An investigation of solute fluxes in enterocytes and the relevance of these fluxes to diarrhoeal disease

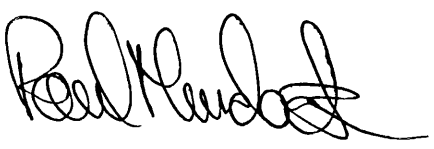
Murdock, P. R.

Award date:
1988

Awarding institution:
University of Bath

AN INVESTIGATION OF SOLUTE FLUXES IN ENTEROCYTES AND THE RELEVANCE OF THESE FLUXES TO DIARRHOEAL DISEASE

Submitted by P.R. MURDOCK
for the degree of Ph.D.
of the University of Bath
1988



P.R. MURDOCK

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

SUMMARY

Enterocytes (intestinal epithelial cells) have been isolated from chickens, rabbits and suckling piglets, and used for solute flux studies. The stability of the isolated enterocytes has been investigated together with their ability to sustain a sodium gradient. It has been found that enterocytes isolated from young chickens, rabbits and suckling piglets provide the enterocyte system most relevant to the physiological state as they are able to maintain their own sodium gradient. Enterocytes isolated from older chickens however, can be used for transport studies providing artificial sodium gradients are produced.

The production of vesicles from the brush border and basolateral surfaces of the enterocyte has been investigated, and brush border vesicles exhibiting good transport properties have been produced.

The heat stable enterotoxin ST_a, secreted by diarrhoeal disease inducing E.coli -431 strains, has been highly purified to a level suitable for enterocyte studies. The subsequent use of ST_a with the target cell (the suckling piglet enterocyte) has shown that piglet enterocytes are unable to maintain physiological cGMP levels once isolated. In whole tissue systems ST_a is thought to increase cGMP. The cGMP analogue 8 Br cGMP has been used to show a decrease in sodium influx into isolated enterocytes, thus showing a possible link between ST_a elevated

cGMP levels and *in vivo* enterocyte sodium flux.

Citrate is an important constituent of the oral rehydration solutions used to combat diarrhoeal disease. The isolated enterocyte system has shown two citrate flux components. The first is best described by a sodium dependent citrate symport with kinetic parameters, K_t.3mM and V_{max}1.3 n moles citrate/minute/mg protein. The second citrate flux is possibly due to a sodium-independent facilitative citrate transporter and is pH dependent. This system has kinetic parameters, K_t.5 mM and V_{max}.32 n moles citrate/minute/mg protein at pH 7.2.

The presence of a Na^+/H^+ exchange system has been discovered and both sodium ion and proton movements through this system have been demonstrated. Experiments were also carried out to test for a link between this system and the citrate fluxes. In addition, proton induced increases in hexose flux through the intestinal hexose (Na^+) symport have been investigated. This system is compared with the pH dependent citrate flux.

The citrate transport system has been discussed with relevance to *in vivo* citrate absorption and the properties of citrate in oral rehydration solutions.

CONTENTS

METHODS

ABBREVIATIONS.

ADP	Adenosine 5' diphosphate
ATP	Adenosine 5' triphosphate
BSA	Bovine Serum Albumin
cAMP	Adenosine 3',5' cyclic monophosphate
cGMP	Guanosine 3',5' cyclic monophosphate
GTP	Guanosine 5' triphosphate
HEPES	N-2-hydroxy ethyl piperazine n-2 ethane sulphonic acid
MES	Morpholino ethane sulphonic acid
Na^+/K^+ ATPase	Sodium,potassium ion activated Adenosine Triphosphatase
PBS	Phosphate Buffered Saline
pH_i	intracellular pH (pH inside cell)
pH_o	extracellular pH (pH outside cell)
Tris	Tris(hydroxymethyl) amino methane
T_4	Thyroxine
T_3	3',5',3'-Tri iodo thyronine

Internally directed Na^+ gradient $\left[Na^+\right]_o > \left[Na^+\right]_i$

Externally directed pH gradient $\left[H^+\right]_o < \left[H^+\right]_i$

suffix: o = outside cell
i = inside cell

INTRODUCTION

Diarrhoeal Disease.

Death from diarrhoeal disease can be caused by changes in solute flux in either the small or large intestine. The small intestine is responsible for the majority of fluid absorption, whilst the physiological regulation of salt and water uptake occurs in the large intestine. Under 'normal' conditions the small intestine accounts for more than 70% of the total water reabsorbed compared with less than 30% for the large intestine. The ability of the large intestine to increase its water uptake allows a limited degree of compensation for losses in efficiency in the small intestine.

Agents which give rise to diarrhoeal disease.

Diarrhoeal disease can be caused by a variety of agents, most notably viruses and bacteria (Fondacaro, 1986). Viral diarrhoea is often due to rotavirus species, and much of their effects originate from cell destruction particularly at the villus tip (Newsome and Coney, 1985). Bacterial diarrhoea, however, is a consequence of bacterial secondary metabolites which interfere with intestinal function without histological damage (Bertshinger,Moon and Whipp, 1972; Whipp et al, 1987).

Human bacterial infection can usually be effectively treated by hospitalisation, a careful diet and the use of antibiotics. However, the dependence of farm animals on their microbial flora and the unjustifiable expense of intensive care results in great suffering and loss of life.

E.coli and diarrhoeal disease.

E.coli is the predominant inhabitant of the mammalian intestine. Certain toxin secreting strains have been shown to cause diarrhoea in calves, piglets, rabbits, foals, lambs and humans (Gerday et al,1984; Banwell et al,1971; Smith and Halls,1967; Smith and Gyles,1970; Potter et al, 1985; Dreyfus et al,1983). The young are particularly vulnerable to infection by toxin secreting E.coli strains and the consequences are invariably more serious than in the adult (Moriaty et al,1986). This susceptibility is probably a consequence of: the absence of an acidic stomach barrier; the absence of an established microbial flora; reduced ability for colonic compensation of small intestinal overspill; and the absence of a fully developed immune system.

Toxin secreting strains of E.coli exert their effects on the small intestine. It is therefore necessary for these bacteria to withstand the small intestine's large fluid movements and vigorous peristalsis. This habitation of the small intestine is usually accomplished by bacterial membrane bound adherence factors. These adherence factors allow specific binding of the bacteria to host receptors (Knutton et al,1985; Rapacz et al,1986). The adherence factors are plasmid encoded and animal species specific (Baldini et al,1983; Mouricout et al,1987).

Toxin producing E.coli strains can secrete any combination of three types of protein based enterotoxin. These enterotoxins are known as heat labile enterotoxin,

(LT) and heat stable enterotoxins type a and b (ST_a and ST_b). The toxins are also plasmid coded, although usually separate from the adherence factors,(Olsvik et al,1985). It has been shown that these plasmids can be transferred to other bacteria,(Takeda et al,1983; So and McCarthy, 1980; Gyles, So and Falkow,1974), and in certain cases with antibiotic resistance (Gyles, Falkow and Rollins, 1978). This may partially explain the failure of antibiotics in certain diarrhoeal disease outbreaks.

ENTEROTOXINS - MECHANISMS OF ACTION

Cholera and heat labile enterotoxins.

Cholera toxin of Vibrio cholerae and the heat labile enterotoxin of E.coli show remarkable similarities (Spicer et al,1981; Robertson et al,1985). They are both high molecular weight, heat labile (65°C), two subunit proteins that exhibit immunological cross reactivity. (Gyles,1971; Carpenter,1972).

Cholera toxin and heat labile enterotoxin (L.T.) have binding subunits which attach to the GMI ganglioside receptor on the external membrane of the small intestine epithelial cell. After a lag period of 15 to 30 minutes, the second subunit penetrates the cell membrane by an unknown mechanism. The host enzymes cause cleavage of the catalytic toxin unit which then exhibits its adenosine diphosphate ribosyl transferase activity. Using this activity and NAD as a substrate, adenosine diphosphate ribose is covalently attached to a GTP binding protein of

adenyl cyclase,(Rodbell,1987). The regulatory function of the GTP binding protein is then lost, which results in chronic stimulation of adenyl cyclase and hence elevation of cAMP levels,(Carpenter,1982).

There is much evidence linking increased cAMP levels to fluid secretion (Gill,Evans and Evans,1976; Donta, Moon and Whipp,1974). However, the intermediatary mechanisms are unknown,(Smith et al,1985). Previous reports of possible intermediatary mechanisms have included cAMP dependent protein kinase induced reductions in enterocyte sodium influx,(Hyun et al,1982). Cytoskeletal 'contractions' have also been proposed, as losses in tight junction adherence between enterocytes is a feature of the effects of cholera toxin (Harrison and Lunt,1980). In addition, it has been reported that elevated cAMP levels may increase intracellular Ca^{++} concentrations. It is then thought that the effects of Ca^{++} may reduce water reabsorption, (Ilundain and Naftalin,1979). In contrast to the afore mentioned reports, it has also been proposed that a neuronal mechanism may precede any effects of cholera toxin or L.T. on intestinal adenyl cyclase activity, (Cassuto,Jodal and Lundgren,1981).

Heat stable enterotoxins.

There are two distinct classes of E.coli enterotoxin that are stable to temperatures in excess of $90^{o}C$. These are consequently known as ST_a and ST_b,(Gonzales et al, 1985; Yashimura et al,1984).ST_a is soluble in methanol, poorly immunogenic, (Thompson et al,1985), and active in neonatal

piglets and infant mice. ST_b is insoluble in methanol and active in piglets greater than three weeks of age. (Burgess et al,1978). The failure to produce a convenient animal model to monitor the biological activity of ST_b has limited the success of purification attempts. Consequently very little is known about the mode of action of ST_b. There are conflicting reports concerning the effects of ST_b on cellular cGMP levels. The initial and most numerous reports indicate an increase in cellular cGMP induced by ST_b (Newsome,Burgess and Mullan,1978; Burgess,1983). However, recently Kennedy et al,(1984) have reported that ST_b does not affect cGMP levels.

Purified preparations of ST_a have been found with aminoacid compositions between 14 and 47 aminoacid residues. However, the majority of reports favour the lower end of this range (Chan and Gianella,1981; Gerday et al,1980). ST_a is an unusual peptide as it invariably contains six cysteine residues. These are all thought to be engaged in disulphide bonds (Alderate and Robertson, 1978). The resulting tightly folded conformation partly explains its extremely poor immunogenicity, heat stability and methanol solubility.

ST_a has recently been found to bind to a single class of membrane bound glycoprotein receptors (Gianella et al, 1983; Dreyfus et al,1984; Frantz et al,1984). The use of a synthetic photoreactive analogue of ST_a has shown two proteins (of 57 and 75 kDa) to be part of, or near to, the

ST_a receptor,(Gariepy and Schoolnik,1986).

After binding, ST_a causes an increase in intracellular cGMP by elevating guanylate cyclase activity,(Newsome, Burgess and Mullan 1978; Guerrantetal,1980). The mechanisms by which cGMP then causes fluid secretion is not understood, although it is well known that cGMP can adversely affect water absorption (Hughes et al,1978; Rao et al,1979).

As previously described for L.T., a role for intracellular Ca^{++} levels has also been implicated in the mechanism of secretion induced by ST_a (Knoop and Abbey, 1981; Donowitz et al,1983). Pharmacologically active agents which affect Ca^{++} metabolism, such as the effects of chlorpromazine on calmodulin, have been found to interfere with the toxins effects (Knoop and Abbey,1981). Recently Henderson et al,(1985) have discovered that there is increased phosphorylation of microvillus membrane proteins in the presence of free Ca^{++} and cGMP, possibly due to protein kinase activity. (c.f. Both cGMP and Ca^{++} levels are thought to play a role in the actions of ST_a (see previous pages)). Further evidence has implicated protein kinase C in the regulation of at least one intestinal sodium influx component (De Jonge,1984; Fondacaro and Henderson,1985).

These findings have led to interest in free intracellular Ca^{++} levels as the common intermediate in the mechanisms of action of the toxins described and numerous other dissimilar secretory substances (Fondacaro,1986).

Oral Rehydration Therapy

Oral rehydration solutions (ORS) represent the major advance in the treatment of diarrhoeal disease (Greenough, 1980; Carpenter,1982; Marin et al,1987).

The salt content of the formulation is designed to optimise the lumenal conditions for transcellular sodium flux,(Mitchell,1985; Wagnir et al,1985). Much attention is paid to the sodium concentration of the solution once inside the lumen (Rolston et al,1987). The second component of a basic oral rehydration solution is the metabolizable substrate (Brown,1985). This serves to elevate mucosal cell energy levels and to aid the restoration of sodium and fluid movements. This is necessary as the majority of dehydrated animals become malnourished (Donowitz et al,1984). In addition to providing an energy source for the intestinal cells, the substrate also increases the nutritional status of the whole animal (Finberg,1985).

Oral rehydration solutions containing high sodium (90mM) cause very rapid rehydration although there are often subsequent problems of hypernatraemia (Finberg,1973). A compromise between optimum conditions for water reabsorption and longer term physiological salt balance is therefore often necessary.

Acidosis is a further problem which accompanies dehydration (Donowitz et al,1984). All of the reasons for this reduction in blood pH are not fully understood, but it is generally accepted that its restoration is an important part of any therapy. It has been found that

bicarbonate can effectively reduce acidosis and it is therefore included in many formulations. (Fondacaro,1986).

The easily formulated World Health Organisation (WHO) solution and the highly developed veterinary therapy 'Lectade' (Beecham) are shown below:

WHO formulation (pre 1985)

Sodium	90 mM
Potassium	20 mM
Chloride	80 mM
Bicarbonate	30 mM
Glucose 40 g/l or sucrose 40 g/l or rice powder 60 g/l	

LECTADE (Beecham)

Sodium	73.2 mM
Potassium	15.6 mM
Chloride	73.2 mM
Phosphate	15.0 mM
Glycine	41.1 mM
Dextrose	113.7 mM
Citrate	1.4 mM

Our understanding of the effects of many of the above constituents justifies their inclusion when designing an oral rehydration solution (see following sections). However in this respect our poor understanding of cellular citrate movement is unusual.

Citrate has been shown to be particularly efficacious at reducing dehydration and acidosis as part of an oral rehydration solution (Bywater,1977; Bywater and Wood,1978;

Hoffman et al,1985; Salazarlindo et al, 1986). Initially it was included in the leading brand of veterinary rehydration solution, but recently (1985) it was included in the WHO formulation (World Health WHO April 1986).

Despite the now widespread acceptance and use of citrate, very little is understood about its biochemical mode of action.

Isolated Enterocytes and Enterocyte Solute Movements.

The highly manipulatable nature of the isolated enterocyte system has allowed the 'in depth' study of transport systems originally discovered in whole tissue. Vesicles produced from the intestinal brush border and basolateral membrane allow further increases in the ability to control cellular conditions. (See following section). The isolated enterocyte system 'bridges the gap' between whole tissue and vesicle methodologies. The isolated enterocyte system therefore reflects a 'compromise' between experimental manipulatability and relevance to the physiological state.

Enterocytes have previously been employed to study both brush border and basolateral membrane transport processes (Kimmich,1970; Rowling and Sepúlveda,1984). The intestinal hexose uptake system has been extensively studied using enterocytes (Kimmich,1970; Kimmich and Randles,1979; Kimmich et al,1977). The majority of reports have added further evidence to the established Crane hypothesis for symport flux (Crane,1977). However, the isolated enterocyte system's high level of manipulatability has lead to the proposition of modifications to this hypothesis, (Kimmich,1970).

The enterocyte hexose symport has also been shown to contribute one of the many sodium influx components to the total enterocyte sodium flux (Brown,Burton and Sepúlveda, 1982). An additional sodium influx component has been demonstrated through the neutral amino acid symport system

(Sepúlveda, Burton and Brown,1982). Two further sodium symport fluxes in the form of the basic and acidic amino acid symports also contribute to enterocyte sodium influx (Lamb et al,1983).

The increased sodium influx due to the four symport systems described necessitate an increase in sodium ATPase 'pump' activity, expelling sodium for potassium (Brown and Sepúlveda,1985). Studies carried out in the isolated enterocyte system show that the increase in sodium efflux is not reflected by an increase in intracellular potassium content (Nellens and Schultz,1976). Brown and Sepúlveda (1985) have shown that this is due to an increase in membrane permeability causing increased potassium efflux. It also appears that this change in permeability may be controlled by inositol triphosphate levels and calcium mobilization (Sepúlveda et al,1987). This discovery of a homeostatic mechanism is a good example of the importance of the isolated enterocyte system's 'compromise' between manipulatability and relevance to the physiological state. It would not be possible to study such a mechanism with either whole tissue or vesicle methods.

A Na^+/H^+ exchange facility has been found using isolated enterocytes thus forming a further route for sodium influx. However, sodium movements have not been studied directly in the enterocyte Na^+/H^+ exchange system (Montrose et al, 1985).

The ability to produce villus-crypt enterocyte gradients

has proved to be a useful adaptation of the enterocyte isolation procedure. This adaptation allows the isolation of enterocytes at the villus tip or near the crypt or at stages inbetween. This technique has allowed the study of cellular differentiation as enterocytes migrate from the Crypt of Lieberkühn to the villus tip (Weizer,1973). In addition, this technique has demonstrated reductions in sodium ATPase activity in enterocytes close to the crypt relative to those at the villus tip (Rowling and Sepúlveda,1984). Investigations of villus-crypt enterocyte differences are not possible with either whole tissue or vesicle methodologies.

An appreciation of the physiological basis of the isolation techniques involved is of great importance when considering the use of the isolated enterocyte system, since different procedures isolate different cell types (Kimmich,1970;Weizer,1973;Ahrens et al,1985).

The small intestine is an undulating epithelium composed of enterocytes at various stages of development and differentiation.

Enterocytes are mitotically produced at the Crypt of Lieberkühn and ascend the villus until they finally migrate to the villus tip after 36-72 hours (Lebland and Messier,1958). The cells are ultimately shed into the intestinal lumen as mature enterocytes and differ in many of their properties from the immature Crypt

enterocytes (e.g.Rowling and Sepúlveda,1984.) The proximity of the enterocyte to the villus tip dictates the ease with which it is removed (Weizer,1973), and therefore the isolation method of choice (Kimmich,1970; Rowling and Sepúlveda,1984; Ahrens et al,1985).

Villus tip enterocytes show many transport and enzymatic properties generally associated with the absorption of nutrients, salts and fluids (Weizer,1973; Rowling and Sepúlveda,1984). Crypt enterocytes, however, show properties generally associated with fluid secretory cells (Fondacaro,1976). An example of this differentiation is the three fold higher sodium ATPase activity (often regarded as the driving force for fluid uptake) in villus tip cells compared to Crypt cells (Rowling and Sepúlveda,1984).

Within the intestine, therefore, there is an equilibrium of salt and fluid movement which results in either net absorption in the 'normal' case or net secretion in the case of secretory diarrhoea. To chose the relevant cell isolation technique it is important to discover whether a diarrhoeagenic agent's actions are due to an increase in crypt cell secretion or a reduction in villus tip cell absorption. A reasonable prediction can be gained from studies with the Ussing chamber, (Ussing, 1949). Ussing chamber measurements allow the calculation of unidirectional fluxes from the mucosal to serosal surfaces and vice-versa. Thus the unidirectional flux can be determined. For example, the bacterial toxin ST_a

causes a marked reduction in mucosal to serosal sodium flux whilst not interfering with the serosal to mucosal flux (Burgess,1983). When studying the effects of ST_a therefore, the most obvious choice would be enterocytes isolated from the villus tip.

A further complication to the choice of enterocyte isolation methodology is the considerable difference in cell morphology at different portions of the small intestine. For example, the mammalian jejunal region is thought to be the area of choice for the study of maximal carbohydrate fluxes,(Semenza et al,1984). Recent reports, however, indicate that jejunal enterocytes are less stable than those from the ileum (Mackenzie et al,1985)

The incorrect choice of intestinal area can therefore produce misleading results (Van Cornen et al,1986; Wahawisan et al,1985). The use of enterocytes from the entire intestine, however, results in the measurement of the 'mean' flux from a population of different cell types. Any effects on this 'mean' flux will almost certainly be smaller than flux effects in cells from the target region.

Careful attention should also be paid to the source of the intestine since there are large differences in the stability of isolated enterocytes from different animal sources (Watford,Lund and Krebs,1979). Both our present knowledge of regional variations in enterocyte properties and our understanding of villus crypt differentiation originates from the mammalian intestine (Lamb et al,1983). Enterocytes isolated from rat tissue are relatively unstable

(Watford et al,1979), although isolated rabbit enterocytes show good stability, good oxygen consumption rates and are the cells of choice particularly for studies of sodium ATPase activity (Brown and Sepúlveda,1985). The relative expense of rabbit tissue, however, has led to the use of chicken intestine, as isolated chicken enterocytes are quite stable (Kimmich,1970). Although there are numerous reports concerning the use of isolated chicken enterocytes (Kimmich,1970; Kimmich,1975; Hyan et al,1982), our understanding of the comparability of intestinal cells from mammalian and avian sources is limited.

Solute Transport In Vesicles.

Vesicles prepared from intestinal tissue retain the high levels of manipulatability seen with the isolated enterocyte system. Vesicles also have the advantage that they can be made specifically from either the brush border or basolateral membrane of the enterocyte. The ability to separate brush border and basolateral membrane events using vesicles has contributed greatly to our understanding of whole tissue processes.

Probably the most widely studied vesicular transport phenomenon is the intestinal hexose uptake system. The production of brush border vesicles has shown the existance of a sodium dependent hexose symport (Kessler et al, 1978), and since this time the proteins responsible have been well studied (Semenza et al,1984). Basolateral vesicles have shown an entirely different facilitative transport system(Wright et al,1980) which has also been

characterised in terms of specificity (Peerce et al,1984) and even to the degree of isolating and gaining molecular weights for the proteins responsible (Vezato et al,1986). This type of in depth study with its ability to distinguish between brush border and basolateral membrane effects is not possible with the isolated enterocyte system. Indeed this thesis would be complemented by knowing on which of these two membranes the sodium dependent and sodium independent citrate fluxes reside. The only way to do this is to use brush border and basolateral vesicles.

Sodium fluxes are of particular relevance to diarrhoeal disease. Brush border vesicles have shown that a significant sodium influx accompanies the hexose symport flux (Kessler et al,1978). Using vesicles, sodium influx components for the neutral amino acid-sodium symport have also been discovered (Freel and Goldner,1981). Similarly, this probably also applies to the basic and acidic amino acid symports (Lamb et al,1983; Storelli et al,1986). A further brush border sodium flux has been found with the di/tri carboxylic acid symport in renal tissue (Kippen et al,1982).

In addition to these brush border sodium influx mechanisms, vesicles have been employed to study sodium/proton (Na^+/H^+) exchange (Soriban,-Sohraby,1986) both at the brush border (Murer et al,1976;Grimstein et al,1986), and basolateral membranes of the cell (Barras et al,1986). Na^+/H^+ exchange has been widely studied in both renal and intestinal brush border. Photoaffinity probes have

been developed for this system (Benos et al,1978, Lazorick et al,1986; Sariban-Sohraby,1986), and the V_{max} enhancing effects of T_4, T_3 and Aldosterone on Na^+/H^+ exchange (Garty,1986; Kinella et al,1986, have also been studied.

Basolateral vesicles have been employed to investigate sodium ATPase activity,(Fondacaro,1986). Recently, vesicles have allowed the distinction between two forms of sodium ATPase activity. The first is the 'classical' ouabain sensitive sodium/potassium ATPase (Jorgensen,1982) and the other an ouabain insensitive electrogenic sodium ATPase not involving potassium (Del Castillo et al,1985 a; Del Castillo et al,1985 b).

Vesicles have also been used to study calcium movements during diarrhoeal disease (Ganguly et al,1985),(the relationship between Ca^{++} and diarrhoeal disease has previously been described). The study of cytoplasmically limited vesicles has shown the specific binding of Cholera toxin subunits to the external brush border membrane and the ADP ribosylation of intracellular GTP binding protein components (Dominguez et al,1985).

Acute viral diarrhoea has also been investigated using brush border vesicles (Butler et al,1974; Kerzner et al, 1977). It has been found that animals infected with TGA virus show a loss in flux through a high affinity sodium hexose symport, leaving only a low affinity symport fully functional (Kelja et al,1986).

It is only 'in depth' studies such as these that reveal

the mechanism of action of diarrhoeagenic agents, and with this knowledge aid our ability to ameliorate the disease symptoms. Brush border and basolateral vesicles were therefore investigated for their potential to complement the bacterial toxin and enterocyte studies.

Citrate Fluxes.

Investigations conducted by Burgess et al (1983) with the Ussing chamber (Ussing, 1949) have shown that citrate is actively transported across rabbit intestine. The sodium ATPase 'pump' inhibitor Ouabain has been seen to rapidly inhibit net citrate absorption and citrate flux through enterocytes has been proposed (Burgess, 1983). Implicit in any mechanism for this transcellular citrate flux must therefore be a route for citrate influx at the brush border surface and citrate efflux at the basolateral membrane. Further studies with the Ussing chamber have shown that citrate in the intestinal lumen increases the rate of intestinal transcellular sodium flux. This finding again indicates a link between the fluxes of sodium and citrate. The inseparability of sodium and fluid movements then hints at a possible explanation of the efficacy of citrate in oral rehydration solutions. However, calculations from the Ussing chamber have shown that there is a vastly dissimilar ratio between the fluxes of citrate and sodium. It has been estimated (Burgess, 1983) that 20 to 100 sodium ions cross the intestinal tissue in the Ussing chamber for the passage of each molecule of citrate. Such a proposition implies that citrate may have many quite different effects on intestinal tissue. These may then lead to a greatly enhanced transcellular sodium flux. If this is the case, then it follows that experimental systems more manipulatable than the Ussing

chamber may separate the multiple sodium flux enhancing effects of citrate.

Citrate fluxes in Kidney Tissue.

Kidney tissue possesses a sodium dependent di and tricarboxylic acid transporter. As there are many similarities between intestinal and renal epithelial tissues (Lamb et al,1983) the renal fluxes could have relevance to intestinal citrate movements (Wright et al,1982). The renal transporter resides in the brush border membrane where its sodium dependence is thought to allow active uptake of citrate from the glomerular filtrate (Kippen et al,1982). The renal brush border vesicles used for these studies have shown that citrate uptake can be competitively inhibited by the majority of citric acid cycle intermediates (Wright et al,1981). Although it is not known whether all of the cycle intermediates are transported, both α-keto-glutarate and succinate are transported at slower rates than citrate. Citrate uptake in this experimental system has also been shown to be pH dependent (Wright et al,1982). It was found that vesicles submitted to media at pH 5.5 transported citrate more rapidly than at pH 7.5. Wright et al, analysed their results assuming that the pH dependance of the renal citrate flux was solely due to substrate ionization. The analysis of the results of Wright et al in this manner led to the implication of citrate $^{I-}$ and citrate $^{2-}$ as the transported species.

Similar analysis by Wright et al for succinate has

shown that renal succinate transport is not affected by pH in the same way as citrate. This was interpreted as succinate being unable to exist in the poorly transported carboxylate $^{3-}$ form. This was further evidence for carboxylate $^{I-}$ and carboxylate $^{2-}$ as the transported species. Voltage sensitive fluorescent dyes have shown that renal sodium citrate transport is electrogenic (Wright et al, 1981). Sodium citrate movement must therefore occur as the movement of an over-all charged moiety. Wright et al, have proposed this moiety to be tri-sodium dianionic citrate ($3Na^{+}$ citrate $^{2-}$, an over all positive charge) at near physiological pH values. To test this hypothesis Wright and his co-workers studied the sodium-citrate stoichiometry of the renal sodium carboxylate carrier. They found that the calculations from several methodologies all produced sodium : citrate ratios of 3 sodium ions : 1 citrate molecule.

THESIS AIMS

The aims of this Thesis were to investigate the properties of enterocytes isolated by the method of Kimmich (1970). Once a suitable preparation procedure was established, it was proposed to prepare purified *E coli* ST_a and study any effects it might have on ion flux in the isolated enterocyte system.

To complement the study of the ST_a diarrhoeal disease processes, it was proposed to study oral rehydration solutions, currently the most effective treatment for diarrhoeal disease. Of particular interest was the oral rehydration constituent citrate (patented for use in ORT by Beecham) since little is understood about the action of this most efficacious component.

It was envisaged that the outlined aims would complement research currently undertaken by Beecham.

MATERIALS.

Enterocyte buffers.

Enterocyte isolation, storage and solute flux studies were carried out in the following media. This section is intended for reference as directed from graph legends.

Media 1 : This media (after Brown and Sepúlveda,1983) was used when enterocyte Na^+ ATPase was to determine the sodium gradient and had the following composition: NaCl 75 mM, $NaHCO_3$ 25mM, $CaCl_2$ 1.3 mM, $MgCl_2$ .5 mM, K_2HPO_4 .36 mM, KH_2PO_4 .44 mM, KCl 5 mM, β-hydroxy butyrate .5mM, D-mannitol 63 mM, HEPES 10 mM, .1% w/v dialysed BSA, 9 mg PMSF/500 ml. The media had a pH of 7.2, osmolarity 316 mOs moles/kg(by depression of freezing point osmometry) and was gassed with 95% O_2 / 5% CO_2.

Media 2 : This media was used for sodium gradient manipulation. Ratios of the isolation and transport media were varied to determine the sodium gradient.

Isolation and cell storage media composition (Na^+ free): mannitol 213 mM, $KHCO_3$ 25 mM, $CaCl_2$ 1.3 mM, $MgCl_2$ .5 mM, K_2HPO_4 .36 mM, KH_2PO_4 .44 mM, KCl 5 mM, HEPES 10 mM, .1% w/v dialysed BSA,30 µM rotenone, 100 µM ouabain, pH 7.2 osmolarity 316 mOs moles / kg. Transport medium (containing sodium): NaCl 75 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 1.3mM, $MgCl_2$ .5 mM, K_2HPO_4 .36 mM, KH_2PO_4 .44 mM, KCl 5 mM, D- mannitol, HEPES 10 mM,30 µM rotenone,

100 µM ouabain, pH 7, osmolarity 316 mOs moles/kg.

Media 3 : This media was used for sustaining pH gradients whilst measuring citrate flux and had the following composition:

Isolation media: as media 2.

Transport media: D-mannitol 63 mM, NaCl 50 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 1.3 mM, $MgCl_2$ 1 mM, KH_2PO_4 1 mM, MES 70 mM, adjusted to pH 6.0 with an identical solution with MES substituted with Tris base.

Media 4 : This media was used for enterocyte storage and experimentation whilst isolation was carried out in media 2. (See methods for equilibration times). This media has low buffering capacity and was designed for use in DMO dye movement studies, and had the following composition: D-mannitol 277 mM, $KHCO_3$ 1mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, HEPES or MES or Tris 5 mM, adjusted to the appropriate pH by varying ratios of the differently buffered solutions.

Media 5 : This media was used for the production of potassium/valinomycin membrane potentials.

Isolation and storage: D-mannitol 100 mM, $KHCO_3$ 25 mM, $CaCL_2$ 1.3 mM, $MgCl_2$ .5 mM, K_2HPO_4 .36 mM, KH_2PO_4 .44 mM, KCl 20 mM, K_2SO_4 27.5 mM, HEPES 10 mM, to pH 7.2 by addition of an identical solution with HEPES substituted by Tris base.

Transport buffer (low potassium): D-mannitol

108mM, $CaCl_2$ 5 mM, $MgCl_2$ 5 mM, KH_2PO_4 1mM, MES or HEPES 150 mM, adjusted to pH6.0 or 7.2 with an identical solution substituting MES or HEPES for Tris base.

Vesicle study buffers.

Brush border and basolateral membrane separation was carried out in the following buffers as described in the methods section:

300 mM mannitol medium: D-Mannitol 300 mM, HEPES 10 mM at pH 7.2 by addition of an identical solution substituting HEPES for Tris base.

50 mM mannitol medium: (cytolysis buffer) D-Mannitol 50 mM, HEPES 10mM, PMSF .1 mM, at pH 7.2 by addition of an identical solution substituting HEPES for Tris base.

Transport medium: NaSCN 150 mM, HEPES 10 mM pH 7.2 by addition of an identical solution substituting HEPES for Tris.

pH correction was carried out as indicated except when marker enzymes were to be assayed. When sucrase activity was to be determined, KOH was used as tris interferes with sucrase activity. When Na^+ ATPase activity was to be measured NaOH was used as potassium interferes with the Na^+ ATPase assay.

Polyacrylamide Gel Electrophoresis.

The following solutions were used for gel electrophoresis as indicated in the methods section:

15% acrylamide gel solution: 1.5 M gel buffer 4 ml, 30% acrylamide stock 8 ml, 75% glycerol 3.97ml, 10% ammonium persulphate 24 µl, TEMED 8 µl.

5% acrylamide gel solution: 1.5 M gel buffer 5 ml, 30% acrylamide stock 3.33 ml, distilled water 11.67 ml, 10% ammonium persulphate 30 µl, TEMED 10 µl. Remove 4 ml of the 5% solution (for use separately if necessary) before addition to the gradient mixer.

Gel buffer: 1.5 M tris base, 0.4% SDS at pH 8.8 with HCl.

Electrode buffer: 15.15 g tris base, 5g SDS, 12.4 g borate made up to 5 l pH 8.3.

Acrylamide stock: 30g acrylamide, 0.8g N'-N' Methylene bis acryladmide in 100 ml 75% glycerol.

Stain: 0.01% Coomassie blue 10% acetic acid, 30% methanol.

Destain: 10% acetic acid, 30% methanol, 60% water.

METHODS

Enterocyte Isolation.

Enterocytes (intestinal epithelial cells) were isolated from rabbits, chickens, rats and piglets.

Rabbits (New Zealand White, 2 - 3 kg) were anaesthetized using a gas mixture of Halothane (1-3%) in 50:50, oxygen: N_2O delivered to the animal at the rate of 1 - 2 litres/ minute. When anaesthetized, the rabbit was killed by cervical dislocation. Alternatively, rabbits were anaesthetized by an intra-vascular injection with sodium pentabarbitone. After removal of the intestine the rabbit was killed with an intra-cardiac injection of Euthatal.

Chickens (Rhode Island Red/Sussex Cross or White Leghorn, aged 4 - 10 weeks) and rats (male, Sprague Dawley, 170 - 190 g) were killed by rapid cervical dislocation.

Suckling piglets (3 - 12 days old) were removed from their sows 3 hours prior to use and anaesthetized using a gas mixture of Halothane (1-3%) in 50:50, oxygen:N_2O delivered to the piglet at the rate of 1-2 litres/minute. For piglet enterocyte isolation the intestine was removed under general anaesthetic. An intra-cardiac injection of 5 ml Euthatal was then administered to kill the animal.

Following the anaesthetic procedure in the case of the piglet, or death in the case of the rabbit, rat and chicken, the entire lower duodenum, jejunum and ileum was removed. After the removal of pancreatic and mesentary tissue the intestine was flushed through with isolation medium dispensed from a wash bottle. The intestine was then slit longitudinally to facilitate eversion and washed in fresh

isolation medium to remove adherent matter. The intestine was cut into 5 cm-lengths and placed into the isolation medium (pregassed when using medium 1 (see materials) with 95% O_2 : 5% CO_2) containing 2 mg/ml Hyaluronidase.

Hyaluronidase (Bovine testes, sigma H3506) catalyses the hydrolysis of β 1 - 4 linkages of the D-glucuronic acid β 1-3 N acetyl glucosamine disaccharides constituting the hyaluronic acid mucopolysaccharide polymer. This most abundant acidic mucopolysaccharide is present in the extracellular 'ground substance' of many tissues and thus its hydrolysis allows the release of enterocytes.

The use of an immersed magnetic stirer in a 37°C water bath and a magnetic follower bar in the isolation container ensured continual agitation and a physiological temperature. When using medium 1 (see materials) the solution was continually gassed with 95% O_2/5% CO_2.

After 30 minutes the contents of the isolation vessel were agitated with a plastic pipette tip and then filtered through a 250 μm nylon gauze, thus separating released cells from the whole intestine. The residual intestinal tissue was then discarded whilst the isolated cells in the hyaluronidase containing medium were placed at 37°C (gassed with 95% O_2/5% CO_2 when using medium 1) and constantly agitated with a magnetic stir bar. After ten minutes the cell suspension was placed in centrifuge tubes. The cells were then lightly sedimented in a bench centrifuge at 200 g

for one minute after which the supernatant was rapidly removed. The cells were then gently resuspended in hyaluronidase free isolation medium and recentrifuged. This procedure was repeated until the cells sedimented uniformly leaving a clear supernatant. Finally the cells were resuspended and stored for up to 2 hours as a 20%-60% v/v cytocrit. The medium used (see materials) and final cytocrit depended on the experimental protocol.

Variations on isolation protocol.

Cells to be ATP depleated were isolated in the presence of 30 μM rotenone and 100 μM Ouabain in all buffers (see materials). All metabolizable substrates (β hydroxy butyrate, glucose etc) were also omitted.

Enterocytes stored in buffers of pH other than pH 7.2 were resuspended in the new buffer and incubated at 37^{o}C for 5 minutes. The cells were then sedimented at 200g and resuspended in the buffer of new pH. This procedure was repeated a further two times to ensure a change in intracellular pH and to wash away the original pH 7.2 isolation buffer. When changing the internal pH of the enterocytes, cytocrit determinations were used to ensure there was no change in cell volume. Protein determinations were also carried out to ensure that the cytocrits compared before and after a pH change contained the same amount of protein.

Inhibitors such as Ouabain, phlorizin, phloretin, 8 Br cGMP, Cytocholasin B and Amiloride were prepared as stock solutions and added to the cells after isolation. Appropriate

controls were prepared to account for any dilution of the cell suspension and the initial dissolving solvent (DMSO, acetone etc.). After an incubation period of 5 minutes at 37°C, the cells were usually stored on ice.

Bacterial toxins such as Cholera toxin, E.coli P16 ST_a, and E. coli 431 ST_a were incubated with the cells at 37°C for up to 30 minutes. Appropriate controls were prepared to account for dilutions and the buffers used to store the toxins. The cells were then stored for limited periods of time at 37°C or at 0°C with appropriate 'warming up' periods before use in transport experiments.

Enterocytes to be used for the study of sodium efflux were isolated in media containing sodium and β-hydroxy butyrate with 0.5 mM glucose present only in the Hyaluronidase medium. Once the cells were isolated,3 µCi / ml ^{22}Na was introduced and the cells were incubated at 37°C for 10 minutes, The cells were then stored on ice until use.

Cells to be treated with Valinomycin were resuspended in isolation medium containing 40 µM Valinomycin and sedimented at 200 g. This valinomycin wash was repeated and the cells were adjusted to the appropriate cytocrit and stored on ice.

TRANSPORT STUDIES.

Sodium influx.

Sodium influx experiments were carried out by one of two methods. The first was adapted from that of Kimmich et al (1980). The low specific radioactivity of sodium in physiological sodium concentration buffers in combination with short flux periods presents special problems. This problem is seen as extremely small levels of radioactive sodium transported into the cells. To overcome this problem a second technique was designed and developed.

Method 1.

This method assumes that the non-transported ^{22}Na contaminating the pellet is negligible.

Sodium influx was initiated by the transfer of 200 µl of the 30% v/v stock enterocyte suspension to 200 µl of transport medium at 37°C containing 3 µCi/ml ^{22}Na. After incubation at 37°C for an accurately measured period of time, 10 ml of ice cold ^{22}Na free transport medium was introduced and the tube centrifuged at 2000g for 15 seconds. A fast accelerating refri~gerated centrifuge was used for this purpose. A 500 µl sample of the supernatant was taken and the remainder of the supernatant was quickly removed. The ^{22}Na activity associated with the pellet was then determined together with the supernatant sample using an LKB Ultrogamma NaI well scintillation counter.

For zero time points, cells were either added after the 10 ml diluting volume or cells were added to ice cold

^{22}Na followed directly by the diluting 10 ml volume.

All γ counts except backgrounds were counted to at least $\pm$ 1% error and usually $\pm$ 0.7%.

Method 2.

This method assumes that the non transported ^{22}Na 'trapped' with the pellet is appreciable. This extracellular 'trapped' ^{22}Na is then accounted for.

Sodium influx was initiated by the addition of 100 µl of the 30% stock cell suspension to 100 µl of transport medium at 37°C for an accurately measured period, 3 ml of ice cold transport medium containing 0.05 µ Ci/ml of ^{14}C labelled B.S.A. (Bovine Serum Albumin) was introduced. The cells were then sedimented in a refrigerated centrifuge at 2000 g for 15 seconds followed by the removal of the supernatant and a 500 µl supernatant sample. The pellet and the 500 µl sample were then γ counted.

After γ counting, 200 µl of 0.5% v/v triton X-100 was added to all samples and those containing cells were vortex mixed to ensure cell lysis and the liberation of Na-22. A 100 µl volume of unused cells was then added to the supernatant samples to act as a protein marker, and 3 ml of 10% trichloro acetic acid (T.C.A.) was added to all samples. The resulting precipitate was prepared by centrifugation at 3,000 g for 2 minutes followed by removal of the Na-22 containing T.C.A. supernatant. The addition of a further 3 ml T.C.A., centrifugation and removal of sodium-22 containing T.C.A. was repeated twice. The protein

pellets were prepared for liquid scintillation counting by adding approximately 5 glass beads and a small amount of a P.P.O. cocktail scintillant. Placing the tube on a vortex mixer allowed the beads to facilitate total solubilization of the protein pellet. Further scintillant was added to give a total volume of 3 ml in the tubes. After allowing 18 hours scintillant/sample equilibration away from fluorescent lightsthe samples in their tubes were β counted.

The zero time points were initiated by adding 3 ml of labelled B.S.A. transport medium before the introduction of the cells. Alternatively the cells were added to ice cold Na-22 followed immediately by the introduction of 3 ml ice cold labelled B.S.A. transport medium.

In addition, labelled B.S.A. free controls were prepared to test for Na-22 carryover to the liquid scintillation counting. (This carryover being typically twice β background).

The γ counts (^{22}Na) were background corrected, whilst β counts (14C B.S.A.) were corrected for Na-22 carryover/background, all determinations except background being counted to at least $\pm$ 1% and usually $\pm$ 0.7%.

Calculations: To correct for the Na-22 carried down with the pellet but not transported into the cells, the labelled B.S.A. is used as an extracellular marker.

Thus from knowing the B.S.A. activity in a 500 µl sample of the supernatant and from the B.S.A. activity in the pellet, it is possible to calculate the contaminating

extracellular volume of Na-22 with the pellet. From the contaminating volume of the pellet and the Na-22 activity in 500 µl of the supernatant, it is possible to calculate the non transported ^{22}Na contaminating the pellet.

Thus by subtraction, the Na-22 which has entered the cells during the transport experiment can be calculated, and thus n moles of sodium. A computer program was written by the author to process this data (see page 36).

Preparation of ^{14}C labelled B.S.A.

Reductively methylated ^{14}C B.S.A. was prepared using ^{14}C formaldehyde (Amersham Int. CFA.343,15 m Ci/m mole) by the method of Jentoff and Dearborn (1979).

An 8 ml volume of reaction mixture(containing 1 mg/ml dialysed B.S.A., 20 mM Na $CNBH_4$, 2 mM hepes corrected to pH 7.4 using NaOH) was added to 0.5 m Ci ^{14}C formaldehyde in a glass vessel. After incubation at 22°C for 48 hours, the reaction mixture was transferred to dialysis tubing.

The mixture was then extensively dialysed at 4°C against sodium free transport buffer.

Samples of the external medium were β counted to monitor the removal of non protein label down to background; this requiring at least a total of seven litres of transport buffer.

After dialysis a sample of the labelled B.S.A. was β counted and frozen in small aliquots such that each was never thawed and refrozen more than once.

EXAMPLE OF THE COMPUTER PROGRAM USED TO ANALYSE THE DATA GENERATED BY SODIUM FLUX METHOD 2

****

+++ CONTROL 1 1/2/85 +++

TIME	C.P.M.	CORRECTED C.P.M.	UG SODIUM	NMOL SODIUM	NMOL SODIUM / MILLION CELLS	CORRECTED NMOL SODIUM / MILLION CELLS
PELLET 00 SECS	G 2471	493.573312	1.08871825	47.3374696	2.75217847	0
	B 3101.25	CONTAMINATING VOLUME =	33.714278 UL			
PELLET 15 SECS	G 3414	1573.02452	3.46975913	150.865127	8.77122831	6.01904984
	B 2887.25	CONTAMINATING VOLUME =	31.3878433 UL			
PELLET 30 SECS	G 4290	2182.49833	4.81412934	209.318344	12.1696712	9.41749269
	B 3305.25	CONTAMINATING VOLUME =	35.9320007 UL			
PELLET 40 SECS	G 5313	3138.54797	6.92297249	301.010843	17.5006304	14.748452
	B 3410.25	CONTAMINATING VOLUME =	37.0734757 UL			
PELLET 50 SECS	G 5309	3477.58886	7.67082492	333.527468	19.3911318	16.6389534
	B 2872.25	CONTAMINATING VOLUME =	31.2247755 UL			
PELLET 60 SECS	G 5713	3796.14745	8.37349774	364.079682	21.1674234	18.4152449
	B 3006.25	CONTAMINATING VOLUME =	32.6815149 UL			
SUPNT 00 SECS	G 11738					
	B 18819.25					
SUPNT 15 SECS	G 11580					
	B 18368.25					
SUPNT 30 SECS	G 11571					
	B 18101.25					
SUPNT 40 SECS	G 11829					
	B 18402.25					
SUPNT 50 SECS	G 11800					
	B 18678.25					
SUPNT 60 SECS	G 11865					
	B 18014.25					

SLOPE = .313301388 INTERCEPT = .690903673

WEIGHTED SLOPE = .337939471 WEIGHTED INTERCEPT = 1.73336155E-32

THIS DATA DOES NOT DIFFER SIGNIFICANTLY FROM A STRAIGHT LINE

VIABLE CELL COUNT = 172 MILLION CELLS / ML (17.2 MILLION CELLS PER PELLET.)

ALL RESULTS BACKGROUND CORRECTED

****

BACKGROUND DETERMINATIONS

DETERMINATION	C.P.M
GAMMA 1	88
BETA 2	38
BETA 3	24
BETA 4	21
BETA 5	40

MEAN GAMMA BACKGROUND = 88
MEAN BETA CARRY-OVER/BACKGROUND = 30.75

Citrate and sugar flux.

1. Standard procedure.

A 200 µl aliquot of the 30% v/v cell suspension was introduced to a 200 µl volume of transport medium. The transport medium contained an appropriate concentration of sugar or citrate and 0.3 µCi of isotopic label at 37°C. After incubation at 37°C with constant agitation for an accurately measured period of time, 10 ml of ice cold isotope free transport buffer was swiftly added. The tube was immediately centrifuged at 3000g for 15 seconds at 4°C followed by prompt removal of the supernatant and a supernatant sample.

