan, Cooke, Blair, Matty & Smith, 1971) and benzoic acid (Jackson, Shiau & Cassidy, 1970).

With benzoic acid, transport is greatest in the jejunum, is increased on addition of the metabolisable sugars glucose and fructose, is inhibited by galactose and metabolic inhibitors and the ratio of unidirectional fluxes is greater than that predicted by Ussing's flux ratio criterion. Ussing's criterion indicates that simple passive forces are not sufficient to account for the observed fluxes. Using classical terms, it might be argued that either an active transport system or a

facilitated-diffusion mechanism exists that is dependent on supplies of ATP, as shown by the action of the metabolisable hexoses and in contrast the action of the non-metabolisable galactose and the inhibitors. As an alternative to the orthodox scheme, it is possible that this increased transfer is mediated by acidification, since all the factors which increase or retard benzoic acid transfer, also have the parallel effect on acidification.

This model of transfer has been proposed for folic acid transfer (Blair, 1972): it must be assumed that the transfer of such a species would increase linearly with concentration until a limiting value was reached when the rate of transport of the unionised species matched the rate at which hydrogen ions could be produced. This form of transfer would be linear with respect to microclimate hydrogen ion concentration, until it reached a saturable level and could easily be mistaken for Michaelis-Menten kinetics. From calculations derived from the in vivo experimental data, it can be calculated that to change the pH of a small section of the jejunum, e.g. three centimetres long, by the proposed mode of ATP hydrolysis, would require only micromolar quantities of ATP. When this is considered in terms of the increased transfer of glucose possible in moving from an unfavourable to the optimal luminal pH and the resultant increase in substrate available for oxidative metabolism, ATP hydrolysis would not seem to be an energetically wasteful

procedure. In the case of producing hydrogen ion that is coupled with the transfer of anion and transport occurs without replacement of hydrogen ion, calculations further show that a means of altering environmental pH would only be of any consequence to compounds which were themselves transferred in micro-molar quantities. Where transfer is of the order of micromoles, it is possible that the concentration ratios across the transporting membrane would become greater than one and indicate 'active transport'. With the acidification model of transfer, the distinction between active and passive transport becomes semantically difficult since one might expect all the criteria of active transfer as dictated in classical carrier-models, yet the system could still be essentially passive in that the underlying physical process would be diffusion.

In the case of propionate, the authors (Barry, Jackson & Smyth, 1966) discount the microclimate as having any effect since the fact that the conditions for equilibrium between the two bulk phases is equal concentrations of non-ionised acid, would not be altered by the interposition of a micro-climate. This may not be the case however when one considers the equilibrium conditions between the microclimate and the bulk phase to which the anion is to be transported. In effect a 'static' microclimate might not affect the equilibrium conditions but a 'dynamic' microclimate with turnover of hydrogen ions definitely would. Propionate transfer was

also increased by mucosal glucose and by serosal glucose in the presence of phlorrhizin, indicating clearly that metabolism stimulated propionate transfer (Barry et al., 1965).

In the studies on caproic acid transfer (Bloch et al., 1972) in which it was shown that caproic acid was transported at greater rates in the jejunum, not explicable by passive processes and could be inhibited by cyanide, the authors concluded that an energy dependent transfer mechanism existed for the middle chain fatty acids. This acid might also be a candidate for acidification-mediated transfer since it has a suitably low pKa around 4.5. The kinetic data available show that in competitive inhibition situations, transfer agrees well with the acidification model. It is reasonable to assume that with similar weak acids, in this case caproic acid and octanoic acid, there will be the likelihood of almost no variation in the affinity of the anions for hydrogen ion. Consequently, where the fatty acids are in equal concentration, the Km for any one acid in the presence of the other ought to be doubled and the constant Ki for the degree of inhibition ought to equal the concentration of the applied inhibitor (Mahler & Cordes, 1966). In the case of caproic acid, this has been shown to be exactly so, where the Ki for equal 50 mM concentrations of both acids was 47 mM and the Km for caproic acid transfer, normally 13.6 mM, became twice as large at 27.5 mM (Bloch et al., 1972). This is kinetically exactly as predicted by an acidification model for enhanced transport.