Pellet samples were prepared for liquid scintillation counting by adding 400 µl of 10% w/v T.C.A., Vortex mixing, and centrifuging at 3000g for 10 minutes. A 300 µl volume of the T.C.A. supernatant was then counted. Alternatively, in cases where pellet volumes varied, a 200 µl volume of water was added, followed by vortex mixing and then vortex mixing with scintillant. Scintran emulsifier cocktail 'T' (BDH Chemicals) was used, with β counting to at least $\pm$ 1% after the recommended 18 hours equilibration period.

2. Cells with an inside negative membrane potential.

This procedure was developed from the method of Hoshi et al,(1986) and adapted for use with cells. This method allows a period of time for the potassium loaded cells to release their potassium through a Valinomycin channel.

After the production of this transient membrane potential,

isotope flux is studied.

A 200 µl aliquot of a 30% v/v cell suspension was added to a 150 µl volume of isotope free transport medium at 37°C. After an accurately measured period (often 2 minutes), a 50 µl volume of isotope at the appropriate specific activity was introduced and constantly agitated for a further period of time. Transport was terminated by the addition of 10 ml ice cold transport medium and the samples were treated as above.

The Determination of Cell Viability.

Cell viability was routinely assessed by two methods; one measuring transport ability and the other cell membrane integrity.

To measure loss in transport ability as the experiment proceeded, a one minute influx time point was measured in duplicate at the beginning of the transport study and at the end of the investigation. Thus any loss in transport phenomena could be observed.

Cell membrane integrity was assessed using the phenomenon that intact cells are able to exclude the dye trypan or direct blue. (Sigma T-0887).

After an appropriate dilution in isotonic medium, the diluted suspension was further diluted 1/10 in 0.1% w/v trypan blue in P.B.S. The cells were allowed one minute to ensure penetration of the dye into lysed cells and were then counted, the initial dilution giving approximately 100 cells on a standard cytometer. Phase contrast optics and a magnification of 40 x were used for visualization,

the viable cells appearing white against the darker background and dark non viable cells.

Measurement of changes in pH gradient.

The measurement of cellular pH changes was carried out using a newly developed centrifugation technique. The development of this new technique was necessitated by the lack of availability of the perfusion apparatus used by Aronson et al,(1980).

Enterocytes were isolated and equilibrated in 5 mM buffers (see materials) of differing pH (as described in 'Variations in enterocyte isolation protocol'), and stored on ice. A 1.5 ml volume of buffer, to become the extracellular medium, was placed in a plastic vessel and held at 37°C with continuous stirring from a magnetic stirrer. A 1 µCi quantity of 14 C Dimethyloxazolidine Dione (DMO) was then added and allowed to mix. Cell suspension warmed to 37°C. 1·5 ml cells added to DMO solution. After 1 minute a 300 µl sample of the suspension was taken and swiftly placed into a microcentrifuge tube. This was followed by centrifugation for 10 seconds at 10,000g, 25°C, and the immediate removal of the supernatant. This process was repeated each minute necessitating a fast accelerating centrifuge and careful organization.

After a certain number of samples, a concentrated unbuffered solution of test additive was introduced (e.g. 97 mM Na_2SO_4) at a set time. Sampling was then resumed at 1 minute intervals for the appropriate period of time.

The 100 µl supernatant samples were counted in a liquid scintillation counter in Scintran cocktail 'T' to ± 1%

after 18 hours equilibration time.

Citrate Chromatography.

In order to determine whether citrate metabolism had taken place, a citrate influx experiment was carried out as previously described using 2 mM citrate and 10 μCi/ml $^{14}_{C}$ citrate.

The enterocyte pellets, with intracellular labelled citrate, were lysed with 500 μl TCA and vortex mixing. After centrifugation at 3000g for 10 minutes to deproteinize the samples, the TCA supernatant was removed and the pellet discarded.

Dowex cat - ion exchange beads were used to remove salt from the samples and to ensure that citric acid cycle intermediates were chromatographically separated as their acidic forms. Dowex cat - ion exchange beads were prepared by placing Dowex 50w -X 8 beads in a ten fold excess of 5 M HCl. After mixing and allowing to stand for 5 minutes, the acid was decanted off and a fresh volume of 5 M HCl added. This procedure was repeated a total of five times. The slurr y was then placed on a glass scinter and washed with double distilled water, until the wash through had a neutral pH.

Washed Dowex beads were then added to the samples (as an amount equivalent in volume to half the sample volume) and the samples were vortex mixed. After removing the TCA liquid phase and adding it to more washed Dowex beads, the samples were ready to be chromatographed.

Standards were prepared by adding washed Dowex beads to

15 mg/ml solutions of citrate, isocitrate, succinate, oxalocetate, 2 oxo glutarate, fumarate, malate, glycine, glutamate and valine.

A 20 µl volume of selected standards and 100µl of the labelled citrate sample were then applied to Whatman N° 1 paper and carefully dried using minimal heat in the standard manner.

The solvent system was prepared by mixing ethyl-acetate : 4.4 M acetic acid in the ratio 1 : 2. After mixing and partitioning, the ethyl acetate saturated with acetic acid (top layer) was decanted and used as the chromatographic solvent.

After running, the chromatogram was dried and sprayed with 0.04% Bromophenol blue in ethanol. When the R_f values had been calculated, the track containing the labelled citrate sample was cut into 40 segments. Each segment was placed in a scintillation vial and the segment was soaked with 500 µl of distilled water. A 5 ml volume of scintillant was added followed by 18 hours equilibration time before counting.

Assay for Enterocyte cGMP Content.

Enterocytes were isolated from piglets as previously described. The cells were allowed to warm to 37°C and appropriate quantities of toxin or control diluent were added. After mixing, a 500 µl sample of the suspension was taken and added to 1 ml of 10% TCA. Further samples were taken after 15 and 30 minutes incubation at 37°C and treated in the same manner. The TCA precipitates

were then vortex mixed and centrifuged for 10 minutes at 3000 g. The supernatants were quantitatively removed and 100 µl 1 M HCl was added to each tube. The TCA samples were then washed with water saturated ether, to remove all traces of TCA which would interfere with the RIA procedure.

Water saturated ether was prepared by shaking ether with double distilled water. After allowing to separate for two hours, the water saturated ether phase was decanted off ready for use. The samples were washed by placing 9 ml of water saturated ether with each of the samples and mixing by inversion. This was repeated 5 times and finally the water saturated ether was removed. The aqueous phase of each sample was then placed in a freeze dryer and each sample was taken to near dryness before adding 0.5 ml of 50 mM tris/HCl, 4 mM EDTA pH 7.5. The pH of each sample was adjusted to pH 7.5 using NaOH. Each sample was made up to 1 ml using 50 mM tris/HCl buffer.

A 100 µl volume of each sample and 100 µl volumes of dilutions of up to 50 fold were then prepared. The cGMP content of the samples and dilutions were determined using the Amersham cGMP radio-immuno assay kit (TRK 500).

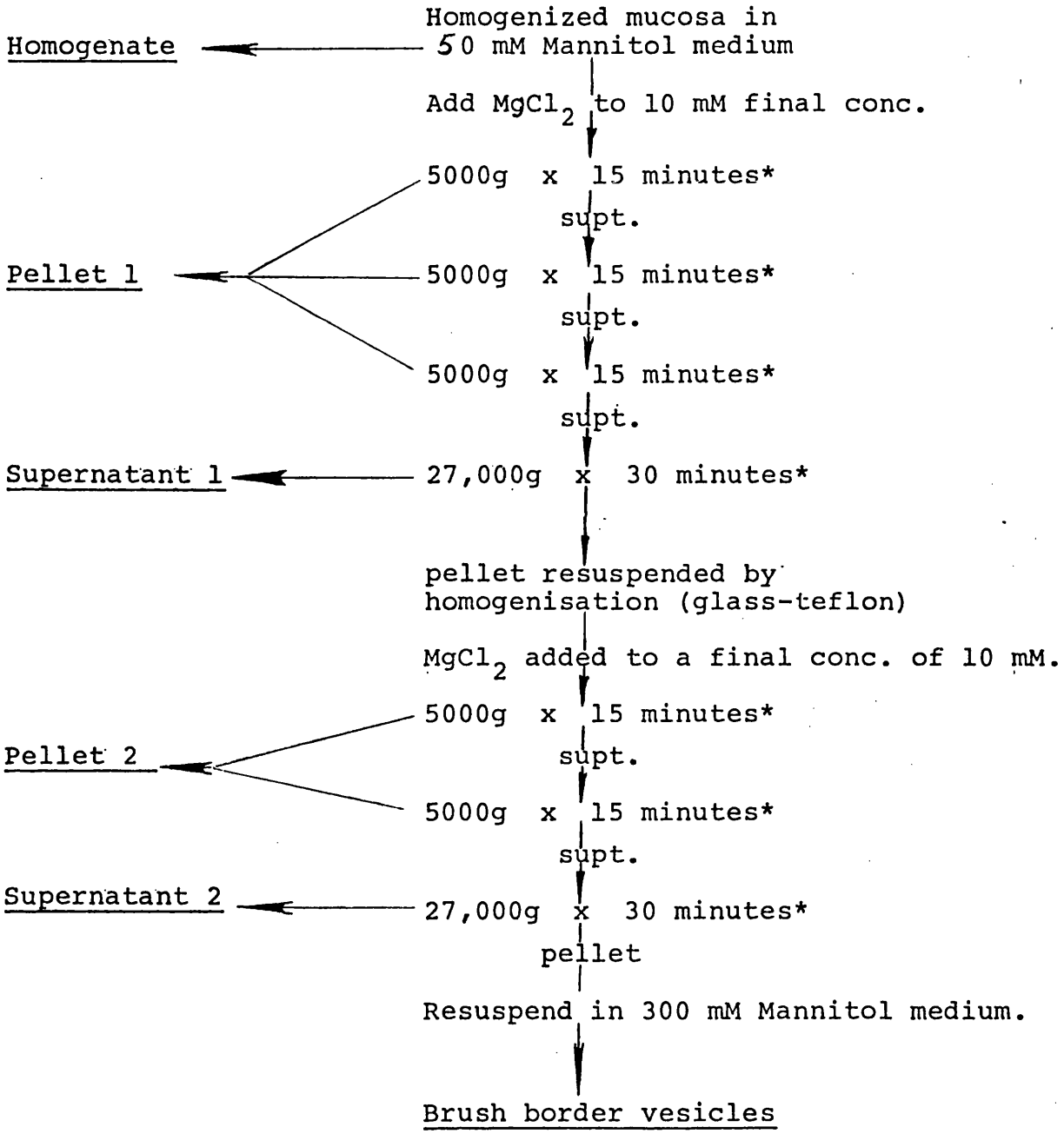
Brush Border Vesicle Preparation.

Brush border vesicles were prepared from rat and rabbit small intestine. (The intestine was used either fresh or after storage at -20°C).

The animal was killed by cervical dislocation and the entire small intestine swiftly removed. The intestine was then flushed with 300 mM mannitol solution (see materials) and stored on ice or rapidly frozen using solid CO_2 for longer storage. The intestine from six rats (male Sprague Dawley, weight 170 - 200 g) or from one rabbit (New Zealand White, 2 - 3 kg) was used for each preparation.

The intestinal tissue was slit longitudinally, rinsed in ice cold 300 mM mannitol medium and placed on paper with the mucosal surface upwards. The mucosal surface was carefully scraped away using a blunt spatula and placed into 500 ml of an ice cold solution of 50 mM mannitol (containing 0.1 mM P.M.S.F.). The mucosal medium was then placed into a Waring commercial blender and homogenized for two minutes at high speed. Magnesium chloride was added to the suspension to give a final concentration of 10 mM $MgCl_2$ and was allowed to stand on ice in centrifuge tubes. The $MgCl_2$ suspension was then centrifuged at 4°C for 15 minutes at 5000 g, followed by the removal of the pellet. This centrifugation procedure was repeated twice. The supernatant was then centrifuged at 27,000 g for 30 minutes. This supernatant was discarded whilst the pellet was resuspended in 10 ml of 300 mM mannitol medium and homogenized using a glass teflon Braun-Melsungen homogenizer for 20

Flow diagram for Brush Border Vesicle Preparation



All stages carried out at $4^{o}C$

* centrifugation data

strokes at 900 rpm. A further 40 ml of 300 mM Mannitol medium was then introduced and magnesium chloride was added to give a final concentration of 10 mM $MgCl_2$. After 15 minutes on ice the suspension was centrifuged at 5000 g for 15 mintues. This was followed by the removal of the pellet and centrifugation of the supernatant at 5000 g for 15 mintues. The pellet was then discarded and the supernatant was centrifuged 27,000 g for 30 minutes. The clear supernatant was discarded, whilst the pellet was resuspended in 3 ml of 300 mM mannitol medium. The resuspended pellet was homogenized in a glass teflon homogenizer for 20 strokes at 9000 rpm. The vesicles produced were then stored on ice until use at a protein concentration of 1 mg/ml.

Marker Enzyme Determinations.

The activities of enzymes characteristic of certain portions of the enterocyte membrane were regularly assessed to follow the separation procedure during vesicle production.

Sucrase Assay.

This brush border membrane marker was assayed either by a modification of the standard Hexokinase assay, or by modifying the discontinuous method of Dahlquist (1964) to produce a continuous assay.

The sucrose was specially purified by preparative paper chromatography before use, to remove the small

quantity of contaminating glucose present.

The modified Hexokinase assay was carried out in a buffer containing 10 mM $MgSO_4$, 50 mM tris/Hepes at pH 7.5. To 915 µl of this buffer was added 10 µl of 500 µM purified sucrose, 10 µl of a 40 mM stock solution of NADP, 10 µl of 100 mM ATP, 50 µl of test sample and 5 µl of glucose 6 phosphate dehydrogenase/Hexokinase enzyme solution, (Bohringer). The production of NADPH was followed at 340 nm using a spectrophotometer. Appropriate controls were constructed for each fraction tested; the most appreciable background rates being due to NADP reduction by enzymes not utilizing sucrose.

In cases where solutions were optically dark or where NADP reduction rates were high the following modified technique was employed.

A 5 µl volume of purified sucrose was added to 975 µl of a solution of peroxidase and glucose oxidase enzymes (Sigma P.G.O. capsules) with previously added σ-dianisidine fom a 2.5 mg/ml solution in a 50:1 ratio. After the addition of 20 µl of membrane fraction the enzymatic rate was followed at 450 nm. Whilst negligible backgrounds were noted in the absence of substrate, contaminants of the sucrose often produced small but significant background rates. These background rates were accounted for in the calculation of sucrase activity. (Tris buffers inhibit sucrase activity - see material section for buffers).

Alkaline Phosphatase.

This second enzymatic brush border marker, (Quigley et al, 1956) is probably less predominantly situated on the mucosal

membrane than sucrase (Quigley et al, 1956). This enzyme was assayed by introducing 40 µl of 6 mM p-nitrophenyl phosphate to 950 µl of a buffer containing 30 mM glycine, 5 mM $MgCl_2$ and 1 mM $ZnCL_2$ adjusted to pH 9.1 with NaOH. After the addition of a 10 µl sample of membrane fraction, the increase in absorbance at 405 nm was followed. The background rates were negligible.

Na^+/K^+ A.T.Pase Activity.

The basolateral membrane marker was assayed by two methods. A 10 µl volume of 6 mM p-nitrophenyl phosphate was added to 900 µl of a buffer which contained: 50 mM $MgSO_4$, 5 mM E.D.T.A., 100 mM tris adjusted to pH 7.8 with HCl. Before the introduction of the membrane fraction sample (20 µl), 40 µl of either 150 mM KCl or 150 mM NaCl was added. The rate of K^+ activated phosphatase (presumed to be another activity of the Na^+/K^+ ATPase) (Kessler et al,1978; Forte et al, 1967) could then be determined by subtracting the phosphatase rates in the presence of Na^+ from those in the presence of potassium. As the pH necessary for the measurement of activity is lower than the ideal for maximum colour intensity of the p-nitrophenyl product, this method is recommended only for comparative studies.

The second method involves seven tubes containing 350µl of a buffer containing 45 mM ATP, 7.5 mM $MgCl_2$, 0.75 mM H_2Na_2EDTA, 30 mM imidazole, 30 mM glycylglycine adjusted to pH 7.4 with sodium hydroxide. To these tubes the following were added:

1) 50 µl 1.5 M NaCl 0.3 M KCl + 50 µl dist. H_2O.

2) 50 µl 1.5 M NaCl 0.3 M KCl + (an extra 50 µl of enzyme extract.)

3) 50 µl 1.5 M NaCl 0.3 M KCl + 50 µl 2.5 mM ouabain.

4) 50 µl 2.5 mM ouabain + 50 µl dist. H_2O.

5) 50 µl NaCl KCl + 10 µM oligomycin.

6) 50 µl 10 µM oligomycin + 50 µl dist. H_2O.

7) 100 µl H_2O (kept on ice for incubation period).

After the addition of 50 µl of membrane fraction to tubes 1 and 3 - 7 and 100 µl to tube 2, samples 1 - 6 were incubated at 37°C for 20 minutes and sample 7 was stored on ice. After this period of time, 700 µl of 10% TCA was added to all tubes followed by centrifugation for 3 minutes at 10,000 g. Volumes of 100 µl from each supernatant were then removed and added to tubes containing 200 µl acid molybdate and 500 µl distilled water. In addition a 'blank' was prepared using 400 µl 10% TCA instead of the enzyme extract and 100 µl of Fiske-Subbarow reagent(Sigma) was added to all tubes. After 10 mintues the absorbance at 650 nm was recorded relative to the TCA blank. Results were only accepted if a doubling in enzyme concentration produced a doubling in rate.

Acid Phosphatase.

This membrane marker is associated predominantly with microsomes (Quigley et al, 1956) and is assayed by the addition of 50 µl of 6 mM p-nitrophenyl phosphate to 900 µl 50 mM citrate buffer adjusted to pH 5.0 with NaOH. After the addition of 50 µl sample the tube is incubated at 20°C for 20 minutes followed by the addition of 1 ml 1M NaOH.

The absorbance at 405 nm relative to substrate blanks can then be measured. However, it must be ensured that the enzyme activity over this 20 minute time period is linear.

Lactate Dehydrogenase.

The assay of this soluble enzyme allows determination of the contamination of extra vesicular non membrane linked cellular constituents. An 840 µl volume of 0.1 M phosphatase buffer pH 7.2 was added to 100 µl of 20 mM lactate and 10 µl of 2 mM NAD^+ followed by the addition of 50 µl of membrane fraction. The increase in absorbance was followed at 340 nm.

Sugar Transport Studies with Brush Border Vesicles.

A 50 µl volume of vesicle suspension was added to 50 µl of vesicle transport medium containing 1 mM glucose and 0.6 µCi U^{14}C glucose or 1 mM galactose and 0.6 µCi 1 ^{14}C galactose. The tube was then incubated at 20^{o}C whilst being continually agitated for an accurately measured period of time. A 1 ml volume of ice cold isotope free vesicle transport medium was then rapidly added. This was followed by the removal of a 1 ml volume of now diluted vesicle/isotope suspension. The 1 ml volume was then placed onto a glass fibre or Millipore membrane filter (0.7µm - 0.22µM) under reduced pressure producing a liquid flow rate of 50 ml/minute. The filter was then washed with 10 ml ice cold isotope free vesicle transport medium and both the filter and a sample from the 100 µl left in the tube were β counted. Samples were allowed the recommended 18 hours equilibration and solublization time in Scintran cocktail 'T' emulsifier

cocktail (BDH chemicals) before counting.

Electron Microscopy.

To investigate brush border vesicle membrane integrity and size variation, transmission electron microscopy was employed. Vesicles were prepared and centrifuged at $27 \times 10^3 g$ for 30 minutes. The pellet was then resuspended in isosmotic buffer, containing 4% glutaraldehyde. This method was used to retain osmolarity until samples were fully fixed. The samples were then left overnight at $4^{\circ}C$. The suspension was then recentrifuged and resuspended in isosmotic buffer without glutaraldehyde. After repeating this wash procedure the vesicles were resuspended in 1 ml of the same buffer. This was followed by incubation at $20^{\circ}C$ in an equal volume of 2% Os O_4 for 1 hour. The suspension was then centrifuged at 27,000g for 30 minutes followed by resuspension in distilled water and recentrifugation as described. The pellet was then disrupted into small fragments and placed into a number of small tubes. A solution of 2% w/v agarose was prepared, liquified, and allowed to cool to $40^{\circ}C$ at which point a small volume was taken up into a pre-warmed pasteur pipette and deposited into a tube of pellet fragments. The agarose and fragments were then swiftly sucked up into the warm pasteur pipette and extruded onto a glass plate just prior to solidifying inside the pasteur pipette. The resulting agarose cylinders were cut into 1 mm lengths and those obviously containing pellet fragments were dehydrated for fifteen minutes in two changes of fresh acetone at concentrations of 30%,50%,70% and 100%v/v.

An equal volume of Epon resin was then added to the acetone agarose blocks and gently rotated for 12 hours. The resin was then removed, replaced with fresh Epon and rotated for a further 8 hours. The resin was replaced once more and after 12 hours rotation the agarose blocks were placed into a sectioning mould. After solidifying the resin in an oven for two days the block faces were trimmed and sections were produced by glass knife microtomy. A high power microscope was used to determine which sections were worthy of further investigation. Selected sections, when mounted on their grids, were further stained with Uranyl acetate and lead citrate to improve contrast when viewing under electron optics.

Polyacrylamide Gel Electrophoresis.

Gradient slab gels of 5 - 15% polyacrylamide were prepared using glass plates cleaned in ethanolic potassium hydroxide and carefully sealed with Pharmacia gel tape. Gel solutions of 15% amd 5% acrylamide (see materials) were prepared and poured into the plates using a standard gradient mixer with peristaltic pump. The gel was then left overnight with a sample well comb to set.

Samples were solubilized by adding 10 µl sample buffer (see materials) per 70 µl membrane sample and allowing to stand for 45 minutes at room temperature. A 5 µl volume of bromophenol blue solution was added to each sample and the appropriate volume (300 µg - 500 µg protein) was introduced to the sample wells using microtips and an automatic pipette.

After running the gel for 14 hours at 27 mA with appropriate molecular weight standards, the gel was carefully removed from the plates and stained for 4 hours (see materials) followed by destaining, until bands were clearly visible.

Purification of E coli - 431 STa

Heat stable enterotoxin was produced on a large scale by Beecham. Their procedure produced 160 litres of E coli -431 culture which was clarified by continuous centrifugation. After concentration, the supernatant was filtered with a 10,000 molecular weight cut off membrane, of which the retentate was discarded, followed by a 1,000 MW cut off membrane. After lyophilization the retentate from the second filter, with its constituents in the M.W. range 1,000 to 10,000, was stored at 20°C for an appreciable period of time and then taken through the following procedure by the author.

The 1,000 to 10,000 M.W. freeze dried retentate was carefully dissolved in de-ionised water at a protein concentration of between 5 and 10 mg/ml. The pH of this suspension was measured and accurately adjusted to 8.15 by the addition of 10 M sodium hydroxide. The volume of the suspension was then recorded and a sample taken for assay of biological activity. The suspension was stirred at 4°C for 30 minutes, after which precooled acetone was added dropwise, from a dropping funnel to give a 5 : 1 v/v ratio of aqueous sample/acetone. The resultant suspension was then stirred at 4°C for 30 minutes, followed by centrifugation at 12,000g and 5°C for 30 minutes using

acetone resistant polypropylene containers. The precipitate was then discarded.

The pH of the decanted supernatant was measured at 20°C and adjusted back to 8.15 with 5 M hydrochloric acid or 10 M sodium hydroxide. The supernatant was then stirred at 4°C whilst precooled acetone was added dropwise, from a dropping funnel, to give a 1 : 1 ratio of aqueous sample/acetone (using the original measured volume as that of the aqueous phase). At this stage the mixture was triphasic, consisting of a solution, a precipitate, and a small volume of an oil-like substance. The precipitate was separated from the other two phases by centrifugation as above and discarded.

The pH of the supernatant and 'oil' was adjusted to 8.15 at 20°C and the mixture was then stirred at 4°C whilst precooled acetone was added dropwise to give a 1 : 9 ratio of aqueous sample/acetone. This again resulted in a triphasic system consisting of a fine precipitate, a solution and a viscous oil-like material which adhered to the walls of the glass vessel. The precipitate was separated from the solution by centrifugation as described and the resultant supernatant was held at 4°C.

The precipitate was dissolved in 20 ml of de-ionised water and its pH recorded at 20°C. After the addition of 0.1 M sodium hydroxide to pH 8.15, the solution was stirred at 4°C whilst 180 ml of precooled acetone was added dropwise. The precipitate was separated by centrifugation (as before) and discarded. The resulting supernatant was combined with the supernatant prepared earlier and held

at 4°C.

The oil-like substance which adhered to the original glass vessel was dissolved in 20 ml of de-ionised water and its pH was adjusted to 8.15 with 0.1M sodium hydroxide. This solution was then stirred at 4°C whilst 180ml of precooled acetone was added dropwise. The 'oil' once again reformed and adhered to the glass vessel. The solution was decanted off from the oil and combined with the pooled supernatant prepared earlier, The 'oil' like material was discarded. The combined supernatant was then evaporated to near dryness by rotary evaporation under reduced pressure at 37°C, diluted back to half its original volume with de-ionised water and stored overnight at 4°C.

The aqueous solution was then stirred at room temperature and 6 volumes of 2 : 1 v/v methanolic chloroform were slowly added (1 volume being the present volume of aqueous phase). The resulting solution was then placed in a separating funnel and 6 volumes of chloroform were added followed by one volume of de-ionised water. The flask was then vigorously shaken, releasing pressure, for 1 minute, and the resultant emulsion was allowed to separate into two phases. After separation of the two phases the methanol/water phase was held at 4°C and the chloroform phase was re-extracted twice using one volume of water each time. After each extraction the aqueous phase was combined with the above methanol/aqueous solution. The chloroform phase was then discarded.

The combined aqueous methanol phase was then concentrated

to approximately 5 mls by vacuum rotary evaporation at 37°C and diluted back to its volume before chloroform treatment with de-ionised water. A sample for Na^+ content and biological activity was then taken.

The solution was dialysed for 72 hours against 5 litres of water at 4°C using Spectra Por 6 (M.W. cut off 2,000) dialysis tubing. The contents of the dialysis bag were then concentrated to half the original volume and stored at -20°C in apropriate small aliquots.

Infant Mouse Bioassay.

The infant mouse bioassay (Gianella, 1976) was used to quantitate the biological activity of ST_a during its production from E coli - 431 crude extract.

Four day old infant MF1 mice were removed from their mothers and randomly assigned to a treatment group. The mice were then orally dosed with a known dilution of the STa fraction in P.B.S from a glass graduated syringe and blunt 21 gauge needle. The infants were given a weight related dose at the rate of 20 ml/kg. After dosing, the infant mice were placed in their treatment groups on absorbent paper and left at 21°C for 3 hours with monitoring for signs of scour at half hour intervals.

After 3 hours the infants were killed by cervical dislocation and the weights of the entire intestine and remaining carcass were determined. The gut weight/body weight ratio calculated thus allowed determination of the biological activity of ST_a fractions, with the amount of ST_a producing a GW/BW ratio of 0.09 being defined as 1 mouse unit (m.u.)

Protein Determination.

Protein was determined by the method of Lowry et al,(1951) using the Folin-Ciocalteau reagent or by a modification of this method using the more convenient recently developed Bicinchoninic acid reagent (Pierce Chemical Co.). The Bradford method (Coomassie) was also employed in certain cases.

Sodium Determinations.

Sodium was determined using an Eel X 117 flame photometer with appropriate filters. Standards were prepared from 'AnalaR' sodium chloride.

Plasma Membrane Separation.

This membrane separation procedure was developed to purify enterocyte plasma membrane and to investigate the feasibility of producing membrane fractions with enriched basolateral membrane activity. It was anticipated that vesicles enriched in basolateral membrane activity could complement the brush border vesicles described on page 43 The technique was also used for photoaffinity labelling of the intestinal hexose transporters.

The enterocytes were isolated from chick intestine as previously described (page 28) and stored on ice. In the case of photoaffinity labelling experiments the labels were introduced at this stage. The photoaffinity label was incubated with cells either in the presence or absence of 'cold' displacing ligand (e.g. glucose). After U.V. irradiation the cells were washed by centrifugation to remove unbound photoaffinity ligand.

A 5 ml volume of 20% v/v cells constituting 20 mg of

of protein, was then added to ice cold cytolysing buffer containing protease inhibitors (see materials). The lysed cells were then homogenized using a Braun-Melsungen homogenizer for 20 strokes at 1500 rpm. The homogenate was centrifuged for 30 minutes and the supernatant was discarded. The pellet was then resuspended and made up to a volume of 4 ml with homogenizing buffer. The resuspension was then carefully layered onto a 3 ml volume of sorbitol of density 1.19 (approx. 52% sorbitol). The step gradient produced was then centrifuged at 44,000 g for 60 minutes at 4^{o}C, after which the cell debris pellet was discarded. The plasma membrane present at the interface was then assayed for marker enzyme activity or solubilized and loaded onto a polyacrylamide gradient gel,(see earlier).

RESULTS

The suitability of enterocytes from different sources to flux studies.

Previous reports of enterocyte isolation have been confined to only a few animal species (Hyan et al, 1982. Brown and Sepúlveda, 1983). The earliest and most numerous reports concern the chicken enterocyte (Kimmich,1970; Kimmich et al,1975). More recently the rabbit has been used for enterocyte production (Brown and Sepúlveda,1985). The results in figure 1 show how chicken enterocytes, unlike rabbit cells, exhibit an age related loss in measurable Na^+ ATPase activity. The histograms indicate the galactose uptake when rabbit and chicken enterocytes determine their own transmembrane sodium gradient. Gradient production is caused by the action of membrane bound Na^+ ATPase activity. In the absence of a sodium gradient, the internal galactose concentration is approaching equilibrium with the external solution after 8 minutes. This occurs in cells where the sodium gradient has been dissipated by the use of the Na^+ ATPase inhibitor ouabain. In the absence of ouabain it can be seen that the sodium gradient causes an increase in galactose uptake of up to three fold for rabbit enterocytes. When comparing the results for enterocytes isolated from chickens of varying ages, there is a marked difference in ouabain sensitive galactose uptake. It can be seen that enterocytes isolated from chickens less than 3 weeks of age show galactose uptakes 2 fold higher than in the presence of ouabain, whereas cells from 10 week old chickens show similar uptakes with or without ouabain.

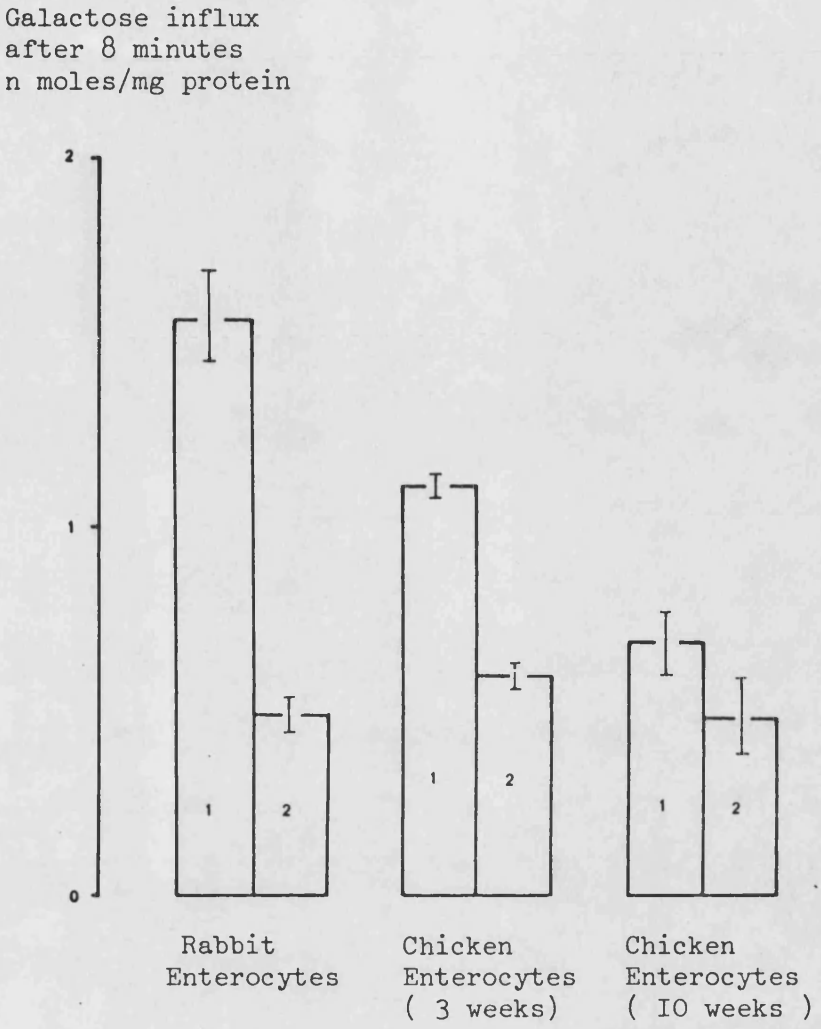


Figure I

The Variation In Sodium Gradient Experienced With Enterocytes From Different Sources And Ages - Shown As Differences In Ability To Accumulate Galactose.

All enterocytes were isolated in medium I containing sodium at pH 7.2 (see materials). After isolation, cells were stored at 37°C for 30 minutes and then the above measurements of I mM galactose influx after 8 minutes were made.
Error bars: ± S.E.

1 control 2 +Ouabain (IOOuM)

Enterocytes isolated from older chickens therefore appear to be unable to maintain their own sodium gradient.

Galactose influx after a short time period(corresponding to initial rate) is shown in figure 2 under conditions where the sodium gradient has been artificially produced. The initial rate of galactose uptake into enterocytes isolated from a 10 week old chicken can be seen to be almost completely inhibitable by phlorizin. This indicates that the enterocytes from older chickens retain their membrane integrity although they suffer from low Na^+ ATPase activity once isolated.

Figure 2 also shows the phlorizin inhibitable galactose uptake for piglet enterocytes under the same conditions. It can be seen that piglet enterocytes show similar high levels of membrane integrity once isolated. In both cases the small galactose flux in the presence of phlorizin probably reflects sodium independent sugar transport.

The efflux of radio labelled sodium from preloaded piglet enterocytes is shown in figure 3. The pre-incubation of cells with radio labelled sodium initially at 37^oC, and subsequently at 0^oC, reduces sodium ATPase activity prior to the efflux studies. This protocol produces cells with similar amounts of intracellular sodium, irrespective of the Na^+ ATPase inhibitor ouabain. When the cells are subsequently placed in a medium containing unlabelled sodium at 37^oC exchange takes place until the specific radio-activity of intracellular sodium is equivalent to that outside the cells. The action of Na^+ ATPase is seen as an increase in sodium efflux. Initially this is seen

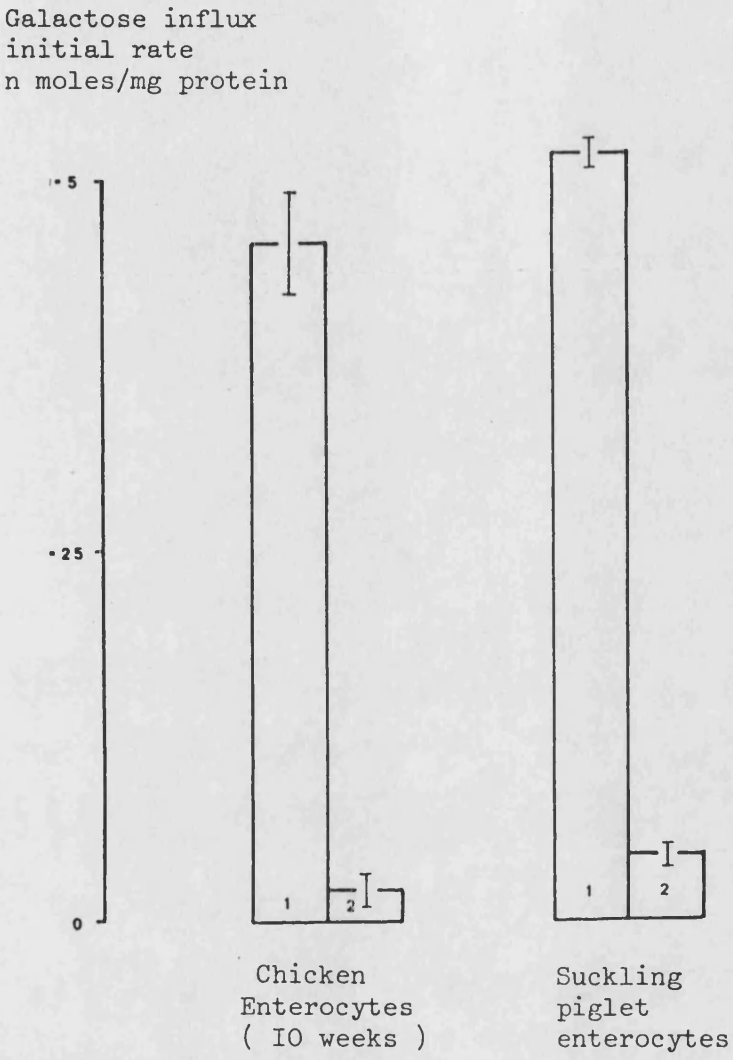


Figure 2 Demonstration Of Enterocyte Membrane Integrety - Shown As The Ability Of Phlorizin To Inhibit Galactose Influx.

Enterocytes were isolated in medium I containing sodium at pH 7.2 (see materials). After isolation, cells were stored for 30 minutes at 37°C and then the initial rate of galactose influx was measured in medium I at pH 7.2 . Error bars: ± S.E.

1 control 2 + Phlorizin (500uM)

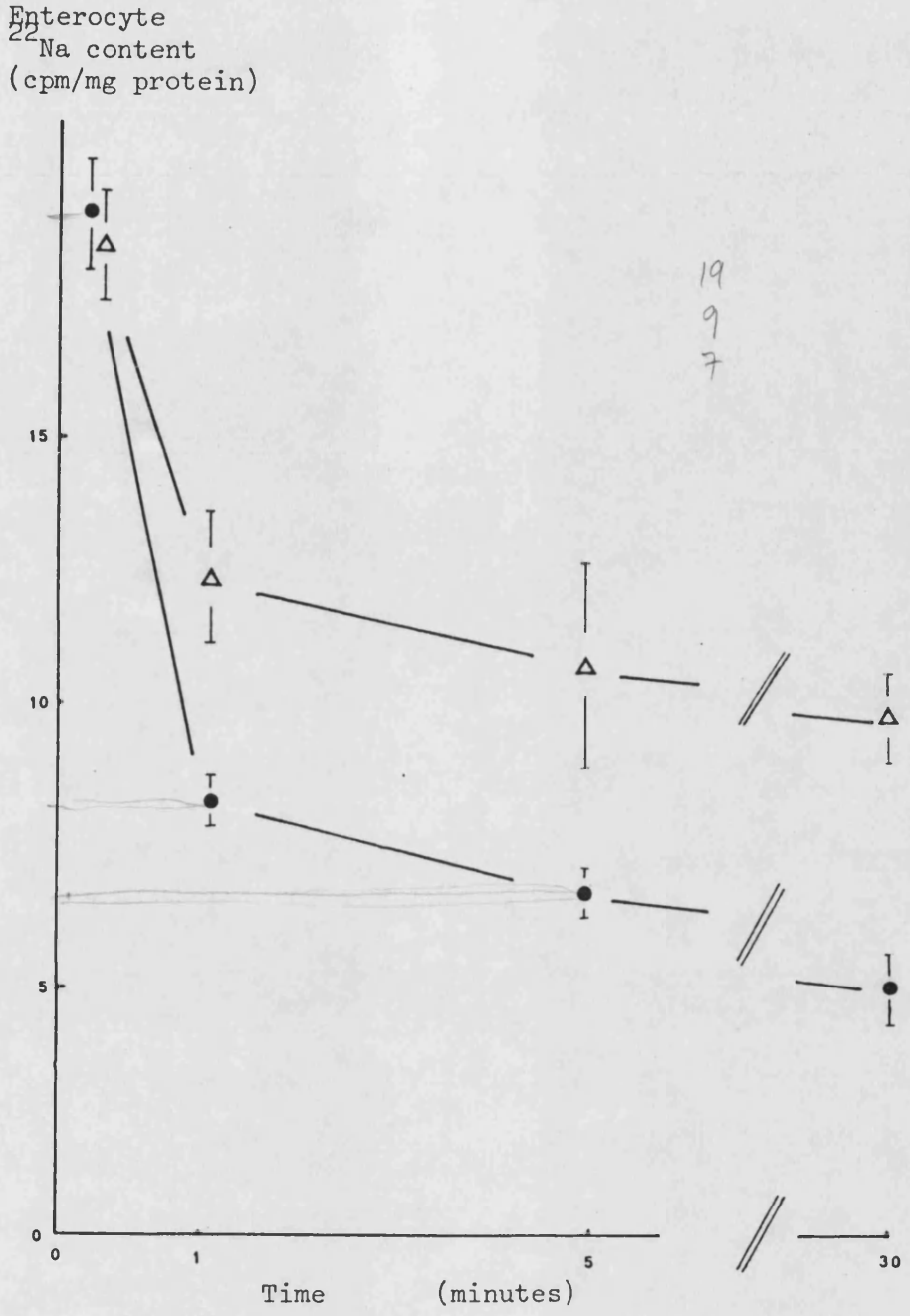


Figure 3 Demonstration Of Suckling Piglet Enterocyte Sodium ATPase Activity - Shown As ^{22}Na Efflux.

Suckling piglet enterocytes were isolated in medium I containing sodium at pH 7.2 (see materials). After incubation with ^{22}Na and with/without ouabain, initially at 37°C and subsequently at 0°C, ^{22}Na efflux was measured in medium I at pH7.2 . Error bars: ± S.E.

● control

△ +Ouabain (500uM)

as an increase in efflux rate and finally as a lower intracellular radioactivity at equilibrium. (The results in figure 3 and 4 are not background corrected.

Piglet enterocytes therefore exhibit sustainable Na^+ ATPase activity and membrane integrity.

Figure 4 shows the sodium efflux profile for rabbit enterocytes under similar conditions to figure 3. The effects of ouabain are very similar to that with piglet enterocytes, showing that both of these mammalian experimental systems are equally competent at producing their own sodium gradients.

The Separation of Brush Border and Basolateral Membranes.

i) The preparation of brush border membrane vesicles. In order to study brush border events in isolation from those at the basolateral border, the feasibility of producing brush border vesicles was studied.

Initial experiments were carried out using frozen rat tissue by a multitude of different protocols. Rat vesicles showed marker enzyme activities compatible with brush border vesicles, but failed to show inhibitable sugar uptakes. After optimizing the membrane separation technique, the use of rabbit tissue produced more acceptable results.

The marker enzyme activities for the fractions at various stages of the isolation procedure for rabbit brush border vesicles are shown in figure 5.

Sucrase is a marker enzyme characteristic of the brush border membrane, and it can be seen that brush border membrane appears in the majority of fractions. However, it is

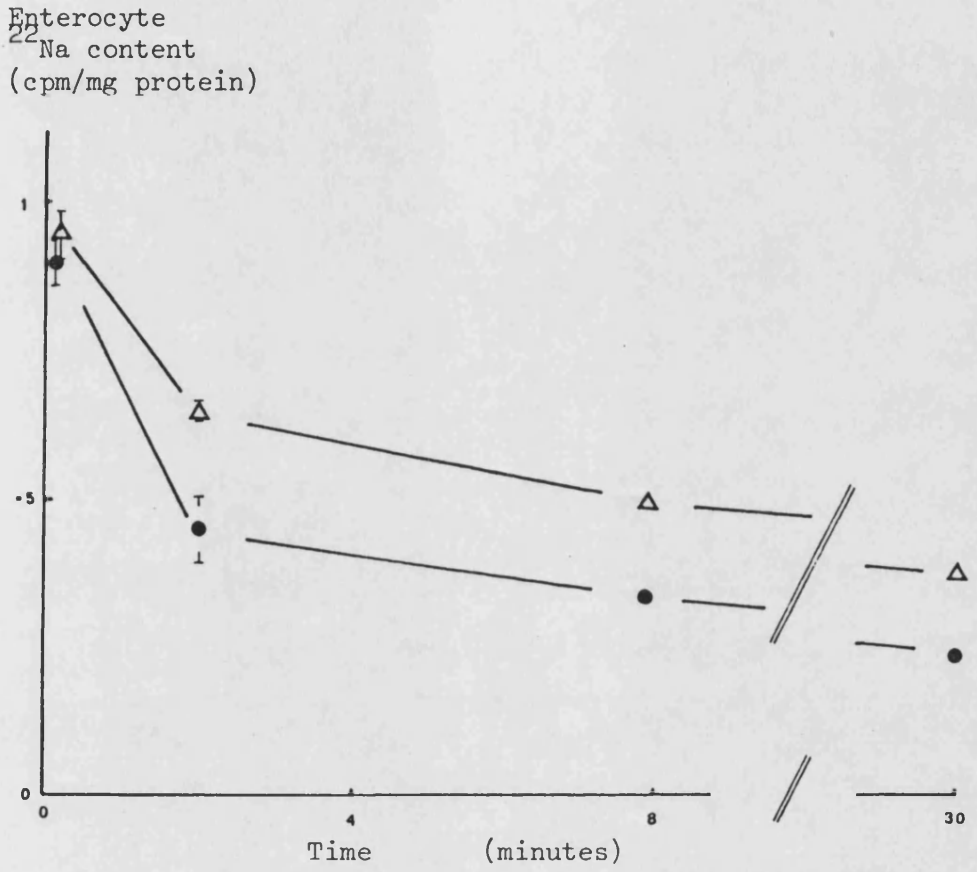


Figure 4 Demonstration Of Rabbit Sodium ATPase Activity - Shown As ^{22}Na Efflux.

Rabbit enterocytes were isolated in medium I containing sodium at pH 7.2 (see materials) . After incubation with ^{22}Na and with/without ouabain, initially at 37°C and subsequently at 0°C, ^{22}Na efflux was measured in medium I at pH 7.2 . Error bars: ± S.E.

● control

△ +Ouabain (500uM)

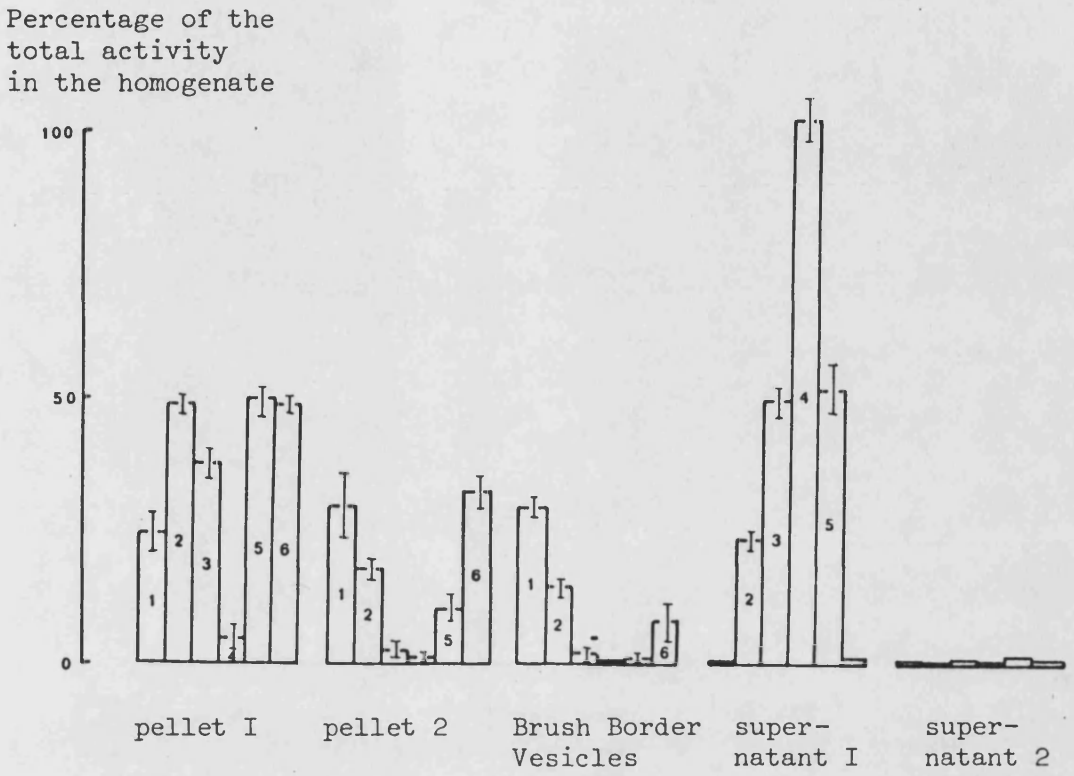


Figure 5 Marker Enzyme Activities Of Fractions During Brush Border Vesicle Production.

1 Sucrase activity (Brush border marker)

2 Alkaline Phosphatase (partly Brush Border)

3 Acid Phosphatase (Microsomal marker)

4 Lactate Dehydrogenase (cytoplasmic contents)

5 Protein

6 Na^+ ATPase activity (Basolateral border)

Error bars: $\pm$ S.E.

only highly enriched in the vesicle fraction, where it appears as a 15 fold increase in specific activity over the initial homogenate. It can also be seen that very little activity appears in the non-pelleted fractions, as would be expected for a solely membrane bound enzyme.

Alkaline phosphatase is a less reliable brush border membrane marker, since an appreciable portion of this activity is not membrane bound, and it is difficult to distinguish the different forms. The majority of the soluble form can be seen in the supernatant 1 fraction, and this obviously suppresses the percentage activity in the brush border vesicle fraction. However, the brush border vesicle fraction shows a significant increase in alkaline phosphatase specific activity.

Acid Phosphatase activity is thought to reside predominantly on the membranes of microsomes. The final brush border preparation has very little acid phosphatase activity and is therefore essentially free of microsomal contamination.

Lactate dehydrogenase is a soluble cytosolic enzyme, and acts as a marker for the efficiency of the washing process. This can be particularly important as lytic enzymes liberated during the homogenization procedure can damage vesicle membrane integrity. All of the lactate dehydrogenase activity appears in the supernatant fractions, as expected for a soluble enzyme, whilst the brush border vesicle preparation is essentially free of soluble cytoplasmic contents.

The protein content of the reactions shows that the protein of the homogenate is equally distributed between the magnesium

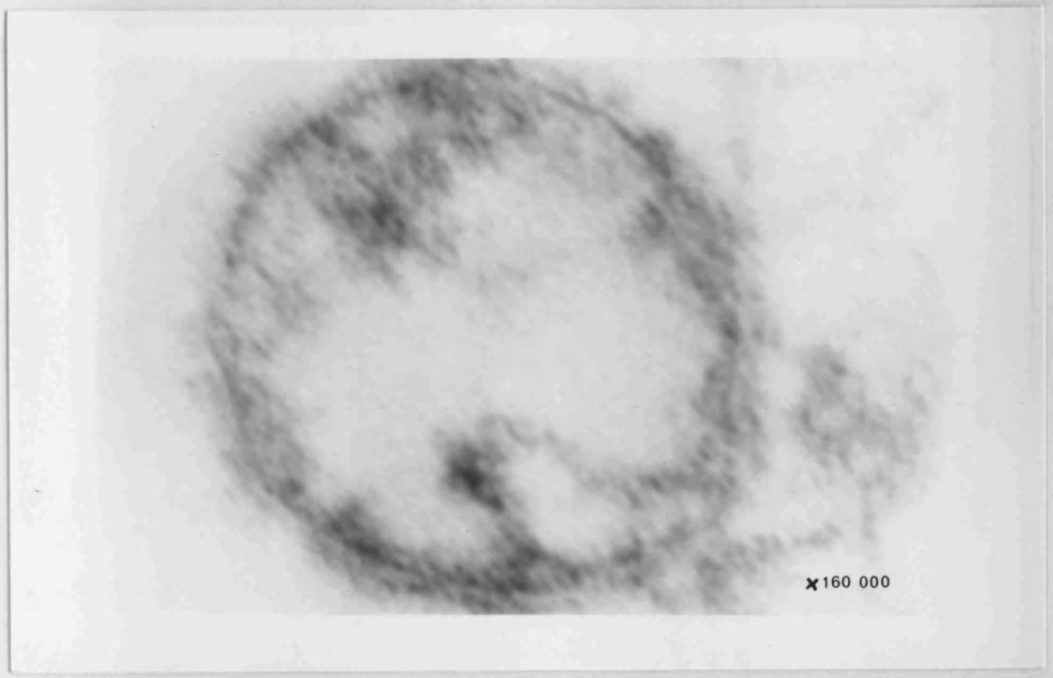
precipitated pellet and the supernatant. The brush border vesicle fraction represents only 2% of the total protein in the homogenate.

The Na^+ ATPase activity acts as a marker for the basolateral border. The largest portion of the Na^+ ATPase activity appears with the magnesium precipitated pellets. These pellets contain the easily sedimented large membrane pieces (usually basolateral border) left after the selective homogenization procedure. The brush border vesicle fraction shows a small portion of the total Na^+ ATPase activity, which represents a small increase in specific activity over the initial homogenate. However, this activity is far less than the large increase in specific activity of the brush border markers. The vesicles produced are, therefore highly enriched in brush border membrane relative to basolateral border.

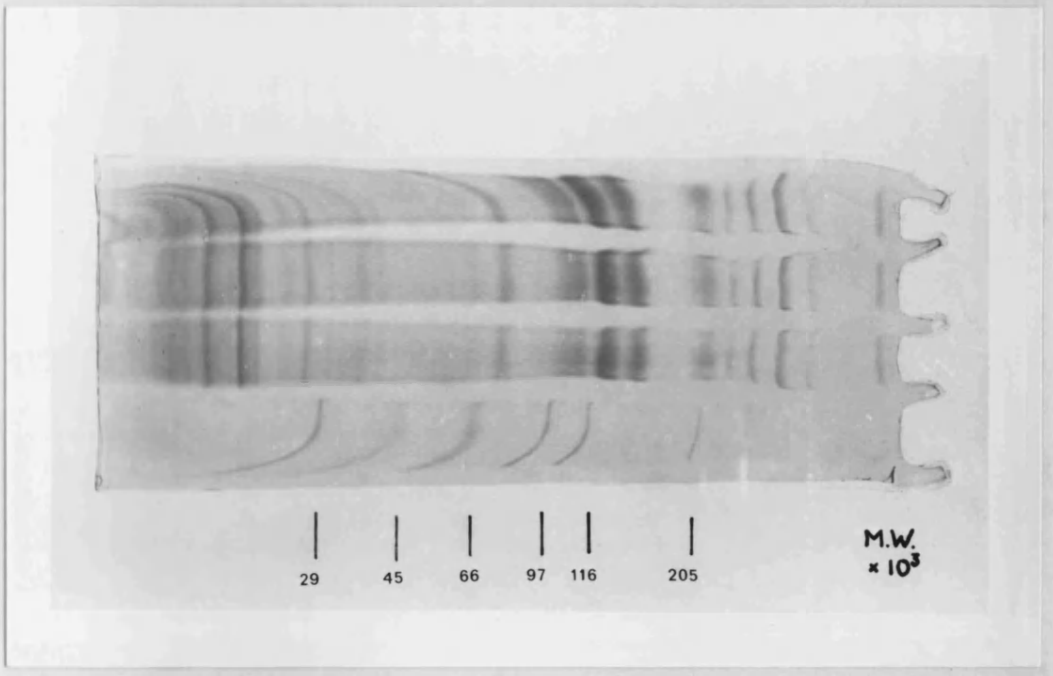
The photographs in figure 6 show further characterization of the brush border vesicles. The electron micrograph shows a typical vesicle, which is totally resealed and spherical. This micrograph also shows the limited intravesicular cytoplasmic contents.

The photograph 6 b shows the separation of brush border vesicle membrane proteins on a gradient polyacrylamide slab gel. The quite distinct bands indicate a more purified preparation over that seen with whole cell components, and compare well with published brush border vesicle gel patterns (Semenza et al, 1984).

The histograms of figure 7 show the uptake properties of the characterized brush border vesicle preparation. The



Electron micrograph showing the limited cytoplasmic contents and intact membrane of the brush border vesicles produced.



Gradient polyacrylamide gel showing separation of brush border vesicle membrane proteins.

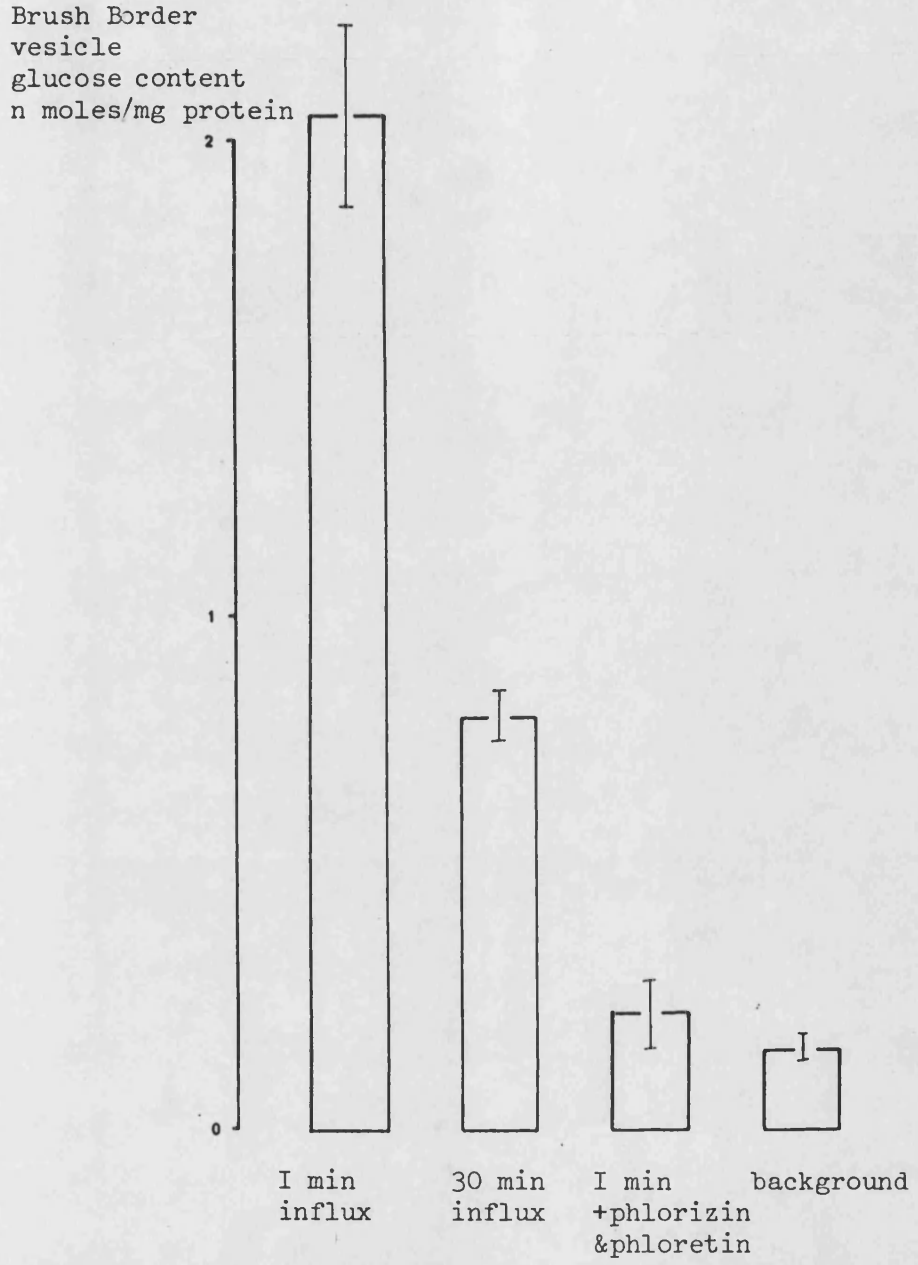


Figure 7 Brush Border Vesicle Glucose Uptakes - Demonstration Of Accumulation And Inhibition Of Glucose.

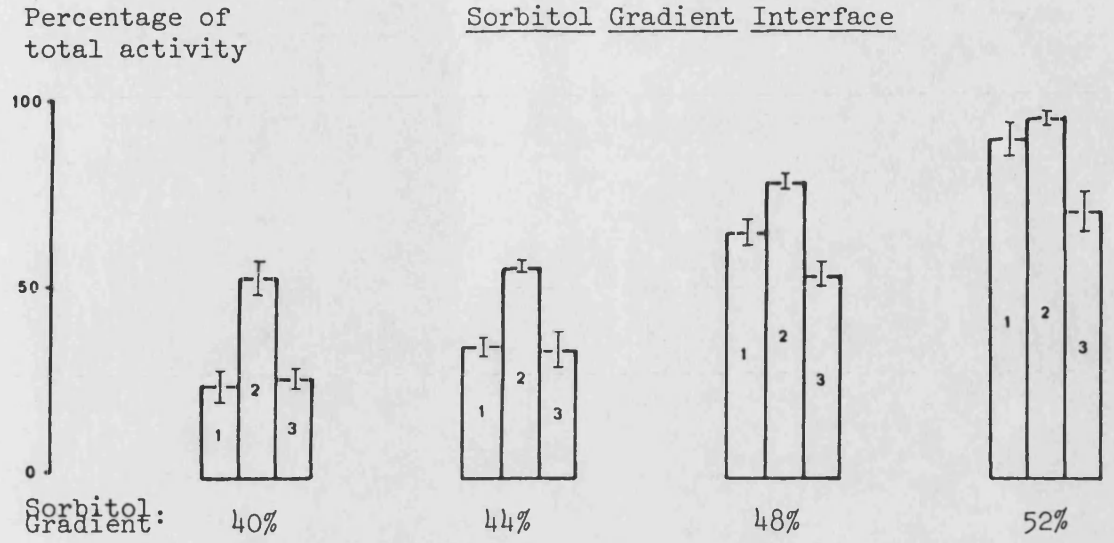
Rabbit Brush border vesicles were prepared in sodium free media (see materials). Vesicles were preincubated with/without inhibitors, and then I mM glucose influx was measured for the indicated time periods in I50mM NaSCN medium . Error bars: ± S.E.

glucose uptake after 1 minute in the presence of a sodium gradient overshoots the final equilibrium point, the equilibrium point being finally reached after 30 minutes. The overshoot reflects the actions of the sodium hexose symport, which results in glucose accumulation. As this symport is a known feature of the brush border membrane, this is further evidence for the vesicles being, at least in part, of brush border origin. The glucose influx after 1 minute can be almost completely inhibited with the classical intestinal sugar uptake inhibitors, showing the excellent resealing properties of rabbit brush border vesicles. The uptake values also compare favourably with the published values for similar preparation procedures (Kessler et al, 1978)

ii) Basolateral membrane separation.

An entirely separate investigation from the diarrhoeal disease studies was proposed to investigate the photoaffinity labelling of the intestinal hexose transporters using novel glucose analogues. This necessitated the development of a technique for purifying plasma membrane in high percentage yield. As part of this development procedure, the feasibility of producing a basolateral membrane enriched fraction was investigated. The basolateral fraction was intended ultimately for vesiculation and flux studies related to diarrhoeal disease.

The histograms of figure 8 show the marker enzyme activities of plasma membrane fractions after lysis and the removal of intracellular contents. The four ultracentrifugation step gradients shown indicate the enzyme activities



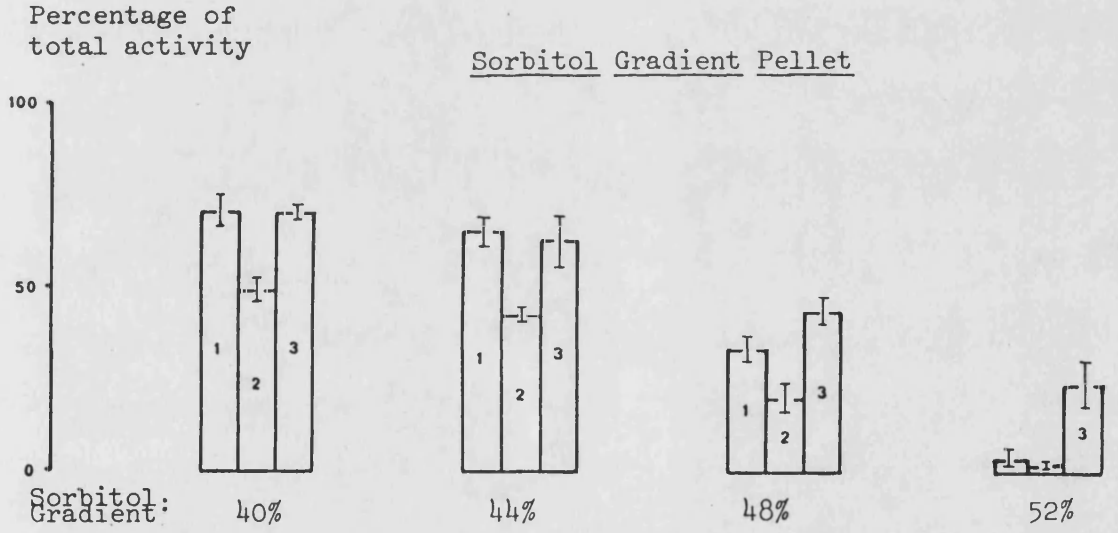


Figure 8 Percentage Distribution Of Enzyme Activities And Protein Between Interface And Pellet Of Sorbitol Step Gradients.

1 Sucrase activity (Brush border marker)

2 Sodium ATPase activity (Basolateral Marker)

3 Protein content

and protein at the interface of the step gradients and the pelleted material which penetrated the gradient.

The 40% gradient shows that the basolateral membrane can be separated from the brush border, seen here as a 2 fold increase in specific activity of the Na^+ATPase relative to sucrase. Further purification, possibly employing a more gradual gradient, allows potential for the production of basolateral membrane enriched vesicles.

The main objective of this investigation, however, was to produce purified plasma membranes with as little loss in yield as possible. The 52% gradient shows that nearly all of the plasma membrane marker enzyme activity can be separated from further contaminating protein (thought to be mainly mitochondrial in origin). This maximal recovery of plasma membrane ensures that small amounts of photoaffinity ligand can be used. The use of the 40% gradient (showing basolateral membrane enrichment) also means that at the expense of protein yield basolateral membrane effects can be amplified relative to those at the brush border.

The graph in figure 9 shows the gradient P.A.G.E. separation of plasma membranes, using the developed technique for enterocytes photoaffinity labelled with DIAB BGPA (Di-Iodo- Azido-Benzoyl Bis-Glucose Propylamine).

This graph shows the extreme reproducibility of the technique with two enterocyte suspensions, treated entirely separately from the very beginning of the developed technique. Extreme reproducibility is necessary, since it must be possible to directly compare the radioactivity of gels in the presence and absence of 'cold' displacing ligand.

Ultimately a specific photoaffinity label could be used

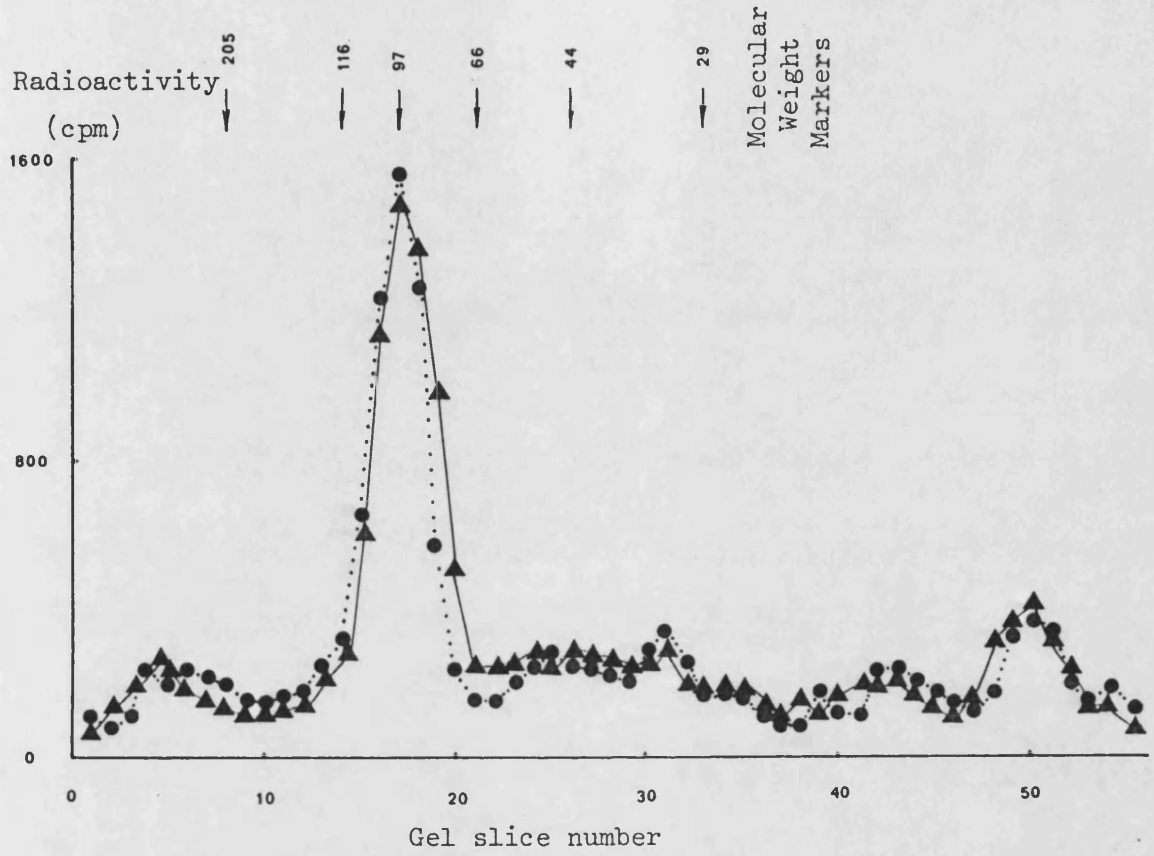


Figure 9 Demonstration Of The Reproducibility Of Photoaffinity Labelling Using The Plasma Membrane Seperation Technique.

Chicken enterocytes isolated in sodium buffer I were photoaffinity labelled, and the plasma membrane seperated by the described technique.

● Photoaffinity glucose analogue

▲ Analogue + excess glucose

with this technique to label the intestinal glucose transporters. However problems experienced in this study with non specific labelling (shown by the inability of glucose to displace the photoaffinity ligand during irradiation), shows that this ligand is inapplicable for enterocyte glucose transporter labelling.

The Purification of E coli -431 ST_a Enterotoxin.

The protein contents of fractions during the purification procedure of E.coli -431 ST_a from the 1,000 - 10,000 MW 'crude' ultrafiltration retentate are shown in figure 10. The initial acetone treatment removed over 40% of the total protein, whilst the second acetone step removed a further 10%. The final 1:9, aqueous:acetone step produced a tri-phasic mixture consisting of a precipitate, supernatant and an immiscible brown oil phase. The protein content of the small pellet produced during this step contained very little protein, however, the oil phase contained 30% of the total protein. The protein content of this oil phase was considerably higher before the two further steps (see methods) to elute away any remaining toxin using a 1:9 aqueous:acetone wash. The pooled supernatants from the initial procedure and the 1:9 aqueous:acetone wash contained the purified toxin and 17% of the total protein. Dialysis of the toxin reduced this to less than 1% of the total protein at a protein level which was almost too low to determine.

The biological activity of ST_a during the purification process was followed with the standardized suckling mouse bioassay (see methods). Figure 11 shows the initial activity

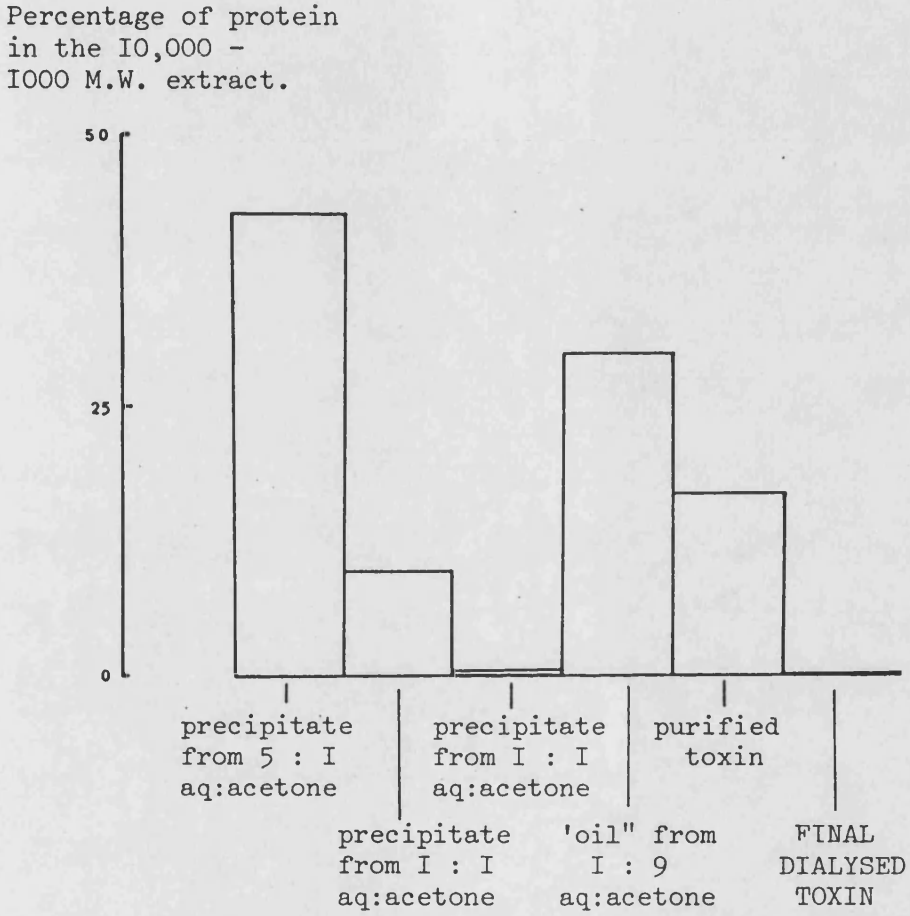


Figure 10 Protein Content Of Fractions During The Purification Of E.coli - 431 ST_a.

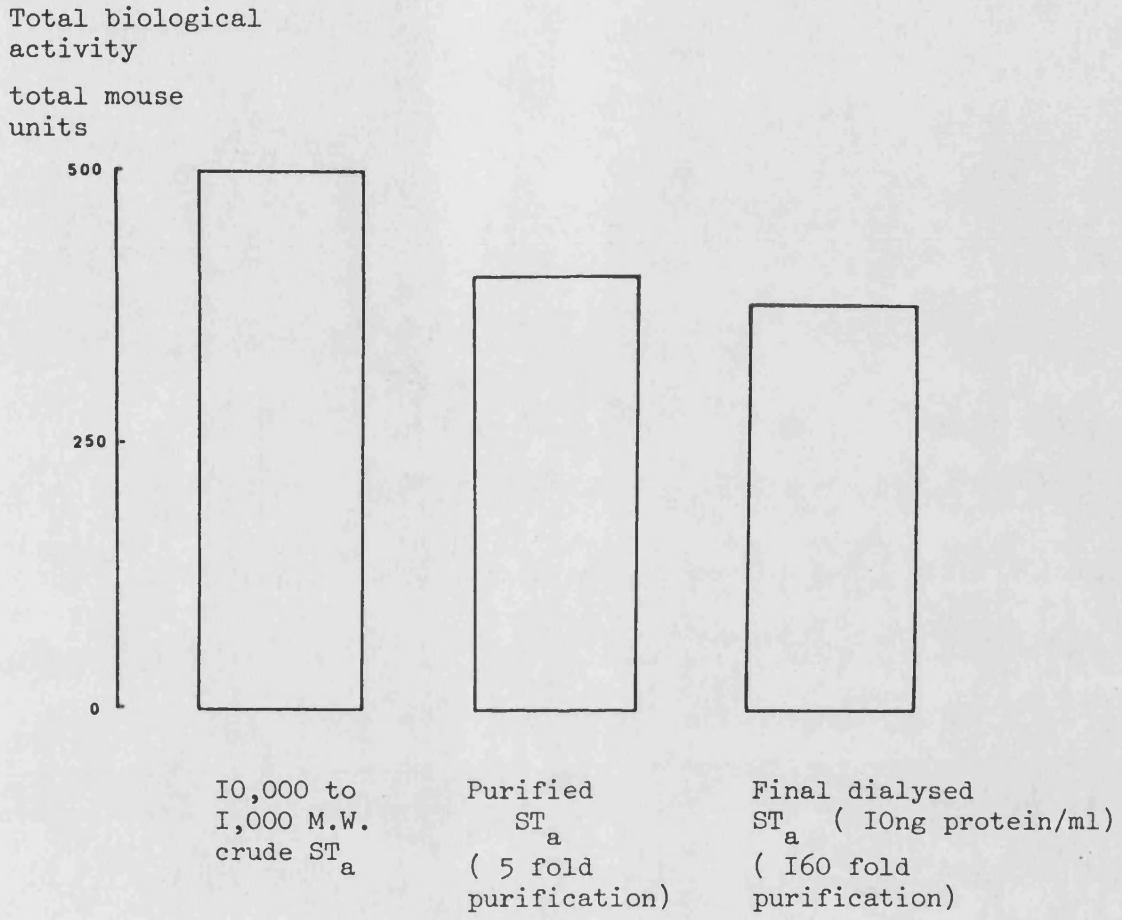


Figure II Biological Activity Of Fractions During The Purification Of E.coli - 43I ST_a

of the 'crude' filtration membrane retentate to contain 500,000 mouse units. The purified toxin shows a five fold increase in specific biological activity, whilst retaining 80% of the initial biological activity. Dialysis of the toxin caused a further 5% reduction in total biological activity, leading to a 160 fold increase in specific biological activity (biological activity/protein). The specific activity relative to the cell free culture supernatant, however, is at least 2 fold higher than this value. This is because the original starting solution contained only the 1000 MW - 10,000 MW residue of the culture supernatant. The continuous nature of toxin production makes exact culture supernatant protein values unavailable.

Finally, the toxin was stored as 1 mouse unit representing 10 ng of protein, and at a concentration of 10,000 mouse units per ml. The sodium content of the toxin was also considerably reduced during the purification procedure to a value of 10 μM.

The dose response curve for the purified E.coli -431 STa is shown in figure 12 with the logarithmically linear portion spanning slightly less than 0.1 to 10 mouse units.

The Effects of STa on Enterocyte Solute Flux.

After purification and biological activity characterization, STa was tested with the newly developed procedure for flux studies using isolated piglet enterocytes. STa has a very specific spectrum of biological activity, related to both ages and species of animal (see introduction). Suckling piglets of ages 3 to 12 days were therefore chosen for enterocyte isolation.

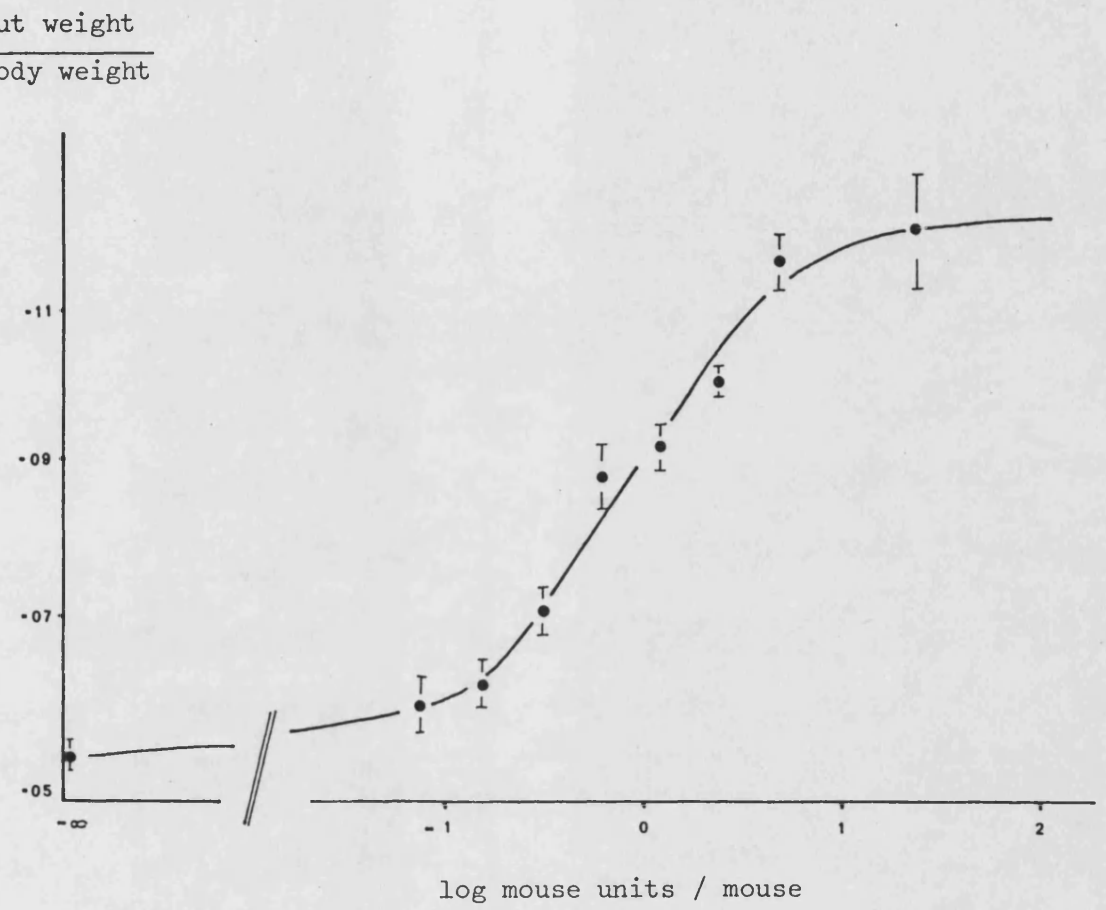


Figure I2

Dose Response Curve For The Infant Mouse Assay Using ST_a Prepared From E.coli - 43I.

Figure 13 shows the sodium influx into piglet enterocytes stored at 37°C, and allowed to maintain their own sodium gradient. Cells incubated at 37°C with and without STa are shown, whilst sodium influx initial rates were studied at 10 minute intervals. It can be seen that incubation of ST_a with the enterocytes has no effect on sodium influx. As the incubation at 37°C proceeds, the loss in uptake with time reflects the reduction in cell viability. This loss in membrane integrity can be completely halted by cell storage at 0°C, and probably reflects the action of cytolic enzymes.

Further experiments also failed to show any effect on sodium influx at any point along the sodium influx progress curve with ST_a concentrations up to 400 mouse units per ml of enterocyte suspension.

After the failure of ST_a to show any effect on sodium influx, cholera toxin was employed to test the new piglet enterocyte model. Figure 14 shows the effects of cholera toxin on the initial rate of sodium influx in enterocytes incubated at 37°C. The conditions were similar to those for ST_a in figure 14, although the sampling was less frequent and the incubation period was extended to 90 minutes. Initially it can be seen that the mere presence of cholera toxin is insufficient to cause a reduction in sodium influx. However, as the incubation at 37°C proceeds, the reduction in sodium influx in cells treated with cholera toxin becomes pronounced. After a period of 30 minutes the sodium influx reduction becomes maximal and remains for up to 90 minutes.

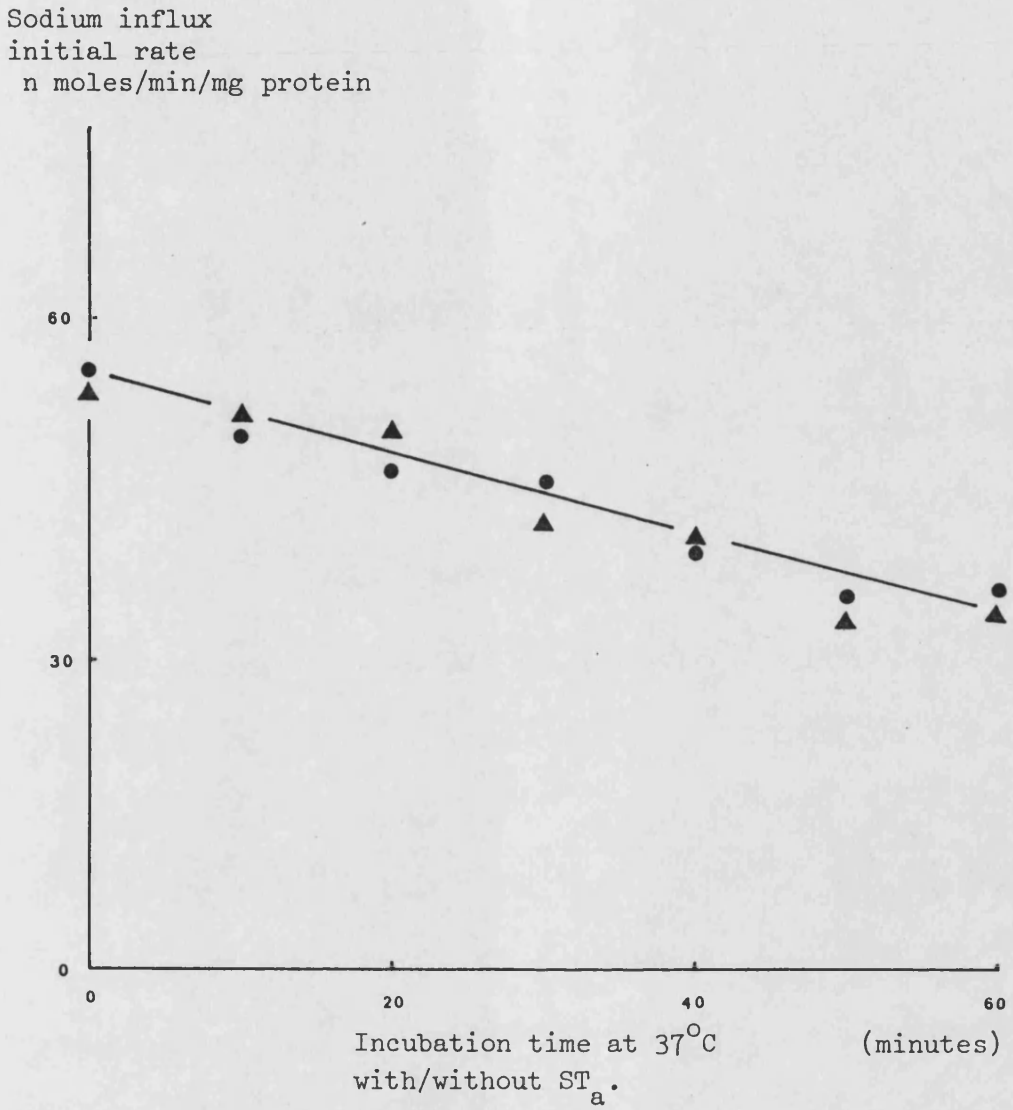


Figure I3 The Effects Of ST_a On Enterocyte Sodium Influx Initial Rate At Various Preincubation Times.

Suckling piglet enterocytes were isolated in medium I containing sodium at pH 7.2 (see materials). After incubation at 37°C as indicated, samples were taken and the influx of sodium measured in medium I at pH 7.2 . Error bars: ± S.E.

● control

▲ ST_a (I00 mouse units/ml)

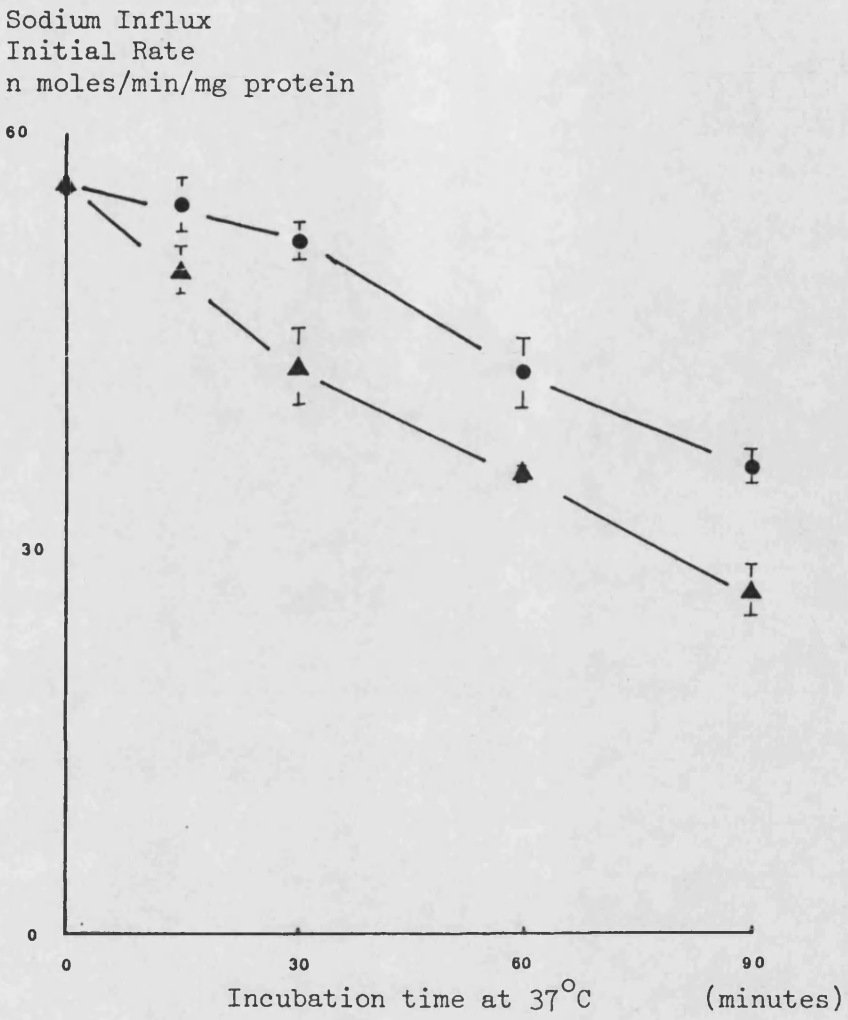


Figure 14 The Reduction In Sodium Influx Initial Rate Caused By Cholera Toxin.

Suckling piglet enterocytes were isolated in sodium medium (I) (see materials). The initial rate of sodium influx was then measured after the incubation periods indicated. Error bars: ± S.E.

● control

▲ Cholera toxin (20ug/ml)

Superimposed on this effect is the loss in cell viability as the enterocytes are stored at 37°C.

The reduction in sodium influx caused by cholera toxin compares well with the previous reports of cholera toxin effects on isolated chicken enterocytes (Hyan et al,1982). The isolated piglet enterocyte system, therefore, compares well with previous enterocyte systems, with the added suitability of being prepared from the target animal for STa's transcellular sodium flux reducing properties (Burgess et al, 1983).

The failure to detect any sodium influx reducing properties of ST_a with isolated piglet enterocytes, led to the investigation of possible ST_a effects on sodium efflux. This rationale was followed since Burgess (1983) has shown that STa significantly reduces the net flux of sodium from the mucosal to serosal surfaces of the intestine. As both cellular sodium influx and efflux give rise to the transcellular sodium movement, a reduction in either could explain the results of Burgess (1983).

The sodium efflux from enterocytes pre-incubated with radiolabelled sodium can be seen in figure 15. The enterocytes were initially incubated with ST_a, ouabain or control additive as appropriate for 30 minutes at 37°C, before cooling to 0°C. The storage at 0°C minimizes cell deterioration as well as ensuring that all preparations begin with similar intracellular sodium concentrations and radioactivity irrespective of Na^+ ATPase activity. Sodium efflux in the presence of Ouabain represents sodium efflux when

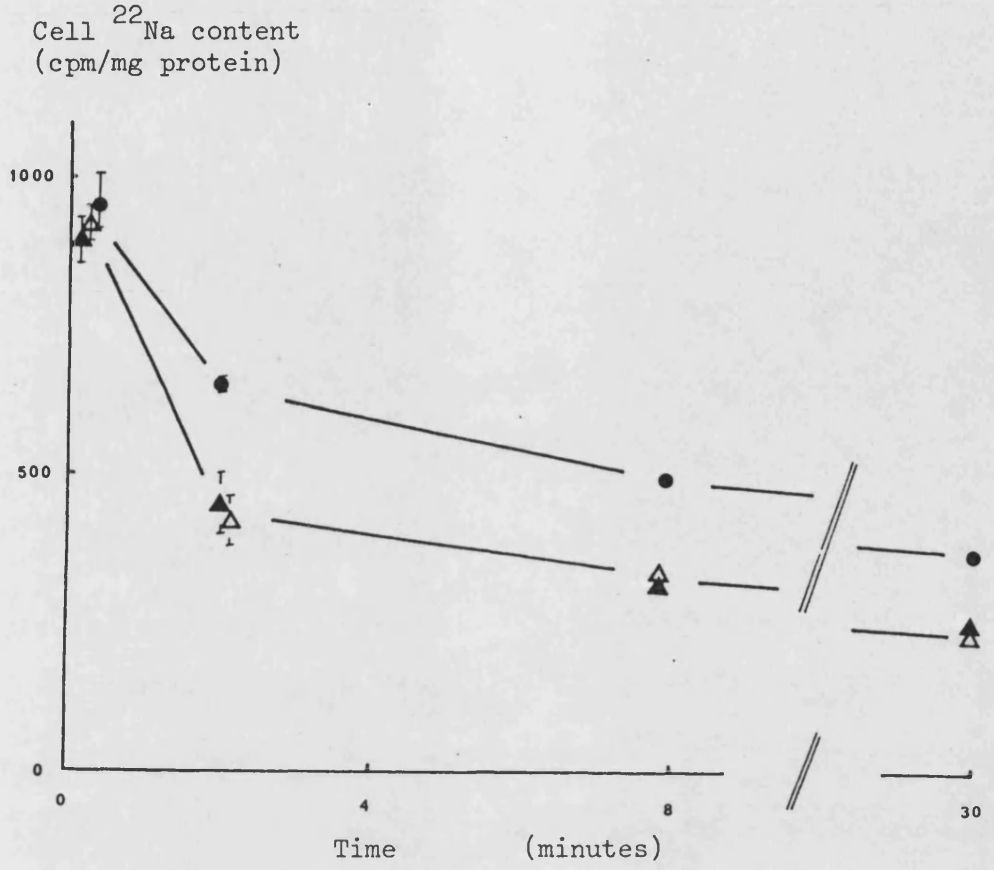


Figure I5 The Effects Of ST_a On Sodium Efflux From Piglet Enterocytes.