The assembled experimental data do not contradict the classical concept of carrier-mediated active transport or facilitated diffusion but it is felt that within certain boundary conditions, acidification processes in the context of a microclimate would readily explain some phenomena seen in the transfer of some weakly ionising substances and could provide an acceptable alternative mode of energetically increased transfer of solutes across the proximal jejunum.

The Clinical Significance of Acidification.

Undoubtedly acidification plays a role in the maintenance of a microclimate and of the normal jejunal pH. It is possible that this role might be the basis of some hitherto inexplicable malabsorption syndromes and be of considerable clinical significance. In some cases of untreated coeliac disease and in idiopathic steatorrhoea, there is severe diminution of jejunal mucosal ATPase activity (Samloff, Davis & Schenk, 1968; Riecken, Stewart, Booth & Pearse, 1966; Cooke, 1968). The deletion of this enzyme in disease states would almost certainly mean a decreased ability of the jejuna to acidify its mucosal contents. In some cases of folate malabsorption, often a high alkaline jejunal pH is also seen (Benn et al., 1971). It is likely therefore that as pH variations can cause reductions in available food folate (Butterworth, 1968) that the reduced ability to acidify causes folate malabsorption. Furthermore, in the enzyme deletion

states, a definite enzyme defect can be demonstrated which though not yet demonstrated to be the cause of malabsorption, is a significant piece of evidence in favour of its deletion having malabsorption as a consequence, and furthermore the same enzyme has been implicated as that causing acidification in the present studies.

It is hoped that the present studies will act as a prelude to further experiments on acidification phenomena in the proximal jejunum, particularly with respect to micro-electrode studies designed to physically establish that a pH microclimate does exist and also clinical studies to investigate the corollaries and consequences of acidification in malabsorption syndromes and to bring acidification firmly into the realm of medical physiology.

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FOREIGN LANGUAGE SUMMARIES.

ZUSAMMENFASSUNG

Die Ansaeurung von Loesungen im proximalen Duenndarm der Ratte wird in ungestuelpten Darmstuecken, Segmenten und in in-vivo perfundierten Darmschlingen untersucht. Die Saeureproduktion, die entlang dem Jejunum abfaellt, steigt mit zunehmender Glukosekonzentration an der Mukosa und erreicht einen Maximalwert. Experimentelle Michaelis-Menten-Konstanten sprechen fuer ein Km von 0.05 mM und eine Vmax von 1.01 ($\mu g/H^+/mg$). Die Saeureproduktion wird durch 10 mM L-β-Phenylalanin nicht gehemmt und bleibt von 10mM Pyridoxinphosphat unbeeinflußt; sie wird durch Glukose-1- und -6-phosphat gesteigert.

Die Saeureproduktion wird durch 10 mM Acetazolamid au der Mukosa sowie Ouabain nicht beeinflußt; daher koennen als Ursachen einer Saeureproduktion Bikarbonatbewegungen ausgeschlossen und aktiver Natriumtransport als unwahrscheinlich angesehen werden. Die Saeureproduktion wird durch Anoxie, Phlorrhizin und Dinitrophenol in Mengen, die oxidative Vorgaenge hemmen, vermindert, und damit ihre Abhaengigkeit vom Stoffwechsel bewiesen. Ebenfalls kann die Anoxie als Ursache der Saeureproduktion ausgeschlossen werden. Die Saeureproduktion wird durch Histamin (10mM) und AeDTA nicht beeinflußt und kann daher von der Magensaeureproduktion physiologisch differenziert werden. Die Unwirksamkeit von Acetazolamid und die Unfaehigkeit der Mukosa, Lactat und Pyruvat zu produzieren, sowie Aminosaeuren abzugeben, machen die bestehende Theorie einer

Verursachung der Saeureproduktion durch Lactatbildung sowie Bicarbonattransport in ungestuelpten Darmstuecken, Segmenten und Schlingen unwahrscheinlich.