Suckling piglet enterocytes were isolated in sodium medium (I) (see materials). Cells were then incubated with radiolabelled sodium and the test additive (see below) at 37°C for 5 minutes, and stored on ice. Sodium efflux was then measured as the cells were added to a diluting volume of the same medium. Error bars: ± S.E. Error bars omitted when smaller than symbols used.

▲ control

● Ouabain (500 uM)

△ ST_a (E.coli-43I)

NA^+ ATPase activity has been inhibited. The action of Na^+ ATPase activity is seen as an increase in sodium efflux. Initially this is seen as an increase in efflux rate and finally as a lower intracellular radioactivity at equilibrium.

The enterocytes pre-incubated with STa show a sodium efflux profile similar to the control cells and unlike those incubated with Ouabain. ST_a therefore, appears to have no effect on the Na^+ ATPase activity under the conditions employed.

At the time of undertaking the work concerning the effects of ST_a on sodium flux with isolated enterocytes, a report of ST_a effects on enterocyte chloride efflux was received (Ahrens et al,1986). Although the ST_a preparation used in Ahrens et al's paper was extremely crude, a similar study was carried out with the highly purified toxin and enterocytes isolated from piglet intestine.

Figure 16 shows the efflux of chloride from enterocytes previously preincubated with either ST_a or a control additive for 30 minutes at 37°C. The chloride efflux rate can be seen to be similar with or without ST_a and is not increased by ST_a as claimed by Ahrens.

Although the mechanism of action of STa is poorly understood, it has been noted by a number of workers that cellular cGMP levels increase in the presence of ST_a (Newsome et al, 1978; Gianella et al, 1979). It was therefore decided to ensure that this was also the case for piglet enterocytes once isolated.

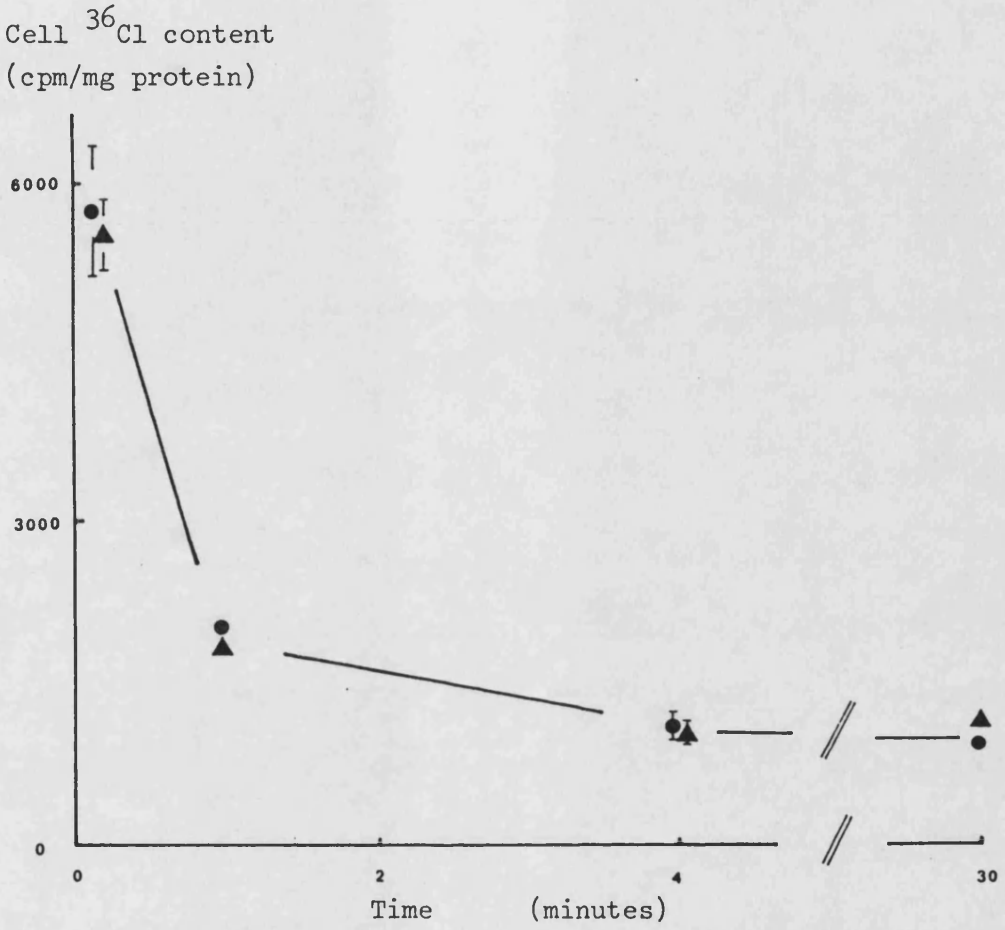


Figure I6 Chloride Efflux From Piglet Enterocytes

Suckling piglet enterocytes were isolated in sodium medium(I) (see materials). Cells were then incubated with radiolabelled chloride and the test additive (see below) at 37°C for I5 minutes. Chloride -36 movement was then measured as the cells were added to a diluting volume of the same medium. Error bars are ± S.E. Error bars omitted when smaller than symbols used.

▲ control

● 400 m.u./ml E.coli-43I ST_a

Figure 17 shows the cGMP levels in piglet enterocytes during incubation with ST_a at 37°C for up to 30 minutes. It can be seen that in all cases the cellular cGMP levels decrease by up to 80% over a 30 minute incubation period at 37°C. All previous sodium influx investigations with STa and piglet enterocytes included an incubation period of up to 30 minutes at 37°C. It therefore appears that rather than ST_a increasing cellular cGMP, there had been an appreciable loss in cGMP over this time period.

The initial intracellular cGMP levels, however, compare well with published values (c.f. piglet enterocytes : 120 p moles/g wet wt. compared with 80-150 for whole intestinal tissue (Steiner et al,1972). It therefore appears that initially the enterocytes maintain their cGMP, and it is only during the subsequent incubation at 37°C that this metabolic incompetency is seen.

The failure to reproduce the ST_a induced reduction in transcellular sodium flux using the enterocyte model, and the cells apparent inability to maintain cGMP levels once isolated, led to the investigation of an artificial cGMP analogue. The analogue chosen was 8 Br cGMP since it has been reported to exert similar effects to ST_a (Burgess 1983).

The histograms of figure 18 show the initial rate of sodium influx into chicken enterocytes, previously incubated with varying amounts of 8 Br cGMP. Concentrations exceeding 50 μM 8 Br cGMP produce a reduction in sodium influx which continues up to 5 mM.

Figure 19 shows the sodium influx timecourse for cells preincubated with 5 mM 8 Br cGMP compared with control cells.

Enterocyte cGMP content
p moles cGMP
/g wet weight

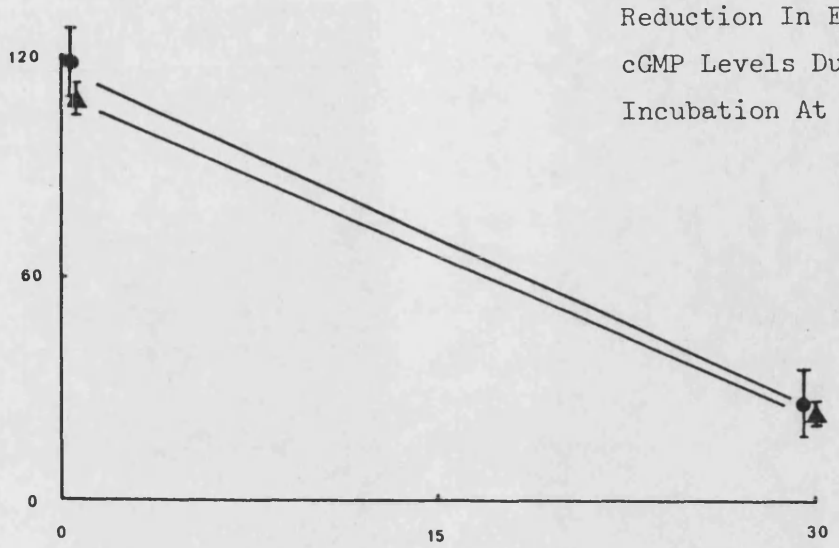
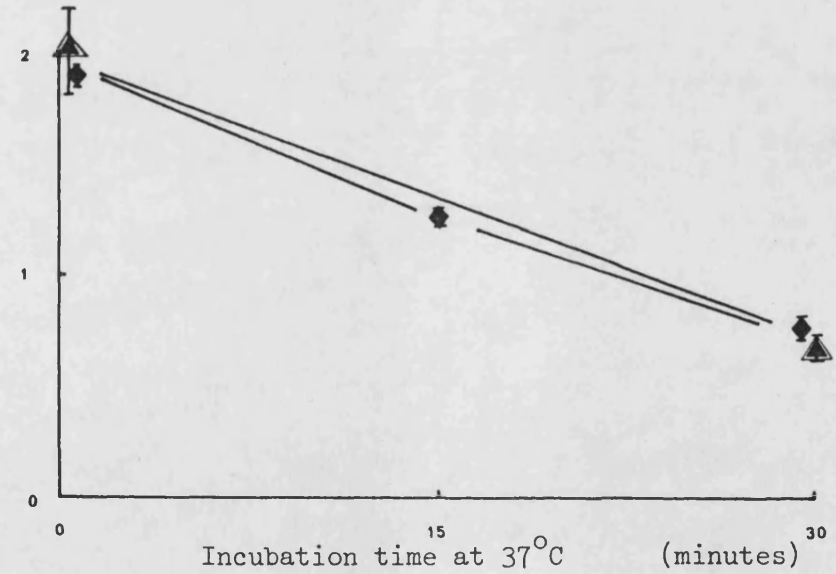


Figure I7

Reduction In Enterocyte cGMP Levels During Incubation At 37°C.

Enterocyte cGMP content
p moles cGMP
/million viable cells.



Suckling piglet enterocytes were isolated in sodium medium (I) and incubated at 37°C with the following test additives:

- ● control ;
- ◆ I00 m.u./ml <u>E.coli</u>-43I ST_a
- ▲ 400 m.u./ml E.coli <u>E.coli</u>-43I ST_a ;
- △ I00 m.u./ml <u>E.coli</u> PI6 ST_a

Sodium Influx
Initial Rate
n moles/min/mg protein

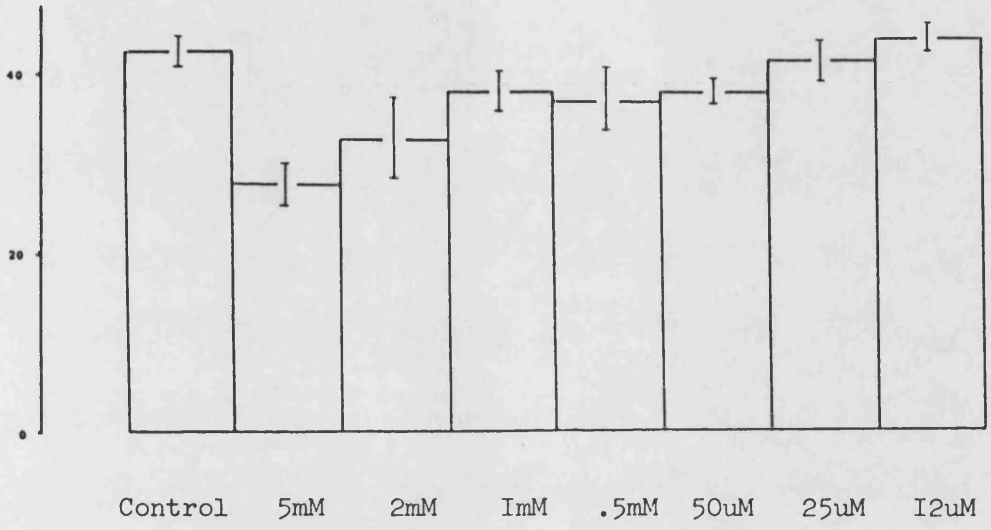


Figure I8

The Reduction In Sodium Influx Initial Rate Caused By Various Concentrations Of 8 Br cGMP.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free medium 2 at pH 7.2 (see materials).
After a 5 minute incubation period with 8 Br cGMP (concentrations as indicated), sodium influx (I00 mM) was measured in media 2.
Error bars: ± S.E.

The chicken cells used for the results in figure 19 were treated with rotenone and ouabain to make them metabolically inert and to remove Na^+ ATPase activity. The initial depression of sodium influx can be seen after one minute and continues until eight minutes, where the intracellular concentration is approaching equilibrium. At equilibrium the sodium concentration and thus radioactivity are equivalent with or without 8 Br cGMP indicating that there is no change in intracellular volume.

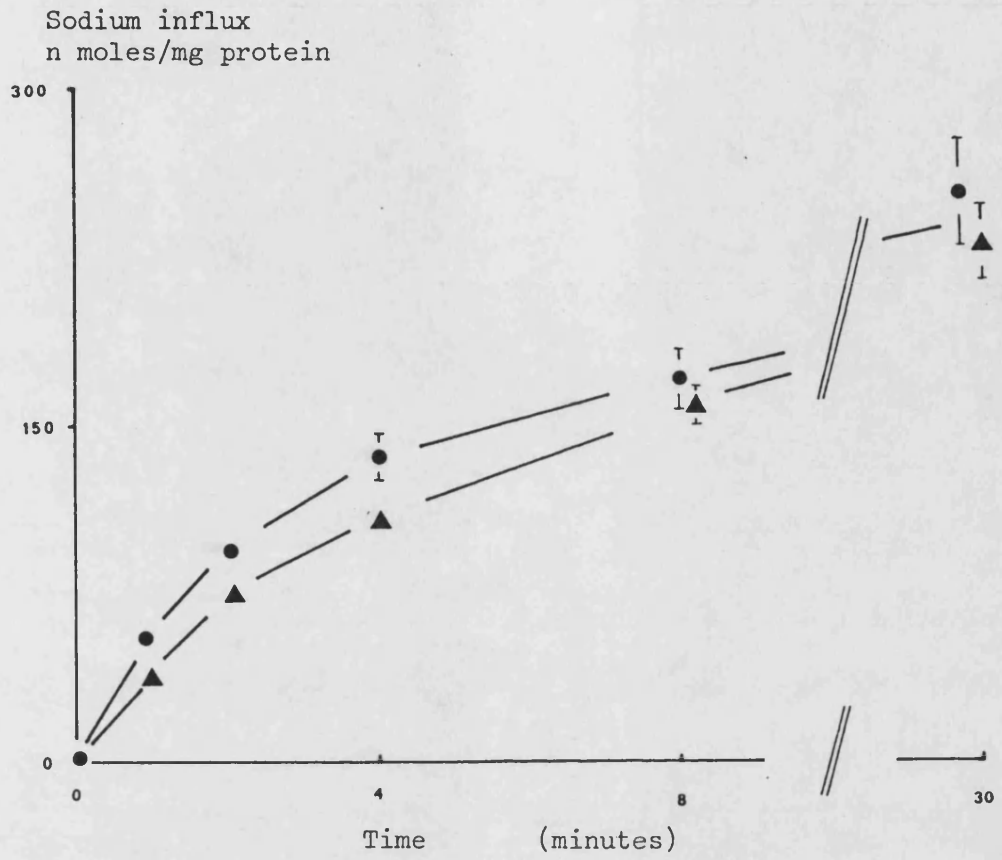


Figure I9 The Effects Of 8 Br cGMP On Enterocyte Sodium Flux.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free medium 2 (see materials). After a 5 minute incubation period with the test additive (see below), the sodium influx (I00 mM) was measured as indicated. Error bars ± S.E. Error bars omitted when smaller than symbols used.

● control

▲ 8 Br cGMP (5 mM)

The Investigation of Citrate Flux in Isolated Enterocytes.

The chicken enterocytes used for the following citrate investigations were treated with ouabain to remove any Na^+ ATPase activity. This was deemed necessary due to the variation in Na^+ ATPase activity with chicken age (figure 1). Sodium gradients were therefore artificially prepared and were highly manipulatable. As citrate is a metabolizable substrate for the cells, it was also felt that enterocytes incubated with citrate might exhibit greater Na^+ ATPase activity than those without. Citrate metabolism was therefore limited by depleting enterocyte ATP levels with rotenone. This allowed transport studies at time points other than initial rates to be investigated without also inadvertently studying citrate metabolism. (The metabolism of citrate was determined under these conditions, and is shown in figure 42).

The results in figure 20 show the influx of citrate into ATP depleted chicken enterocytes free from intracellular sodium. Under intracellular and extracellular sodium free conditions the influx of citrate is slow, and the intracellular concentration of citrate is still increasing after 8 minutes. When a sodium gradient is applied there is a marked increase in initial rate of citrate influx, which results in a maximal citrate content after 8 minutes. Further results (figure 25) indicate that this plateau represents near equilibrium of internal and external citrate, and that the applied sodium gradient is lost in the absence of Na^+ ATPase activity after 8 minutes (figure 33).

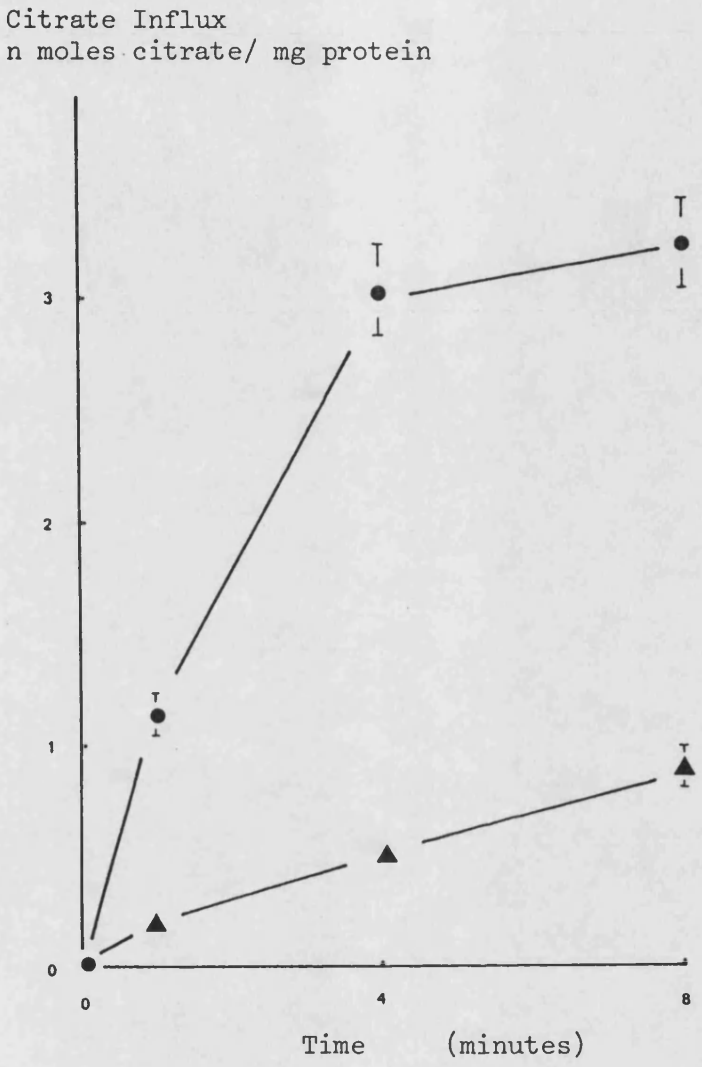


Figure 20 Effects Of A Sodium Gradient On Enterocyte Citrate Influx.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free medium 2 at pH 7.2 (see materials). Cells were then introduced to medium 2 containing 2 mM citrate and with/without 100 mM sodium at pH 7.2 . Error bars: ± S.E. Error bars omitted when smaller than symbols used.

● Sodium gradient

▲ Sodium free media

Figure 21 shows the change in initial rate of enterocyte citrate influx with different sodium gradients. In the absence of sodium the rate of citrate influx corresponds to the citrate influx in the absence of sodium seen in figure 20. As the external sodium concentration is increased the initial rate rises until it reaches its maximum at concentrations in excess of 50 mM.

The influx of citrate after 4 minutes in the presence of different sodium gradients is shown in figure 22. A similar sigmoid profile can be seen to the initial rates in figure 21. Figure 22 also shows that the intracellular citrate concentration is maximal after 4 minutes at initial sodium gradients in excess of 50 mM. Only initial sodium gradients above 10 mM appear to elevate the intracellular citrate concentration over that due to the sodium independent citrate flux.

A consideration of the multiple ionization forms of citrate led to the investigation of citrate influx rates in media of different pH value. Figure 23 shows the initial rates of citrate influx into ATP and sodium depleated cells at different extracellular pH values. Enterocytes with a 100 mM sodium gradient, previously seen to have the maximal initial rate of citrate influx, can be seen to show a further increase in initial rate with a pH gradient. In the absence of sodium, an internally directed pH gradient also increases the sodium independent citrate flux. It can also be seen that the sodium dependent and independent fluxes appear to be increased by a near equal amount, and not by similar proportions.

Figure 24 shows the effects of varying the pH gradient,

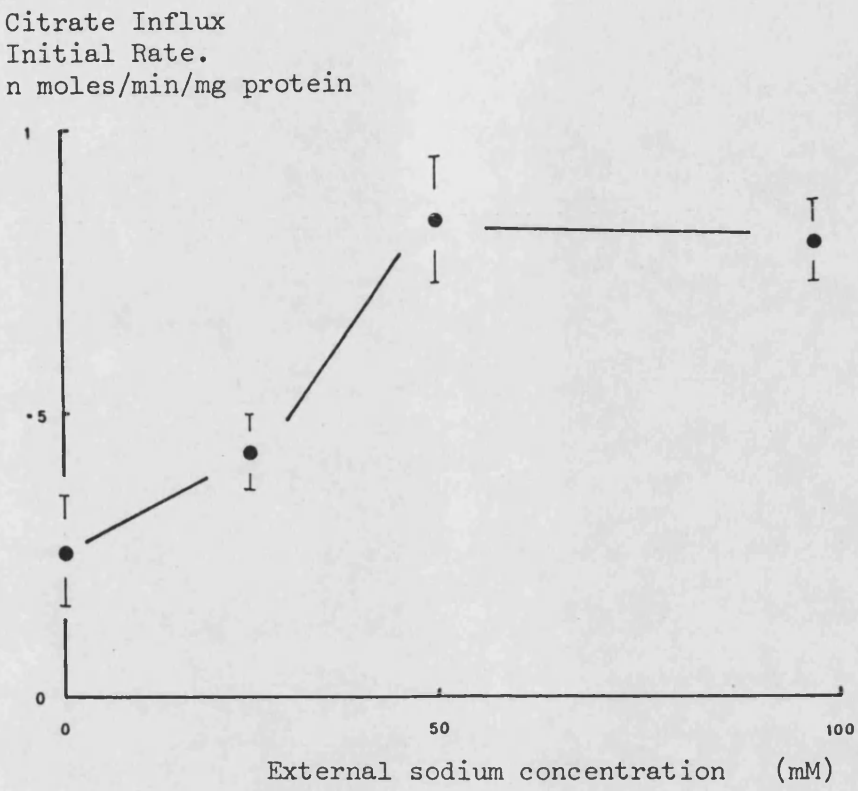


Figure 2I Effect Of Sodium Gradients On The Initial Rate Of Citrate Influx.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free medium 2 at pH 7.2 (see materials). Cells were then introduced to medium 2 containing 2 mM citrate and the indicated sodium concentrations.
Error bars : ± S.E.

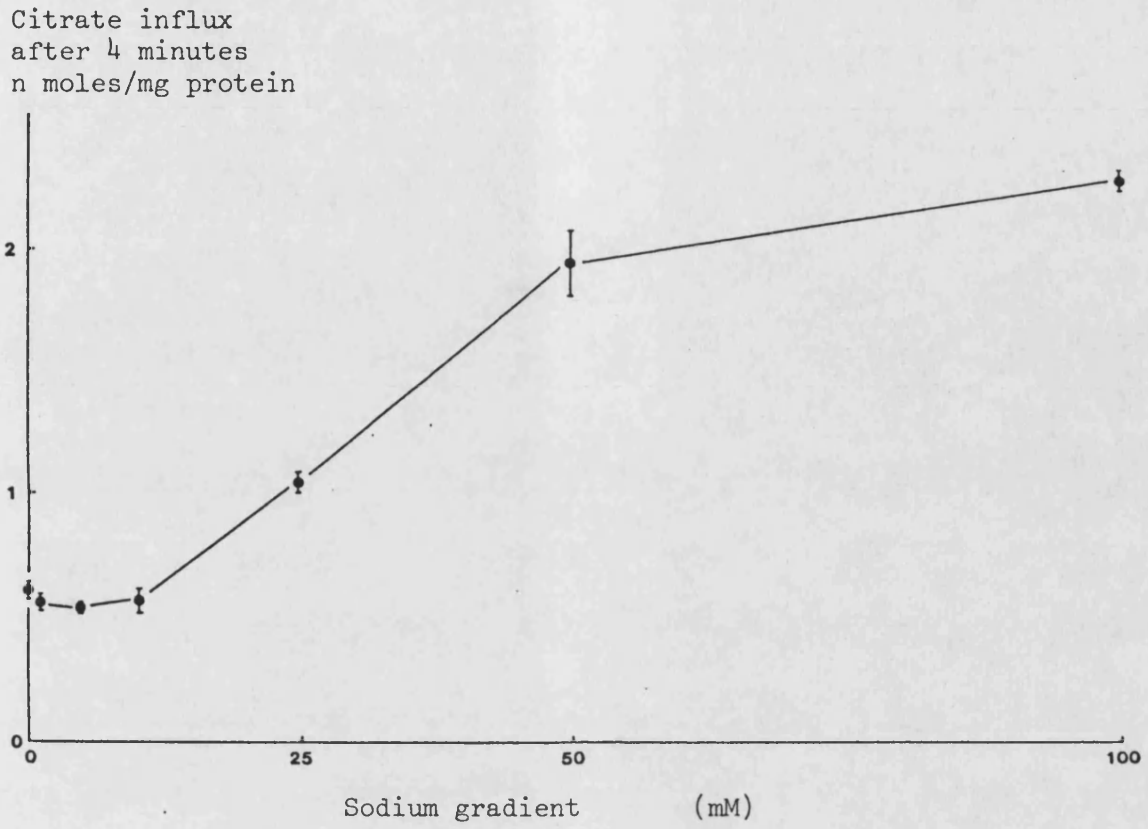


Figure 22 The Increase In Enterocyte Citrate Content With Various Sodium Gradients.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free medium 2 (see materials). Cells were then incubated at 37°C for 4 minutes in buffer 2 containing 2 mM citrate and the indicated sodium concentrations. Error bars : ± S.E.

across ATP and sodium depleted enterocytes, on the four minute intracellular citrate content. In sodium free media, the citrate content after 4 minutes increases in a sigmoid manner with the internally directed pH gradient. Enterocytes with a sodium gradient also show a consistently higher intracellular citrate concentration at any one pH value compared to the citrate content of cells in sodium free conditions.

Figure 25 shows the sodium dependent citrate influx time course for ATP and sodium depleted cells. In the absence of an internally directed pH gradient citrate flux gradually approaches equilibrium without causing citrate accumulation. However, citrate accumulation is seen when both a sodium and pH gradient are applied together. (It should be noted that although a pH and sodium gradient together cause limited accumulation, a pH gradient alone does not (figure 24).

The time course for sodium independent citrate influx is shown in figure 26. In the presence of an inwardly directed pH gradient it can be seen that the initial rate of citrate influx is increased relative to that in the absence of a pH gradient. It also appears that the intracellular citrate concentration after 4 minutes is a simple reflection of this initial rate, although from this graph it is not possible to determine whether the same equilibrium will be attained.

The demonstration that the initial rates of sodium independent citrate influx vary with the imposed pH gradient

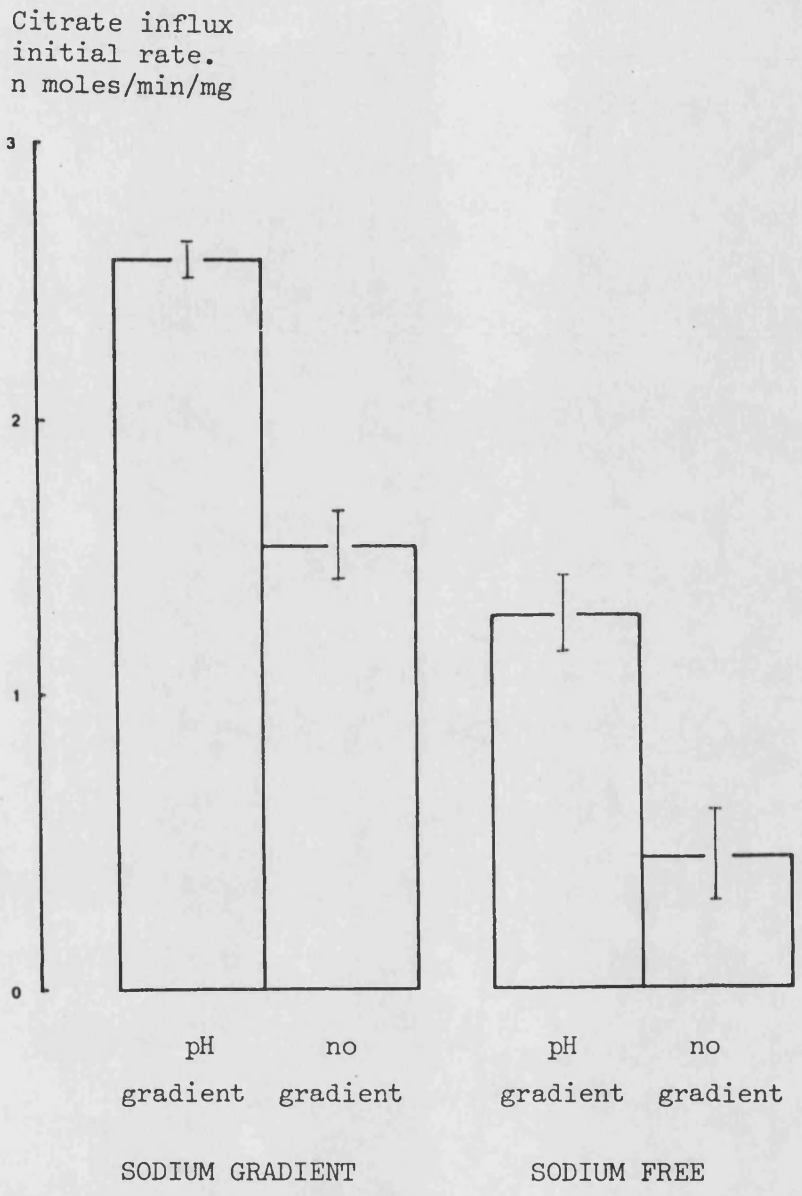


Figure 23 Effects Of pH And Sodium Gradients On The Initial Rate Of Citrate Influx.

Rotenone and ouabain treated chicken enterocytes were isolated and stored in sodium free medium 2 at pH 7.2 (see materials). Cells were then introduced to medium 2 with/without sodium (50 mM) and at pH 6.0 or Ph 7.2 . Error bars: ± S.E.

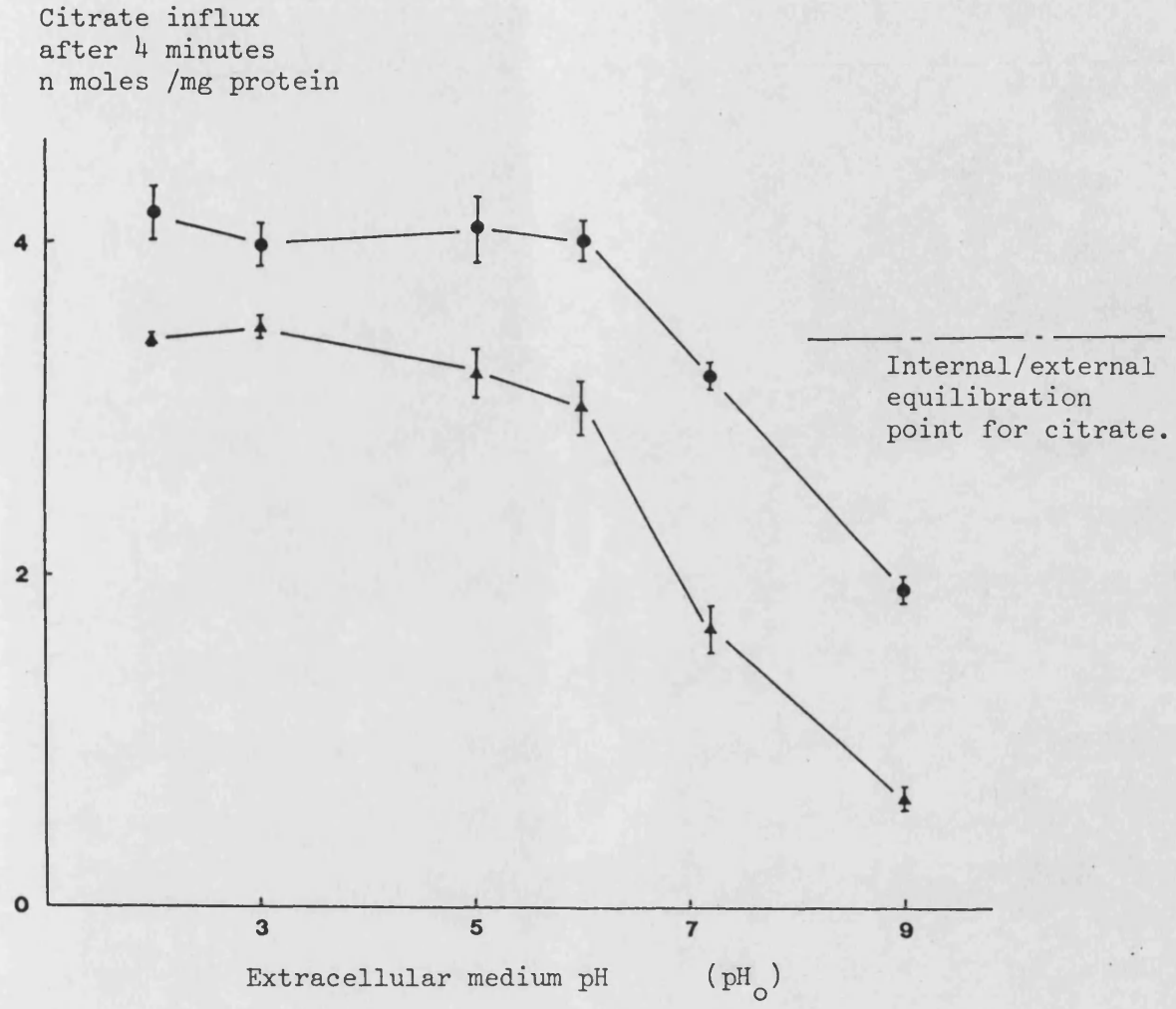


Figure 24 Effects Of pH Gradients On Enterocyte Citrate Content.

Rotenone and ouabain treated chicken enterocytes were isolated and stored in sodium free medium 2 at pH 7.2 (see materials). Cells were then introduced to highly buffered media at the indicated pH values containing 2 mM citrate for 4 minutes at 37°C. Error bars : ± S.E.

● 50 mM sodium media

▲ sodium free

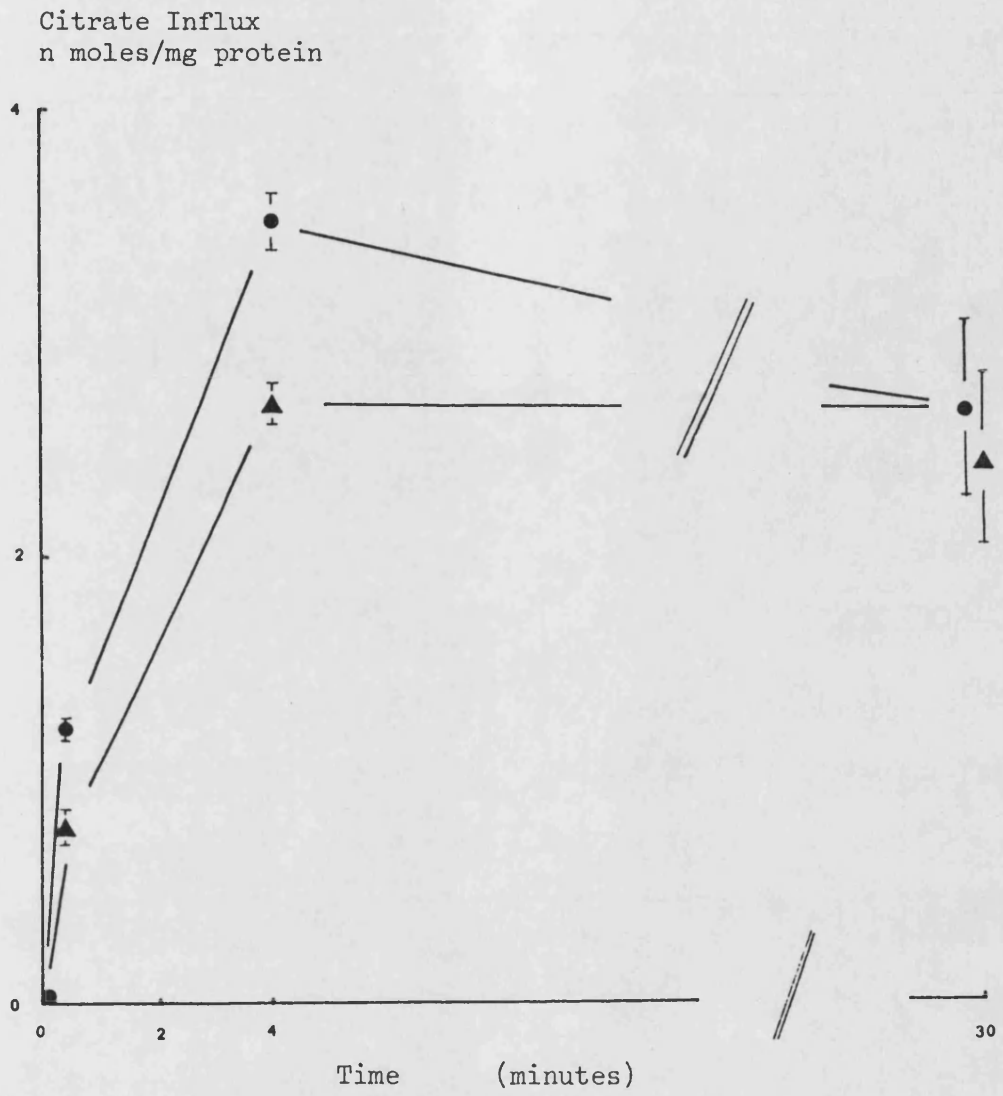


Figure 25 The Effect Of A pH Gradient On Citrate Flux In The Presence Of Sodium.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media 2 at pH 7.2 (see materials). Cells were then introduced to medium 2 containing 50mM sodium and 2 mM citrate at either pH 6 or pH 7.2 . Error bars: ± S.E.

● pH_o 6.0

▲ pH_o 7.2

implies that these effects cannot be explained by pH gradient induced swelling. However, in addition to this, cytocrit determinations show that the cells do not measurably swell, and galactose influx is unaffected by a pH gradient.

Figure 27 shows the analogous galactose influx under similar conditions to figure 26. It can be seen that the influx of galactose is not dependent on the pH gradient across the enterocyte. In this case the comparison of influx profiles of galactose and citrate under similar conditions is probably the best control for eliminating the possibility of changes in cell volume.

Figure 28 shows the influx of citrate into ATP depleted enterocytes in the absence of a pH gradient. The citrate concentration was maintained at a 'minimum' to determine whether it was possible to accumulate citrate with a transient sodium gradient alone. It can be seen that after only 1 minute the internal citrate concentration equals that in the external medium. After 4 minutes citrate has been accumulated. This accumulation is then maintained for a further 4 minutes. At this time the sodium gradient has been found to dissapate (e.g. see figure 33). After 30 minutes the internal citrate concentration has decreased to point close to the equilibrium level. Citrate can, therefore, be accumulated by a sodium gradient alone. However, it appears that with higher citrate concentrations this does not occur (possibly because the transient sodium gradient may be dissipated before accumulation can take place.)

Figure 29 shows the variation in initial rate of citrate

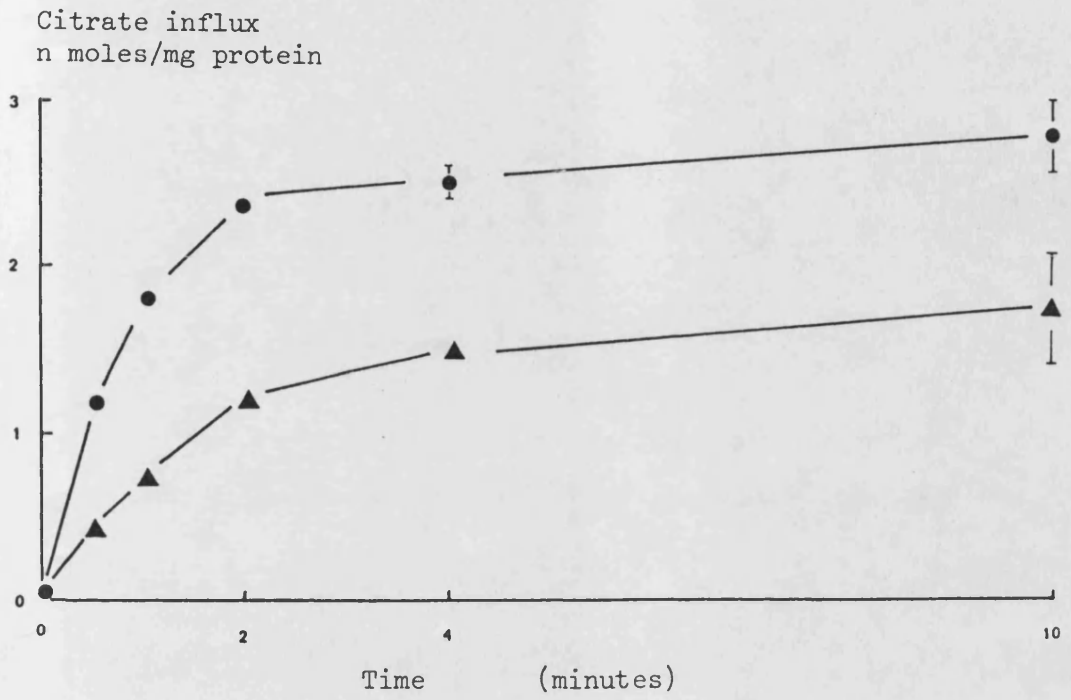


Figure 26 The Effect Of A pH Gradient On Sodium Independent Citrate Flux.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media 2 at pH 7.2 (see materials). Cells were then introduced to sodium free medium 2 containing 2 mM citrate at either pH 6.0 or pH 7.2. Error bars: ± S.E. Error bars ommited when smaller than symbols used.

● pH gradient (pH_o 6 -- pH_i 7.2)

▲ no pH gradient (pH_i=pH_o=7.2)

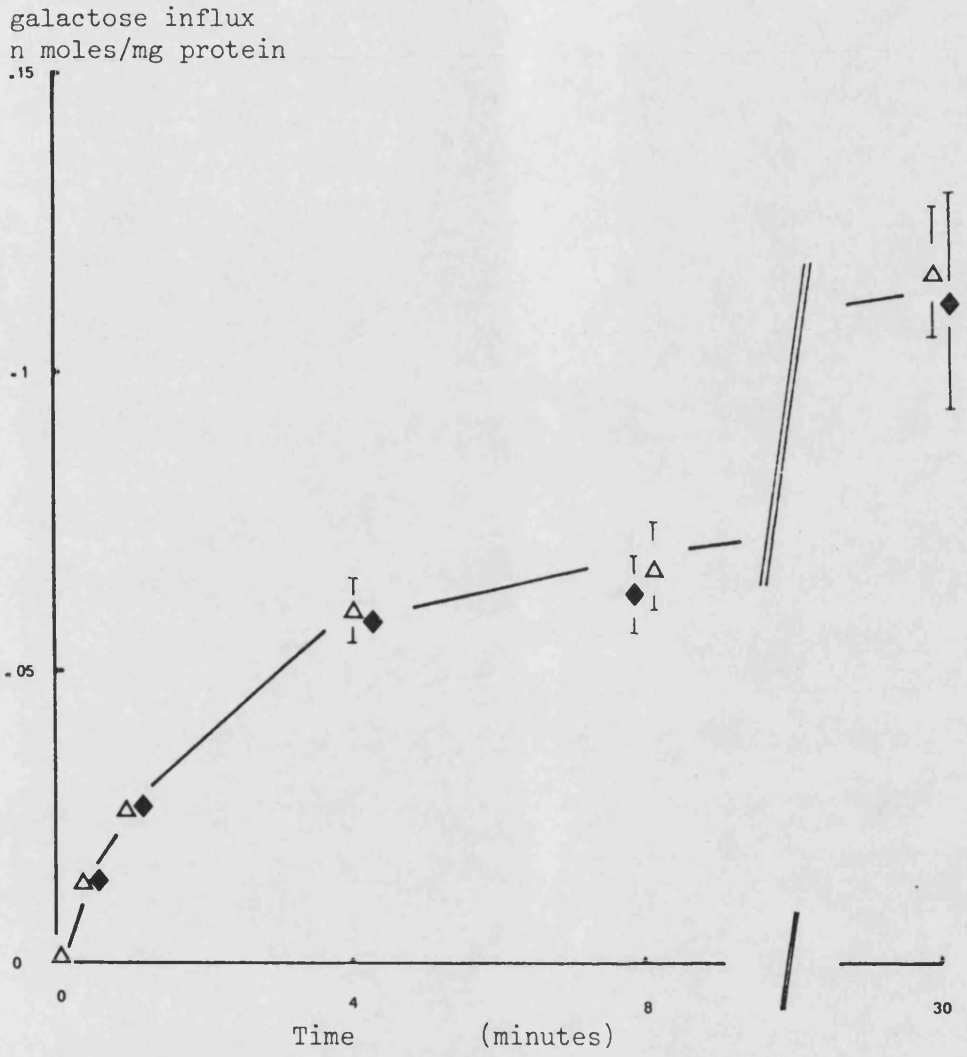


Figure 27 Galactose Influx Under Comparable Conditions To Citrate Influx In Figure 26.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media 2 at pH 7.2 (see materials). Cells were then introduced to sodium free medium 2 containing galactose (100 uM) at either pH 6.0 or pH 7.2 . Error bars: ± S.E. Error bars omitted when smaller than symbols used.

△ pH gradient (pH_o 6 -- pH_i 7.2)

◆ no pH gradient (pH_i=pH_o=7.2)

influx with citrate concentration for ATP and sodium deprived enterocytes. The cells in figure 29 were exposed to media containing 100 mM sodium at pH 7.2. Evidence of saturation can be seen at citrate concentrations above 250 μM, with prominent saturation above 750 μm. The data presented in the Double Reciprocal Plot (figure 30) shows good correlation to saturation kinetics, with a K_t value calculated from a Direct Linear Plot of 324 μM. (Median K_t value : 324 μM, K_t range : 126-411 μM).

Figure 31 shows the saturation of the sodium independent citrate flux for ATP depleted cells without a pH gradient. The initial rates can be seen to be considerably lower than those for the sodium dependent flux. Figure 32 shows the Double Reciprocal Plot of this data and a Direct Linear Plot determines the K_t to be 504 μM. (Median K_t value : 504 μM, K_t range 379 - 1151 μM).

The sodium dependence of one of the citrate fluxes and the ability of sodium to cause citrate accumulation led to the investigation of the effects of citrate on sodium flux. In order to maximise any effects of citrate on sodium influx, investigations were carried out with an internally directed pH gradient. These conditions had previously been shown to cause maximal citrate uptake (see figure 23).

The sodium influx into ATP and sodium depleted enterocytes in the presence of a pH gradient is shown in figure 33. Citrate can be seen to increase sodium influx, particularly at between 2 - 4 minutes. Time periods below

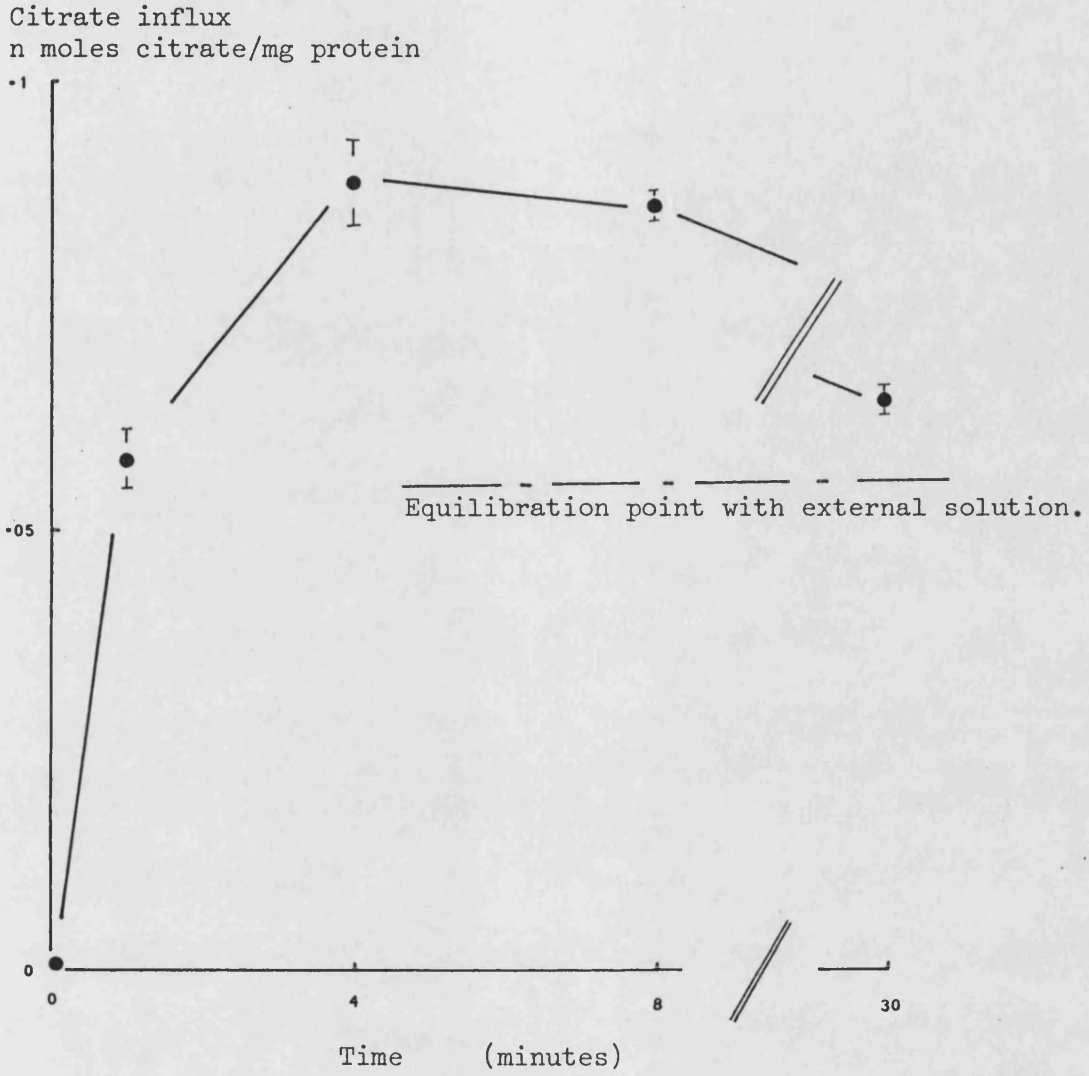


Figure 28 The Effect Of A Transient Sodium Gradient On The Uptake Of Low Citrate concentrations.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media 2 at pH 7.2 (see materials). Cells were then introduced to medium 2 containing 100 mM sodium and 20 uM citrate at pH 7.2 .
Error bars: ± S.E.

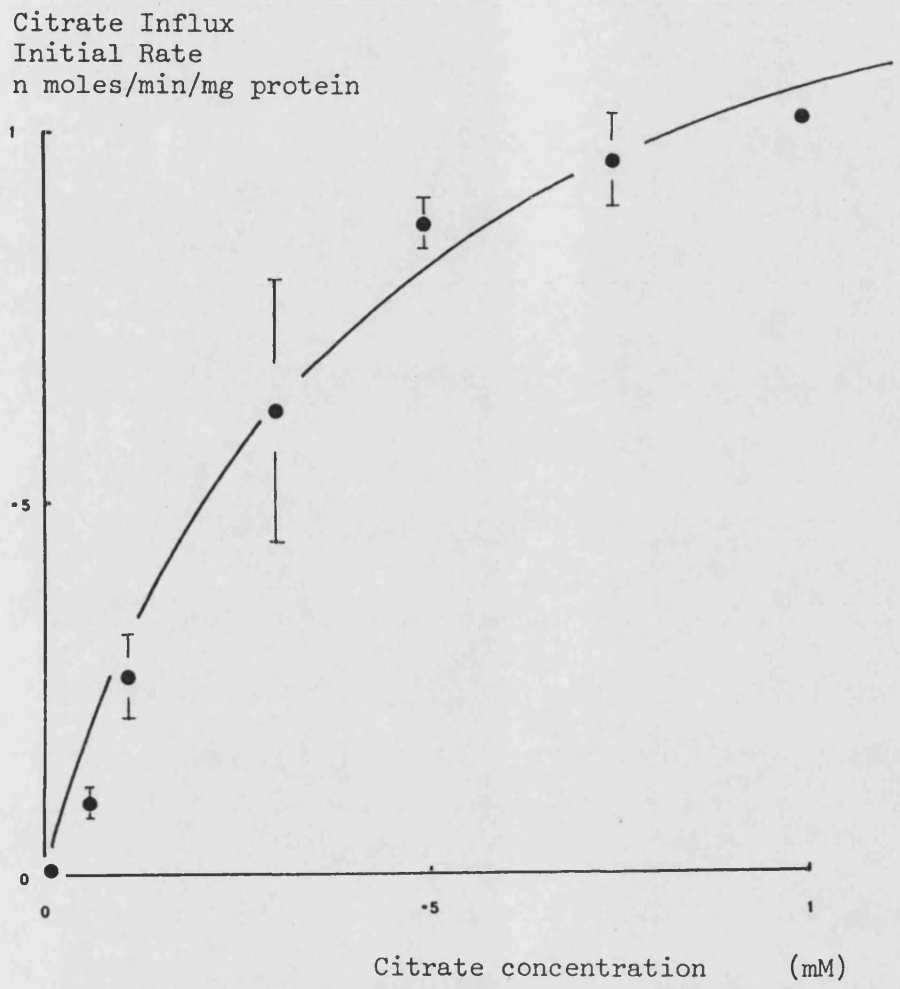


Figure 29 The Saturation Of Sodium Dependent Citrate Flux By Citrate Concentration

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media 2 at pH 7.2 (see materials). Cells were then introduced to medium 2 containing 100 mM sodium and the indicated citrate concentration at pH 7.2 . Computer calculated curve with K_t 324 uM. Error bars: ± S.E.

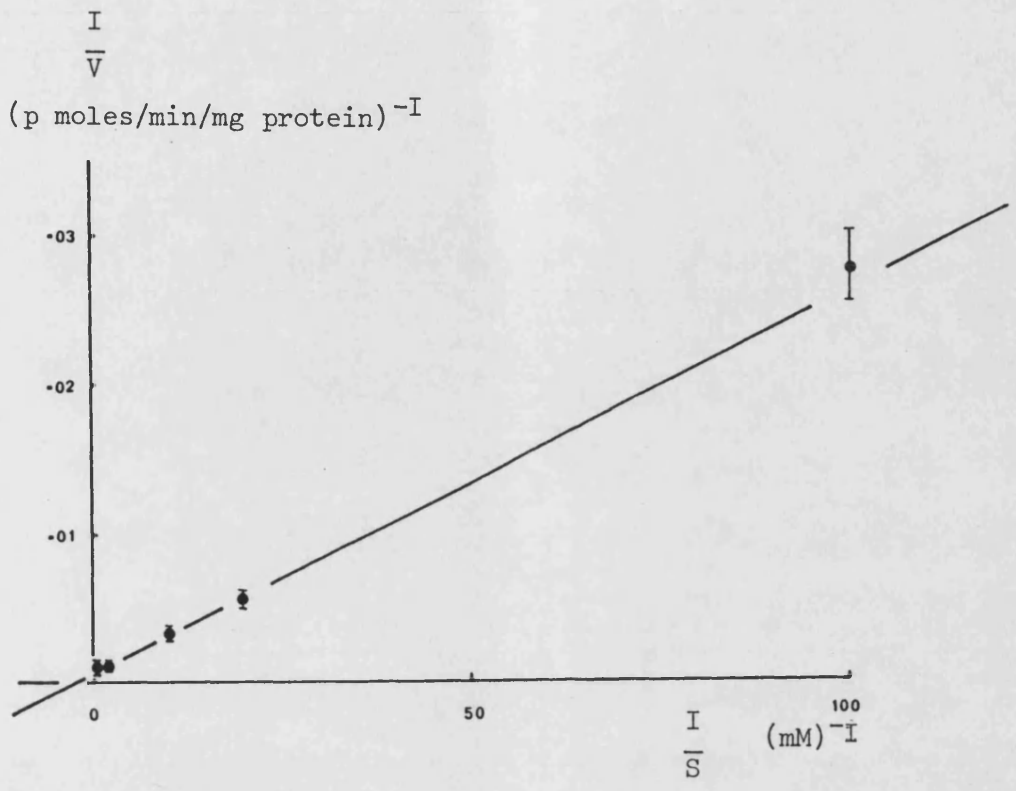


Figure 30 Sodium Dependent Citrate Influx Kinetics.

Double reciprocal plot of sodium dependent citrate influx data. (see previous figure (29) for experimental details).

K_t 504 uM

V_{max} I.3 n moles/min/mg

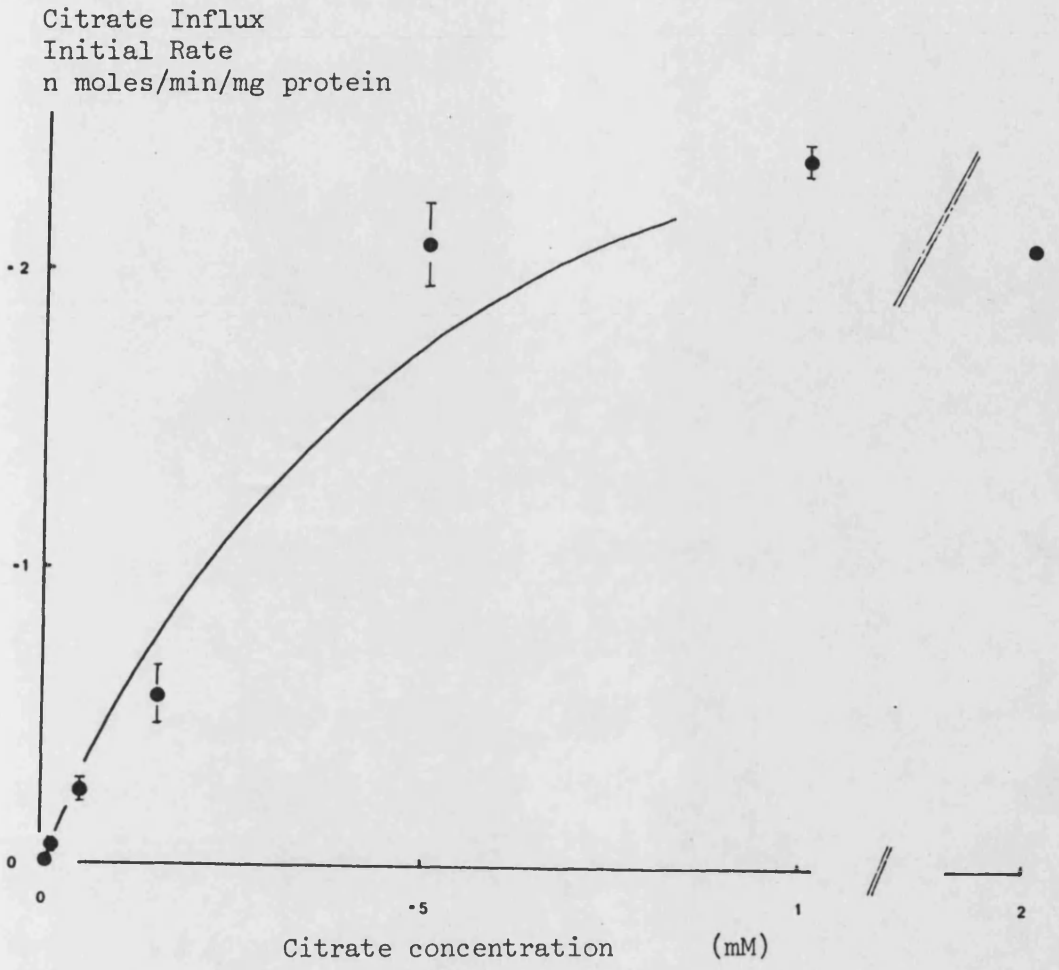


Figure 3I The Saturation Of Sodium Independent Citrate Flux By Citrate Concentration

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media 2 at pH 7.2 (see materials). Cells were then introduced to sodium free medium 2 containing the indicated citrate concentration at pH 7.2 . Computer calculated curve with K_t 504 uM. Error bars: ± S.E.

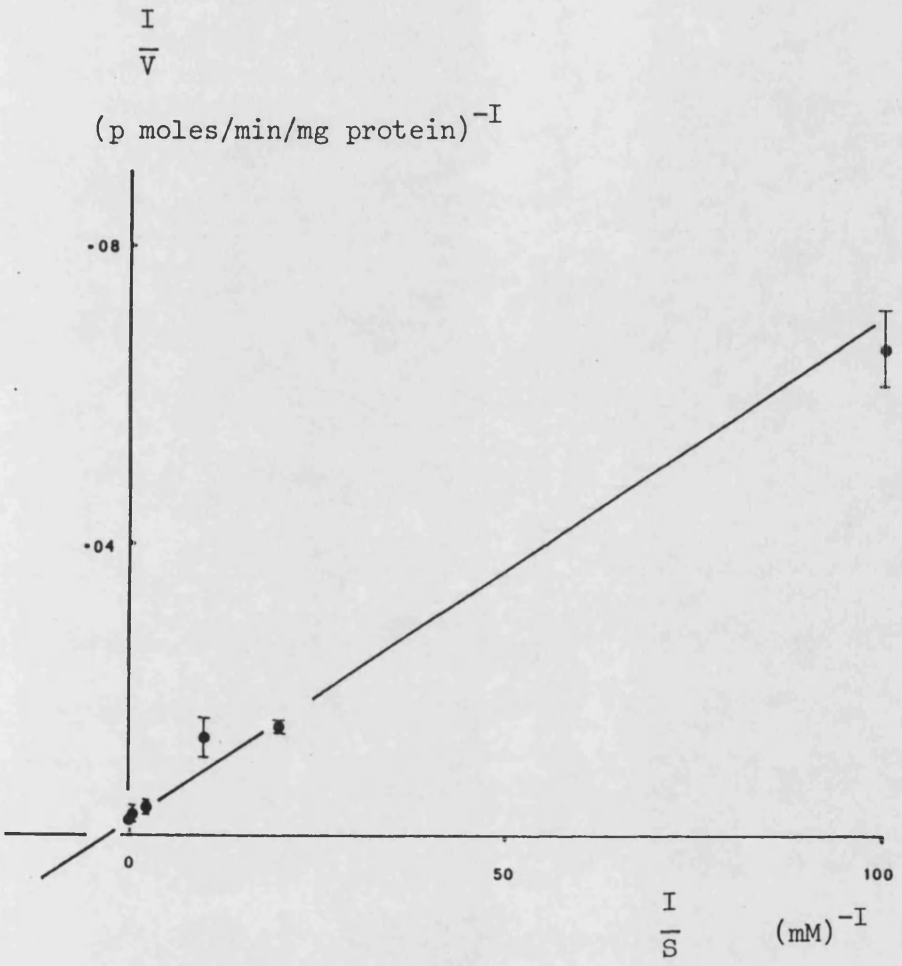


Figure 32 Sodium Independent Citrate Influx Kinetics.

Double reciprocal plot of sodium independent citrate influx data. (see previous figure for experimental details)

K_t 324 uM

V_{max} .32 n moles/min/mg

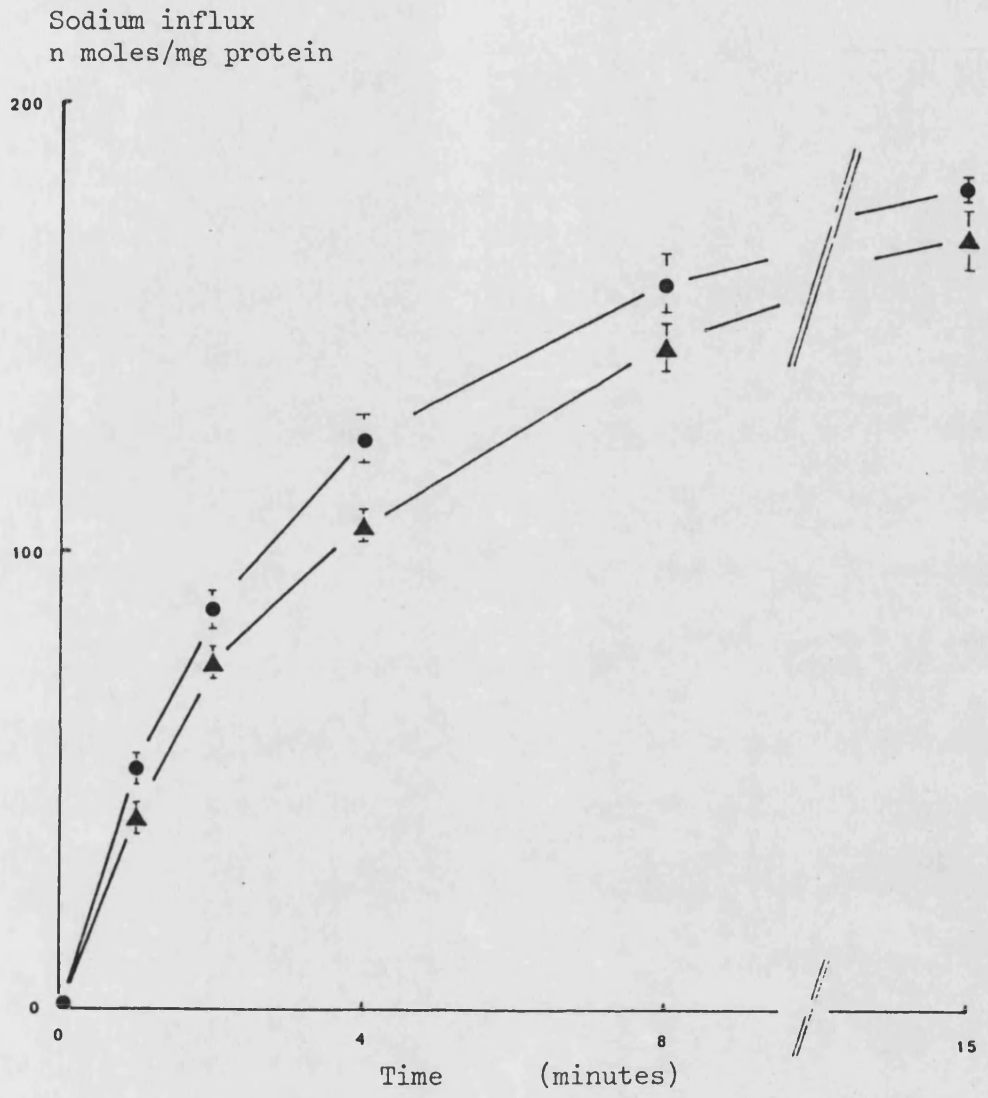


Figure 33 Effects Of Citrate On Sodium Flux.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free medium 2 at pH 7.2 (see materials). Cells were then introduced to medium 2 containing 50 mM sodium, with/without citrate at pH 6. Error bars: ± S.E.

● + CITRATE (6mM)

▲ control

2 minutes show smaller effects of citrate on sodium influx than might be expected from the 2 - 4 minute values. This suggests possible effects after citrate has been transported. After 4 minutes the effect of citrate on sodium flux diminishes. This may reflect the reaching of the maximal intracellular citrate levels seen previously (figure 20).

Although it was appreciated that the smaller effects of citrate on sodium influx at short time periods, could be an artefact of high sodium and pH gradient production, this finding led to the consideration of 'delayed' citrate effects on sodium influx.

One such mechanism, bearing in mind the pH gradient sensitivity of citrate flux, was that Na^+/H^+ exchange played a role in the interaction of sodium and citrate. Na^+/H^+ exchange systems had been found in intestinal brush border vesicle preparations (Murer et al,1976; Kinsella et al, 1956). However, Na^+/H^+ exchange had not been previously demonstrated in the isolated enterocyte.

Figure 34 demonstrates enterocyte Na^+/H^+ exchange. The closed circle symbols represent ATP and sodium depleted enterocytes isolated at pH 7.2 and introduced to an external medium containing sodium. This case therefore represents the sodium influx in the absence of a pH gradient. The closed triangles represent the sodium influx into ATP and sodium depleted enterocytes which were previously equilibrated in sodium free medium at pH 6. When placed in a medium at pH 7.2, the resulting outwardly directed pH gradient can be seen to increase sodium influx. The exchange

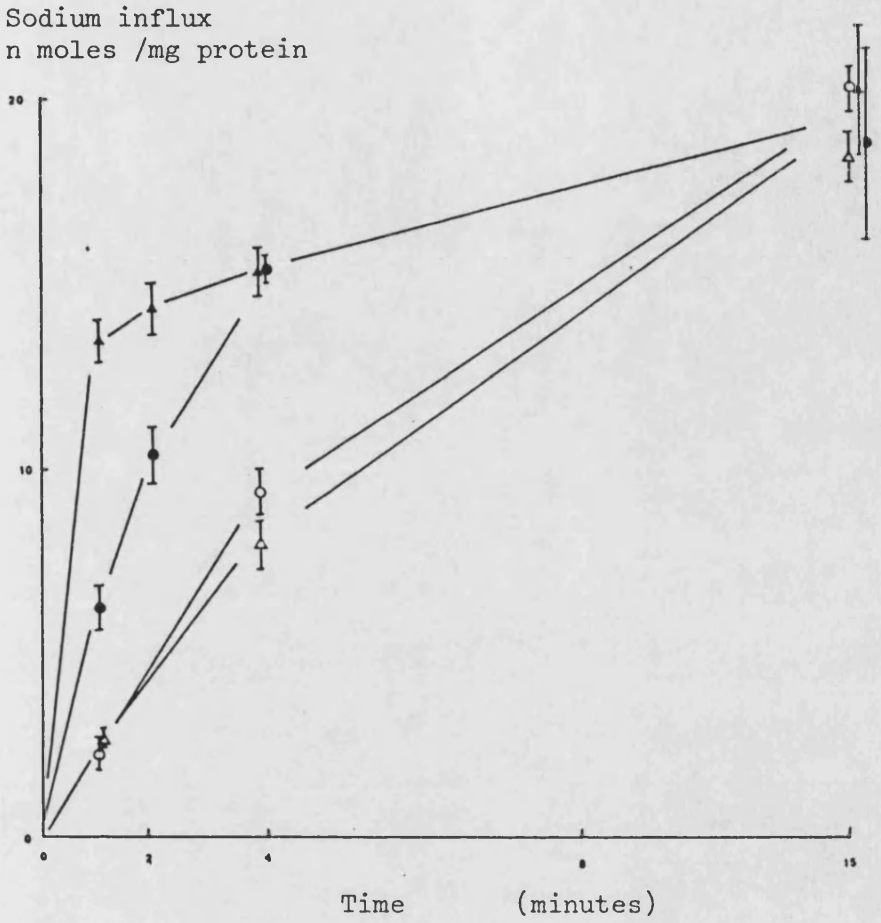


Figure 34 Demonstration Of Enterocyte Na^+/H^+ Exchange - Effects Of H^+ Movements On Sodium Influx.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free medium 2 at pH 7.2 (see materials). Cells were then equilibrated in sodium free media at pH 7.2 (● , o), or at pH 6.0 (▲ , △). Sodium influx (IO mM) was then measured into the cells with varying internal pH values. Open figures show cells pre incubated with amiloride (IOOuM). Error bars: ± S.E.

Summary:

▲ pH_i 6, pH_o 7.2

● $pH_i = pH_o = 7.2$

△ as above but pre incubated

o with amiloride

of protons for sodium ions can be seen to be very rapid, causing a marked increase in the initial rate of sodium entry. After a period of 4 minutes, the pH and sodium gradients can be seen to have dissipated at this particular external sodium concentration. The initial pH gradient can also be seen to have little effect on the final sodium equilibrium value. Cytocrit and cell protein determinations also ensured that there had been no initial cell swelling.

The open circles and triangles represent identical conditions to the closed symbols, with the exception that the cells had been preincubated with amiloride. Amiloride is a specific Na^+/H^+ exchange inhibitor and can be seen to entirely remove the effect of the pH gradient on sodium entry. In addition to proving that the effects demonstrated are due to Na^+/H^+ exchange, amiloride can be seen to reduce the sodium influx into cells without a pH gradient. This indicates that sodium entry through the Na^+/H^+ exchange system is appreciable even in the absence of any pH gradient. In this case the influx of sodium through the Na^+/H^+ exchange system must cause the ejection of protons from the cells. Thus an inwardly directed sodium gradient may ultimately give rise to an inwardly directed pH gradient. As this is an exchange system, when the Na^+ and H^+ ion gradients are equal in direction and magnitude each ion gradient will tend to reduce the influx of the other ion.

Figure 35 shows that this is the case and that sodium influx in the presence of an inwardly directed pH gradient

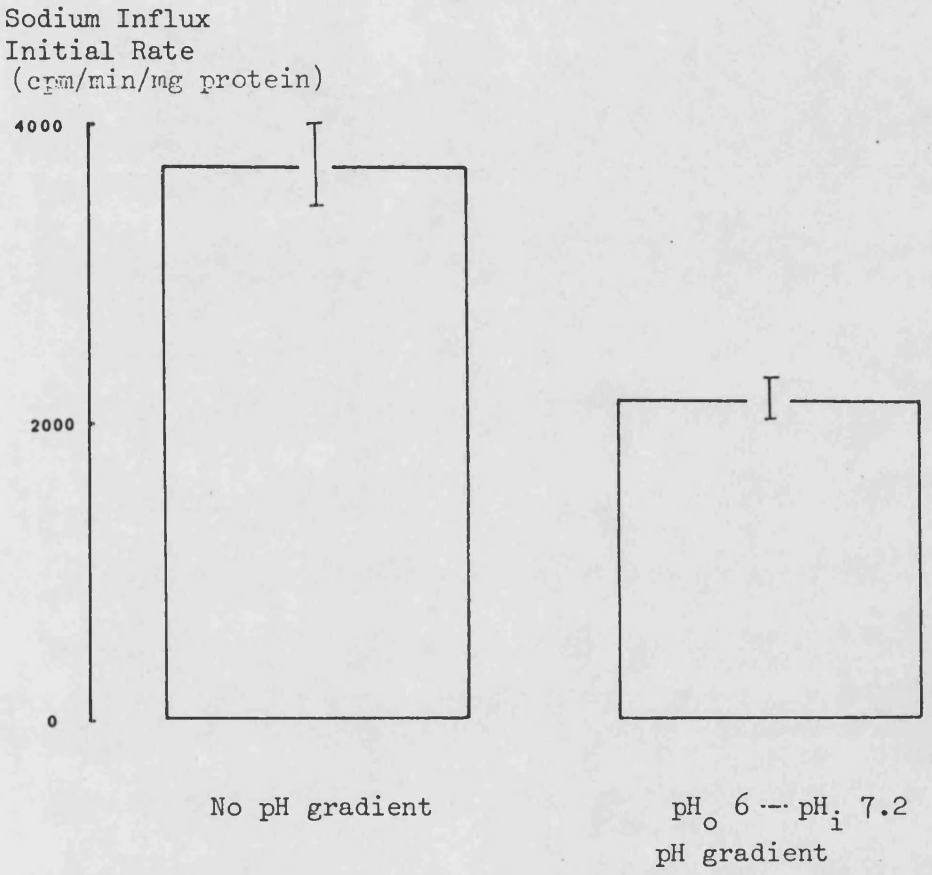


Figure 35 The Effect Of An Inwardly Directed pH Gradient On Sodium Influx.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free medium 2 at pH 7.2 (see materials). Cells were introduced to medium 2 containing 50 mM sodium at either pH 6.0 or pH 7.2 . Error bars: ± S.E.

is less than in the absence of a pH gradient,(c.f. an inwardly directed pH gradient was used for the studies of citrate effects on sodium influx).

The Na^+/H^+ exchange system was further studied by developing a new technique for determining proton sensitive radiolabelled dye distribution. The dye used was dimethyloxazolidine dione. Ultimately the aim was to employ this method for studying citrate and proton relationships.

Dimethyloxazolidine dione (D.M.O.) is a lipid soluble dye with weak acid properties. DMO has a pK_a of 6.13 and the protonated and dissociated forms show differences in their lipid solubility as the dissociated form is charged. When DMO is added to a cell suspension the dissociation equilibrium is set up in the extracellular medium depending on pH. The undissociated form, being lipophilic, then crosses the cell membrane with the appropriate redistribution of the external dissociation equilibrium. Once inside the cells the undissociated DMO then reaches an internal dissociation equilibrium depend ent on intracellular pH. After an immeasurably rapid period of time the whole system reaches an equilibrium. Any changes in intracellular and extracellular pH are then reflected in the amount of dye inside and outside the cells. Increases and decreases in DMO in the extracellular medium can then easily be measured (^{14}C DMO).

(It should be noted that DMO dye distribution is independent of the numerical pH value. Thus it is not possible to distinguish between cells of pH_i 6, pH_o 6 and cells of pH_i 7, pH_o 7.)

Figure 36 shows the use of the DMO dye technique to demonstrate Na^+/H^+ exchange. The three treatment profiles shown are; enterocytes with an outwardly directed pH gradient (pH_i 6, pH_o 7.5) and the addition of mannitol (▲), an outwardly directed pH gradient and the addition of sodium (●), and no pH gradient with the addition of sodium (Δ). It can be seen that initially the three treatments begin at two different radioactivity levels. This corresponds to a pH gradient for the ● and ▲ symbols and no pH gradient for the Δ symbols. As samples cannot be taken in duplicate, sampling was continued until, after 5 minutes, either unbuffered mannitol or unbuffered sodium (as NaCl or Na_2SO_4) was added. For cells with a pH gradient, it can be seen that the addition of mannitol (▲ in figure 36) caused a change in the DMO distribution such that the radioactivity in the external medium was reduced. This does not represent a variation in the dissociation equilibrium, but merely a change in internal/external dye distribution as the external volume was increased.

The cells without a pH gradient (Δ) experience a similar change, leading to a reduction in the radioactivity of the external medium. This also represents a change in distribution of dye (due to dilution) and not a change in

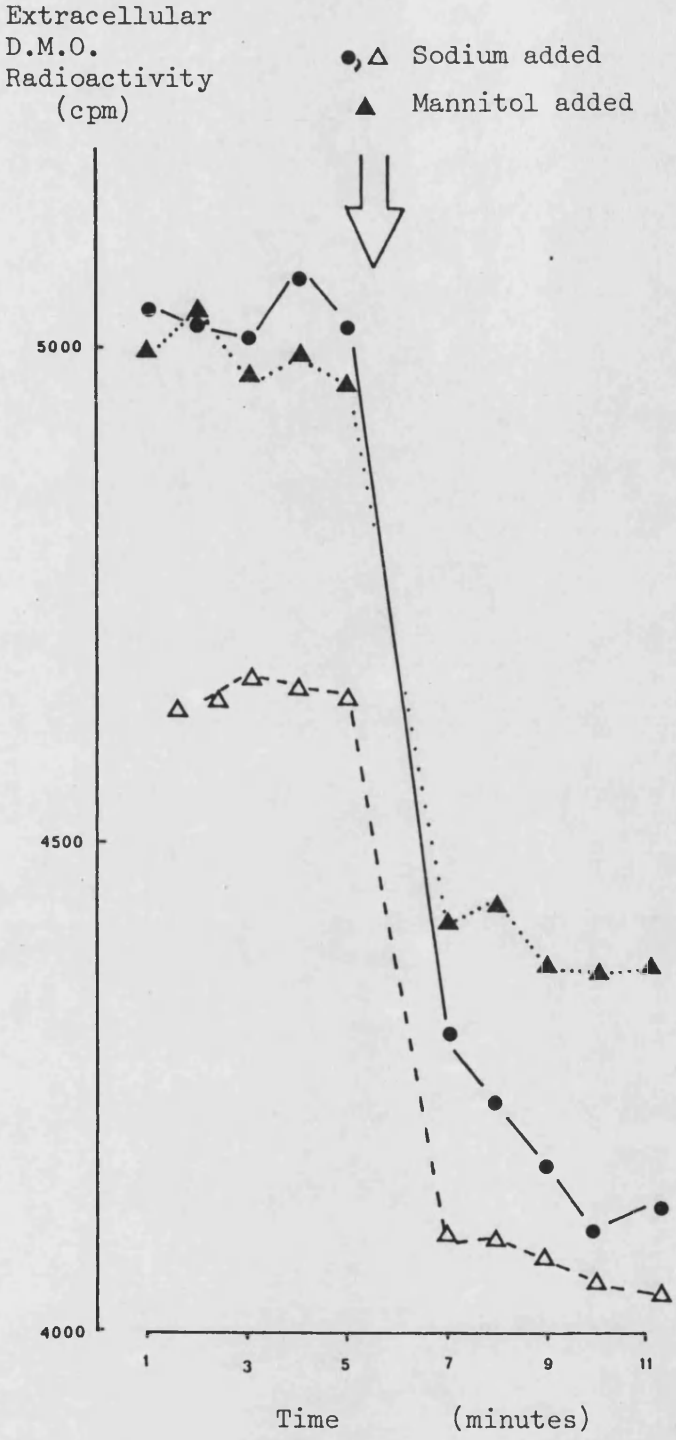


Figure 36

Demonstration Of Enterocyte Na^+/H^+ Exchange - Effects Of Sodium On Proton Movements.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media and equilibrated in low buffering capacity solutions at pH 6.0 . Cells were then introduced to low buffering capacity solutions at pH 6.0 (△) or pH 7.5 (●,▲). Sodium or mannitol was then added after 6 minutes.

Summary:

● and ▲ pH_i 6, pH_o 7.5 ; △ pH_i 6, pH_o 6

dissociation equilibria. When comparing the changes, with and without a pH gradient, caused by the addition of mannitol (▲ and △ respectively), it can be seen that the profiles are exactly superimposable. This is an indication that there has been no change in pH gradient, and only a constant change in external radioactivity from the original starting point.

The addition of sodium to cells with a pH gradient, however, shows a marked difference in profile to the aforementioned profiles, (● in figure 36). It can be seen that the two sets of cells with a pH gradient begin at initially identical levels of radioactivity. The introduction of sodium then causes a change in extracellular radioactivity that finally resembles the values for cells without a pH gradient. This indicates the breakdown of the pH gradient by the addition of sodium. It can be seen that this is a gradual process taking approximately 5 minutes.

Figure 37 shows the effect of the specific Na^+/H^+ exchange inhibitor amiloride. The three cell suspensions shown begin with similar distributions of DMO, since all have a pH gradient (pH_i 6, pH_o 7.5). The closed triangles (▲) represent cells with unbuffered mannitol added after 5 minutes and show the effect of dilution, (as seen in figure 36). The ability of sodium to cause pH gradient breakdown is represented in this figure by open triangles, (same profile as the closed circles of figure 36). The remaining profile (●) represents the inhibition of sodium induced pH gradient breakdown by amiloride. It can be seen

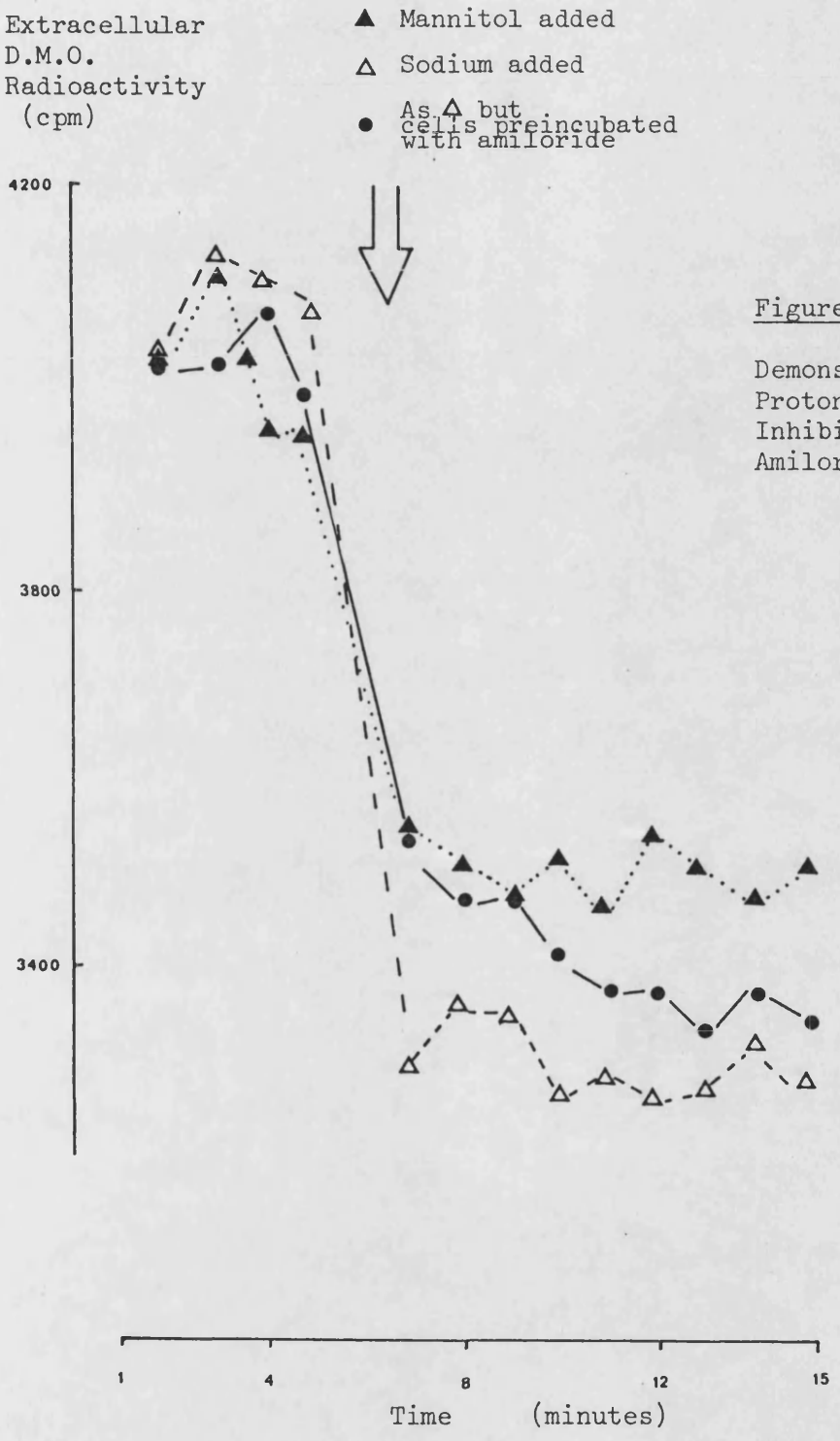


Figure 37

Demonstration Of Proton Movement Inhibition By Amiloride.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media and equilibrated in low buffering capacity solutions at pH 6.0 . Cells were then introduced to a sodium free solution at pH 7.5 and after 6 minutes either mannitol or sodium was added. Therefore all cells pH_i 6 and pH_o 7.5 .

that after the addition of sodium, amiloride has almost entirely inhibited the pH gradient breakdown. This is shown by the 6 - 8 minute points resembling cells with a pH gradient. As the incubation continues, however, the amiloride inhibited profile can be seen to approach that showing gradient breakdown in the presence of sodium. Finally the amiloride inhibited profile reaches a value close to that for pH gradient breakdown although this does take a considerable period of time. This represents the competitive nature of amiloride inhibition, and thus amiloride retards rather than stops Na^+/H^+ exchange.

Figure 38 shows the effects of citrate on intracellular pH under similar conditions to those showing an increase in sodium influx by citrate (figure 33). The DMO concentration in the external medium can be seen to rise as sodium and citrate enter the cells. This corresponds to a breakdown of the imposed pH gradient (pH_o 6, pH_i 7.2). Citrate can be seen to exert little effect on the DMO distribution, and the pH gradient can be seen to have dissipated quite considerably after 5 minutes.

The inset histogram shows the sodium uptake after 4 minutes under the same conditions as those for the DMO distribution investigation. It can be seen again that citrate increases sodium influx, even when citrate has no effect on H^+ ion movement.

Figure 39 shows the effects of amiloride on the citrate induced increase in sodium influx. When the enterocytes are preincubated with amiloride the sodium influx values can be seen to decrease. However, the increase in sodium

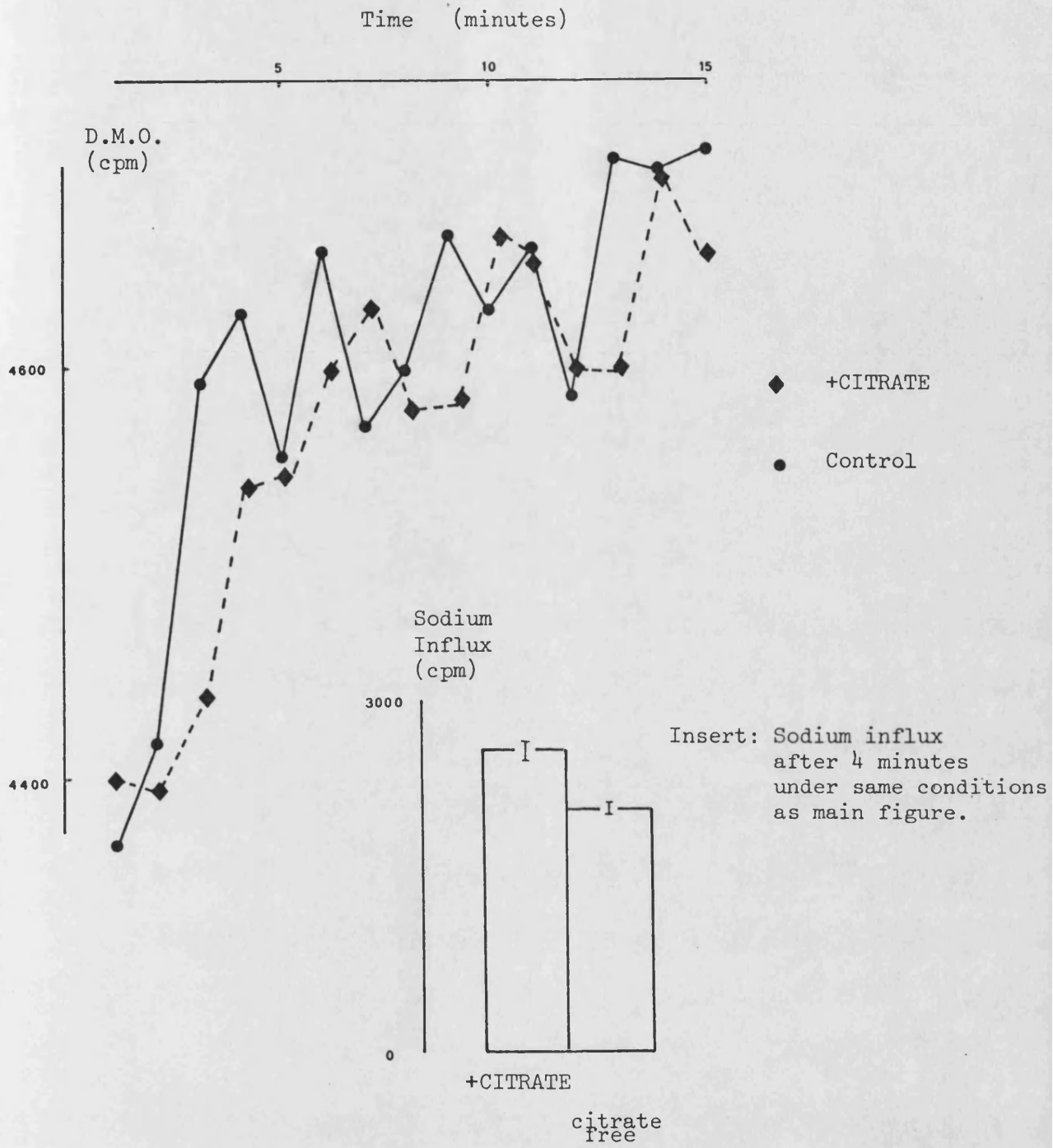


Figure 38 The Effects Of Citrate On Proton Movements.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free medium 4 at pH 7.2 (see materials). Cells were then introduced to a medium containing 50 mM sodium at pH 6.0, with/without citrate.