Als Stimuli der Saeureproduktion wirken die metabolisierbaren Zucker, Glukose, Mannose und Fruktose, dagegen nicht die nicht-metabolisierten Zucker 3-O-Methylglukose und Galaktose. Diese Ergebnisse zeigen die Herkunft der Wasserstoffionen aus dem Stoffwechsel. ATP (10mM) an der Mukosa, das nicht in die Zellen transportiert wird, verursacht eine Steigerung der Saeureproduktion, die durch die Faehigkeit von ATP, Chelatbindungen einzugehen, nicht erklaert wird. Die Saeureproduktion wird am deutlichsten gehemmt durch an der Mukosaseite befindliches Amionophyllin (10mM) und zeigt damit die Bedeutung des ATP-stoffwechsels innerhalb des Adenylcyclase-Phopsphodiesterase-Systems. Imidazol (10mM) hat keinen Einfluß.

Es wird angenommen, daß die Saeureproduktion kein passiver Vorgang ist, sondern das Ergebnis der Hydrolyse von ATP an der Oberflaeche der Epithelzellen. Diese Annahme wird in Bezug auf die 'Mikroklima-Hypothese geprueft. Es werden die Auswirkungen der Saeureproduktion auf den Transport der schwach-ionisierten Substanzen diskutiert.

SOMMAIRE

On a etudie l'acidification de solutions par le jejunum proximal du rat, ce sur des poches retournees, des segments, et des boucles vivantes aynt subi une perfusion. L'acidification diminue a mesure que l'on descent le long du jejunum et croit en meme temps que la concentration de glucose muqueux, atteignant une valeur maximum. En derivant des donnees de Michaelis-Menten, on obtient un Km apparent de 0,05mM et une Vmax de 1.01 (ugH/mg). L'acidification n'a pas ete entravee par 10 mM de L-β-phenilalanine, ni stimulee par 10mM de phosphate pyridoxal muqueux: par contre elle a ete stimulee par les glucose-1 et-6 phosphates.

L'acidification n'a pas ete affectee par 10mM d'acetazolamide ni par 10mM d'ouabaine, ce qui exclut comme agent probable d'acidification un deplacement de bicarbonate ou un transfert anormalement haut de sodium actif. L'acidification a diminue sous l'influence de l'anoxie, de la phlorrizine et du DNP en des concentrations qui empechaient un processus d'oxidation: ceci indique une interdependance metabolique et exclut l'anoxie comme cause d'acidification. Non affectee par l'histamine (10mM) et par l'EDTA (10mM), l'acidification est physiologiquement distincte de la production d'acide gastrique. L'absence d'effet de l'acetazolamide et l'impuissance a rendre entierement compte de l'acidification par la production d'acide lactique muqueux et d'acide pyruvique etpar la perte d'acide amine diminuent la probabilite de la theorie actuelle selon laquelle l'acidification est produite par

le deplacement de bicarbonate et par la production d'acide lactique.

Les sucres non transformables par metabolisme, tels que le 3-O-methy glucose et le galactose, n'activent par l'acidification, et les sucres transformables, tels le glucose, le mannose et le fructose l'activent, ce qui indique que l'origine des protons est de nature metabolique. Pareillement, l'ATP muqueux (10mM), qui ne penetre pas dans les tissus, cause un accroissement de l'acidification qui n'est pas du a la propriete de chelation de l'ATP. L'acidification est entravee de facon significative par l'aminonhylline (10mM), ce qui montre l'importance de l'ecoulement de l'ATP A travers le systeme d'adenyl cyclase-phosphodiesterase. L'imidazol (10mM) ne produit aucun effet. L'on suggere que l'acidification n'est pas un processus passif, mais le resultat d'une hydrolyse externe de l'ATP à la surface des cellules epitheliales. Nous examinons cette proposition en relation avec l'hypothese du microclimat et debattons la question de l'implication de l'acidification dans le transfert des substances a faible puissance d'ionisation.