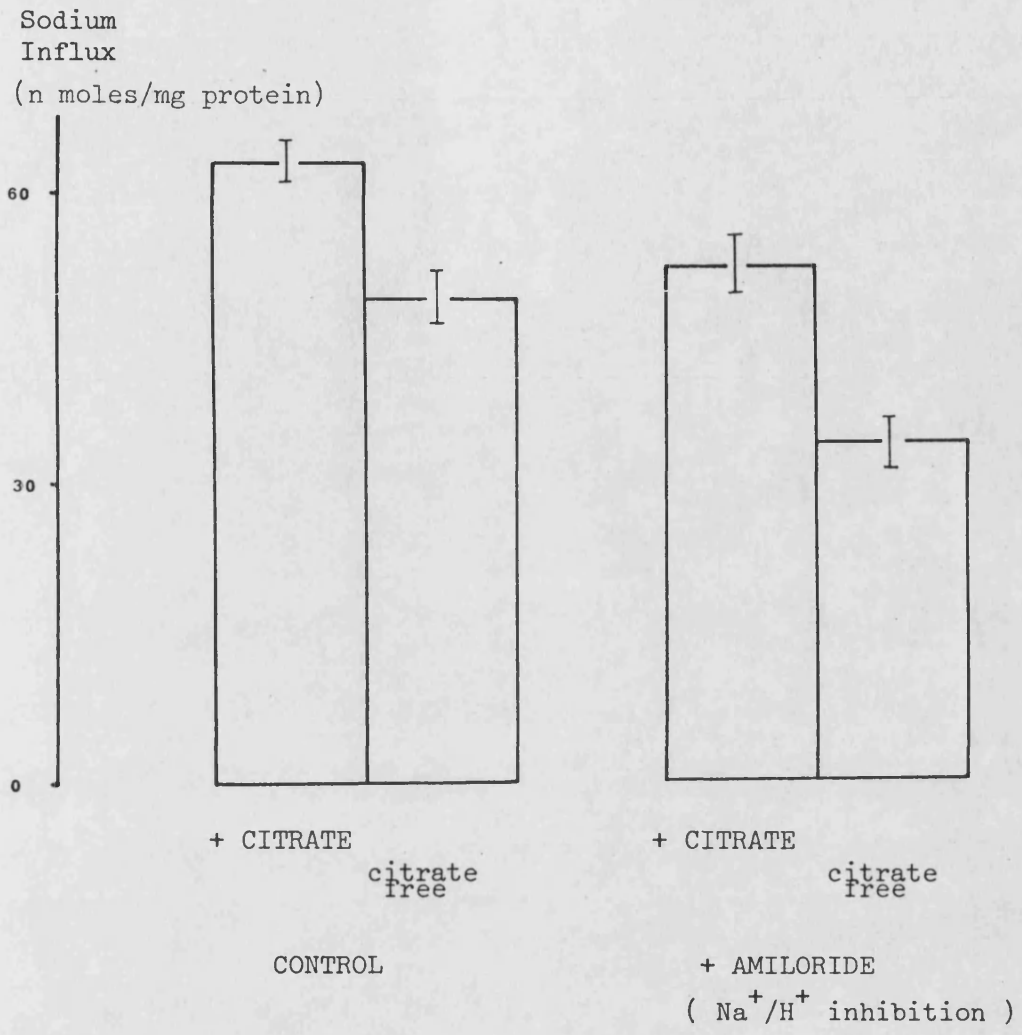


Figure 39 The Effects Of Citrate On Sodium Influx And The Role Of Na^+/H^+ Exchange.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media 2 at pH 7.2 . (see materials). Cells were then introduced to medium 3 containing 50 mM sodium at pH 6.0 with/without 6 mM citrate for 4 minutes.
Error bars: ± S.E.

influx in the presence of citrate is of similar magnitude irrespective of amiloride.

Figure 40 shows the sodium influx time course in the presence of amiloride, for enterocytes incubated with and without citrate. Amiloride has no effect on the citrate induced increase in sodium influx,and again the maximal effects on sodium influx are seen at time periods exceeding 1 minute.

Figure 41 shows that the inhibition of Na^+/H^+ exchange has no effect on citrate influx.

This is further evidence against the involvement of Na^+/H^+ exchange in the actions of citrate on sodium influx.

Figure 42 shows the investigation of citrate metabolism with ouabain and rotenone treated enterocytes. Figure 42a shows the influx of citrate into enterocytes from which duplicate points at 4, 8 and 30 minutes were taken for citrate metabolism analysis. It can be seen that this profile reaches a plateau after 4 minutes, and there is little increase in intracellular citrate after 30 minutes. This in itself is indicative of very little metabolism of citrate. The zero time point forms a relatively small proportion of the 4 to 30 minute influx values. This indicates that extracellular citrate contributes very little to the citrate radioactivity seen in the following chromatograms.

Figure 42b shows the separation by paper chromatography of the non TCA precipitatable intracellular contents after 4 minutes. The only appreciable radioactivity peak corresponds to citrate with all other points being within the

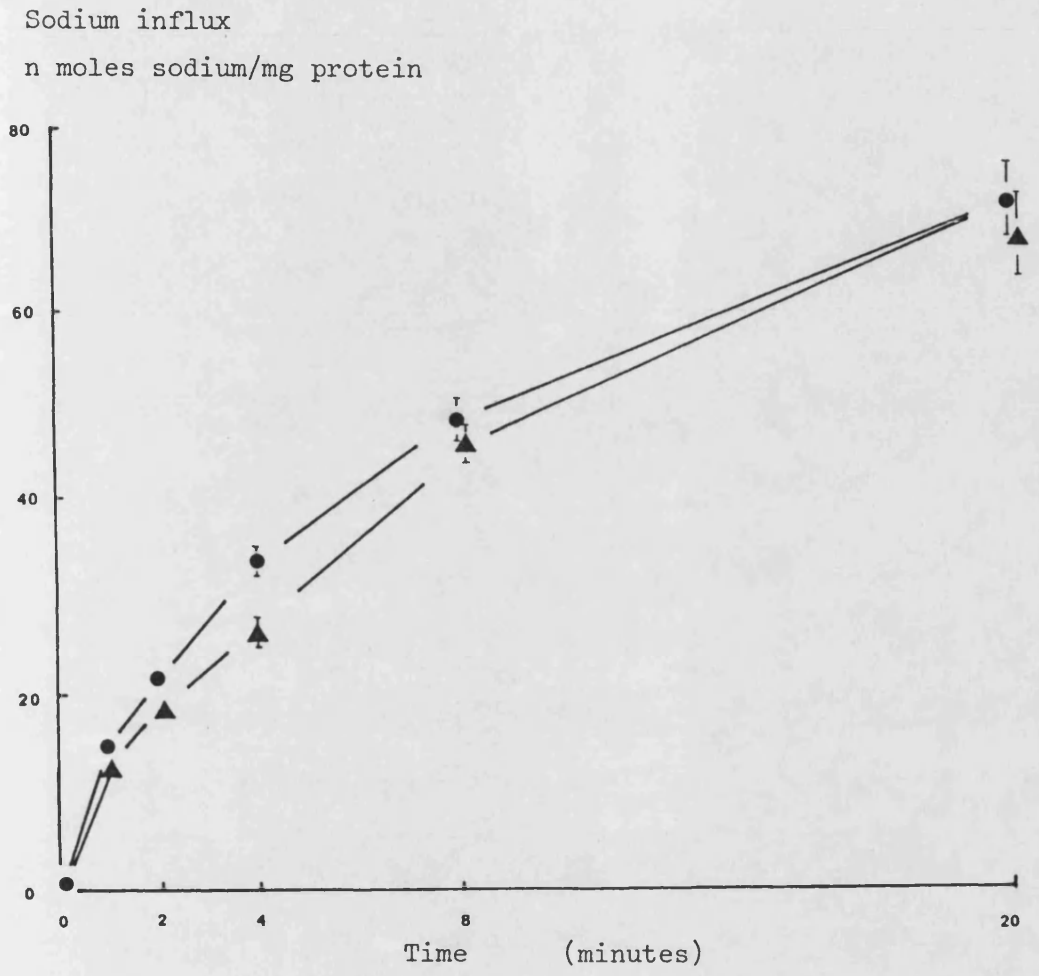


Figure 40 The Effects Of Citrate On Sodium Influx When Na^+/H^+ Exchange Is Inhibited.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media 2 at pH 7.2(see materials). Cells pre-incubated with IOOuM amiloride, were then introduced to medium 2 containing 50 mM sodium at pH 6 with/without 6mM citrate.Error bars: ± S.E.
Error bars are omitted when smaller than symbols used.

● + 6 mM Citrate

▲ control

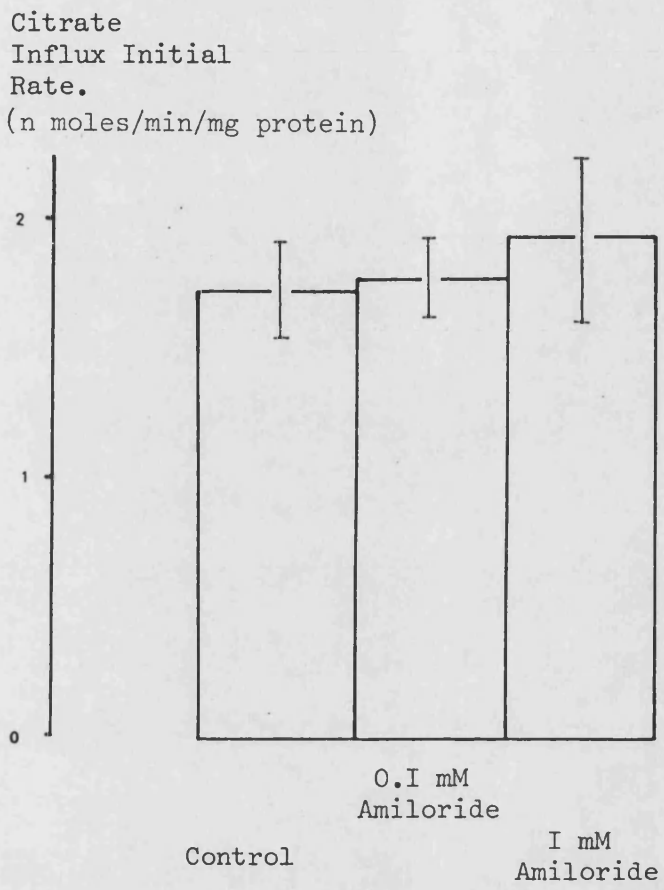


Figure 41 The Effect Of Inhibiting Na^+/H^+ Exchange On Citrate Influx.

Rotenone and ouabain trateted chicken enterocytes were isolated in sodium free media 2 at pH 7.2 (see materials).After pre-incubation with/without amiloride cells were introduced to a medium containing 50 mM sodium and 6 mM citrate at pH 6.0 . Error bars: $\pm$ S.E.

Determination Of The Extent Of Citrate Metabolism
With Rotenone And Ouabain Treated Enterocytes.

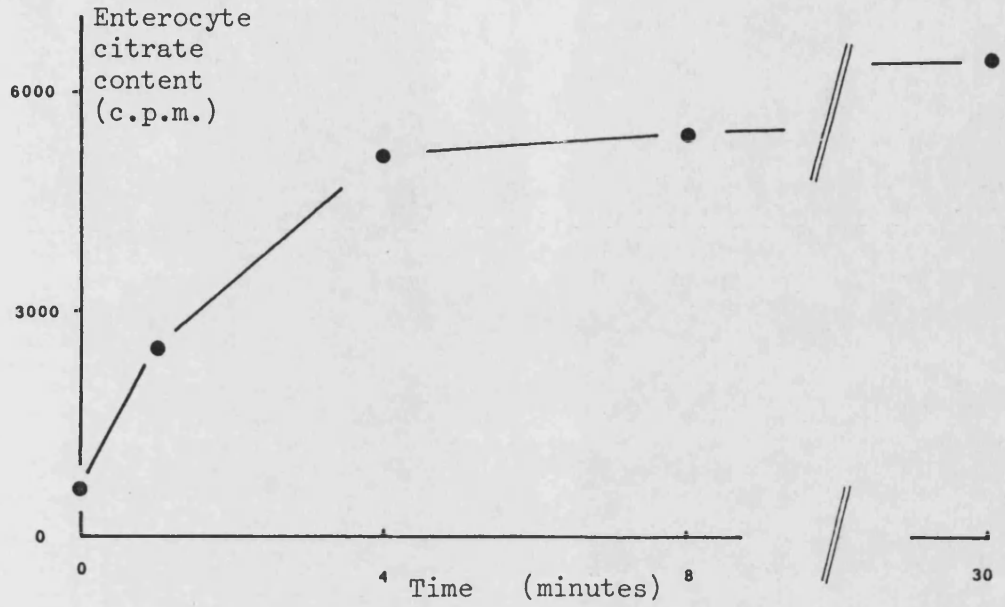


Figure 42 a Citrate influx profile -(points from which chromatography samples were taken).

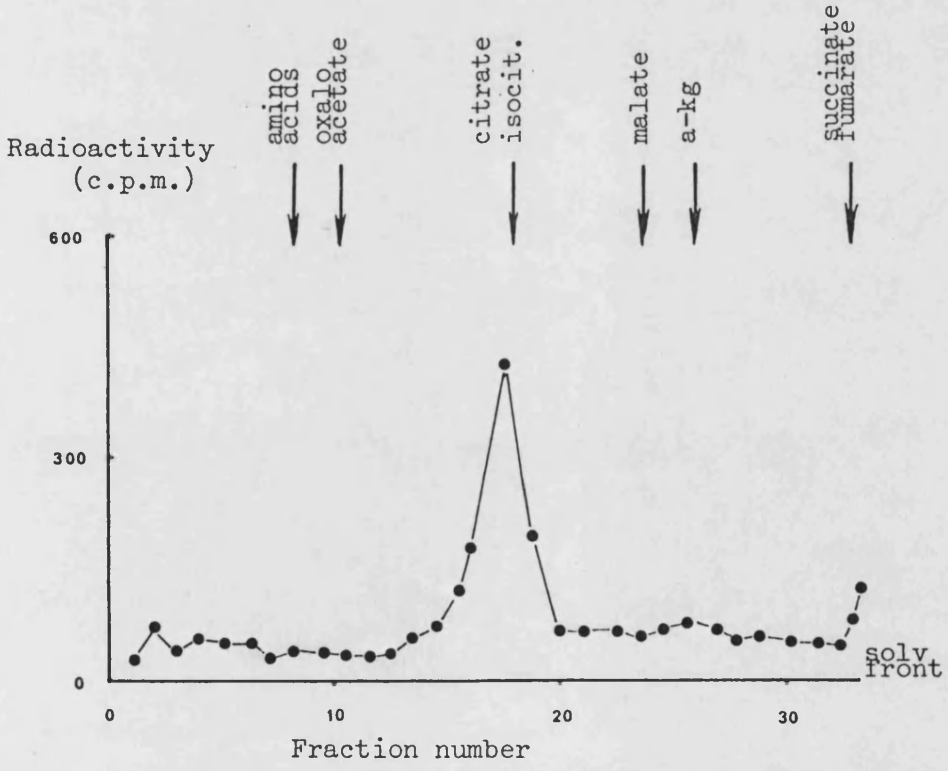


Figure 42 b Chromatographic Seperation Of The 4 Minute Citrate Influx Time Point.

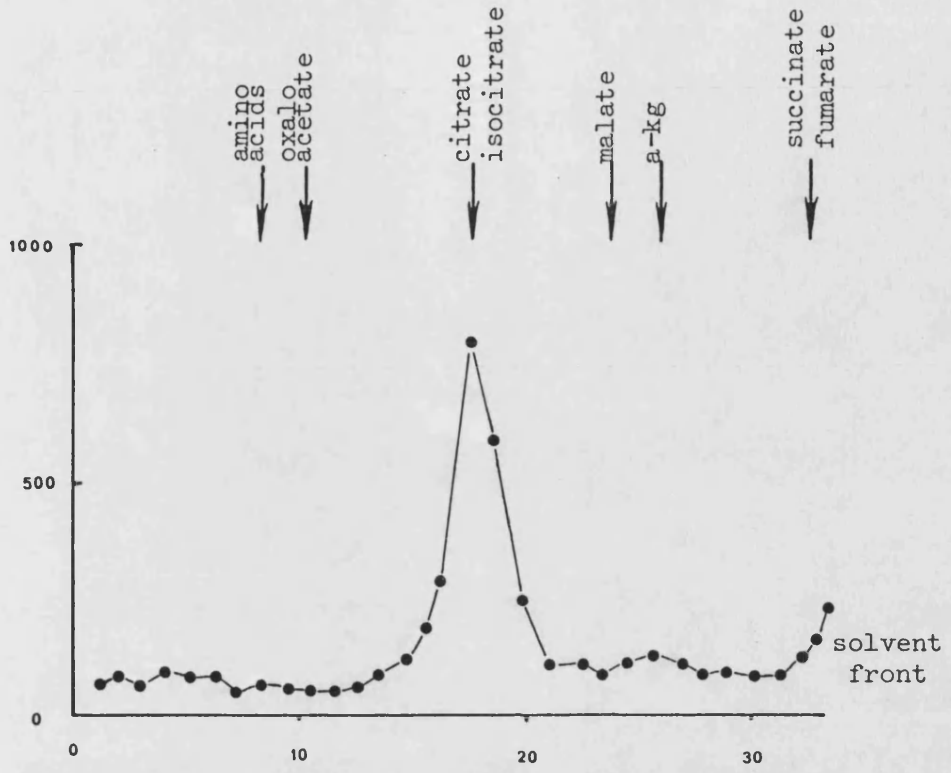
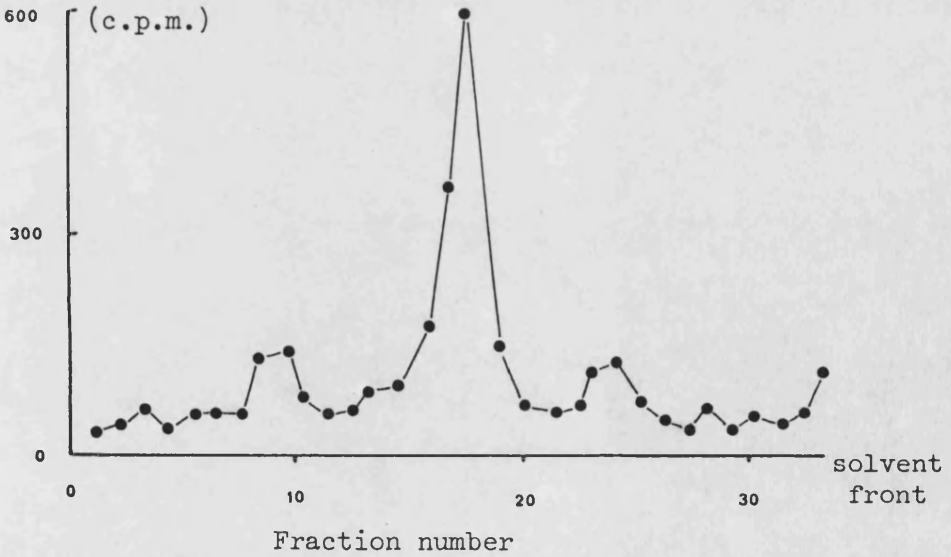


Figure 43 a Chromatographic Seperation Of The 8 Minute Citrate Influx Time Point.

Figure 43 b Chromatographic Seperation Of The 30 Minute Citrate Influx Time Point.



background variation.

A further peak was seen to run close to the solvent front, corresponding to the R_f value for succinate or fumarate. Labelled citrate from the stock bottle also showed this peak close to the solvent front. However, when samples were run using TLC plates only one peak was seen, with an R_f value equivalent to that for citric acid. Unfortunately the lower R_f values and inability to gain so many 'slices' from TLC plates made their use impractical. When stock bottle samples of labelled citrate were run on the TLC plates after adding NaCl to the samples, a large peak appeared near the solvent front corresponding to that seen earlier.

As the citrate-salt peaks seem to correspond to the solvent front peaks seen previously, then the salts found on paper and not on TLC plates may explain the solvent front peaks. A further point to be remembered is that this method was originally designed for the detection of non-radiolabelled krebs cycle intermediates using weak acid dyes. It is therefore only the acidic forms that would be visualized with the original method, and only the use of radioactive citrate would show the salt form.

Figure 43a shows the radioactivity profile for the intracellular contents of enterocytes after an 8 minute influx period. The only major peak corresponds to citrate, all other points again showing the background variation. Differences in peak radioactivity values between the 4 minute and 8 minute chromatograms simply reflect differences in sample loading. It therefore appears that there is

negligible citrate metabolism after 8 minutes.

Figure 43b shows the intracellular radioactivity after 30 minutes. This radioactivity profile has multiple peaks, although by far the largest corresponds to citrate. The other peaks correspond to citric acid cycle intermediates and aminoacids. It therefore appears that after 30 minutes there is citrate metabolism, although non citrate radioacitivity still only accounts for less than 25% of the total intracellular radioactivity.

The Effects of pH Gradients on Galactose Flux.

(Ultimately intended for a comparison with the pH dependent citrate flux.)

Consideration of the relationship between sodium gradients and pH gradients on citrate flux (see previous section) aroused interest in a new report by Hoshi et al,(1986). This Paper described a competition between sodium ions and protons, such that either a sodium or a pH gradient could 'drive' the intestinal hexose symport system.Although the majority of this Paper concerned physiological measurements, there was a single graph showing the effects of a pH gradient on glucose uptake into brush border vesicles. This graph was remarkably similar to that shown in figure 26 for pH gradient dependent citrate flux.

It was therefore decided to investigate the reported pH dependent hexose symport flux in the enterocyte system for a comparison with the pH dependent citrate flux (described in the previous section). The comparison between these two pH dependent fluxes and the conclusions that can be drawn are presented in the discussion section (page 164).

Figure 44 shows the influx of galactose into ATP and sodium depleted enterocytes. In the presence of sodium an initial rate faster than that in sodium free media can be seen. After 4 minutes a peak intracellular galactose content is seen. This is followed by a gradual decline to a lower equilibrium level, indicating that galactose accumulation had taken place. In the presence of phlorizin (the specific inhibitor of sodium-hexose symport) it can be seen that both this high initial rate and the accumulation are lost.

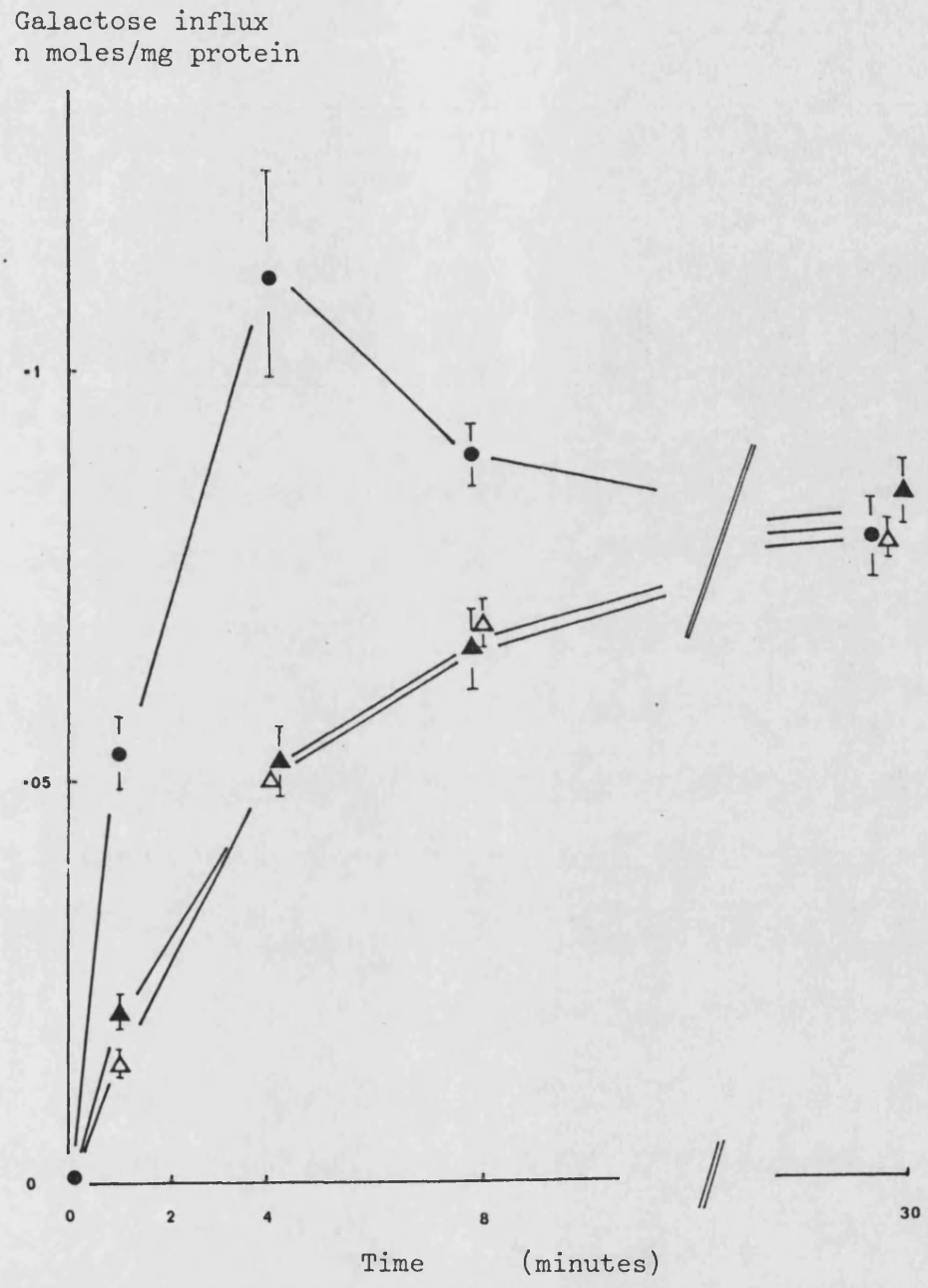


Figure 44 The Effects Of Sodium On Galactose Influx.

Rotenone and ouabain treated chicken enterocytes were isolated in sodium free media 2 at pH 7.2 (see materials). Cells were then introduced to media 2 at pH 7.2 containing 25 uM galactose and the following test additives. Error bars: ± S.E.

● 50 mM Sodium

△ 50 mM Sodium + I00uM Phlorizin

▲ Sodium free

The sodium independent galactose flux can be seen to be similar to the phlorizin inhibited sodium dependent galactose flux. The majority of this sodium independent flux probably represents flux through the basolateral, facilitative hexose transport system.

Figure 45 shows the sodium independent galactose influx into ATP depleted enterocytes, with and without a pH gradient. The results in this graph were obtained under similar conditions to those in figure 26, where a pH gradient increased the initial rate of citrate influx. The inability of the pH gradient to increase galactose influx is a marked difference between the citrate and galactose influx systems.

However, the results of Hoshi et al, with brush border vesicles were obtained in the presence of a membrane potential. This membrane potential was induced with Valinomycin and an outwardly directed potassium gradient. The rapid efflux of potassium results in a negative membrane potential which it is argued is necessary to 'amplify' the pH gradient effects on galactose uptake. It is also pointed out in this Paper that in vivo enterocytes have a membrane potential.

Figure 46 shows the influx of galactose in the presence of a potassium gradient alone, and a potassium/Valinomycin membrane potential. In the presence of a potassium gradient alone, it can be seen that an inwardly directed pH gradient has little effect on galactose influx. Phlorizin can be seen to reduce the initial rate of galactose influx to half of its original value, irrespective of the new pH gradient.

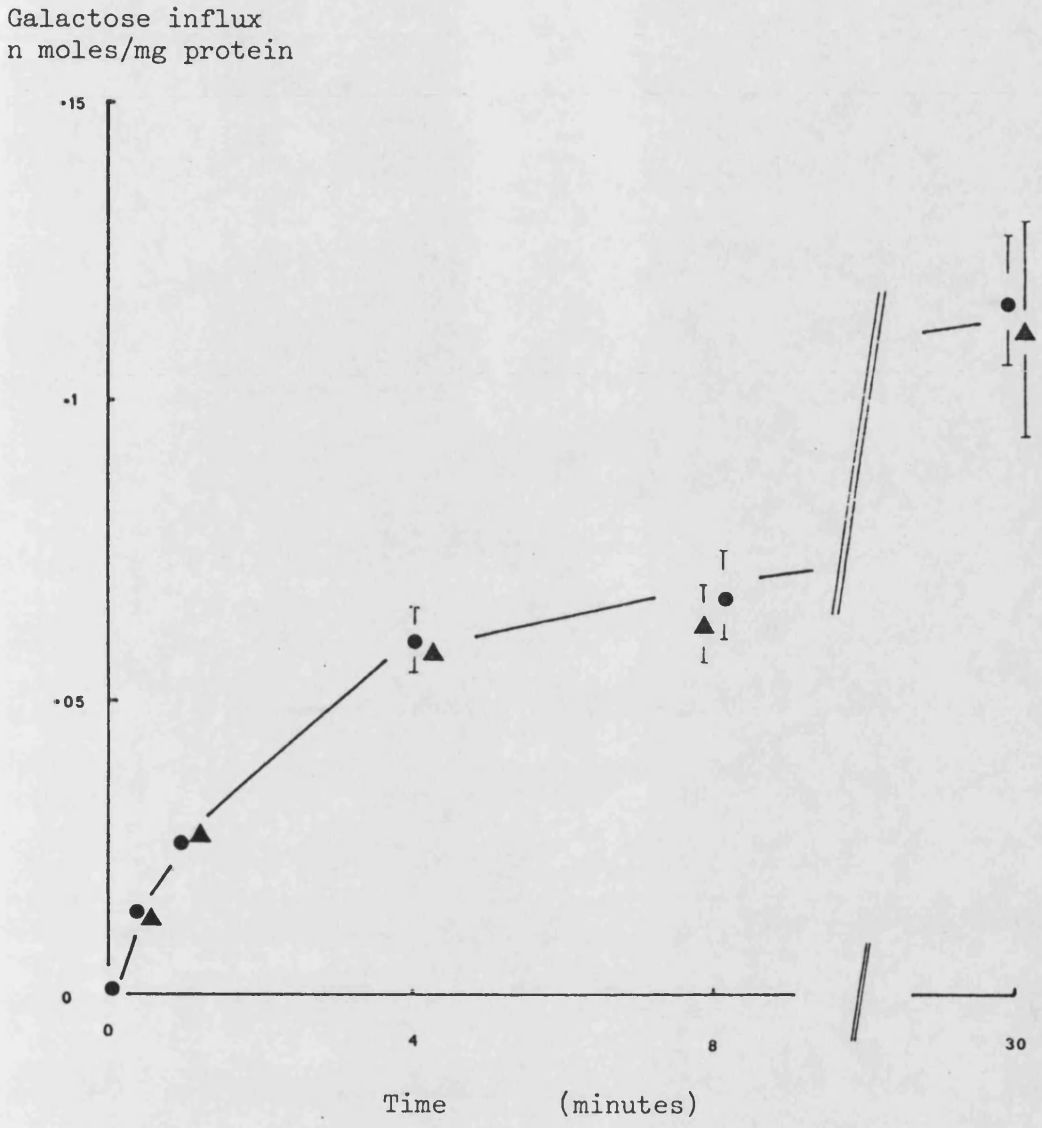


Figure 45 The Effect Of A pH Gradient On Sodium Free Galactose Influx.

Chicken enterocytes were isolated in sodium free medium 2 at pH 7.2 (see materials). Cells were then introduced to sodium free medium 2 containing 100 uM galactose at pH 6.0 or pH 7.2 .
Error bars: ± S.E. Error bars omitted when smaller than symbols used.

● pH_o 7.2

▲ pH_o 6.0

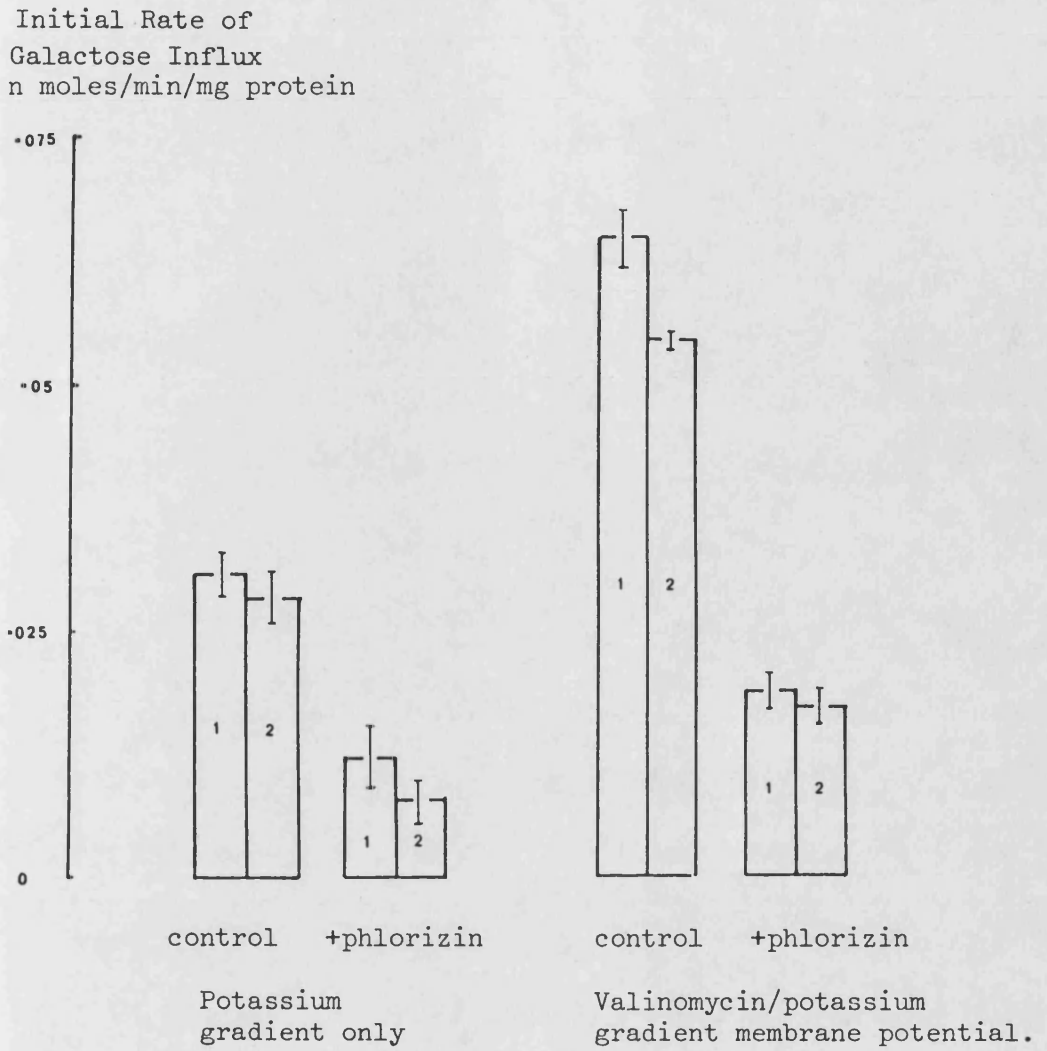


Figure 46 The Increase In Phlorizin Inhibitable Galactose Influx Produced By A Membrane Potential.

Chicken enterocytes were isolated in sodium free, high potassium medium 4 at pH 7.2 (see materials). Certain cells (as indicated) were then subjected to a valinomycin wash (see methods). Galactose influx was then measured in low potassium medium 4 at either pH 6.0 or 7.2 .
Error bars: ± S.E.

1 pH_o 6.0

2 pH_o 7.2

When the cells are pretreated with Valinomycin, a pH dependent increase in initial rate of galactose flux can be seen. The applied membrane potential increases all of the initial rates including those for cells pretreated with phlorizin. It can also be seen that the increase in initial rate due to the pH gradient is entirely lost in the presence of phlorizin. This increase in rate must therefore be a hexose (Na^+) symport effect.

Cytocrit determinations to investigate cell volume changes indicate that the potassium/Valinomycin membrane potential induces considerable swelling. This swelling is independent of the pH gradient and after 5 minutes accounts for a 15% increase in cell volume. Although initial rates should be unaffected by changes in cell volume, a certain proportion of the initial rate increases may be linked with the potassium/Valinomycin conditions.

One of the advantages of working with a characterised system is the availability of specific inhibitors. Figure 47 compares the initial rate of galactose influx when cells have been pre-incubated with the basolateral facilitative hexose transport system inhibitor, Cytocholasin B. Cytocholasin B can be seen to have no effect on the pH gradient induced increase in galactose uptake, although there is a decrease in overall initial rates. This is presumably since the proportion of the initial rate due to the facilitiative transport system has been removed. The pH gradient induced increase in galactose influx is therefore not an effect on the basolateral facilitative hexose flux system.

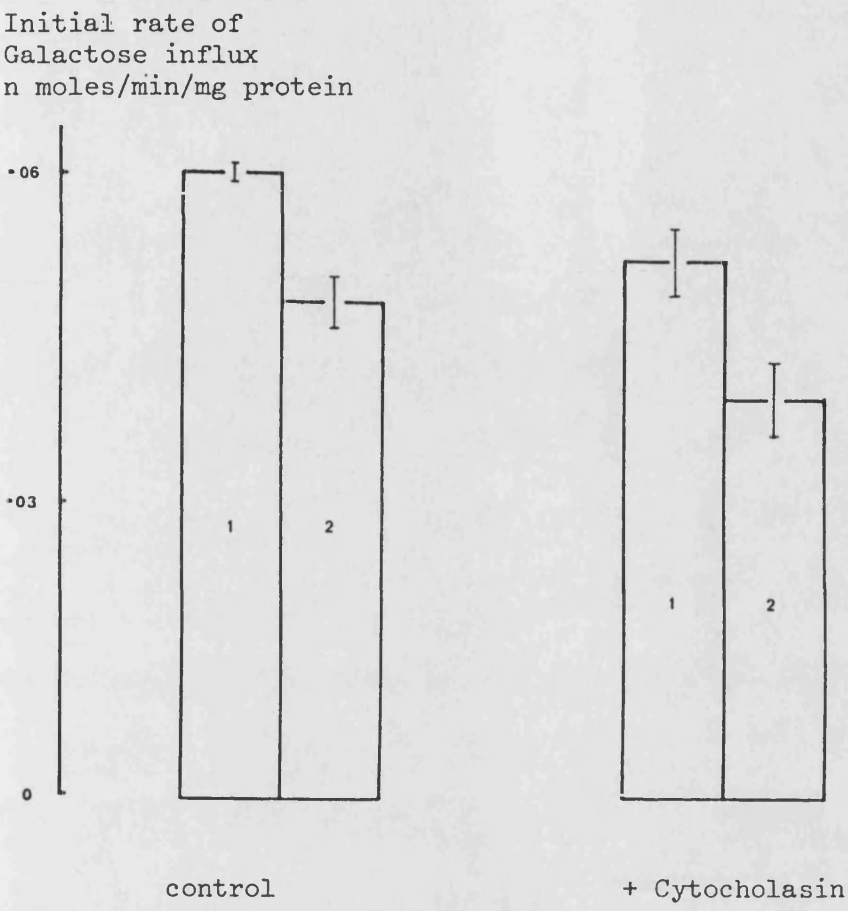


Figure 47 The Effect Of Basolateral Membrane Galactose Flux inhibition On pH Dependent Galactose Influx.

Chicken enterocytes were isolated in sodium free, high potassium buffer 4 at pH 7.2 (see materials). After incubation with/without cytocholasin B (IOOuM), galactose influx was measured at pH 6.0 or pH 7.2 . Error bars: ± S.E.

1 pH_o 6.0

2 pH_o 7.2

A more conclusive indication of a proton driven galactose flux through the hexose (Na^+) symport would be galactose accumulation in the presence of a pH gradient. Figure 48 shows that the rates of galactose influx in the absence of sodium are slow. These slow rates result in the reaching of equilibrium after 30 minutes. However, it is unlikely that the conditions necessary for accumulation (membrane potential, pH gradient) can be maintained for 30 minutes in order to allow the cell galactose content to exceed the equilibrium point. It is therefore not possible to assess whether a pH gradient can lead to galactose accumulation. Without showing accumulation it is not possible to attribute the pH dependent increase in hexose symport flux to a 'proton driven' hexose flux. Figure 48 does however show that the difference in intracellular galactose content continues for at least 8 minutes. This influx time course with and without a pH gradient is very similar to that originally shown by Hoshi et al,(1986) for brush border vesicles.

In order to investigate whether the pH_o 6 $\rightarrow$ pH_i 7 gradient was maximal for galactose influx, a range of pH gradients were investigated.

Figure 49 shows the initial rate of galactose influx into ATP depleted enterocytes treated to produce a potassium/ Valinomycin membrane potential. As the inwardly directed pH gradient is increased from an external value of pH 9 to pH 6, increases in initial rate can be seen. However, at external pH values lower than pH 6 the initial rates fall

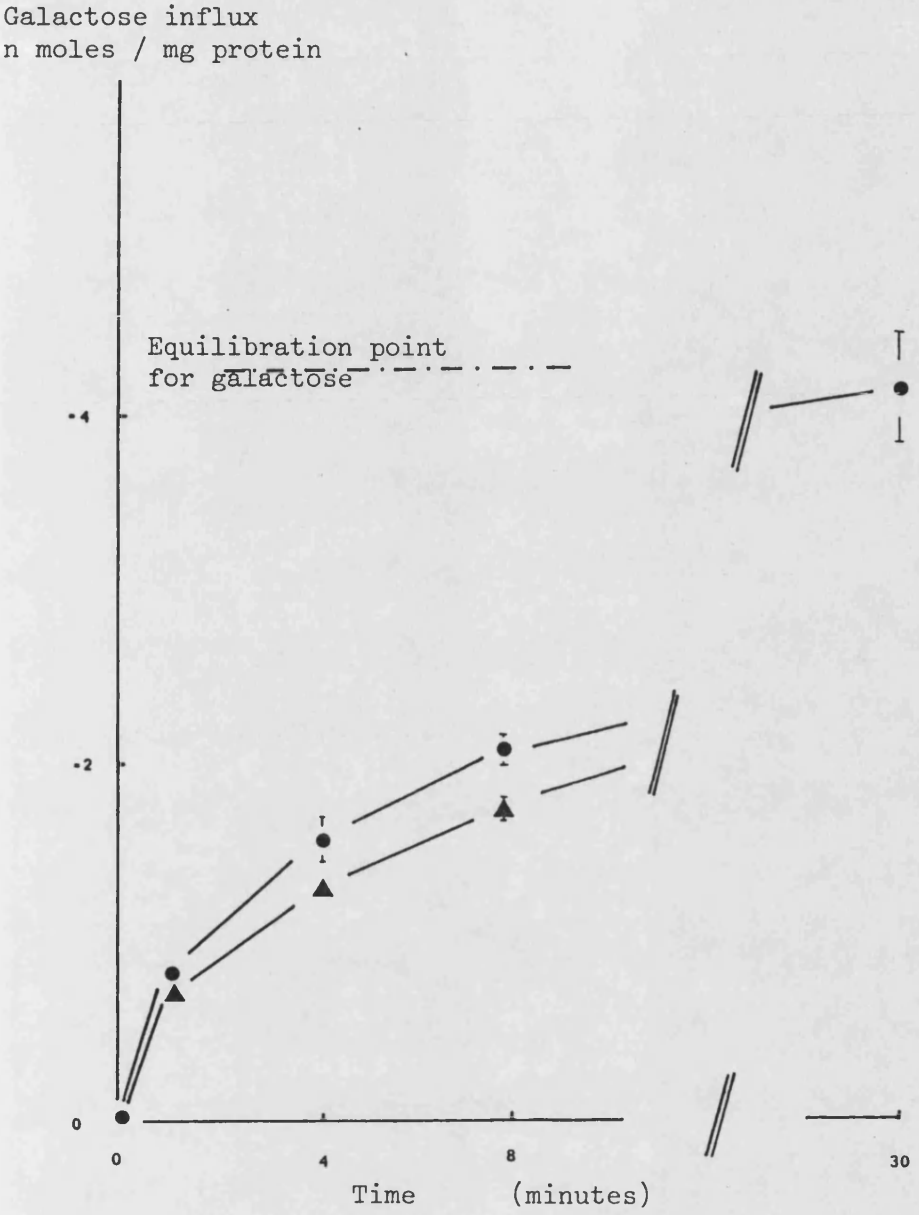


Figure 48 Demonstration Of The Inability Of A pH Gradient To Cause Galactose Accumulation.

Chicken enterocytes were isolated in sodium free, high potassium medium 4 at pH 7.2 (see materials). Galactose influx(IOOuM) was then measured in low potassium medium 4 at pH 6.0 or pH 7.2 . Error bars: ± S.E. Error bars omitted when smaller than the symbols used.

● pH_o 6.0

▲ pH_o 7.2

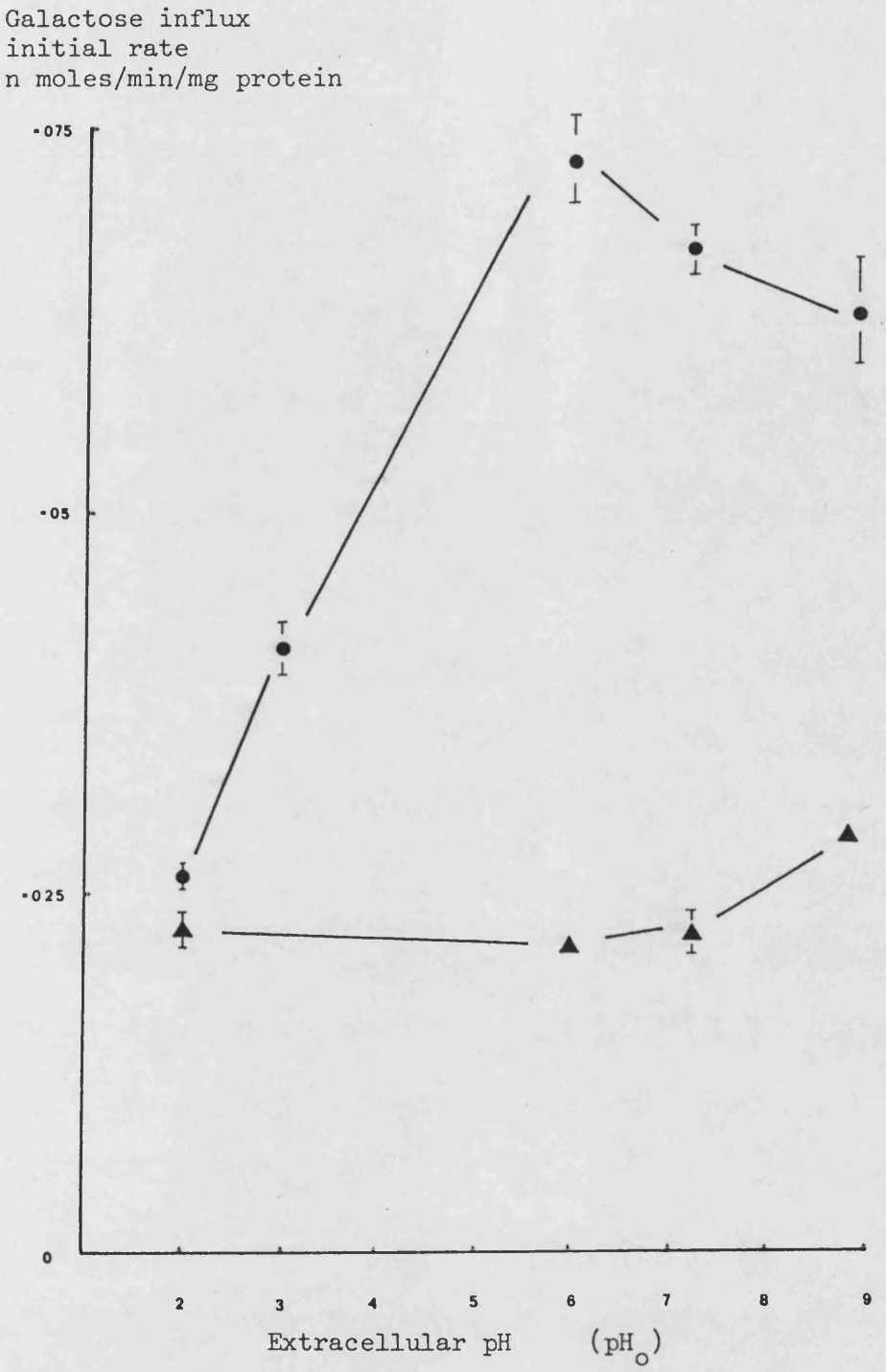


Figure 49 The Effect Of Various pH Gradients On Sodium Free Galactose Influx.

Chicken enterocytes were isolated in sodium free, high potassium medium 4 at pH 7.2 (see materials). Aftre incubation with valinomycin, cells were introduced to low potassium medium 4 containing IOOuM galactose at the pH values indicated. Error bars: ± S.E.

● control

▲ cells preinc. with 500 uM phlorizin

markedly. At the lowest external pH the initial rate is equivalent to that in the presence of phlorizin, a situation where there is little symport flux at all.

It therefore appears that an extracellular pH value of 6 is the optimal pH for sodium free galactose influx. It can be seen that this pH gradient gives a higher initial rate when compared to any other gradient, and not only pH_i 7 to pH_o 7 used by Hoshi et al,(1986).

Figure 50 shows the pH variation in initial rate of galactose influx into Na^+ and ATP depleted enterocytes in the presence of a Na^+ gradient. The rate of galactose influx can be seen to rise as the extracellular pH is reduced from 9 to 7 and the rate to decrease as the extracellular pH is lowered below 7. This variation in initial rate of galactose influx is almost identical to the pH profile in the absence of sodium. However, the only notable difference between these two pH profiles is that the pH optimum for sodium independent galactose flux is pH 6 and the optimum for sodium galactose flux is pH 7.(The phlorizin controls ensure that these effects are due to hexose symport fluxes). This increase in isolated enterocyte(sodium free) galactose flux at pH 6 compared to pH 7 seems fragile evidence to support the hypothesis of a H^+/hexose symport flux. However, physiological methods provide further evidence for a H^+/hexose symport flux (Hoshi et al,1986).

The comparison between the H^+/hexose galactose flux and the pH dependent citrate flux and the conclusions that can be drawn are presented in the discussion section (page 164).

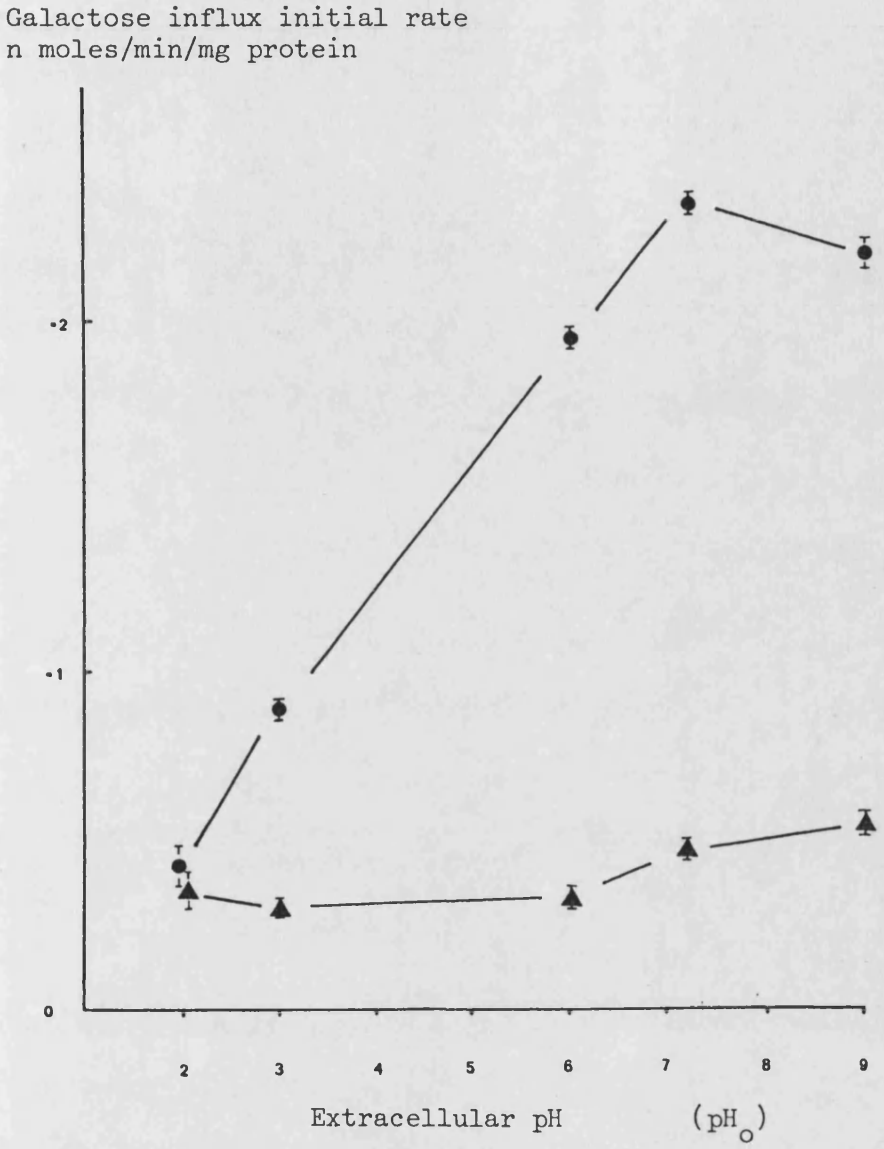


Figure 50 The Effects Of Various pH Gradients On Sodium Galactose Influx.

Chicken enterocytes were isolated in sodium free, high potassium medium 4 at pH 7.2 (see materials) . Cells were then introduced to medium 4 at the pH values indicated containing IOOuM galactose and 50 mM sodium. Error bars: ± S.E.

● control ; ▲ Cells pretreated with 500uM phlorizin

DISCUSSION

Enterocyte Stability and Suitability to Flux Studies.

i) Avian Enterocytes.

Isolated enterocytes offer a highly manipulatable experimental system for the study of numerous intestinal phenomena. However, the use of isolated intestinal cells does have its limitations, which must be appreciated and in certain cases avoided.

The variation in sustainable isolated chick enterocyte sodium ATPase activity with chicken age is one such limitation. Young chickens (approximately 4 weeks of age) were found to exhibit sustainable sodium ATPase activity, and to maintain a sodium gradient for up to one and a half hours after isolation. However, older chickens (10 weeks of age) showed little sustainable sodium ATPase activity after isolation. Chickens between these ages showed progressively less ability to sustain a sodium gradient. The reason for this variation has not been fully investigated, although a likely explanation may be that there are differences in enterocyte ability to maintain ATP 'energy' levels once isolated. This problem has been previously experienced with certain mammalian preparations (Bradford and McGiven,1982; Towler et al,1978).

Whatever the reason for this age related variation in sodium ATPase activity, ways of avoiding the problem had to be considered. Previous reports have avoided this problem, presumably knowingly, by using chickens less than 6 weeks of age and stating that teams of workers used the enterocytes within 1 hour of isolation,(Hyun et al,1982; Kimmich,1970;

Carter Su et al,1979).

Unfortunately, lower cell yields from young chickens made the use of chickens greater than 4 weeks old necessary. The use of a narrow age range (greater than 4 weeks and less than 6 weeks) presented storage and supply problems. A further limiting factor was the inability to improve yields from less than 4 week old chicks by increasing enterocyte isolation times, as this results in the isolation of cells other than those of the villus tip (Rowling and Sepúlveda, 1985, (for mammals)). However, the high levels of membrane integrity irrespective of chicken age (shown by phlorizin inhibition of galactose influx (fig.2)) allowed greater age range flexibility.

The method of Hyun et al,(1982) allows this dilema to be resolved by depleting the intracellular sodium content of chicken enterocytes. Although this method has advantages, since it allows sodium gradient manipulation, it does mean that only initial rates can be measured. Measuring only initial rates can be restrictive as it is often useful to determine whether fluxes have resulted in accumulation. The inhibition of Na^+ ATPase activity with ouabain extends this methodology, allowing progress curves to be followed rather than initial rates alone. Ouabain . . . removes all Na^+/K^+ ATPase activity from the enterocytes, making flux studies in enterocytes from chickens of various ages comparable. A further progression of this rationale is to ATP deplete the cells with rotenone, thus ensuring sodium 'pump' inhibition (Montrose et al,1985; Cassel et al,1986).

The routine combination of these methodologies, as reported

in this Thesis, removes the problem of sodium ATPase variation with chicken age. It also allows greater control of enterocyte flux conditions, similar to the control available in vesicle studies.

Employing this methodology, chickens of up to 10 weeks of age were used. Over this age range none of the transport phenomena reported here were found to vary between experiments.

A further advantage of ATP and sodium depleted enterocytes stored at $0^{o}C$, was that greater levels of viability could be maintained. This allowed experimentation up to 2 hours after isolation. This was a significant improvement for the single operator when using chicken cells. In addition, the ATP depletion allowed the study of fluxes of otherwise metabolizable substrates.

The ATP and sodium depleted isolated enterocyte system therefore allows further flexibility for the operator to dictate enterocyte conditions. However, although increases in manipulatability are highly advantageous to certain studies, other investigations require an enterocyte system more relevant to the physiological state. For this reason not all of the studies reported in this Thesis used the ATP and sodium depleted isolated enterocyte system.

ii) Mammalian Enterocytes.

The successful isolation of enterocytes from suckling piglets has not previously been reported. Intestinal cell isolation from older pigs has been reported, although the more harsh vibration method of isolation was used (Ahrens and Panichkrangkrai,1985). The piglet enterocytes used for

this Report show high levels of membrane integrity. This can be seen by the near complete inhibition of galactose influx with the hexose symport specific inhibitor phlorizin.

Enterocytes isolated from suckling piglets showed little variation in sodium ATPase acitivity over the piglet age range used. This allowed the high levels of sustainable sodium ATPase activity to be used for cell sodium gradient production. The piglet enterocytes, therefore presented a more phyiologically relevant system than the sodium and ATP depleted chicken enterocytes. For this reason piglet enterocytes were used for the toxin studies.

Enterocytes isolated from rabbit tissue also showed high levels of membrane integrity and consistently high sodium ATPase activity. Rabbit enterocytes are probably the most widely used enterocytes for sodium ATPase studies (Brown and Sepúlveda,1985; Hyun et al,1985), and the sodium ATPase studies shown in this Thesis indicate that rabbit and suckling piglet cells compare favourably.

These characteristics make the isolated suckling piglet enterocyte an excellent system for the study of porcine intestinal disease.

Brush Border and Basolateral Membrane Enrichment and Vesiculation.

One of the limitations of isolated enterocytes is the loss of the ability to distinguish between brush border and basolateral membrane events once the cells are isolated. Vesicles produced from specific areas of the enterocyte membrane allow this problem to be resolved.

The brush border membrane vesicles shown in this Thesis

exhibit hexose transport properties typical of the brush border membrane (Semenza et al,1984).

The glucose accumulation 'overshoot' in the presence of a sodium gradient supports previous findings (Murer and Hopfer,1974) and is as would be predicted from Crane's co-transport mechanism (Crane,1977). The hexose uptake values also compare favourably with those found previously for similar preparation procedures (Kessler et al,1978). The ability to totally inhibit sugar flux shows the high degree of resealing. Failure to reseal is the problem most often experienced with other vesicle methodologies (Gains and Hauser,1984; Christiansen et al,1981).

The development of the brush border vesicle preparation procedure required repeated characterisation of separated fractions by assaying marker enzymes. Many of the previous brush border membrane isolation procedures have used lengthy discontinuous marker enzyme assays (Kessler et al,1978). The sucrase assay of Dahlquist (1964 and 1966) was found to be particularly lengthy as used by Schmitz (1973). The modification of this method from a discontinuous to a continuous assay proved to be more convenient, although interference was experienced in certain 'crude' samples. The preferable method for the assay of sucrase activity has not been previously used for brush border characterization, and was adapted from the hexokinase/glucose 6 phosphate dehydrogenase assay for glucose concentration. (Methods in Enzymology). This assay as described in the methods section produced reliable results in a fraction of the time taken for the widely used discontinuous methods (Dahlquist and

Messer,1966).

The widely used sodium ATPase assay of Quigley and Gotterer,(1969) measuring the release of inorganic phosphate (Fiske and Subbarow,1925), also proved very lengthy. A preferable method was developed from that of Forte et al, (1967), and is described in the methods section. This continuous assay measures the potassium activated p-nitro phenyl phosphatase activity and is totally inhibitable with ouabain.

The modified membrane characterization assays offer particular advantages to the single operator in both time and convenience. With the developed assays it is possible to undertake a vesicle separation procedure and assay the key marker enzymes on the same day, which is not practical with the previously used discontinuous assays (Dahlquist et al,1966; Quigley and Gotterer,1969).

Complementary to the brush border vesicles are vesicles originating from the basolateral membrane, and there are numerous methodologies for their production (Vezoto et al, 1986; Del Castillo et al,1985; Mircheff et al,1976). Wright et al,(1980) show that the brush border and basolateral membranes differ in their densities sufficiently to allow separation. The results shown in this Thesis indicate that this is indeed the case, and fractions enriched in basolateral membrane relative to brush border have been shown.

Unfortunately the inability to detect ST_a toxin effects in isolated enterocytes resulted in no need for 'in-depth' toxin studies using vesicles. However, both brush border

and basolateral vesicles offer much potential for distinguishing between brush border and basolateral processes. Indeed, it is interesting to note that vesicle studies to distinguish between brush border and basolateral citrate fluxes would be the next logical step for citrate flux investigations, (see later).

The Investigation of Changes in Enterocyte Solute Flux Caused by ST_a.

The purification procedure of Alderete and Robertson (1978) as modified by Burgess (1983) produced a highly pure ST_a preparation for enterocyte studies. The specific activity of ST_a produced by this procedure (10 ng protein/mouse unit) compares well with the original procedure (6.1 ng protein/mouse unit - Alderete and Robertson 1978), and with that of Burgess (mean - 13.1 ng protein/mouse unit, 1983). In addition, the 75% recovery of biological activity for the procedure in this Thesis is similar to the 70% recovery for the original extraction method of Alderete and Robertson (1978).

The enterocyte system chosen for the study of ST_a induced changes in ion movements was the suckling piglet enterocyte. This system was chosen primarily since the suckling piglet is one of the target animals for *E coli* strains secreting ST_a (Newsome,1980). Piglet intestine has also been found to respond well to ST_a (Newsome *et al*,1978; Merritt *et al*,1985). Other considerations included the high levels of membrane integrity and sustainable sodium ATPase activity with the isolated piglet enterocytes. This allowed the use of sodium containing isolation buffers and produced

cells that could maintain their own sodium gradient.

The findings of Burgess et al,(1980) that ST_a, purified by the method of Alderete and Robertson (1978), caused decreases in net transcellular sodium flux led to the consideration of sodium flux studies. Calculations from Ussing chamber fluxes for individual cellular sodium fluxes implicated reductions primarily in enterocyte sodium influx (Burgess,1983). For this reason sodium influx was initially investigated.

When piglet enterocytes were exposed to ST_a there was little change in the initial rate of sodium influx. Various concentrations of toxin were tested with pre-incubation periods of up to 60 minutes. Any effects of ST_a should have been visible over this incubation period.

Previous reports have shown very short lag periods before ST_a induced effects are seen. Gianella et al, (1979) have reported increases in cGMP after less than 5 minutes, whilst Newsome et al,(1978) have found maximal ST_a induced cGMP levels after less than 40 minutes.

The inability to detect the effects of ST_a on sodium influx led to doubts concerning the suitability of the piglet enterocyte system to the study of the effects of ST_a. Cholera toxin was therefore employed to test the suitability of the piglet enterocyte system to toxin effects on sodium influx. On exposure to Cholera toxin the piglet enterocytes showed a similar reduction in the rate of sodium influx to that reported by Hyan et al, (1982). In addition,the Cholera toxin effect was seen to appear only after 15 minutes incubation with the piglet entero-

cytes, with maximal effects after 30 minutes. This is consistent with the lag period reported by Finkelstein et al, (1985) for Cholera toxin.

It therefore appeared that the piglet enterocyte system was suitable for the study of at least certain toxins.

In the light of Ussing chamber effects of ST_a on net sodium flux (Burgess,1983), the efflux of sodium from piglet enterocytes was considered. A reduction in either sodium influx or sodium efflux could potentially reduce transcellular sodium flux, and thus changes particularly in sodium ATPase activity were of relevance to the reported effects of ST_a (Burgess,1983). Further justification of this rationale can be appreciated in terms of the reported reductions in sodium ATPase activity by protein kinase phosphorylation, the presence of cGMP dependent protein kinases (Henderson et al,1985), and the involvement of cGMP elevation in the actions of ST_a (Newsome et al,1978; Gianella et al,1979).

The sodium efflux studies, however, showed no effect of ST_a on sodium efflux or sodium ATPase activity. As this was the case for various ST_a concentrations, this led to concern about the comparability of conditions for the isolated enterocyte investigations and the Ussing chamber studies of Burgess et al,(1983).

Burgess had also noted a need for chloride in the bathing medium for his whole tissue studies on ST_a. This led to the implication of a role for chloride/bicarbonate exchange in the actions of ST_a (Burgess,1983). A further report by Ahrens and Panichkrangkrai (1985) of ST_a effects on chloride

movements using isolated cells, aroused interest in the study of chloride movements in the piglet enterocyte system. However, Ahrens and Panichkrangkrai's ST_a preparation was not purified, being primarily a bacteria free culture broth supernatant. They found that when the crude broth was incubated with the cells, the subsequently measured chloride efflux was increased.

When the chloride efflux measurements were repeated under similar conditions to those used by Ahrens and Panichkrangkrai,(1985) with the piglet enterocytes and highly purified ST_a, no increase in chloride efflux could be detected.

The limited knowledge concerning the mechanism of action of ST_a left little scope for determining whether the conditions used for the isolated enterocyte system were inappropriate to studying ST_a . However, studies of cGMP levels in intestinal whole tissue have shown that cGMP levels increase after exposure to ST_a (Newsome et al,1978; Gianella et al,1979). Exogenously added cGMP analogues have also been seen to show ST_a like effects on whole tissue ion fluxes (Burgess,1983).

When assaying the cGMP content of the piglet enterocytes in the presence and absence of the ST_a toxin it was found that all enterocytes irrespective of ST_a showed a massive loss in cGMP once isolated and incubated at $37^{o}C$. This loss exceeded 80% of the initial cGMP level over a period of 30 minutes.

It therefore appears that once piglet enterocytes are isolated they are unable to maintain their cGMP levels. Under such circumstances it can be appreciated that if cGMP

is a true intermediate in the actions of ST_a, then any cGMP induced effects would be abolished. Such a problem may be related to the exclusion of high energy yielding metabolizable substrates from the bathing medium.

A similar problem, presumably with ATP levels, in isolated chicken enterocytes has been previously mentioned in this Thesis, and has been experienced by other workers with certain mammalian enterocytes (Bradford and McGivan,1982).

The inclusion of high energy yielding substrates such as glucose may help to ameliorate this problem, although sodium symport fluxes will obviously lead to an increase in 'background' sodium influx. Increases in 'background' sodium influx would be detrimental to the measurement of any effects ST_a might have on sodium influx.

A different approach to this problem is the use of cGMP analogues which are not readily broken down and can be added exogenously to the cell suspension. Hughes et al,(1978) have shown that 8 Br cGMP is a good cGMP analogue for mouse intestine. Other workers have shown that 8 Br cGMP appears to elicit similar actions to ST_a (e.g. Burgess,1983).

Exogenous 8 Br cGMP was found to reduce sodium influx into the enterocyte system under similar conditions to the investigations with ST_a. This finding explains the reduction in whole tissue transcellular sodium flux seen with 8 Br cGMP (Burgess,1983).

These effects may also be similar to those which might have been seen with ST_a, had the isolated piglet entercytes been capable of sustaining their cGMP levels.

Enterocyte Citrate Fluxes

i) Sodium dependent citrate flux.

The in vivo enterocyte sodium gradient is of paramount importance to the efficient functioning of the intestine (Fondacaro,1986). It has been previously found that the sodium gradient, maintained at great cost to the cell, is employed to 'drive' the uptake of metabolizable substrates (Tucker et al, 1973; Ghishan et al,1985). In view of the high energy yielding potential of citrate, the discovered link between citrate and sodium may be a further example of this phenomenon.

The external sodium concentration considerably increases the initial rate of enterocyte citrate influx. The rate of citrate influx becomes maximal at a sodium concentration of 50 to 100 mM. This concentration of sodium is exceeded by the body fluids (Sokucu et al,1985), and usually approached by the lumenal contents of the small intestine, (Lamb et al,1983). Under normal conditions therefore, there is potential for maximal stimulation of this citrate flux by sodium.

Kidney tissue also exhibits a sodium dependent citrate flux with a reported maximal initial rate of citrate flux at sodium concentrations slightly less than 100 mM (Wright et al,1982). A further similarity between the renal and intestinal sodium dependent citrate transport systems, is their transport saturation constants. Wright et al,(1981) report a K_t value of 180 μM for the renal system compared to the value of 300 μM reported here for the intestinal

system.

The sodium dependent citrate transporter of kidney brush border appears to be a symport system (Wright et al,1982), and exhibits the ability to accumulate citrate (Kippen et al, 1982).

The intestinal sodium dependent citrate flux also shows the ability to accumulate citrate. However, enterocyte citrate accumulation by a transient sodium gradient was only seen at low citrate concentrations. This phenomenon could be explained by losses in the transient sodium gradient. The Crane hypothesis (1977) shows that a Na^+ gradient is necessary for metabolite accumulation by a Na^+ symport system. Citrate concentrations considerably below the K_t for Na^+ dependent citrate flux will produce different fractional filling rates from citrate concentrations considerably above the K_t. This is a consequence of transporter saturation. Therefore if the transient sodium gradient breaks down before intracellular/extracellular citrate equilibration has taken place, then accumulation will not be seen. The results show that Na^+ gradient differences could explain why citrate accumulation is seen only at citrate concentrations lower than the K_t value for sodium dependent citrate transport.

Citrate accumulation in the presence of a Na^+ gradient is the single most definitive piece of evidence concerning the nature of the Na^+ citrate flux component. Symport systems between sodium and metabolites have become a characteristic of enterocytes, (Fondacaro,1985) and it appears that the Na^+ citrate flux may be a further example.

A further property of sodium symport systems is the ability of the symported metabolite to increase enterocyte sodium influx. This property has been shown by many other symport systems (Brown and Sepulveda,1983), including the renal sodium citrate symport (Kippen et al,1982), and is also seen with the enterocyte sodium citrate symport. However, the citrate induced initial rate of sodium influx is smaller than might be expected from the citrate induced difference in intra-cellular sodium concentration after 2 - 4 minutes. When enterocytes have previously been used to study the hexose and amino acid symport induced increases in Na^+ flux (Brown and Sepúlveda,1983), the increases in sodium influx initial rates have been in proportion to the increases in intra-cellular sodium concentration studied during the time course. However, in these studies the enterocyte sodium gradients were maintained by physiological sodium ATPase activity and not artifically manufactured. There are two possible explanations for the relatively small effects of citrate on the initial rate of sodium flux. A reduced initial rate of sodium influx may result from the sodium and ATP depletion and the imposed pH gradient. Alternatively, the 2 - 4 minute cellular sodium content may be increased by the appearance of citrate in the intra-cellular medium. (This possibility has been further investigated, see Na^+/H^+ exchange (page 161)).

Previous workers have found that membrane potentials can modulate (either increase or decrease) the initial rate of flux through symport systems. It is interesting

to note that Wright et al,(1982) amplify the initial rate of sodium flux through the renal sodium citrate symport by using an outwardly directed membrane potential.

Wright et al,(1982) have also shown that 2.8 sodium ions are transported with each molecule of citrate. Approximations from the enterocyte sodium induced citrate flux and the citrate induced sodium flux indicate that 3 sodium ions are transported with each molecule of citrate.

It can therefore be seen that there are marked similarities between the renal sodium citrate symport and the sodium dependent citrate flux described in this Thesis. This is therefore a further example to complement the numerous reports of transporter similarities between renal and intestinal epithelial tissues (e.g. Murer, Hopfer and Kinne,1976).

ii) Sodium Independent Citrate Flux.

The isolated enterocyte system allowed the separation of the sodium dependent and sodium independent fluxes. In a less manipulatable experimental system this would not be possible.

The pH dependence of the sodium independent flux is marked. A 1 pH unit reduction in external pH(from pH 7) leads to an initial rate similar to that seen with the sodium dependent flux. This flux therefore can be appreciable and cannot be ignored simply because it contributes only a small proportion of the total citrate flux at pH 7.2. It was not possible to produce a pH dependent accumulation of citrate with the sodium independent flux. (As previously mentioned, this is unlike the sodium dependent flux where a sodium gradient could produce citrate

accumulation).

It is interesting to note that the investigations of Wright et al,1981) show no such sodium independent flux in renal brush border vesicles. However, brush border vesicles are produced from only one membrane face of the enterocyte and these epithelial cells exhibit gross differences in brush border and basolateral membrane transport processes, (e.g. Kessler et al,1978; Wright et al,1980). The sodium independent citrate flux could therefore be present in the basolateral membrane.

The reports of Wright et al,(1981) also include discussion of the role of the renal sodium dependent citrate symport with regard to net citrate reabsorption from the glomerular filtrate. However, the renal sodium symport system alone does not facilitate net citrate absorption. Burgess et al,(1983) have shown an intestinal net transcellular citrate flux in the Ussing chamber. However, the intestinal sodium-citrate symport described in this Thesis alone does not allow net citrate absorption. If a sodium independant citrate transport system was present on the enterocyte basolateral membrane a net citrate flux would be possible.

The described pH effects on the sodium independent flux component could originate from either transport protein or substrate ionization, or a proton driven flux. Transporter ionization would imply that residues with a pK_a less than 7 need to be protonated for maximal activity. If the pH effect is solely due to substrate ionization then it is interesting to note that the citrate influx initial rate

appears to increase in the region of one of the ionization points of citrate. This would imply the transport of cit $^{2-}$ and possibly also cit $^{1-}$ species. These two citrate species are those proposed by Kippen et al,(1982) for renal citrate symport transport. However, the presence particularly of magnesium ions in the buffers used for the experiments in this Thesis means that the citrate pK_a values may be quite different to published ionization points, (Morris, 1978). In addition, part of the study by Kippen et al, (1982) includes the measurement of fluxes at both pH 7.5 and pH 5.5 using the buffering capacity of Hepes to maintain these pH values. However, Hepes has little buffering capacity at pH 5.5.

A further consequence of substrate ionization under the conditions used for the study of citrate effects on sodium flux, is that the transport of citrate $^{2-}$ or citrate $^{1-}$ (at an external pH of 6 to an internal pH of 7.2) would result in the liberation of protons inside the cell. The resulting citrate induced proton flux would tend to reduce the intracelular pH.

A similar reduction in intracellular pH would result if this pH dependent flux was described by a proton driven symport. (Proton driven symports have been found for succinate/citrate transport in B.subtilis (Gutowski et al,1975)).

It can be seen that two out of the three possibilities to explain the pH dependence of the Na^+ independent citrate flux could lead to a citrate induced decrease in intracellular pH. This has been investigated and is discussed in detail in a following section. (page 161). It has been found that

citrate flux does not measurably reduce intracellular pH. This leaves the conclusion that the pH effects on this flux represent transporter protein ionization (or that the H^+ flux is too small to be measured by the DMO technique).

Having considered the reasons for the pH dependence of the Na^+ independent flux, it is of value to consider whether this flux represents a different transporter to the sodium dependent citrate symport.

Does Enterocyte Citrate Transport Represent Two Separate Citrate Transporters or One Transporter with Two Fluxes?,

1) Two separate citrate transporters.

It is possible that the Na^+ independent flux represents a totally separate saturatable transporter from the Na^+ - citrate symport system. A sodium independent citrate transporter has been found in Streptococcus diacetylactus, and exhibits a remarkably similar pH dependence and K_t value to that described here (Harvey et al,1962).

The loss in polarity of enterocytes once they are isolated removes the ability to distinguish whether the sodium independent citrate flux appears on one or both of the brush border and basolateral membranes of the cell.

It is difficult to appreciate why a sodium independent citrate flux would appear at the brush border membrane since it would reduce the efficiency of citrate absorption carried out by the Na^+ - citrate symport.

The only remaining possibility, therefore, is that this flux component lies on the basolateral membrane of the cell. If this were the case, there are several consequences:-

Firstly, a sodium dependent species would be found on

the brush border face of the cell with a sodium independent species on the basolateral membrane. This is known to be the case to facilitate the uptake of other useful metabolites, such as hexoses and amino acids (e.g. Kinsella et al,1978; Wright et al,1980).

Secondly, a net flux of citrate could be observed to flow across the cell. This is known to take place in intestinal tissue (Burgess,1983).

Finally, If vesicles were prepared from the brush border membrane by the method of Kessler et al, (1982), only the sodium dependent flux would be expected. Renal vesicles prepared by this method show only a sodium dependent citrate influx (Wright et al,1982).

It therefore appears that a sodium independent flux on the basolateral membrane explains all of the results presented in this Thesis together with our previous knowledge of the intestine and other similar tissues. However, it is not possible to shed further light on the reasons for the pH dependence other than those already proposed. It does seem difficult to justify a need for a proton driven citrate symport on the basolateral membrane, leaving only the possibilities of transporter and substrate ionization as feasible alternatives.

2) One transporter - two citrate fluxes.

It is possible that the pH dependent citrate flux component of the total citrate flux represents a sodium independent flux through the already described sodium citrate symport system. If this is the case, then the separation of this flux from the sodium dependent flux simply reflects

the manipulatability of the isolated enterocyte system.

There are three explanations for the possible pH dependence of a flux through the citrate symport system. As previously mentioned these are substrate ionization, transporter protein ionization, or an H^+ 'driven' citrate flux directly analogous to the Na^+ 'driven' flux.

If substrate ionization or transporter ionization explains the proposed pH dependence of the citrate symport, then accumulation could be expected in the presence of a pH_o 6 to pH_i 7 gradient. Accumulation could take place since transport of citrate from pH 6 to 7 would be fast, whereas the backflux from pH 7 to 6 would be slow. The avian intestine has just such a pH gradient throughout its entire length (Montrose et al,1985). However, the mammalian small intestine does not. If a single transporter with sodium independent and sodium dependent fluxes is a true reflection of in vivo citrate flux, then this system appears to optimize the avian intestinal environment. As the mammalian intestinal environment is different, it could be suggested that the mammalian citrate transport system would also be different. This suggestion is in opposition to the similarities previously found between mammalian and avian enterocytes (e.g. Kimmich, 1975; Tucker et al,1978).

There is however, a real possibility that a sodium-proton competition exists for the single type of symport system. This would allow both Na^+ and H^+ ion gradient to cause accumulation. Both types of symport have previously been found (Semenza et al,1974; Gutowski et al,1975), although the proton driven symport is usually a bacterial feature.

However, even in intestinal symport systems where a sodium-proton competition has been reported, the pH dependent flux is always the minor component of the total flux, (Hoshi et al,1986).

Particular difficulty was experienced in assessing the possibility of a sodium-proton competition for a single type of citrate symport. It was therefore decided to compare the citrate results with those for the reported sodium-proton competition in the intestinal sodium-hexose symport. The comparison between these two systems is presented in a following section (page 164).

A further point worthy of consideration is that both substrate ionization or a proton symport, as discussed, would lead to a decrease in intracellular pH (under the conditions of a pH gradient). The presence of a Na^+/H^+ exchange system in the enterocyte could link citrate induced changes in intracellular pH with sodium flux.

The presence of an enterocyte Na^+/H^+ exchange system and the ability of citrate to reduce intracellular pH were therefore explored.

Na^+/H^+ Exchange and the Effects of Citrate on Intracellular pH.

Na^+/H^+ exchangers have previously been found in numerous biological systems (Escabales et al,1986; Kinsella et al, 1980; Benos et al,1978; Lacoz-Vievia,1986). The mammalian small intestine and kidney brush border membranes have been seen to show Na^+/H^+ exchange systems (Murer et al,1975). However, at the time that this work was conducted there were no reports of Na^+/H^+ exchange in the isolated chicken

enterocyte system.

The Na^+/H^+ exchange induced increases in sodium influx seen with the enterocyte system are similar to those shown by Murer et al,(1975) for intestinal brush border vesicles. The use of amiloride to specifically abolish pH dependent Na^+ influx provides further proof that these effects are due to Na^+/H^+ exchange. The D.M.O. dye distribution technique, specially developed for this work, also proved to be able to clearly demonstrate sodium effects on proton movements. The dye technique was also easier and required less specialised equipment than previous techniques. (Kinsella et al,1980; Murer, Hopfer and Kinne,1975).

The discovery of the enterocyte Na^+/H^+ exchange system allowed further investigation of the effects of citrate on sodium flux, and the possible involvement of Na^+/H^+ exchange. One particular anomalous result provoked interest in Na^+/H^+ exchange. This was the discovery that the relatively small citrate induced increases in initial rate of sodium influx resulted in large differences in intracellular sodium content after 2 to 4 minutes, (see page 110). This time delay between maximal citrate influx and the maximal citrate induced effects on the sodium influx progress curve could be linked with Na^+/H^+ exchange.

The possibility that citrate flux could reduce intracellular pH has already been discussed (page 157).

The resulting lower internal pH could then be restored to the original intracellular pH value via Na^+/H^+ exchange. (pH homeostasis is one of the proposed roles of Na^+/H^+ exchange systems,(Moalenaar,1986)).

This hypothesis would explain the time delay between citrate transport and maximal citrate induced sodium influx effects.

The D.M.O. dye technique, however, failed to show any citrate induced changes in proton movements even in lightly buffered media. This study was carried out in conjunction with the measurement of sodium influx and under similar conditions to the dye studies the effects of citrate on sodium influx were still seen. The use of amiloride to specifically inhibit Na^+/H^+ exchange also failed to abolish the effects of citrate on sodium influx.

It therefore appears that Na^+/H^+ exchange plays little role in the demonstrated effects of citrate on sodium influx. The citrate induced increases in sodium influx therefore probably reflect the actions of the sodium-citrate symport as first thought.

Although citrate seems to cause little direct effect on Na^+/H^+ exchange, the metabolism of citrate (proposed by Bywater,(1977) to explain part of citrate's effects) may lead to increased Na^+/H^+ exchange.

As citrate is metabolized to CO_2 the proton released to produce bicarbonate in water at intracellular pH, will be available for Na^+/H^+ exchange.

Thus citrate metabolism <u>in vivo</u> may lead to increased sodium influx via Na^+/H^+ exchange. (See diagramatic representation - page 167).

The Intestinal H^+/Hexose Symport.

A Comparison Between The pH Dependent Hexose Symport Galactose Flux and The pH Dependent Citrate Flux.

A report by Hoshi et al,(1985) described a proton-sodium competition for the intestinal hexose (Na^+) symport. Hoshi et al had found that a pH gradient could compete with a sodium gradient to increase sugar uptake by the intestinal hexose symport system. The enterocyte citrate fluxes presented in this Thesis were found to exhibit similar characteristics to the sugar fluxes reported by Hoshi et al. It was therefore decided to investigate enterocyte H^+/hexose symport sugar flux and compare it with the enterocyte pH dependent citrate flux.

The results in this Thesis show that a pH gradient alone is unable to increase galactose flux through the enterocyte hexose symport system. This finding is unlike that for the pH dependent citrate flux, where a reduction in external pH from pH 7 increases citrate influx. However, Hoshi et al, used an artificial membrane potential for their experiments, created with a potassium gradient and Valinomycin. When these conditions were reproduced with the isolated enterocyte system, a pH gradient was seen to increase the initial rate of galactose influx. The potassium gradient/Valinomycin membrane potential also significantly increased the galactose flux in the absence of a pH gradient, as well as causing appreciable cell swelling. The use of specific hexose flux inhibitors allowed the pH gradient induced increase in galactose flux to be specifically attributed to the hexose symport. However, the pH gradient induced increases in

galactose flux were significantly smaller than those seen with citrate, even in the presence of a membrane potential.

Investigation of enterocyte galactose influx at various extracellular pH values indicated that the maximum pH dependent galactose flux through the hexose symport occurred at pH 6. However, maximal sodium dependent galactose influx occurred at pH 7. This is unlike the finding for citrate fluxes where both sodium independent and sodium dependent citrate fluxes are greater at pH 6 than at pH 7.

The report by Hoshi et al describes how the pH dependent hexose symport flux is insignificant in the presence of high sodium concentrations (50-100 mM). This finding has been reproduced with the enterocyte pH dependent galactose flux. However, the pH dependent citrate flux and the sodium dependent citrate flux appear to be additive, even at high sodium concentrations.

The pH dependent citrate flux and the pH dependent galactose flux through the hexose (Na^+) symport therefore appear to be quite different. Although it is not possible to conclusively disprove the existence of a sodium-proton competition for the citrate symport, this does not appear to be the best explanation of the citrate fluxes presented in this Thesis.

Conclusions concerning the Two Components of Enterocyte Citrate Flux.

The results presented in this Thesis together with a consideration of our previous knowledge of other citrate fluxes and other intestinal fluxes, leads to the drawing of tentative conclusions. These conclusions also lead to

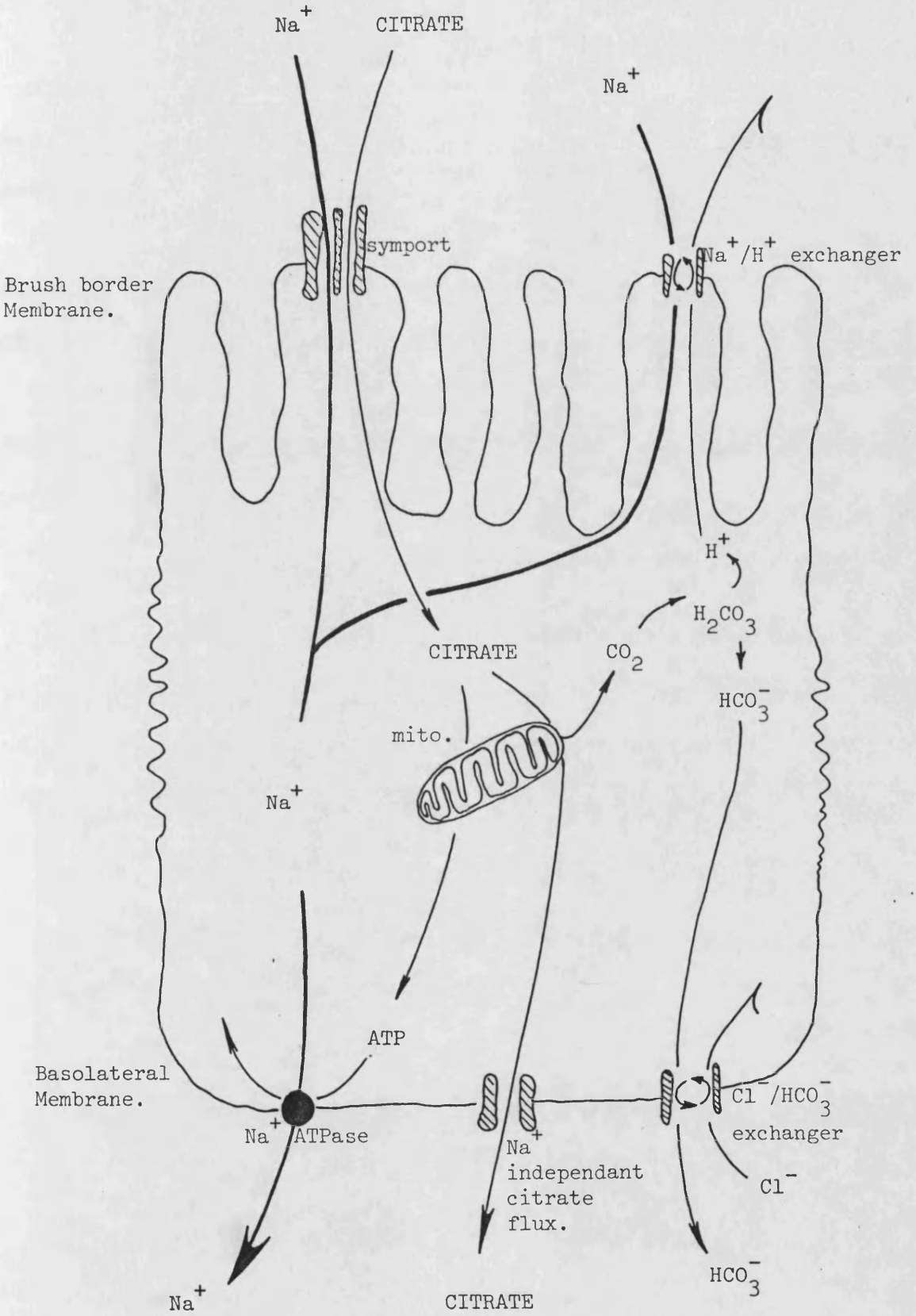
the proposition of a hypothesis to explain the citrate fluxes. This hypothesis can then be applied to examine the in vivo effects that citrate might have. This examination is particularly valuable to the understanding of the role of citrate in oral rehydration therapy.

The conclusions which can tentatively be drawn place the sodium dependent flux on the brush border membrane in the form of a sodium dependent citrate symport. The sodium independent citrate flux, in contrast, is best described by a citrate facilitative transport system on the basolateral side of the cell.

Citrate and its Anti-Diarrhoeal Properties.

The increase in water reabsorption caused by citrate was first reported by Bywater (1977) and subsequently rationalized as a citrate induced increase in net trancellular sodium flux by Burgess (1983). The hypothesis proposed here further rationalizes the citrate induced increase in net sodium flux in terms of cellular processes.

The presence of sodium and citrate in the intestinal lumen allows the transport of Na^+ -citrate into the enterocyte via the Na^+ -citrate symport. In the healthy animal this symport flux will lead to only a small increase in total enterocyte sodium influx. This is the case as enterocytes in the well nourished animal will experience sodium influx events mediated by other symport systems (Fonacaro,1986; Semenza et al,1984; Freel et al,1981; Tucker et al,1973). However, in the dehydrated and malmourished animal the sodium flux through this symport system may lead to a significant increase in total enterocyte sodium influx.



THE MODEL - Citrate Absorption.

(A single enterocyte is shown)

Once citrate has entered the cell it may either be metabolised or cross the cell (as seen for other absorbed metabolites, e.g. glucose, Lamb et al,1983).

For the proportion of citrate that is matabolised via citrate-ATP-lyase and subsequently the mitochondrial enzymes of the TCA cycle, both CO_2 and ATP will be produced.

The CO_2 dissolved in the intracellular fluid as H_2CO_3 (enzymatically aided by carbonic anhydrase) will ionize, giving rise to bicarbonate and a proton. The potentially harmful decrease in intracellular pH will instantly be rectified via Na^+/H^+ exchange and the cell's sodium gradient. The proton will therefore be ejected to acidify the lumen, whilst another sodium influx event takes place. Two routes of citrate induced sodium influx have therefore now been identified.

The ATP produced by the oxidation of citrate is now available to increase the cellular energy levels. In the well nourished animal probably little of the citrate will be metabolised, and any that is will cause little change in the already ATP saturated cellular processes. However, in the dehydrated and malnourished animal probably much of the citrate will be metabolised and help to restore ATP levels close to those of the control animal. Reduced cellular ATP levels can reduce sodium ATPase activity and thus ATP level restoration may restore sodium ATPase activity.

Thus three separate events have now been identified, each individually having the potential of increasing the net transcellular sodium flux. Burgess (1983) has reported that the absorption promoting effects of citrate on sodium move-

ments are in excess of the 3 sodium ions per citrate molecule proposed by Wright et al,(1982). The three separate transcellular sodium flux promoting events in this hypothesis may well explain the findings of Burgess.

The bicarbonate released as a consequence of citrate metabolism (described earlier) is available as additional buffering capacity to the cell, (Elliott et al,1985). This buffering capacity is of particular importance since dehydration invariably results in dangerous levels of acidosis (Booth et al,1987; Brugere-Picoux,1985). A reduction in blood plasma pH is also invariably seen which may be ameliorated by the bicarbonate which can be made available to the blood via basolateral HCO_3^-/Cl^- exchange (Liedke et al,1982.)

In summary the model shows that three separate sodium fluxes can be increased by citrate. It can be seen that in the dehydrated and malnourished animal this may make a significant increase to the transcullular sodium flux. However, in the well nourished (control) animal any changes due to citrate will probably be insignificant. This is a highly desirable phenomenon for a pharmaceutical compound. The easily metabolizable nature of citrate to release much ATP per citrate molecule leads to quick restoration of cellular ATP levels. Citrate metabolism also indirectly results in bicarbonate production. The bicarbonate is then available to the cell and the blood (via HCO_3^-/Cl^- exchange) to reduce the acidosis which accompanies dehydration.

CONCLUSIONS

Enterocytes isolated from chicken, rabbit and suckling piglets have proved to be highly manipulatable systems for the study of intestinal ion fluxes. However, after isolation, enterocytes from older chickens showed a rapid loss in sodium ATPase activity and thus their ability to sustain a sodium gradient is also lost. However, these cells can be used providing a transient sodium gradient is artificially produced. The use of young chickens, rabbits or piglets overcame this problem entirely and resulted in a system more relevant to the physiological state.

The purification of E.coli -431 ST_a by the modified method of Alderete and Robertson (1978) produced a highly pure preparation with high biological activity and in a concentrated form suitable for enterocyte studies.

The use of enterocytes isolated from the target animal for ST_a diarrhoeal disease (suckling piglet) failed to show any ST_a induced effects on ion fluxes. Subsequently it was found that the enterocyte cGMP levels necessary for ST_a activity (Newsome et al,1978; Gianella et al,1980) could not be maintained after enterocyte isolation. However the use of an exogenously added cGMP analogue showed a reduction in sodium influx. This reduction was presumably similar to that which would have been detected if cGMP level maintenance had not been a limitation of the isolated piglet enterocyte system. Any future studies in this area would require the use of an isolated enterocyte system capable of sustaining cGMP levels.

The study of the role of citrate in oral rehydration formulations using the isolated enterocyte system, revealed the presence of two citrate fluxes. The characteristics of the sodium dependent flux are best described by a sodium-citrate symport. This system shows numerous similarities to the renal sodium-citrate symport (Wright et al,1982), and likewise is probably situated on the brush border membrane. The sodium independent flux is best described by a facilitative transport system on the basolateral membrane of the cell. Future studies concerning these two fluxes would be best undertaken using brush border and basolateral vesicles. This would probably allow separation of the two citrate fluxes. The hypothesis to explain the action of citrate in oral rehydration therapy, shows three separate mechanisms for increasing transcellular sodium flux. These mechanisms are: an increase in enterocyte sodium influx via a Na^+- citrate symport; an increase in enterocyte sodium influx via Na^+/H^+ exchange and protons resulting indirectly from citrate metabolism; and increases in cellular ATP levels from citrate metabolism leading to increases in Na^+ ATPase activity. The three separate mechanisms explain the gross disproportionality between citrate flux and the effects of citrate on sodium flux, first described by Burgess, (1983). This ability of citrate to massively increase enterocyte sodium flux in vivo, explains the rehydrating properties of citrate in the life saving solutions used for oral rehydration therapy.

ACKNOWLEDGEMENTS.

I would like to thank my supervisors Dr. Geoffrey Holman, Dr. Robin Bywater and Dr. Peter Newsome for their help and guidance during my PhD.

I also wish to express my gratitude to Beecham Pharmaceuticals for their financial support throughout this CASE award. I would also like to thank the S.E.R.C. for their financial contribution and the opportunity of attending their business and administration course.

In addition, I would also like to thank Dr. Robin Bywater and his staff for their constant co-operation and liason throughout my PhD and particularly during my period at Walton Oaks. In particular I would like to thank Dr. Malcolm Burgess for his advice concerning toxin production, Dr. Robert Dupe and Mr. Mike Goddard for their instruction and assistance with the anaesthetic techniques, and Mrs. Kate Powell for her advice on the electron microscopy.

Finally, I would like to thank my parents and Janice for their constant support during my PhD studies. I am also very grateful to my parents for their tireless patience and help in the preparation of the typed manuscript.

REFERENCES.

Ahrens, F.A. and W. Panichkrangkrai (I985).
Effects of E.coli Heat Stable Enterotoxin, Atropine, Clonidine and Morphine On Chloride Efflux From Isolated Enterocytes.
Am. J. Vet. Res. 46,IO,Oct I985,p 2067-207I

Alderete, J.F. and D.C. Robertson (i978).
Purification and Chemical Characterisation of the Heat Stable Enterotoxin Produced by Porcine Strains of Enterotoxigenic E.coli
Infect. Immun. I9,3,March I978,p IO2I-IO3O

Baldini M.M., J.B. Kaeper, M.M. Levine (I985).
Plasmid Mediated Adhesion In Enteropathogenic E.coli.
J. Pediat. Gastroent. and Nut. 2,(3),p 534-538

Banwell, J.G., S.L. Gorbach, N.F. Pierce, R. Mitra and A. Monda (I97I).
Acute Undifferentiated Human Diarrhoea In The Tropics: Alterations In Intestinal Fluid And Electrolyte Movements.
J. Clin. Invest. 5O,I97I,p 89O-9OO

Barros, F., P. Dominguez, G. Velasco and P.S. Lazo (I986).
Na^+/H^+ Exchange Is Present In Basolateral Membranes From Rabbit Small Intestine.
B.B.R.C. I32,2,Jan 29 I986,p 827-834

Benos, D.J. and L.J. Mandel (I978).
Irreversible Inhibition Of Sodium Entry Site
In Frog Skin By A Photosensitive Amiloride Analogue
Science I99,I7 March I978,p I205-I206

Bertschinger, H.U., H.W. Moon and S.C. Whipp (I972)
Association Of E.coli With The Small Intestinal
Epithelium: Comparison Of Enteropathogenic and
Non-Enteropathogenic Porcine Strains In Pigs.
Infect. Immun. 5,I972,p 595-605

Booth A.J. et al (I987)
Correction Of Metabolic Acidosis In Diarrhoeal
Calves By Oral Administration Of Electrolyte
Solutions With Or Without Bicarbonate.
J.A.V.M.A. I987, I9I,(I),p 62-70

Bradford, N.M., and J.D. McGivan (I982)
The Transport Of Alanine And Glutamine Into
Isolated Rat Intestinal Epithelial Cells.
B.B.A. I982,689,p 55-62

Brown, J.D. (I985)
Oral Rehydration Therapy For Diarrhoea.
Milit. Med. I985,I50,(II),p 577- 583

Brown P.D., K.A. Burton and F.V. Sepúlveda (I983)
Transport Of Sugars Or Amino Acids Increases
Potassium Efflux From Isolated Enterocytes.
F.E,B.S. lett. I63,2,Nov I983,p 203-2I0

Brown P.D. and F.V. Sepúlveda (I985).
Potassium Movements Associated With Amino Acid
And Sugar Transport In Enterocytes Isolated From
Rabbit Jejunum.
J. Physiol I985,363,p 27I-285

Brown P.D., and F.V. Sepúlveda (I985)
A Rabbit Jejunal Isolated Enterocyte Preparation
Suitable For Transport Studies.
J. Physiol. I985,363,p 257-270

Brugere-Picoux, J. I985.
Use Of Solutes In The Treatment Of Dehydration
And Acid-Base Disorders In Adult Cattle.
Recueil Med. Vet. Ecole Alfort I985,I6I,3,p 23I-245

Burgess, M.N. (I983)
The Biochemical, Biological and Chemical Characterisation
Of A Heat Stable Enterotoxin Secreted By
Enteropathogenic Strains Of E.coli.
Ph.D. Thesis University of Bath I983

Burgess, M.N., Bywater R.J., C.M. Cowley, N.A. Mullan,
and P.M. Newsome (I978)
Biological Evaluation Of A Methanol Soluble Heat
Stable E.coli Enterotoxin In Infant Mice, Pigs,
Rabbits and Calves.
Infect. Immun. I978,2I,p 526-53I

Burgess, M.N., N.A. Mullan and P.M. Newsome (I980)
Heat Stable Enterotoxins From E.coli PI6.
Infect Immun. 28,3,June I980,p I038-I040

Burton K.A., A. Ilundain, J.A. O'Brien and F.V. Sepúlveda (I987).
Calcium Mobilization By Inositol I-4-5 Tri-Phosphate In Chicken Enterocytes.
J. Physiol. I987, 378, 77P

Butler D.G., D.G. Gall, M.H. Kelly and J.R. Hamilton
Transmissable Gastroenteritis: Mechanisms For Diarrhoea In A Acute Viral Enteritis In Piglets.
J. Clin. Invest. I974,53,p I335-I342

Bywater R.J. (I972)
Dialysis and Ultrafiltration of a Heat Stable Enterotoxin From E.coli.
J. Med Microbiol. 5, I972, p337-343

Bywater R.J. (I985)
Overcoming Antibiotic Resistance.
Vet. Times I2 Sept I985, p I5-I8

Bywater R.J. (I977)
Evaluation of an Oral Glucose Glycine Electrolyte Formulation and Amoxycillin For The Treatment Of Diarrhoea In Calves.
Am. J. Vet. Res. 38,p I983-I987

Bywater R.J., and G.N. Woode (I980)
Oral Fluid Replacement By A Glucose Glycine Electrolyte Formulation In E.coli And Rotavirus Diarrhoea In Pigs.
Vet. Rec. I06,I980,p 75-78

Carter-Su C. and G.A. Kimmich (I979)
Membrane Potentials And Sugar Transport By ATP depleated Intestinal Cells: Effects Of Anion Gradients.
AM. J. Phys I979,237,(I),C67-C74

Carter-Su C. and G.A. Kimmich (I98O)
Effect Of Membrane Potentials on Sodium Dependent Sugar Transport By ATP Depleated Intestinal Cells.
AM. J. Phys 238,I98,C73-C8O

Cassel D. and M. Katz (I986)
Depleation Of Cellular ATP Inhibits Na^+/H^+ Exchange In Cultured Cells.
J. Biol. Chem. I986,26,(I2),p546O-5466

Cassuto J., M. Jodel, R. Tuttle and O. Lundgren (I98I)
The Role Of Intramural Nerves In The Pathogenesis Of Cholera Toxin Induced Intestinal Secretion.
Scand. J. Gastroent. I98I,I6,p 377-384

Carpenter C.C.J. (I982)
The Pathophysiology Of Secretory Diarrhoea.
Med. Clin. I982,66,p 597-6IO

Carpenter C.C.J. (I982)
Oral Rehydration - Is It As Good As Parental Therapy.
New Engl. J. Med 3O6, I982, p IIO3-IIO4

Carpenter C.C.J. (I972)
Cholera And Other Enterotoxin Related Diarrhoeal Diseases
J. Infect. Dis. I26,I972,p 55I-564

Chan S. and R.A. Gianella (I98I)

Amino Acid Sequence Of Heat Stable Enterotoxin Produced By E.coli Pathogenic For Man.

J. Biol. Chem. I98I 25,p 7744-7746

Christiansen K. and J. Carlsen (I98I)

Microvillus Membrane Vesicles From Pig Small Intestine: Purity and Lipid Composition.

B.B.A. 647,I98I,p I88-I95

Crane R.K. (I977)

The Intestinal Sodium Dependent Sugar Carrier.

Rev. Physiol. and Bochem Pharmacol. I977,78,p 99-I59

Dahlqvist A. (I964).

Method For The Assay Of Intestinal Disaccharides.

Anal. Biochem. 7,I964, p I8-25

Dahlqvist A. and M. Messer (I966)

A One Step Ultramicro Method For The Assay Of Intestinal Disaccharides.

Anal. Biochem. I966, I4, p 376-392

DeJonge H.R. (I984)

The Mechanism Of Action Of E.coli Heat Stable Enterotoxin.

Biochem. Soc. Trans. I984,I2,(I2),p I8O-I84

DelCastillo J.R. and J.W.L. Robinson (I985)

Magnesium ATP Dependent Sodium Transport In Inside out Basolateral Plasma Membrane Vesicles From Guinea Pig Small Intestinal Epithelial Cells.

B.B.A. 8I2,I985,p 4O2-4I2

DelCastillo J.R. and J.W.L. Robinson (I985)
Sodium Stimulated ATPase Activities In Basolateral Plasma Membranes From Guinea Pig Small Epithelial Cells.
B.B.A. I985, 8I2,p 4I3-422

Dominguez P., F. Barros and P.S. Lazo (I985)
The Activation Of Adenylate Cyclase From Small Intestinal Epithelium By Cholera Toxin.
Eur. J. Biochem. I985,I46, p 533-538

Donowitz M., J. Wicks, S. Cusolito and G.W. Sharp. (I984)
Pharmacotherapy Of Diarrhoeal Diseases: An Approach Based On Physiological Principles.
in: Mechanisms Of Intestinal Electrolyte Transport and Regulation Of Calcium. Pages 329-359.
I984 Alan R. Liss Inc. I50 Fifth Ave N.Y.

Donowitz M.J., J. Wicks, J.L. Madara and GW. Sharp (I983)
Studies On THe Role Of Calmodulin In Calcium Regulation Of Rabbit Ileal Sodium and Chloride Transport.
Am. J. Phys. I983,248, G780-785

Donta S.T., H.W. Moon and S.C. Whipp (I974)
Detection Of Heat Stable E.coli Enterotoxin With The Use Of Adrenal Cells In Tissue Culture.
Science I974,I83,p 334-336

Dreyfuss L.A., J.C. Frantz and D.C. Robertson. (I983)
Chemical Properties Of Heat Stable Enterotoxins Produced By Enterotoxigenic E.coli Of Different Host Origins.
Infect Immun. 42,2,Nov I983,p 539-548

Dreyfus L.A. and D.C. Robertson. (I984)

Solubilization And Partial Characterisation Of The Intestinal Receptor For E.coli Heat Stable Enterotoxin.

Infect. Immun. Nov I984,46,2,p 537-543

Dupont H.L. (I985)

Diarrhoeal Diseases - An Overview

Am. J. Med. Jun 28 I985, 78,p 63-64

Eidels L., R.L. Proia and D.A. Hart (I983)

Membrane Receptors For Bacterial Toxins.

Microbiology Reviews Dec I983,47,4,p 596-620

Elliott E.J., M.J. Kelly, A.J.M. Watson and J.A. Walker

Effect Of Bicarbonate On The Efficacy Of Oral Rehydration Therapy In A Rat Model Of Secretory Diarrhoea.

Gut Oct I985,26,IO,p II42-II43

Escobales et al (I986)

Amiloride Sensitive Na^+/H^+ Exchange.

J. Memb. Biol. I986 9O,(I),p 2I-3I

Finberg, L. (I985)

Oral Glucose Electrolyte Solutions.

Amer. j. Dis. Child. I985 I39,6,p 552-553

Finch, M.H. et al (I987).

Oral Rehydration Therapy

Southern Med. J. I987,8O,5,p 6O9-6IO

Finberg, L. (I973)

Hypernatraemic Dehydration In Infants.

New Engl. J. Med. I973,289,p I96-I98

Finklestein, R.A, et al. (I985)

Cholera Enterotoxin (Choleragen).

Pharmacol. Ther. I985,27,I,p 37-49

Fisher S.E. (I987)

Alanine Enhances Jejunal Sodium Absorption In

The Presence Of Glucose: Studies Of Piglet

Viral Diarrhoea.

J. Pediat. Gastro ent. Nutr. I987,6,4,p 654-660

Fiske and Subbarow (I925)

Measurement Of Inorganic Phosphate In Biological

Systems.

J. Biol. Chem. I925,66,p 375-400

Fondacaro J.D. (I986)

Intestinal Ion Transport And Diarrhoeal Disease.

Am. J. Phys. I986,250,GI-G8

Fondacaro J.D. and L.S. Henderson (I985)

Evidence For Protein Kinase C AS A Regulator Of

Intestinal Electrolyte Transport.

AM. J. Phys I985, 249,G422-G426

Forte, J.G., G.M. Forte and P. Saltman I967

The Measurement Of Na^+/K^+ ATPase Activity

Using para Nitro Phenyl Phosphate.

J. Cell Phys. I967,69,p 293-304

Frantz, J.C., L. Jaso-Friedman and D.C. Robertson. (I984)
Binding Of E.coli Heat Stable Enterotoxin To Rat Intestinal Cells And Brush Border Membranes.
Infect. Immun. Feb I984,43,2,p 622-630

Frantz, J.C., and D.C. Robertson(I98I)
Immunological Properties Of E.coli Heat Stable Enterotoxins: Development Of A Specific Radio-Immunoassay For Heat Stable Enterotoxins With Suckling Mouse Assay Activity.
Infect. Immun. July I98I,33,I,p I93-I98

Freel, R.W. and a.m. Goldner (I98I)
Intestinal Amino Acid Uptake.
Am. J. Phys I98I,24I,G45I-G460

Ganguly, U. and S. Talukder (I985)
Effects Of E.coli Heat Stable Enterotoxin (S.T.) On Calcium Uptake By Rat Brush Border Membrane Vesicles.
F.E.M.S. Microbiol. lett. I985,26,p 255-257

Gariepy, J. and G.K. Schoolnik. (I986)
Design Of A Photoreactive Analogue Of The E.coli Heat Stable Enterotoxin ST_a : Use In Identifying Its Receptor In Rat Brush Border Membranes.
Proc. Nat. Acad. Sci. I986, 83,p 483-487

Garty, H. (I986)
Mechanisms Of Aldosterone Action In Tight Epithelia.
J. Memb. Biol. I986, 90,p I93-205

Gains, G. and H. Hauser. (I984)
Leakiness Of Brush Border Vesicles.
B.B.A. I984 772,pI6I-I66

Gerday, C., M. Hernan, J. Olivy, N. Geradin-Otthiers, D. Art, A. Kaeckenbeek and J. VanBeeumen. (I984)
Isolation And Characterisation Of Heat Stable Enterotoxin From A Pathogenic Bovine Strain Of E.coli.
Vet. Microbiol I984 9,p 399-4I4

Gerday, C., J. Olivy, F. Daniel, F. Schoenaers and A. Kaeckenbeek. (I980).
Isolation And Characterisation Of Heat Stable Enterotoxin Of E.coli From Bovine Origin.
ANN. Med. Vet. I980 I24,p 527-533

Gianella, R.A. (I976)
Suckling Mouse Model For Detection Of Heat Stable E.coli Enterotoxin: Characteristics Of The Model.
Infect. Immun. I976 I4,p 95-99

Gianella, R.A. and K.W. Drake (I979)
The Effects Of Purified E.coli Heat Stable Enterotoxin On Intestinal Cyclic Nucleotide Metabolism And Fluid Secretion.
Infect. Immun. I979 24,pI9-23

Gianella, R.A., K.W. Drake and M. Luttrell. (I98I)
Development Of A Radioimmunoassay For E.coli Heat Stable Enterotoxin: Comparison With The Suckling Mouse Bioassay.
Infect. Immun. July I98I , p I86-I92

Gianella, R.A., M. LUttrell and M. Thompson (I983)
Binding Of E.coli Heat Stable Enterotoxin To Receptors On Rat Intestinal Cells.
Am. J. Phys I983 245,p G492-G498

Gill, D.M., D.J. Evans and D.G. Evans. (I976)
Mechanism Of Activation Of Adenylate Cyclase in vitro By Polymyxin Released, Heat Labile Enterotoxin Of E.coli.
J. Infect. Dis. I976 I33,p IO3-IO7

Gonzales, E.A. et al (I985)
Comparison Between Enterotoxigenic Activity And Methanol Solubility In Heat Stable Enterotoxins (ST_a and ST_b) From E.coli Of Human, Porcine And Bovine Origins.
Bact. Microbiol (Zbl Bakt Mikrobiol Hyg A-Med) 260, PI

Greenough, W.B. (I980)
Principles And Prospects With Treatment Of Cholera And Dehydrating Diarrhoeas.
in:Cholera and Related Diarrhoeas.
Ed: O.Ouchterlony and J. Holmgren, Basel S. Karger

Grienstein et al (I986)
Mechanisms Of Regulation Of Na^+/H^+ Exchange.
J. Memb Biol. I986 90,(I),pI98

Guerrant, R.L., J.M. Hughes, B. Chang, D.C. Robertson and F. Murad.
Activation Of Intestinal Guanylate Cyclase By Heat Stable Enterotoxins Of E.coli : Studies Of Tissue Specificity, Potential Receptors and Intermediates. J. Infect. Dis. I42,2, p 220-227

Gutowski, S.J. and H. Rosenberg (I975)
Succinate Uptake And Succinate Induced Proton Uptake In E.coli.
Biochem. J. I975 I52,647,p 647

Gyles, C.L. (I97I)
Heat Labile And Heat Stable Forms Of The Enterotoxin From E.coli Strains Enteropathogenic For Pigs.
Ann N.Y. Acad Sci I97I I76,p 3I4-322

Gyles, C.L., S. Falkow and L. Rollins (I978)
In vivo Transfer Of An E.coli Enterotoxin Plasmid Possessing Genes For Drug Resistance.
Am. J. Vet. Res. I978 39,p I438-I44I

Gyles, C., M. So and S. Falkow (I974)
The Enterotoxins Plasmids Of E.coli.
J. Infect. Dis. I974 I3O.p 4O-49

Harrison, R. and G.G. Lunt. (I98O)
in: Biological Membranes - Their Structure and Function.
Blackie and Sons Ltd, London WCIV 6BX

Harvey, R.J. and E.B. Collins (I962)
Citrate Transport Systems Of Streptococcus diacetilactis.
J. Bacteriol. I962 83,p IOO5-IOO7

Henderson, L.S., D. Naseisky and J.D. Fondacaro (I985)
Calcium And Cyclic Nucleotide Dependent Phsphorylation Of Intestinal Microvillus Membranes.
Federation Proc. I985 44,p 7O4

Hoffman, S.L. et al (I985)
Rehydration And Maintenance Therapy Of Cholera Patients In Jakarta: Citrate Based Oral Rehydration Salt Solution.
J. Infect. Dis. I985 I52,(6),p II59

Hoshi, T., N. Takuwa, M. Abe and A. Tajima. (I986)
Hydrogen Ion Coupled Transport Of D-Glucose By Phlorizin Sensitive Sugar Carrier In Intestinal Brush Border Membranes.
B.B.A. I986 86I,p 483-488

Hughes, J.M., F. Murad, B. Chang and R.L. Guerrant
Role Of Cyclic GMP In The Action Of Heat Stable Enterotoxin Of E.coli.
Nature I978 27I,p 755-756

Hyun, C.S., E.J. Cragoe J$^{nr.}$ and M. Field. (I985)
a_2 Adrenergic Receptor Mediated Regulation Of Intestinal Calcium Transport.
Am. J. Phys. I985 249,CII7-CI23

Hyun, C.S. and G.A. Kimmich (I982)
Effects Of Cholera Toxin On cAMP Levels And Sodium Influx In Isolated Epithelial Cells.
Am. J. Phys. Sept I982 243,(3),CIO3-CII5

Ilundain A., J.A. O'Brien, K.A. Burton and F.V. Sepúlveda. (I986).
Inositol Tri-phosphate and Calcium Mobilization In Permeabilized Enterocytes.
B.B.A. I986 896,pII3-II6

Ilundain, A. and R.J. Naftalin (I979)
Role Of Calcium Dependent Regulatory Protein
In Intestinal Secretion.
Nature I979 279,p 446-448

Jentoff, N. and D.G. Dearborn (I979)
Labelling Of Protein By Reductive Methylation
Using Sodium Cyanoborohydride.
J. Biol. Chem. I979 254,p II

Jorgensen, P.L. (I982)
Intestinal Sodium/potassium ATPase Activity.
B.B.A. I982 694,p 27-68

Keljo, D.J., R.J. MacLeod, M.H. Perdue, D.G. Butler
and J.R. Hamilton. (I985)
D-Glucose Transport In Piglet Jejunal Brush
Border Membranes: Insights From A Disease Model.
Am. J. Phys. I985 249,G760-G75I

Kennedy, D.J., R.N. Greenberg, J.A. Dunn, R.Abernathy,
J.S. Ryerse and R.L. Guerrant. (I984)
Effects Of E.coli Heat Stable Enterotoxin ST_b
On Intestine Of Mice, Rats, Rabbits and Piglets.
Infect. Immun. I984 46,3,p 639-643

Kerzner, B., M.H. Kelley, D.K. Gall, D.G. Butler
and J.R. Hamilton. (I977)
Transmissable Gastroenteritis: Sodium Transport
And The Intestinal Epithelium During The Course
Of Viral Enteritis.
Gastroent. I977 72,p 457-46I

Kessler, M., O. Acuto, C. Storelli, H. Murer,M.Muller and G. Semenza. (I978)
A Modified Procedure For The Rapid Preparation Of Efficiently Transporting Vesicles From Small Intestinal Brush Border Membranes: Their Use In Investigating Some Properties Of D-Glucose And Choline Transport Systems.
B.B.A. I978 5O6,p I36-I54

Kimmich, G.A. (I97O)
Preparation And Properties Of Mucosal Cells Isolated From Small Intestine Of The Chicken.
Biochemistry I97O 9,I9,p 3659-3668

Kimmich, G.A. (I97O)
Active Sugar Accumulation By Intestinal Epithelial Cells. A New Model For Sodium Dependent Metabolite Transport.
Boichemistry I97) 9,I9,p 3669-3677

Kimmich, G.A. (I973)
Coupling Between Sodium And Sugar Transport In The Small Intestine.
B.B.A. I973 3OO,p 3I-7O

Kimmich, G.A. (I975)
Preparation And Characterisation Of Isolated Intestinal Epithelial Cells And Their Use In Studying Intestinal Transport.
in: Methods In Membrane Biology
Ed: E. Korn New York Plenum I975,p 5I-II5

Kimmich, G.A. and J. Randles (I975)
A Sodium Independent, Phloretin Sensitive Monosaccharide Transport System In Isolated Intestinal Epithelial Cells.
J. Memb. Biol. I975 23,p 57-76

Kimmich, G.A., J. Randles and J.S. Brand (I975)
Assay Of pico Mole Amounts Of ATP, ADP and AMP Using The Luciferase Enzyme System.
Anal. Biochem. I975 69,p I87-2O6

Kimmich, G.A., C. Carter-Su and J. Randles (I977)
Energetics Of Sodium Dependent Sugar Transport By Isolated Intestinal Cells: Evidence For A Major Role For Membrane Potentials.
Am. J. Phys. I977 233,E357-E362

Kimmich, G.A. and J. Randles (I979)
Energetics Of Sugar Transport By Isolated Intestinal Epithelial Cells: Effects Of Cytocholasin B.
Am. J. Phys. I979 237,(I),C56-C63

Kinsella, J.L. and P.s. Aronson (I98O)
Properties Of The Na^+/H^+ Exchanger In Renal Villus Membrane Vesicles.
Am. J. Phys. 238,F46I-F468

Kinsella, J.L., T. Cujdik, and B. Sacktor (I986)
Kinetic Studies On The Stimulation Of Na^+/H^+ Exchange Activity In Renal Brush Border Membranes Isolated From Thyroid Hormone Treated Rats.
J. Memb. Biol. I986 p I83-I9I

Kippen, I., B. Hirayama, J. Klinenberg and E.M. Wright
Transport Of Tricarboxylic Acid Cycle
Intermediates By Membrane Vesicles From Renal
Brush Border.
Proc. Nat. Acad. Sci. July I979,76,7,p 3397-3400

Klichelbein, R., P.S. Aronson, W. Atherton and
J.W. Dobbins. (I983)
Sodium And Chloride Transport Across Rabbit Ileal
Brush Border: Evidence For Na^+/H^+ Exchange.
Am. J. Phys. I983 245,G5O4-G5IO

Knoop, F. and D.M. Abbey (I98I)
A Possible Role For Calcium In Cyclic Nucleotide
Mediated Fluid Secretion.
Medical Hypothesis. I98I 7,p 7I9-727

Knutton, S. et al (I985)
Adhesion Of Enterotoxigenic Escherichia coli
To Human Small Intestinal Enterocytes.
Infect. Immun I985 48,(3),p 824-83I

Lacoz-Vieira F. (I986)
Sodium Flux In The Apical Membrane Of The Toad
Skin: Aspects of Its Regulation And The Importance
Of The Ionic Strength Of The Outer Solution On
THe Reversibility Of Amiloride Inhibition.
J. Memb. Biol. I986 92,p 27-36

Lamb, J.F., C.G. Ingram, I.A. Johnston, and
R.M. Pitman (I983)
Essientials Of Physiology.
Blackwell Scientific Publications, London

Lazorick, K., C. Miller, S. Sariban-Soraby and
D. Benos. (I985)
Synthesis And Characterisation Of Methyl
Bromoamiloride, A Potential Biochemical Probe
Of Epithelial Sodium Channels.
J. Memb. Biol. I985 86,p 67-77

Leblond, C.P. and B. Messier (I958)
Villus Cell Migration.
Anat. Rec. I958,I32,p 247-259

Liedke, C.M. and U. Hopfer (I982)
Mechanism Of Cl^- Translocation Across Small
Intestinal Basolateral Border Membrane: Absence
Of Na^+ Cl^- CoTransport.
Am. J. Phys. I982 242,G 263-G 27I

Long-Krug, S.A., C.S. Weikel, K.T. Tiemens,
E.L. Hewlett, M.N. Levine and R.L. Guerrant.
Does Enteropathogenic E.coli Produce Heat Labile
Enterotoxin, Heat Stable Enterotoxins, Or
Cholera Toxin A Subunits ?
Infect. Immun. Nov I984,46,(2),p 6I2-6I4

Lowry, O.H., N.J. Rosenbrough, A.L. Farr and
R.J. Randall (I95I)
Protein Measurement With The Folin Phenol
Reagent.
J. Biol. Chem. I95I I93,p 265-275

Mackenzie, N.M. (I985)
Comparison Of Metabolic Activities Of Enterocytes
Isolated From Different Regions Of The Small
Intestine Of The Neonate.
Biol. Neonate. I985 48, p 257-268

Marin L. et al (I987)
Oral Rehydration Therapy In Neonates And Young
Infants With Infectious Diarrhoea.
Acta. Pediat. Scand. I987 76,(3),p 43I

Merritt, A.M. et al (I985)
Effect Of Lidmidine-HCl On E.coli Heat Stable
Enterotoxin Induced Jejunal Water And Electrolyte
Secretion In Neonatal Piglets.
J. Vet. Pharmacol. Therapy I985 8,(2),p I5O-I57

Mircheff, A.K. and E.M. Wright (I976)
Analytical Isolation Of Plasma Membranes Of
Intestinal Epithelial Cells: Identification Of
Na^+,K^+ ATPase Rich Membranes And The Distribution
Of Enzyme Activities.
J.Memb. Biol. I976 28,p 3O9-333

Mitchell, A.R.

Composition Of Oral Rehydration Solutions.

Vet. Rec. I985 II7,(20),p 535

Montrose, M.H., G. Bebernitz and G.A. KImmich. (I985)

Evaluation Of Ion Gradient Dependent H^+ Transport Systems In Isolated Enterocytes From The Chick.

J. Memb. Biol. I985 88,p 55-66

Moolenaar, W.H. (I986)

Regulation Of Cytoplasmic pH By Na^+/H^+ Exchange.

T.I.B.S. I986 II,(3),p 141

Morris, J.G. (1980)

A Biologists Physical Chemistry.

Pub. Edward Arnold, London

Mouricout, M.A. et al (I987)

Pilus Mediated Binding Of Bovine Enterotoxigenic E.coli To Calf Small Intestinal Mucins.

Infect. Immun I987 55,(5),p 1216

Moriarty, K.J. et al (1986)

Bacterial Toxins And Diarrhoea.

Clin. Gastroent. I986 I5,(3),p 529

Mullen, N.A., M.N. Burgess and P.M. Newsome. (I978)

Characterisation Of A Partially Purified, Methanol Soluble Heat Stable Escherichia coli Enterotoxin In Infant Mice.

Infect. Immun. March I978 I9,(3),p 779-784

Murdock, P.R., G.D. Holman and R.J. Bywater (I987)
The Role Of Citrate In Oral Rehydration Therapy.
Biochem. Soc. Trans. I987 vol 15,5,p 913 - 914.

Murer, H., U. Hopfer and R. Kinne (I975)
Na^+/H^+ Antiport In Brush Border Membrane Vesicles Isolated From Rat Small Intestine And Kidney.
Biochem. J. I975 I54,p 597-604

Murer, H., U. Hopfer, E. Kinne-Saffran and R. Kinne.
The Intestinal Na^+ Dependent Glucose Carrier.
B.B.A. I974 345,p I70-179

Nellans, H.N. and S.G. Schultz (I976)
Relations Among Transepithelial Sodium Transport Potassium Exchange and Cell Volume In Rabbit Ileum.
J. Gen. Phys. I976 68,p44l-463

Newsome, P.M. (I980)
Escherichia coli Enterotoxins In Colibacilliosis: A Review.
Proc. Brit. Cattle Vet. Assoc. I980,p 91-100

Newsome, P.M., M.N. Burgess, M.R. Burgess, K.A. Coney M.E. Goddard and J.A. Morris. (I987)
A Model Of Acute Infectious Diarrhoea
J. Med. Microb. I987 23,(1),p I9

Newsome P.M., M.N. Burgess, R.J. Bywater, C.M. Cowley and N.A. Mullan. (I978)
Studies Of The Biological Activities And Mechanism Of Action Of Heat Stable E,coli Enterotoxins.
Proceed. Second Int. Symp. On Neonat. Diarr. Oct 3-5 I978 Univ Of Saskatchewan.

Newsome P.M., M.N. Burgess and G.D. Holman. (I983)
Stimulation Of IOn Absorption By Sodium Citrate.
Scand. J. Gastroent. I983 I8,Supp 87,p II9-I21

Newsome P.M., M.N. Burgess and N.A. Mullan. (I978)
Effect Of E.coli Heat Stable Enterotoxin On cGMP Levels In Mouse Intestine.
Infect. Immun. Oct I978 22,I,p 290-291

Newsome P.M. and K.A. Coney (I985)
Synergistic Rotavirus And E.coli Diarrhoeal Infection Of Mice.
Infect. Immun. Feb I985 47,(2),p 573-574

Nord E.P., A. Hafezi, E.M. Wright and L.G. Fine.
Mechanisms Of Na^+ Uptake Into Renal Brush Border Membrane Vesicles.
Am. J. Phys. 247, F548-F554

Olsvik O., R. Solsberg and T. Bergan. (I(85)
Characterisation Of Enterotoxigenic E.coli
Serotypes, Enterotoxins, Adhesions and
The Presence Of Plasmids.
Infect. Immun. I985 2I,526,p 78

Peerce B.E. and E.M. Wright.
Sodium Induced Conformational Changes In
The Glucose Transporter Of Intestinal Brush
Borders.
J. Biol. Chem. Nov 25 259,22,p I4IO5-I4II2

Potter M.E. et al (I985)
Diarrhoea Due To E.coli OI57:H7 In The infant
Rabbit.
J. Infect. Dis. I985 I52,(6),p I341

Quigley J.P. and G.S. Gotterer (I956)
ATPase Activity By Measuring Release Of Inorganic
Phosphate.
B.B.A. I969 I73,p 456-468

Rao M.C., S. Guandalini, P.L. Smith, and M. Field (I978)
Heat Stable E.coli Enterotoxin ST_a Effects On
Guanylate Cyclase: Tissue And Subcellular
Specificity And Relation To Ion Transport.
Clin. Res. I979 27,456A

Rapacz J. et al (I986)

Polymorphism And Inheritance Of Swine Small Intestinal Receptors Mediating Adhesion Of Three Serological Variants Of E.coli Producing K88 Pilus Antigen.

Anim. Genet. I986 I7,(4),p3O5

Rhoads J.M. et al (I986)

Alanine Enhances Jejunal Sodium Absorption In The Presence Of Glucose - Studies In Piglet Viral Diarrhoea.

Pediat. Res. I986 2O,(9),p 879

Robertson D.C. et al (I985)

E.coli Heat Labile Enterotoxin.

Pharmacol. and Therap. I985 28,(3),p303

Rodbell M. (I987)

Role Of GTP Binding Proteins In Hormone And Drug Action: Adenylate Cyclase And Other Transduction Processes.

Drug Develop. Res. I987 Io,(4),p I95-2O4

Rolston D.D.K. et al (I987)

Efficacy Of Oral Rehydration Solutions In A Rat Model Of Secretory Diarrhoea.

J. Pediat. Gastroent. and Nutr. I987 6,(4),p 624

Ronnberg B., T. Wadstrom and H. Jornwell (I983)
Structure Of A Heat Stable Enterotoxin Produced
By A Human Strain Of E.coli.
F.E.B.S. lett. May I983,I55,2,p I83-I86

Rowling p.J.E. and F.V. Sepúlveda. (I984)
The Distribution Of ($Na^+ + K^+$) ATPase Along
The Villus-Crypt Axis In The Rabbit Small
Intestine.
B.B.A. I984 77I,p 35-41

Sack R.B., S.L. Gorbach, J.C. Banwell, B. Jacobs,
B.D. Chaterjee and R.C. MItra (I970)
Enterotoxigenic E.coli Isolated From Patients
With Severe Cholera Like Diseases.
J. Infect Dis. I970 I23,p 378-385

Saeed A.M.K., N.S. Magnuson, C.C. Gay and R.N Greenberg.
Characterisation Of Heat Stable Enterotoxin
From A Hypertoxigenic E.coli Strain Pathogenic
For Cattle.
Infect. Immun. Aug I986 53,(2)p 445-447

Sariban-Sohraby S. and D.J. Benos (I986)
Detergent Solubilisation, Functional Reconstitution
And Partial Purification Of Epithelial Amiloride
Binding Protein.
Biochem. I986 25,p 4639-4646

Salazarlindo E. et al (I986)
Bicarbonate Versus Citrate In Oral Rehydration Therapy In Infants With Watery Diarrhoea - A Controlled Clinical Trial.
J. Pediat. I986 IO8,(I), p 55

Semenza G., M. Kessler,M. Hosang, J. Weber and U. Schmidt. (I984)
Biochemistry Of The Sodium D-Glucose CoTransporter Of The Small Intestinal Brush Border Membrane: The State Of The Art In 1984.
B.B.A. I984 779,p 343-379

Sepúlveda F.V., K.A. Burton and P.D. Brown (I982)
The Relation Between Sodium Coupled Amino Acid And Sugar Transport And Sodium/Potassium Pump Activity In Isolated Intestinal Epithelial Cells.
J. Cell. Phys. I982 III,p 303-308

Sepúlveda F.V., and W.T. Mason (I985).
Single Channel Recordings Obtained From Basolateral Membranes Of Isolated Rabbit Enterocytes.
F.E.B.S. Lett. I985, I9I, p87-9I

Sepúlveda F.V., (I985).
Uptake Of Ca^{++} By Isolated Rabbit Small Intestinal Cells With Highly Permeable Plasma Membranes.

Sepúlveda F.V., K.A. Burton and Brown P.D. (I986)
Calcium Movements Accompanying The Transport
Of Sugar and Amino Acids By Rabbit Enterocytes.
J. Pysiol. I986, 856, pI85-I87

Sepúlveda F.V. and S.M. Smith (I987)
Calcium Transport By Permeabilized Rabbit Small
Intestinal Epithelial Cells.
Pflugers Arch : Eur J. Physiol 408 (3), p23I-238

Siegenbeek Van Heukelom J. (I986)
Physiological Aspects Of Absorption And Secretion
In The Intestine.
Vet. Res. Comm. I986 IO,p 341-354

Smith H.W. and C.L. Gyles. (I970)
The Effect Of Cell Free Fluids Prepared From Cultures
Of Human And Animal Enteropathogenic Strains Of
E.coli On Ligated Intestinal Segments Of Rabbits
And Pigs.
J. Med. Microbiol I970 3,p 403-409

Smith H.W. and S. Halls. (I967)
Observations By Ligated Intestinal Segment And Oral Inoculation Methods On E.coli Infections In Pigs, Calves, Lambs and Piglets.
J. Path. Bact. I967 93,p 499-529

Smith H.W. and M.A. Linggood (I97I)
Observations On The Pathogenic Properties Of The K88, HLY and ENT Plasmids Of E.coli With Particular Reference To Porcine Diarrhoea.
J. Med. Microbiol. I97I 4, p 467-485

Smith G.S., G. Warhurst, A. Tonge and K.A. Turnberg.
Prostaglandins Are Not Mediators Of The Intestinal Response To Cholera Toxin.
Gut. I985 26,p 680-682

So M. and B.J. McCarthy (I980)
Nucleotide Sequence Of The Bacterial Transposon T_n I68I Encoding A Heat Stable (ST) Toxin And Its Identification In Enterotoxigenic E.coli Strains
Proc. Nat. Acad. Sci. I980 77,p 40ll-4015

Sokucu S., L. Marin, H. Gunoz, A. Aperia, O. Neuzi and R. Zetterstrom. (I985)
Oral Rehydration Therapy In Infectious Diarrhoea.
Acta. Pediatr. Scand. I985 74,p 489-494

Soriban-Sohraby S. and D.J. Benos. (I986)
The Amiloride Sensitive Sodium Channel.
Am. J. Phys I986 250,(2),CI75-CI83

Spicer E.K., W.M. Kananaugh, W.S. Dallas, S. Falkow
W.H. Konigshaw and D.E. Schafer. (I98I)
Sequence Homologies Between A Subunits Of E.coli
and Vibrio cholerae Enterotoxins.
Proc. Nat. Acad. Sci. I98I 78,p 50-54

Storelli C., S. Vilella and G. Cassaro. (I986)
Amino Acid Transport In Eel Intestinal Brush
Border Membrane Vesicles.
Am. J. Phys I986 251,(3),part 2,R463-R470

Takeda T., M. Kitamura, Y. Takeda and T. Miwatani.(I983)
In Vitro Transfer Of ENT Plasmid From E.coli
To Shigella dysenteriae and Salmonella sp.
F.E.M.S Microbiol. lett. I983 I8,p 253-256

Thierry J., P. Poujeal and P. Ripoche. (I98I)
Interactions Between Na^+ Dependent Uptake Of
D-Glucose, Phosphate and L-Alanine In Rat Ranal
Brush Border Membrane Vesicles.
B.B.A. I98I 647,p 203-210

Thompson M.R., M. Luttrell, G. Overmann and R.A. Gianella.
Biological And Immunological Characteristics Of
^{125}I - 4 Tyr and I - I8 Tyr E.coli Heat Stable
Enterotoxin Species Purified By HPLC.
Anal. Biochem. I985 I48,p 26-36

Towler C.M., G.P. Pugh-Humphries and J.W. Porteous (I978)
Characterisation Of Columnar Absorptive
Epithelial Cells Isolated From Rat Jejunum.
J. Cell Science. I978 29,p 53-75

Tucker A.M. and G.A. Kimmich. (I973)
Characterisation Of Amino Acid Accumulation By
Isolated intestinal Epithelial Cells.
J. Memb. Biol. I973 12,p I-22

Uezato T. and M. Fujita. (I986)
Cytocholasin B Binding Proteins Related To Glucose
Transport Across The Basolateral Membrane Of The
Intestinal Epithelial Cell.
J. Cell. Sci. I986 85,p I77-I85

Ussing H.H. (I949)
The Distinction By Means Of tracers Between Active
Transport an diffusion.
Acta. Physiol. Scand. I949 I9,p 43-56

Van Cornen et al (I986)
Enterocyte Isolation Procedure Affects ATP Dependent
Calcium Transport.
Cell Calcium I986 7,(2),p 89-9I

Wahawison R., T.S. Gaginella and L.J. Wallace. (I985)
Jejunal - Ileal Differences In Dopaminergic
But Not a Adrenergic Anti-Secretory Effects.
Am. J. Phys. I985 248, G332-G336

Wapnir R.A. et al (I985)
Osmolarity And Solute Concentration- Their Relationship With Oral Rehydration Solution Effectiveness - An Experimental Assessment.
Pediat. Res. I985 I9,(9),p 894

Watford M., P. Lund and H.A. Krebs. (I979)
Isolation and Metabolic Characteristics Of Rat and Chicken Enterocytes.
Biochem. J. I979 I78,p 589-596

Weiser M.W. (I973)
Intestinal Epithelial Cell Surface Membrane Glycoprotein Synthesis.
J. Biol. Chem. April IO I973,248,(7),p 2536-2451

Whipp S.C. et al (I987)
Functional Significance Of Histological Alterations Induced By E.coli Pig Specific, Mouse Negative Heat Stable Enterotoxin.
Vet. Res. Commun. I987 II,(I),p 41

Wright S.H., I. Kippen, J.R. Klinenberg and E.M. Wright.
Specificity Of The Transport System For TCA Cycle Intermediates In Renal Brush Border.
J. Memb. Biol. I980 57,p 73-82

Wright S.H., S. Krasne, I Kippen and E.M. Wright.
Sodium Dependent Transport Of Tricarboxylic Acid Cycle Intermediates By Renal Brush Border Membranes. Effexts On Fluorescence Of A Potential Sensitive Cyanine Dye.
B.B.A. I98I 640,p 767-778

Wright S.H., I. Kippen and E.M. Wright. (I982)
Effect Of pH On The Transport Of Krebs Cycle
Intermediates In Renal Brush Border Membranes.
B.B.A. I982 684,p 287-290

Wright S.H., I. Kippen and E.M. Wright. (I982)
Stoichiometry Of Na^+ - Succinate CoTransport
In Renal Brush Border Membranes.
J. Biol. Chem. Feb 25 I982,257,(4),p 1773-1778

Wright E.M. and B.E. Peerce.
Identification Of The Intestinal Proline Carrier.
J. Biol. Chem. 259,(24),p I4993-I4996

Wright E.M., C.H. Van Os and A.K. Mircheff. (I980)
Sugar Uptake By Intestinal Basolateral Membrane
Vesicles.
B.B.A. I980 597,p II2-I24

Yoshimura S., T.Takao, H. Ikemura, S.Aimoto, Y. Shimonishi,
S. Hara, T. Takeda, Y Takeda and T. Miwatani. (I984)
Chemical Synthesis Of A Fully Active And Heat
Stable Fragments Of Heat Stable Enterotoxin
Of Enterotoxigenic E.coli Strain I8D.
Bull. Chem. Soc. Jpn. I984,57,p I38l-I387

AUTHOR'S PUBLICATIONS

Published Paper: The Role Of Citrate In Oral Rehydration Therapy.

Biochem. Soc. Transactions Oct I987
vol I5,5, p 913-914

Authors: P.R. Murdock, G.D. Holman and R.J. Bywater.

Published: October I987

Presentation: at 622nd. Meeting of The Biochemical Society.

Leicester meeting, University of Leicester.

Title: The Role Of Citrate In Oral Rehydration Therapy.

Poster abstract No. 101

Authors: P.R. Murdock, G.D. Holman and R.J. Bywater.

Presented by P.R. Murdock April 1987